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SUMMARY

AGRICULTURAL BIOTECHNOLOGY

1. EVALUATION OF THE GENETIC DIVERSITY OF *Pleurotus ostreatus* STRAINS USING COMBINATORIAL TUBULIN BASED POLYMORPHISM (cTBP) AND ISSR MARKERS - **Gabriela POPA, Matilda CIUCĂ, Călina Petruța CORNEA, Bogdan NICOLCIOIU, Radu TOMA** 11
2. STUDY ON THE ADAPTABILITY OF GIANT BAMBOO UNDER SOIL-CLIMATIC CONDITIONS SPECIFIC TO ROMANIAN - **Giovanni BEZZE, Davide VITALI, Damiano PAGNINI, Maurizio VALERI, George COJOCARU, Ricuța-Vasilica DOBRINOIU, Silvana DĂNĂILA-GUIDEA** 17
3. SELECTION AND CHARACTERIZATION OF NEW ENDOPHYTIC BACTERIAL STRAINS ISOLATED FROM POTATO TUBER USEFUL IN BIOCONTROL STRATEGIES - **Oana-Alina BOIU-SICUIA, Florica CONSTANTINESCU, Călina Petruța CORNEA** 23
4. DOWNY MILDEW IN SUNFLOWER-THE MANAGEMENT OF *Plasmopara halstedii* PATHOGEN - **Gabriel Florin ANTON, Maria JOIȚA-PĂCUREANU, Luxița RÎȘNOVEANU, Călina Petruța CORNEA, Mihaela POPA** 29
5. SELENIUM BIOFORTIFICATION TREATMENT OF CAULIFLOWER ENHANCES THEIR CONTENT IN CHEMOPREVENTIVE COMPOUNDS AND *IN VITRO* ANTITUMORAL ACTIVITY - **Elena UTOIU, Anca OANCEA, Alexandra GASPARGASPAR, Ana-Maria SECIU, Laura M. ȘTEFAN, Viorica COROIU, Oana CRĂCIUNESCU, Cristinel Dumitru BADIU, Florin OANCEA** 33
6. EFFECT OF EXOGENOUS ABSCISIC ACID ON SOME QUALITY ATRIBUTES OF SWEET POTATO (*Ipomoea batatas* L.) - **Evelina GHERGHINA, Florentina ISRAEL-ROMING, Daniela BĂLAN, Gabriela LUȚĂ** 41
7. *IN VITRO* PROPAGATION OF BITTER GOURD (*Momordica charantia* L.) - **Sevil SAGLAM** 46
8. EFFECTS OF SUCROSE CONCENTRATIONS ON INCREASE IN BULB SIZE OF *IN VITRO* REGENERATED HYACINTH (*Hyacinthus orientalis* L.) BULBLETS - **Suleyman KIZIL, Tahsin SOGUT, Umit ACAY, Berfin SARIHAN, Ugur SESIZ, Khalid Mahmood KHAWAR** 51
9. SURFACE RESPONSE OPTIMIZATION OF SUBMERGED BIOMASS PRODUCTION FOR A PLANT BIOSTIMULANT *Trichoderma* STRAIN - **Valentin ZAMFIROPOL-CRISTEA, Iuliana RĂUT, Tatiana Eugenia ȘESAN, Bogdan TRICĂ, Florin OANCEA** 56
10. OBTAINING MINITUBERS BY APPLYING METHOD OF CULTURE ON SUBSTRATES INDUSTRIAL - **Andreea TICAN, Nicoleta CHIRU, Mihaela CIOLOCA, Carmen BĂDĂRĂU** 66
11. ACHIEVEMENTS IN WINTER PEAS BREEDING PROGRAM - **Ancuța CRÎNGAȘU (BĂRBIERU)** 72
12. POTENTIAL USE OF KOMBUCHA CRUDE EXTRACT IN POSTHARVEST GRAPE MOULDS CONTROL - **Bogdan MATEI, Florentina MATEI, Camelia DIGUȚĂ, Ovidiu POPA** 77
13. ANTIMICROBIAL ACTIVITY OF NEWLY ISOLATED *Bacillus* sp. AND *Pseudomonas* sp. STRAINS AND THEIR POTENTIAL USE AS BIOCONTROL AGENTS - **Mariana-Grațiela SOARE (VLADU), Caterina TOMULESCU, Maria PETRESCU, Irina LUPESCU, Mișu MOSCOVICI, Ovidiu POPA, Narcisa BĂBEANU** 81
14. THE INFLUENCE OF THE OPEN POLLINATION ON THE INDUCING RATE ON TOP AND BOTTOM OF THE EAR ON DH TECHNOLOGY - **Ana Raluca COJOACĂ (BIȚICĂ)** 87

15. VARIABILITY OF COLEOPTILE LENGTH IN MUTANT/RECOMBINANT WHEAT DH (DOUBLED HAPLOID) LINES - Steliana Paula BARBU, Gabriela ȘERBAN, Călina Petruța CORNEA, Aurel GIURA	91
16. STUDIES ON THE INFLUENCE OF SEVERAL ABIOTIC FACTORS ON SOME NEWLY ISOLATED ANTAGONISTIC STRAINS - Mariana-Grațiela SOARE (VLADU), Caterina TOMULESCU, Liliana-Claudia BLASS, Narcisa BĂBEANU	96
17. IDENTIFICATION OF MAIN VOLATILE COMPOUNDS FROM THE MUST OF HYBRID GRAPES GROWN IN ROMANIA - Luminița Valerica VIȘAN, Ricuța Vasilica DOBRINOIU, Diana GROPOȘILĂ-CONSTANTINESCU, Silvana DĂNĂILĂ-GUIDEA, Radiana TAMBA-BEREHOIU	99
18. PHENOLIC CONTENT AND POTENTIAL INHIBITORY ACTIVITY OF ROMANIAN BEE POLLEN ON DIFFERENT PLANT PATHOGENIC STRAINS - Roxana SPULBER, Mariana-Grațiela VLADU, Ovidiu POPA, Narcisa BĂBEANU	104
19. EMERGING TECHNOLOGIES FOR MARA SEA BUCKTHORN (<i>Hippophae rhamnoides</i> L.) BERRIES VALORIFICATION - Cătălin STOIAN, Oana LIVADARIU, Mihaela TURTURICĂ, Nicoleta STANCIUC, Liliana MIHALCEA	109

BIOTECHNOLOGY IN VETERINARY MEDICINE

1. MICROBIOLOGICAL QUALITY CONTROL OF A NEW PLANTS MIX EXTRACT FOR VETERINARY USE - Tomina PURCARU, Camelia DIGUȚĂ, Florentina MATEI	115
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FOOD BIOTECHNOLOGY

1. EFFECT OF COLD STORAGE ON ANTIOXIDANTS FROM MINIMALLY PROCESSED HERBS - Giorgiana M. CĂTUNESCU, Ioan ROTAR, Roxana VIDICAN, Ancuța M. ROTAR	121
2. ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACTS FROM <i>Agrimonia eupatoria</i> L. AND <i>Epilobium hirsutum</i> L. HERBA - Alina Ioana NICU, Lucia PÎRVU, Adrian VAMANU	127
3. USING CENTRIFUGE AND ULTRAFILTRATION AS DOWNSTREAM PROCESSES FOR THE CONCENTRATION OF MICROBIAL β -MANNANASE FERMENTATION MEDIA - Ercan YATMAZ, Irfan TURHAN, Mustafa GERMEC, Ercan KARAHALIL	133
4. STATISTICAL MODEL FOR INDUSTRIAL IMPROVING OF WHEAT FLOURS WITH CALCIUM LACTATE - Radiana-Maria TAMBA-BEREHOIU, Ciprian-Nicolae POPA, Mira Oana TURTOI, Luminița Valerica VIȘAN	137
5. BINDERING AND THE STABILITY OF BETA CAROTEN FROM <i>Neurospora sitophila</i> - Jhondri JHONDRI, Abun ABUN, Denny RUSMANA, Rachmat WIRADIMADJA, Handi BURHANUDDI	143
6. INFLUENCE OF TECHNOLOGICAL FACTORS ON ACRYLAMIDE LEVEL FROM BISCUITS - Mioara NEGOIȚĂ, Adriana Laura MIHAI, Enuța IORGA	149
7. ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACTS MADE OF MUSHROOM MYCELIA DEVELOPED IN SUBMERGED CULTURE - Mihai Bogdan NICOLCIOIU, Gabriela POPA, Florentina MATEI	159
8. NUTRIENT COMPOSITION OF PARTIALLY DEFATTED MILK THISTLE SEEDS - Livia APOSTOL, Corneliu Sorin IORGA, Claudia MOȘOIU, Gabriel MUSTĂȚEA, Șerban CUCU	165

INDUSTRIAL AND ENVIRONMENTAL BIOTECHNOLOGY

1. INFLUENCE OF CULTURE CONDITIONS ON GROWTH OF KERATINOPHILIC FUNGAL STRAINS - Mariana CĂLIN, Iuliana RĂUT, Diana CONSTANTINESCU-ARUXANDEI, Mihaela BADEA DONI, Melania-Liliana ARSENE, Nicoleta-Olguța CORNELI, Gelu VASILESCU, Luiza JECU, Mariana Ștefania BUCUR, Veronica LAZĂR 173
2. EVALUATION OF *Trichoderma* spp. AS A BIOCONTROL AGENT AGAINST *Phytophthora parasitica* - Iuliana RĂUT, Florin OANCEA, Ana Belén López Santísima TRINIDAD, Mariana CĂLIN, Diana CONSTANTINESCU-ARUXANDEI, Mihaela BADEA DONI, Melania- Liliana ARSENE, Gelu VASILESCU, Tatiana Eugenia ȘESAN, Luiza JECU 179
3. PRELIMINARY STUDIES ON YEAST-PLANT SYSTEMS WITH APPLICATIONS IN PHYTOREMEDIATION - Ortansa CSUTAK, Alexandra SIMON-GRUIȚĂ, Viorica CORBU, Nicoleta CONSTANTIN, Daniela POJOGA, Tatiana VASSU, Georgiana DUȚĂ-CORNESCU 183
4. PRELIMINARY RESEARCH ON ENERGETIC CAPITALIZATION OF LIGNOCELLULOSIC MATERIALS IN FORM OF BIOETHANOL - Diana GROPOȘILĂ-CONSTANTINESCU, Gabriela MĂRGĂRIT, Radu-Cristian TOMA, Dana BARBA, Luminița VIȘAN, Marius HANGAN 190
5. ISOLATION OF FUNGAL MICROBIAL STRAINS FROM GIURGIU NORD TECHNOLOGICAL PARK WASTEWATER TREATMENT PLANT - Ovidiu IORDACHE, Iuliana DUMITRESCU, Floarea PRICOP, Elena VĂRZARU, Cornelia MITRAN, Andreea CHIVU, Steliana RODINO 194
6. POLYAROMATIC HYDROCARBONS UTILIZATION BY A *Pseudomonas* STRAIN - Mihaela Marilena STANCU 200
7. RESEARCHES CONCERNING THE ENZYMATIC ACTION OF BYPRODUCT GRAPES - Mihaela DUMITRU, Ștefana JURCOANE 206
8. SELECTION OF MICROALGAL STRAINS WITH LOW STARCH CONTENT AS POTENTIAL HIGH LIPID- CONTAINING ISOLATES - Ana Valentina ARDELEAN, Marinela CÎRNU, Ioan I. ARDELEAN 210
9. EFFICIENCY OF POWDER INOCULUM AND MICROORGANISM ENCAPSULATION ON HYDROLYZATE SUGAR FERMENTATION OF NEWSPAPER CELLULOSE FOR BIOETHANOL PRODUCTION - Ratu SAFITRI, Mia MIRANTI, Ismi Dwi ASTUTI, Lien KARTIAWATI, Jetty NURHAYATI 216
10. A REVIEW ON THE ENZYMATIC INDICATORS FOR MONITORING SOIL QUALITY - Maria-Mihaela MICUȚI, Liliana BĂDULESCU, Florentina ISRAEL-ROMING 223
11. SALT TOLERANCE OF BACTERIAL STRAINS ISOLATED FROM HYPERSALINE WATER LOCATED IN LOPĂTARI, ROMANIA - Irinel Gabriel PROCA, Florentina MATEI, Camelia Filofteia DIGUȚĂ, Ștefana JURCOANE 229
12. *Camelina sativa* OIL - A REVIEW - Alina-Loredana POPA, Ștefana JURCOANE, Brândușa DUMITRIU 233
13. PRODUCTION AND OPTIMIZATION OF EXTRACELLULAR AMYLASE FROM A NEWLY ISOLATED STRAIN OF *Bacillus mycoides* - Caterina TOMULESCU, Mișu MOSCOVICI, Bujor ALBU, Roxana STOICA, Claudia SEVCENCO, Delia JITEA, Radu TAMAIAN, Adrian VAMANU 239

FOOD SAFETY

1. CRITICAL REVIEW ON PROCESSING EFFECT ON NUTRITIONAL COMPOSITION OF FOOD PRODUCTS - Georgiana-Aurora ȘTEFĂNOIU, Elisabeta Elena POPA, Amalia Carmen MITELUȚ, Mona Elena POPA 249

2. MARKET RESEARCH REGARDING THE DEMANDS OF THE BUSINESS OPERATORS ON THE SUPPLY CHAIN LOGISTICS - Mihaela Cristina DRĂGHICI, Paul Alexandru POPESCU, Adina BAICU, Mona Elena POPA	260
3. OPTIMIZATION AND EVALUATION OF ELISA IMMUNOASSAY FOR MYCOTOXIN DETECTION OF BREAKFAST CEREALS - Irina SMEU, Elena Mirela CUCU, Alina Alexandra DOBRE, Enuța IORGA	265
4. CONTAMINATION OF POULTRY FEED BY POTENTIALLY TOXIGENIC FUNGI - Vesna KRNJAJA, Aleksandar STANOJKOVIĆ, Zorica BIJELIĆ, Violeta MANDIĆ, Miloš LUKIĆ, Zdenka ŠKRBIĆ, Veselin PETRIČEVIĆ	270
5. FUNCTIONAL PROPERTIES OF CAMEL MILK AND THEIR INFLUENCES ON TECHNOLOGICAL APPLICATIONS - Selda BULCA	275
6. SIMULTANEOUS DETECTION OF THREE FORBIDDEN ANIMALS (PORCINE, CANINE, AND RAT) IN HALAL FOOD BY USING HIGH RESOLUTION MELTING ANALYSIS - Anat DENYINGYHOT, Chirapiphat PHRAEPHAISARN, Mongkol VESARATCHAVEST, Winai DAHLAN, Suwimon KEERATIPIBUL	284
7. WHY HARMONIZE FOOD REGULATIONS AND WHAT IS NEEDED TO MAKE IT WORK? - Huub LELIEVELD	289

MISCELLANEOUS

1. PROFILE OF HIGH RISK WASTING FOOD CONSUMER IN ROMANIA - Sorin Corneliu IORGA, Livia APOSTOL, Nastasia BELC, Claudia Elena MOȘOIU, Lavinia Mariana BERCA, Oana Mihaela NICULAE, Mona Elena POPA	301
2. GM PLANTS AS BIOFATORIES OF PHARMACEUTICAL PROTEINS: PRESENT STATE AND FUTURE DEVELOPMENTS - Oscar VICENTE, Monica BOSCAIU	308
3. EUROPEAN FOOD LAW, YOUNG GOVERNANCE - Adina-Alexandra BAICU, Mona Elena POPA, Elisabeta Elena POPA, Amalia Carmen MITELUȚ	314
4. TOTAL PHENOLIC ANALYSIS, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF SOME MUSHROOM TINCTURES FROM MEDICINAL AND EDIBLE SPECIES, BY <i>IN VITRO</i> AND <i>IN VIVO</i> TESTS - Emanuel VAMANU, Alina VOICA	318
5. SELECTIVE FRACTIONS OBTAINED FROM PLANT SPECIES CULTIVATED IN ROMANIA WITH POTENTIAL EFFECT ON COUNTERACTING DISEASES ASSOCIATED WITH AGING PROCESSES - Svetlana COLCERU-MIHUL, Corina BUBUEANU, Ecaterina Anca ȘERBAN, Alice GRIGORE, Domnica RUGHINIS, Cristina BAZDOACĂ, Sultana NIȚĂ	325
6. EFFECTS OF ROSEHIP SEED FLOUR ON THE RHEOLOGICAL PROPERTIES OF BREAD DOUGH - Hülya GÜL, Hicran ŞEN	330
7. THE INFLUENCE OF ROSEHIP SEED FLOUR ON BREAD QUALITY - Hülya GÜL, Hicran ŞEN	336
8. HPTLC IDENTIFICATION OF BIOACTIVE COMPOUNDS AND ANTIOXIDANT ACTIVITY OF <i>Pleurotus ostreatus</i> AND <i>Lentinus edodes</i> EXTRACTS - Corina BUBUEANU, Alice GRIGORE, Ecaterina ȘERBAN, Gabriela POPA, Călina Petruța CORNEA	343
9. DRY AND FRESH HERBA OF <i>SATUREJA MONTANA</i> L.: A COMPARATIVE STUDY REGARDING CHEMICAL COMPOSITION AND ANTIOXIDANT CAPACITY OF VOLATILE OILS - Cristian MOISA, Lucian COPOLOVICI, Georgeta POP, Dana COPOLOVICI	349

AGRICULTURAL BIOTECHNOLOGY

EVALUATION OF THE GENETIC DIVERSITY OF *Pleurotus ostreatus* STRAINS USING COMBINATORIAL TUBULIN BASED POLYMORPHISM (cTBP) AND ISSR MARKERS

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Abstract

Pleurotus ostreatus is one of the most widely cultivated and popular edible fungi from the genus *Pleurotus*. In the last years, genetic variation of wild strains and cultivated varieties was investigated by use molecular markers and revealed a reduction of the diversity in commercial cultures. In this paper are presented the results obtained by application of combinatorial tubulin based polymorphism (cTBP) and inter simple sequence repeat (ISSR) markers, in order to investigate the genetic variability in several commercial strains of *P. ostreatus* collected from different geographical locations and their genetic relationships with wild isolates. Five polymorphic PCR products were obtained with primers designated for intron 1 of beta-tubulin gene, and 12 polymorphic amplicons were detected when primers for intron 2 of the same gene were used. Molecular analysis performed with ISSR primers displayed a polymorphism at intra specific level. The data achieved with the cTBP and ISSR markers revealed a reduced genetic differentiation among the commercial lines dispersed in Europe. However, comparing with commercial lines originated from USA, differences at molecular level were observed, suggesting a possible influence of environmental conditions. Regarding the wild strain of *P. ostreatus*, the molecular data indicated similarity with all European commercial lines. The results demonstrated that the applied molecular methods could be considered efficient tools to evaluate the genetic diversity in *Pleurotus ostreatus*.

Key words: genetic diversity, ISSR markers, *Pleurotus ostreatus* polymorphism, β -Tubulin, cTBP molecular markers.

INTRODUCTION

The genus *Pleurotus* (Jacq. Fr.) Kumm. (*Pleurotaceae*, *Basidiomycetes*) comprises a wide group of edible mushrooms with high nutritional value, therapeutic properties, and various environmental and biotechnological applications (Cohen et al., 2002).

Pleurotus is one of the most diverse groups among cultivated fungi with many taxonomic problems (Vilgalys et al., 1996).

In addition, the cultivated lines of *Pleurotus* may undergo a drastic loss of diversity resulting from human selection (Hamrick and Godt, 1989; Iracabal et al., 1995).

To clarify the taxonomic status of species in the genus *Pleurotus*, researchers started to classify these fungi also by genetic criteria (Bao et al., 2004).

In the last years, the interest in the genetic structure of natural populations has increased the necessity to expand the knowledge of genetic variation in cultivated species.

Currently, new approaches for assessing genetic variability of the wild and cultivated of *Pleurotus* strains represent an important goal of molecular genetics research.

Molecular approaches included techniques based on random amplified polymorphic DNA (RAPD) markers (Wang et al., 2003), amplified fragment length polymorphism (AFLP) markers (Pawlik et al., 2012), inter simple sequence repeat (ISSR) markers (Zhao et al., 2013) and they are currently used to analyse genetic variation in basidiomycetes.

ISSR markers were frequently used for the analysis of genetic variability and population structure in many types of organisms

(Kausserud and Schumacher, 2003; Wang et al., 2012). In 1999, Thon and Royse developed a set of primers for the β -tubulin genes which are coding for basidiomycete β -tubulin proteins and showed that these genes may have a great potential for phylogenetic studies of mushroom species. Tubulin-Based-Polymorphism (TBP) was introduced as a novel method for assaying genetic diversity of some plant species and varieties (Bardini et al, 2004), and is based on the analysis of amplification products resulted with primers directed to the first intron from the coding region of the β -tubulin gene family. The disadvantage of this method is the low number of molecular markers due to limited variation in the nucleotides sequence of this intron of β -tubulin gene family. To solve this inconvenient Breviaro et al (2007) proposed a new set of primers targeted to the second intron of the β -tubulin genes. The combined application of the primers for these introns caused increased number of the molecular markers and allowed a more reliable evaluation of relationships between species/varieties (Breviaro et al., 2007). No information about this approach was encountered for *Pleurotus* genetic analysis. In this respect, the aim of present study was to evaluate both cTBP and ISSR markers for the investigation of the genetic diversity in several commercial strains of *Pleurotus* collected from different geographical locations and their genetic relationship with natural isolates.

MATERIALS AND METHODS

Mushrooms samples. Seven *P. ostreatus* commercial lines collected from different geographic regions (Romania, Belgium, Germany and USA) and one *P. ostreatus* natural isolated were used for genetic diversity studies (Table 1). Mostly of the fungal material was kindly provided by Ph.D. Eng. Ioana Tudor and Eng. Paul Covic.

The stock cultures of fungal strains were maintained on PDA (potato dextrose agar) slants at 4°C.

DNA isolation. Mycelia developed on PDA medium in Petri dishes with cellophane were used for DNA extraction. The total DNA was extracted using a Plant Genomic DNA

Miniprep Kit (Sigma) according to the protocol specified by the manufacturer. The purity and quality of the genomic DNA were determined spectrophotometrically and by 0.8 % agarose gel electrophoresis. The DNA solution in TE buffer was stored at -20°C.

Table 1. Fungal strains used in experiments

No.	<i>P. ostreatus</i> strains	Source
1	<i>P. ostreatus</i> – wild isolate	Chitila forest
2	<i>P. ostreatus</i> var. Florida2125	Belgium
3	<i>P. ostreatus</i> var. Florida	Romania
4	<i>P. ostreatus</i> 1220	USA
5	<i>P. ostreatus</i> 50	Romania
6	<i>P. ostreatus</i> HK35	Belgium
7	<i>P. ostreatus</i> 375	Belgium
8	<i>P. ostreatus</i> 2191	Belgium

cTBP - PCR Amplification. Two pairs of primers for the introns of beta-tubulin gene proposed by Breviaro et al. (2007) were used for PCR amplification (Table 2).

Table 2. The sequences of primers used for cTBP-PCR amplification

TBP markers	Primers	Nucleotide sequence (5'-3')
Intron 1	TBP fex1	AACTGGGCBAAARGGNCAAYAYAC
	TBP rex1	ACCATRCAYTCRTCDGCRTTYTC
Intron 2	TBP fin2	GARAAYGCHGAYGARTGYATG
	TBP rin2	CRAAVCCBACCATGAARAARTG

PCRs were performed in a 20 μ l total volume which consisted of 1 \times PCR buffer (10 mM Tris.HCl (pH 8.8), 50 mM KCl, and 0.1% v/v Triton X-100), 0.2 mM each dNTP, 2.5 mM MgCl₂, 50 ng of template DNA, 1 μ M each primer, and 1 U *Taq* polymerase 360 (*AmpliTaq360DNA Polymerase-Promega*). All amplification reactions were performed in a thermal cycler (Eppendorf, Germany) with the following program: 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 40 s, and 72 °C for 90 s. The reactions were held at 15 °C after a final extension at 72 °C for 8 min (Bardini et al., 2004). Highlighting products was performed on agarose gel (1.5 %), staining with ethidium bromide and visualization under UV light using UVP BioDocIt system.

PCR Amplification with ISSR primers. For PCR amplifications were used six ISSR primers (Table 3).

Table 3. ISSR primers used for PCR amplification

ISSR Primers	Nucleotide sequence (5'-3')
HB15	(GTG) ₃ GC
17898A	(CA) ₇ -AC
17898B	(CA) ₇ -GT
UBC810	(GA) ₈ - T
UBC866	(CTC) ₆
UBC876	GAT AGA TAG ACA GAC A

The reactions for the amplification of the DNA were carried out in a thermocycler (Eppendorf) under the following conditions: 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 44 °C for 30 s, and 72 °C for 90 s. The DNA fragments were separated by 1.0 % agarose gel electrophoresis, in 1× TBE buffer. The gels were stained with ethidium bromide and examined using UVP BioDocIt system.

Data analysis. Amplicons generated by PCR amplification were scored as either present (1) or absent (0) across all accessions to build separate binary data matrices. The dendrograms were constructed with UPGMA cluster analysis using TREECON for windows software (version 1,3b). UPGMA cluster analysis was based on Nei and Li's (1979) genetic distance.

RESULTS AND DISCUSSIONS

cTBP analysis. The electrophoretic patterns of the DNA fragments obtained for both the first and the second intron of beta-tubulin gene from eight lines of *P. ostreatus* were analysed. Clear five polymorphic bands with 340 bp, 430 bp, 500 bp, 780 bp and 970 bp were detected for the first intron (Figure1).

Differences regarding the presence/absence of some amplicons were observed among the mushrooms varieties, both wild type (natural isolate) and commercial lines.

Among the European varieties the differences regarding the first intron were reduced. However, the electrophoretic pattern of the amplicons obtained from USA variety presented some differences: few supplementary amplicons were scored.

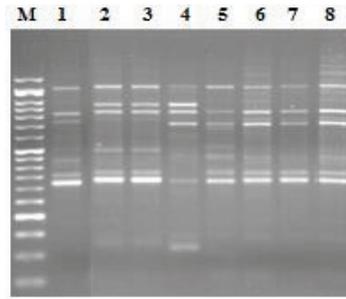


Figure 1. Electrophoretic pattern of amplicons generated with primers for the first intron of β -tubulin gene

Legend:

M = Molecular marker standard (GeneDirex 50 bp); 1 = *P. ostreatus* (Romania - Chitila forest); 2 = *P. ostreatus* var. Florida 2125 (Belgium); 3 = *P. ostreatus* var. Florida (Romania); 4 = *P. ostreatus* 1220 (USA); 5 = *P. ostreatus* 50 (Romania); 6 = *P. ostreatus* HK 35 (Belgium); 7 = *P. ostreatus* 375 (Belgium); 8 = *P. ostreatus* 2191 (Belgium).

The confirmation of the molecular variability among *P. ostreatus* varieties at beta-tubulin gene was performed by analysing the electrophoretic patterns of the amplicons generated with primers for the second intron of this gene. Clear 12 polymorphic bands were evaluated for this intron: 370 bp, 550 bp, 600 bp, 740 bp, 875 bp, 900 bp, 950 bp, 1000 bp, 1100 bp, 1200 bp and two bands > 1500bp (Figure 2).

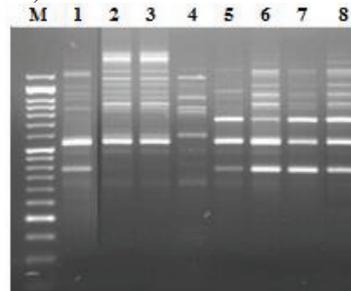


Figure 2. Electrophoretic pattern of amplicons generated with primers for the second intron of β -tubulin gene (Please repeat the legend)

The results obtained with the primers for the second intron were similar with those generated with the primers for the first intron: all the commercial European varieties were similar and comparable with the Romanian natural isolate. Variations were observed among *P. ostreatus* var. Florida, *P. ostreatus* from USA and European commercial/wild type lines.

These data suggest a possible influence of environmental conditions on the molecular variability in *P. ostreatus*. The reduced genetic variability among the commercial lines could be explained by the successive breeding cycles applied for mushrooms in order to increase the productivity or for other characteristics. In contrast, the molecular polymorphism observed at beta-tubulin gene level of the natural isolates of *P. ostreatus* suggests new sources for valuable genes, useful in commercial approaches.

Cluster analysis. The genomic relationships between the studied *Pleurotus* strains based on the data obtained from cTBP - PCR amplification with intron 1 and 2 primers are presented in the dendrogram constructed with an UPGMA cluster analysis (Nei and Li's, 1979)(fig. 3). Two main clusters comprising eight *Pleurotus* strains were obtained. In the first cluster are included five strains of *P. ostreatus*: three strains originated from Belgium (*P. ostreatus* HK 35, *P. ostreatus* 375, and *P. ostreatus* 2191) that were similar at beta-tubulin amplicons profiles level, and two other varieties, *P. ostreatus* wild strain and *P. ostreatus* 50 commercial strain. The second cluster included *P. ostreatus* 1220 strain (USA) and two cultivated strains of *P. ostreatus* var. Florida, one from Belgium and another from Romania. The higher genetic similarity between the commercial strains *P. ostreatus* var. Florida 2125 from Belgium and *P. ostreatus* var. Florida from Romania suggests the possibility that these varieties are identical and have the same origin (Figure 3).

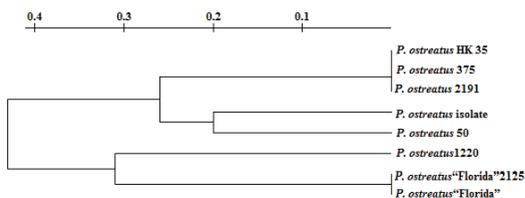


Figure 3. UPGMA dendrogram of genetic relationships among *Pleurotus* strains based on intron1 and intron2 polymorphisms

Analysis of genetic distance between the eight *P. ostreatus* varieties showed a lower variation between the European varieties: 0.44 (*P. ostreatus* natural isolate vs. *P. ostreatus* var.

Florida), 0.30 (*P. ostreatus* natural isolate vs. *P. ostreatus* 50) and 0.27 (Romanian natural isolate vs. Belgian *P. ostreatus*). However differences at molecular level were found between Romanian *P. ostreatus* wild strain and USA originated *P. ostreatus* 1220 (0.58).

The combinatorial version of original TBP method has increased the possibility of detection the variability by addition of the second intron present within the vast majority of beta-tubulin genes (Breviario et al, 2007). Preliminary experiments performed on different plant species such as bean (*Phaseolus vulgaris*), tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*) and rice (*Oryza sativa*) confirmed that the second intron of the β -tubulin genes also could be a reliable source for DNA polymorphism, and could be used for studies in more plant or even mushroom species. The multifunctional and essential role of the β -tubulin proteins is the conservation of the regions with the main sequence of amino acids from a polypeptide chain. This data are relevant in the studies on the evolution of the genes for β -tubulin and for investigation of the relationships between basidiomycete species (Begerow et al., 2004).

ISSR-PCR amplification. Six ISSR primers (HB15, 17898A, 17899B, UBC 810, UBC 866, and UBC 876) were used for detection molecular polymorphism among *P. ostreatus* varieties. Molecular analysis performed with HB15 primer displayed a polymorphism at intraspecific level: distinctive PCR product of 850 bp was observed at *P. ostreatus* wild strain and *P. ostreatus* 50 commercial strain (fig.4). PCR amplification with UBC866 primer highlighted a genetic polymorphism for *P. ostreatus* 1220 (USA), *P. ostreatus* 50 (Romania), and *P. ostreatus* 2191 (Belgium) (fig.4). The amplicons obtained with 17898A, 17899B, UBC810 and UBC876 ISSR primers also displayed an intraspecific polymorphism in the group of *P. ostreatus* var. "Florida" 2125 (Belgium), *P. ostreatus* var. "Florida" (Romania), *P. ostreatus* 1220 (USA) and *P. ostreatus* 50 (Romania and the wild strain of *P. ostreatus* (Figure 4).

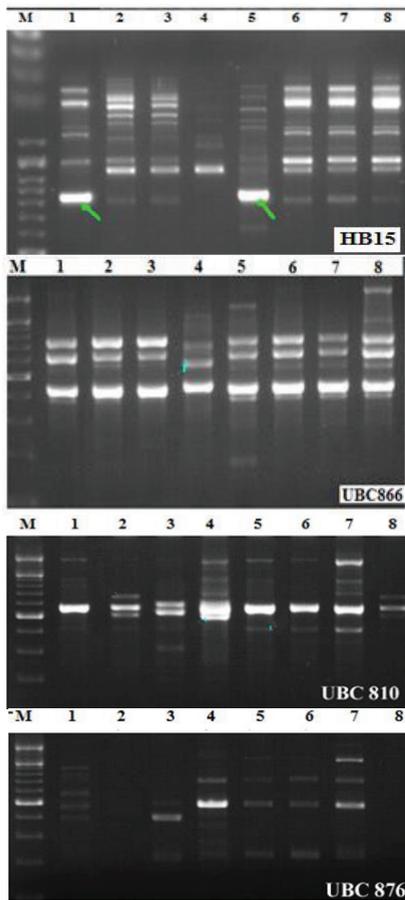


Figure 4. PCR amplification profiles of ISSR primers
 Legend:
 M = Molecular marker standard (GeneDirex 50 bp); 1 = *P. ostreatus* (Romania - Chitila forest); 2 = *P. ostreatus* var. Florida 2125 (Belgium); 3 = *P. ostreatus* var. Florida (Romania); 4 = *P. ostreatus* 1220 (USA); 5 = *P. ostreatus* 50 (Romania); 6 = *P. ostreatus* HK 35 (Belgium); 7 = *P. ostreatus* 375 (Belgium); 8 = *P. ostreatus* 2191 (Belgium).

Similar intraspecific polymorphism was observed by other authors: according to Santos Araújo et al. (2016) the difference in the profile of amplification of the primers is related to the kind of DNA sampled. This is possible because using DNA samples in bulk, alleles with low frequency in the population cannot be amplified (Yanaka et al. 2005).

In our studies the data obtained with the ISSR markers revealed a lower genetic differentiation among mushroom varieties dispersed in Europe and a higher genetic diversity between

European varieties and American ones. Based on such observations, specialists consider that mushroom populations must be divided into groups according to their geographical origins which indicate that the genetic diversity is closely related to the geographical distribution (Zhao et al., 2013).

CONCLUSIONS

In order to investigate the genetic diversity in *P. ostreatus* commercial and natural varieties collected from different geographical locations, cTBP and ISSR markers were used. The results revealed a polymorphism at intraspecific level. Molecular analysis of *P. ostreatus* strains both with primers directed to beta-tubulin gene or ISSR primers showed molecular differences according to their geographical origins. Among the European *P. ostreatus* varieties the genetic variation is reduced. On the contrary, higher genetic diversity was found between European mushroom varieties versus *P. ostreatus* 1220 strain originated from USA. However, the wild isolate of *P. ostreatus* shows similarity with the European commercial strains.

The results obtained allowed the conclusion that the markers generated by cTBP and ISSR primers are very useful to detect the genetic polymorphism among varieties of *P. ostreatus*. According to our knowledge it is the first study concerning the use of cTBP and ISSR markers to investigate the genetic diversity in *Pleurotus ostreatus*.

ACKNOWLEDGEMENTS

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STUDY ON THE ADAPTABILITY OF GIANT BAMBOO UNDER SOIL-CLIMATIC CONDITIONS SPECIFIC TO ROMANIA

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Abstract

Giant bamboo, acknowledged as a specific culture of China than Europe, was introduced in Romania by the Italian Consortium of Bamboo, Only Moso International, in order to popularize this plant among farmers in the EU. Cultivated at this time only for decorative purposes, bamboo is a real industry in Asia, where it is recovered almost entirely: trunk, root, rhizome, the leaves and buds are really hunted for Asian restaurants.

Year 2014 represented the debut year in the cultivation of bamboo in Europe, with the first plantation in Italy, where currently there are 1.400 hectares planted.

Phyllostachy pubescens specie is a giant perennial graminaceae, ages 80-100 years and height of stems from 14 to 25 meters, with a diameter of 8 to 15 cm. It can adapt easily in temperate climates, provided precisely to achieve specific technological link, this plant can resist in areas where winter minimum temperatures do not fall below -25°C.

In Romania, giant bamboo get interest already for many farmers, especially those of researchers within the UASVM Bucharest, where he founded an experimental plantation in order to test the adaptability of species to the specific soil-climatic conditions.

As a result of the observations and determinations made on giant bamboo plants in various stages of vegetation, we can say with certainty that that specie has adapted surprisingly well to conditions specific to Romania, successfully resisting the extreme lows that have exceeded the values of -23°C, recorded during winter 2016.

In these conditions, the cultivation of giant bamboo may represent a new opportunity for Romanian agriculture and at the same time a new paradigm for business.

Key words: giant bamboo, rhizomes, sprouts, eco-friendly, vegetarians, investment.

INTRODUCTION

Bamboo wood is an excellent raw material for a wide range of products, its multiple and varied uses, from this plant being valorized all vegetative organs: bamboo shoots, highly appreciated due to its nutritional value and which have multiple uses in cosmetics, bamboo and wood can be used to heat homes or as constructive material (Bello and Espilo, 1995; Dransfield and Widjaja (eds.), 1995).

Bamboo can be drawn and can also be used for fiber and clothing as well as for the manufacture of beverages.

While a commercial plantation of wood needs 20-30 years to produce high-quality timber, a bamboo plantation timber provides clearly superior quality in a much shorter period of

time, without such an effort and can produce between 50 and 70 tons of wood per year.

Bamboo not only has many uses in terms of capitalizing on the timbers, buds and Rhizome, but this plant brings many benefits to the environment by reducing emissions of greenhouse gases, generating an annual significant quantity of oxygen.

Among more than 1.200 species and 80 genera of bamboo grown in China, there has been selected the species *Phyllostachys pubescens* due to its qualitative characteristics, productive, and his plasticity (Gonzales and Umali, 1995), the latter making possible the cultivation ways in various soil-climatic zones without the risk of damaging the culture.

Development and implementation of cultivation technologies for giant bamboo through a sustainable management and the use

of raw materials resources derived from this species, can stimulate economic development in rural areas, with the foundations for the growth of living standards in deprived communities, through the creation of new jobs in rural and suburban areas.

MATERIAL AND METHOD

In pursuing the degree of its adaptability to the soil-climatic conditions specific to Romania, an experiment was mounted within the Experimental Teaching and the University of Agronomical Sciences and Veterinary Medicine of Bucharest, during which comments were made and biometric measurements concerning the growth and morphological development of bamboo plants belonging to the species *Phyllostachys pubescens*.

Culture was established in April 2016 by planting seedlings in rows at an equidistant area of 2.88 m, thus ensuring a culture density of 1.200 plants/ha.

Before running the basic soil (plough), was conducted on the base of soil fertilization, using for this purpose the manure well fermented, EC was administered at a dose of 20 tonnes/ha, by uniform soil surface scattering and subsequent incorporation with making the plough.

Technological itinerary began carrying out the work of tilling to a depth of 25-30 cm, followed by disking with a paper GD 3,2, at 10-12 cm deep, perpendicular to the direction of the plough.

Upon completion of the disking work followed by picketing the land operation, operation that aimed to determine the place which they will handle future giant bamboo plants.

For the purpose of planting seedlings were dug holes for planting, the sizes of 20/20 cm, work that was carried out in the growing day.

After planting was mounted drip irrigation system, which is absolutely necessary, at least during the first 2 years after planting knew that this species is susceptible to hydric deficit in the early years after planting, when the root system is not sufficiently well developed, the plant not being well anchored in the soil, so as to be able to take over from reserve of soil total amount of water it needs.

An important work carried out immediately after planting was the land humectation, mulch layer being made of chopped wheat straw the size of 10 cm, which are placed on the rows of plants, in a thick layer of 15 cm. Through the operation of soil humectation was achieved both weed whose growth was inhibited but keeping the ground water reserve due to avoid rapid evaporation at the surface of the soil.

The humectation operation of the soil had a direct impact on the growth of bamboo plants resistance to hydric stress conditions, encountered frequently in Romania, especially in the months of July-August.

Throughout the growing season of the crop were carried out observations and biometrical measurements plants morphological development, during which the number of stems on the plant, the number of parent shoots trained during the whole period of vegetation, their height, diameter of stems and the degree of branching.

Experimental results obtained were centralized in synthetic tables, analysed and interpreted in terms of dynamic analysis method, during which the degree of adaptability of the species in condition soil-climatic conditions specific to the Romanian Plain.

RESULTS AND DISCUSSION

Analyzing the number of stems formed on the parent plant, we note that this biometric parameter recorded average values between 7 and 19 stems/plant, number of stems per plant growing parent formats with advancing plant vegetation of giant bamboo.

Thus, if at the time of planting seedlings number of stems on the plant was 7 stems/plant, calculations performed after 3 months from the moment of planting have highlighted the fact that their number has increased, reaching 11 stems/plant.

With the advancement in the growing season, plants showed a strong capacity for differentiation of main stems, the average number of stems formed reaching 15 stems/plant, 6 months at the time of planting, 18 stems/plant, after 9 months of planting, in the early spring, the average values of this parameter have been 19 stems/plant.

This phenomenon can be put on genetic species which once rooted manifests a fantastic capacity of differentiation of new stems, but a pace emphasized growth and development. Note so that the process of differentiation and growth of principal stems and continued as long as during low temperatures were recorded

so that, in the interval between the entrance and the exit of winter was more differentiated average 1 stem/plant.

We can thus conclude that the plant continues its morphological processes as long as temperatures remain positive though low, however.

Table 1. Number of stems per plant and height of stems

TIME OF DETERMINATIONS	STEMS/PLANT		DIF. (No.)	HIGH OF STEMS		DIF. (cm)
	(No.)	(%)		(cm)	(%)	
Time of planting (Control)	7	100.0	Control	27.3	100.0	Control
3 months after planting	11	157.1	4.0	45.4	166.3	18.1
6 months after planting	15	214.3	8.0	46.8	171.4	19.5
9 months after planting	18	257.1	11.0	59.6	218.3	32.3
12 months after planting	19	271.4	12.0	68.3	250.2	41.0

Average height of stems present on parent plant has been an upward curve, this biometric indicator recording averages of 27.3 cm at the time of planting, 45.5 cm, at 3 months after planting, 46.8 cm, at 6 months of vegetation, 59.6 cm at 9 months after planting, maximum heights of stems being recorded at the conclusion of a cycle it's vegetation,

respectively at 12 months after the time of planting. It can be seen that, as bamboo plants is advancing, isolates had an accelerated growth rate, increasing their height by more than 10 cm per month, the differences recorded versus time of planting, took control of the experience, being covered by 18.1 and 41.0 cm.

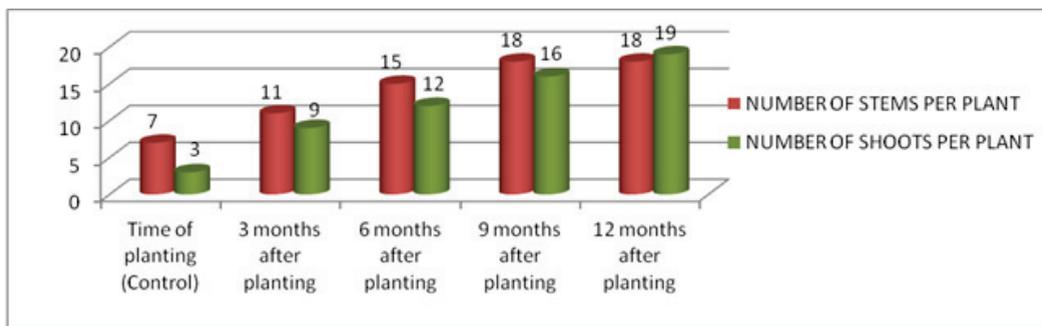


Figure 1. Number of stems and shoots per plant

We can thus conclude that with the parent plant, rootedness the root system develops powerful herb lashed into the ground very well and are thus able to explore an increasingly large volume higher than the ground, so that the plants are able to extract nutrients essential for differentiation of stems and their growth and development.

The root system of bamboo plants represented by the rhizomes (underground stems), which penetrate into the soil up to a depth of 40 cm, and monopodial growing characteristics of the species due to increased branching, the species ability to give rise to new shoots, respectively new plants forming their own roots and differencing its self being able to synthesize single feed (Ramoran et al., 1993).

Table 2. Number of the shoots per plant and height of the shoots

TIME OF DETERMINATIONS	SHOOTS/PLANT		DIF.	HIGH OF SHOOTS		DIF.
	(No.)	(%)	(No.)	(cm)	(%)	(cm)
Time of planting (Control)	3	100.0	Control	7.4	100.0	Control
3 months after planting	9	300.0	6.0	35.6	481.1	28.2
6 months after planting	12	400.0	9.0	50.9	687.8	36.0
9 months after planting	16	533.3	13.0	52.3	706.8	44.9
12 months after planting	19	633.3	16.0	67.0	905.4	59.6

Trained in these shoots during the period of vegetation in turn recorded accelerated growths, their stems growing in height with a few cm per day.

Analyzing the number and height of shoots mother plants trained on during the period of vegetation is observed that the two biometric parameters recorded a upward curve, with the advancement in bamboo plant vegetation.

Thus, if at the time of planting were present on the parent plants, on average 3 shoots/plant, after the 3 months from the moment of planting their number has tripled, reaching 9 shoots/plant, arriving after a cycle of vegetation

at 19 shoots/plant, which once again confirms the strong offspring capacity of this species.

The results are spectacular when considering the average height of shoots in various phenostages of vegetation.

Note so that if you are making a comparison with the control group (planting) when trained on the mother plant shoots have reached heights of 7.4 cm environments, subsequently increases are becoming more intense, 35.6 cm respectively in 3 months from the moment of planting, 50.9 cm at 6 months, 9 months 52.3 cm and 67.0 cm 12 months after planting These offshoots reaching and even exceeding the present values of main stems on the plants.

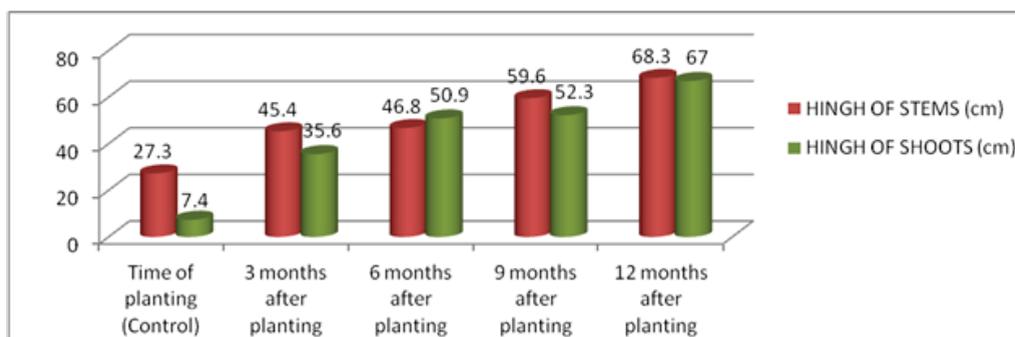


Figure 2. High of stems and shoots

In the first year of vegetation, the diameter of the main stems present on the parental plants has been recorded values between 0.1 and 0.3 mm, minimum values being recorded at the time of planting (version control), and highs after 9 and 12 month block area at the time of the establishment of the plantation.

Analyzing the average number of branches formed on the parental plant, it becomes

apparent that if at the time of planting were differentiated 2 branchers/plant, after 3 months from the time of planting, their number has risen to 5, after 6 months number of branches differentiated per plant was 6, and when temperatures decreasing, plants have slowed the pace of growth, the number of branchers present on the parental plants was 6.5 branchers/plant.

Table 3. Diameter of stems and branches on the stem

TIME OF DETERMINATIONS	STEMS DIAMETER		DIF.	BRANCHERS PER STEM		DIF.
	(mm)	(%)		(No.)	(%)	
Time of planting (Control)	0.1	100.0	Control	2.0	100.0	Control
3 months after planting	0.2	200.0	0.1	5.0	250.0	3.0
6 months after planting	0.2	200.0	0.1	6.0	300.0	4.0
9 months after planting	0.3	300.0	0.2	6.5	325.0	4.5
12 months after planting	0.3	300.0	0.2	6.5	325.0	4.5

The diameter of the shoots had registered lower values compared to the diameter of the main stems, this indicator having values between 0.1

and 0.2 mm, maximum values being recorded after 9 and 12 months from the time of planting.

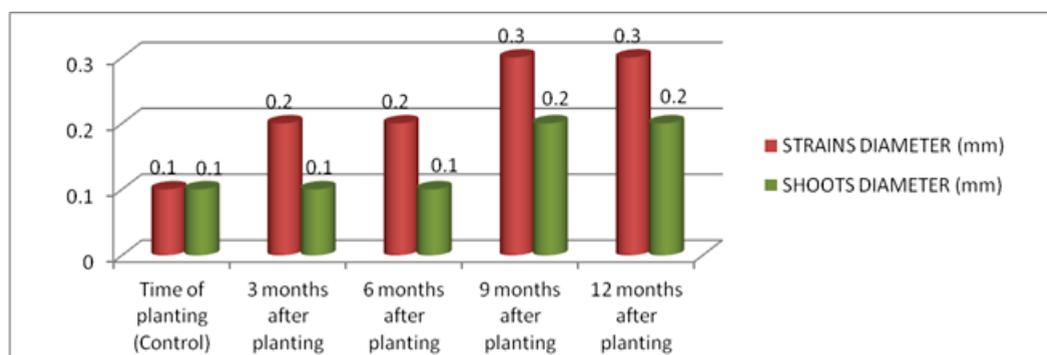


Figure 3. Diameter of stems and shoots

Table 4. Diameter of shoots and branches on the shoot

TIME OF DETERMINATIONS	SHOOTS DIAMETER		DIF.	BRANCHERS PER SHOOT		DIF.
	(mm)	(%)		(No.)	(%)	
Time of planting (Control)	0.1	100.0	Control	1.5	100.0	Control
3 months after planting	0.1	100.0	0.0	5.5	366.7	4.0
6 months after planting	0.1	100.0	0.0	6.0	400.0	4.5
9 months after planting	0.2	200.0	0.1	6.5	433.3	5.0
12 months after planting	0.2	200.0	0.1	8.0	533.3	6.5

In terms of the number of branches formed during the period of vegetation on the young shoots bands during the same period, notes that this biometric parameter recorded values between 1.5 and 8.0 branchers/shoot, this issue highlights once again the differentiation of secondary branching out and the strongly growth of that specie.

Based on the observations and determinations carried out we can say with certainty not to err, that specie *Phyllostachys pubescens* has been successfully adapted to the soil and climate conditions specific to Romania, despite to the extreme minimum temperatures over -23°C , recorded in the winter of 2016 and may represent a new opportunity for farmers in the context of a sustainable rural development.

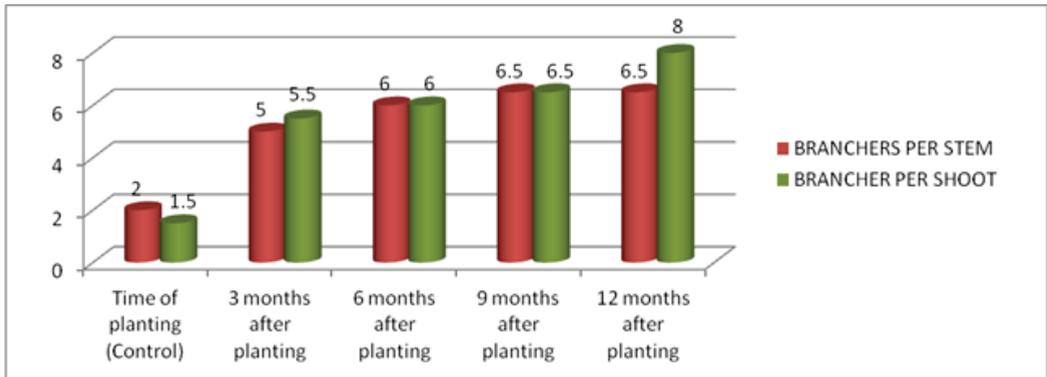


Figure 4. Number of branchers per stem and shoot

CONCLUSIONS

Bamboo giant leverages very well degraded land, considered unsuitable for other species of crop cultivation.

Increase and developing very well, provided the optimum planting times (early spring, March-April).

Mulching of the soil provide resistance in the hydric stress conditions, thus avoiding loss of water as a result of evaporation from soil surface thereof.

Does not require additional maintenance works apart from irrigation and weed control, just in the first years after planting.

Do not submit specific pests and diseases, as a result unnecessary expenditure on plant-protection products to combat them.

After installing culture in the field (rooting) species has large capacity for diseases, starting from the shoots nodes present on the underground stems (rhizomes).

It can be a successful business for farmers in the area of soil-climatic from Romania, provided to ensure water demand, especially in the first two years after plantation establishment

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SELECTION AND CHARACTERIZATION OF NEW ENDOPHYTIC BACTERIAL STRAINS ISOLATED FROM POTATO TUBER USEFUL IN BIOCONTROL STRATEGIES

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Abstract

*Endophytic bacteria are plant-associated bacteria colonizing the internal plant tissue, living in symbiotic association with their host. Such microorganism could contribute to plant growth promotion and defence against biotic and abiotic stress. Our study aimed to describe new endophytic bacteria from potato tubers. Therefore, a group of 20 endophytic bacteria was isolated from seven Romanian varieties of healthy potato tubers. Four of the isolated strains revealed antifungal activity against three important pathogens of potato, *Fusarium solani* involved in tubers dry rot, *Rhizoctonia solani* involved in stem canker and black scurf of potato, and *Alternaria solani* causing early blight of potato plants. Among all twenty isolates obtained, 13 were Gram positive bacteria. Most of the newly isolated endophytes (65%) expressed phosphatase and protease activity, and 55% presented amylases, however only 10% revealed cellulose degrading enzymes. Based on preliminary laboratory analysis, the isolate 6T4 identified as *B. atrophaeus/subtilis* revealed promising perspectives for biocontrol strategies.*

Key words: bacterial endophytes, antifungal activity.

INTRODUCTION

The environmental friendly approaches of plant growth promotion and plant protection, sustain the use of microorganisms as an alternative to chemicals. The most studied plant beneficial microorganisms are the rhizobacteria, but in the meantime the microbial endophytes gained a special attention, due to their understudied benefits for their hosts, including plant defence against biotic and abiotic stress. The particular interest for endophytes, is due to their attributes regarding plant growth promotion (Abbamondi et al., 2016; Liaqat and Eltem, 2016), and plant defence against biotic and abiotic stress (Choudhary and Johri, 2009; Rania et al., 2016; Akbari et al., 2016).

This study was focused on the endophytic bacteria from Romanian potato tubers, as potato culture is an important crop for Romanian agriculture. The aim of the study was to describe new endophytic strains with plant beneficial attributes.

MATERIALS AND METHODS

Isolation of bacterial endophytes

Seven varieties of potato tubers were used for bacterial endophytes isolation: Tâmpa 5, Zamolxis 5, Kronstad 5, Rustic 5, Christian 5, Cumidava 5 and Roclas 5, kindly provided by INCDCSZ Braşov (Romania). In order to remove the adhering soil particles and decrease the microbial load of epiphytic microorganisms, the potato tubers were vigorously washed with NaOCl 0.4% based detergent and rinsed with tap water (Anjum and Chandra, 2015). For surface disinfection they were immersed in 70% ethanol and flamed (Zinniel et al., 2002).

Using aseptic procedures the tubers were sliced with a sterile scalpel in laminar air flow cabinet and approximately 1 cm³ of potato pulp was harvested and crushed in sterile phosphate saline buffer using a sterile mortar and pestle. After 15 minutes of diffusion, 100 µl of homogenate was plated on Luria Bertani (LB) agar and incubated at 28±0.5°C in order to

recover bacterial endophytes. Bacterial cultures were observed after 18, 24 and 40h of incubation, however morphologically different bacterial colonies were selected only after 48h of growth. To achieve pure bacterial isolates the selected bacteria were subcultured on the same solid medium. All selected isolates were stored in glycerol at -80°C. Before use the bacterial isolates were two times cultured in LB agar and, if necessary, maintained at 4°C till further used.

Characterization of bacterial isolates

Endophytic bacterial isolates were characterized based on colony morphology, Gram reaction, swimming and swarming motility, King B fluorescent growth in UV light, enzymes production (amylase, cellulase, protease, and phosphatase) and Voges-Proskauer reaction.

Antifungal activity evaluation

Isolated bacterial endophytes were analyzed for their antifungal activity against three fungal

pathogens of stored potato tubers (*Fusarium solani*) or potato plants (*Rhizoctonia solani* and *Alternaria sp.*). The test was performed *in vitro*, on PDA medium, by dual culture technique (Soria et al., 2012).

Bacterial identification

Four of the studied endophytic bacterial isolates were identified using the Biolog GEN III technique according to the manufacturer protocol.

The identification was made using the semi-automatic Biolog Microbial Identification System, by analyzing 71 carbon source utilization capacity and 23 chemical sensitivity assays.

RESULTS AND DISCUSSIONS

Endophytic bacteria

All seven varieties of potato tubers hosted endophytic bacteria, from which twenty isolates were obtained (table 1). Most of them (13 isolates) are Gram positive bacteria.

Table 1. Endophyte bacterial isolates

Bacterial isolate	Hosting potato variety	Gram reaction	Motility		Enzymes production				VP reaction
			swimming	Swarming	phosphatase	amylase	cellulase	protease	
1T1	Tâmpa 5	-	±	-	+	-	-	-	+
1T2		+	+	+	+	-	+	+	+
1T3		+	-	-	±	+	-	+	-
2T1	Zamolxis 5	-	-	-	±	+	-	+	-
2T2		+	-	-	+	+	-	+	-
3T1	Kronstad 5	+	-	-	+	+	-	+	-
3T2		+	-	-	±	+	-	+	-
3T3		-	-	-	+	+	-	+	-
4T1	Rustic 5	+	-	-	+	+	-	+	-
4T2		+	-	-	-	-	-	-	-
4T3		+	±	-	+	-	-	-	-
5T1	Christian 5	+	-	-	-	+	-	+	-
5T2		+	-	-	-	-	-	-	-
6T1	Cumidava 5	-	+	+	+	-	-	+	+
6T2		-	+	+	-	-	-	-	-
6T3		+	-	-	+	+	-	+	-
6T4		+	+	+	+	+	+	+	-
7T1	Roclas 5	-	+	-	-	-	-	-	-
7T2		-	-	-	-	+	-	+	-
7T3		+	-	-	-	-	-	-	-

One of the isolated strains 6T1 was remarked for its orange pigmentation, abundantly synthesized and diffused on various nutritional substrates. This strain revealed swimming and swarming motility, protease activity, and

positive VP reaction. Another strain, 6T4 was also highly distinctive among the other isolates especially due to its rapid colonization of soaked agar media. This Gram positive strain also presented swimming and swarming

motility on soft agar plates, and produced various hydrolytic enzymes such as amylase, cellulose, protease and phosphatase.

The bacterial strain 6T2 was the only one producing fluorescent pigment on King B medium, exposed in UV light.

This strain was not able to express any of the studied enzymes, was negative to VP and Gram reactions, but revealed very good swimming and swarming motility.

Among phosphate solubilizing bacteria, 1T1 isolate was the first initializing the hydrolysis, but 1T2 isolate expressed the largest and most clear zone of phosphate solubilization of all isolates (3mm).

Antifungal potential

The antifungal properties of the endophytic strains was analyzed *in vitro* against three important pathogens of potato: *Fusarium solani*, involved in tubers dry rot, *Rhizoctonia solani* involved in stem canker and black scurf of potato, and *Alternaria* spp. causing early blight of potato plants. Among all twenty selected isolates only 1T2, 6T1, 6T2 and 6T4 expressed inhibitory activity against the mentioned fungi.

The 6T1 and 6T4 strains were active against all three pathogenic fungi. However, 1T2 inhibited only *Rh.solani* and 6T2 reduced *Alternaria* sp. and *Rh.solani* growth.

Analyzing the microbial interactions, it was noticed that 6T1 maintained clear inhibiting zones, restraining each fungal growth at minimum 5mm (*F.solani* and *Rh.solani*), or 2mm distance (*Alternaria* sp.) from the bacterial colony edge, indicating diffusible antifungal compounds (figure 1).



Figure 1. Bacterial interaction of 6T1 strain with *Fusarium solani* hyphae reveal clear inhibition zone of the fungal growth.

Analyzing the interactions of 6T1 strain under the optical light microscope alterations in the morphology of the fungal hyphae was observed. The main alterations of *Alternaria* sp. growth consisted of curly growth of hyphae and irregular cells formations (figure 2).



Figure 2. Bacterial interaction of 6T1 strain with *Alternaria* sp. hyphae observed under the optical light microscope. Details illustrate irregular cells formations in the presence of 6T1 bacterial exudates.

Curly growth of the mycelia was also seen on *F.solani* towards the 6T1 colony (figure 3).

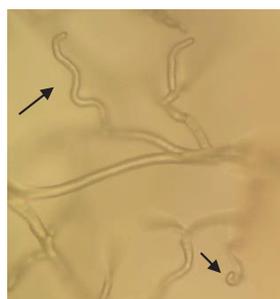


Figure 3. Curly growth of *Rh.solani* hyphae in the presence of 1T2 bacterial strain

On *Rh.solani*, the main alterations consisted of an increased number of vacuoles in the fungal cell, increased vacuole size, disruption of the tonoplast membrane or cytoplasmic coagulation within the hyphae (figure 4). Similar changes of cytoplasmic coagulation were also reported on eugenol treated fungal cultures of *Botrytis cinerea* (Wang et al., 2010). Moreover, fungal growth alterations with increased number of vacuoles in the fungal cell, and increased vacuole size were also reported in *Rosellinia necatrix* exposed to the biocontrol bacteria *Pseudomonas chlororaphis* PCL1606 (Calderón et al., 2014).

Rh.solani fungal growth was visible affected also by the presence of 6T4 bacterial strain. The

main alterations of fungal growth consisted of an increased number of vacuoles present within the hyphae; and an increase thickness of part of the mycelia (figure 5). Similar symptoms were

also described by Giorgio et al. (2015) in *Sclerotinia sclerotiorum* exposed to *Bacillus* spp. USB2103 strain, and by Calderón et al. (2014) in other microbial interactions.

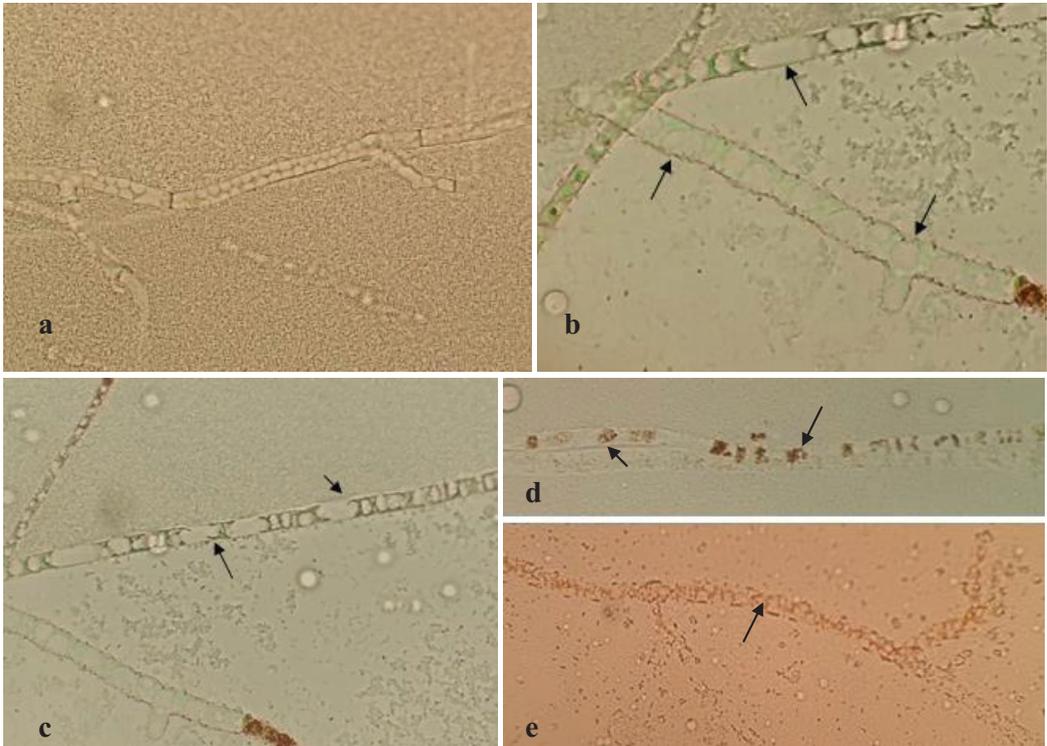


Figure 4. Bacterial interaction of 6T1 strain with *Rhizoctonia solani* hyphae observed under the optical light microscope. Details in a) illustrate an increased number of vacuoles in the fungal hyphae due to 6T1 bacterial exudate. The black arrows in picture b) indicate some of the vacuoles with an increased size. In picture c) there are indicated several internal disruptions in the fungal cells. Cytoplasmic coagulation within the hyphae is illustrated in d) and e) images revealing severely affected fungal hyphae due to bacterial exposure.

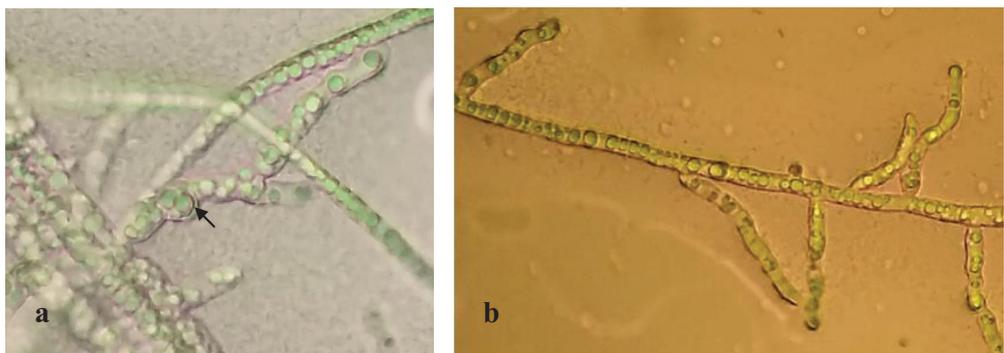


Figure 5. Bacterial interaction of 6T4 strain with *Fusarium solani* hyphae observed under the optical light microscope. Details in a) illustrate clear inhibition zone of the fungal growth. Details in b) reveal an increase number of vacuoles in the fungal hyphae due to 6T1 bacterial exudate exposure.

The fungal vacuoles are dynamic organelles which undergo extensive expansion and remodeling during morphogenetic transitions and moreover, they are involved in several cellular functions, contributing to the cellular homeostasis and storage of irons and molecules (Richards et al., 2012), and also act as a recycling centre for worn-out organelles and macromolecules especially under nutrient starvation (Raven et al., 1999; Klionsky et al., 1990). Therefore, the increased number of vacuoles in the fungal cells or their increased volume could be a correlated to a fungal stress reaction, which might be generated by the antifungal metabolites released by the endophytic bacterial cells which repress mycelia growth and fungal access to the nutritional substrate. Several studies describing similar perturbations of the fungal growth correlated these disturbances with the presence of different antifungal metabolites such as 2-hexyl,5-propyl resorcinol produced by *Ps. chlororaphys* (Calderón et al., 2014) and various volatile organic compounds produced by *Pseudomonas* spp. and *Bacillus* spp. strains (Giorgio et al., 2015). Based on these data it could be assumed that the modifications detected in the new bacterial isolates from potato are related to similar compounds.

Identification

Five of the newly isolated endophytic bacteria were selected for Biolog GEN III identification, 1T1 due to its rapid activation of phosphatase; 1T2 for its high phosphatase activity and inhibitory action against *Rh.solani*; 6T1 for its antifungal potential against the three analysed potato pathogens and orange pigmentation; 6T2 due to UV fluorescence on King B medium, correlated to siderophore production, and antagonistic activity against *Alternaria* spp. and *Rh.solani*; 6T4 due to its rapid colonisation potential and wide spectrum of antifungal activity. The Biolog GEN III identification revealed bacterial species commonly found as endophytes (table 2).

Table 2. Identification of selected endophytic isolates

Endophytic bacterial isolates	Biolog GEN III identification
1T1	<i>Klebsiella oxytoca</i>
1T2	<i>Bacillus endophyticus</i>
6T1	<i>Pseudomonas marginalis</i>
6T2	<i>Ps. Viridilivida</i>
6T4	<i>B. atrophaeus/subtilis</i>

Based on the biochemical identification, some of the isolates were identified as potential plant or human pathogens, like: *Pseudomonas viridilivida* reported as pathogenic to lettuce (De Vos et al., 1985), *Pseudomonas marginalis* described as post-harvest pathogen able to cause soft rot in many legumes (Achbani et al., 2014), and *Klebsiella oxytoca* sometime involved in human bacteremia (Lin et al., 1997), however highly appreciated for its potential of nitrogen fixation in cereals (Bao et al., 2013).

Although 6T1 and 6T2 were identified as potential plant pathogenic species, they did not induce any disease symptoms in the potato tubers used for endophytes isolation.

However, the data obtained with the isolate 6T4 identified as *B. atrophaeus/subtilis* are very promising for biocontrol strategies. Further molecular analyses are necessary for confirmation the identification of bacterial isolates performed by Biolog system.

CONCLUSIONS

The endophytic bacteria isolated from Romanian potato tubers were symptomless colonizers, harvested from healthy plant material.

Out of twenty newly isolated endophytic bacteria, five strains (1T1, 1T2, 6T1, 6T2, and 6T4) presented beneficial properties for plant protection and/or growth promotions.

The identification of these strains revealed bacterial species commonly found as endophytes: *Bacillus endophyticus*, *B. atrophaeus/subtilis*, *Pseudomonas marginalis*, *Ps. Viridilivida* and *Klebsiella oxytoca*.

The *B. atrophaeus/subtilis* 6T4 strain was selected due to its biocontrol potential, high colonization competitiveness and enzymatic activity, which could bring several benefits for the host plant.

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DOWNY MILDEW IN SUNFLOWER - THE MANAGEMENT OF *Plasmopara halstedii* PATHOGEN

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Abstract

The virulence of the downy mildew pathogen increased during the last period and new virulent races of this fungus appear. Thus it is essential to know data about the presence and distribution of the pathotypes. In Europe, an increasing number of the pathotypes, have been identified.

In Romania we identified eight races of the pathogen. They are not present in all areas cultivated with sunflower, in some areas being present only five races, in other six or seven.

*Using the sources of resistance to *Plasmopara halstedii* pathogen, for the most virulent races present now in Europe we have introduced genes of resistance in the best lines from our germplasm collection.*

The testing of some sunflower hybrids with different degree of resistance to downy mildew has showed that there is difference in the pathogen attack degree, depending on the climatic conditions in the years and locations.

Key words: sunflower, downy mildew, *Plasmopara halstedii*.

INTRODUCTION

The sunflower downy mildew, caused by *Plasmopara halstedii* (Farl.) Berl. and de Toni is one of the most devastating diseases for this crop, over the world. However, this pathogen could be controlled by the resistant hybrids or treatments with different fungicides. There are some factors that make difficult this disease control: pathogen variability (Gulya et al., 1998) and pathogen resistance or tolerance to fungicides, as metalaxyl - mefenoxam (Albourie et al., 1998; Molinero-Ruiz et al., 2000).

The virulence of the downy mildew pathogen increased lately and new virulence races of this fungus appear. Thus it is essential to know data about the presence and distribution of the pathotypes. In Europe, an increasing number of the pathotypes, each with a distinct virulence structure, have been identified. In Romania, five pathotypes of the pathogen have been identified, before 2006 year (Pacureanu et al.,

2006), in the last years, being identified other two. Since fungal diversity of this kind has consequences in both disease epidemiology and breeding for resistance, there is a need to identify the virulence of the local fungal populations and to monitor the changes over the time. Currently, there are at least 36 pathotypes of *Plasmopara halstedii* worldwide (Gulya, 2007) but number is increasing rapidly (Virányi and Spring, 2011; Türkmen and Çalışkan, 2016), considering the fact that in most sunflower producing countries, just 12 well distinguished virulence pathotypes exist. Planting downy mildew-resistant hybrids is very important to manage downy mildew. However, due to the development of new races, resistance may not be a sufficient management in all fields (Virányi et al. 2015).

Crop rotation has a minimal effect on downy mildew management. Overwintering oospores could survive in the soil up to 10 years, rendering crop rotation practices inefficient for

downy mildew (Friskop, 2009). Downy mildew races in our growing region are assessed by the resistance screening program conducted by NARDI Fundulea and some seed companies. This paper presents the data about the *Plasmopara halstedii* pathogen virulence in Romania and the management of its control by genetic resistance.

MATERIALS AND METHODS

In order to study the pathogen races infecting Romania fields, the international sunflower differential set has been used. Several sunflower lines with good agronomic traits, which have been introduced into conversion process for resistance to downy mildew, have been used. The behaviour of some sunflower hybrids have been studied in natural infection conditions, during four years, in two locations. Infected sunflower plants from different fields placed in all important areas of sunflower cultivation, in Romania, have been collected and used for artificial infections. Pathogen inocula were directly recovered either from infected leaves, by brushing the fungal

structures, either from infected leaves pre-incubated in humid chambers at 18 to 20°C in the dark for 24 to 48 h.

Thirty to forty pregerminated seeds of each differential line (three replicates per line) were inoculated by the whole-seedling immersion technique. After 12 days, plants were maintained at 20°C and 100% relative humidity for 24 to 48 h, in order to enhance pathogen sporulation and evaluate its susceptibility (sporulation on cotyledons and/or first true leaves) or resistance (absence of sporulation or weak sporulation only on cotyledons) reactions.

RESULTS AND DISCUSSION

For the identification of the pathogenic races, eight isolates coming from eight areas cultivated with sunflower in Romania have been used. The results presented in table 1 are showing that all isolates have attacked the differentials carrying the P11 and P15 genes. The differential carrying the gene P12 was not attacked by isolates coming from Constanta, Iasi and Tulcea areas. The differentials PM 13 and PM 17 were attacked by isolates coming from Fundulea, Craiova and Timis.

Table 1. Results from the sunflower differentials set testing for resistance to *Plasmopara halstedii* pathogen races, Fundulea, 2016

Differentials	Isolates							
	Braila	Slobozia	Fundulea	Craiova	Constanta	Iasi	Tulcea	Timis
	Infection degree (%)							
HA 265 (P11)	56.0	47.4	45.7	57.9	36.6	49.7	38.3	59.4
AD 66 (P11)	51.3	44.1	52.7	24.4	33.9	41.4	32.2	42.7
DM2 (P15)	49.0	35.4	33.7	31.8	35.4	48.7	36.4	47.3
RHA-274 (P12)	33.6	24.8	11.3	2.9	0.0	0.0	0.0	31.5
PM 13 (P1?)	0.0	0.0	4.0	7.9	0.0	0.0	0.0	6.0
PM 17 (P1?)	0.0	0.0	1.9	2.0	0.0	1.3	0.0	2.0
803-1 (P1?)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
RHA 419 (P1?)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
RHA 340 (P18)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.5
HA-335 (P16)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
HA 304	74.7	58.4	51.7	64.3	38.9	43.8	42.4	65.7

The *Plasmopara halstedii* pathotypes, from different areas of sunflower crop in Romania are presented in table 2. In case of Fundulea, Craiova and Timis isolates, 8 pathogen races are present.

In case of Constanta and Tulcea isolates, there were present only 5 races, missing the 310, 330 and 314 races.

In case of Iasi isolates, there were present 6 races of the pathogen, however 310 and 330

races were missing. In case of Braila and Slobozia isolates there were present 7 races of *Plasmopara halstedii*.

Using different sources of resistance to the attack of *Plasmopara halstedii*, specially carrying the gene P16, we have introduced resistance to this pathogen, in some of our best sunflower inbred lines.

The results are presented in table 3.

Table 2. The pathotypes of the pathogen *Plasmopara halstedii*, identified in the sunflower crop, in Romania

Pathotypes								
Races	100	300	714	703	310	330	710	314
Location								
Braila	X	X	X	X	X	X	X	
Slobozia	X	X	X	X	X	X	X	
Fundulea	X	X	X	X	X	X	X	X
Craiova	X	X	X	X	X	X	X	X
Constanta	X	X	X	X			X	
Iasi	X	X	X	X			X	X
Tulcea	X	X	X	X			X	
Timis	X	X	X	X	X	X	X	X

Table 3. Results of the improvement for resistance to downy mildew, for some sunflower genotypes

CMS lines resistant to <i>Plasmopara halstedii</i>	
Source of resistance	Number of resistant lines
HA 335	21
AS - 110	12
Populations	78
Restorer lines resistant to <i>Plasmopara halstedii</i>	
Source of resistance	Number of resistant lines
CRF- 821	27
RHA 340	9
Populations	109

Testing some experimental sunflower hybrids, regarding the resistance to *Plasmopara halstedii*, in two locations and four years, showed that the attack degree is depending on the climatic conditions of the years and locations, as it is presented in figures 1 and 2.

In the years 2015 and 2016, when the air temperatures were low and it was enough rain in the beginning of sunflower development, there have been good conditions for the pathogen development. This it could to be seen in the values of the infection degree with this pathogen.

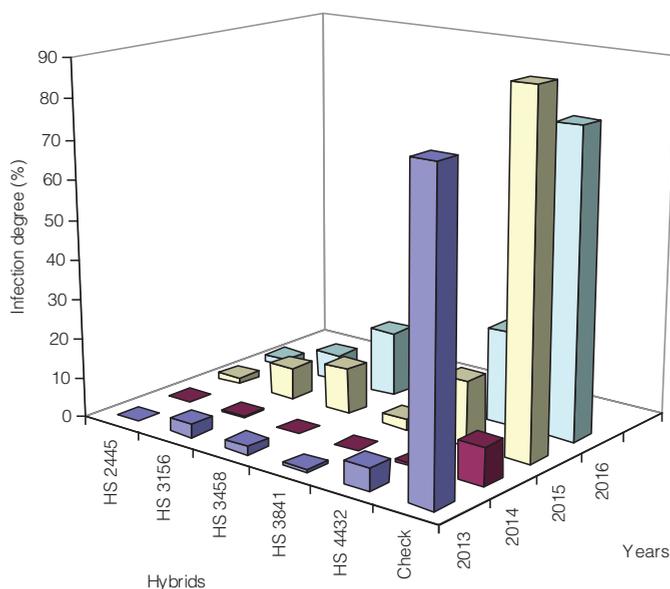


Figure 1. The behaviour of some sunflower hybrids, regarding the resistance to the attack of *Plasmopara halstedii* pathogen (Fundulea location)

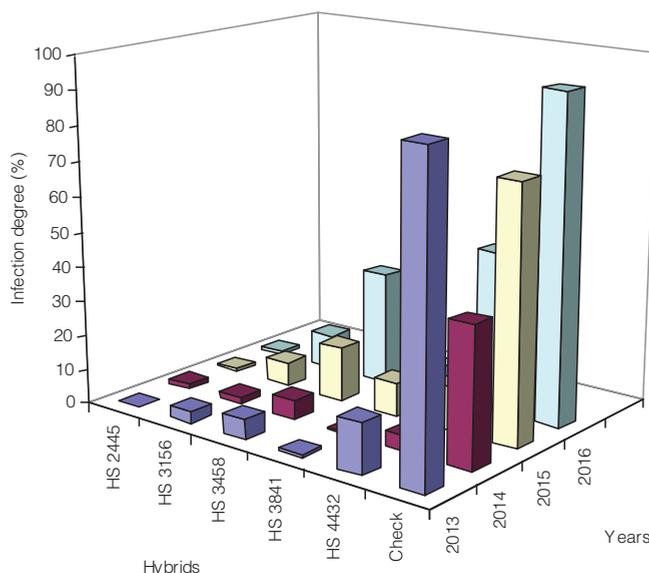


Figure 2. The behaviour of some sunflower hybrids, regarding the resistance to the attack of *Plasmopara halstedii* pathogen (Braila location)

Regarding the hybrids, HS 2445 hybrid was the most resistant, in all years and in both locations. In Braila area the attack degree of the pathogen was higher in all years, comparing with Fundulea location. Braila area gave more suitable conditions for the development of *Plasmopara halstedii*.

CONCLUSIONS

The downy mildew has become very dangerous for sunflower crop in almost all areas cultivated with sunflower over the world.

It is of a great importance to identify the races of the pathogen in the important areas cultivated with sunflower as well as to identify the sources of resistance. In the sunflower crop in Romania there have been identified eight races of this pathogen. In some areas are present only five races.

Using the sources of resistance to the new races of this pathogen it has been transferred genes for resistance in the best sunflower inbred lines from our institute germplasm collection.

The attack degree of the pathogen which produces downy mildew in sunflower is high influenced by the climatic conditions in the years and in different locations.

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SELENIUM BIOFORTIFICATION TREATMENT OF CAULIFLOWER ENHANCES THEIR CONTENT IN CHEMOPREVENTIVE COMPOUNDS AND *IN VITRO* ANTITUMORAL ACTIVITY

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Abstract

Cruciferous vegetables are known as food with chemopreventive effect due to their high content in bioactive compounds, such as mineral nutrients, including selenium, antioxidants, vitamins and glucosinolates, which were shown to inhibit cancer cell growth, both in vitro and in vivo testing. The aim of this study was to evaluate the effect of a new selenium-based composition, applied on experimental field conditions to cauliflower plants (Brassica oleracea L.), on their chemopreventive compounds level and antitumoral activity. Treated plants, cultivated both in normal watered and water stress conditions, were compared for total selenium and sulforaphane contents, determined by electrothermal atomic absorption spectrometry (ETAAS) and high performance liquid chromatography (HPLC), respectively. In vitro cytotoxicity of cauliflower extracts was evaluated in NCTC fibroblast cell line, while their antitumoral activity was tested in Caco-2 human adenocarcinoma cell line using MTT colorimetric assay. The results indicated that the applied biofortification treatments increased the selenium intake, allowed formation of bioactive glucosinolates and enhanced the antitumoral activity of cauliflower plants cultivated in both normal watering and water stressed conditions. In conclusion, this new biotechnological approach on cauliflower cultivation, using a treatment with a novel selenium-based composition, could be considered promising step for obtaining functional food from cauliflower crops.

Key words: antiproliferative activity, cauliflower, glucosinolate, selenium, sulforaphane.

INTRODUCTION

Selenium (Se) is an important element for human and animal nutrition because it plays critical roles in a variety of physiological processes (Rayman, 2012). Statistical studies on human subjects have revealed constant correlations between the physiological response, determined by the expression of major selenoproteins and seleno-chemopreventive compounds, and the risk of mortality from chronic diseases, including cancers (Bleys et al., 2008; Rocourt and Cheng, 2013). The dietary intake of Se on a specific area is determined by the mean value of Se in

soil. Worldwide such value is 383 ± 255 $\mu\text{g}/\text{kg}$, when not affected by deficits or excesses (Kabata-Pendias and Pendias, 2001).

In Romania, Se level in soil stands at the deficit limit. Various pathologies caused by Se deficiency were reported for animals from different regions of the country (Serdaru et al., 2003; Lăcătușu et al., 2012). Compared to the international known mean value, the Se content is reduced with 30% - 63% in different regions of Romania (Lăcătușu et al., 2010; Lăcătușu et al., 2012). A low level of Se in soil reduces Se dietary intake and indicates the need of supplementation, to achieve the optimal level of Se, beneficial for reducing the risk of

chronic diseases (Mehdi et al., 2013; Steinbrenner et al., 2013). However, Se supplementation treatment for a better human health shall be related also to the very narrow Se physiological window, wherein the difference between the recommended daily human dose for prevention of chronic diseases and the dose producing pathophysiological effects is very small (Oancea et al., 2014).

Selenium agronomic biofortification, i.e. Se-treatment applied during plants cultivation period, have been considered an effective solution for producing functional foods, beneficial for both animal and human health. Such biotechnology applied to largely consumed vegetables show several advantages, like supplementation by controlled levels of highly bioavailable seleno-compounds and a wider availability to different categories of people at risk of chronic diseases, including those with low income (White and Broadley, 2009; Fageria et al., 2012).

On the other hand, Se biofortification treatments allow the valorization of Se protective and stimulatory effects on plants (Feng et al., 2013). Experimental studies have shown that Se is a beneficial microelement for plants, stimulating their growth (Hartikainen and Xue, 1999; Sajedi et al., 2011) and playing a role in plant protection against infestation caused by insects or phytopathogenic agents (Hanson et al., 2003), oxidative stress (Xue et al., 2001) and hydric stress (Wang et al., 2011). It was reported that Se-based treatments applied to plants have also improved their response to drought stress, involving both water and oxidative stress (Kuznetsov et al., 2003; Yao et al., 2009). Due to this protective effects against biotic and abiotic stresses selenium was included among inorganic compounds acting as plant biostimulants (Du Jardin, 2015).

Consumption of cruciferous vegetables increased lately, due to their high content in bioactive compounds, such as mineral nutrients, including Se, antioxidant compounds, vitamins and glucosinolates, which form a unique class of sulfur compounds (Samec et al., 2016). The known role of chemopreventive food was attributed to cruciferous mainly due to studies indicating that glucosinolates have acted as potent inducers of phase II enzymes,

which inactivated carcinogenic metabolites and inhibited cancer growth in vitro and in vivo (Park et al., 2014; Tortorella et al., 2015). Vegetables from *Brassica* spp., such as broccoli, Brussels sprouts and cabbage were the main crops on which Se biofortification treatments were applied (White and Broadley, 2009). However, per our knowledge, few studies were done on cauliflower Se biofortification (Avila et al., 2014, Oancea et al. 2015).

We developed previously a new composition for selenium biofortification treatment, intended not only to increase accumulation of (organo)selenium compounds on *Brassica* crops, but also to enhance protective effects of selenium treatment on cultivated plants, especially against water stress (Oancea et al., 2014, Oancea et al., 2015).

The aim of the study was to investigate this new selenium-based composition, applied on experimental field conditions to cauliflower plants, on accumulation of chemopreventive compounds (Se, glucosinolates) into edible parts, cytotoxicity and antitumoral activity of plant extracts and cauliflower plant resistance to water stress.

MATERIALS AND METHODS

Biological material. Seedlings of cauliflower (*Brassica oleracea* L. var. *botrytis* cv. Adelanto F1) were transplanted and cultivated on an experimental field, located on Ștefan cel Mare, Călărași, Romania (40° 59' N latitude, 27°40' E longitude, 54 m altitude), according to the recommended cultivation technology. The calcareous kastanic chernozem soil was fertilized with 160 kg ha⁻¹ N, 120 kg ha⁻¹ P and 120 kg ha⁻¹ K, 5 days before cauliflower seedlings transplantation. The total selenium content in the upper soil was 67 μg/kg, representing 40% lower value than the average content in soils unaffected by Se deficiencies (Lăcătușu et al., 2010). The transplants were placed on 25 cm, in rows done at 70 cm one from another.

During 9 weeks of cultivation, there were recorded higher monthly temperatures (+1.3°C in May; +0.4°C in June; +2.7°C in July) and lower monthly precipitations (-31.5 mm in May; -22.7 mm in June; -34.9 mm in July) than the multi-annual average.

Plant treatment. Plants received two subsequent treatments with the new selenium based mixture, consisting of 10 μM Na_2SeO_4 (Sigma), 5 mM betaine (Sigma) and 1% spraying adjuvant (Teso Spec Srl). The treatments were applied by foliar spraying, at 3 and 6 weeks after crop establishment by seedling transplant. The spraying adjuvant was obtained from rapeseed oil by transesterification in the presence of potassium hydroxide, neutralization of excess alkali with oleic acid, and final addition of lecithin and

nonionic emulsifier (Vladulescu et al., 2012). Plants were grown in normal watering conditions (watered daily, at 80% field capacity) and in water stress conditions (watered once every two days, at 80% field capacity). After 9 weeks of cultivation, normally watered and water stressed cauliflower crops were separately harvested and weighed, for the establishment of the marketable yields. Controls were obtained from untreated plants, cultivated in similar conditions and without Se-based treatment (Table 1).

Table 1. Experimental treatments done on field grown cauliflower

Sample label	Water supply	Se -based mixture used for cauliflower treatment
C1	normal watering, control	-
C2	normal watering	10 μM Na_2SeO_4 + 5 mM betaine + 1% spaying adjuvant
C3	water stressed control	-
C4	water stressed	10 μM Na_2SeO_4 + 5 mM betaine + 1% spaying adjuvant

Determination of total Se content. Total Se content was measured using an atomic absorption spectrometer (Agilent AA-1475, with Vapor Generation Accessory, VGA 76, and Agilent Se- hollow cathode lamp). The measurements were undertaken after electrothermal atomization of each sample in a graphite oven SR EN ISO 15586:2004. The results were reported in $\mu\text{g/g}$ dry weight (d.w.).

Analysis of sulforaphane content. Sulforaphane extraction was performed using the method described by Campas-Baypoli et al. (2010). Briefly, fresh cauliflower plants were weighed (0.15 g) and incubated with 4 ml of acidic water (pH 6) for 2.5 h at 45°C. The mixture was extracted with 20 ml dichloromethane and the resulting solution was filtered through Whatman no. 5 paper. The sulforaphane was purified with Chromabond SPE silica gel (SiOH) columns. Prior to use, the silica gel column was conditioned with dichloromethane after which the organic extract was loaded. The column was washed with ethylacetate and the sulforaphane was eluted with methanol. The methanol extract was dried at 45°C using a rotary evaporator and re-dissolved with 1 ml acetonitrile. The resulting solution was filtered with a PTFE membrane of 0.45 μm and stored at -4°C until HPLC analysis.

The chromatographic analysis was performed using an Agilent 1200 HPLC system, equipped with a photodiode array detector. HPLC

identification and quantification of sulforaphane was carried out using a Zorbax XDB C18 (4.6 x 150 mm) column (Agilent) and 70% acetonitrile as mobile phase, at a flow rate of 0.6 ml/min. Twenty microliters of sample were injected into the HPLC system and the sulforaphane was detected at 202 nm.

Standard solutions of sulforaphane were prepared in acetonitrile in the range of 5-100 $\mu\text{g/ml}$. The chromatograms were processed with ChemStation Agilent software and the sulforaphane was quantified from the peak areas, in correlation with sulforaphane standard concentration. Calibration curves were built for concentrations ranging between 5-100 $\mu\text{g/ml}$.

Cell culture experiments. Total extracts of control and treated plants were obtained by incubation of fresh cauliflower plants (30 g) in deionized water, at 45°C, for 24 h. The samples were centrifuged at 2500 rpm, for 10 min and the supernatant was sterile filtered through 0.2 μm membranes. The resulting solutions were stored at -20°C until cell culture analysis.

In vitro experiments were performed using a normal cell line of mouse fibroblasts (NCTC clone L929) and a tumor cell line derived from human colorectal adenocarcinoma (Caco-2), provided by ECACC. The cells were maintained in MEM culture medium containing 10% fetal calf serum (FCS) and antibiotics, at 37°C, in humid atmosphere with 5% CO_2 . For the experiment, cells were seeded in 96-wells

culture plates, at a cell density of 5×10^3 cells/well, for 24 h, to allow cell adhesion. Then, different concentrations (0-2 mg/ml) of cauliflower extracts were added in each well and the plates were incubated in standard conditions, for 72 h.

Evaluation of cytotoxicity and antiproliferative activity. Cytotoxicity and antiproliferative activity of cauliflower extracts were evaluated using MTT assay, as previously described (Moldovan et al., 2008). Briefly, at the end of incubation period, the culture medium from each well was replaced with 500 μ l MTT solution (0.25 mg/ml) in fresh culture medium and the plates were incubated in standard conditions (5% CO₂ air, 37°C), for 3 h. After discarding the culture medium, 500 μ l isopropanol were added to dissolve formazan crystals by gently shake, at room temperature, for 15 min and the optical density (OD) was read at 570 nm using a microplate reader (Tecan, Austria). The results were reported as cell viability percent from control sample (cells incubated in culture medium), considered 100% viable. The samples were tested in triplicate.

Statistical analysis. The results were expressed as mean of 3 values \pm standard deviation (SD). Statistical analysis of the results was performed using paired Student's t-tests. Significant differences were considered at values of $p < 0.05$.

RESULTS AND DISCUSSIONS

Crop yield of Se-treated cauliflower field plants

Normally watered and water stressed cauliflower crops were separately harvested after 9 weeks of cultivation (Figure 1) and weighed to calculate the obtained yields.



Figure 1. Se-treated cauliflower plants, cultivated in normal watered (A) and water stressed (B) conditions. The cauliflower crop yield variation was analyzed related to normal watering or water

stress conditions of cultivation. It was observed that similar crop yields were obtained for Se-treated cauliflower plants, in both normal watering conditions (equivalent to 23.80 tones ha⁻¹) and water stressed (equivalent to 23.40 tones ha⁻¹) conditions. In turn, the corresponding controls presented a lower yield in water stressed conditions (18.62 tones ha⁻¹) than in normal watering conditions (24.10 tones ha⁻¹). These values allowed us to conclude that the Se-based treatment mixture provided protection of cauliflower plants against hydric stress.

The protective effect of Se-based biofortification was also reported for other vegetables and cereal crops (Hanson et al., 2003; Feng et al., 2013).

Total Se content in biofortified cauliflower plants. In our study, a biostimulant mixture of 10 μ M sodium selenate, 5 mM betaine and 1% spraying adjuvant was selected for the treatment of cauliflower plants, based on previous experiments (Oancea et al., 2015; Oancea et al., 2016). The mixture was planned to contain sodium selenate, as the main form of inorganic Se used for crops biofortification (Hawkesford and Zao, 2007), betaine, as a plant osmoprotectant and a modulator of S-Adenosyl-Methionine cycle, overused by selenium assimilation (Oancea et al., 2015) and a spraying adjuvant, based on methyl esters of rapeseed fatty acids, as an enhancer of foliar fertilizers penetrability. A parallel experiment involving normal watered and water stressed plants was performed, as described in "Experimental part" section, in order to observe the effect of Se-based treatments on plant response to water stress. After 9 weeks of cultivation, the results of total Se content analysis showed that, in normal watered plants, the level of total Se content significantly increased ($p < 0.05$) (1.35-fold) compared to control plants (Table 2). Similar variation of total Se content was obtained for cauliflower field plants cultivated in water stressed conditions, the level of Se being significantly increased ($p < 0.05$) (1.34-fold) in treated plants compared to untreated control plants (Table 2).

The registered values were slightly lower than those of plants cultivated in normal watering conditions, indicating the positive effect of Se-based treatment on cauliflower plants

cultivated in water stress conditions. Other studies reported that foliar application of sodium selenate increase the fruit yield in olive trees cultivated under water stress conditions (Proietti et al., 2013) and the antioxidants level in the leaves of lettuce (Rios et al., 2008).

Table 2. Variation of Se content in Se-treated cauliflower field plants, determined by electrothermal atomic absorption spectrometry*

Sample	Selenium content ($\mu\text{g/g d.w.}$)	
	Normal watering	Water stressed
Control, field grown plants	0.080 ± 0.004	0.076 ± 0.003
Se-based treatment, field grown plants	$0.108 \pm 0.010^*$	$0.102 \pm 0.005^*$

*Results represent mean of 3 determinations \pm SD.

Sulphoraphane content in Se-treated cauliflower plants. 4-methylsulfinybutyl glucosinolate (glucoraphanin) and its hydrolysis product, sulforaphane, are the most studied compounds with chemopreventive activity in *Brassica* phytochemicals research (Samec et al., 2016). In our study, sulforaphane was extracted from cauliflower plants, grown in field conditions, treated with Se-based (biostimulant) mixture and cultivated in normal and water deficit conditions. Identification of sulforaphane in treated plant extracts was performed by HPLC analysis and comparison of the retention time with that of the standard solution. As indicated in (Figure 2) and (Figure 3), sulforaphane peak was present in the recorded profiles at ≈ 4.9 min, in both treated plants cultivated in different conditions of water stress.

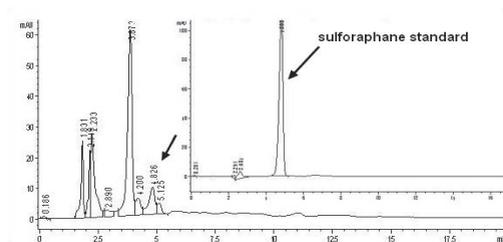


Figure 2. HPLC identification of sulforaphane in Se-treated cauliflower plants cultivated in normal watering conditions

Sulforaphane content in each cauliflower sample was determined using the method of plotting the calibration curve of sulforaphane standard by linear regression analysis of the integrated peak area versus concentration.

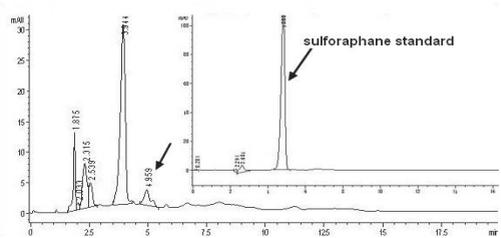


Figure 3. HPLC identification of sulforaphane in Se-treated cauliflower plants cultivated in water stressed conditions

The results of quantitative calculations obtained for all cauliflower plant extracts are presented in (Table 3). The values of sulforaphane content obtained for cauliflower plants treated with Se-based mixture, in normal watering and water stressed conditions are presented in (Table 3).

Table 3. Content of sulforaphane in cauliflower plant extracts*

Sample	Sulforaphane ($\mu\text{g/g d.w.}$)	
	Normal watering	Water stressed
Control, field grown plants	34.83 ± 5.88	27.65 ± 5.44
Se-based treatment, field grown plants	25.39 ± 4.12	18.20 ± 4.11

*Results represent mean of 3 determinations \pm SD.

The results indicated that the treatment did not significantly ($p > 0.05$) affect the accumulation of glucosinolates degradation product. The Se-treated plants presented sulforaphane values like those of corresponding control plants.

Previous studies reported the content of six main glucosinolates in 7-day-old cauliflower seedlings treated with $50 \mu\text{M}$ sodium selenate and indicated a significant variation in two of three cauliflower cultivars (Avila et al., 2014). The differences in total glucosinolate levels in relation to cultivars were explained by their genotype or genotype-environment interaction (Farnham et al., 2004).

The biochemical results of this study indicated that the used of Se-based mixture induced high levels of total Se and allowed accumulation of glucosinolates in cauliflower field plants cultivated in both normally watered and water stressed conditions. This is probably due to betaine from the treatment mixture, which could influence and compensate the cross-talk between selenium and sulphur metabolism. Hsu et al. (2011) also showed that it was possible to produce Se-biofortified broccoli that

concomitantly accumulated high levels of Se and glucosinolates.

In vitro cytotoxicity of cauliflower extracts. The cytotoxicity of cauliflower extracts was tested in a normal cell line of fibroblasts to determine the biocompatible range of concentrations for *in vitro* experiments. The results reported to the cell viability of untreated fibroblast cells (considered 100% viable) are presented in (Figure 4, Figure 5). It was observed that the extracts of Se-treated plants and cultivated in normal watering conditions induced a decrease in cell viability of fibroblast cells proportional with the tested concentrations. Still, they were biocompatible in the range of 0-1500 µg/ml, with values of cell viability higher than 75%. Only at 2000 µg/ml extract concentration it was recorded a decrease in cell viability up to 64.92%. The same trend was recorded for control plants and for the Se-treated plants cultivated in water stressed conditions. The Se-treated plants induced significantly higher ($p < 0.05$) cell viability, in comparison with that of control plants, at certain values of concentration (Figure 3). This is the first study reporting the cytotoxicity of cauliflower extracts in a cell line of normal fibroblasts.

In vitro antiproliferative activity of cauliflower extracts. The antitumoral activity of several vegetables was previously demonstrated as accumulation of bioactive food components, like glucosinolates and methyl Se amino acids (Shankar et al., 2013; Bera et al., 2013).

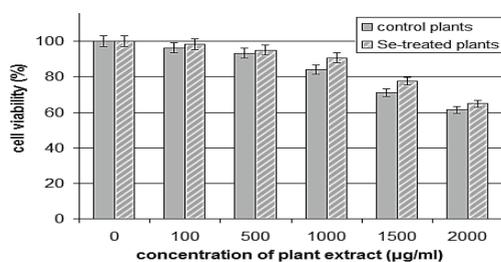


Figure 4. Effect of plant extracts from cauliflower cultivated in normal watering conditions on NCTC fibroblast cells viability after 72 h of cultivation, evaluated by MTT assay.

In our study, the effect of Se biofortification of cauliflower field plants on their capacity to inhibit adenocarcinoma cells growth was investigated in cell culture experiments.

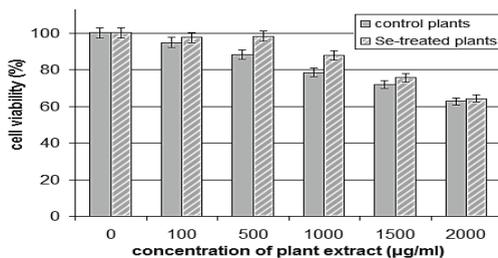


Figure 5. Effect of plant extracts from cauliflower cultivated in water stressed conditions on NCTC fibroblast cells viability after 72 h of cultivation, evaluated by MTT assay. Values are expressed as mean of three determinations \pm SD and reported to the control, considered 100% viable

After 72 h of cultivation of Se-treated cauliflower extracts in tumor cell culture, the normally watered variant has induced a decrease in cell viability below 75% at concentrations of 1500 µg/ml (68.64%) and 2000 µg/ml (60.62%) (Figure 6). The water stressed plants presented lower values of cell viability, reaching 61.35% and 52.91% at concentrations of 1500 µg/ml and 2000 µg/ml, respectively (Figure 7). These values were significantly lower ($p < 0.05$) than those of control plants tested at the same concentrations.

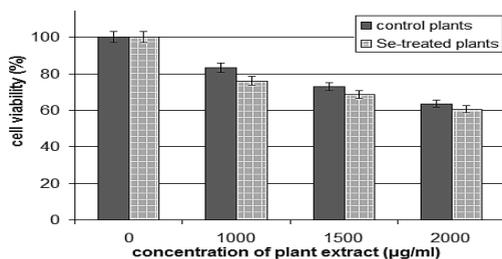


Figure 6. Effect of plant extracts from cauliflower cultivated in normal watering conditions on Caco-2 human adenocarcinoma cells viability after 72 h of cultivation, evaluated by MTT assay

These results showed that Se treated cauliflower plants presented a higher antitumoral activity than control plants. This activity is probably due to the high content of total Se and glucosinolates, which we determined in the cauliflower plants during the biochemical screening. Previously, it was reported that broccoli sprouts treated with selenate presented an enhanced antiproliferative effect in human prostate cancer cell lines, in a dose-dependent manner (Abdulah et al., 2009). Also, Se-

enriched broccoli extracts induced a greater growth inhibition of human colon cancer cells than untreated extracts (Tsai et al., 2013).

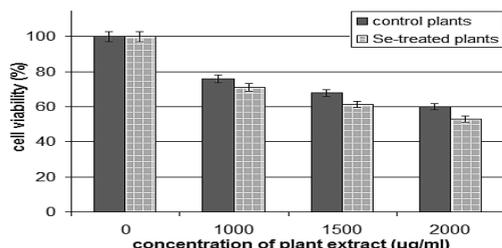


Figure 7. Effect of plant extracts from cauliflower cultivated in water stressed conditions on Caco-2 human adenocarcinoma cells viability after 72 h of cultivation, evaluated by MTT assay.

Our new Se-based composition enhance the biostimulant effect of selenium, related to an improved response to water stress and to a higher quality of the treated plants. Plant stimulants are characterized by the effects on nutrients uptake and nutrient use efficiency, response to abiotic stress and edible yield quality (Du Jardin, 2015). This new Se-based composition reduce the effects of water stress on cauliflower yield, increase the level of the main chemopreventive compounds and the antitumoral effects.

CONCLUSIONS

We have used a mixture of 10 µM Na₂SeO₄, 5 mM betaine and 1% adjuvant as biostimulant composition for treating cauliflower crops in the field.

The obtained results demonstrated that the proposed approach of Se-based biofortification is protective against drought cultivation conditions, resulting in increased plant tolerance to water stress, probably by modifying plant physiological processes. In the same time, the treatment had stimulatory effect, resulting in an increased Se intake by treated cauliflower plants and allowing accumulation of bioactive glucosinolates. Equilibrate formation of Se and glucosinolates in cauliflower crops treated with this Se-based biostimulant composition could provide characteristics of functional food for this vegetable. The selected cyto-compatible concentrations of biofortified cauliflower extract presented higher antitumoral activity in

Caco-2 adenocarcinoma cell line. The new biotechnology consisting of Se-based biostimulant treatment of cruciferous field crops could be further tested using in vivo experimental models.

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EFFECT OF EXOGENOUS ABSCISIC ACID ON SOME QUALITY ATTRIBUTES OF SWEET POTATO (*Ipomoea batatas* L.)

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Abstract

Sweet potato (Ipomoea batatas L.) is an important food crop around the world, being cultivated in more than 100 countries as the crop can be a rich source of energy and have been recognized as healthy foods because of their significant content of phytonutrients. In recent years, the use of bioregulators in sustainable agriculture has been growing because it leads to higher content of nutrients in the plant tissues and positive metabolic changes. It appears that using abscisic acid in controlling the mechanisms of plant evolution is a good alternative for an ecologic agriculture given that recent studies on the mechanism of the abscisic acid action have shown its great importance as a bioregulator for plants. The objective of this work was to study the influence of the abscisic acid treatment on some quality characteristics of sweet potato. For this purpose abscisic acid solutions in different concentrations were used as foliar treatments on sweet potato leaves and comparative results concerning some morphological and biochemical changes in the tuberous roots were studied. The experiment was conducted starting with the first leaves appearance until the harvesting of the roots, while determination of the sugars and proteins content were made on the tuberous root harvested in the maturity stage.

Key words: abscisic acid, phytohormones, proteins, sugars, sweet potato.

INTRODUCTION

Sweet potato (*Ipomoea batatas* L.) is an important food crop around the world, being cultivated in more than 100 countries (Wu et al., 2008). The crop can be a rich source of energy as well as of carbohydrates. The carbohydrates concentration in sweet potato roots varies widely between individual lines (Hill et al., 1992). Despite the high carbohydrate content, sweet potato has a low glycemic index, indicating low digestibility of the starch (ILSI, 2008) and it is the only starchy staple, which contains appreciable amounts of amino acid lysine that is deficient in cereal-based diets like rice (Bradbury et al., 1985). Also, orange-fleshed cultivars have been recognized as healthy foods because of their significant content of phytonutrients, such as β -carotene, anthocyanin (Mohamad-Zahari, 2016), phenolic acids, minerals, vitamins and dietary fibre (Turner, 2001; Tumuhimbise et al., 2009). An important reason for the popularity of sweet potato is its adaptability to a range of agroecological conditions (Horton, 1988). In recent years, the use of bioregulators in sustainable agriculture has been growing. The

application of bioregulators leads to higher content of nutrients in the plant tissues and positive metabolic changes.

The mode of action of bioregulators is often unknown and difficult to clarify because they contain multiple bioactive components, for instance plant hormones, that together may contribute to specific effects in plants (Ertan et al., 2011).

It appears that using abscisic acid in controlling the mechanisms of plant evolution is a good alternative for an ecologic agriculture given that recent studies on the mechanism of the abscisic acid action have shown its great importance as a bioregulator for plants.

Besides environmental factors (water, nutritive substances, temperature, light) phytohormones play an essential role in controlling the plant growth, cells differentiation and development. Phytohormones are a group of naturally occurring, organic substances which influence physiological processes at low concentrations (Davies, 1987).

The main hormonal groups acting in the plant tissues are auxins, gibberellins, cytokinine, which are considered growth promoters,

together with abscisic acid and ethylene, known as growth inhibitors. However, it is interesting to note that hormones do not act alone, such as the final effect on the plant development is the result of a hormonal balance, which controls many processes that impact on crop performance and yield (Wilkinson et al., 2012).

Phytohormones with inhibitory properties can operate on the whole plant as well as on some tissues and organs causing the inhibition of some metabolic processes like: biosynthesis of nucleic acids, photosynthesis of chloroplasts and of some physiological processes, especially the division and elongation of cells and the buds opening (Davies, 1987).

Abscisic acid (ABA) is often referred to as an inhibitory rather than stimulatory hormone. ABA accelerates the process of ageing and the fruit and leaves abscission by causing break down of proteins and nucleic acids. It is involved in the closure of stomata, bud and seed dormancy, response to drought stress (Bassaganya-Riera, 2010) and is known to inhibit other hormonal actions.

The objective of this work was to study the influence of the abscisic acid treatment on some quality characteristics of sweet potato. For this purpose solutions of abscisic acid in different concentrations were used as foliar treatments on sweet potato leaves and comparative results concerning some morphological and biochemical changes in the tuberous roots were studied. The experiment was conducted starting with the first leaves appearance until the harvesting of the roots, while determination of the sugars and proteins content were made on the tuberous root harvested in the maturity stage.

MATERIALS AND METHODS

Biological materials

Sweet potato (*Ipomoea batatas* L.) Porto-Rico variety, a common variety largely spread in countries where sweet potatoes is traditionally consumed, was investigated. Bush-type Porto Rico is an ideal variety for smaller gardens, which also produces good yields of medium-sized potatoes with tapered roots that are great for baking because the delicious sweet flavor of the orange flesh.

The experiment was conducted starting with the first leaves appearance until the harvesting

of the tuberous roots (meaning for 6 months, from May to October). During the experiment development the sweet potatoes plants were treated with solutions of 10^{-5} mol/L, 1.5×10^{-5} mol/L, 2×10^{-5} mol/L and 3×10^{-5} mol/L ABA by foliar applications once every two weeks, according to horticultural practice (Ifrim, 1997). Determination of the sugars and proteins content were made on the tuberous root harvested in the maturity stage. The biochemical analyzes were made in triplicate, using fresh tuberous roots of sweet potatoes.

Determination of reducing sugars was performed according to the Nelson-Somogyi method (Iordachescu, 1988; Somogyi, 1952). The reducing glucids when heated with alkaline copper tartrate reduce the copper from the cupric to cuprous state and thus cuprous oxide is formed. When cuprous oxide is treated with arsenomolybdic acid, the reduction of molybdic acid to molybdenum blue takes place. The measurements of absorbance were achieved at 620 nm with a UV/Visible ThermoSpectronic Helios spectrophotometer.

Determination of crude protein was made after the digestion of the vegetal material by Kjeldahl method (Iordachescu, 1988; Kjeldahl, 1883). The content in total nitrogen was measured by volumetrical method and converted in crude protein content.

RESULTS AND DISCUSSIONS

The molecular action mechanism of ABA in plants is not fully elucidated. It is known that ABA has a biochemical and physiological action against substances that stimulate growth, resulting in a biochemical balance that causes the growth and development of the plant in different phenophases and seasons.

The objective of this work was to study the influence of the abscisic acid treatment on some quality characteristics of sweet potato. For this purpose solutions of abscisic acid in concentrations of 10^{-5} mol/L, 1.5×10^{-5} mol/L, 2×10^{-5} mol/L and 3×10^{-5} mol/L ABA were used as foliar treatments on sweet potato leaves followed by observations on some morphological characteristics of the plants and also by determination of the sugars and proteins content of the tuberous root harvested in the maturity stage.

Influence of ABA on some morphological characteristics

Leaf area is a plant biometric index important to crop production. It was found that foliar treatment of sweet potato plants with ABA induced changes in the leaf aspect. Thus, the leaves were smaller and irregular shaped (figure 1B) at the plants treated with concentrations of 10^{-5} mol/L, 1.5×10^{-5} mol/L and 2×10^{-5} mol/L ABA compare to the leaves of the control plants, which were wide, large and smooth (figure 1A).

On the contrary, the 3×10^{-5} mol/L concentration of ABA induced a total inhibition of the plants growth and development, so that the plants died after one month of ABA treatment.



A)



B)

Figure 1. Aspect of the leaves of control plants (A) compare to plants treated with 10^{-5} mol/L ABA solution (B)

Previous studies reported also that treatment with abscisic acid influenced the plant growth.

Thus, ABA in concentrations of 0.01, 0.1, 1.0 or 10.0 mg l^{-1} inhibited axillary bud and root development at the sweet potatoes plant and subsequent plantlet growth. ABA at 10 mg l^{-1} completely inhibited axillary shoot development but did not affect the viability of the plants (Jarret, R.L. et al., 1991). Researches made on bell pepper found that plant stem diameter was lowest in ABA treatment; marketable yield was highest in ABA (Díaz-Pérez et al., 2014).

Instead, regarding the tuberous roots it was noticed an increase in size and number as results of the treatment with 10^{-5} mol/L ABA (figure 2B) compare to the control plants (figure 2A).



A)



B)

Figure 2. Aspect of the tuberous roots of control plants (A) compare to plants treated with 10^{-5} mol/L ABA solution (B)

Considering that hormones have been suggested to play a prominent role in the control of tuberization, it was assumed that

ABA is a promoting hormone in potato tuberization. Scientific literature reports that exogenous ABA stimulated tuberization and reduced stolon length in potatoes (Xu et al., 1998).

Influence of ABA on some biochemical parameters

The soluble sugar is the main substance for plant growth and fruit quality formation. It has been demonstrated that sugar accumulation in sink organs (flowers, fruits, roots etc.) is stimulated by application of some plant hormones including ABA (Brenner et al., 1989; Daie, 1985).

The treatment with ABA of the sweet potato plants determined an increase of the sugars content together with a decrease in the protein content at the plants treated with concentrations of 10^{-5} mol/L, 1.5×10^{-5} mol/L and 2×10^{-5} mol/L ABA (Figure 3), indicating a change in the photosynthesis development in the plant leaves. It is known that photosynthetically active tissues, such as mature leaves, export fixed C (primarily as sucrose) to non-photosynthetic tissues such as fruits or reproductive organs, tubers, meristems, roots (Koch, 2004).

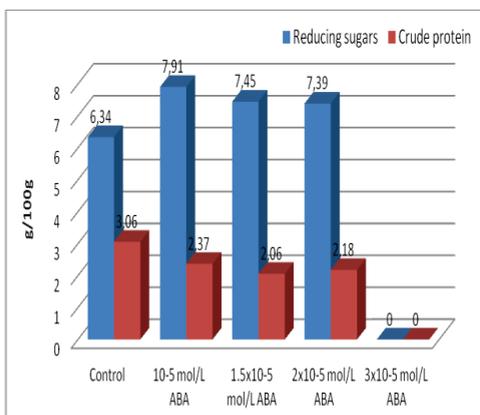


Figure 3. Influence of the ABA treatment on the content in reducing sugars and crude protein of the tuberous roots

Previous studies have found an increase in sugar content of the root of bean seedlings which appears to be the consequence of an ABA-induced stimulation of sugar transport

from the shoot to the root (Karmoker and Van Steveninck, 1979).

Similar results are noticed in recent papers concluding that an exogenous ABA treatment increased sugar content also in other plant species as *Citrus* fruit (Kojima et al., 1996) and peaches (Kobashi et al., 2001).

CONCLUSIONS

It was found that foliar treatments of sweet potato plants with 10^{-5} mol/L ABA induced changes in the leaf aspect: smaller and irregular shaped at the plants treated with ABA compare to the leaves of the control.

Regarding the tuberous roots it was noticed an increase in size and number as results of the treatment with 10^{-5} mol/L ABA compare to the control plants. Considering that hormones have been suggested to play a prominent role in the control of tuberization, it was assumed that ABA is a promoting hormone in potato tuberization.

The 3×10^{-5} mol/L concentration of ABA induced a total inhibition of the plants growth and development.

The treatments with ABA of the sweet potato plants determined an increase of the sugars content together with a decrease in the protein content indicating a change in the photosynthesis development in the plant leaves. It appears that using abscisic acid in controlling the mechanisms of plant growth is a good alternative for an ecologic agriculture given that recent studies on the mechanism of the abscisic acid action have shown its great importance as a bioregulator for plants.

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***IN VITRO* PROPAGATION OF BITTER GOURD (*Momordica charantia* L.)**

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Abstract

Bitter gourd (Momordica charantia L.) belongs to the family Cucurbitaceae. It is widely consumed as a vegetable and especially as a folk medicine in Asia. This review outlines the work done on the tissue culture of Momordica charantia L.. Commonly known as karela or bitter melon and contains bright red seeds due to high lycopene, a pigment that can be used as an artificial food colorant. Bitter gourd protein has been reported to have HIV inhibitor properties. The fruits of bitter gourd contain nutritionally useful essential minerals and amino acids. It has hypoglycemic activity which reduces the blood glucose, antitumor activity and antispermatogenic and androgenic activities. It is a common food item of the tropics and is used for the treatment of cancer, diabetes, AIDS and many ailments. It has also include alkaloids, insulin like peptides, and a mixture of steroidal sapogenins known as charantin. Bitter gourd is tolerant to a range of limiting factors of the environments and can be grown in tropical and subtropical climates. Improvement of this crop and development of new varieties are obviously necessary which could be done through the applications of modern techniques of biotechnology. Plant tissue culture is one of the biotechnological technique to culture plant cells or tissues under controlled aseptic conditions on artificial medium and is used to some degree in the improvement of almost every major agronomic, vegetable and fibre crop species. It has value in basic research like cell biology, genetic transformation studies and biochemistry for the production of medicinally valuable secondary metabolites. Some limited efforts have been made for the improvement of this crop using biotechnological techniques like: type of explants (auxin, cytokinin), media composition, growth conditions, genotypes.

Key words: *Bitter gourd, Momordica charantia L., propagation, tissue culture.*

INTRODUCTION

Bitter melon (*Momordica charantia* L.) is an annual, climbing, medicinal vine with small yellow flowers. It has cucumber-shaped fruits, 10-20 cm in size. The fruit has a warty structure and turns from green to orange-red color as it becomes ripe. Its seeds are 7-10 mm in length, flat and coarse. Its fruits and leaves are rich in iron, calcium, phosphorus and vitamin B. Its homeland is East India, and it is also grown in tropical regions of Caribbean, Africa and South America and in the Mediterranean countries including Turkey and Italy (Thiruvengadam et al., 2006). In Turkey, it is generally cultivated in Yalova, Bursa, in the Gazipaşa county of Antalya and the Silifke county of Mersin.

It is used in the treatment of stomach ulcer, eczema and other skin diseases, in viral diseases such as HIV and bacterial diseases. It has the ability to prevent tumor growth due to its lutein and lycopene content. It is also

regarded as an insulin plant because of its ability to regulate the control of blood sugar (Simina et al., 2016).

Plant tissue cultures are used for plant breeding, commercial production and basic biological researches (Agarwal M., 2015). Callus and suspension cultures are extremely useful methods to produce herbal products with economic importance in terms of raw materials, especially to the pharmaceutical industry for the production of secondary metabolites. Auxines are growth regulators that are frequently used in callus formation from explants.

The auxin group hormones are divided into two groups, "natural auxins" and "synthetic auxins". Natural auxines are indole-3-acetic acid (IAA), 4-chloro-indole acetic acid (4-CPA) and phenyl acetic acid (PAA). Synthetic auxins are naphthalene-acetic acid (NAA), β -naphthoxyacetic acid (BNOA), indole-3-butyric acid (IBA), 3-chlorophenoxypropionamide (3-CPA), 2,4-dichlorophenoxyacetic acid (2,4-D),

2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2-(2,4,5-trichlorophenoxy) propionic acid (2,4,5-TP) etc. (George et. al., 2008). 2,4-D is the most widely used auxin derivative for this purpose. Although *in vitro* propagation studies in plants (Al Munser et. al., 2007; Ananya et al., 2009; Nikam et. al., 2009, Malek et. al., 2010; Safdar et. al., 2013) have already been studied in bitter melon. Other techniques such as: *in vitro* callus regeneration, somatic embryogenesis, organogenesis and regeneration studies in bitter melon have not yet been conducted in Turkey. This research is the first study in this regard. In this study, *in vitro* and *in vivo* germination capacities of two genotypes of *Momordica charantia* L. and the effects and *in vitro* propagation of 2,4-D at different doses for callus formation on leaf and stem explants isolated from these conditions were investigated.

MATERIALS AND METHODS

The bitter melon seeds used in the study were obtained from local growers from Gazipaşa and Silifke in Turkey. Sodium hypochlorite (NaOCl) (commercial name ACE) was used for surface sterilization of seeds. The seeds were soaked for 15 minutes in solution at 20% NaOCl for *in vitro* cultivation and for 3 minutes at 10% NaOCl for *in vivo* cultivation. The stem and leaf explants were rinsed 3 times for 5 minutes each and the sterilization was completed. The seeds were then inoculated in MS (Murashige and Skoog, 1962) basal medium, each magenta vessel containing 5 seeds and 5 explants. Each application was carried out in 3 repetitions. Leaf and stem explants were cut to 1 cm size for using. As callus-forming medium, MS medium was supplemented with 2 mg/l, 4 mg/l, 6 mg/l and 8 mg/l 2,4-D. Also, 30 g/l sucrose and 5 g/l agar were added to the media. The pH of the medium was adjusted to 5.7 and sterilized at 121°C for 20 minutes under 1.5 atmosphere pressure autoclave conditions. The cultures were kept in the climate chamber at 25°C for 16 hours of light and 8 hours of darkness. Lighting was provided with white fluorescent lamps under 2000 lux light.

RESULTS AND DISCUSSIONS

In *in vitro* and *in vivo* germination studies conducted with the Gazipaşa and Silifke bitter melon genotypes, the seeds, following the sterilization, were cultured in MS nutrient media and pots (Figure 1) in climate chambers. The first germination time was at 8 days after the start of culturing in both media. Germination development was recorded on the 8th, 15th, 25th and 35th days.



Figure 1. The Gazipaşa and Silifke bitter melon genotypes growing in the pot in the climate (*in vivo*) room

The effect of different germination times on the germination rates (%) of the seeds of Gazipaşa and Silifke genotypes were analyzed using analysis of variance, germination rates (%) at different times were given for *in vitro* (Table 1) and *in vivo* (Table 2).

Table 1. Germination rates at different times under *in vitro* conditions (%)

Germination Time (days)	Germination ratio (%)	
	Gazipaşa	Silifke
8	17.0 ^c	12.0 ^b
15	17.0 ^c	16.0 ^a
25	22.0 ^b	16.0 ^a
35	28.0 ^a	16.0 ^a

Letters show different groups at 0.01 level.

As seen in Table 1, germination ratios ranged from 12 to 28% under *in vitro* conditions. Germination ratios were higher in the Gazipaşa genotype than those in the Silifke genotype in all time periods observed under *in vitro* conditions. The germination rates and duration in the germination medium increased in both

genotypes. This increase was more evident in the Gazipaşa genotype.

Under *in vivo* conditions, germination rates varied between 20 and 50% in the pots (Table 2). Germination rates under *in vitro* conditions were higher in the Gazipaşa genotype than those in the Silifke genotype in all time periods observed under *in vitro* conditions.

Table 2. Germination rates at different times under *in vivo* conditions (%)

Germination Time (days)	Germination ratio (%)	
	Gazipaşa	Silifke
8	40.0 ^c	20.0 ^c
15	47.0 ^b	23.0 ^b
25	47.0 ^b	27.0 ^a
35	50.0 ^a	27.0 ^a

Letters show different groups at 0.01 level.

In callus culture studies conducted with the Gazipaşa and Silifke bitter melon genotypes, leaf and stem explants were isolated from 9-10 day old plantlets growing *in vitro* and *in vivo* media, and cultured in nutrient media containing 2,4-D at different concentrations (2 mg/l, 4 mg/l, 6 mg/l and 8 mg/l) (Figure 2). Analysis of variance was performed with the data obtained at the end of four weeks and the results of callus formation rates are given in Table 3 (*in vitro*) and Table 4 (*in vivo*).

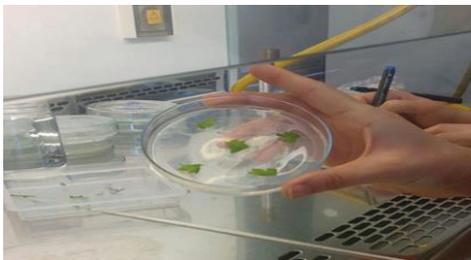


Figure 2. Cultivation of leaf explants of Gazipaşa bitter melon genotypes in MS + 2,4-D media

Table 3. Callus formation rate (%) after four weeks in leaf explants isolated from *in vitro* conditions

2,4-D (mg/l)	Callus Formation Rate (%)	
	Gazipaşa	Silifke
Control (MS0)	0.00 ^c	0.00 ^c
2.00	100.00 ^a	100.00 ^a
4.00	0.00 ^c	93.00 ^b
6.00	100.00 ^a	100.00 ^a
8.00	66.00 ^b	100.00 ^a

Letters show different groups at 0.01 level.

As shown in Table 3, the callus formation rate of leaves in the Gazipaşa and Silifke genotypes developed under *in vitro* conditions ranged from 0% to 100%. The highest callus formation rate in the Gazipaşa genotype was observed in MS(1962) nutrient media containing 2% and 4 mg/l 2,4-D at 100%. The highest callus formation rate in the Silifke genotype was also obtained from MS medium containing 2, 6 and 8 mg/l 2,4-D with 100%.

Table 4. Callus formation rate (%) after four weeks in stem explants isolated from *in vitro* conditions

2,4-D (mg/l)	Callus Formation Rate (%)	
	Gazipaşa	Silifke
Control (MS0)	0.00 ^c	73.00 ^b
2.00	0.00 ^c	47.00 ^c
4.00	33.00 ^b	47.00 ^c
6.00	73.00 ^a	87.00 ^a
8.00	0.00 ^c	67.00 ^b

Letters show different groups at 0.01 level.

As shown in Table 4, the callus formation rate of stems in the Gazipaşa and Silifke genotypes developed under *in vitro* conditions ranged from 0% to 100%. In both of the Gazipaşa and Silifke genotypes, the highest callus formation rate was obtained on MS medium containing 6 mg/l 2,4-D with 73% and 87%, respectively.

One of the somatic embryos developed from the callus of the Silifke genotype in the control group [MS0] nutrient medium from the stem explant formed plantlets and this plantlet were adapted to the external conditions in the climate chamber (Şekil 3.). In both genotypes, callus formation occurred at the maximum level from medium containing 6 mg/l 2,4-D and a decrease in callus formation was observed in the Silifke genotype at 8 mg/l 2,4-D dose.

Callus formation was not observed in the Gazipaşa genotype on MS0 and MS medium containing 8 mg/l 2,4-D.

As shown in Table 5, the callus formation rate of leaves in the Gazipaşa and Silifke genotypes developed under *in vivo* conditions ranged from 0% to 87%.



Figure 3. Adapting the plantlet obtained from the stem explants of the Silifke genotypes through indirect somatic embryogenesis from the callus formed in the MS0 nutrient medium to the external conditions in the pot

The maximum callus formation rate was observed in the Silifke genotype with 87%. In the same genotype, there was a continuous decrease in callus formation after a dose of 2 mg/l 2,4-D, and no callus formation was observed in the medium containing 8 mg/l 2,4-D. In the Gazipaşa genotype, callus formation was observed only in the MS medium containing 2 mg/l 2,4-D.

Table 5. Callus formation rate (%) after four weeks in leaf explants isolated from *in vivo* conditions.

2,4-D (mg/l)	Callus Formation Rate (%)	
	Gazipaşa	Silifke
Control (MS0)	0.00 ^b	0.00 ^d
2.00	53.00 ^a	87.00 ^a
4.00	0.00 ^b	40.00 ^b
6.00	0.00 ^b	7.00 ^c
8.00	0.00 ^b	0.00 ^d

Letters show different groups at 0.01 level.

As shown in Table 6, the callus formation rate of stems in the Gazipaşa and Silifke genotypes developed under *in vivo* conditions ranged from 0% to 73%.

Table 6. Callus formation rate (%) after four weeks in stem explants isolated from *in vivo* conditions

2,4-D (mg/l)	Callus Formation Rate (%)	
	Gazipaşa	Silifke
Control (MS0)	20.00 ^c	67.00 ^b
2.00	47.00 ^a	60.00 ^c
4.00	40.00 ^b	73.00 ^a
6.00	40.00 ^b	60.00 ^c
8.00	0.00 ^d	73.00 ^a

Letters show different groups at 0.01 level.

The highest callus formation rate in the Gazipaşa genotype was observed in MS food media containing 2% and 4 mg/l 2,4-D with 73%. In the Gazipaşa genotype, the highest callus formation occurred with 47% in the medium containing 2 mg/l 2,4-D, and callus formation started to decrease after this concentration. Callus formation was not observed on the medium containing 8 mg/l 2,4-D.

CONCLUSIONS

The difference in the germination rates of the Gazipaşa and Silifke bitter melon genotypes under *in vitro* and *in vivo* conditions may be due to seed shell hardness. The seeds of the Gazipaşa genotype are small, wrinkled and soft, while the seeds of the Silifke genotype are large, flat, and the shells are harder than those of the Gazipaşa bitter melon seed. The height of the plantlets growing under *in vivo* conditions in the pot were observed to be higher than that of the Silifke genotype. The germination rates of Gazipaşa and Silifke bitter melon seeds were high in both species under *in vivo* conditions. Also, under both conditions, the seeds of the Gazipaşa genotype showed higher germination.

In terms of callus formation rates, the media in which the explant was cultivated, 2,4-D concentrations, genotypes and explants were compared and a number of differences were observed in the obtained data. The highest callus formation was 100% in the explants obtained from *in vitro* media, whereas this rate was detected as 87% in the explants from *in vivo* media. The most effective 2,4-D concentration in explants taken from *in vitro* media was determined to be 6 mg/l in both genotypes and both used explants (leaf and stem). At the same time 2 mg/l 2,4-D in leaf explants caused maximum callus formation. The most effective concentration of auxin was found to be 2 mg/l in both types of leaf explants under *in vivo* conditions. The most effective concentration of auxin in the stem explant was 2 mg/l in the Gazipaşa genotype, 4 and 8 mg/l 2,4-D in the Silifke genotype, resulting in maximum callus formation. Also, it was observed that the calli of the Gazipaşa

genotype were more easily dispersed than those of the Silifke genotype. In both *in vitro* and *in vivo* media, callus formation was found to be 73% and 67% in Silifke genotype, respectively, whereas it was 20% in Gazipaşa genotype under *in vivo* conditions.

The callus formation in control group was not detected at leaf explants for both culture conditions. Callus formation was observed higher in the leaf explant than that in the stem explant in both media from which the explant was taken.

However, some plantlets were obtained by indirect somatic embryogenesis from callus developed from the stem explants of genotype Silifke and not from the leaf explants.

Although this is the case, one plantlet was obtained from callus formed from the stem explants of the Silifke genotype through indirect somatic embryogenesis and not from the leaf explants. The obtained plantlet was taken from *in vitro* conditions to the pots and transferred to external conditions and adaptation was maintained.

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EFFECTS OF SUCROSE CONCENTRATIONS ON INCREASE IN BULB SIZE OF *IN VITRO* REGENERATED HYACINTH (*Hyacinthus orientalis* L.) BULBLETS

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Abstract

In vitro regenerated bulblets of *Hyacinthus orientalis* L., improved their size using MS medium containing different sucrose concentrations. The sucrose concentrations showed higher growth and better development of the bulblets on MS medium stored at 24 °C. Sucrose showed the highest performance rate at 9 % concentration for both small and large bulblets. Sucrose treatments showed positive effects of induction of bulb diameter, number of shoots per bulb, shoot length and bulb weight in the culture medium.

Key words: *Hyacinthus orientalis*, bulb size, carbohydrate, growth, micropropagation.

INTRODUCTION

Hyacinthus species, mostly natives of the mild climate of the Mediterranean region, are cultivated for their strong fragrant flowers (Nowak and Rudnicki, 1993).

Among the three species of *Hyacinthus*, *H. orientalis* is the only commercially important one (Rees 1992, Nowak and Rudnicki (1993) but the natural production rates of their bulblets are very low and the number of the bulblets developed in the scale segments of mother bulbs are also very small (Bach et al., 1992).

Under natural conditions, axillary bulbs develop at the base of the mother bulb; small numbers and could start to bloom only after 2–3 years of cultivation under ideal conditions of no biotic and abiotic stress. (Smigielska and Jerzy, 2013).

The techniques and methodologies used in plant tissue-culture are playing a very important role in basic and applied scientific studies (Brown and Thorpe, 1995).

Therefore, there is need to use these techniques in effective way for increased and rapid multiplication of plants in parallel to the traditional multiplication techniques.

This could offer alluring options for commercial multiplication of numerous geophytes especially bulbous plants (Bach and Sochacki, 2013).

Plant tissue additionally offers an exceptionally useful pathway for rapid clonal multiplication of slow multiplying elite plant species like *hyacinth*.

Explants under *in vitro* culture conditions require exogenous carbon as energy source for their growth and differentiation because they lack complete autotrophism. Although sucrose is the most generally used carbon source with the concentration of 2-3% under *in vitro* studies, other types of carbon sources are also used (Huang and Okubo, 2005).

Sucrose is often assumed to be the best choice of carbon source in cell and tissue culture media because it is the main sugar translocated in the phloem of many plants (Peterson et al., 1999). However, there are a number of plants that can grow on other sources of carbons for plant regeneration. Regeneration via organogenesis or somatic embryogenesis was stimulated in a number of plants on the media containing glucose, fructose, maltose, mannose or sorbitol (Bach, 1992, Hossain et al., 2013). There are some reports that compared the response to carbon sources for growth and development of some bulbous plant species like Bach et al. (1992), who reported that glucose and sucrose containing medium was superior to fructose containing medium in induction of shoots and bulblets.

The aim of this study was to understand, the role of different sucrose concentrations in increasing bulblet development in hyacinth.

MATERIALS AND METHODS

Plant materials and experiments

The study made use of *in vitro* regenerated *H. orientalis* bulblets obtained in the previous study (Kizil et al., 2016). (Figure 1a) regenerated on MS medium containing 0.05 mg/l TDZ and 0.10 mg/l NAA or 0.10 mg/l TDZ and 0.10 mg/l NAA under *in vitro* conditions (primary medium).

To obtain desired increase in bulblet size, they were cultured on MS basal medium (Murashige and Skoog, 1962) containing with 0.00, 30, 60 or 90 g/l sucrose (w/v) and solidified with 6.2 g/l agar (w/v) in Magenta GA⁷ vessels. The pH of all cultures medium was adjusted to 5.6 - 5.8 with 0.1 M KOH or 0.1 M HCl before autoclaving at 121°C, 104.5 kPa for 20 min.

Hardening and acclimatization

Well-developed bulbs were washed thoroughly in running tap water transferred to 250 ml plastic pots containing sterilised peat moss under controlled greenhouse conditions at temperature (24° ± 1°C) and light 3000 lux (16/8 h photoperiod) conditions for sprouting and growth.

Potted plantlets were covered with transparent plastic bags to ensure high humidity and each

bulb was given 100 ml water every day for 15 days during acclimatization.

All cultures were placed in Fitotron growth chamber (Fitotron SGC 120; Epinal Way, Loughborough, UK) with 16 h of cool white fluorescent light (Philips lamps TLD 36 W/54, Hungary) at a photon flux density of 35 μmol/ m²/s per day.

Statistical analysis

All experiments made use of 60 explants equally divided into 10 replications. Statistical analysis was performed using IBM SPSS 22 program for windows by comparing means using One Way ANOVA. All values expressed in percentage were arcsine transformed before statistical analysis (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

Results

It was made sure to measure initial bulblet diameter before culturing using different concentrations of sucrose as mentioned in Materials and Methods (Table 1 – Figure 1a). Thereafter, 9 weeks on the culture medium the final increase in bulb diameter and weight was made (Figure 1b, c). The results showed that cultured small and large bulblets of hyacinth on MS medium supplemented with different concentration of sucrose (control, 30, 60 and 90 g l⁻¹) showed increase in all growth parameters that were significantly ($p \leq 0.05$) different except for shoot length (Table 1).

Table 1. Bulbils diameter and weight at the beginning and end of the study

Sucrose amount (g l ⁻¹)	Initial				Final							
	Bulb diameter (cm)		Bulb weight (g)		Bulb diameter (cm)		Number of shoots per bulb		Shoot length (cm)		Bulb weight (g)	
	Small	Large	Small	Large	Small	Large	Small	Large	Small	Large	Small	Large
Control (0)	0.46	0.60	0.32	0.95	0.46 c	0.59 d	2.48 c	4.33 b	0.67	1.14	0.43 c	1.16 d
30	0.43	0.58	0.25	0.93	0.47 c	0.76 b	3.25 c	4.83 b	0.68	1.17	0.44 c	1.52 c
60	0.43	0.64	0.26	1.01	0.75 b	0.69 c	5.00 b	4.86 b	0.75	1.18	0.50 b	1.73 b
90	0.50	0.68	0.32	1.08	0.87 a	1.21 a	18.16 a	6.83 a	1.06	1.26	0.54 a	2.02 a
Mean	0.46	0.63	0.29	0.99	0.63	0.81	7.22	5.21	0.79	1.19	0.48	1.61

Means within a column followed by the same letter are not significantly different according LSD test at $p \leq 0.05$.

Bulblets were categorized depending on their size, those ≥ 0.5 cm (0.58 -0.68 cm) were called as large bulblets and those ≤ 0.5 cm (0.43-0.50 cm) were called small bulblets. The weight of small bulblets changed between 0.25 and 0.32

g and those of large ones changed between 0.93 - 1.08 g.

Irrespective of sucrose concentrations used in the study, Sucrose at 90 g l⁻¹ concentration exhibited maximum gain in bulb diameter

compared to the other concentrations tested for enhancing diameter of bulblets of *H. orientalis*. Increase in bulb diameter for small bulblets was determined between 0.46 - 0.87 cm, while for large bulblets, the bulb diameter was determined between 0.59 - 1.21 cm. Bulb diameter for both sizes increased depending on sucrose concentration in the culture medium. Moreover, shoot length for small size bulbils varied between 0.67 - 1.06 cm, and for large size bulbils between 1.14 - 1.26 cm. Bulb weight gain is very important for healthy bulbs production. During two months duration, bulblets, the small bulbs had difficulty in weight gain compared to the large bulbs. The results showed an average gain of 0.19 g weight for small size over initial bulb weight compared to the large sized bulblets that had mean weight gain of 0.62 g bulblets if compared to initial weight at the start of the experiment. This could also be said that the large bulbs were more prone to weight gain compared to small bulbs.

Hardening and acclimatization

These bulbs rooted during hardening on peat moss without treatments with any auxin (Figure 1d). Thakur et al. (2002) reported that for hardening *in vitro* rooted bulblets of *Lilium*, peat moss gave 100% survival. Gong et al. (1996) obtained 80–90% survival rate of tissue culture plants of *Lilium* × Connecticut King when transplanted in potting peat moss. The present work was the first attempt to increase bulb diameter and acclimatise them under semi arid conditions with 100% survival rate.

Discussion

In the present study bulblets induced on 0.05 and 0.1 mg l⁻¹ TDZ and 40 and 80 g sucrose amount in previous studies were used (Kizil et al., 2016).

Sucrose is the most common carbon source as well as an osmotic agent for plant tissue and organ culture. It also supports the maintenance of osmotic potential and the conservation of water in cells. However, high sucrose concentration in the media restricts the photosynthetic efficiency of cultured plants by reducing the levels of chlorophyll, key enzymes for photosynthesis and epicuticular waxes promoting the formation of structurally

and physiologically abnormal stomata (Hazarika, 2006).

Variable concentrations of sucrose are used for increase of bulb diameter and weight gains. The reason of this argument should be that sucrose is stored in bulb scales in the form of starch and therefore the availability of this carbohydrate source in the medium may account for the weight increase of bulbs (Santos et al., 2006).

There are many positive results on different bulb increment using different sucrose concentrations. Sun et al. (2012) reported that *Lilium davidii* var. *unicolor* had 100% percent gain in bulblet formation at the sucrose concentration of 100 g l⁻¹, while the diameter and weight increment of bulblets came to the maximum.

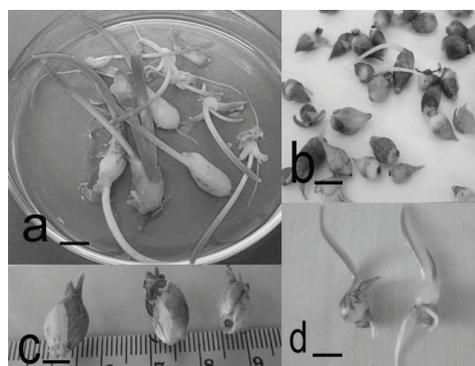


Figure 1. Increasing bulblet diameter of *H. orientalis* under *in vitro* conditions (a) The *in vitro* regenerated *H. orientalis* bulblets regenerated on MS medium containing 0.05 mg/l TDZ (b, c) enhanced bulblet diameter after 9 weeks on the culture medium containing 90 g l⁻¹ (d) rooted bulbs after hardening on peat moss without treatments with any auxin. Bar; Fig 1 a, c = 0.5 cm, Fig 1 b, d = 0.7 cm

Bach et al. (1992) have reported that concentration and variety of sugar affected shoot and bulblet regeneration and glucose- and sucrose-containing medium was superior to fructose-containing medium in induction of shoots and bulblets.

The bulbs belonging to many species develop dormancy to survive under unfavourable surrounding conditions during a period that starts from late autumn to winter and both under *in vitro* and *ex vitro* conditions. This dormancy could be broken by low temperature or vernalisation treatment (Langens-Gerrits et

al., 2003). During this period levels of endogenous abscisic acid (Djilianov et al., 1994; Yamazaki et al., 2002) and sucrose (Hobson and Davies, 1978; Aguetaz et al., 1990) in bulbs are linked with the development of dormancy and its release.

Santos et al. (2006) noted that if bulbs produced on IBA and IBA+BA containing media (irrespective of their size) were transferred onto the same basic medium without growth regulators by increasing sucrose level from 3% to 6%, they promote both rooting and further growth of the bulbs. Furthermore, they found that bulbs attained a mean diameter of 8 mm and medium with 6% sucrose was the most adequate medium to promote bulb enlargement with well-developed root system.

CONCLUSION

Medium without growth regulators and with sucrose increased to 9% (90 g l⁻¹) led to the growth of the bulbs and roots as well in 9 weeks. Thereafter, the bulbs obtained on the MS medium containing different sucrose were planted in a soil mixture in pots. Where the transplanted bulbs showed leaves above the soil, indicating that the bulb dormancy had been broken. Transplantation success was high; with an average survival rate 80-90%.

In conclusion, the data obtained in this study showed that sucrose doses increased from 6-9% could be usefully used to increase the bulb size of this important plant and could be used to overcome the problem of low natural bulb size obtained during multiplication.

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SURFACE RESPONSE OPTIMIZATION OF SUBMERGED BIOMASS PRODUCTION FOR A PLANT BIOSTIMULANT *Trichoderma* STRAIN

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Abstract

We describe the optimization of cultivation medium composition for biosynthesis of *Trichoderma asperellum* T36 biomass. This *Trichoderma* strain is a multifaceted one, being antagonist against major plant pathogens, stimulating vegetables growth, enhancing bioactive accumulation into nutraceutical crops and promoting development on early stages of the plants cultivated into high residues system. To improve the eco-efficiency of T36 based bioproducts production, a maximum conversion of cultivation medium components into fungal biostimulant biomass is required. We optimize the cultivation medium through a designed experiment, based on surface response methodology, wherein several components of cultivation medium were modified in the same time. The studied components were: glucose (carbon source), ammonium sulphate (inorganic nitrogen and sulphur source), soymeal and yeast extract (complex, organic nitrogen source and growth factors source), potassium mono- and di-hydrogen-phosphates (as phosphorus source and buffering ingredients). The optimal composition of cultivation medium, resulted after experimental results analysis, is: 34.2 g. l⁻¹ glucose, 0.37 g. l⁻¹ ammonium sulphate, 0.8 g. l⁻¹ yeast extract, 2.7 g. l⁻¹ soymeal, 1.2 g. l⁻¹ K₂HPO₄, 1.7 g. l⁻¹ KH₂PO₄. We compared the tolerance to dry-flowable formulation of *T. asperellum* T36 biomass produced on optimized liquid media and on liquid Weidling media. The biomass produced on optimized media has a better tolerance to dry-flowable formulation than biomass produced on Weidling media. Also, the preservation of biological activities, specific to T36 strain, is better on dry-flowable formulation produced with biomass resulted from optimized medium than that with biomass resulted from liquid Weidling medium. Higher tolerance to dry-flowable formulation and better survival rate, of formulated T36 propagules, suggest that optimized medium promote formation of more resilient fungal resting structures. A techno-economic analysis was performed for the optimized cultivation medium.

Key words: *Trichoderma*, biostimulant strain, cultivation medium, optimization, surface response methodology.

INTRODUCTION

Despite positive effects associated with the use of agrochemicals on costs and yield efficiency in various agriculture systems, it is now recognized that this practice presents major environmental sustainability issues (Carvalho, 2006; Gomiero *et al.*, 2011).

Among alternatives to agrochemicals, with significant lower potential impact on environment, are included microbial based bioproducts (Pérez-García *et al.*, 2011).

Strains from *Hypocrea* genera, usually known by the name of their anamorph forms, *Trichoderma*, are the most used fungal active ingredients into such agricultural microbial

products, being marketed as biopesticides, biofertilizers, plant strengtheners and plant biostimulants (Woo *et al.*, 2014).

Initially *Trichoderma* strains, selected on their *in vitro* antagonism against fungal plant pathogens, were proposed to be used as biopesticides/biofungicides, due to their proven high biocontrol efficacy on field trials (Sesan *et al.*, 1999; Elad, 2000; Benitez *et al.*, 2004). However, the plant protection effects of fungal strains from *Trichoderma* genera are not related only to biocontrol through microbial antagonism. *Trichoderma* was considered also an opportunistic avirulent plant symbiont, which activate plant defence mechanisms (Harman *et al.*, 2004). Stimulation of natural

mechanisms involves both jasmonic acid-ethylene (JA/ET) and salicylic acid (SA) signalling pathways (Nawrocka and Malolepsza, 2013) and is done through the action of various elicitors, including MAMPs, microbial-associated molecular patterns, and DAMPs, damage-associated molecular patterns, (Hermosa et al., 2013).

Several *Trichoderma* strains have been marketed as biofertilisers (Kaewchai et al., 2009), mainly due to their effects on phosphate and micronutrients bioavailability (Altomare et al., 1999) or on nutrients (mainly nitrogen) use efficiency (Harman, 2011). However, plant growth promotion by *Trichoderma* strains results also from other mechanisms, beside enhanced nutrient availability / use efficiency. Such mechanisms include production of plant hormones, as indole acetic acid, indole-3-carbaldehyde, indole-3-acetaldehyde, indole-3-ethanol (Contreras-Cornejo et al., 2009), plant hormones modulators like ACC-deaminase (Zhang et al., 2017) or bioactive secondary metabolites – e.g. harzianolide and 6*n*-pentyl-6*H*-pyran-2-one (6PP) (Vinale et al., 2008), chrysophanol (Liu et al., 2016), cremonolide (Vinale et al., 2016).

The above mentioned multilevel actions on plants of plant beneficial fungal *Hypocrea* / *Trichoderma* strains was proposed to be better described as plant biostimulants activities (Lopez-Bucio et al., 2015). Plant bio-stimulants represent a new category of agricultural inputs, which enhance plant tolerance to biotic and abiotic stress, promote plants growth, improve nutrient uptake and nutrient use efficiency, influence yield quality (Brown and Saa, 2015; du Jardin, 2015). Despite their regulatory status, which is not fully defined on important markets, e.g. European Union, plant biostimulant market will reach \$2,241million by 2018, with a compound annual growth rate of 12.5% (Calvo et al., 2014).

Our group focused on the last years on selection and applications of *Hypocrea* / *Trichoderma* plant biostimulants strains (Oancea et al., 2014; Răut et al., 2015; Şesan et al., 2015). We developed new formulations for our selected plant biostimulants *Trichoderma* strains, a dry-flowable formulation (Oancea et al., 2016a) and a hydro-gelified and film forming formulation (Oancea et al., 2016b),

intended to be used mainly on conservation agriculture/high residues farming and for nutraceutical crops.

The holistic approach of sustainability for agro-systems (Arodudu et al., 2017) involves the eco-design of used inputs, aiming an increased eco-efficiency, with a lower carbon footprint. For manufacturing process of bioproducts, especially for those based on *Trichoderma* biostimulant strains and intended to be used into low inputs agricultural systems, eco-efficiency requires also a maximum conversion of low costs cultivation media ingredients into fungal biomass / fungal propagules.

The main objective of this study was to optimize, in term of submerged fungal biomass / fungal propagules productivity, the composition of a liquid cultivation media, for a *Trichoderma* biostimulant strain from our collection, *T. asperellum* T36, through a designed experiment, based on surface response methodology. A first derived objective was to establish the influence of submerged cultivation, into resulted medium with optimal composition, on T36 propagules tolerance to formulation, T36 propagules survival during storage of formulated bioproduct and preservation of T36 specific activities during bioproduct storage. Final derived objective was to make a techno-economic analysis of the biostimulant *Trichoderma* biomass production, on the optimized medium.

MATERIALS AND METHODS

Plant biostimulant Trichoderma strain. We used on our study a strain from ICECHIM collection, *T. asperellum* T36 NCAIM F 001434, a multifaceted, plant biostimulant strain. This strain is antagonist for major plant pathogens (Răut et al., 2014b), produces bioactives volatile compounds, including 6PP (Răut et al., 2014a), protects and stimulates vegetables growth (Răut et al., 2016), accelerate the degradation of lignocellulose material, enhances bioactive accumulation into nutraceutical crops and promotes development on early stages of the plants cultivated into high residues system (Răut et al., 2015).

Optimization of cultivation medium composition. We optimize the cultivation

medium through a designed experiment, based on surface response methodology, wherein several components of cultivation medium were modified in the same time. These studied components were: glucose (carbon source), ammonium sulphate (inorganic nitrogen and sulphur source), soymeal and yeast extract (complex organic nitrogen and growth factors source), potassium mono and di-hydrogen-phosphates (as phosphorus source and buffering ingredients). Design of Experiments (DoE) approach was utilized for optimization of biomass production. Cultivation medium components, considered as independent factors into DoE, were coded as following: glucose-X1; ammonium sulphate-X2; dipotassium phosphate-X3; mono-potassium phosphate-X4; yeast extract-X5; soymeal-X6, were assigned as independent variables, as shown in Table 1. Each independent factor was tested at three levels, with -1 designated as the lower level, 0 as the centre and +1 as the upper level. Twenty individual experiments were performed with different factor configurations. We used a specialised software, Design Expert® v.10.0 (Stat-Ease, Minneapolis, MN, USA), as support for experiments design.

Table 1. Independent factors considered for the biomass optimization of *T. asperellum*

Factor (g/l)	Name	Factorial levels		
		-1	0	1
X1	glucose	15	25	35
X2	(NH ₄) ₂ SO ₄	0.20	0.30	0.40
X3	K ₂ HPO ₄	1	1.2	1.4
X4	KH ₂ PO ₄	1	1.4	1.8
X5	yeast extract	0.5	0.7	0.9
X6	soymeal	1	2	3

To ensure reliable data collection and evaluation, several non-variable factors were taken in consideration. The *T. asperellum* T36 strain was cultivated in a submerged medium under continuous aeration and stirring. On each experiment 100 ml of liquid medium was distributed in 500 ml Erlenmeyer flasks, sealed with cotton plugs. The flasks were incubated at 25°C and agitated at 100 rpm. After 7 days, the resulted biomass was separated from the culture medium using an ultra-filtration membrane. The resulted biomass was weighted after drying at 105°C for 4 hours.

Dry-flowable formulation. We used a process for dry-flowable formulation, described in details elsewhere (Oancea *et al.*, 2016a). Briefly, this process includes the following main steps: (i) encapsulation of biostimulants *Trichoderma* biomass into soft alginate microbeads; (ii) drying encapsulated fungi with antioxidant protection and (iii) mixing the resulted spray-dried flowable powder, with polyvinyl alcohol, as adhesive agent, lecithin as wetting agent and a CO₂ generating dispersant system, based on polyacrylic acid, citric acid and sodium bicarbonate. We applied this dry-flowable formulation process to T36 wet biomass, obtained after cultivation for 7 days, at 25°C and 100 rpm, from resulted DoE optimal media or from liquid Weidling medium, as was already presented (Sesan and Oancea, 2010).

Assay of water activity on stored dry-flowable formulation. We determined the water activity on the dry-flowable formulations, made with T36 biomass produced on resulted DoE optimal medium and on liquid Weidling medium, by using a water activity meter (4TE, AquaLab, Pullman, WA, USA). These determinations were done each two month, for bioproducts maintained on room temperature, on closed dark plastic bottles.

Determination of viable propagules. We performed monthly determination of viable propagules, on the stored dry-flowable formulations, made with T36 biomass produced on DoE optimal medium and on liquid Weidling medium. The determination was done by cultivation on a selective media (Williams *et al.*, 2003), which contains, beside the minimal nutrients sources, biocides with selective action toward *Trichoderma* strains: 1.5 ml.l⁻¹ formulated propamocarb (Previcur 607 SL, Bayer Crop Science, 607 g active ingredient per litre); 0.15 g.l⁻¹ Roz Bengal (sodium salt, R3877 Sigma, Sigma-Aldrich), 0.2 g.l⁻¹ pentachloronitrobenzen (quintozene, P2205 Sigma, Sigma-Aldrich), 9 ml.l⁻¹ of stock solution of streptomycin (1% weight/volume, S6501 Sigma-Aldrich), 0.25 g chloramphenicol (C0378 Sigma-Aldrich). For these determinations, we used, beside the reagents supplied by mentioned producers, reagents from EMD Millipore.

Determination of in vitro antagonistic characteristics. We determine the antagonistic activity, resulted from volatiles produced by dry-flowable formulations based on T36, grown on potato-dextrose agar, by using the double plate sandwich confrontation assay (Räut *et al.*, 2014a). Into this confrontation assay the used plant pathogen fungal strain was *Fusarium graminearum* DSM 4527. We made these determinations monthly, on the room temperature stored T36 formulations, obtained with biomass produced on DoE optimal medium or on liquid Weidling medium.

Assay of 6PP production. We sampled 0.1 g of each dry-flowable formulations, immediately after formulation process and after 12 month of storage. The formulation samples were cultivated on potato-dextrose broth for 10 days. We homogenized with a blender (Waring[®], Laboratory Blender, Fischer Scientific, Waltham, MA, USA) the resulting mycelium within culture medium. We determined the biomass from homogenate gravimetrically, after filtration on filter paper Whatman No. 1 and drying at 105°C. The homogenate was extracted twice with dichloromethane, CHCl₂, 1 part of homogenate to 1 part of CHCl₂. We reunited the extracts, we vacuum dried (Rotavapor, Buchi, Flawil, Switzerland) and we re-suspended in 0.1 ml CHCl₂. We determined the 6PP content by gas-chromatography (El-Hasan *et al.*, 2007), using an Agilent 700 gas chromatograph, equipped with quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA) and a standard curve, made with pure 6PP (Sigma-Aldrich).

Preservation of plant material degradation activity. We organized an experiment wherein we recorded the oxygen consumption and release of various compounds from lignocellulose material, treated with the tested dry-flowable formulations, made with biomass produced on DoE optimal medium or on liquid Weidling medium. We introduced aseptically into sterile Erlenmeyer flasks the following: 0.1 g of lignocellulose material (wheat stem, dried, grounded to 0.25 mm and sterilized by gamma-irradiation), 19 ml of sterile water, pH 5.5, and 0.1 g of flowable formulation, with 5×10^9 cfu per g. We determined the oxygen consumption after incubation for 48 hours on room temperature, by using an oxygen

fluorescence probe (Ocean Optics, Halma, Amersham, UK). We recovered the supernatants and we determined: reducing carbohydrates, with DNS reagent (King *et al.*, 2009); Total Organic Carbon (TOC), with a HT Formacs apparatus (Scalar Analytical, Breda, Netherlands), using the batch method (Trulleyová and Rulík, 2004) and total soluble phosphorus (Self-Davis and Moore Jr, 2000). We performed the experiments with a control, which include same determinations, done on the lignocellulose material not-inoculated with dry-flowable formulations based on *Trichoderma* plant biostimulant strains. We made these determinations on the beginning and on the end of 12 months' storage period.

Techno-economic analysis. We used a model for *Trichoderma* industrial cultivation previously developed for cellulases production (Klein-Marcuschamer *et al.*, 2012), based on a cascade of batch biosynthesis, each bioreactor providing 5% inoculum to the next bioreactor in the cascade. Stoichiometry of the conversion of culture medium components into plant biostimulant *T. asperellum* T36 biomass is that calculated per bellow Equation 1. The residence time for each bioreactor was 96 h. We considered the costs of the main agro-industrial raw materials, glucose, soymeal and yeast extract, used for the *Trichoderma* growing media, as being the average price for such agricultural commodities on European Union during the last year (EC, 2017). For inorganic ingredients of cultivation medium, ammonium sulphate and potassium mono- and di-hydrogen-phosphates, we considered the price for fertilizers (EC, 2017). Costs were expressed in USD.

Statistical analysis. We made all experiments in triplicate, in complete randomized blocks. Colony forming units, cfu, per g or per ml, were log-transformed, with the calculation of standard errors. We used comparison on average survival percentage and not in log cfu. g⁻¹, because propagules producing biomass was produced from different batches. We established statistical relevance by ANOVA and linear mixed model (Bolker *et al.*, 2009). We used a specialised software, Design Expert[®] v.10.0 (Stat-Ease, Minneapolis, MN, USA), for analysis of the results from designed experiments based on surface response

methodology. We used Excel software (Office 365 - Excel 2016, Microsoft, Redmont, WA, USA) to make calculations and to draw figure.

RESULTS AND DISCUSSIONS

We performed twenty individual experiments with different factor configurations, based on independent factors, codes as in Table 1, which produced different quantities of biomass, expressed as dry biomass, $g.l^{-1}$ (Table 2). Even on the most effective independent factor combination *Trichoderma* dry biomass yield is still under threshold of $10 g.l^{-1}$. We analysed the experimental data containing the independent factors, by using a specialised software, Design Expert® v.10.0 (Stat-Ease).

Table 2. Independent factors and corresponding three level design space

Test	Independent variables						Biomass ($g.l^{-1}$)
	X1	X2	X3	X4	X5	X6	
1	-1	-1	-1	-1	-1	-1	6
2	1	-1	-1	-1	1	-1	6,1
3	-1	1	-1	-1	1	1	7,8
4	1	1	-1	-1	-1	1	8,4
5	-1	-1	1	-1	1	1	7,2
6	1	-1	1	-1	-1	1	7,4
7	-1	1	1	-1	-1	-1	9
8	1	1	1	-1	1	-1	7,9
9	-1	-1	-1	1	-1	1	6,5
10	1	-1	-1	1	1	1	6,9
11	-1	1	-1	1	1	-1	8,6
12	1	1	-1	1	-1	-1	7,5
13	-1	-1	1	1	1	-1	7,8
14	1	-1	1	1	-1	-1	6,3
15	-1	1	1	1	-1	1	9
16	1	1	1	1	1	1	9,1
17	0	0	0	0	0	0	7,3
18	0	0	0	0	0	0	7,1
19	0	0	0	0	0	0	7,2
20	0	0	0	0	0	0	7,2

We established a full factorial DoE, with two levels. We arrive to a reduced 3FI design model, by imposing a constraint of 20 levels, which include 4 centre points. Model effects were determined from the half-normal plot. These include: X1, X2, X3, X4, X6, X1:X3, X1:X4, X1:X6. The last three terms show that the glucose content in the beginning interacts statistically with the K_2HPO_4 , KH_2PO_4 and soymeal levels. We can also observe that the yeast extract content (X5) does not interact significantly with the glucose content.

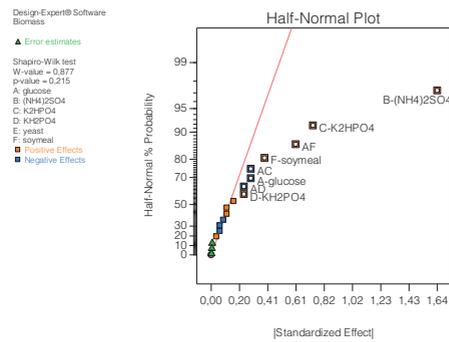


Figure 1. Half-normal plot generated by Design Expert 10 for determining individual term effects

We made an ANOVA analysis, to assess how well the proposed statistical model fits. In Table 3 we can see the calculated p-value for the model and for each individual term.

Table 3. P-values for model and individual terms

Source	Sum of Squares	df	Square	Value	p-value
Model	16.12	8	2.01	28.03	< 0.0001
X1	0.33	1	0.33	4.6	0.0551
X2	10.73	1	10.73	149.2	< 0.0001
X3	2.18	1	2.18	30.27	0.0002
X4	0.23	1	0.23	3.14	0.1041
X5	0.6	1	0.6	8.36	0.0147
X1*X3	0.33	1	0.33	4.6	0.0551
X1*X4	0.23	1	0.23	3.14	0.1041
X1*X6	1.5	1	1.5	20.88	0.0008
Residual	0.79	11	0.072		
Lack of Fit	0.77	8	0.096	14.45	0.0252
Pure Error	0.02	3	6.67E-03		
Cor Total	16.91	19			

The values are considered acceptable and validate the data resulted for the optimal composition of cultivation medium used for biosynthesis of *T. asperellum* T36 biomass. The coefficient of determination (R^2) is high enough (0.9532) for using the model for optimization purposes. The standard deviation is 0.27, while the coefficient of variation is 3.57, which further validates the model.

The final equation regarding actual factors was calculated and is presented here as Eq. 1.

$$Biomass = 4.558 - 0.013X1 + 0.082X2 + 0.08X3 + 0.238X4 - 0.242X6 - 1.438E - 003 \cdot X1 \cdot X3 - 5.938E - 003 \cdot X1 \cdot X4 + 0.01 \cdot X1 \cdot X6$$

Eq. 1

The optimal composition of cultivation medium, resulted after experimental data analysis with Design-Expert® v10.0, is: 34.2 g. l^{-1} glucose, 0.37 g. l^{-1} ammonium sulphate, 0.8

g. l⁻¹ yeast extract, 2.7 g. l⁻¹ soymeal, 1.2 g. l⁻¹ K₂HPO₄, 1.7 g. l⁻¹ KH₂PO₄. This composition is closed to the upper level of the considered independent factors and is on agreement with others recently reported optimization of biosynthesis processes based on *Trichoderma* strains, wherein low costs meals, from oleaginous seeds, were used as complex nitrogen sources of cultivation media (Gao *et al.*, 2013; Almeida *et al.*, 2015).

We further used the optimal medium composition, resulted from designed experiments based on surface response methodology (DoE optimal medium), for dry-flowable formulation.

The formulation process was done by using an already published process (Oancea *et al.*, 2016a), which includes the following main steps: (i) encapsulation of biostimulants *Trichoderma* biomass; (ii) drying encapsulated biomass; and (iii) mixing the resulted spray-dried flowable powder with formulation ingredients. We applied this dry-flowable formulation process on T36 wet biomass, obtained on DoE optimal medium, after cultivation for 7 days, at 25°C and 100 rpm, or on liquid Weidling medium, as was already described (Sesan and Oancea, 2010).

We determined on stored dry-flowable bioproducts, obtained with T36 biomass produced on DoE optimal medium or liquid Weidling medium, the water activity, the number of propagules and the antagonistic activity resulted from the production of volatiles compounds. A slightly increasing on water activity, which remains well below the threshold requested for xerophyte microorganisms (i.e. osmophilia yeasts) development into solid substrates, was demonstrated for both bioproducts stored for 12 months - Figure 2. Both bioproducts are stable and without risk to develop spoilage microorganisms.

Low water activity in our dry-flowable formulations is in concordance with others reports regarding formulation of spray-dried *Trichoderma* biomass (Jin and Custis, 2011; dos Santos *et al.*, 2015).

The survival of propagules from *T. asperellum* T36 biostimulant strain was good, for the tested period of storage of 12 months, on both bioproducts. During this storage period the T36 strain formulated into bioproducts maintained

its antagonistic activity toward *F. graminearum*, resulted from the production of volatiles compounds active against fungal pathogens (Figure 3).

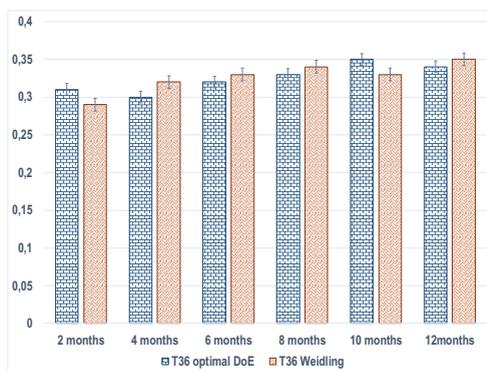


Figure 2. Evolution of the water activity of bioproducts, dry-flowable formulations of *T. asperellum* T36 biomass produced on optimal DoE medium or on Weidling medium

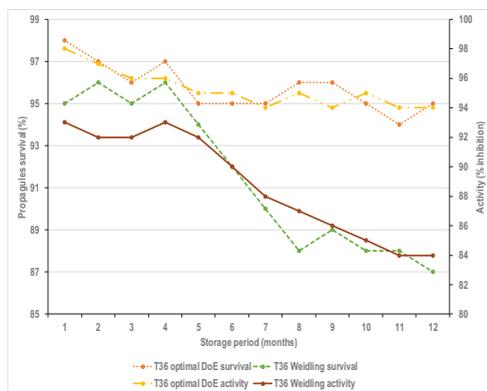


Figure 3. Survival and biological activity (% inhibition of growth of *F. graminearum* DSM 4527 by volatile) during storage of T36 propagules, produced on DoE optimal medium or on liquid Weidling medium and formulated as dry-flowable products

The survival rate and the biological activity against *F. graminearum* was significantly better for bioproduct which was made with biomass from DoE optimal medium. We determined also the preservation of the capacity to produce one of the major bioactive volatile compound, 6PP, 6*n*-pentyl-6*H*-pyran-2-one, which is toxic for fungal pathogens (El-Hasan *et al.*, 2007) and have a stimulant effect on plants (Vinale *et al.*, 2008). Both tested bioproducts retained their ability to produce 6PP after storage for 12 months – Table 4. *Trichoderma* propagules resulted from DoE

optimal medium preserved better the capacity to produce 6PP after storage as formulated bioproduct. The data suggest that plant-based, complex nitrogen sources, with high content of other bioactives ingredients, are more effective in promoting accumulation of fungal resting forms.

Table 4. Production of 6*n*-pentyl-6*H*-pyran-2-one (mg.g⁻¹ dry mycelia) by T36 strain recovered from bioproducts made with propagules produced on DoE optimal medium or on liquid Weidling medium

<i>T. asperellum</i> T36 DoE optimal		<i>T. asperellum</i> T36 Weidling	
Initial	After 12 months	Initial	After 12 months
84.38±14.65	74.12±9.27	82.41±12.38	54.64±10.78

Production of 6PP by our *T. asperellum* T36 biostimulant strain, recovered from dry-flowable bioproducts, is on the same level with those already reported for other *Trichoderma* strains (Kalyani *et al.*, 2000; Serrano-Carreón *et al.*, 2004; El-Hasan *et al.*, 2007). Cultivation on DoE optimal medium promote formation of propagules which maintain better the ability to produce 6PP after long term storage on dry-flowable formulation – Table 4.

Our plant biostimulants T36 strain was intended to be used for treatment of plant residues which are covering the soil on conservation agriculture systems (Räut *et al.*, 2015), thus we tested also the preservation of the capacity of propagules, produced on DoE optimal medium or on liquid Weidling medium, to degrade lignocellulose material. The obtained results demonstrate that DoE optimal medium produce propagules which are preserving better the ability to degrade lignocellulose material specific to CA system, i.e. wheat straw – Table 5.

Table 5. Capacity of *Trichoderma* biostimulants strains, formulated as dry-flowable products, to degrade lignocellulose material

Determination	<i>T. asperellum</i> T36 DoE optimal		<i>T. asperellum</i> T36 Weidling	
	Initial	After 12 months	Initial	After 12 months
Oxygen consumption (mg.dm ⁻³)	2.29±0.22	2.18±0.27	2.24±0.28	1.52±0.24
Soluble phosphorus (mcg.dm ⁻³)	18.76±2.12	17.84±1.54	18.58±1.74	14.92±1.81
Total organic content on supernatant (mcg.dm ⁻³)	4.37±0.48	4.18±0.34	4.54±0.63	3.83±0.23
Soluble reducing carbohydrates (mcg.dm ⁻³)	18.24±1.73	18.42±2.28	18.42±2.35	15.40±1.84

The results demonstrated that the biomass produced on optimized media have a better

tolerance to dry-flowable formulation than biomass produced on Weidling medium, preserving better the specific activities.

Trichoderma strains produce different types of propagules, mycelial fragments, conidia, (aerial or liquid), chlamydo spores, micro-sclerotia, with different bio-efficacy and/or storage stability (Mishra *et al.*, 2012). Usually, the *Trichoderma* bioproducts are made with aerial conidia, produced on moisten grains, by solid substrate fermentation (Woo *et al.*, 2014). Better survival rate and formulation stability was recently reported for chlamydo spores, produced on liquid medium, containing a plant-based complex nitrogen source, i.e. cornmeal (Li *et al.*, 2016), and for microsclerotia and submerged conidia, produced on liquid media, with high carbon concentration (36 g.L⁻¹) and with plant-based complex nitrogen source, i.e. cotton seed flour (Kobori *et al.*, 2015).

DoE optimal medium presents important characteristics related to accumulation of more resistant propagules: the level of carbon is high and contains a plant-based complex nitrogen source. This low costs, plant-based nitrogen source, is supplemented by an additional source of growth factors and complex / organic nitrogen, yeast extract, with a proven stimulatory effect on growth and development of *Trichoderma* strains (Rossi-Rodrigues *et al.*, 2009).

The breakdown of the optimised cultivation medium ingredients costs, resulted from the techno-economic analysis is presented in figure 4. To produce 1 kg of *T. asperellum* T36 biostimulant strain the total costs for medium ingredients is 2.45 USD. More than 75% of this cost with medium ingredients is for glucose, which represent almost 81.5% from the weight of the cultivation medium ingredients.

Medium ingredients represent 28% of the total operation costs for manufacturing bioproducts based on *Trichoderma* (Klein-Marcuschamer *et al.*, 2012). Thus, the cost for industrial production of biostimulant *Trichoderma* biomass is under 10 USD per kg. The value of formulation ingredients in on average on the same order. The price for the commercialisation for the field crop of such a bioproduct was considered 20 USD. kg⁻¹, with a 50% production margin (sale price double

than production costs). The treatment with a dose of 2 kg/ha of bioproduct based on biostimulant *Trichoderma* is around 40 USD/ha. The value of the additional yield resulted from such treatment on conservation agriculture systems is at least 100 USD.ha⁻¹ (Oancea, 2011), which make it profitable even for field crops.

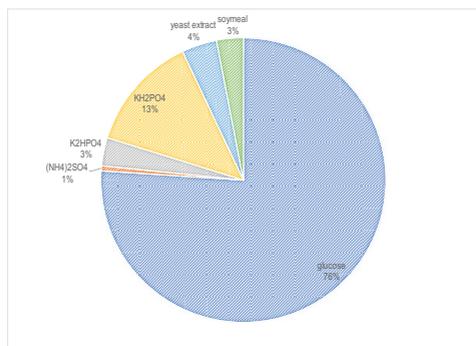


Figure 4. Breakdown of the optimised cultivation medium ingredients costs

Liquid Weidling medium contain a reach nitrogen source, bactopectone, produced by enzymatic treatment of animal protein. Our results demonstrate that a plant-based, complex nitrogen sources, with high content on other bioactives, are more effective in promoting accumulation of fungal resting forms more resistant to formulation, than nitrogen reach animal-based product. is on agreement with others recently reported optimization of biosynthesis processes based on *Trichoderma* strains, wherein low costs meals, from oleaginous seeds, were used as complex nitrogen sources of cultivation media (Gao et al., 2013; Almeida et al., 2015).

CONCLUSIONS

We used a designed experiment, based on surface response methodology, for the optimization of medium composition for submerged biosynthesis of biomass from a biostimulant strain, *T. asperellum* T36. The studied medium components were: glucose (carbon source), ammonium sulphate (inorganic nitrogen and sulphur source), soymeal and yeast extract (organic nitrogen and growth factors source), potassium mono- and di-hydrogen-phosphates (as phosphorus source

and buffering ingredients). The optimal composition of cultivation medium, resulted after experimental data analysis, is: 34.2 g. l⁻¹ glucose, 0.37 g. l⁻¹ ammonium sulphate, 0.8 g. l⁻¹ yeast extract, 2.7 g. l⁻¹ soymeal, 1.2 g. l⁻¹ K₂HPO₄, 1.7 g. l⁻¹ KH₂PO₄. We compared the tolerance to dry-flowable formulation of *T. asperellum* T36 biomass produced on optimized liquid media and on liquid Weidling media. The biomass produced on optimized media has a better tolerance to dry-flowable formulation than biomass produced on Weidling media. It seems that plant-based, complex nitrogen sources, with high fibres content, are more effective in promoting accumulation of fungal resting forms more resistant to formulation, than nitrogen reach animal-based products. The costs of the optimized medium ingredients determine a cost of biostimulant *Trichoderma* biomass production lower than 10 USD.kg⁻¹.

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OBTAINING MINITUBERS BY APPLYING METHOD OF CULTURE ON SUBSTRATES INDUSTRIAL

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Abstract

National Institute of Research and Development for Potato and Sugar Beet Brasov in 2016 INCDCSZ investigated two hydroponic systems to see the behavior of different potato Romanian varieties (Braşovia, Castrum, Marvis and Sarmis) regarding the following parameters: the number of minitubers/plant and weight of minitubers/plant. As hydroponic systems it was used one with circulating nutrient solution and another one with static layer of nutrient solution and for both cases the substrate used was perlite. Regarding the average weight of minitubers/plant, the plants culture on circulating nutrient solution had a beneficial influence comparative with culture on static stratum of nutrient solution, which recorded a highly significant difference in minituber weight compared to the first mentioned, statistically assured. Analyzing the number of minitubers using the hydroponic culture, relative to control variety (Braşovia with 5.10 minitub./pl.) shows that the Castrum variety gets the best results with a positive significant distinct difference (+5.90 minitub./pl.) followed by variety Marvis with a positive significant difference (+5.10 minitub./pl.). Varieties influence on weight minitub./pl. shows that the difference is very significant positive for Marvis variety (+42.49 g), compared to control variety and insignificant for the other varieties. From the obtained data we recommend using hydroponic system with nutrient solution circulating.

Key words: hydroponics, minitubers, nutrient solution, perlite, plantlets.

INTRODUCTION

The world's population increased greatly in last few decades. The improvement of living standard in many countries increased with the great demand for high value crops, off season supply and high quality products. Therefore, quality of life of people increased considerably. In this regard, protected agriculture which is a labor intensive industry can produce higher amount of food for the increased population of the world. The efficiency and quality of the agricultural produce can be increased through the modifications of the environmental controls, management of culture systems and use of technological innovations. The greatest advantage of soilless culture is that it allows direct control of the nutrient solution, possible to modify composition and concentration to achieve predictable results in relation to dry matter content, nitrate content or other organoleptic and structural features of the crop produce (Elia A. and colab. 1999 cited by, Asaduzzaman and colab. 2015). The soil is usually most available growth medium for plants. For a successful plant growth, soil

provides nutrients, air, water, etc. Changing the soil with another alternative growth medium tends to be expensive.

Sometimes, the soil may create limitations on the growth of plants. Some of these are: the presence of disease-causing organisms and pests, inadequate soil reaction, unfavorable soil compaction, poor drainage, degradation due to erosion, etc. (Hussain et al., 2014).

Hydroponic method application has increased significantly in recent years, worldwide, because it allows a more efficient use of water and fertilizer, and better control of climate and nuisance factors (Trejo-Téllez and Gómez-Merino, 2012).

By using the hydroponic method increases crop quality and productivity, leading to increased competitiveness and economic income (Trejo-Téllez and Merino Gómez-2012). Using hydroponic systems offers an excellent alternative by reducing the potential risk of roots contamination with pathogens and tubers from the soil and eliminating chemical disinfectants, which are generally very harmful to human health and the environment (Scherwinski-Pereira et al., 2009).

Horticultural crops "without soil" is now a true peak of high-performance technologies that have already gained a leading position in world agriculture crop production (Atanasiu, 2007). In conditions of our country, in recent years there has been increased interest in these unconventional technologies culture that open attractive prospects for professional growers (Atanasiu, 2007).

Advantages of hydroponic culture: cultures can be grown where there is no suitable soil or where the soil is contaminated with pathogens; watering and other traditional practices are largely eliminated; possible maximum yields, which makes the system without soil to be economically feasible in high density; conservation of water and nutrients is a feature for all systems; this will reduce pollution; a more complete environmental control is a general feature of the system.

MATERIALS AND METHOD

The study was done in 2016 in Laboratory of Vegetal Tissue Culture, National Institute of Research and Development for Potato and Sugar Beet Brasov.

The biological material used in the study consisted in: meristems for obtain a material free of virus. After 6-8 months biological material, by *in vitro* subculture, was represented by plantlets, from Braşovia, Castrum, Marvis and Sarmis varieties.

Potato seed starts from a virus-free meristem located in the apex growth (meristem dimensions are 0.1-0.2 mm). Meristematic explants are inoculated in test tubes containing Murashige-Skoog (1962) medium.

After a period of 6-8 months, depending of genotypes, from meristems are developed vitroplants (plantlets) (fig. 1).

To ensure the phytosanitary accuracy DAS ELISA test is performed.

The infected plantlets are eliminated and we multiplied *in vitro* only the healthy ones. *In vitro* multiplication is performed in sterile conditions, by segmenting at every internode and minicuttings obtained are inoculated on fresh Murashige-Skoog medium for the formation of new plantlets.

After obtaining a sufficient number of plantlets, these plants are planted in hydroponic system

(fig. 2) with circulating nutrient solution (a) and static stratum of nutrient solution (b) in protected space "insect-proof".

The experience was bifactorial, 4x2 and number of repetitions was 5: experimental factor A, type of culture in hydroponic system with 2 graduations: a₁- culture on circulating nutrient solution; a₂ - culture on static layer of nutrient solution; experimental factor B, cultivar with 4 graduate: b₁- Braşovia; b₂- Castrum; b₃- Marvis; b₄- Sarmis.



Figure 1. Plantlets



a



b

Figure 2. Planting *in vitro* material in hydroponic culture: with circulating nutrient solution (a) and static stratum of nutrient solution (b)

Other materials used in experience: a recirculating hydroponic system which can reuse unabsorbed nutrient solution during the process of irrigation, containing: basin, tray, pots, pump, supply system; culture tanks

(trays) for pots with industrial substrate made of galvanized sheet with sides 0.9 m and height of 10cm ferry with a stopper drain and refresh with new solution; perlite used as substrate for plant rooting; fertilizers: nutrient solutions for culture without soil „Universol“.

In the first stage our aim was to have a higher concentration of nitrogen, then in the second stage to have a higher concentration of phosphorus and potassium, respecting levels specified in the technical prospectus in accordance with the needs of plants in N, P₂O₅, K₂O, MgO, and micro elements, namely: Yellow Universol 12 + 30 + 12 + 2 MgO + micro elements (at a concentration of 0.5 to 1.0 g/l); Violet Universol 9 + 9 + 27 + 9 MgO + micro elements (at a concentration of 0.5 to 1.5 g/l).

Phases of potato seed production program by using hydroponic culture method are:

In the first year: tubers of the best clones of varieties are selected; the tubers are washed and kept in laboratory conditions, to light for sprouting; in February-March meristems are taken from tubers shoots and placed on the growth medium in tubes; subcultivation of meristems; in August and September from meristems are formed plantlets by multiplying stem cuttings; meristematic plantlets are tested; healthy cuttings are multiplying further (and kept in tubes).

In the second year: multiplication of plants from January to April; transferring of plantlets in "insect-proof" space is performed in May, on industrial substrates; the growth medium is treated with preventive insecticide, as a precaution against aphids; is perform regular control of system to ensure accurate phytosanitary of plants is achieved ELISA; in the beginning of August the drip system is interrupted to stop the growth of plants; in late August - beginning of September is harvested minitubers; minitubers are located in the storage space (at a low temperature: 4⁰C), the net bag;

The third year: minitubers are planted in isolated field (clonal field).

Haulms were cut two weeks before harvesting, then was performed harvesting, counting, and determining the average weight of minitubers/plant.

RESULTS AND DISCUSSIONS

Using method of analysis of variance, results obtained for variants planted in two variants of hydroponic system (Table 1) were interpreted statistically. For the first parameter, the average number of minitubers, the analyzed values were similar, the difference was not significant, statistically assured (-2.90 for minitubers obtained in system with static stratum of nutrient solution).

Table 1. Influence of hydroponic system used on the minitubers number obtained

Hydroponic system	The average number of minitubers produced/plant		Differences	Significance
	Number	%		
System with circulating nutrient solution (Ct)	9.80	100.00	-	-
System with static stratum of nutrient solution	6.90	70.40	-2.90	ns

DL 5%= 2.96;

DL 1%= 4.96;

DL 0.1%= 9.17

Table 2. Influence of hydroponic system used on the weight of minitubers obtained/plant

Hydroponic system	The average weight of minitubers produced/plant		Differences	Significance
	g	%		
System with circulating nutrient solution (Ct)	57.91	100.00	-	-
System with static stratum of nutrient solution	26.39	45.58	-31.52	ooo

DL 5%= 7.05 g; DL1%= 11.67g; DL 0.1%= 21.84 g

For the two types of systems in which minitubers were produced (Table 2), the difference in system with static stratum of nutrient

solution reached -31.52 minitubers/plant, compared to the circulating solution, which is very significant negative, statistically assured.

For obtain minituber with high weight/plant it is recommended the system with circulating nutrient solution.

Regarding of the influence of variety (Table 3), by comparison with the control variety Brasovia is noted that Castrum variety had a distinct significant difference, positive by +5.90 minitub./plant and Marvis variety had a significant differences positive +5.10 minitubers/plant.

About the weight of minitub./plant (Table 4), statistical interpretation presents a very significant positive difference, statistically assured for Marvis variety (42.49 g).

In case of combined influence of hydroponic system and varieties studied (Table 5), a

significant difference positive for number of minitubers, statistically assured was obtained for Marvis variety (+7.4 minitub/pl.) in case of culture with circulating nutrient solution. In hydroponic system with static stratum of nutrient solution Castrum variety recorded a significant difference positive (+7.2 minitub./pl.). Between the two hydroponic system models Marvis variety recorded a very significant negative difference in case of nutrient solution with static stratum (-7.20 minitub./pl). It can be seen that the values are higher for system with circulating nutrient solution, only Castrum variety presented the same number of minitubers in both cultures.

Table 3. The influence of variety on the average number of minituber obtained in hydroponic system

Variety	Number of minitubers/plant		Differences	Significance
	Number	%		
Brasovia (Ct)	5.10	100.00	-	-
Castrum	11.00	215.69	+5.90	**
Marvis	10.20	200.00	+5.10	*
Sarmis	7.10	139.22	+2.00	ns

DL 5% = 3.87; DL1% = 5.26; DL 0.1% = 7.04

Table 4. The influence of variety average weight of minitubers/plant in hydroponic system

Variety	The average weight of minitubers produced/plant		Differences	Significance
	g	%		
Brasovia (Ct)	31.54	100.00	-	-
Castrum	28.57	90.58	-2.97	ns
Marvis	74.03	234.71	+42.49	***
Sarmis	34.46	109.27	+2.92	ns

DL 5% = 16.75; DL1% = 22.77 ; DL 0.1% = 30.51

Table 5. Influence of variety and hydroponic system on the average number of minituber obtained/plant

Hydroponic system / Variety	System with circulating nutrient solution, a1			System with static stratum of nutrient solution, a2			Dif. a2-a1
	Number of minitub/plant	Dif	Sig	Number of minitub/plant	Dif.	Sig	
Brasovia b ₁	6.40	-	-	3.80	-	-	-2.60 *
Castrum b ₂	11.00	+4.6	ns	11.00	+7.2	*	0.00 ns
Marvis b ₃	13.80	+7.4	*	6.60	+2.8	ns	-7.20 ooo
Sarmis b ₄	8.00	+1.6	ns	6.20	+2.4	ns	-1.80 ns

DL 5% =5.47 (minitub.)
DL 1% = 7.44 (minitub.)
DL 0,1% =9.96 (minitub.)

DL 5% =2.18 (minitub.)
DL 1% =3,12 (minitub.)
DL 0,1% =4,61 (minitub.)

Statistical analysis on the influence on weight minitubers obtained (Table 6) shows that Marvis variety has a very significant positive difference of + 80.9 g. Comparing the two systems, only Sarmis variety obtain minitubers with a higher weight/pl., recording a significant

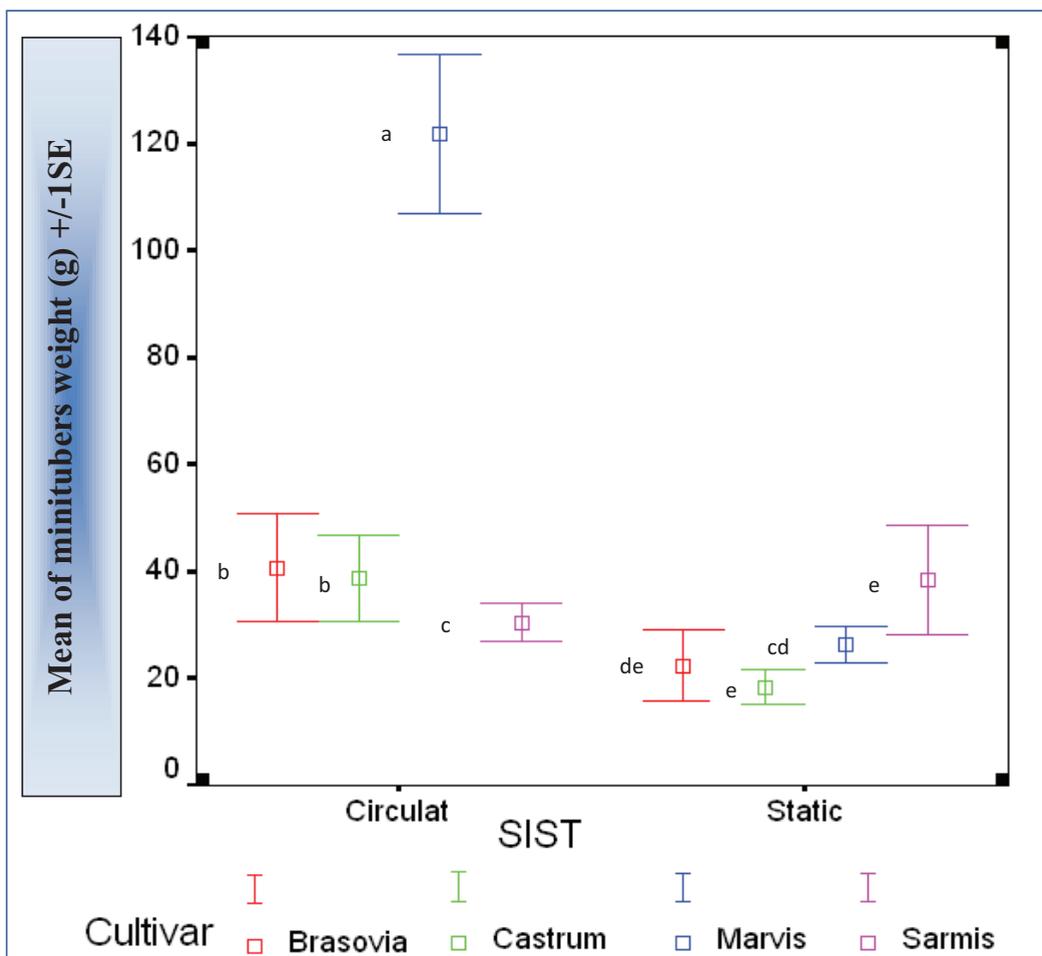
positive difference (+7.92) in case of culture on static stratum; for the other varieties, the differences are very significant negative, this aspect showing us the beneficial influence of system with circulating nutrient solution regarding the weight of minitubers.

Table 6. Influence of variety and hydroponic system on the average weight of minituber/plant (g)

Hydroponic system / Variety	System with circulating nutrient solution, a1			System with static stratum of nutrient solution, a2			Dif.
	Average weight of minituber (g)	Dif.	Sig	Average weight of minituber (g)	Dif.	Sig	a2-a1
Brasovia b ₁	40.70	-	-	22.38	-	-	-18.32 ooo
Castrum b ₂	38.81	-1.89	ns	18.31	-4.07	ns	-20.5 ooo
Marvis b ₃	121.60	+80.9	***	26.44	+4.06	ns	-95.16 ooo
Sarmis b ₄	30.50	-10.2	ns	38.42	+16.04	ns	+7.92 *

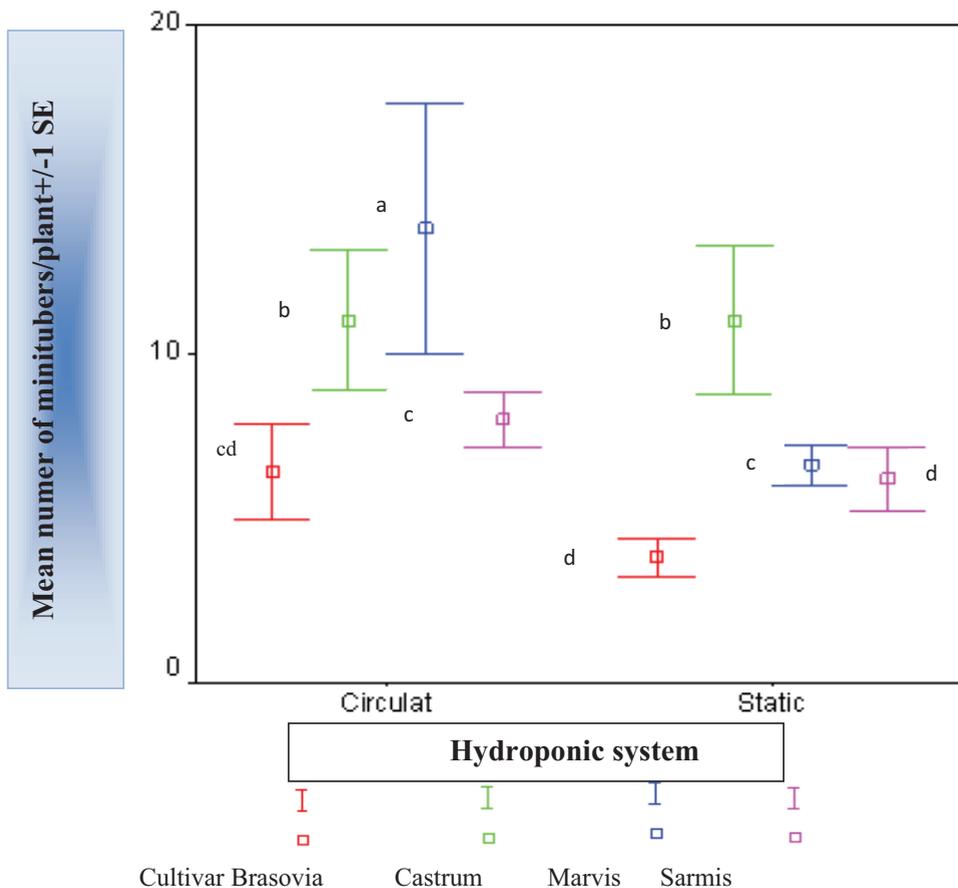
DL 5% =23.70 (g)
DL 1%= 32.21 (g)
DL 0,1%=43.14 (g)

DL 5% =6.55 (g)
DL 1%=9.26 (g)
DL 0,1%=12.87 (g)



The letters indicate differences between variants according of the ANOVA and Duncan test (P <0.05)

Figure 3. Hydroponically number of minitubers/variety was higher in system with circulating nutrient solution than in static stratum



The letters indicate differences between variants according of the ANOVA and Duncan test ($P < 0.05$)
 Figure 4. Hydroponically weight of minitubers/variety was higher in system with circulating nutrient solution than in static stratum

CONCLUSIONS

In conclusion, results of the presented study show that system with circulating nutrient solution can be a suitable system of producing potato pre-basic seed and its optimization may be considered as a strategic investment with the aim of promoting a more efficient and sustainable production of high quality potato minitubers.

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ACHIEVEMENTS IN WINTER PEAS BREEDING PROGRAM

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Abstract

The development of the winter pea crop represents a major challenge to expand plant protein production in temperate areas. Breeding winter cultivars requires the combination of freezing tolerance as well as high seed productivity and quality. In this paper we present data obtained from the F3 and F4 lines of winter peas from the four hybrid combinations (Specter/F95-927; F98-492/Windham; F95-927/CHECO; Specter/CHECO) obtained in the NARDI-Fundulea. A number of 121 lines (81 F3 lines and 40 F4 lines descendants from F3) selected from winter/winter and winter/spring crosses pea genotypes, have been tested in two years 2015 and 2016 in the field of NARDI Fundulea. At these lines were determined winter hardiness, earliness, yield and plant height comparing with the winter peas control (Specter, Checo and Windham). The results of this preliminary study suggested that it is possible in the breeding of winter peas to realize, in the same time, a genetic progress for high yield, good level of winter hardiness, plant height and earliness.

Key words: winter pea, breeding, winter hardiness, yield.

INTRODUCTION

Field pea (*Pisum sativum* L.) is one of the most important grain legumes in the world. Its grain is a major source of plant-based dietary protein for animals.

Kosev (2010) said that field pea can provide protein-rich feed and improve the sustainability of organic systems. The share of agricultural land that is under organic agriculture approaches 4% in EU and 7% or more in Scandinavian countries, Italy, Austria and Greece, and it may reach 25% in EU by 2030.

Field pea continues to be an important crop worldwide both for food and feed and as a rotational crop with other cultures. The pea breeding programs are based on yield and yield components. Pea grain yield is a quantitative trait which is affected by many genetic and environmental factors.

Cultivars adapted to winter sowing have been developed and deployed in Europe and north-west USA giving the potential for better yields because of a longer growing season, higher biomass production, and earlier maturity to avoid late season drought and heat stress (Hanocq et al., 2009). The introgression of the

Hr allele, which delays flower initiation until after the main winter freezing period have passed, permitted to obtain some cultivars with notably improved winter hardiness (Lejeune-Hénaut et al., 2008).

Delayed floral initiation helps some forage pea genotypes to escape the main winter freezing periods, as susceptibility to frost increases during the transition to the reproductive state (Lejeune-Hénaut et al. 1999). Numerous studies describe the physiological and phenological effects of the main loci governing the transition to flowering in pea, such as *Lf* and *Hr*, known to delay floral initiation of autumn-sown peas until a longer day length is reached in the following spring (Lejeune-Hénaut and Delbreil 2009). The oldest winter pea cultivars carry the dominant allele, *Hr*, but some of them have *hr* allele (Bourion et al. 2002). A study of one population of recombinant inbred lines (RILs) allowed detection of six quantitative trait loci (QTL) for frost tolerance, which is in agreement with oligogenic determinism of frost tolerance in pea. In this population, the most explanatory QTL was found to colocalize with the *Hr* locus (Lejeune-Hénaut et al. 2008). Further studies in

the same genetic background gave an insight in the genetic determinism of physiological traits potentially involved in cold acclimation, showing for example the colocalization of QTLs for raffinose concentration or RuBisCO activity with QTLs for frost tolerance on linkage groups 5 and 6 (Dumont et al. 2009).

Winter peas have a good level of frost resistance, and are also characterized by a large foliage development in spring which favours lodging and fungal diseases in humid conditions (Lejeune-Heaut et al., 1999).

In temperate climate, winter crops are the majority and frost damage is an important factor reducing crop yields, especially in regions where winter is regularly severe (Biarnès et al., 2016). The winter forage pea varieties are suitable for arid regions. The utilization of winter forage pea in the conditions with a high water deficit, results in higher and more stable aboveground biomass and protein yields, enhancement of the ratio between symbiotic and fertilizer nitrogen in organic farming and the possibilities for more economic use of the agricultural land (Castel et al., 2017).

Winter peas may soon become an alternative increasingly more reliable and profitable crop canola. Genetic variation has been reported for vegetative-stage frost tolerance (Swensen and Murray, 1983; Bourionet et al. 2003), and there has been some genetic analysis of differences for frost tolerance, and six chromosomal regions affecting winter frost tolerance were identified. Genetic analysis of frost tolerance is a prerequisite for the development of lines that are tolerant to frost at reproductive stages (Shafiq, 2012).

The aim of this work was to appreciate the yield performance and other traits, mainly the winter hardiness of several winter pea lines in the climatic conditions from NARDI-Fundulea.

MATERIALS AND METHODS

In this study the plant material used initially consisted in 19 lines from each of the four hybrid combinations (Specter/F95-927; F98-492/Windham; F95-927/CHECO; Specter/CHECO) and 5 parents, tested in two years (2015 and 2016) at NARDI Fundulea. In 2015, the first 81 F3 peas lines were tested in four preliminary micro-cultures, each of

them with 25 variants, one rep besides checks (parents of these lines: Specter, Windham, Checo, F95-927 and F98-492), on a plot of 6m² harvested area.

In 2016, 40 lines of peas F4 generation (descendants from F3 generation presented above) were tested in preliminary micro-cultures, each of them with 25 variants, one rep besides (parents of these lines), on a plot of 6m² harvested area. The lines from F4 generation are more advanced and more stable than lines from F3 generation.

The two years of testing were climatically different, so that, 2015 winter was slight enough, with a short period with negative temperatures of - 20°C (the end of January), with a snow layer of 20 cm which has protected the crop. There are no damages registered due to frost. The early spring was normal, fact that led to restart the vegetation under optimum conditions.

In contrast to the 2015, the 2016 winter was more severe, with a short period with negative temperatures of - 14,5°C (the beginning of January), without snow, which realised a good differentiation of genotypes regarding the winter hardiness. The early spring, allowed the restart the vegetation in the beginning of February, with an average air temperature higher with 9,5 °C than normal during 50 years. Under these conditions, the peas genotypes were highly differentiated versus other years, regarding flowering precocity depending their reaction to daylight. The varieties peas originating from USA, Specter and Windham cultivars, with a requirement of a longer photoperiod – 12 hours/day - did not pass to the generative stage, in spite of the high temperature registered in the beginning of February.

The level of resistance to winter hardiness was estimated in the field, early in the spring, in a scale 1 to 9, where score 1 is very resistance and 9 very susceptible. Plant height was measured in cm, total length of plant from the ground till the top to the end of flowering time. The earliness was appreciated like number of days from 1st January till the end of flowering time and yield as kg/ha.

The statistical analyses of data have been evaluated by ANOVA, correlations and linear regressions between study traits.

RESULTS AND DISCUSSIONS

Yield performances of 40 peas lines of F4 generation and 81 lines F3, tested in advance trials and respectively in preliminary trials in 2015 and 2016, (Figure 1 A and B) showed a large variation.

Among of F3 lines (bulk) 27% of them and 31% from the F4 lines, have achieved superior yields than the winter checks, Windham, Specter and Checo. There are several lines which realized 4.5 till 5 t/ha. That means it is possible to be realize an important genetically progress for yield from such germplasm.

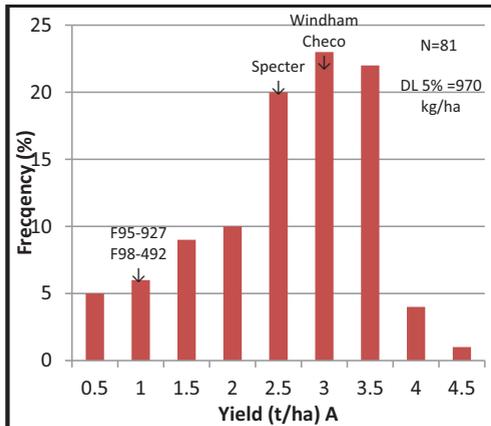


Figure 1.A. Distribution of 81 F3(A) winter pea lines after their yield performances (t/ha) in 2015

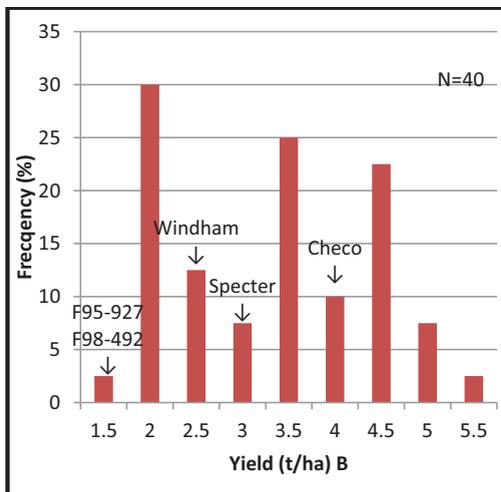


Figure 1.B. Distribution of 40 F4 winter pea lines after their yield performances (t/ha) in 2016

Also, it is very important to select the perspective lines with good yield potential but in the same time to recombine an acceptable earliness for Romanian climate conditions. The distribution of the 40 of F4 lines for precocity (Figure 2), divides the breeding material in two groups: late forms which take this trait from American peas cultivars Windham and Specter, and early forms similar with spring peas parents F98-492 and F95-927 and respectively winter peas cultivar Checo.

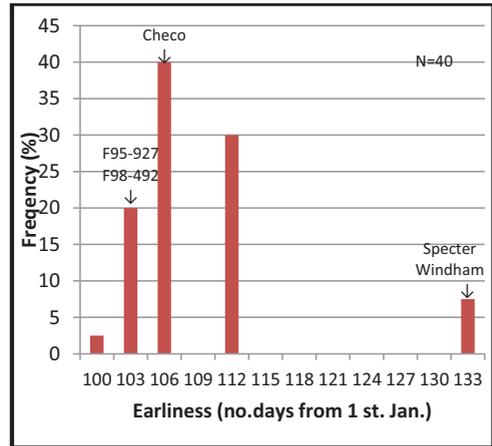


Figure 2. Distribution of 40 F4 winter pea lines after their earliness

Based on molecular assay, using molecular marker AA175, the parents used in this study have split into two groups: Specter and Windham carry *Hr* allele and Checo, F95-927, F98-492 carry *hr* allele (data not shown).

According to this distribution, the most of the lines, 82.5% approximately, were in the early group.

The distribution of the 40 lines from F4 generation for plant height (Figure 3) indicated the presence of pea lines with different plant height, from 80 cm till 210 cm. Most of them are shorter than tallest parent Specter, only a few have been the same plant high like Specter. What it is interesting that many of the lines had intermediate plant height, between short plant parents, Windham and Checo and height plant parent Specter. That means the possibility to realize the new varieties with different plant height, that being important for end use of this varieties, for seed or for silage.

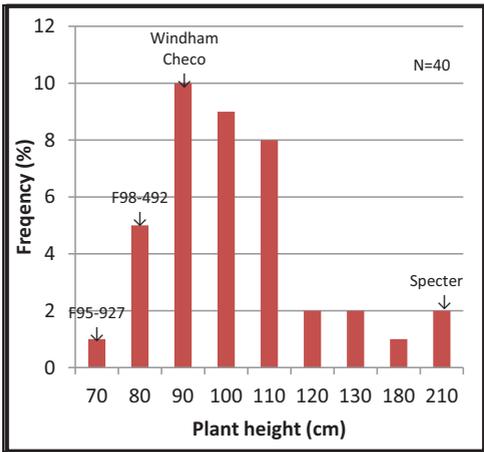


Figure 3. Distribution of 40 F4 winter pea lines after their plant height (cm)

The data for winter hardiness presented in figure 4, suggest that it is easier enough to recover the winter hardiness level like winter parents, in the many lines, even from the winter/spring crosses. From the distribution of the 40 F4 lines, it is possible to see that 60% of them are the same level of winter hardiness like winter parents, Checo, Windham and Specter.

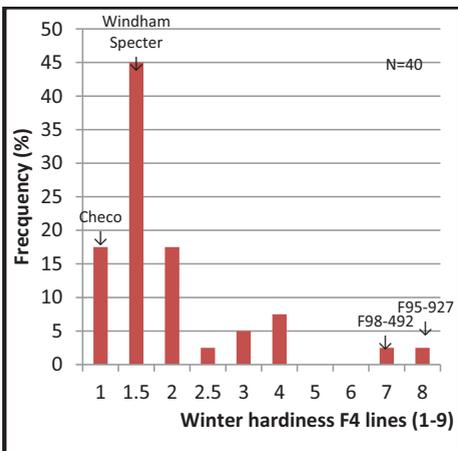


Figure 4. Distribution of 40 F4 winter pea lines after their winter hardiness

Having in view that winter hardiness in winter peas is a very important trait, there was necessary to highlight in what way this trait could be recombined with other important agronomical characteristics, like earliness to

flowering, plant height, grain yield as well as the relationship among other traits as plant height/earliness or yield/earliness. Correlations between different characteristics in 81 F3 lines from 2015 and 40 F4 lines from 2016 were presented in Table 1.

The correlation between winter hardiness and yield in F3 and F4 lines was very distinct significantly negative ($r=-0.54$ and $r=-0.60$) (Table 1 and figures 5 A and 5 B), what means that in winter peas is absolutely necessary to cultivate genotypes with good level of winter hardiness, to realize high and stable yields. Also relationship between plant height and earliness should be positively strong enough in some case, what means that it quite easily to be recombine such characteristics. The correlation, between plant height and winter hardiness, was only in F4 lines negative significantly ($r=-0.32$), but in the F3 lines, was not significant, that suggests possibility to select the genotypes which recombine both traits.

Table 1. Correlation coefficients among different traits in F3 and F4 lines of the four hybrid combinations

The generation genotypes	Correlation between different characters	The correlation coefficient
81 F3 lines tested in micro-cultures in 2015	Winter hardiness/yield	-0,54***
	Winter hardiness/earliness	-0,05ns
	Winter hardiness/plant height	-0,02ns
	Plant height/earliness	0.45***
	Yield/earliness	0.19ns
40 F4 lines tested in micro-cultures in 2016	Winter hardiness/yield	-0.60***
	Winter hardiness/earliness	-0.21ns
	Winter hardiness/plant height	-0.32*
	Plant height/earliness	0.28ns
	Yield/earliness	-0.32*

The relationship between yield and earliness, in F3 and F4 peas lines was insignificant and negative significant, (0.19 and -0.32), what means that later types can realize high yield than earlier types.

Also, the relationship between winter hardiness and earliness, in the all cases, was not significant, that means it is too easy to select the winter pea form with both traits.

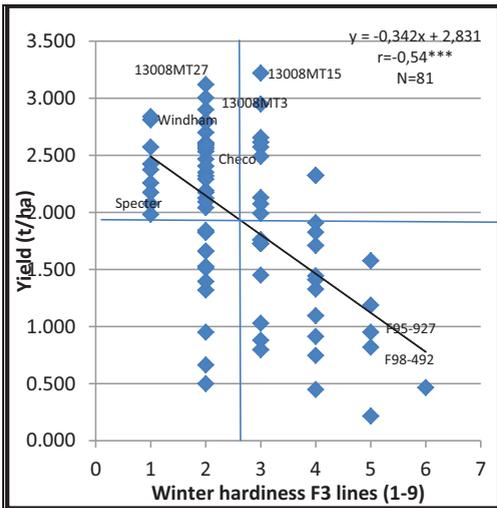


Figure 5.A. Relationship between winter hardness and yield data of 81 F3 lines

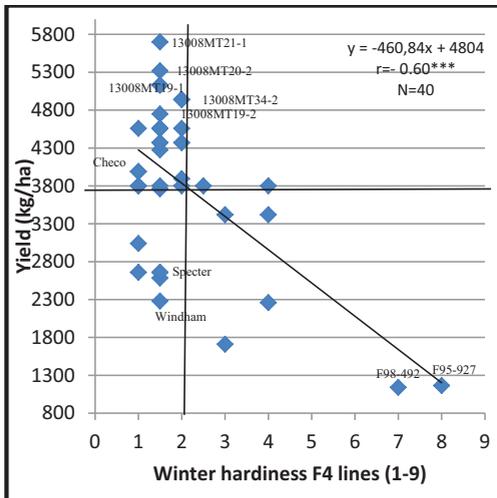


Figure 5.B. Relationship between winter hardness and yield data of 40 F4 lines

The correlation distributions between yield and winter hardness of 81 F3 lines and of the 40 F4 lines tested in 2015 and 2016 respectively, presented in Figure 5A and 5B, indicated, in the both cases, that there are lines with the same level of winter hardness like winter parents but with higher yield than this. Such lines are from combination hybrids F95-927/Checo, from F3 were 13008MT2, 13008MT1 and 13008MT5

and from F4, 13008MT21-1, 13008MT20-2, and 13008MT19-1.

CONCLUSION

The data obtained in this study on the F3 and F4 random lines from four hybrid combinations (Specter/F95-927; F98- 492/Windham; F95-927/CHECO; Specter/CHECO) suggested the possibility to achieve simultaneously a genetic progress for high yield, good level of winter hardness, plant height and earliness, useful in the breeding of winter peas.

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POTENTIAL USE OF KOMBUCHA CRUDE EXTRACT IN POSTHARVEST GRAPE MOULDS CONTROL

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Abstract

Postharvest diseases of fruits and grapes are caused by fungi and bacteria and the losses in this step increase several folds than in the field. In the case of the grapes, most damages are due to the presence of filamentous fungi belonging to species like Botrytis sp., Penicillium sp. or Aspergillus sp. Several non-chemical treatments have been proposed for fungal decay control, including acetic acid. Our experiments have targeted the potential inhibitory effect of different tea sourced Kombucha crude extracts on the most common moulds of the grapes in pre and postharvest steps. Kombucha is known mainly as a consortium (SCOBY - symbiotic acetic/lactic bacteria and yeast). Because of the presence of acetic bacteria the final content in acetic acid of Kombucha suspensions vary between 8.5 and 17 g/l. Kombucha tea suspension has been prepared starting from three different source of tea plants, respectively green tea, green tea with Melissa officinalis L. and oolong tea. The most significant inhibition has been registered in the case of Botrytis cinerea (38 -55%), less significant on Penicillium expansum (4-8%) and not significant on Aspergillus flavus and Aspergillus carbonarius. It is proposed further to investigate the inhibition of Kombucha extracts, in vivo, on artificially infected grape berries with Botrytis cinerea and to validate the in vitro results.

Key words: grape, postharvest, mould, Kombucha crude extract.

INTRODUCTION

Postharvest diseases of fruits and grapes are caused by fungi and bacteria and the losses in this step increase several folds than in the field (Coates et al., 1997; Scholberg, 2009; Meneses et al., 2014). In the case of the grapes, most damages are done by the presence of filamentous fungi belonging to species like *Botrytis* sp., *Penicillium* sp., *Aspergillus* sp. or *Rhizopus* sp. (Scholberg, 2009). Postharvest diseases of the grapes are controlled by the application of sulphur dioxide, either by weekly fumigation in storage rooms or by packing grapes in polyethylene-lined boxes with sulphur dioxide generator pads (Hafez, 2008). Several non-chemical treatments have been proposed for fungal decay control. Although these approaches have been shown to reduce postharvest rots of fruit or grapes each has limitations that can affect their commercial applicability (Tripathi et al., 2004). The exploitation of natural products to control fruits postharvest diseases and to prolong their storage life has received special attention in the last decade. Biologically active natural

products such as flavour compounds, acetic acid, propolis, chitosan or plant extracts, have the potential to replace synthetic fungicides in postharvest (Tripathi et al., 2004; Xu et al., 2007; Scholberg, 2009; Meneses et al., 2014). Acetic acid can be employed with no restriction as preservative and represents a possible substitute to sulphur dioxide. The use of acetic acid in postharvest treatments have been reported for citrus fruits (Venditti et al., 2009), stone or berry fruits (Scholberg, 2009; Radi et al, 2010), as well as for table grapes (Hafez, 2008; Venditti et al., 2012). Kombucha is known mainly as a consortium (SCOBY - symbiotic acetic/lactic bacteria and yeast) used to prepare from green tea a slightly acidulous beverage with several reported positive health effects, but these effects still have to be scientifically demonstrated. In the recent years, the interest in cultivating Kombucha consortium is linked to the production of bacterial cellulose of very diverse use (medical, textile, industrial, etc) because of its versatile structure; in this process are

involved acetic bacteria from the SCOBY, like by *Gluconacetobacter* sp. or *Acetobacter* sp. (Dutta et al, 2007; Nguyen et al. 2008). Acetic acid concentration in Kombucha range from 8.5 to 17-18 g/L if the tea is allowed to ferment more than 21 days to 30 days (Jayabalan et al., 2014; Chakravorty et al., 2016). In the case of cellulose production, the suspension, otherwise used as beverage, is a residue and its possible uses are under screening. Because of the acetic acid levels, may be used also as treatment in postharvest fruits or grapes.

In this context, the main aim of our research it was to test the effects of different tea sourced Kombucha extracts on the growth of the most common moulds which lead to the decay of the grapes in the pre and postharvest steps.

MATERIALS AND METHODS

Kombucha filtrate preparation. The Kombucha SCOBY consortium has been procured from Medica Farmimpex S.R.L., Otopeni, Romania through the kindly help of Dr. Ionut Moraru.

Kombucha tea suspension has been prepared starting from three different source of tea plants, respectively green tea *Camellia sinensis*, green tea with *Melissa officinalis* L. and oolong (dark green *Camellia sinensis*) tea. The sweetened tea (90 g sucrose/L and 10 g/L plant) has been fermented with Kombucha SCOBY during 18 days at 28°C. After the fermentation, the suspension has been centrifuged at 1000 rpm during 10 minutes to separate the debris, followed by sterile filtration through Millipore membrane of 0.2 µm pores size.

Measurement of pH and acetic acid content. After sterilisation, the pH has been determined with an electronically pH meter and the crude extract (100%) has been used in the antagonistic tests. The acetic acid was determined in Kombucha crude extract by high performance liquid chromatography (HPLC). The mobile phase was 20 mM potassium dihydrogen phosphate, pH 2.4 with a flow rate of 1.0 mL/min and running time of 40 min. The column temperature was maintained at 28°C and the detection was carried out at 220 nm by

comparing the retention time of the standard compounds. The concentration of acetic acid was quantified from standard curves.

Fungal pathogens. Four different moulds have been taken into account in the experiments, being known as most common contaminants of the grapes, respectively *B. cinerea* MI-Aligote H, *P. expansum* MI-BB H, *A. flavus* MI-35 and *A. carbonarius* MI-32. All moulds strains have been isolated from grapes and identified in the laboratory of Applied Microbiology from UASMV Bucharest Faculty of Biotechnologies, Romania. Cultures are maintained on PDA (potato-dextrose-agar) medium at 4°C.

Inoculum preparation. Targeted moulds have been cultivated on PDA plates during 14 days at 20°C in the case of *B. cinerea* and at 28°C for the other moulds. Spore suspension has been prepared by flooding the 2-weeks plates with a small volume of sterile distilled water containing 0.05% (v/v) Tween-80, and spores were removed by gently scraping with a glass spatulum. By the aid of a hemacytometer has been determined the spore content in the suspension. Further dilutions with sterile water were performed to obtain a spore concentration of 10⁶ CFU/mL (Karabulut *et al.*, 2005).

Antifungal assay has been performed on synthetic medium on plate. For the *in-vitro* tests MEA (Malt-Extract-Agar) has been employed in Petri dishes. The medium has been flooded with 1 mL of different Kombucha crude extracts (prepared as described above) than, in the centre have been placed 10µl of fungal spore suspension. The cultures were incubated during 7 days at 20°C for *B. cinerea* and 28°C for the other three fungi (*P. expansum*, *A. flavus* and *A. carbonarius*). As control has been used MEA plates inoculated with same fungal spores suspension without Kombucha crude extract. The radial mycelial growth was measured daily, and the percentage of inhibition was calculated on the basis of growth in control plates as follows:

$$\frac{\text{mycelial growth in control} - \text{mycelial growth in Kombucha}}{\text{mycelial growth in control}}$$

The experiment design has taken into account three replicates for each sample.

Statistical analysis

Analysis of variance was performed. To determine differences in radial growth between samples and controls, Duncan's and Tukey's multiple pairwise comparisons tests were applied to the results (p -levels at 0.01 and below were considered significant).

RESULTS

The Kombucha crude extract has been prepared as described above; for all Kombucha samples the final pH after 18 days of cultivation has reached values of 2 ± 0.15 which is in agreement with other reports (Jayabalan et al.,

2014; Chakravorty et al., 2016). Regarding the acetic acid content, the results were close to the one obtained by Jayabalan et al. (2014), respectively 10 ± 0.5 g/L acetic acid in the crude extract.

In the *in vitro* tests have been taken into account four of the most common moulds which affect the grapes in pre- and postharvest steps, respectively *B. cinerea*, *P. expansum*, *A. flavus* and *A. carbonarius*.

The radial growth of the moulds have been measured after 7 incubation days at optimal temperature for each mould (table 1).

Table 1 - Growth and inhibition of moulds on synthetic medium in the presence of different Kombucha crude extracts

	Radial growth (mm)				Inhibition %		
	Control	K1	K3	K4	K1	K3	K4
<i>B. cinerea</i> MI-Aligote H	47.2	20.9*	21.6*	29.0*	55.6	54.3	38.6
<i>P. expansum</i> MI-BB H	50.0	46.0*	46.0*	48.0	8.0	8.0	4.0
<i>A. flavus</i> MI-35	48.3	47.0	47.2	48.0	2.7	2.3	0.6
<i>A. carbonarius</i> MI-32	49.1	48.5	48.7	49.0	1.2	0.8	0.2

Values represent means of measurements made on three independent plates per treatment; * $p \leq 0.01$ vs. respective control.

K1 - Kombucha on green tea; K3- Kombucha on green tea with *Melissa officinalis*; K4 Kombucha on oolong tea

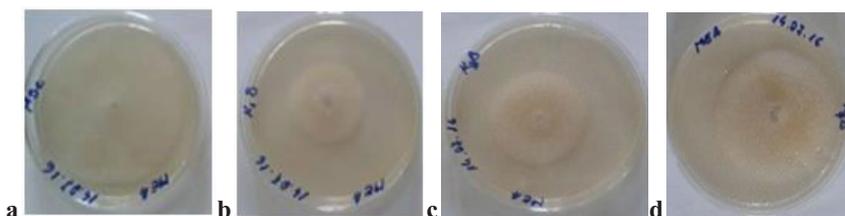


Figure 1. On-plate aspects of *Botrytis cinerea* growth's inhibition by different Kombucha crude extracts: **a** - control on MEA; **b** - K1 (Kombucha made of green tea); **c** - K3 (Kombucha made of green tea with *Melissa officinalis*); **d** - K4 (Kombucha made of oolong tea)

The most significant inhibition, statistically assured, has been registered in the case of *B. cinerea*, respectively 54-55% inhibition in the case of green tea Kombucha and 38.6% in the case of oolong Kombucha. Aspects of the mycelium growth inhibition of *B. cinerea* are presented in figure 1.

Positive reports on *B. cinerea* inhibition by the use of acetic acid, mainly as vapours, have been described for different fruits, like strawberries (Hassenberg et al., 2010), kiwifruit (Lagopodi et al., 2008) or grape (Venditti et al., 2008) and our results are in accordance. The reports on using Kombucha extracts for the inhibition of *B. cinerea* are rather limited (Hafez, 2008) and reveal the high inhibitory effect on the spore germination on table grapes

(80% to 100%). In this context it has been suggested that Kombucha treatments may be used as an alternative natural solution to replace the pre and postharvest chemical treatments.

Meanwhile, in our tests, less significant has been the inhibition of all the extracts on *P. expansum* growth (4-8%), while in the case of *Aspergillus* sp. the inhibition was not significant. Radi et al. (2010) have reported the inhibition of *P. expansum* by acetic acid on red apples, but only as heated solutions as 50°C. The reports on the effect of *Aspergillus* sp. are rather limited and are linked mainly to food safety and micotoxin production in food commodities; Hassan et al. (2015) reported the inhibition of *A. flavus* of 45.21% for a

concentration of 10 g/L in acetic acid; this data are not in range with our results and further analysis should be performed in this respect.

CONCLUSIONS

The exploitation of natural products to control fruits postharvest diseases and to prolong their storage life has received special attention in the last decade. The use of acetic acid in postharvest treatments have been reported for citrus fruits, stone or berry fruits, as well as for table grapes.

Kombucha suspensions, in crude extracts, as residue when preparing bacterial cellulose, contain 8.5 to 17 g/L acetic acid, depending on the cultivation time.

Our experiments have targeted the potential inhibitory effect of different tea sourced Kombucha crude extracts on the most common moulds of the grapes in pre and postharvest steps. The most significant inhibition has been registered in the case of *B. cinerea* (38 -55%), less significant on *P. expansum* (4-8%) and not significant on *A. flavus* and *A. carbonarius*. While the results regarding the inhibition on *B. cinerea* are in total agreement with other reports, the data on the other moulds are far to be similar. It is proposed further to investigate the inhibition of Kombucha extracts *in vivo* on artificially infected grape berries with *B. cinerea* and to validate the *in vitro* results.

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ANTIMICROBIAL ACTIVITY OF NEWLY ISOLATED *Bacillus* SP. AND *Pseudomonas* SP. STRAINS AND THEIR POTENTIAL USE AS BIOCONTROL AGENTS

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Abstract

Most of the plant diseases are caused by microorganisms. Among these, most often mentioned in the literature are bacteria and fungi. Diseases caused by phytopathogens like *Erwinia carotovora* and *Xanthomonas campestris* lead to lower production and quality, causing significant economic losses. In order to prevent the diseases can be used microbial antagonists which, besides protection capability, can stimulate plant growth by degrading the substrate and releasing of certain compounds needed for growth.

After a preliminary screening, from a total of 25 microorganisms isolated from plant materials, best antimicrobial activities were registered with bacteria B1 and Bm belonging to the genera *Pseudomonas* sp. respectively *Bacillus* sp.. Following biochemical tests in conjunction with microscopy studies and MALDI-TOF MS, selected bacteria were identified as *Pseudomonas putida*, respectively *Bacillus mycoides*. Their antimicrobial activity was comparable to that of microorganisms belonging to the same genera, from the collection of the National Institute for Chemical Pharmaceutical Research and Development-ICCF. When they were grown on agar media with different compositions, significant differences regarding antimicrobial activity have not been observed. However, substantial differences were recorded in terms of antagonistic ability between *Pseudomonas putida* and *Bacillus mycoides*, the last one making the biggest area of inhibition against both phytopathogen *X. campestris* ICCF 274 (40 mm) and *E. carotovora* ICCF 138 (20 mm).

These results suggest the possibility of using newly isolated antagonists to prevent diseases caused by *Erwinia carotovora* and *Xanthomonas campestris*.

Key words: antagonists, antimicrobial activity, biocontrol, phytopathogens.

INTRODUCTION

Diseases caused by phytopathogens from the genera *Erwinia* and *Xanthomonas* affects many species of plants, causing significant economic losses. Bacteria from *Xanthomonas* genus, for example, can affect over 300 species of plants. Moreover, even if some organisms may look less affected, the bacteria can locate at the seeds level and can be passed on to other susceptible plants.

Bacteria of the genus *Erwinia* have a rich enzymatic equipment of amylases, cellulase, xylanases, polygalacturanases and pectin-methyl esterases that cause maceration of the tissues affected (Opara and Asuquo, 2016).

Species of *Erwinia carotovora* attack and infects a variety of vegetables and plants including carrots, potatoes, cucumbers, onions, tomatoes, lettuce and ornamental plants like iris (Wood, 1998). Some strains of the genus, like *Erwinia carotovora* are bacteriocine producers. These substances, released in the medium, have antibacterial action and enable the bacteria to compete with other microbial species that occupy the niche. These, together, are attributes that make those bacteria so adaptable and persistent in the environment.

Although researches in the field of chemical synthesis had resulted in discovering of new pesticides it is proved that the phytopathogens become resistant (McManus et al., 2002) to

them and harder to control. In addition, arises increasingly stronger, the problem of the environmental and crops pollution affecting people's health (Horrihan et al., 2002).

In this context, we need to find new solutions for combating the phytopathogens using beneficial microorganisms that can prevent plant illnesses through their own mechanisms such as: competition for occupying the niche, production of secondary metabolites (substances with bactericidal and fungicidal effect, enzymes), direct parasitism of the phytopathogens (hyperparasitism) etc.

Among the microorganisms with recognized activity in biocontrol are bacteria from the genera *Bacillus* and *Pseudomonas*. The most important mechanism of their action is to produce substances with bactericidal and fungicidal effect. *Bacillus* sp. produces and releases during sporulation or in stationary phase of growth, a number of lipopeptides with antibiotic role (Kalai-Grami et al., 2016; Ongena and Jacques, 2008) like iturins, fengycin, surfactin. These bacteria also produce lytic enzymes like cellulases, glucanases, proteases, chitinases, or volatile compounds like hydrogen cyanide. Many species of the genus have been utilized in agriculture and are considered as safe microbes (Fravel, 2005).

Besides the bioprotection role it seems that these microorganisms can stimulate plant growth by producing phytohormones and by increase the availability of mineral compounds with low solubility.

Moreover the secondary metabolites, like biosurfactants can have various industrial applications (Banat et al., 2010; Pathak and Keharia, 2014).

Many studies have demonstrate that *Pseudomonas* strains are in a close relationship with the suppressive soils (Raaijmakers et al., 1997). Some strains that produce the antimicrobial metabolite 2,4-diacetylphloroglucinol (Shanahan et al., 1992) were isolated from the soil, roots of various plants and even from different tissues of plant (Turner et al., 2013). Other antimicrobial substances produced by strains of *Pseudomonas* are phenazines, hydrogen cyanide, pyrrolnitrin (Nandi et al., 2015).

In addition, many strains of *Bacillus* sp. and *Pseudomonas* sp. seem to have the capacity to

induce systemic resistance in plants (Pieterse et al., 2001; Bargabus et al., 2002) by chemical elicitors like salicylic acid, siderophore, 2,3-butanediol, lipopolysaccharides.

The main objective of our research was to isolate and identify microorganisms capable of controlling the phytopathogens *Erwnia carotovora* and *Xanthomonas campestris*. Also antagonistic activity was examined in relation to the optimal growth medium for phytopathogens and respectively antagonists, and with inoculation moment.

MATERIALS AND METHODS

The microorganisms studied for antagonistic activity have been isolated from various plant materials (hay, beans).

The plant material from which microbial strains were isolated was collected from different areas of Romania (Vâlcea, Ilfov) and kept in a refrigerator in sterile containers until processing. Approximately 1g of plant material was inoculated onto liquid growth medium, specific to each type of microorganism as follows: IPS medium broth for bacteria, YMPG for yeasts and fungi strains. The IPS broth containing (% g/v) glucose 1.00%, corn extract 1.50%, KH_2PO_4 1.00%, NaCl 1.00%, MgSO_4 0.05%, and YMPG containing (% g/v) yeast extract 3.00%, malt extract 3.00%, peptone 5.00%, glucose 10.00%, were sterilized at 115°C, for 20 minutes. The plant material was inoculated into these media in 500 ml shake flasks containing 100 ml medium and left to develop, in an incubator at 30±1°C and 220 rpm for 24 h. Serial dilution did follow and pour plated onto NA (nutrient agar), YMPG (Yeast Malt Peptone Glucose) and PDA (Potato Dextrose Agar). In order to obtain single colonies, streak plating technique was used.

A number of 25 microorganisms isolated in pure culture were grown and maintained on their specific media as follows: the bacteria (20 strains) on nutritive agar, the yeasts (3 strains) on YMPG and the fungus (2 strains) on PDA media.

For strains identification, microscopy studies in conjunction with biochemical tests and MALDI-TOF MS were done.

Morphological characterization was performed by microscopic examination using a Novex

microscope. Biochemical assays were done according to literature on diverse media for testing the capacity of the microbes to utilize or produce various compounds.

Microflex LT (MALDI-TOF mass-spectrometer manufactured by Bruker Daltonics Inc. - Billerica, MA, USA), equipped with nitrogen laser, was used to acquire the mass-spectra from fresh colonies. The equipment identify a microorganism by analyzing the expression of the most abundant ribosomal proteins from the acquired mass spectra and by comparing the specificity of his mass spectrum with a large number of reference patterns from its database (Tomulescu et al., 2015).

In order to establish their antagonistic capacity, newly isolated microorganisms were grown on agar medium along with the phytopathogenic strains *Erwinia carotovora* ICCF 138 and *Xanthomonas campestris* ICCF 274 by dual cultures method. During experiments were tested, in the same conditions, two other strains *Bacillus subtilis* ICCF 84 and *Pseudomonas putida* ICCF 391. *Erwinia carotovora* ICCF 138, *Xanthomonas campestris* ICCF 274, *Bacillus subtilis* ICCF 84 and *Pseudomonas putida* ICCF 391 belong from Culture Collection of Industrial Importance Microorganisms (CMII) of the National Institute for Chemical-Pharmaceutical Research and Development, Bucharest.

To ensure optimal conditions for development, the antagonists and the phytopathogens were grown on various types of agarized medium: NA, YMPG, PDA, M44. Medium M44 (containing (%g/v): yeast extract 1.00%, bacteriological peptone 1.00%, glycerol 5.00%, agar 2.00%) was the most appropriate for the majority of the strains and YMPG was the best for *X. campestris*. On these media were performed all subsequent experiments. For our research were used bacterial strains after 48 hours of development on their specific agaric medium. The broth medium for bacterial development has the same composition as mentioned above. After 24 hours at 30°C and 220 rpm the culture was appropriate for pour plate inoculation. For carrying out the method of dual cultures, one ml of inoculum from the broth culture of phytopathogens was added by pipette to the center of the Petri dish, over the agar medium (cooled, but still molten) and

rotated gently, to ensure that the culture and medium are thoroughly mixed. After solidification, 100µL of inoculum from the broth culture of antagonists was put in the middle of the same plate and allowed to be adsorbed in medium. After 48-72 hours of incubation at 30±1°C the inhibition zones were checked.

RESULTS AND DISCUSSIONS

From a number of 25 microorganisms, were isolated in pure culture 20 strains of bacteria, 3 of yeasts and 2 of fungus. Two bacterial strains, who have registered the best antimicrobial action against *Erwinia carotovora* ICCF 138 and *Xanthomonas campestris* ICCF 274 when they were cultured together by dual cultures method, were further investigated for identification.

These strains noted B1 and Bm, were identified as *Pseudomonas putida* and respectively *Bacillus mycoides* through microscopy studies in conjunction with biochemical tests and MALDI-TOF MS (see figures and tables below).



Figure 1. Macroscopic and microscopic aspect of B1 strain



Figure 2. Macroscopic and microscopic aspect of Bm strain

Regarding microbiological and biochemical characteristics (shown in Table 1), the results were similar to those reported for *Pseudomonas putida* and *Bacillus mycoides* in ABIS online Encyclopedia and in the works of other researchers (Borah et al., 2002; Egamberdiyeva, 2005).

Table 1. Microbiological and biochemical characteristics for B1 and Bm strains

MICROBIOLOGICAL CHARACTERISTICS	RESULTS	
	B1	Bm
Gram staining	-ve	+ve
Rod shaped	+ve	+ve
Spore forming	-ve	+ve
Motility	+ve	-ve
BIOCHEMICAL TESTS		
Citrate utilization	+ve	-ve
H ₂ S production	-ve	-ve
Gas production	-ve	-ve
Gelatin utilization	-ve	+ve
Catalase production	+ve	+ve
Urea hydrolysis	+ve	-ve
Starch hydrolysis	+ve	+ve
Glucose fermentation	+ve	+ve
Glycerol utilization	+ve	+ve

Note: +ve indicates positive and -ve indicates negative results

By MALDI-TOF MS analysis the strain noted as B1 was identified as *Pseudomonas putida* (data not shown).

For growth and development of the microorganisms tested, were investigated some culture media: NA, YMPG, PDA, M44. From these, medium M44 was the most appropriate for the majority of the strains involved in this study and YMPG was the best for *X. campestris*.

As it shows in figures below, Bm presented the best action against *X. campestris* ICCF 274 (a 40 mm inhibition area) and *E. carotovora* ICCF 138 (a 20 mm inhibition area). It was followed, in descending order, B1 (20 mm against *X. campestris* ICCF 274 and 8 mm against *E. carotovora* ICCF 138), *B. subtilis* ICCF 84 (20 mm against *X. campestris* ICCF 274 and 8 mm against *E. carotovora* ICCF 138) and *P. putida* ICCF 391 (16 mm against *X. campestris* ICCF 274 and 8 mm against *E. carotovora* ICCF 138).



Figure 3. Dual cultures method - front view:
1) Bm, 2) Control, 3) *B. subtilis*, 4) B1,
5) *P. putida* -against *Erwinia carotovora* ICCF 138



Figure 4. Dual cultures method - reverse view:
1) Bm, 2) Control, 3) *B. subtilis*, 4) B1,
5) *P. putida* - against *Erwinia carotovora* ICCF 138

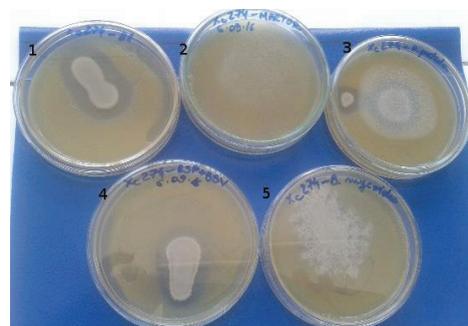


Figure 5. Dual cultures method - front view:
1) B1, 2) Control, 3) *P. putida*, 4) *B. subtilis*,
5) Bm - against *Xanthomonas campestris* ICCF 274

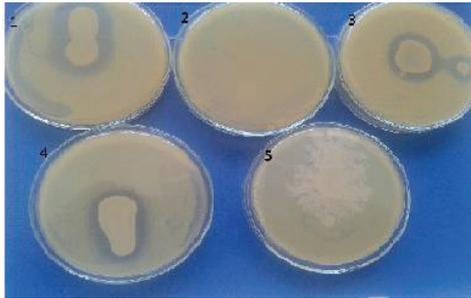


Figure 6. Dual cultures method - reverse view:

- 1) B1, 2) Control, 3) *P. putida*, 4) *B. subtilis*,
- 5) Bm - against *Xanthomonas campestris* ICCF 274

CONCLUSIONS

Microscopy studies in conjunction with biochemical tests and MALDI-TOF MS led to the conclusion that the strains noted B1 and Bm are *Pseudomonas putida* and respectively *Bacillus mycoides*.

Optimal conditions for development of the phytopathogens were obtained with *X. campestris* on medium YMPG and *E. carotovora* on M44. Medium M44 was best for most bacteria used in this research.

Among the microorganisms isolated, Bm and B1 recorded the best antimicrobial activity against phytopathogens *Erwinia carotovora* ICCF 138 and *Xanthomonas campestris* ICCF 274. It was followed *Bacillus subtilis* ICCF 84 and *Pseudomonas putida* ICCF 391. These strains will be used in further studies for obtaining microbial origin products for plant protection.

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THE INFLUENCE OF THE OPEN POLLINATION ON THE INDUCING RATE ON TOP AND BOTTOM OF THE EAR ON DH TECHNOLOGY

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Abstract

Accelerating the development of homozygous lines and consequently hybrids is an important aspect of the maize breeding programs. Doubled haploid technology has successfully replaced the traditional method of obtaining homozygous lines in maize breeding programs in Europe, North America, China and Central America, due to the clear advantages in terms of timing and important reduction of the workload and costs by eliminating controlled pollination and relatively simple methodology.

Haploid inducing efficiency is influenced by the genotype of the population submitted to the induction, haploid inducing capacity of the inducer and the inducing protocol (time and type of pollination). The study was carried out in special climatic conditions of the year 2016 (the absolute maximum temperature was 35.8°C for June and 35.7°C for July. The results showed that the source genotype used in the induction nursery influenced the anthocyanin coloration in both aleurone and embryo. Clear variability regarding the anthocyanin coloration was observed among the ears from the same genotype and even between aleurone and embryo from the same ear. Comparing the PHK (putative haploid kernels) number from the top and bottom of the ear, the top average is distinct significantly positively over the bottom average PHK number. Similarly to controlled pollination, the highest percentage of PHK remains on top of the ear in the case of this experiment with open pollination induction nursery.

Key words: DH technology, inducers, open pollination, haploid kernels.

INTRODUCTION

The *in vivo* maternal haploid induction scheme is based on a dominant anthocyanin color marker, known as *RI-Navajo (RI-nj)*, that expresses in the aleurone as well as in the embryo of the haploid inducer, unlike the source populations, where the coloration is usually missing in both aleurone and embryo (Prassana et al., 2012). However, it must be noted that the size and intensity of the anthocyanin coloration of the *RI-nj* color marker might vary significantly depending on the genetic background of the source genotype (from which we want to obtain haploid forms) and haploid inducer, as well as environmental factors (Chase, 1952; Röber et al., 2005; Kebede et al., 2011; Prigge et al., 2011).

It is known in the literature that in case of controlled pollination, the highest frequency of haploid forms is at the top of the ear. Studies realized by Sarmaniuc and published in 2015 in her doctoral thesis titled "Improving the

technology of creating homogeneous lines of maize (*Zea mays L.*)" showed that the rate of haploid kernels is much higher at the top of the ear after 2, 3 and 4 days from the controlled pollination, but in evolution of female inflorescence development and maturation, the index decreases in both versions - "top" and "bottom". Poor pollination occurs because of delayed silk emergence, after pollen shedding was complete; drying pollen or silk - all these situations occurring in periods of drought and heat. At temperatures of 35°C, maize pollen loses its viability in 1-2 hours and silks begin to dry at temperatures exceeding 32-33°C. In an isolation of induction with open pollination, donor sources should be grouped depending on the silking period and the inductor must be planted at different planting times for optimal pollination (Prassana et al., 2012). The year 2016, was extreme dry with high temperatures during pollination that affected both pollen and silk viability. The average for daily maximum temperatures was 29,4°C in June and 31,4°C in

July. The absolute maximum temperature was 35.8⁰C for June and 35.7⁰C for July. The aim of this study was to check if in case of open pollination (when we don't know exact the time of pollination) the highest percentage of haploids remains on top of the ear.

MATERIALS AND METHODS

The study was carried out at the National Institute of Research and Development Fundulea in 2016. A number of 15 F1 maize breeding populations from different heterotic groups were used as female sources. Each female source was crossed with the inducer MHI (Moldavian Haploid Inductor), in the field in an induction isolation nursery. Ten ears from each population were divided in two, top and bottom and for each of these two parts putative haploid kernels (PHK) were counted. Grains resulted from crosses were divided in 3 categories in both parts (top and bottom) based on the expression of the anthocyanin coloration coded by *R1-nj* gene on the kernel as follows: category 1, kernels with no coloration on both aleurone and embryo; category 2, kernels with coloration of both aleurone and embryo and category 3 considered as PHK with purple coloration only on the aleurone and uncolored embryo. A scale 0-4 was used for visual assessment of the intensity of anthocyanin coloration on aleurone and embryo from category 2 (kernels with coloration in both aleurone and embryo): 4=intense pigmentation and 0=lack of pigmentation (Sarmaniu et al., 2013).

RESULTS AND DISCUSSION

Many researchers have highlighted that the source genotype influence both the anthocyanin coloration as well as the rate induction (Coe, 1994; Eder and Chalyk, 2002; Kebede et al., 2011; Bitica et al., 2016). Variation in the size and intensity of the anthocyanin coloration is presented in table 1; all 15 populations showed high variability of the expression of anthocyanin coloration for both embryo and aleurone, appreciated on average with scores between 1 (identification of haploid is possible but errors could occur due to very weak staining in the embryo) and 4 (level that allows

easy identification of PHK). Clear variability was observed also among the ears from the same genotype and even between aleurone and embryo from the same ear. A good example is the genotype L537 appreciated on average with scores between 3,3 - 4 for aleurone and 1- 2,3 for embryo.

Table 1. The anthocyanin coloration for aleurone and embryo, 15 genotype (10 ears for each genotype), NARDI Fundulea 2016

Genotype	Ear	1	2	3	4	5	6	7	8	9	10
L502	A	3	3	2.7	3	2.5	3	3	3	3	3
	E	3	3	3	3	3	3	3	3	3	2.7
L507	A	2	2.5	2	2	2	2.5	2.5	2	2	3
	E	3	3	3	3	3	3	3.5	2.5	2.5	2.7
L508	A	2.7	2.5	3	2	3	2.5	2.7	3	2.7	2.5
	E	3	2	2.5	3	2.7	2.5	3	3	3	2.7
L519	A	2	3	2.3	2	1.7	2	1.3	3	3	1
	E	3	3.3	2.7	2.3	2	2.3	2	3.7	3.3	2.0
L529	A	3	3	3	3	3	3	2.7	3	3.3	3
	E	3	3	3	3	3	3	3.3	3	3	3
L535	A	4	3.7	3.7	3.7	3.7	3.7	3.7	3.7	3.7	3.7
	E	2	2.7	2.7	3	2.7	2.7	3	3	3	2.7
L537	A	4	4	4	4	3.7	4	4	3.3	4	3.7
	E	2	1.7	2	2	2.3	1.7	1	1	1	1.3
L552	A	2	3	3.3	3	3	3	3	2.7	3.3	3.3
	E	3	3	3	3	3.3	3.3	3.7	2.7	3.7	4
L560	A	3	2.3	3	3.7	3	3.3	3.3	3	3	3
	E	3	2.7	2.7	3.7	3.7	3.7	3	3	3.7	3.7
L567	A	3	3	3.7	2.7	2.7	3	3.3	3.3	3.7	3.3
	E	4	3.3	2.7	3	2.7	3.7	3	3	3	3
L573	A	3	3.3	3	2.7	3	3	3	3.3	3.7	3
	E	3	3	3	3	3	2.7	3.3	3	3	3
L576	A	3	4	4	3.7	4	4	3.3	4	3.7	4
	E	3	2.7	3	3	4	3.7	4	3.3	3.3	3
L584	A	4	4	4	4	4	3.3	4	3.7	4	3.7
	E	4	3	3	3	4	2.7	4	3	4	3
L586	A	3	2.7	2.3	3	2.7	3	2.7	3	3	3
	E	3	2.3	2.3	2.3	2	2.7	2.7	3	2.3	2.7
L587	A	3	2.3	3.3	3.3	3.7	4	4	3	3.3	3.3
	E	3	3	3.3	2.7	4	4	4	4	3.3	2

*A= aleurone, E= embryo

Furthermore, the analyses of variance for anthocyanin coloration intensity of the aleurone and embryo (table 2) confirmed that all the variance sources - genotype, kernel components (aleurone and embryo) as well as the interaction between genotype and kernel

components have significant effect on the anthocyanin coloration intensity.

Table 2. ANOVA for the anthocyanin coloration intensity of the aleurone (A) and embryo (E), 15 maize populations submitted to induction, NARDI Fundulea, 2016

Source of variation	Degrees of freedom	Sum of squares	Mean square	F Value	Probability
Replications	9	1,510	0.168	0.844	
Genotype(G)	14	34,220	2,444	12.295	0.0000 (***)
Error(G)	126	25,049	0.199		
Aleurone/Embryo(AE)	1	2,576	2,576	29.601	0.0000 (***)
GxAE	14	34,845	2,489	28.599	0.0000 (***)
Error	135	11,749	0.087		
Total	299	109,949			

As it can be seen in table 3, showing the anthocyanin coloration for aleurone and embryo for the genotypes submitted to induction, the aleurone expressed much better anthocyanin coloration than the embryo. However, there were genotypes distinct significantly positively compared to the average experience as populations L537, L576, L584 regarding the aleurone anthocyanin coloration and significant, populations L584 and L586 for the embryo.

There were also genotypes with coloration for the aleurone was very poor compared to the average experience as L507 and L519. The most stable genotypes regarding the anthocyanin coloration are L576 and L584. In case of population L537 the anthocyanin is much better expressed in the aleurone than in the embryo.

Table 3. Anthocyanin coloration for aleurone and embryo, 15 genotypes, NARDI Fundulea, 2016

Genotype	Score for anthocyanin coloration			
	Aleurone (A)	Embryo (E)	A-E	Average
L502	2.92	2.97	-0.05	2.95
L507	2.25***	2.92	-0.67 ^{SSS}	2.59 ^{&&}
L508	2.66*	2.74	-0.08	2.7 ^{&}
L519	2.13***	2.66	-0.53 ^{SSS}	2.395 ^{&&&}
L529	3.00	3.03	-0.03	3.02
L535	3.7**	2.78	0.92 ^{SSS}	3.24
L537	3.87***	1.6***	2.27 ^{SSS}	2.735 ^{&}
L552	2.99	3.27	-0.28 ^S	3.13

L560	3.03	3.29	-0.26 ^S	3.16
L567	3.17	3.11	0.06	3.14
L573	3.10	2.97	0.13	3.04
L576	3.8***	3.30	0.5 ^{SSS}	3.55 ^{&&&}
L584	3.87***	3.37*	0.5 ^{SSS}	3.62 ^{&&&}
L586	2.81	2.53*	0.28 ^S	2.67 ^{&}
L587	3.32	3.30	0.02	3.31 ^{&}
Average	3.11⁺⁺⁺	2.92		3.02
LSD for factor A (genotypes) average=5%=0.28; 1%=0.37; 0.01=0.48 (&&&-significant for 5, 1, and 0.1 level, respectively)				
LSD for factor B (kernel components, aleurone and embryo) average: 5%=0.02 1%=0.03; 0.01=0.04 (+++-significant for 0.1 level)				
LSD for factor B (kernel components, aleurone and embryo) at the same level of factor A (genotypes)-horizontal comparison: 5%=0.26; 1%=0.35; 0.01%=0.44 (\$,\$,\$\$-significant for 5, 1, and 0.1 level, respectively)				
LSD for factor A (genotypes) at the same level of (kernel components, aleurone and embryo)-vertical comparison: 5%=0.39 1%=0.51; 0.01=0.66 (*,\$,\$,\$\$-significant for 5, 1, and 0.1 level, respectively)				

Recent researches related to the rate of induction have highlighted that the highest percentage of haploid forms is found at the top of the ear induced and is influenced by time of pollination. In case of open pollination, analysis of variance (table 4) for the PHK number showed that all the sources of variation (genotype, position on the ear-top/bottom) as well as the interaction between genotype and position on the ear have significant effect on the number of haploid forms.

Table 4. ANOVA for PHK (Putative haploid kernel) number at the top and bottom of the ear, 15 genotypes submitted to induction, NARDI Fundulea, 2016

Source of variation	Degrees of freedom	Sum of squares	Mean square	F Value	Probability
Replications	9	54,883	6,098	1,540	0.1409 ns
Genotype(G)	14	138,567	9,898	2,499	0.0036 **
Error (E)	126	498,967	3,960		
Part of the ear (T/B)	1	180,963	180,963	98,887	0.0000 ***
G x T/B	14	56,487	4,035	2,205	0.0104 ***
Error	135	247,050	1,830		
Total	299	1176.92			

*T= top, B= bottom

The evaluation of the PHK number for the top and bottom of the ear have shown that some genotypes like L502, L573, L576 were significantly positively for the top PHK

number as compared to the experiment average. Comparing the PHK number from the top and bottom of the ear, the top average is distinct significantly positively over the bottom average PHK number. At some genotypes the PHK number from the top was much higher than the PHK number from the bottom of the ear as genotypes L502, L507, L573 and L576.

Table 5. PHK number at two positions on the ear (top and bottom), 15 genotypes, NARDI Fundulea, 2016

Genotype	PHK number			
	Top (T)	Bottom (B)	T-B	Average
L502	4.8*	2.0	2.8 ^{SSS}	3.4
L507	3.4	1.2	2.2 ^{SSS}	2.3
L508	4.4	2.5	1.9 ^{SS}	3.5
L519	2.4	1.1	1.3 ^S	1.8
L529	3.0	2.4	0.6	2.7
L535	2.4	1.2	1.2 ^S	1.8
L537	2.7	1.2	1.5 ^S	2.0
L552	2.8	1.9	0.9	2.4
L560	2.4	1.2	1.2 ^S	1.8
L567	2.2	1.3	0.9	1.8
L573	5*	1.2	3.8 ^{SSS}	3.1
L576	4.6*	2.3	2.3 ^{SSS}	3.5
L584	2.3	1.6	0.7	2.0
L586	1.7	1.0	0.7	1.4
L587	3.3	2.0	1.3 ^S	2.7
Average	3.2⁺⁺⁺	1.6		2.4
LSD for factor A (genotypes) average: 5%=1.25; 1%=1.65; 0.01=2.12				
LSD for factor B (position on the ear(top, bottom) average: 5%=0.31 1%=0.41; 0.01=0.53 (+++significant for 0.1 level)				
LSD for factor B (position on the ear (top, bottom) at the same level of factor A (genotypes)-horizontal comparison: 5%=1.20; 1%=1.58; 0.01%=2.03 (\$,\$\$, \$\$\$-significant for 5, 1, and 0.1 level, respectively)				
LSD for factor A (genotypes) at the same level of (position on the ear (top, bottom)-vertical comparison: 5%=1.22; 1%=1.99; 0.01=2.56 (*-significant for 5 level)				

CONCLUSIONS

Anthocyanin coloration is influenced by the genotype of the population submitted to the induction; a good coloration on the aleurone

and embryo allows easy identification of the PHK forms.

Moreover, similarly to controlled pollination, the highest percentage of PHK remains on top of the ear in the case of this experiment with open pollination induction nursery. For this reason it is recommended to know the silking period of the sources, and flowering of the inducer to ensure optimal induction by handling planting time of the parents.

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VARIABILITY OF COLEOPTILE LENGTH IN MUTANT/RECOMBINANT WHEAT DH (DOUBLED HAPLOID) LINES

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Abstract

Most of the modern wheat cultivars carry GA (gibberellic acid) insensitive height reducing gene, Rht-B1b, that reduces stem elongation but improves yield potential, lodging resistance and increased harvest index. Recent cultivars released in Romania are semi-dwarf, carrying the Rht-B1b allele which confers an advantage in excessive temperate environmental conditions. However, such cultivars are characterized as having short coleoptile that influences stand establishment and seedling vigor, especially in dry autumns. Genotypes carrying Rht gene-alleles can contribute to increasing coleoptile length, and having semi-dwarf characteristics can be used as sources in advanced breeding programs. A set of 86 mutant/recombinant wheat doubled haploid (DH) lines, was obtained through a complex protocol including two genotypes, two irradiation cycles with gamma rays (200Gy, 100Gy), direct and reciprocal hybridization, rapidly homozygosity attained by using Zea system. This study was aiming to explore the genetic variability for the coleoptile length and plant height, produced by mutagenesis and recombination, in the selected 86 mutant/recombinant DH lines, and compared to the parents. The analyses performed in this study, reveal that are lines which registered a longer coleoptile than parents, and also one line was highlighted for a semi-dwarf stature and a long coleoptile, that can be used in wheat breeding programs.

Key words: coleoptile, mutant/recombinant, Rht-B1b, semi-dwarf;

INTRODUCTION

The climate changes from the last period and the unpredictable weather conditions makes very difficult to maintain yield stability through good and acceptable levels of resistance against biotic and abiotic stress factors.

Drought stress has become a major factor that influences negatively the plant growth and the productivity in the last years. For this reason the breeders have manifested a real interest for obtaining a resistant material against abiotic stress factors.

Semi-dwarf cultivars, with longer coleoptile could ensure an early and good seedling emergence, a better ground coverage that confers an advantage in competition with weeds, and finally an efficient soil weather use by reducing evaporative water loss. If the coleoptile is short and sowing is deeper, the first leaf may suffer by pushing through the soil and emerge in a dark environment, being also exposed to soil crusting and diseases (Gulnaz et al., 2011). Consequently, the length of

coleoptile is very important for crop establishment, allowing also a deeper sowing to utilize efficiently the soil moisture and uniform seed germination (Budak et al., 1995).

In wheat, more than 20 genes were described for reducing plant height (Mc. Intosh et al., 2013), from which, the *Rht-B1b* was used prevalently in breeding program at NARDI Fundulea. The most widely used genes in wheat breeding programs all over the world are *Rht-B1b*, *Rht-D1b*, *Rht-D1c* and *Rht8* (Korzun et al., 1988; Worland et al., 1998; Li et al., 2012). The *Rht-B1b* and *Rht-D1b* insensitive alleles at gibberellic acid (GA) induce lower sensitivity of vegetative tissue to endogenous gibberellin, and reduce cell elongation (Rebetzke et al., 2001).

The *Rht-B1b* and *Rht-D1b* alleles can increase yield by 6.1 and 14.1%, respectively; *Rht-B1* can also produce more productive tillers and higher yield per plant (Sial et al., 2002).

Rht8 is quoted as having contradictory effects on yielding in different environments (Sial et al., 2002; Kovalski et al., 2016).

Serban (2012) identified in a set of mutant/recombinant DH lines, with large variability for plant height and for coleoptile length, some semi-dwarf genotypes with longer coleoptile, that constitute an important breeding material. In Romania, the modern cultivars carry *Rht-B1b* gene, several yield tests revealing real advantages of *Rht-B1b* carriers in most environment (Saulescu, 2001, Serban, 2012). In some comparative tests performed in different wheat growing areas with isogenic lines carrying either *Rht-B1b* or *Rht8* showed that *Rht-B1b* carriers were superior for yielding capacity (Mustatea et al., 2000).

It is desirable to use as genitors *Rht-B1b* carriers and, if it is possible, with a long coleoptile. Improving stand establishment in dry autumns and at deep sowing by obtaining new genotypes with superior value of coleoptile length, without increasing plant height, is the main concern in the wheat breeding program at NARDI Fundulea.

The aim of this paper is to present the results obtained for plant height and coleoptile length for a selected set of 86 mutant/recombinant wheat DH lines, released at NARDI Fundulea (Giura, 2011), comparative with the two parents and identification of valuable semi-dwarf genotypes with short coleoptile.

MATERIAL AND METHODS

A number of 86 mutant/recombinant DH lines, derived from a complex protocol that included two modern genotypes, two irradiation cycles (100 Gy and 200Gy) and *Zea* system application for material homozygosis (Giura, 2011), were analyzed in laboratory and field conditions and compared with the two parents.

The 86 genotypes are part of a set with over 550 mutant and mutant/recombinant DH lines. A first set with 172 lines were analyzed in 2012 and the results proved that mutagenesis could generate extensive variability affecting not only the plant height, but also coleoptile length (Serban, 2012). It was noted the line *BiII 294* with approximately 2.5 mm longer coleoptile than both parental forms. This line has already been extensively used in NARDI Fundulea wheat breeding program.

The parents of this set of lines combines short stature with good winter hardiness and very

good filling grain, due to a higher resistance to drought and heat.

F0628G1-34 parent - has in genealogy a line of triticale which conferred tolerance to rust, a greater length of coleoptile and good resistance to frost.

Izvor parent- variety with superior behavior under water stress conditions due to high osmotic adjustment capacity.

The 86 mutant/recombinant DH lines were planted in the autumn of 2014, in the field in plots of 1 m length, 20 cm between rows and 50 cm among genotypes for plant height determination. Plant height measurements were made, in June 2015, at complete heading, when plant growth ceased.

To determine the coleoptile length, seeds with uniform appearance, without any diseases and pest traces, were planted at uniform depth (10 mm) in pots with commercial soil (Flora Sol) to germinate, watered to field capacity, in December 2015. After a 3-days period, at 1°C, for obtaining uniform seed imbibition, trays were introduced in a growth chamber at 20°C in dark conditions (Serban, 2012). When coleoptile growth ceased and the first leaf appeared (figure 1), coleoptile length was measured with a ruler (figure 2). A number of 21 mutant/recombinant DH lines, best for coleoptile length measured at 20°C germination were retested after the same protocol, but germinated at 17°C.



Figure 1. Plantlets developed in dark conditions at 20°

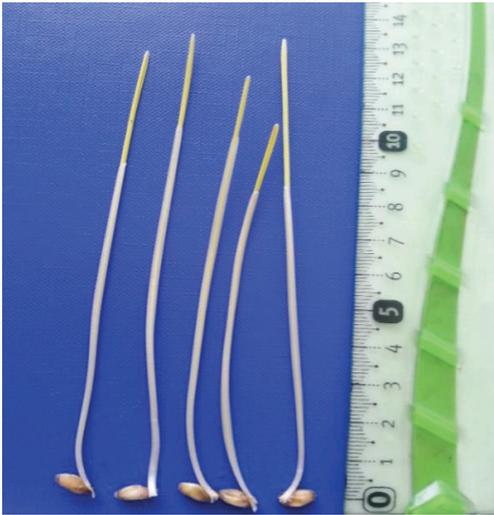


Figure 2. Coleoptile length measuring

RESULTS AND DISCUSSIONS:

Coleoptile length and plant height analysis for 86 lines included in study, revealed a large variability for both characters; thus, plant height varied between 85-113 cm (28 cm amplitude) and coleoptile length was in the range 4.23-6.6 cm (Table 1).

Table 1. The results registered for plant height and coleoptile length

No.	Genotype	Plant height (cm)	Coleoptile length (cm) at 20°
1	AiII - 214	100	5.44
2	AiII - 215	95	5.45
3	AiII - 216	95	5.62
4	AiII - 217	100	5.40
5	AiII - 218	95	5.75
6	AiII - 219	105	4.85
7	AiII - 220	100	5.35
8	AiII - 221	102	5.51
9	AiII - 222	105	5.09
10	AiII - 223	105	5.83
11	AiII - 224	97	5.70
12	AiII - 225	100	5.50
13	AiII - 226	93	5.18
14	AiII - 227	94	5.60
15	AiII - 228	102	5.05
16	AiII - 229	109	5.73
17	AiII - 230	110	5.50
18	AiII - 231	105	5.68
19	AiII - 232	101	4.76
20	AiII - 233	95	5.45
21	AiII - 234	100	5.11
22	AiII - 235	100	5.49
23	AiII - 236	97	6.09
24	AiII - 237	95	5.68

25	AiII - 238	97	4.95
26	AiII - 239	91	5.58
27	AiII - 240	95	6.51
28	AiII - 241	100	5.24
29	AiII - 242	87	4.90
30	AiII - 243	92	5.94
31	AiII - 244	105	5.61
32	AiII - 245	110	5.64
33	AiII - 246	110	5.27
34	AiII - 247	101	5.78
35	AiII - 248	86	5.48
36	AiII - 249	103	6.59
37	AiII - 250	101	6.36
38	AiII - 251	104	6.11
39	AiII - 252	100	5.73
40	AiII - 253	95	5.63
41	AiII - 254	90	5.89
42	AiII - 255	106	6.57
43	AiII - 256	91	6.28
44	AiII - 257	95	6.47
45	AiII - 258	102	6.27
46	AiII - 259	94	5.65
47	AiII - 260	115	5.79
48	AiII - 261	100	5.79
49	AiII - 262	105	5.26
50	AiII - 263	90	6.28
51	AiII - 264	102	5.30
52	AiII - 265	108	5.63
53	AiII - 266	97	4.33
54	AiII - 267	105	5.51
55	AiII - 268	103	6.22
56	AiII - 269	104	5.34
57	AiII - 270	85	4.23
58	AiII - 271	92	4.96
59	AiII - 272	93	4.93
60	AiII - 273	97	5.16
61	AiII - 274	100	5.80
62	AiII - 275	91	4.96
63	AiII - 276	95	4.23
64	AiII - 277	112	5.18
65	AiII - 278	100	5.89
66	BiII - 127	113	5.76
67	BiII - 128	95	5.93
68	BiII - 129	100	6.29
69	BiII - 130	107	5.44
70	BiII - 131	105	5.71
71	BiII - 132	110	6.12
72	BiII - 133	103	5.87
73	BiII - 134	91	4.99
74	BiII - 135	90	4.88
75	BiII - 136	101	6.41
76	BiII - 137	100	5.97
77	BiII - 138	102	5.00
78	BiII - 139	102	6.02
79	BiII - 140	93	5.16
80	BiII - 141	93	5.91
81	BiII - 142	94	5.98
82	BiII - 143	88	6.51
83	BiII - 144	88	5.34
84	BiII - 145	97	6.22
85	BiII - 146	113	6.23
86	BiII - 147	85	6.60
87	IZVOR (Check)	99	5.92
88	F0628G1-34 (Check)	97	6.30

CONCLUSIONS

The line BII143 represents a valuable genotype for breeding programs, recording the best results in our study, a semi-dwarf stature and a long coleoptile.

Even if the differences between the superior parent for coleoptile length and the best mutant/recombinant DH lines are non-significant (maximum 0.55cm), the material represent a valuable quantitative accumulation for wheat breeding programs.

Lines that revealed long coleoptile and semi-dwarf plants will be studied further for a more accurate determination of their potential, either for direct use as varieties or as genitors in the breeding programs for the creation of new varieties adapted to the conditions of Romania.

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STUDIES ON THE INFLUENCE OF SEVERAL ABIOTIC FACTORS ON SOME NEWLY ISOLATED ANTAGONISTIC STRAINS

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Abstract

The microbial antagonists represent natural means to combat the phytopathogens in order to obtain healthy crops. The abiotic factors such as temperature, pH, NaCl concentration in the growth medium and medium composition have decisive influence on the existence and development of microorganisms. Here are presented the results of the influence of these factors on development of *Pseudomonas* sp. and *Bacillus* sp. strains. Two of these strains, B1 and Bm, identified as *Pseudomonas putida* respectively *Bacillus mycoides* were recently isolated and showed remarkable activity in *in vitro* biocontrol of the phytopathogens *Erwinia carotovora* and *Xanthomonas campestris*. During experiments were tested, in the same conditions, two other strains *Bacillus subtilis* ICCF 84 and *Pseudomonas putida* ICCF 391 from Culture Collection of Industrial Importance Microorganisms (CMII). To study the influence of abiotic factors were chosen five values of temperature, five for the pH and five values for concentration of NaCl from medium. The best results regarding microbial growth were obtained for temperature values of 28 and 32°C, NaCl concentration in the culture medium between 0.5-4% and the pH between 5 and 8. Regarding the composition of the culture medium, of the four types of media, the best for the antagonists growth and development proved to be the medium M44 containing yeast extract, peptone and glycerol.

Key words: abiotic factors, antagonists, biocontrol, phytopathogens.

INTRODUCTION

Biological control of phytopathogenic microorganisms that can induce plants diseases and cause important economic losses can be accomplished by using microbial antagonists (Pamfil et al., 2008).

In order to be able to exercise their action in combating the phytopathogens, the microbial antagonists or beneficial microorganisms require certain conditions for growth and development (Davies and Whitbread, 1989). The abiotic factors such as temperature, pH, NaCl concentration in the growth medium and medium composition have decisive influence on the existence and development of microorganisms (Strickland and Rousk, 2010; Gao et al., 2012). In this situation is needed a good knowledge of abiotic factors which favors or unfavors the biocontrol agents (Ownley, 2003) in supporting the health and growth of the plants (Nihorimbere et al., 2011).

Here we investigated several factors that have decisive influence in development of some strains from the genera *Pseudomonas* and *Bacillus* that showed remarkable antagonistic activity in *in vitro* experiments.

MATERIALS AND METHODS

Growth media and cultivation conditions

Studies on the influence of abiotic factors such as temperature, pH, NaCl concentration in the growth medium and medium composition, were performed in laboratory conditions.

For temperature were chosen the values: 5°C, 16°C, 28°C, 32°C and 37°C.

The influence of pH on strains development was studied for values between 5 and 9. The NaCl concentration in the growth medium had next values: 0.5%, 1.0%, 2.0%, 4.0%, and 8.0%. Regarding the composition of the medium were investigated four recipes, detailed in CMII catalog:

- NA (0.5 g % peptone, 0.3 g % yeast extract, 1.5 g % agar, 0.5 g % NaCl),
- YMPG (yeast extract 0.3 g%, malt extract 0.3 g %, peptone 0.5 g %, glucose 1.0 g %, agar 2.0 g %),
- PDA (potato infusion 20.0 g %, dextrose 2.0 g %, agar 2.0 g %),
- M44 (yeast extract 1.00%, bacteriological peptone 1.00%, glycerol 5.00%, agar 2.00%).

A broth medium with the same composition was used for submerged fermentation. Specific parameters like optical density, pH and the biomass amounts were regularly controlled.

All culture media were prepared with distilled water, pH adjusted and sterilized for 15 minutes at 121°C.

Biologic material

For experiments were used four microbial strains (that showed remarkable antagonistic activity in *in vitro* experiments) as it follows:

- B1 and Bm, newly isolated and identified as *Pseudomonas putida* respectively *Bacillus mycoides*.

- *Bacillus subtilis* ICCF 84 and *Pseudomonas putida* ICCF 391 from Culture Collection of Industrial Importance Microorganisms (CMII) of the National Institute for Chemical-Pharmaceutical Research and Development, Bucharest.

For studying the influence of the temperature, pH and NaCl concentration, the bacterial strains were developed for 48-72 hours on NA medium. When the strains development on different recipes of medium was studied, the microorganisms were allowed to grow for 48-72 hours at 30°C.

RESULTS AND DISCUSSIONS

All the abiotic factors included in this study are very important for microbial growth and development. For best action in controlling the phytopathogenous microorganisms, the biocontrol agents should have certain characteristics like: resistance under extreme temperature (frost or heat), development at different values of pH and of NaCl concentrations, versatility to metabolize the nutrients from the environment.

As it shows in table 1, the optimal temperatures for the growth of the antagonists are situated

between 28-32°C, but there have grown as well at temperatures below 28°C, and also at 37°C.

Table 1. The influence of *temperature* on growth and development of the microbial antagonists

Microbial strain	Temperature				
	5°C	16°C	28°C	32°C	37°C
B1	+	++	+++	+++	+++
Bm	+	++	+++	+++	+++
<i>P. putida</i>	+	++	+++	+++	++
<i>B. subtilis</i>	+	++	+++	+++	+++

Legend: "+"=minimal growth; "++"=good growth; "+++ "=very good growth.

For all five values of pH, the strains Bm and *B. subtilis* ICCF 84 were very good developed (see table 2).

Table 2. The influence of *pH* on growth and development of the microbial antagonists

Microbial strain	pH				
	5	6	7	8	9
B1	+	++	++	+	-
Bm	+++	+++	+++	+++	+++
<i>P. putida</i>	+	++	+++	++	++
<i>B. subtilis</i>	+++	+++	+++	+++	+++

Legend: "+"=minimal growth; "++"=good growth; "+++ "=very good growth; "-" =no growth.

At concentrations of NaCl in the growth medium between 0.5-4.0%, all the strains were developed well (see table 3), but none was developed at the 8.0% concentration.

Table 3. The influence of *NaCl concentration* on growth and development of the microbial antagonists

Microbial strain	NaCl concentration				
	0.5%	1%	2%	4%	8%
B1	++	++	++	+	-
Bm	+++	+++	+++	+++	-
<i>P. putida</i>	+++	+++	+++	++	-
<i>B. subtilis</i>	+++	+++	+++	+++	+++

Legend: "+"=minimal growth; "++"=good growth; "+++ "=very good growth; "-" =no growth.

Regarding the composition of the culture medium (see table 4), of the four types of

media (NA, YMPG, PDA, M44), the best for the antagonists growth and development proved to be the medium M44 containing yeast extract, peptone and glycerol. On this medium all the strains grew in less than 48 h.

A broth medium with the same composition (as M44) was used for submerged fermentation (data not shown). Interestingly those after 62 h the values of optical density were still growing, and at the end of the bioprocess were obtained significant biomass amounts.

Table 3. The influence of the *culture medium* on growth and development of the microbial antagonists

Microbial strain	Culture medium			
	NA	YMPG	PDA	M44
B1	+	++	+	+++
Bm	+++	+++	+++	+++
<i>P. putida</i>	++	++	++	+++
<i>B. subtilis</i>	+++	+++	+++	+++

Legend: “+”=minimal growth; “++”=good growth; “+++”=very good growth.

CONCLUSIONS

The results of the influence of abiotic factors on the development of strains B1 and Bm, identified as *Pseudomonas putida* respectively *Bacillus mycoides*, were very good in terms of adaptability at different values of temperature, pH, concentration of NaCl. During experiments were tested, in the same conditions, two other strains *Bacillus subtilis* ICCF 84 and *Pseudomonas putida* ICCF 391 for which were obtained similar results.

The best results regarding microbial growth were obtained for temperature values of 28 and 32°C, NaCl concentration in the culture medium between 0.5-4% and the pH between 5 and 8. Regarding the composition of the culture medium, of the four types of media, the best for

the antagonists growth and development proved to be the medium M44 containing yeast extract, peptone and glycerol. These results encourages us to affirm that all strains used in this study could be suitable as antagonists as they can resist in such conditions of the environment similar to those studied here.

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IDENTIFICATION OF MAIN VOLATILE COMPOUNDS FROM THE MUST OF HYBRID GRAPES GROWN IN ROMANIA

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Abstract

Grape must originated from American hybrids *Vitis labrusca* (first generation) were analyzed by Gas Chromatography/Mass Spectrometry (GC/MS) and Gas Chromatography/Olfactometry (GC/O) to identify the main compounds that characterize aroma of these vine varieties.

Were detected a number of 29 compounds, from which 23 were identify as: volatile aldehydes and acids, ketones, esters, alcohols, terpenes. The responsible compounds with the specific character of grape *labrusca* are *o*-Aminoacetophenone, compound which give that foxy aroma of these hybrids and 2,5-dimethyl tetra hydro (2H)-3-furanone, known for having that sweet candy and candy-floss aroma. Other compounds identified in higher concentrations were the hydroxyl esters, they contributed at that "fruity" character of *labrusca* grapes.

Key words: Chromatography/Mass Spectrometry, Olfactometry, grape hybrids, *Vitis labrusca*.

INTRODUCTION

Romania is well known and appreciated for her wines, occupying 13th in world rankings of winemakers, with approximate 5 million hl of annual wine product. Cultivated area of noble vine has 243.000 ha, of which about 200.000 ha is an area of vines dedicated for wine production. However a huge area of vines, cultivated exclusively on amateur winegrowers lands (in particular from village areas) is represented by older vines belonging to the American species (interspecific hybrids).

The interspecific hybrids divided by generation, in American old hybrids (first generation), represented by varieties brought from the American continent, created before the invasion of phylloxera in Europe: *Noah*, *Isabelle*, *Lidia*, *Delaware*, *Othello* etc; Euro x American hybrids (second generation, result of crossing between hybrids from first generation with noble vines): *Seibel 1*, *Seibel 1000*, *Terras 20*, *Rayon d'or* etc, to which adds varieties with biological resistance, resulted from multiple crossings between noble vines with American hybrids (Grecu V., 2010).

The biggest areas belong to amateur winegrowers from villages, are cultivated with hybrids from first and second generation. There

are many studies about grape flavor of *Vitis vinifera*, as those which aim the interspecific hybrids cultivated on the American continent, but there is no research on flavor of older interspecific hybrids cultivated in Romania (Allen M. et al., 1991; Visan L. et al., 2007; 2015).

Gas Chromatography/Olfactometry methods (GC/O) have been used in aroma research and enable the determination of odor-active compounds in food (Williams P. J. and Allen M. S., 1996; Tominaga T. et al. 1998; 2000). Olfactometric techniques, aroma extract dilution analysis (AEDA) and Charm analysis are commonly applied in characterization of aromatic compounds in foods, inclusively in juices, grape must and wine (Serot Th., 2001).

In AEDA dilutions in series of aromatic extract are analyzed through Gas Chromatography/Olfactometry methods (GC/O) to determine the dilution factor of flavor.

Our studies have focused on identifying and characterizing of aromatic volatile compounds from first generation of hybrids must, resulting from crossing American species *Vitis labrusca* with *Vitis vinifera* (*V. labrusca* x *V. vinifera*), using the continuous extraction liquid-liquid and identification of the aromatic compounds specific of these hybrids through AEDA method.

MATERIALS AND METHODS

Juice Preparation

Grapes from *Vitis labrusca* hybrids, cultivated in southern Romania (Dambovita) were harvested at full maturity on 18 September 2016; maturity was estimated by content in sugars (degrees Brix), titrable acidity and berry size.

After harvesting, grapes were crushed and pressed with a laboratory winepress. The must was homogenized, filtered and stored at -18°C prior to extraction of volatile compounds.

Extraction of Volatile Compounds

At extraction of the volatile compounds was used a continuous extractor liquid-liquid. Two hundred milliliters of juice (containing internal standard IS: 1-heptanol) placed in a conical flask, was extracted with 5 mL of distilled dichloromethane (Merck, Darmstadt, Germany) by stirring for 30 min at 0°C and then centrifuged for 15 min at 10000 g.

The extract was dried with 4 g sodium sulfate and stored at -18°C until analysis (Chandary S. et al., 1984; Baek H.H., 1997).

Gas Chromatography/Mass Spectrometry (GC/MS)

The GC/MS system includes a Hewlett Packard 5890 Series II gas chromatograph and a Hewlett Packard 5971 mass spectrometer. Each extract was injected 1 µl in the spitless mode (200°C injector temperature, 60 sec valve delay) into a capillary column (DB-Wax, 30 m length x 0.32 mm internal diameter x 0.5 µm film thickness).

The flow rate of helium (carrier gas) was 1 mL/min. Oven temperature was programmed from 50 to 200°C at a rate of 3°C/min with initial and final hold times of 5 and 50 min (Guth H., 1997).

Mass spectrometer, conditions were: ion source temperature: 280°C; ionization energy: 70 eV; mass range: 30-350 a.m.u.; electron multiplier voltage: 2100 V; scan rate: 2.2 sec⁻¹.

Volatile compounds identification was based on comparison of GC retention indices (RI), mass spectra (comparison with MS spectra database and internal library of the laboratory) and odor properties (Tranchant J. et al., 1995; Le Guen S., 2000; Campeanu et al., 2001).

Gas Chromatography/Olfactometry (GC/O)

The GC/O system consisted of a 3300 Varian GC, equipped with a flame ionization detector (FID) and a sniffing port. Each extract (2 µL) was injected in the spitless mode (250°C injector temperature) into a capillary column. The flow rate of helium (carrier gas) was 1mL/min. The oven temperature was programmed from 50 to 200°C at a rate of 5°C/min (Serot Th., 2001).

Aroma Extract Dilution Analysis

The AEDA method is commonly applied in characterization of aromatic compounds from foods, juices, grapes and wine must. Serial dilutions (1:3) were prepared using as thinner dichloromethane. From each dilution was injected 1 µL in a FSOT column, conditions being the same as for GC/MS (Rapp A., 1998; Guth H., 1993; Ullrich F. and Grosch W., 1988). Odor description as perceived by panelists during olfactometry analysis.

RESULTS AND DISCUSSIONS

Identification of the compound

Volatile compound identification was based on comparison of GC retention indices (RI), mass spectra (comparison with MS spectra database and internal library of the laboratory) and odor properties. By using the chromatographic analysis, 23 compounds were identified and their quantity was evaluated by the method of the internal standard. The results of olfactometric analysis are summarized in table 1.

In the must of *labrusca* grapes were detected a number of 29 volatile compounds, from which 23 aromatic compounds were identified and dosed (table 2).

Aldehydes and acids

Were identified 4 aldehydes, with characterized flavors as green apples, sour (acetaldehyde), grass, green (hexanal), rose, floral aroma (phenyl acetaldehyde).

Compounds with 6 atoms of carbon, as hexenal and hexanal, are responsible for that raw, greenery smell; these compounds are formed during production and processing of the must

by the enzymatic action of lipases, lipoxygenase and alcohol dehydrogenase on the unsaturated fatty acids.

In the analyzed must were identified both compounds, but in the highest concentration was found the hexenal, which was characterized by a rough flavor, of raw, greenery, grass, cucumber.

The aroma of phenyl acetaldehyde (pure substance) can be described as honey-like, rose, green, grassy and is added to fragrances to impart hyacinth, narcissi, or rose nuances (Chisholm M. G. et al., 1994).

In the analyzed must, compound was characterized as having a sweet aroma, floral, rose (figure 1). Between acids were identified: 3-methylbutanoic acid, with a characterized aroma as dried fruits, hay and caprylic acid, with an unpleasant aroma of wax, tallow, fatty flavor (Guedes de Pinho P., 1995).

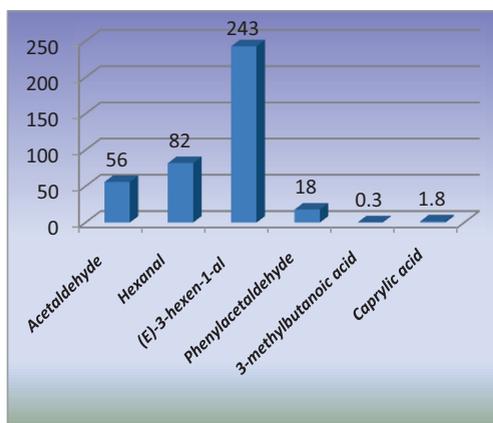


Figure 1 Aldehydes and acids identified in must of *labrusca* grapes (ppm)

Esters

In *labrusca* grapes were identified 9 esters, represents 40% from the identified compounds. This were characterized by having pleasant smells, fruitiness and floral, as Ethyl butyrate, Ethyl 2-methyl butanoate, Ethyl hexanoate, Ethyl decanoate, ethyl 3-hydroxy butanoate and Phenyl acetate, but heavy odor, unpleasant: Ethyl acetate, Ethyl caproate (pungent, sour, cheese), and Ethyl-2-hydroxy-3-phenylpropanoate. The hydroxyl esters contributed to the “fruity” character of *labrusca* grapes (Schreier P., 1980). In the analyzed must the hydroxy esters were identified in high concentrations, way beyond threshold (figure 2).

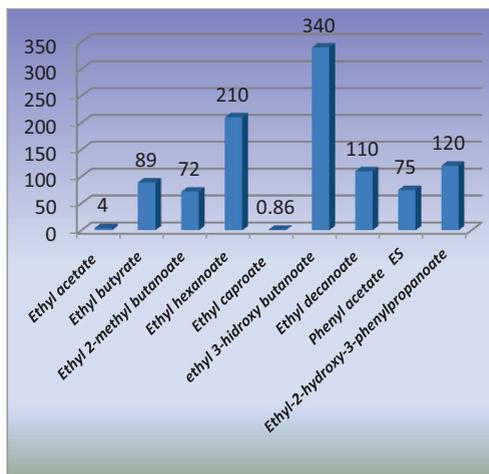


Figure 2 Esters identified in must of *labrusca* grapes (ppm)

Alcohols

Alcohols identified: 2-methyl 3-buten-2-ol with sweet, oily, fruity, herbal aroma and 2-phenylethanol (rose, floral).

2,5-dimethyl tetrahydro (2H)-3-furanone

In *Vitis vinifera* wines, furaneol is found in reduced concentrations. In contrary in must and wines of American hybrids have caramel flavor or strawberries and is found in big quantity, above his sensory perception threshold, of 30-300 µg/l (Rapp et al., 1980).

In the analyzed must, the 2,5-dimethyl tetrahydro (2H)-3-furanone is found in large quantity, the compound being characterized as having a sweet candy and candy floss aroma (Acree T. and Lavin E, 1990). Furaneol is one of the compounds responsible with the specific character of *labrusca*.

Among ketones were identified *o*-Aminoacetophenone, 2-pentanone, with a rough aroma, of solvent; 1-octene-3-ona with a mushroom, earth, oxidized metal aroma; 6-methyl-1-heptenone with green grass aroma;

o-Aminoacetophenone

Rough smell of foxy compound, cat urine, were identified in higher concentration, being considered together with 2,5-dimethyl tetrahydro (2H)-3-furanone aromatic compounds, predominantly in hybrid must, responsible for the foxy odour of *Vitis lambrusca* (Rogers I.M. and C.J. van Wyk, 2000).

Table 1. Odor-Active Compounds detected in the grape must by Olfactometric Method

No. peak	Compound	RI DB-Wax column	Odor description
1	Acetaldehyde	705	green apples, sour
2	Ethyl acetate	851	sweet unpleasant smell
3	unknown	915	caramel, pleasant
4	2-pentanone	982	acetone
5	Ethyl butyrate	1045	fruity, pineapple
6	Ethyl 2-methyl butanoate	1062	green apples, fruity
7	2-methyl 3-buten-2-ol	1070	sweet, oily, fruity, herbal
8	Hexanal	1084	grass, green
9	unknown	1112	unpleasant, mice urine
10	(E)-3-hexen-1-al	1145	grass, green, cucumber
11	unknown	1198	bitter, greenery
12	Ethyl hexanoate	1250	green apples, fruity
13	Ethyl caproate	1259	cheese, oil, pungent, sour
14	1-octene-3-ona	1315	mushroom, earth, metal
15	6-methyl-1-heptenone	1368	grass, green
16	ethyl 3-hidroxy butanoate	1520	sweaty
17	Phenylacetaldehyde	1652	rose, floral, sweet
18	Ethyl decanoate	1672	brandy, grape, pear
19	3-methylbutanoic acid	1681	dried fruits, hay
20	unknown	1695	green, foxy
21	unknown	1752	dark chocolate
22	Phenyl acetate	1832	sweet, floral, honey, spice
23	Ethyl-2-hydroxy-3-phenylpropanoate	1995	fat, fruit, pungent
24	2-phenylethanol	2011	rose, floral
25	4-hydroxy-2,5-dimethyl-3(2H)-furanone	2052	sweet candy, candy-floss
26	Caprylic acid	2072	wax, tallow, fatty flavor
27	o-Aminoacetophenone	2195	foxy, cat urine
28	unknown	2235	smell channel
29	β -mircen	2241	floral, pleasant

Table 2. Concentrations of volatile compounds identified in *labrusca* must

No. peak	Compound	ppm	
1	Acetaldehyde	25	56
2	Ethyl acetate	-	4
3	2-pentanone	-	0.045
4	Ethyl butyrate	-	89
5	Ethyl 2-methyl butanoate	1.12	72
6	2-methyl 3-buten-2-ol	300-330	147
7	Hexanal	10	82
8	(E)-3-hexen-1-al	-	243
9	Ethyl hexanoate	76	210
10	Ethyl caproate	240	0.86
11	1-octene-3-ona	0.03-1.12	0.8
12	6-methyl-1-heptenone	10	trace
13	ethyl 3-hidroxy butanoate	-	340
14	Phenylacetaldehyde	1.1	18
15	Ethyl decanoate	-	110
16	3-methylbutanoic acid	0.7	0.3
17	Phenyl acetate	-	75
18	Ethyl-2-hydroxy-3-phenylpropanoate	-	120
19	2-phenylethanol	25-105	59.8
20	4-hydroxy-2,5-dimethyl-3(2H)-furanone	25	292
21	Caprylic acid	10	1.8
22	o-Aminoacetophenone	0.5	0.7
23	β -mircen	36-461	2.4

CONCLUSIONS

In musts from *labrusca* grapes were identified 29 volatile compounds, from which 23 are aromatic compounds.

The identified aldehydes were hexenal, in higher concentration, characterized with a rough aroma, of raw, greenery, grass, cucumber, hexanal, acetaldehyde and phenyl acetaldehyde with a sweet aroma, floral, of roses.

Among acids were identified 3-methylbutanoic acid, with a characterized aroma as dried fruits, hay and caprylic acid with an unpleasant aroma of wax, tallow, fatty flavor.

In the *labrusca* grapes were identified 9 esters characterized as having pleasant smells, floral and fruitiness (Ethyl butyrate, Ethyl 2-methyl butanoate, Ethyl hexanoate, Ethyl decanoate, ethyl 3-hidroxy butanoate and Phenyl acetate) and even heavy scents, unpleasant: Ethyl acetate, Ethyl caproate (pungent, sour, cheese), and Ethyl-2-hydroxy-3-phenyl propanoate.

In analyzed must, the hydroxy esters were identified in higher concentrations, far over threshold.

In must of *labrusca* grapes the 2,5-dimethyl tetra hydro (2H)-3-furanone is found in higher quantity, compound being characterized as having aroma of sweet candy, candy floss, one of the compounds responsible of specific character of *labrusca*.

o-Aminoacetophenone characterized as having a heavy smell of foxy, cat urine, were identified in higher concentration being together with 2,5-dimethyl tetra hydro (2H)-3-furanone aromatic compounds responsible for the foxy character of *Vitis lambrusca*.

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PHENOLIC CONTENT AND POTENTIAL INHIBITORY ACTIVITY OF ROMANIAN BEE POLLEN ON DIFFERENT PLANT PATHOGENIC STRAINS

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Abstract

*Bee pollen is a fine powder gathered from different plant species, enriched and transformed by bees into a complex bee product. Most bioactive properties of bee pollen, including antimicrobial activity, have been attributed to phenolic compounds. The aim of this study was determination of botanical origin, total phenolic content, flavonoid content and antimicrobial properties of three different pollen types from Romania against some plant microbial pathogens. Due to the fact that bioactive compounds of bee pollen depends strongly on the plant source, we tested extracts of fresh monofloral and polyfloral bee pollen. The ethanol 70% was used for pollen extraction. In this study, three microbial agents were tested: two bacterial strains represented by *Erwinia carotovora* (subsp. *carotovora*) ICCF 138, *Xanthomonas campestris* ICCF 274 and one fungal strain represented by *Aspergillus niger* ICCF 92. Furthermore, total phenolic and flavonoid contents were carried out using Folin-Ciocalteu procedure and aluminum chloride spectrophotometric method, respectively. Qualitative screening of pollen extracts antimicrobial activity was tested by disc diffusion method. The palynological investigations allowed us to identify the botanical origin of bee pollen loads, represented by species of eight different genera. Pollen sample extracts revealed high values for phenolic and flavonoid contents and also demonstrated that possess antimicrobial activities. We tried to established if the total phenolic or flavonoid content were related to the phytopathogenic antimicrobial activity. Our results indicated that bee pollen could be considered a promising natural source of plants protection.*

Key words: bee pollen, phenolic content, antimicrobial activity.

INTRODUCTION

Bee pollen is a fine powder gathered from different plant species, enriched and transformed by bees into a complex bee product. Composition of bee collected pollen presents variations from species to species, however, the major components are proteins and amino acids, sugars and lipids (Pawar et al., 2014).

The minor components are represented by vitamins, minerals and flavonoid glycosides (Bogdanov, 2004). The chemical constituents and also the secondary phenolic metabolites (carotenoids, phenolic compounds and in particular, flavonoids) play an important role in biological activities assigned to bee pollen. The bioactive compounds, even are present in small quantities, contributed to beneficial properties

(Guiné, 2015). These secondary metabolites have a potential for many biological activities which include antimicrobial property (Diao, 2015). The therapeutic properties of bee pollen have been widely investigated (Carpes et al., 2007; Jannesar et al., 2014; Mărgăoan et al., 2016) and also has been extensively used in food (Khider et al., 2013; Solgajová et al., 2014). In recent years, bee pollen, has gained increased attention not only for its high nutritive value and apitherapeutic properties, it was demonstrated the efficiency as a seed protectant agent for plant disease, as well (Basim, 2006).

Among other bacteria species, *Xanthomonas campestris* and *Erwinia carotovora* are incriminate that causes severe damage to agricultural crops, between 20-40% of global crop totals are lost annually. Also, *Aspergillus*

spp. causes significant health hazards and foodborne infections. (Pandey et al., 2017) The purpose of this study was to investigate the contents of biologically active compounds and antimicrobial potential of Romanian pollen against plant pathogens.

MATERIALS AND METHODS

Collection of samples

The bee pollen loads were collected from different apiary using pollen traps. We selected fresh monofloral and polyfloral samples with different botanical origins. To determine the plant sources, pollen preparation was made and observed under optical microscope after protocol proposed by Barth O. (Barth et al., 2010). The pollen grains were investigated for morphological characterisation. Exine surface sculpture has different physiological and structural adaptations which forming a characteristic pattern for each species. (Chwil, 2015)

Preparing of bee collected pollen extract

Each samples (5 g) were milled and extracted individually using 50 mL ethanol 70%. This mixture was sonicated for 15 min. and left overnight at room temperature. The extracts were filtered through Whatman paper no. 5 and stored at 4-6°C until use.

Determination of total polyphenol and flavonoid contents

For total phenolic and flavonoid contents we used ethanolic extracts of fresh bee pollen. Total phenolic content was quantified using Folin-Ciocalteu method (Rebiai and Lanez, 2012) and flavonoid content was determined with the help of colorimetric method measured at 430 nm by comparing with standard curve of quercetin. (Rebiai and Lanez, 2013)

Antimicrobial activity testing

Qualitative antimicrobial screening of pollen extracts was tested against two bacterial strains represented by *Erwinia carotovora* (subsp. *carotovora*) ICCF 138, *Xanthomonas campestris* ICCF 274 and one fungal strain represented by *Aspergillus niger* ICCF 92. We used two comparative methods: disc diffusion and cylinder method with different volumes of pollen extract, 10µL and respectively, 100µL. Final concentrations of bacteria cultures were 10⁹ cfu/mL and 10⁶ cfu/mL for *Aspergillus niger*. All tests were done in triplicate.

RESULTS AND DISCUSSIONS

The identification of pollen types was based on shape, morphological characteristics, size and also was used the reference collection slides from bee product chemistry laboratory of the Institute for Apicultural Research and Development and pollen atlases (Ricciardelli, 1997; Bucher, 2004).

Almost all the taxa determined in the pollen samples came from insect-pollinated plants and only two species from wind-pollinated plants belonging to *Poaceae*.

Samples were considered monofloral with more than 90% of a unique pollen type or heterofloral batches represented by three or more botanical species (Freitas, 2013).

Due to bees forage different plants, none of the sample occurrence over 92% of one pollen type. Samples A and B represented in Figure 1 have between two and three accessory pollen types, no more than 3%. This minor pollen is attributed to surface contamination from other pollen pellets (Stimec et al., 1997). Results showed that *Asteraceae* and *Brassicaceae* families were detected in sample A and *Boraginaceae*, *Asteraceae* and *Chenopodiaceae* families were present in sample B.



Fig. 1. Bee pollen loads: A-*Prunus sp.*; B-*Rubus sp.*; C-polyfloral

Prunus sp. occurred in sample A, Figure 2, as dominant pollen type (92%) and *Rubus sp.* was found in sample B, Figure 2, (91%) both of which were classified as monofloral. Macroscopic aspect of sample C presented in

Figure 1 (C), suggests a wide variety of pollen types, confirmed by microscopic analysis. *Asteraceae* (*Carduus* type), *Fabaceae*, *Rosaceae*, *Brassicaceae* and *Salicaceae* were the main families identified in this sample.

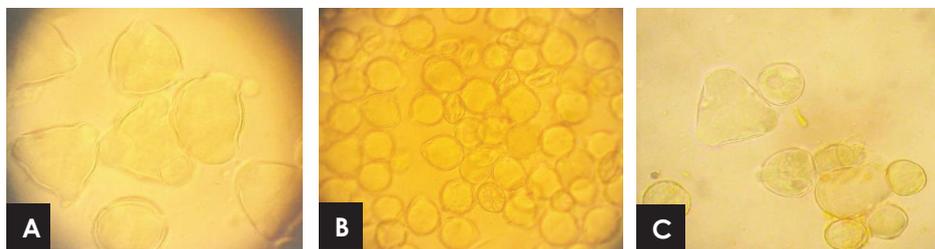


Fig.2 Morphological aspects of bee pollen types tested, optical microscopy (40X): A-*Prunus sp.*;B-*Rubus sp.*; C-polyfloral

High levels of phenolic constituents are often accompanied by a high antimicrobial activity (Carpes, 2007)

Total phenolic content was highest in polyfloral pollen extract (25.33 mg GAE/g pollen), followed by *Prunus sp.* extract (22.64 mg GAE/g pollen) and *Rubus sp.* extract (21.1 mg GAE/g pollen). In the case of flavonoids was observed a decreased content in the same order: polyfloral >*Prunus sp.*>*Rubus sp.*. The results indicated that polyphenol and flavonoid contents mainly depend of the plant origins (Figure 3). A similar conclusion was reached by Zhang et al. (2015), who reported that total polyphenol and flavonoid contents varied significantly according to the floral species. (Zhang et al., 2015)

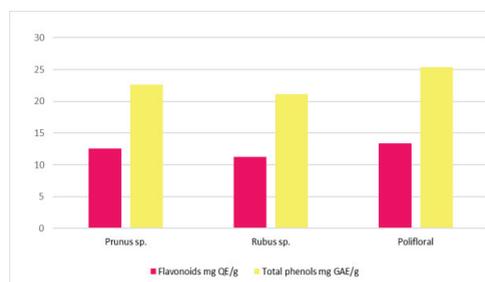


Fig. 3 Graphical representation of flavonoid and total phenol contents of pollen extracts

The antimicrobial activity of bee pollen ethanolic extracts was analysed according to the disk diffusion assay and cylinder method and the results are shown in Table 1.

Table 1 Antimicrobial activity of bee pollen ethanolic extracts (PEE)-inhibition zone diameter in mm

Pollen extract (PEE)	<i>Erwinia carotovora</i> (subsp. carotovora) ICCF 138	<i>Xanthomonas campestris</i> ICCF 274	<i>Aspergillus niger</i> ICCF 92
Disc diffusion method (10µL)			
<i>Prunus sp.</i>	1	1	0
<i>Rubus sp.</i>	1	2	0
Polyfloral	2	2	0
Control (EtOH)	0	0	0
Cylinder method (100µL)			
<i>Prunus sp.</i>	11	4	0
<i>Rubus sp.</i>	11	9	0
Polyfloral	12.5	5	0
Control (EtOH)	0	0	0

The obtained results characterize Romanian bee pollen as a product with antibacterian effect. The strongest antimicrobial effect was shown by cylinder method against bacteria

strains. Very good inhibitory effect of polyfloral bee pollen was found against *Erwinia carotovora* (Table 1, Figure 4).

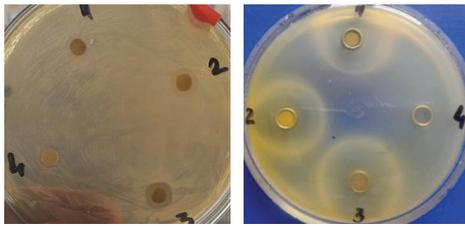


Fig.4 Inhibition of *Erwinia carotovora* by pollen extracts noted as follow (1-*Prunus sp.*, 2-*Rubus sp.*, 3-polyfloral, 4-ethanol, control);(disc difusimetric method/cylinder method)

There was found a similar antibacterial effect of polyfloral and *Rubus* pollen to *Xanthomonas campestris* by disc diffusion method. The same situation was observed for *Prunus* and *Rubus* bee pollen to *Erwinia carotovora*. (Table 1, Figure 5).

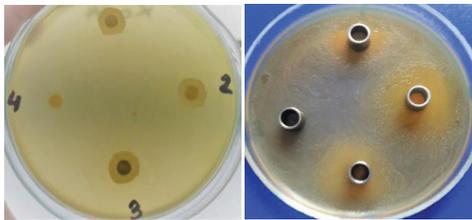


Fig. 5 Inhibition of *Xanthomonas campestris* by pollen extracts (disc difusimetric method/cylinder method)

None of the bee pollen ethanol extracts showed inhibition against *Aspergillus niger* (Table 1, Figure 6).

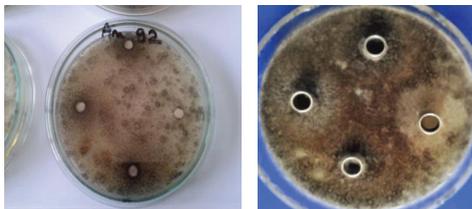


Fig. 6 *Aspergillus niger* growth on all bee pollen extracts (disc difusimetric method/cylinder method)

Negative control (ethanol) represented by no. 4 in Figures 4, 5 and 6, did not show an inhibitory effect on the tested bacterial strains. In the study of Basim et al. (2006), was demonstrated antimicrobial activity of Turkish bee pollen against 13 different bacterial species pathogens for plants.

He reported that all bee pollen extracts have an inhibitory effect against all pathogens. The diameters (in mm) of the clear zones of growth

inhibitions around the spots in the case of *Erwinia carotovora* (subsp. *carotovora*) and *X. campestris pv. campestris* were larger than those obtained in our study. The differences between the results may be attributed to the pollen type and the solvent used, methanolic extracts being more effective (Khider et al., 2013).

CONCLUSIONS

In our study, polyfloral bee pollen contains the highest phenolic compounds and an inhibitory effect on plant pathogenic strains. Bee pollen extracts exhibited different antimicrobial activities related to phenolic compounds.

The results of antimicrobial tests showed an interesting activity against *Erwinia carotovora* and *Xanthomonas campestris* but no effect on *Aspergillus niger*, also we found that pollen extracts exert different selectivity for each microorganism.

Our results revealed that the antimicrobial activity increased with the content of phenolic compounds in pollen but is needed more studies regarding antibacterial activities of pollen against plant pathogenic microorganisms.

This work also indicated that bee pollen could be considered a promising natural source for plants protection.

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EMERGING TECHNOLOGIES FOR MARA SEA BUCKTHORN (*Hippophae rhamnoides* L.) BERRIES VALORIFICATION

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Abstract

*This paper aims to assess the viability of using novel oil extraction methods for Sea Buckthorn (*Hippophae rhamnoides* L.). Supercritical fluid extraction (SCFE) although extensively used for oil extraction in other countries, is not commercially used in Romania at the moment. Cost constraints, as well as the ease of use, more established methods such as solvent extraction and cold pressing have delayed the implementation of such technologies. Three oil sources were investigated: oils extracted from dry berries using SCFE and cold pressing, and oil extracted from druff (residues after juice extraction) using SCFE. The oils have been analyzed using a HPLC unit, and their carotenoid levels were compared. The results show a slight variation in the carotenoid composition in relation to the extraction methods. This suggests that the SCFE method is viable to be used for large scale Sea Buckthorn oil production.*

Key words: Sea Buckthorn, supercritical fluid extraction, cold pressing, oil, Romania, carotenoids.

INTRODUCTION

Recent trends in nutrition are starting to integrate novel foods into diets. Because of their so called “nutraceutical” values, Foods such as Sea Buckthorn are seen as a viable alternative to conventional sources of vitamins, minerals and other essential elements of a healthy lifestyle (Yang and Kallio, 2001; Upadhyay et al., 2011).

Traditional products from the Sea Buckthorn berries include juices, liqueurs, wine, jams, candy, and ice-cream. However, the berry’s unique chemical and nutritional composition has offered economic potential as a health food (Krejcarová, 2015, Suryakumar and Gupta, 2011). Sea Buckthorn oil contains a large amount of fatty acids, liposoluble vitamins and sterols, and are considered the most valuable part of the berries (Cenkowski et al., 2006; Górnaś et al., 2016).

The method used to extract oil from oil-bearing materials is the limiting factor of its quality, as heat or solvents interfere with its purity and chemical composition. Chemical solvent extraction is at the moment the preferred method for obtaining Sea Buckthorn oil. Its very high extraction efficiency is economically viable,

however, unless properly executed using sophisticated equipment, proper hygienic conditions and strict parameter process control, it has the potential to leave traces of residual chemical solvents in the finished product, making it unfit for human consumption (Bargale et al., 1999).

The SCFE method removes these concerns, as it uses CO₂ in its supercritical state (liquid) to extract the oils from the source material (Jose, 2015; Walker et al., 2007). The main advantage of the method is the ease of the solvent’s recovery after extraction, ensuring that the finished product is 100% pure (Fornari, 2016; Mohamed and Mansoori, 2002).

This paper aims to investigate the differences between different extraction methods, seeing that the nutraceutical market is gaining more and more recognition from Romanian consumers, and demand for alternative nutrient sources is due to rise.

MATERIALS AND METHODS

Raw materials

Ripe berries of Mara sea buckthorn (*Hippophae rhamnoides* L.) were collected from the Biofarmnet plantation, Ialomita county, located

in the South region of Romania. Harvesting was done by cutting whole branches off the shrubs in September through October, then quickly freezing them at -38°C. This allows the berries to be removed from the branch with minimal damage by shaking. The berries are sorted and graded by hand then frozen at -18°C. Stored at this temperature, the berries maintain their physico-chemical properties and can safely be kept for up to 6 months without any significant damage such as freezer burn or spoilage.

Whole berries were dried in a discontinuous drier at 40°C for six days. The dried berries were extracted by cold pressing and supercritical carbon dioxide. For higher valorification of raw material, after the juice processing, the residues (draff) were extracted by supercritical carbon dioxide.

Supercritical carbon dioxide extraction (SFE) of sea buckthorn berries

Extractions were carried out using a pilot-plant sized supercritical carbon dioxide extractor (Natex, Prozesstechnologie GesmbH, Austria, Fabr. no. 10-023/2011) designed with a single cylinder extraction vessel and two separators. The extraction was carried out for 1000 g of raw material.

The extractor basket was filled with ~ 0.300 kg of ground dried sea buckthorn in three batches. During extraction, the solvent (technical CO₂, 99.99% purity supplied by Messer S.A., Romania) was constantly chilled to remain liquid and able to be recirculated. The solvent was brought to supercritical conditions at 7.30 MPa, and a flow rate of 20 kg/h, as indicated by the data sheets from ABB software (ABB - Mannheim, Germany). The extraction conditions were carried out using Xiang Xu et.al. (2008) experimental parameters (pressure of 27.6 MPa, temperature of 34.51°C and extraction time of 82.0 min). The oil was collected at the end of the process, weighed and analysed using the HPLC method.

Cold pressing extraction of sea buckthorn berries

Sea buckthorn Mara seeds were cold pressed on site by Biofarmnet SRL. The dried berries were cleaned and sorted. 10 kg of seeds were added in the receiving funnel of the cold

presser. Soon after, 5 kg of oil was obtained. The resulting oil was subjected to a sedimentation period of three days, in order to remove impurities and protein residues.

The oil was then filtered using a 5 micron cloth, then moved into brown glass bottles to protect against oxidation.

Pressing was done using a FARMER 20 cold press with the Farnet Duo screw press attached by FARMET.

Sea buckthorn juice process

The juice was obtained with a domestic slow juicer (Greenis Slow Juicer, model F-9007) made from BPA free plastics. The juicing strainers are made from GE-Ultem plastic and the super slow 65 rpm speed reduces oxidation with minimal loss of nutrients. The juice was used for jelly production and the residues were dried at 40°C prior to the SFE.

Carotenoids identification by high-performance liquid chromatography (HPLC) analysis

The sea buckthorn oil samples obtained by supercritical carbon dioxide extraction were analyzed by HPLC in order to identify and quantify the carotenoid levels.

The system used was an HPLC from Thermo Finnigan Surveyor (Finnigan Surveyor LC, Thermo Scientific, SUA), controlled by Xcalibur software system. The carotenoids from each sample were analyzed at 450 nm on a Lichrosorb RP-18 (5 µm) Hibar RT 125-4 column. The elution solvents were 90% acetonitrile (A) and 100% ethyl acetate (B). The injection volume was 20 µL, and the flow rate was maintained at 0.500 mL/min.

The elution profile used was: 0–16 min, isocratic on 15% B; 16–54 min, linear gradient from 15% to 62% B, 54–56 min, isocratic on 62% B; 56–60 min, linear gradient from 62% to 15% B; 60–70 min, isocratic on 15% B (Pop et al., 2014). The quantification of carotenoids was done using a β-carotene calibration curve. The calibration curve for the β-carotene standard was prepared using six different concentrations (0.04–0.1 mg/ml) and dissolving it in ethyl acetate before the analysis. The linear regression factor of the calibration curve for this standard was 0.988.

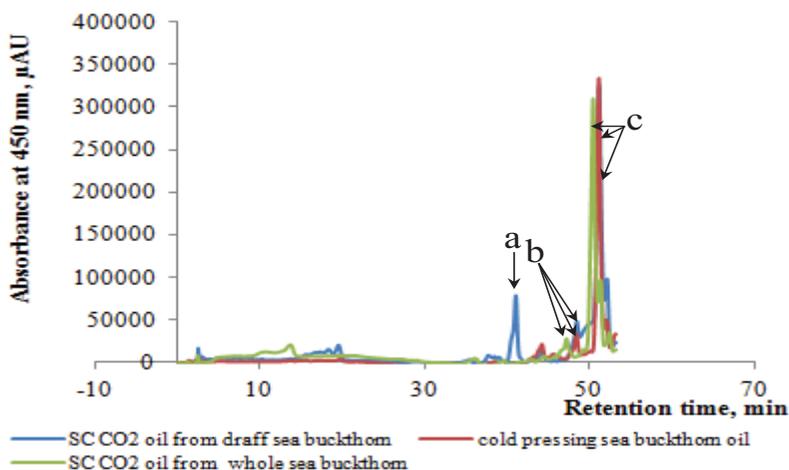


Figure 1. Representative HPLC chromatogram of carotenoids from MARA sea buckthorn oil sample, recorded at 450 nm

RESULTS AND DISCUSSION

A quantitative HPLC analysis of sea buckthorn oils was used to identify the carotenoids. Figure 1 shows the HPLC chromatogram for all oil samples. Carotenoids identification was made based on their retention time and comparison with literature data.

β -criptoxantina (peak a) and lycopene (peak b) were identified in all samples. The quantitative evaluation indicated a variation of carotenoid content in the range 0,028 – 0,33 mg/g, while β -criptoxantina was present only in the sea buckthorn draff oil (0,54 mg/g). Among the carotenoids, β -carotene (peak c) was identified in all oils samples with a small variation in the range (2,65 – 3,07) mg/g.

The carotenoid composition varied largely due to the varieties and extraction methods.

CONCLUSIONS

After analyzing the results, it has been concluded that further research is needed in order to definitely state that SCFE is a reliable and cost efficient method of extracting Sea Buckthorn oil in Romania. This paper only assessed the feasibility of obtaining oil using such technology; however it has not covered aspects such as costs or market research. It is currently unknown if using such technologies is economically viable in the Romanian market,

as traditional technologies are both established and cost efficient due to their much lower implementation costs.

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**BIOTECHNOLOGY
IN VETERINARY
MEDICINE**

MICROBIOLOGICAL QUALITY CONTROL OF A NEW PLANTS MIX EXTRACT FOR VETERINARY USE

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Abstract

*The microbiological control of veterinary products needs an integrated approach, being part of the quality assurance in the pharmaceutical industry. During the production of a phytopharmaceutical product of veterinary use it is compulsory to have a standardized method for the quantification of the microbial charges (fungi and bacteria) from the raw vegetal material to the final product. Our work has been focused on the microbial charges of a new phytoimmunomodulator veterinary product based on *Inula* sp., *Eupatorium* sp. and *Helleborus* sp. For the product standardisation several attempts have been done and one part of the work was related to microbiological criteria fulfilment. The microbial charges have been quantified according to adapted method developed by the authors and correlated to limits recommended by European Pharmacopeia. In the case of the raw dried and grounded plants the total mesophilic aerobic bacteria load is much higher than the recommended limits, while the fungal load has reached almost the maximum recommended limits. Acceptable contents of coliforms and no traces of *Salmonella* have been detected in the final product. The phytopharmaceutical company to patent and produce the new veterinary product, should make efforts especially in the raw material procurements, as long as their actual sources comes with a much more higher content in aerobic bacteria than the recommended limits. Supplementary measures should be taken to avoid in this context the cross-contamination.*

Key words: good manufacturing practices, microbial charge, veterinary phytopharmaceutical product.

INTRODUCTION

The medicinal plants represents, by centuries, the main raw material for the old but always new phytopharmacy. The medicinal plants' extracts have been demonstrated to have different effects on human, but also on animal health, like antimicrobial and antioxidant activity, resistance against toxins or stimulate the enzymatic activity and nitrogen absorption (Viegi et al., 2003, Burcea et al. 2007).

A special attention have been given in the last decades to develop mix products made of different plants with medicinal effects for a better prevention or cure of human and animal diseases. Because our work has taken into account a mix made of *Inula*, *Eupatorium* and *Helleborus*, these plants will be shortly presented for their phytopharmaceutical potential.

Relatively recently, the studies have demonstrated that *Inula* sp. shows different positive biologic activities, respectively:

anticancerigenic (Dorn et al., 2006), antimicrobial (Cohen et al. 2002, Diguta et al., 2014; Zhao et al., 2010), hepato-protector or anti-inflammatory. Empirically, dried roots of *Inula* were used for the cows for a better and safety milk production of for the sheep and pigs to keep away their illness (Khuroo et al., 2007, Davidovic et al., 2012).

Also, the literature reported *Eupatorium* having different pharmacological effects such as antimicrobial (Purcaru et al., 2015), antiinflammatory, immunoregulatory, liver damage protection, blood glucose decrease (Kazuo, et al.: 1979; Xu et al., 1998; Yan et al., 2003). Moreover, extracts of *Eupatorium lindleyanum* are proposed to be used as food additive (Li et al, 2008), while essential oil of *Eupatorium cannabinum* can be employed during food storage against *Aspergillus* development and aflatoxin formation (Kumar et al, 2007).

Meanwhile, in the case of *Helleborus* have been proven its antibacterial activity (Puglisi et

al, 2009) or antineoplastic properties (Wang et al, 2004).

The microbiological control of veterinary products needs an integrated approach, being part of the quality assurance in the pharmaceutical industry. The quality control must be applied along the whole technological flux, from the raw material reception to the final product, ready to be delivered. Each phytopharmaceutical company should elaborate internal procedures according to the recommendation of the *Pharmacopoeias*.

During the technological process it is necessary to minimize as much as possible the microbial contamination by following the principles of *Good Manufacturing Practices (GMP)*. It is very important to be taken into account the fact that most of the physical or chemical interventions taken for the microbial load reduction may affect negatively the active principles of the plants.

The main contamination source is even the plant as raw material and the initial microbiological control is a must, as well as after a preliminary processing to be able to avoid the cross-contaminations and the final product to have a minimal microbial load, under the recommended limits. In the case of non-aqueous products, the contamination issue is diminished because the microorganisms can't survive in environments with low water activity (aw).

In the case of phytopharmaceutical products, including the one of veterinary use, the European *Pharmacopeia* delimits the following microbiological quality indicators: total number of aerobe mesophilic bacteria and fungi, as well as the presence of specific pathogens, like *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* or *Salmonella* (Table 1).

Table 1 – Acceptability limits for microbial loads in phytopharmaceutical products in CFU /g or CFU /ml (source: *European Pharmacopeia* 8.0)

Microbiological indicator	Dried plant	Pretreated extracts 1 (treatments which can lead to the decrease of microbial load)	Pretreated extracts 2 (treatments which can't lead to the decrease of microbial load)
Total number of mesophilic aerobic bacteria	10 ⁷	10 ⁴	10 ⁵
Fungi	10 ⁵	10 ²	10 ⁴
<i>E.coli</i>	10 ³	Absent (in 1 g or 1 ml)	Absent (in 1 g or 1 ml)
<i>Salmonella</i> spp.	Absent (in 25 g)	Absent (in 25 g or 25 ml)	Absent (in 25 g or 25 ml)

The final phytopharmaceutical product of veterinary use is proposed to be made of three different plants, as described above (*Inula*, *Eupatorium* and *Helleborus*), this is why the microbiological indicators have been analysed for the plants as raw materials, as well as for the final mix product, according to the *European Pharmacopeia*.

MATERIALS AND METHODS

The **biologic material** consisted in dried and grounded parts of *Inula*, *Eupatorium* and *Helleborus* plants cultivated under ecological conditions in Brasov county, Bod area. Also, the analysis have been applied to a mix of these plants extracted (0.25 g/ml) in 20% ethanol (v/v). The recipe of the product is subject to a patent and data can't be disclosed.

The **media** used in the testing are the one recommended by *European Pharmacopeia*, respectively, for the bacterial counting has been used nutrient agar, for the fungal load YGP (Yeast Extract-Glucose-Peptone), for the coliforms both BBLV and GEAM Levine media.

Sample preparation: 5 g of each sample (plants and mix) have been suspended in 45 ml of buffered peptone water followed by an agitation at 100 rpm/30 min for a total microorganisms recovery. Supplementary decimal dilutions (10⁻³ to 10⁻⁷) have been applied to the samples, according to former preliminary results. For the analysis last two dilutions have been employed and made it in triplicate.

In the case of aerobe bacteria and fungi counting has been used the classical

inoculation method, respectively plate spreading technique. The **incubation** temperature for bacteria was 35°C/24-48 hours, while for the fungi 28°C/2-3 days.

After the incubation the colony-forming units have been counted and the results have been compared to the recommended limits.

In the case of *E.coli* analysis the samples have been inoculated in triplicate in fermentation tubes with BBLV medium and incubated at 37°C/48 hours; positive samples (forming gas) are laid into plate with GEAM medium at 37°C/24 hours for the confirmation.

For *Salmonella* spp. presence classical steps have been followed: pre-enrichment in buffered peptone water at 35°C/6 hours, enrichment in Muller-Kauffmann at 35°C/24 hours, isolation by plating on deoxycolate medium and incubated at 35°C/48 hours; if positive colonies would appear the confirmation is made by plating on TSI (Triple Sugar Agar) medium at 35°C/48 hours.

In the case of *E.coli* and *Salmonella* spp., have been used test microorganisms, respectively *Escherichia coli* ATCC 8739 and *Salmonella typhimurium* ATCC14028.

RESULTS

For the herbal products registration there are some regulatory challenges to be faced, including the microbiological loads in respect to the recommended limits.

In the recent past years have been developed a veterinary immunomodulatory product (under patent) made of three plants mix, respectively *Inula*, *Eupatorium* and *Helleborus*.

For the product standardisation several attempts have been done and one part of the work was related to microbiological criteria fulfilment.

In this regard, microbiological analysis have been performed for the raw materials and for the final mix and compared with limits recommended by the *European Pharmacopeia*. The results are presented as average of three different analysis and can be followed in table 2. In the case of the raw dried and grounded plants the total mesophilic aerobic bacteria load is much higher than the recommended limits, while the fungal load has reached almost the maximum recommended limits.

Table 2-Microbial load of raw materials and plant mix of a phytoimmunomodulator veterinary new product (CFU/g)

Medicinal plant	Mesophilic aerob bacteria	Fungi	<i>E. coli</i>	<i>Salmonella</i>
<i>Eupatorium spp.</i>	5.2x10 ⁷	1.5x10 ⁴	0.1x 10 ³	-
<i>Helleborus spp.</i>	TNBC*	5.0x10 ⁴	0.8 x 10 ³	-
<i>Inula spp.</i>	2.2x10 ⁷	2.5x10 ⁴	0.6 x 10 ³	-
Mix product	2.8x10 ³	-	-	-

*TNBC - too numerous to be counted
- not detected

No thermal process have been applied to the raw plants, but in the mix have been added ethanol (20% v/v); in this context, the total mesophilic aerobic bacterial and fungal loads have registered values under the *European Pharmacopeia* recommended limits.

In the case of all the samples the coliforms loads are positively under the recommended limits, while the presence of *Salmonella* has not been registered in any of those.

In terms of microbial diversity, all three medicinal plants have shown moderate diversity; for instance have been isolated four different bacterial species (to be identified) and two main fungal species of which one was macroscopically identified as *Aspergillus* sp.

(figure 1). The presence of fungi should be carefully investigated and/or monitored, since some common species produce toxins, especially aflatoxins. Aflatoxins in herbal drugs can be dangerous to health even if they are absorbed in minute amounts.



Fig.1 Aspects regarding the fungal load of dried medicinal plant (left) and of the plants' mix (right)

CONCLUSIONS

The microbiological control of veterinary products needs an integrated approach, being part of the quality assurance in the pharmaceutical industry and must be applied from the raw material reception to the final product. Medicinal plants may be associated with a broad variety of microbial contaminants, represented by bacteria, fungi, and viruses. Inevitably, this microbiological background depends on several environmental factors and exerts an important impact on the overall quality of herbal products and preparations. Herbal drugs normally carry a number of bacteria and molds, often originating in the soil. Poor methods of harvesting, cleaning, drying, handling, and storage may also cause additional contamination, as may be the case with *Escherichia coli* or *Salmonella* spp.

In the case of the new veterinary immunomodulator product made of three plants *Inula*, *Eupatorium* and *Helleborus*, same principle should be followed, in line with the appropriate *Pharmacopeia*.

According to our results, the phytopharmaceutical company to patent and produce the new veterinary product, should make efforts especially in the raw material procurements, as long as their actual sources comes with a much more higher content in aerobe bacteria than the recommended limits. Supplementary measures should be taken to avoid in this context the cross-contamination. However, as a positive aspect, the company is conducted by the principles of GMP which has lead to acceptable contents of coliforms and no traces of *Salmonella* spp. in the final product.

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FOOD BIOTECHNOLOGY

EFFECT OF COLD STORAGE ON ANTIOXIDANTS FROM MINIMALLY PROCESSED HERBS

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Abstract

Vitamin C and total polyphenols are quality markers used to assess the effect of treatments and storage on foodstuffs. Although, the effect of other conventional shelf-extension method is well established, refrigeration was taken for granted and rather neglected. The present study aims to describe its influence on three minimally processed herbs stored at 4°C for 12 days: parsley (*Petroselinum crispum*), dill (*Anethum graveolens*) and lovage (*Levisticum officinale*). The content of ascorbic acid and total polyphenols was determined on methanolic extracts. Ascorbic acid was separated, identified and dosed using HPLC coupled with an UV-VIS detector. Total polyphenols were determined spectrophotometrically, following Folin-Ciocalteu method. On the first day of storage, the content of vitamin C was above 170 mg/100 FW for the three herbs: dill had the highest content, followed by parsley and lovage, statistically similar. During the 12 days of storage, the content of vitamin C decreased by 18% for parsley, by 8% for lovage and by 3% for dill. At the beginning of the study, lovage had the highest content of total phenols followed by parsley and dill. On day 5 of storage, the content increased, reaching the maximum values for the three herbs and then it decreased below the levels of the first day. It was noted that during the 12 days of study, the evolution of total polyphenols at refrigeration temperature was given by a function of second degree. Thus, the present study confirms that vitamin C can be successfully used as a quality marker for herbs due to its low stability during storage. The evolution of total phenols is polynomial, reaching its peak during the shelf-life of herbs.

Key words: ascorbic acid, dill, lovage, parsley, phenols.

INTRODUCTION

Consumers are becoming more literate about the benefits of fresh products containing bioactive compounds (Parfitt et al., 2010). Aromatic herbs are believed to provide antioxidant compounds; vitamins, phenolic acids, flavonoids, sterols and coumarins are the compounds with functional properties in herbs (Charles, 2012; Santos et al., 2014). But, the continuous race against time of modern society has led to an increasing demand for ready-to-eat products. This generated a steady increase of minimal processing industry of fruit and vegetables (Parfitt et al., 2010). The commodities are fresh-like containing all the valuable phytochemicals and consumers perceive them as convenient, of high quality, less wasteful and with a reasonable price (Alvarez et al., 2013). Although minimal processing keeps the products alive, every operation in the production chain promotes senescence

processes (ethylene production, respiration and browning) and reduces resistance to microorganisms (Alvarez et al., 2013). This lowers quality, shortens shelf-life and enhances microbial contamination.

Hydrosoluble vitamins, such as vitamin C, are the compounds with the highest variation during the storage of minimally processed products, because the changes are more intense in the water fraction (Santos et al., 2014). Thus, vitamin C and total polyphenols are quality markers traditionally used to assess the effect of treatments and storage on vegetables. Although, the effect of other conventional shelf-extension method is well established (Śledź et al., 2013; Mezeyová et al., 2016), refrigeration was taken for granted and rather neglected. Herbs from *Lamiaceae* family (basil, lemongrass, marjoram, mint, oregano, rosemary, thyme) are thoroughly studied (Blasa et al., 2010; Śledź et al., 2013; Curutchet et al., 2014; Santos et al., 2014), while *Apiaceae* family (coriander, dill, lovage, parsley) has

been less considered (Mezeyová et al., 2016; Tsamaidi et al., 2016). Moreover, parsley, dill and lovage are the main herbs consumed in Romania (Glăman et al., 2015).

The aim of this study was to assess the evolution of the content of antioxidants (vitamin C and total polyphenols) in three minimally processed herbs stored at 4°C for 12 days: parsley (*Petroselinum crispum* (Mill.) Fuss var. neapolitanum), dill (*Anethum graveolens* L.) and lovage (*Levisticum officinale* Koch.).

MATERIALS AND METHODS

Preparation of samples. Fresh parsley (*Petroselinum crispum* (Mill.) Fuss var. neapolitanum), dill (*Anethum graveolens* L.) and lovage (*Levisticum officinale* Koch.) were purchased from a local market and minimally processed as previously described (Cătunescu et al., 2012b; Cătunescu et al., 2016) by cutting of stems to approximately 6 cm, washing with tap water, draining and packing in polyethylene bags (Krupps Vacupack Plus F380). The samples were stored at 4°C for 12 days and analyzed in the 1st, 5th, 8th and 12th day (Santos et al., 2014).

Extraction procedure. The content of antioxidants was determined on methanolic extracts. The extraction procedure proposed by Cătunescu et al. (2012b); Cătunescu et al. (2017) was followed: 1 gram of fresh herbs was manually extracted with 10 ml of acidified methanol (99.09:0.01 v/v, MeOH:HCl) in a mortar, at shade. The extracts were later filtered, dried in a vacuum rotary evaporator at 37°C and redissolved in 5 ml of pure methanol. The samples were stored at -20°C in opaque containers.

Determination of ascorbic acid content. A previously proposed method was employed (Cătunescu et al., 2012b; Roman et al., 2013; Cătunescu et al., 2017): the extracts were filtered (Teknokroma Syringe Nylon Filters 0.45 µm; 13 mm diameter) and the ascorbic acid was separated, identified and dosed in a HPLC Agilent 1200 system coupled with UV-

VIS detector (DAD). The mobile phase was water/acetonitrile/formic acid (94/5/1; v/v/v) and it isocratically eluated an Eclipse XDB-C18 column (5 µm; 150 x 4.6) at a flow rate of 0.5 ml/min. The chromatograms were registered at a wavelength of 240 nm. A calibration curve of L-ascorbic acid (Sigma 99%) was used to dose the ascorbic acid in the samples. All results were presented as mg/100 g of fresh herbs (FW).

Determination of total polyphenols content.

The Folin-Ciocalteu method proposed by Roman et al. (2013) and Cătunescu et al. (2017) was used. A calibration curve was plotted using 5 concentrations of a 1 mg/ml solution of gallic acid in 40% ethanol. One ml of each dilution, 5 ml of Folin-Ciocalteu reagent and 60 ml of distilled water were mixed. A volume of 15 ml of 7.5% Na₂CO₃ solution were added after 1 minute and the absorbance was measured after 2 hours at a wavelength of 750 nm (Biotek multidetector UV-Vis spectrometer). The same procedure was used for the samples prepared in the wells of spectrophotometric plates as follows: 2.375 ml of distilled water; 0.025 ml of methanolic extracts; 0.150 ml of Folin-Ciocalteu reagent and 0.450 ml Na₂CO₃. The blanks contained 0.025 ml of 40% ethanol instead of extracts. The content of total polyphenols in the samples was expressed in mg GAE/100 g FW using the calibration curve.

Statistical analyses. The results were obtained in triplicate and expressed as mean ± SD. XLSTAT software (Addinsoft, New York, USA, Version 2016.03.31333) was used to compute one-way ANOVA (p < 0.05), Fisher pairwise comparisons (LSD, p = 0.05) and correlational analyses (Pearson coefficient).

RESULTS AND DISCUSSIONS

Effect of cold storage on vitamin C content.

Ajayi et al. (1980) concluded that the content ascorbic acid was close to 100% of the total content of vitamin C in leafy vegetables, thus, it was approximated as such for the current study (Cătunescu et al., 2012b).

Table 1. Vitamin C content of minimally processed parsley, dill and lovage during a 12-days storage (mg /100g fresh herbs)

Storage day	parsley	dill	lovage
1	179.75±5.92 ^{A, b}	204.53 ± 5.06 ^{A, a}	173.49 ± 3.37 ^{A, b}
5	161.67 ± 1.15 ^{B, c}	198.00 ± 2.65 ^{AB, a}	171.83 ± 1.53 ^{A, b}
8	154.67 ± 4.16 ^{BC, c}	193.67 ± 2.52 ^{BC, a}	171.33 ± 5.48 ^{A, b}
12	147.91 ± 3.71 ^{C, c}	188.53 ± 3.18 ^{C, a}	168.76 ± 1.66 ^{A, b}

Note: Different uppercases represent significant statistical differences for each herb during the storage period; while different lowercases among the three herbs for the same storage day (Fisher (LSD), $p < 0.5$).

Dill had the highest content of vitamin C on the first day (Table 1) (Cătunescu et al., 2012b), similar to 116 to 186 mg/100 g FW previously reported (Lisiewska et al., 2003; Lisiewska et al., 2006), but 3 times higher than the content obtained by Galoburda et al. (2012).

Parsley contained less ascorbic acid, similar to other reports (Cătunescu et al., 2012b; Stan et al., 2014; Cătunescu et al., 2017), higher than 133 mg/100 g found in other studies (Daradkeh and Essa, 2016), while lower than 310 mg/100 g reported by Lisiewska et al. (2003) for Hamburg variety and 257 mg/100 g for plain leaf parsley.

Lovage had a content of vitamin C similar to parsley's (Cătunescu et al., 2012b).

Thus, the three minimally processed herbs are all a "good source" of vitamin C, as defined by the Food and Drug Administration (FDA), because one table spoonful (10 g) provides the 10-19% of the recommended dietary allowance (RDA)(e-CFR, 2016).

During the 12 days of storage the content of vitamin C decreased linearly by 18% for parsley ($r = -0.970$, $p < 0.05$), 8% for dill ($r = -0.998$,

$p < 0.05$) and 3% for lovage ($r = -0.978$, $p < 0.05$). The linear regression of vitamin C content (y) and storage (x) are showed in equation 1 for parsley, equation 2 for dill and equation 3 for lovage.

$$y = -2.86 x + 179.56; R^2 = 0.94 \quad (1)$$

$$y = -1.45 x + 205.63; R^2 = 0.98 \quad (2)$$

$$y = -0.41 x + 174.03; R^2 = 0.96 \quad (3)$$

Similar, Howard et al. (1999) showed that the content of vitamin C decreases linearly during storage of vegetable at refrigeration temperatures. Lisiewska et al. (2003) observed that the dill stored at refrigeration temperatures gradually lost its vitamin C. Hydrosoluble

vitamins – vitamin C included - are mainly lost because of water losses during handling or storage, temperature fluctuations and enzymatic oxidation (Mezeyová et al., 2016).

Thus, vitamin C, due to its low stability, is a good quality marker of herbs, both fresh and minimally processed during storage.

The content of vitamin C showed a significant correlation with sensory scores for taste, odor, aroma and overall quality (Cătunescu et al., 2012a) in the cases of dill and lovage (Table 2). These correlations can be attributed to the role that vitamin C can play as an aroma intensifier and as a general antioxidant. However, vitamin C is lost linearly with storage, as are other sensory attributes and correlation does not necessarily mean causation.

Table 2. Correlation of vitamin C content with some quality attributes of parsley, dill and lovage

Quality attribute	Pearson correlation coefficient (r)		
	parsley	dill	lovage
taste	0.45	0.94	0.96
odor	0.38	0.86	0.98
aroma	0.41	0.90	1.00
quality numbers	0.58	0.98	0.99
overall quality	0.54	0.98	1.00

Note: Values in bold are different from 0 with a significance level $\alpha = 5\%$.

Effect of cold storage on total polyphenols content.

The content of total polyphenols was determined on methanolic extracts. Acidified pure methanol was showed to optimally solubilize phenols, compared with a 50% acetone solution or 70-80% methanol (Parry et al., 2006).

Lovage had the highest content of total polyphenols (Table 3).

Table 3. Total polyphenols content of minimally processed parsley, dill and lovage during a 12-days storage (mg GAE/100g fresh herbs)

Storage day	parsley	dill	lovage
1	281.36 ± 7.55 ^{C, b}	292.01 ± 10.49 ^{A, b}	417.43 ± 6.37 ^{B, a}
5	337.45 ± 3.15 ^{A, b}	246.42 ± 11.26 ^{B, c}	413.35 ± 5.25 ^{B, a}
8	324.75 ± 4.25 ^{B, b}	243.00 ± 1.78 ^{B, c}	467.14 ± 7.62 ^{A, a}
12	203.33 ± 0.87 ^{D, c}	296.33 ± 9.38 ^{A, b}	386.94 ± 4.89 ^{C, a}

Note: Different uppercases represent significant statistical differences for each herb during the storage period; while different lowercases among the three herbs for the same storage day (Fisher (LSD), $p < 0.5$).

Lisiewska et al. (2003) found a similar content of phenols in dill leaf 248 ± 9 mg/100 g FW.

The content of total polyphenols varies with the species, but also within the same group depending on external factors (pedo-climatic) and internal (intra-species variability), similar to other bioactive compounds (Bravo, 1998). Zheng and Wang (2001) found a slightly different content of phenols in the three herbs: 263 mg GAE/100 g FW for lovage, 112 mg GAE/100 g FW for parsley and 312 mg GAE/100 g FW for dill.

Al-Mamary (2002) reported 193.46 ± 4.22 mg GAE/100 g FW for parsley and established that the herbs from *Apiaceae* family were the richest in phenols. Zheng and Wang (2001) indicated, however, a very high content of phenols in oregano and marjoram (1,180 and 1 respectively, 165 mg GAE/100 g FW). But, parsley, lovage and dill are relatively rich in polyphenols when compared with other herbs known for their antioxidant activity such as: bay leaves, rosemary, thyme and lemon balm (Zheng and Wang, 2001).

Similar, the three herbs stand out when compared with other vegetables and fruit traditionally recognized as a “good source” of phenols. Bravo (1998) reported 100-2025 mg GAE/100 g FW for onions, 27-298 mg GAE/100 g FW apples, 140-1200 mg GAE/100 g FW for blackcurrant, 135-280 mg GAE/100 g FW for blackberries, 50-490 mg GAE/100 g FW grapes and 38-218 mg GAE/100 g FW strawberries.

Culinary herbs are, generally, rich in flavonoids, with much higher concentrations compared to other vegetables (Blasa et al., 2010; Daradkeh and Essa, 2016). Parsley contains apigenin, phenolic acids, especially caffeic acid, and reduced quantities of quercetin and luteolin (Hedges and Lister, 2007;

Daradkeh and Essa, 2016). Jipa et al. (2008) indicated 225.93 mg of apigenin; 8.08 mg myricetin; 1.49 mg kaempferol; 0.33 mg luteolin and 1.24 mg quercetin per 100 g DW of parsley. Lovage is one of the richest herbs in quercetin (170 mg/100 g FW), but also contains kaempferol (7 mg/100 g FW); while dill contains quercetin (48-110 mg/100 g FW) kaempferol (16-24 mg/100 g FW) and isorhamnetin (5-72 mg /100 g FW) (Hedges and Lister, 2007).

The content of total polyphenols increased on the 5th day of storage, reaching the highest levels: it increased by 20% for parsley, 18.5% for dill and 12% for lovage when compared to the beginning of the study. But, it decreased on the 12th day by 28% for parsley and 7% for lovage, while remaining at approximately the level of the 1st day for dill.

The variation of total polyphenols during the 12 days of study followed the curve of a 2nd degree function. The curves of phenols content (y) and storage (x) are showed in equation 4 for parsley, equation 5 for dill and equation 6 for lovage.

$$y = -3.17x^2 + 34.31x + 249.13; R^2 = 0.99 \quad (4)$$

$$y = -1.77x^2 + 22.77x + 224.75; R^2 = 0.99 \quad (5)$$

$$y = -1.36x^2 + 13.85x + 410.71; R^2 = 0.71 \quad (6)$$

A similar variation was described for apples, beans, white grapes and celery. Schmitz-Eiberger and Matthes (2011) observed an increase in the content of phenols up to 34% during storage of apples, followed by a decrease, but the values fluctuated. Granito et al. (2008) reported a slight increase, by 7%, in the content of total phenols in beans up to the 90th day of storage, followed by a 76% decrease in the 150th day. Similar the content of phenols increased in white grapes and hawthorn by 24% and 59%, respectively, on the 9th day of storage and decreased subsequently

up to the 17th day (Šamec and Piljac-Žegarac, 2011).

The increase of total polyphenols at the beginning of storage is generated by the physiological processes of the plant senescence processes. The aromatic amino acids, precursors of phenolic acids deaminate and the conjugated polyphenols hydrolyze resulting phenolic acid. Similarly, proteins and complex carbohydrates degrade, releasing phenolic compounds (Šamec and Piljac-Žegarac, 2011).

CONCLUSIONS

On the 1st day of storage dill had the highest content of vitamin C, followed by parsley and lovage. These values, above 170 mg/100 FW, indicated that herbs had a significantly higher content compared to other vegetables. During the 12 days of storage, vitamin C showed a substantial decrease during storage similar to other plant products stored at refrigeration temperature. It was reduced linearly, by 18% for parsley, 8% for lovage and 3% for dill.

Thus, vitamin C, due to its low stability, is a quality marker for herbs, both fresh and processed.

For dill and lovage, a positive correlation was observed between the content of vitamin C and taste, odor, flavor and overall quality ($r = [0.89...0.99]$, $p < 0.05$).

The content of total polyphenols was evaluated for methanol extracts. Lovage had the highest content of phenols, followed by parsley and dill.

The three herbs had a content similar to other fruit and vegetables known for their polyphenols. The content of total polyphenols increased on the 5th day of storage, reaching the maximum levels and later decreased until the end of the study. It was noted that the evolution of total phenols during storage at refrigeration temperature was given by a function of second degree.

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ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACTS FROM *Agrimonia eupatoria* L. AND *Epilobium hirsutum* L. HERBA

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Abstract

Given the growing concern regarding bacterial resistance to antibiotics, it is important to investigate alternative antibacterial compounds, such as phenols and flavones from natural sources. In this context, the aim of the present study was to evaluate the antibacterial activity of two indigenous medicinal plants from Romania against some Gram positive and Gram negative bacteria. Ethanolic extracts (70% v/v) from the aerial parts of *Agrimonia eupatoria* L. and *Epilobium hirsutum* L. were obtained, their total phenols content was determined using Folic-Ciocalteu assay as it is described by the Romanian Pharmacopoeia. The qualitative assay of the two ethanolic extracts was done by high performance thin layer chromatography (HPTLC) and their antibacterial activity was assessed using the agar diffusion method and minimum inhibitory concentration determination against four pathogenic bacteria: *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538 and *Staphylococcus epidermidis* ATCC 12228. Our results showed that both extract contain caffeic acid and some of its derivatives, however the flavones content differs: while *A. eupatoria* contains several quercetin and luteolin derivatives, *E. hirsutum* is rich in myricetin derivatives. The antibacterial activity tests showed better results against the Gram positive bacteria such as *Staphylococcus epidermidis*, especially in the case of great willowherb (*Epilobium hirsutum* L.).

Key words: antibacterial activity, phenols, plant extract.

INTRODUCTION

Although the use of medicinal plant is known for centuries, mainly starting in the second part of the XXth century the scientific research was focused on the mechanisms through which these plants exert their biologic activity. In doing so, it was proven that plant polyphenols can interact with numerous biomolecules, thus leading to different biological activities. In addition, they are non-narcotic, biodegradable, they have very few to none side effects and are not toxic to the environment (Biswas et al., 2013; Shrestha et al., 2013). In a recent study, Farias et al. (2013) showed that between 1981 and 2007 half of the new drugs approved by the FDA contained natural compounds such as flavones, phenols, lactones or saponins. Also, it was estimated that 74% of the plant derived pharmacologically active components now used as therapeutics were discovered by studying plants that were used in traditional medicine (Gibbons, 2003).

Considering this recent increasing interest in medicinal plants, we focused our attention on two indigenous plants from Romania, which are used in traditional medicine. First, *Agrimonia eupatoria* L. (fam. *Rosaceae*), commonly known as agrimony – used primarily as a mild antiseptic and astringent, recommended for sore throat and gastro-intestinal disorders. It also has good antioxidant properties and was proven to help with lipid metabolism in young healthy humans (Ivanova et al., 2013). Agrimoniin, an important flavone found in agrimony has anti-tumor activity, increases the production of interleukin-1 and has antibacterial activity against *Helicobacter pylori* and *Campylobacter jejuni* (Murayama et al., 1992; Funatogawa et al., 2004; Cwikla et al., 2010; Ad’hiah Ali et al., 2013).

Epilobium hirsutum (fam. *Onagraceae*), commonly known as great willowherb is known in the Romanian traditional medicine for its high concentration of flavones (Barakat et al., 1997) and is used for treating fever or pain, but also

for benign prostatic hyperplasia, the extract from this plant being useful for inhibiting the proliferation of PZ-HVP-7 human prostate cell line (Tita et al., 2001; Vitalone et al., 2003; Miano et al., 2008). Further studies determined that *E. hirsutum* contains acidic saponins, anthocyanidines, vitamin C, several minerals and microelements thus having immune-stimulating effects, antimicrobial and anti-cancer properties (Battinelliet al., 2001; Pakravan et al., 2011). A more recent study, Celik et al. (2016) suggests that the extract from the great willowherb may interfere with the activity of CYP P450 enzyme and recommends caution when combining it with other drugs.

Considering all of this, the aim of the present study was to evaluate the antibacterial activity of *A. eupatoria* and *E. hirsutum* and to determine the phytochemical constituents that are responsible for this activity.

MATERIALS AND METHODS

Plant material

Agrimonia eupatoria L. was purchased from a Romanian Plant Product Company, while *Epilobium hirsutum* L. was harvested from the Sinaia region in August. Both specimens were identified by the botanists at the *National Institute for Chemical and Pharmaceutical Research and Development* (ICCF) and voucher specimens are deposited at ICCF *Plant Material Storing Room*.

Ethanolic extracts – obtaining and characterisation

Briefly, 100 g of powdered vegetal material (aerial parts) were twice heat assisted (1 hour, continue stirring) extracted in 1000 ml 70% ethanol. Afterwards, the extracts were filtered through filter paper and used as such for analytical studies. For microbiological investigations, the two extracts were concentrated at residue and then solved in 20% propylene glycol to a final concentration of 5 mg total phenols (gallic acid equivalents) per 1 ml sample (5 mg GAE/ml).

Total phenols content determination

Total phenols content was estimated by the Folin-Ciocalteu assay as described in the *Romanian Pharmacopoeia*. Briefly, 50-100 μ l of vegetal sample was mixed with 200 μ l Folin-Ciocalteu reagents and completed to a final

volume of 5 ml with sodium carbonate 5%. After mixing the solution and 5 min incubation at room temperature in the dark, the optical density was measured at a wavelength of 750 nm. Gallic acid standard calibration curve was used ($r^2=0.9989$) and the results were expressed as gallic acid equivalents/ ml sample (GAE/ml).

HPTLC assay

For the qualitative determination, the HPTLC method was used, as previously described (Nicu et al., 2016). Briefly, volumes measuring from 0.5 to 3 μ l vegetal extract, as well as reference samples were loaded as 8 mm band length in the 10 \times 10 cm silica gel 60F HPTLC plate (Merck, Darmstadt, Germany) using Linomat 5 CAMAG instrument (Muttentz, Switzerland). Afterwards, the plates were kept in a TLC twin developing chamber at 18–19°C with the mobile phase (ethyl acetate–acetic acid–formic acid–water/100:12:12:26) until it reached a length of 90 mm. The developed plate was then dried and immersed in identification reagents (Natural Product followed by PEG4000). Finally, the plate was disposed in a photo-documentation chamber, and the images were taken at UV 366 nm. Spots' assignment was done using reference compounds data and plant product literature (Wagner and Bladt, 1996; Reich and Schibli, 2008)

All chemicals and reagents for these experiments were purchased from Fluka and Sigma-Aldrich Co (Bucharest, Romania).

Antibacterial activity

Four bacterial strains were used in this study: two Gram negative - *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027; two Gram positive – *Staphylococcus aureus* ATCC 6538 and *Staphylococcus epidermidis* ATCC 12228. All strains were purchased from Mecconti (Merck Romania S.R.L.) and were activated by culturing the bacterial cells on casein soya agar medium (Merck Romania S.R.L.) (CaSoA) and incubated for 24 h at 35°C.

The agar diffusion method was performed as described in our previous work (Nicu et al., 2016), briefly 15-20 ml of culture medium inoculated with 10^4 - 10^5 CFU/ml (colony forming units) of the respective test bacteria was poured in a Petri dish with a 90 mm diameter. After the medium solidified at room temperature, 4 stainless steel cylinders (8mm diameter) were placed on the surface of the medium and the

tests samples were added in the cylinders (0.2 ml sample/ cylinder). Finally, the Petri dishes were incubated at 35°C for 24 h and then the growth inhibition zones were measured. The interpretation of the results was made after the *Romanian Pharmacopoeia* as follows: <10 mm – no activity, 10-15 mm – weak activity, 16-20 mm – good activity, >20 mm – certain antibacterial activity.

The minimum inhibitory concentration (MIC) was determined using the microdilution method. In 96-well plates, 80 µl of casein soya broth medium (Merck Romania S.R.L.) (CaSoB) were added, previously inoculated with 10⁴-10⁵ CFU/ml of the respective test bacteria and 80 µl of the test sample. Serial dilutions were obtained for every extract and bacteria combination from 2500 to 156.25 µg GAE/ml. The plates were then incubated for 24 h at 35°C and the optical density was read at 600 nm.

Statistical analysis

Results were expressed as mean values of three measurements ± standard deviation (SD).

RESULTS AND DISCUSSIONS

Characterisation of the extracts

As mentioned before, the two extracts were analysed by HPTLC in order to determine the phenolic and flavonoid components. The results for *Agrimonia eupatoria* L. are shown in Figure 1.

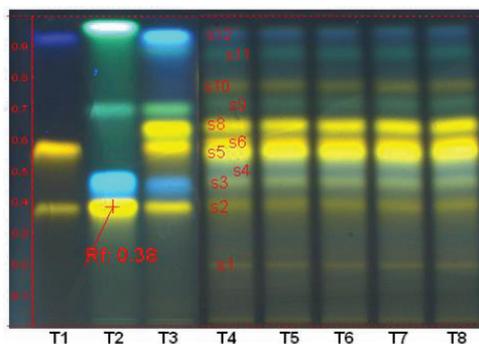


Figure 1: Polyphenols profile of the *Agrimonia eupatoria* ethanolic extract (Pirvu et al., 2016b)

T1 track – quercetin-3-O-rutinoside/ rutin, quercetin-3-O-galactoside/ hyperoside and protocatechuic acid (ref.); T2 track – rutin, chlorogenic acid, hyperoside, luteolin-7-O-glucoside/ cynaroside, apigenin-8-C-glucoside/ vitexine and caffeic acid (ref.);

T3 track – rutin, chlorogenic acid, apigenin-7-O-glucoside/ cosmosiin and kaempferol (ref);

T4-T8 tracks: *Agrimonia eupatoria* ethanolic extract.

As it can be seen in Figure 1, there are several flavonoid compounds observed, such as the yellow-orange fluorescent spots (s2, s6, s10) that were attributed to quercetin derivatives such as rutin, hyperoside and quercetin; the yellow fluorescent spots (s3, s5, s8) attributed to isorhamnetin and orientin and also apigenin (green fluorescent spot s9) or kaempferol (blue-green fluorescent spot s11). As far as phenolic compounds are concerned, they can be seen in smaller quantities in spots 4 and 12 – neochlorogenic (blue fluorescent) and caffeic acid (blue-marine fluorescent).

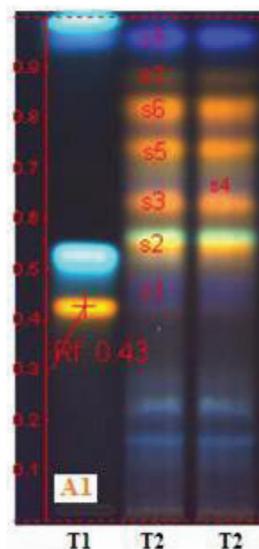


Figure 2: Polyphenols profile of the *Epilobium hirsutum* ethanolic extract (after Pirvu et al., 2014)

T1 track – rutin, caffeic acid, chlorogenic acid

T2 track – *Epilobium hirsutum* ethanolic extract

Based on Figure 2 and the measured Rf, it can be stated that five red-orange fluorescent spots (s2, s3, s5, s6, s7) were identified as myricetin derivatives, while the indigo fluorescent ones (s1, s4, s8) and the blue fluorescent spot are caffeic and gallic acids derivatives.

Antibacterial activity

All antibacterial activity tests were performed using extracts in 20% propylene glycol at a final concentration of 5 mg GAE/ml vegetal sample. The results for the agar diffusion assay are presented in Table 1.

Table 1: Antibacterial activity of plant extracts

Sample	Bacterial strain	Inhibition zone (mm)
<i>Agrimonia eupatoria</i> extract	<i>Staphylococcus aureus</i> ATCC 6538	<8
	<i>Staphylococcus epidermidis</i> ATCC 12228	15±0.16
	<i>Escherichia coli</i> ATCC 8739	<8
	<i>Pseudomonas aeruginosa</i> ATCC 9027	<8
<i>Epilobium hirsutum</i> extract	<i>Staphylococcus aureus</i> ATCC 6538	17±0.15
	<i>Staphylococcus epidermidis</i> ATCC 12228	17.66±0.577
	<i>Escherichia coli</i> ATCC 8739	17±0.15
	<i>Pseudomonas aeruginosa</i> ATCC 9027	18.33±0.577

Diameter of the inhibition zones is given here as mean ± standard deviation.

As it can be seen in Table 1, the polyphenolic extract from *Epilobium hirsutum* has a more potent activity against all four bacterial strains tested than *Agrimonia eupatoria*. It is interesting to mention that although in general Gram positive bacteria tend to be more susceptible to antibacterial agents due to the lack of an outer membrane that can act as a barrier, in this case *E. hirsutum* shows virtually the same potency against both Gram positive and negative bacteria.

Due to the fact that the agar diffusion assay is known to have some limitations, such as the very high probability that the components of a mixture exhibit different diffusion rates and therefore can give uncertain results (Silva et al., 2005), this method is recommended only as a preliminary screening and so MIC determination was also carried out in this study. It is important to mention that some of this results were previously published before (Pirvu et al., 2014; Pirvu et al., 2016b), however the results are shown here for an easier comparison between the two plants in discussion and for an easier transition to the MIC determination part of the study, the results of which are shown in Table 2. We chose MIC determination because is a more sensitive method than the diffusion assay and it allows the use of small quantities of extract (Langfield et al., 2004).

Table 2: MIC determination for plant extracts, expressed as phenolic compounds value

Sample	Bacterial strain	MIC (µg/ml)
<i>Agrimonia eupatoria</i> extract	<i>Staphylococcus aureus</i> ATCC 6538	625
	<i>Staphylococcus epidermidis</i> ATCC 12228	625
	<i>Escherichia coli</i> ATCC 8739	1250
	<i>Pseudomonas aeruginosa</i> ATCC 9027	312.5
<i>Epilobium hirsutum</i> extract	<i>Staphylococcus aureus</i> ATCC 6538	156.25
	<i>Staphylococcus epidermidis</i> ATCC 12228	625
	<i>Escherichia coli</i> ATCC 8739	625
	<i>Pseudomonas aeruginosa</i> ATCC 9027	312.5

Regarding MIC determination, it can be easily seen from Table 2 that the lower values were determined also in the case of *E. hirsutum*, with emphasis on *S. aureus* and *P. aeruginosa*, proving once again that this extract has good antibacterial activity against both Gram positive and Gram negative bacteria.

What is surprising is that *A. eupatoria* presents a MIC value of 312.5 µg/ml against *P. aeruginosa* although it showed no activity when the agar diffusion method was used. This could probably be due to some compounds that cannot migrate in the agar medium, however further studies are necessary in order to test this theory. The results obtained against the other three bacterial strains are in correlation with the first results, showed in Table 1.

Other study reports confirm our findings, in the sense that low MIC values are not always correlated with high activity in the agar diffusion assay (Lourens et al., 2004). Furthermore, another recent study obtained slightly different results in the MIC determination of *Epilobium hirsutum* ethanolic extract against some of the same bacteria, such as *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228 or *Escherichia coli* ATCC 8739 (Pirvu et al., 2016a). However, the differences are not major and they may be due to the different method used for MIC determination, the conclusion being the same, that the great willow herb is a promising source for natural antibacterial products.

CONCLUSIONS

The present study aimed at investigating the antibacterial activity of two indigenous species of medicinal herbs from Romania: *Agrimonia eupatoria* L. and *Epilobium hirsutum* L. We proved that the ethanolic extracts from these two species contain several different phenolic compounds, some which are common such as caffeic acid and its derivatives, while some others are specific to just one plant, such as the myricetin derivatives from the great willowherb or the quercetin and apigenin derivatives found in agrimony.

The qualitative assay showed some differences in the chemical composition of the two extracts, differences that were observed also in the second part of the study, determining the antibacterial activity. While agrimony only showed weak activity against *Pseudomonas aeruginosa* and a MIC of 312.5 µg/ml, the great willowherb had moderate activity against all four bacterial strains used for testing and lower MIC values, especially against *Staphylococcus aureus* (156.25 µg/ml) and *Pseudomonas aeruginosa* (312.5 µg/ml), thus proving efficient on Gram negative and Gram positive bacteria.

These results are a good basis for justifying the need for further studies in order to better understand the mechanism through which these two plants exert their antibacterial activity and how to better use them in treating infections.

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USING CENTRIFUGE AND ULTRAFILTRATION AS DOWNSTREAM PROCESSES FOR THE CONCENTRATION OF MICROBIAL β -MANNANASE FERMENTATION MEDIA

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Abstract

*Enzymes are protein-based catalyzers which organize the specific chemical reactions. Therefore, they could be used for lots of industrial applications. For the industrial grade, enzymes were extracted by different physical or chemical methods from the plant or animal tissues formerly. But these techniques are very hard to apply and provide enough enzymes for the industry. The production of the microbial enzymes could be done by a series of operations, which can be divided into two groups (upstream and downstream processes). Production of bulk or partial concentrated enzyme solutions are easier than the production of pure enzyme extracts. In this study, the effects of centrifuge and ultrafiltration on the production of concentrated β -mannanase enzyme extract from fermented carob pod medium by recombinant *Aspergillus sojae* were investigated. For this purpose, combination of time (5, 10, and 15 min) and speed (7000, 10000, and 15000 rpm) were tested for centrifuge assays. Then, ultrafiltration processes were performed with 10kDa and 30kDa membranes by using 1000 ml fermented media. Because the enzyme molecular weight was 50-60 kDa. Results showed that the centrifuge was not statistically important at β -mannanase enzyme purification in carob medium in point of enzyme activity. But both ultrafiltration membranes were help to improve the specific enzyme activity from 2176.65 U/mg (initial) to 2582.92 U/mg for 10kDa and 2718.89 U/mg for 30kDa ($p < 0.05$). It was obviously seen from the results that 100ml concentrated enzyme extract was collected from the retentate.*

Key words: Centrifuge, Enzyme, Ultrafiltration, β -mannanase.

INTRODUCTION

The chemical reactions are catalyzed in vivo or in vitro by the proteins which are called enzymes. Different extraction strategies for enzyme purification from animal or plant cell tissues were used in the past. But today industrial enzyme requirements could not be adequate by traditional methods from the animal or plant cell tissues.

Therefore, new biotechnological methods have been used to produce industrial enzymes by an economical and environmental ways. Amylases, pectinases, hemi-cellulases, invertases or etc. are the most produced industrial enzymes.

They have lots of different usage all over the world. β -mannanase is also one of the important industrial enzyme.

It has been used for the detergent formulas, pharmaceutical applications, paper and pulp production, animal feeds, instant coffee

production, manno-oligosaccharides production etc. (Van Zyl et al., 2010; Lu et al., 2014).

The production of value-added products by biotechnological processes has been increasing day by day. But the downstream processing is also important for the purified value-added products to use for specific reactions.

There are lots of different ways to get purified value added products from fermented medium. But it is hard and not economical for some of the industrial usage.

So, partial purified or concentrated fermented medium could be used for general industrial applications.

Centrifuge, membrane filtration and other processes have been generally used to produce bulk enzyme solutions.

For this purpose, centrifuge and ultrafiltration were performed to get partial purified/concentrated bulk β -mannanase enzyme solution from fermented media by recombinant *Aspergillus sojae* in this research.

MATERIALS AND METHODS

Microorganism and medium

Recombinant *Aspergillus sojae* from Prof. Dr. Z.B. Ogel Laboratory (METU, Ankara, Turkey) (Duruksu et al., 2009) was used for β -mannanase production. Microorganism was sub-cultured on PDA (Potato Dextrose Agar) at 30°C for 4-5 days bimonthly and stored at 4°C (Ozturk et al., 2010).

Fermentation medium and fed-batch β -mannanase fermentation

Fed-batch fermentations were performed in a stirred tank bioreactor with addition microparticle agent (talcum). Carob pod extract was prepared as explained by Turhan et al., (2010). And fermentation media was prepared by enhancing 4 °Bx carob pod extract with 4 g/L of yeast extract, 0.5 g/L of MgSO₄.7H₂O, and 1 g/L of K₂HPO₄ (Ozturk et al. 2010). Then, fermentation was started with %1 inoculation of spore suspension (prepared with sterile salt-tween solution to be 10⁷ spores/ml). The fermentation conditions were 400 rpm, no pH control, 1 lpm aeration, and 30 °C. Whole fermentations were performed for 9 days. 10°Bx carob pod extract was also prepared to feed the media twice when the sugar concentration was under 5 g/L.

Concentration of fermented medium by centrifuge

Three different reverse speeds (7000 rpm, 10000 rpm, and 15000 rpm) and time (5 min, 10 min, and 15 min) were combined to concentrate the fermented medium. All processes were performed triplicated. After the centrifuge process, samples were taken from the supernatant. Enzyme activity, residual sugar and total protein analyses were performed to calculate the purification coefficient.

Concentration of fermented medium by ultrafiltration

Sartocon Slice 200 model (Sartorius Stedim Biotech, Goettingen, Germany) ultrafiltration system was used to ultrafiltrate fermented medium. For this purpose, 10 kDa and 30 kDa ultrafilters were used. Ultrafiltration process were started with 1000 ml fermented medium and stopped when the retentate was 100 ml. All

processes were performed triplicated. Then permeate and retentate samples were analysed to calculate enzyme activity, residual sugar and total protein.

Analysis

Enzyme activity was determined by DNSA method and calculated from the mannose standard curve (Puchart et al., 2004, Ozturk et al., 2010). DNSA method was also used to determine the residual sugar as described by Miller (1959). Total protein analyse was performed with Thermo Scientific Coomassie (Bradford) Protein Assay Kit. The supernatants and other samples were treated with SDS-PAGE protocols to carry out molecular weight of β -mannanase enzyme (Karaoglan et al., 2016). Data were subjected to analysis of variance using the General Linear Models procedure of the Statistical Analyses System software (Version 7, SAS Institute Inc., Cary, NC). Differences among the mean values of the various treatments were calculated and the significance was defined at p<0.05 (Yatmaz et al., 2016).

RESULTS AND DISCUSSIONS

Concentration of fermented medium by centrifuge

Centrifuge processes were used not only for concentration of β -mannanase but also for filtration of filamentous fungi from fermented medium. So, different reverse speed-time combinations were performed to carry out the effect of centrifuge process on concentration of β -mannanase enzyme. Samples were taken from the supernatant and results were given in Table 1.

Table 1. Specific activity and purification coefficient for centrifuge assays

Sample	Specific β -mannanase act. (U/mg)		Purification coefficient
	Initial	After process	
7000 rpm-5 min	2149.86	2030.25 ^b	0.94 ^b
7000 rpm-10 min	2149.86	2006.44 ^b	0.93 ^b
7000 rpm-15 min	2149.86	2208.04 ^{ab}	1.03 ^{ab}
10000 rpm-5 min	2149.86	2180.70 ^{ab}	1.01 ^{ab}
10000 rpm-10 min	2149.86	2177.50 ^{ab}	1.01 ^{ab}
10000 rpm-15 min	2149.86	2041.01 ^b	0.95 ^b
14000 rpm- 5 min	2149.86	2173.58 ^{ab}	1.01 ^{ab}
14000 rpm- 10 min	2149.86	2342.59 ^a	1.09 ^a
14000 rpm- 15 min	2149.86	2364.98 ^a	1.10 ^a

*The differences between the values were statistically significant (p<0.05)

It was obviously seen from the results that 14000 rpm and its time combinations were always given the higher specific activities than initial fermented medium.

The highest specific activity value was 2364.98 U/mg for 14000 rpm-15 min. It was also given the highest purification coefficient to be 1.10.

The results showed that the centrifuge process was not statistically significant for concentration process.

So, the centrifuge process could just be used to separate filamentous fungi from fermented medium instead of filter paper.

But for this aim, continuous centrifuge system should be chosen to filter higher amounts of fermented medium.

Concentration of fermented medium by ultrafiltration

Before the ultrafiltration processes SDS-PAGE analyse were performed to determine the molecular weight of the enzyme. So, ultrafiltration cartridge with 10 kDa and 30 kDa cut-off value was chosen for the ultrafiltration processes.

1000 ml fermented medium were used for each ultrafiltration processes. The ultrafiltration was ended when the retentate was concentrated to be 100 ml. So, 900 ml permeate and 100 ml retentate was taken from the 1000 ml fermented medium. Then total protein, total enzyme activity, specific enzyme activity, and purification coefficients were calculated to carry out the best ultrafiltration process for concentration of β -mannanase. The results were given in Table 2.

Table 2. Specific activity and purification coefficient for ultrafiltration assays

Samples	Total protein (mg)*	Total enzyme activity (U)*	Specific activity (U/mg)*	Volume (ml)	Purification coefficient*
Initial	178.02 ^a	387480.80 ^a	2176.65 ^b	1000	1 ^b
10 kDa UF retentate	38.38 ^b	99132.81 ^b	2582.92 ^a	100	1.19 ^a
10 kDa UF permeate	0 ^c	0 ^c	0 ^c	900	0 ^c
30 kDa retentate	35.91 ^b	97642.61 ^b	2718.89 ^a	100	1.25 ^a
30 kDa permeate	0 ^c	0 ^c	0 ^c	900	0 ^c

*The differences between the values were statistically significant (p<0.05)

The initial specific enzyme activity was 2176.65 U/mg for fermented medium.

The highest specific activity value was calculated to be 2718.89 U/mg for 30 kDa retentate. It was also given the highest purification coefficient (1.25).

And, there were no protein and enzyme activity determined in the permeate solutions for 10 kDa and 30 kDa. So, the β -mannanase concentration by ultrafiltration processes was carefully done by 10 kDa or 30 kDa ultrafiltration cartridges. And, ultrafiltration results were statistically significant for concentration processes (p<0.05).

The SDS-PAGE analyse was also performed again to carry out the ultrafiltration performance.

The results were given in Fig 1.

It was obviously seen from the Figure 1 that β -mannanase molecular weight was 50-60 kDa. There wasn't seen any band for the initial of the fermentation.

The end of the fermentation β -mannanase protein band was appeared. Then the ultrafiltration processes were performed with

different cartridges, and the β -mannanase protein band became bigger and clearer.

Because the protein was concentrated from 1000 ml fermented medium to 100 ml concentrated bulk enzyme.

So, the same amount of the samples of retentate samples was given the bigger and clearer band from the initial.

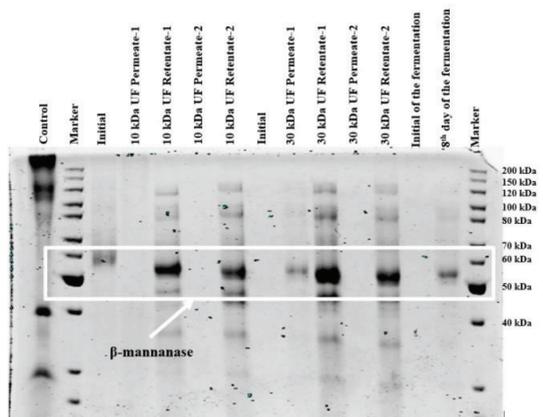


Figure 1. SDS-PAGE analyse for UF and fermentation

CONCLUSIONS

The production of value-added products by biotechnological processes has been increasing day by day. There are lots of different ways to get purified value added products from fermented medium. But it is generally hard and not economical for some of the industrial usage. So, partial purified or concentrated fermented medium could be used for general industrial applications. Consequently, the results showed that the centrifuge process were not statistically significant for concentration of β -mannanase ($p < 0.05$). It could be just used for filtration of filamentous fungi from the fermented medium. But ultrafiltration process was statistically significant for β -mannanase concentration ($p < 0.05$), and there was not any protein residue in the permeate. So, the results showed that the best ultrafiltration process was 30 kDa, and it could be used to concentrate the β -mannanase from the fermented medium.

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STATISTICAL MODEL FOR INDUSTRIAL IMPROVING OF WHEAT FLOURS WITH CALCIUM LACTATE

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Abstract

*The aim of this research was to identify the main changes that the addition of calcium lactate induces to rheological properties of dough, in industrial environment. In this regard, a number of 62 wheat flours, coming from Romanian wheat, were additivated with variable amounts of calcium lactate, ranging from 10 to 300 g/100 kg. Both the control flours and the additivated flours with calcium lactate have been evaluated in terms of rheology, using the alveographic method. The results showed that treatment with calcium lactate caused a very significant increase of following parameters: Resistance (P, mm; $t=4.864^{***}$), dough capacity to absorb Mechanical work (W, 10^4 J/gram; $t=6.990^{***}$) and the ratio between Resistance and Extensibility (P/L; $t=7.174^{***}$). Elasticity parameter increased significantly in dough treated with calcium lactate, compared to control flours. Also, treatments with calcium lactate caused a very significant decrease of some parameters, such as: Extensibility (L, mm; $t=-2.751^{**}$, and gluten extensibility index (G). There were significant correlations between the amounts of added calcium lactate and the modification of Resistance ($r=0.66^{***}$), Extensibility ($r=-0.56^{***}$) and the P/L ratio ($r=0.85^{***}$).*

Key words: alveogram, bread dough, calcium lactate, wheat flours.

INTRODUCTION

Calcium lactate (calcium 2-hydroxypropanoate, E 327) is used in bakery products, either as a source of calcium for flour fortification, or to compensate the anti-nutritive effect of phytic acid. In all cases, calcium lactate has a technological effect of acidity reviser, due to its property to operate as a buffer system (Krupa-Kozak, 2015; Tamba Berehoiu, 2015).

By modifying the ionic strength of the dough, calcium lactate also exerts a significant effect on the conformation of gluten proteins (Basset et. al., 2014). Changing the gluten proteins conformation involves the proteic globules unfolding, so a large number of chemical groups become available to interact with other active groups of proteins or with water (Anderssen, 2007). In this way, spaces are created where water molecules penetrate, causing the swelling of the proteins.

Also, as the number of positive charges decrease (acidic pH), the electrostatic repulsions intra- and inter-molecular decrease too, it is therefore favored the emergence of new bonds, whose effects are the aggregation

of molecules and finally, precipitation of the proteins (Maher et al., 1978).

This may explain why at the isoelectric pH, swelling, solubility, viscosity and osmotic pressure of the gluten proteins are minimal (Mathason, 1983; MacRitchie, 1992). Rheological effect is to increase the resistance and stability of dough. Therefore, increases the dough tolerance in kneading, fermentation and improves bread volume (Popa, 2007; Sudha et. al., 2008; Salinas et al., 2014).

This technology could be used to improve defective flours, such as those derived from wheat attacked by wheat bedbugs (*Eurygaster* sp.), which is a relatively common problem in the case of Romanian crops (Popa et. al., 2008). The aim of this study was not to detect a dose-effect relationship, concerning the calcium lactate treatments for a standardized flour, because the effect of calcium lactate on the rheological properties of such flours is relatively well known. What we were interested in was the effect of variable treatments on some flours with variable alveographical parameters. This variability is important to research in industry, where the flours of the same type

never have similar values of quality parameters. We aimed to find out what is the predictable variability part of treatments with calcium lactate, in industrial variability conditions, on the rheological properties of dough, as described in alveographic method.

As known, alveographic parameters of dough are strongly correlated with the technological behavior. Therefore, the results will provide an important tool, in order to dose the amounts of calcium lactate, in correspondence with the technological needs of flours used in production flows.

MATERIALS AND METHODS

The tests were performed on 62 type 650 wheat flours (according to Romanian Industrial classification, with an ash content expressed on dry matter of max 0.65%), coming from Romanian wheat harvests in the years 2014 and 2015. Flours were obtained from Farinsan SA., a manufacturer of milling industry. For each 62 flour samples were carried out alveographic analyzes, using a Chopin alveograph, according to the method described by SR ISO 27971: 2008.

There were determined the average values of the following parameters: the dough **resistance** to extension (P, mm); dough **extensibility** (L, mm), **extensibility index** (G) whose value is calculated based on the length of the curve (L), using formula $G=2.226\sqrt{L}$; the dough **absorbed energy** by stretching (W, 10^{-4} /Joules/gram dough); the dough **index of elasticity** (ie %), calculated as a ratio between resistance of dough at 40 mm at the beginning of the alveographic curve (P_{200}) and the maximum resistance (P); and finally **P/L ratio**, calculated as the ratio of the two parameters P and L, showing the extent to which the dough is more extensible, or more resistant.

Flours which were alveographic analyzed have been treated with variable amounts of calcium lactate, ranging from 10 to 300 g/100 kg of flour (100 - 3000 ppm).

The calcium lactate amounts applied for treatments were chosen according to the needs to improve mechanical work and P/L ratio parameters for each flour separately, taking into account the technical specifications of the respective flour.

The trade name of used **Calcium lactate** was Puracal PP/FCC, a fermentation product, dedicated to food industry, delivered by the French company Corbion. The product is characterized by a calcium content between 13.4 and 14.5% and has a minimum of 96% stereochemical purity (L isomer). The dosage of calcium lactate was made after weighing with analytical balance, directly in the mixing bowl of alveograph. Before the proper analysis, flour and calcium lactate were homogenized in the mixing bowl of alveograph for 2 minutes.

The results were statistically interpreted using the professional software "Statistica". Statistical analysis highlighted the average effect of the calcium lactate treatments on the alveographic parameters of flours in industrial environment.

RESULTS AND DISCUSSIONS

In industrial environment, due to large daily quantities of flours with different technical specifications, is suitable an average approach of quality parameters and of improving treatments.

The results of alveographic analyzes performed for control flours are shown in Table 1. (statistical variability estimates).

Table 1. Variability parameters for alveographic quality indices of the flours (n = 62)

Parameter	$\bar{X} \pm s_x$	Range of variation (min. – max.)	Coefficient of variation (%)
Resistance (P, mm)	76.758 ± 8.028	62 - 99	10.459
Extensibility (L, mm)	70.758 ± 12.023	42 - 82	16.992
Gluten extensibility index (G)	18.647 ± 1.687	14.4 – 20.2	9.047
Mechanical work (W, 10^{-4} /Jouli/g)	175.548 ± 47.010	78 - 240	26.779
P/L	1.080 ± 0.223	0.82 – 1.71	20.648
Dough elasticity index (Ie, %)	46.714 ± 7.841	29.6 – 56.8	16.785

We observed in Table 1. that the studied flours were characterized by a higher variability of some alveographic parameters, namely: the mechanical work (W) and the resistance/ extensibility ratio (P/L). The other quality

parameters are relatively homogeneous. In terms of average values of the analyzed parameters, it can be considered that the flours properties were characterized by modest bakery properties (alveographic mechanical work - below 200 W, P/L ratio greater than 0.65). It can be noticed, from the variation ranges analysis of alveographic parameters, that in the test samples were included flours with very low bakery potential (with mechanical work $W=78$, or extensibility, $L=42$). The average effects of the addition of calcium lactate to the flours are shown in Table 2.

Table 2. Variability parameters for alveographic quality indices of flours treated with calcium lactate

Parameter	$\bar{X} \pm s_x$	Range of variation (min. -max.)	Coefficient of variation (%)
Resistance (P, mm)	87.145 ± 14.545	52 - 123	16.690
Extensibility (L, mm)	64.790 ± 12.137	35 - 85	18.733
Gluten extensibility index (G)	17.831 ± 1.732	13,2 - 20,5	9.713
Mechanical work (W, 10^{-4} Joules/g)	200.081 ± 43.930	80 - 278	2.876
P/L	1.408 ± 0.438	0.82 - 2.89	31.11
Dough elasticity index (Ie, %)	49.006 ± 11.248	0 - 62.7	22.952

In Table 2, we observed that resistance parameter (P, mm) of dough had averaged an increase of 10.4 mm in the samples treated with calcium lactate, compared to control. The increase is statistically very significant $t=4.864$, $p=0.000001$, (Figure 1).

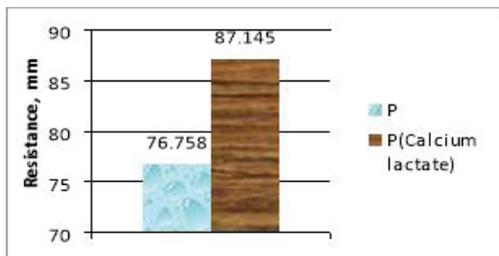


Figure 1. The effect of calcium lactate treatment on dough resistance (P) parameter

Figure 2, shows the regression equation between the amount of lactate used for the

flours treatment and the values of resistance parameter. We noticed that the addition of calcium lactate contributed very significantly to increase of dough resistance ($r=0.66^{***}$). In fact, 43% of the variation in resistance alveographic parameter (P, mm) is due to the addition of calcium lactate ($r^2 = 0.43$).

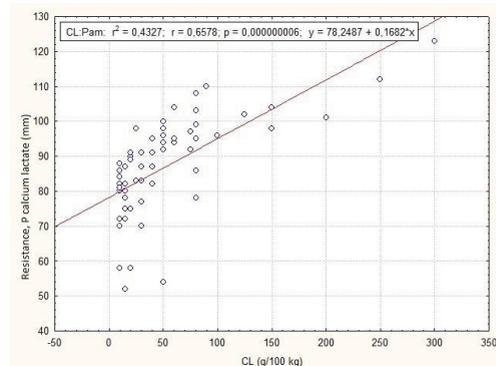


Figure 2. Regression between alveographic resistance (EMP) and the amount of added calcium lactate (CL, g/100 kg)

The addition of calcium lactate caused a significant decrease of Extensibility (L, mm) parameter value ($t=-2.751^{**}$). The decrease was on average, of 5.97 mm (Figure 3).

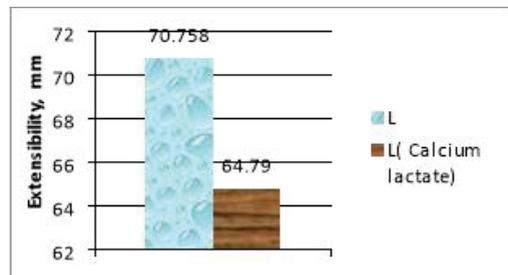


Figure 3. The effect of calcium lactate treatment on dough extensibility (L) parameter

Almost 31% of extensibility parameter variation can be explained on account of calcium lactate dose variation ($r^2=0.31$). The regression equation that describes the decrease of dough extensibility parameter, due to the increase of calcium lactate amount, is shown in Figure 4.

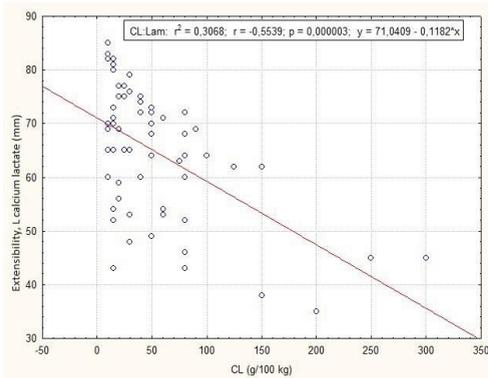


Figure 4. Regression between alveographic extensibility (L, mm) and the amount of added calcium lactate (CL, g / 100 kg)

As it was natural, given that it is a quality indicator derived from extensibility values, the extensibility index of gluten (G) registered a significant decrease in the flours treated with calcium lactate, compared to flours untreated ($t = -5.751$, $p = 0.000001$). This reduction was of 0.816 points.

The ability of dough to absorb mechanical work (W), namely the alveographic parameter that correlates most strongly with bread volume, as supports data in the literature, recorded a very significant increase in the flours treated with calcium lactate, from $175.5 \cdot 10^{-4}$ Joules/ gram dough, to $200.1 \cdot 10^{-4}$ Joules/ gram dough ($t = 6.990$ ***, $p = 0.0000001$, (Figure 5).

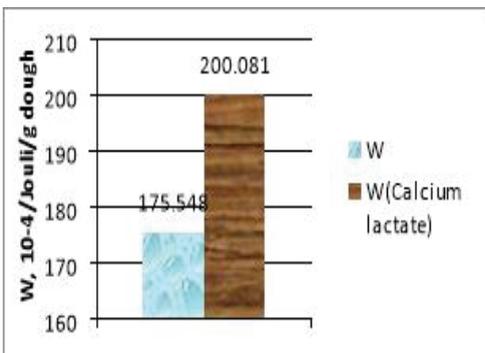


Figure 5. The effect of calcium lactate treatment on dough mechanical work (W, 10^{-4} /Joules/gram dough)

An interesting aspect of our results is the lack of a linear correlation between the amounts of calcium lactate used for flour treatments and the values of alveographic mechanical work parameter ($r = 0.094ns$).

Although the treatments with calcium lactate increase significantly the values of this parameter, as mentioned previously, the mechanism by which calcium lactate acts on the mechanical work, involves probably factors that have not been included in this analysis.

The most important of these appears to be the initial values of mechanical work in the treated flours, because if we introduce those values in a multiple regression model, then it can be explained 72% of the mechanical work variability. Multiple correlation coefficient of the regression equation was $r = 0.85$ (Figure 6).

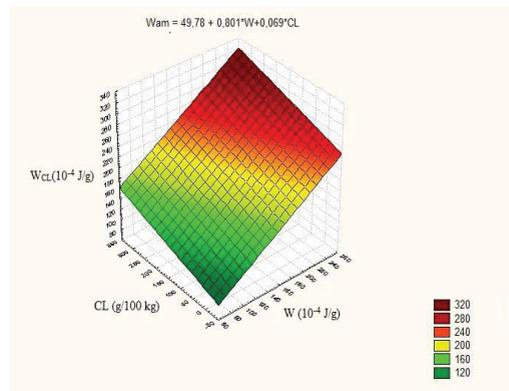


Figure 6. The relationship between the alveographic mechanical work (Wam) of treated flours, the amount of added calcium lactate (CL, g / 100 kg) and the initial alveographic mechanical work (W) of untreated flours

Relationship between dough resistance and extensibility (P/L ratio) was the alveographic parameter that registered the most spectacular growth, after treatments with calcium lactate, from an average value of 1,080 to an average value of 1.408 ($t = 7.174$ ***, $p = 0.0000001$) (Figure 7).

This is due to an increase of dough resistance and a concomitant decrease of dough extensibility.

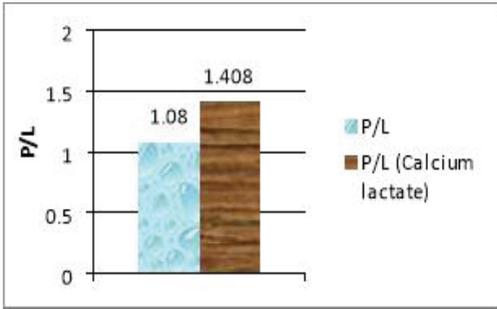


Figure 7. The effect of calcium lactate treatment on the dough P / L ratio

The P/L parameter showed the strongest correlation with the added amount of calcium lactate ($r=0.85^{***}$). In fact, 73% of its variation may be explained by variation of the added amount of calcium lactate ($r^2 = 0.73$).

Figure 8 shows regression between P/L and the added amount of calcium lactate.

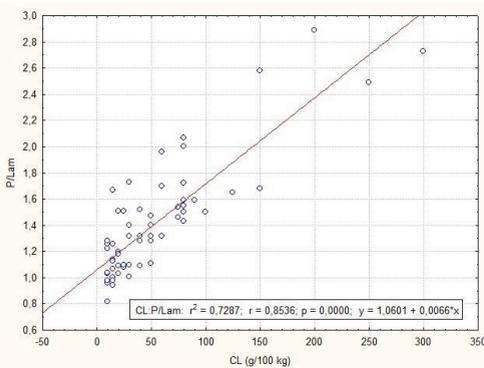


Figure 8. The regression between the resistance/extensibility (P/L) ratio and the amount of added calcium lactate (CL, g / 100 kg)

The correlation between the amount of added lactate and the average of P/L ratio in the treated flours, was strong enough and allowed to obtain a descriptive model, faithful to the relationship between dose and response, on account of the initial value of the P/L ratio in the treated flours.

Thus, Figure 9 shows a graphical model that describes the relationship between the initial value of P/L ratio in untreated flours, the dose of added calcium lactate and the value of P/L ratio in treated flours.

Multiple correlation coefficient of the model was 0.98 and describes about 96% of the P/L

ratio value in the treated flours, on account of calcium lactate amount variation and on the initial value of P/L ratio (Popa et al., 2009).

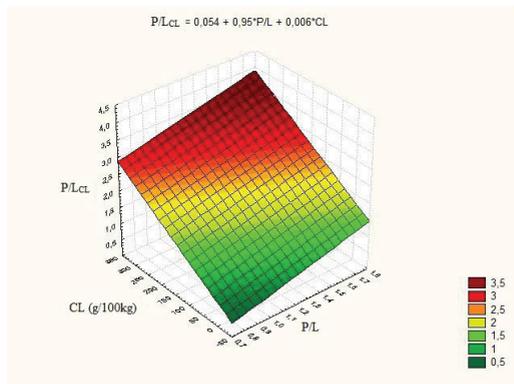


Figure 9. The relationship between the resistance/extensibility (P/L_{CL}) ratio in the treated flours, the amount of added calcium lactate (CL, g / 100 kg) and the initial value of P/L ratio in untreated flours

Dough elasticity index (Ie, %) increased significantly in the flours treated with calcium lactate, compared to control flours (from 46.7% to 49.0%, $t=2.121^*$, $p=0.038$) (Figure 10).

The Ie parameter value of analyzed flours increased significantly with increase of added calcium lactate amounts ($r=0.35^*$, $p=0.05$).

However, only about 12% of the Ie parameter variation may be associated with the variation of added calcium lactate amount. By introducing in the model the initial value of elasticity index in untreated flours (as we did for the alveographic mechanical work and the P/L ratio), the explained variation increase to 43%.

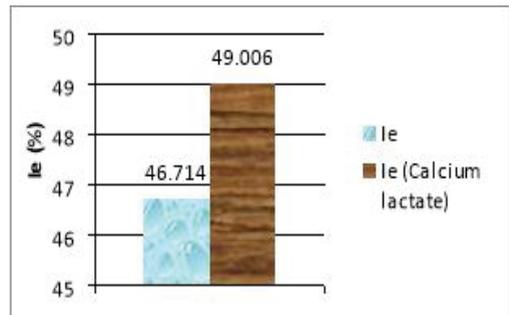


Figure 10. The effect of calcium lactate treatment on the dough Elasticity index (Ie, %)

Most changes in the elasticity index of flours treated with calcium lactate, as we can see, are due to some factors that have not been included in this analysis.

As our analyzes have shown, the effects of calcium lactate addition on the alveographic parameters, generally refers to increase of resistance and mechanical work.

CONCLUSIONS

In terms of average values of the analyzed parameters, it may be considered that control flours were characterized by modest bakery properties (alveographic mechanical work W , below 180, P/L ratio greater than 1.0).

Calcium lactate treatments led to a very significant increase of resistance parameter (P , mm), of dough capacity to absorb mechanical work (W , 10^{-4} J/gram) and of the resistance/extensibility ratio (P/L).

Elasticity index increased significantly in dough treated with calcium lactate, compared to control flours.

Calcium Lactate treatments led to a very significant decrease of some parameters, namely: extensibility (L , mm), and index of gluten extensibility (G).

Highly significant correlations were established between the amount of calcium lactate-dough resistance (positive correlation, $r=0.66^{***}$), and the amount of calcium lactate-extensibility (negative correlation, $r=-0.55^{***}$).

Multiple regression between the initial value of the dough mechanical work (W), the added amount of calcium lactate and the final amount of mechanical work, describes 72% of the mechanical work variability in the flours treated with calcium lactate ($r=0.85$), in industrial practice.

Multiple regression between the initial value of the flours P/L ratio, the added amount of calcium lactate and the final value of P/L ratio, describes 96% of the the P/L ratio variation, in the flours treated with calcium lactate ($r=0.98$).

Our results authorize the effects anticipation of calcium lactate dosage on dough rheology in industry, where the flours technical specification are very different.

These results can be used for the defects correction of the flours alveographic parameters, and for maximizing the dough potential in the case of normal or fortified flours.

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BINDERING AND THE STABILITY OF BETA CAROTEN FROM *Neurospora sitophila*

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Abstract

Neurospora sitophila, a species of fermentation fungi was reported containing the β -carotene pigments, the secondary metabolite of the yellow, orange or red-orange pigments groups. On this research, the carotenoid pigments of *Neurospora sitophila* has been bindered by a Oligosaccharide of gelatin-maltodextrins. The bindered products were dried by a spray drier and the β -carotene powder was determined its stability to the storage influence at Relative Humidity 20-30%. Bindered product of β -carotene extract obtained the GME powder with BY value ± 50 %. Bindered of this extract β - carotene was increased the water solubility and stable at Relative Humidity 20-30% condition, the stability the powder (GME) was decreased start at the third week. HPLC analysis of GME powder showed the decreasing of β -carotene about 30%, after storage for 5 weeks.

Key words: Bindering, β -carotene, spray-drier, stability.

INTRODUCTION

Oncome is a food rich in carotene pigment derived from fungal fermentation process substrate oncome on solid media.

Oncome yellow to red color produced by the fungus *Neurospora sitophila*, and the strain has orange, red, and pink.

Colors are caused by the existence of a kind of secondary metabolites carotenoid called β -carotene.

Carotenoid compounds have a range of color variations that are very attractive, especially when used as pigments for foodstuffs, feedstuffs, cosmetics, and pharmaceuticals. But generally these secondary metabolites, sensitive to light, heat, and oxygen, as well as having properties of low water solubility.

So that the compound is water insoluble, it is necessary through binding reaction (Reaction Mailard) β -carotene compound with other compounds that can give the effect of increased water solubility and color stability. β -carotene or natural dyes produced from fermentation by molds become a promising alternative to commercial-scale production, because in addition to cultivated quickly and provide a more pure product.

Research on β -carotene pigment produced by fungi, has been developed in Europe, and is recommended as a safe food additive and has a beneficial effect on health (Avalos, 2003). The result of this development is further used by the industry for the production of β -carotene in biotechnology and the development of new natural products rich in β -carotene.

The active compound binding reaction carotene can be made by dissolving the active compound and copolymer (binder) in an organic solvent, and then evaporated and diluted with water. The active compound can be bound to the particles when the solvent is replaced with water (Pan, et al., 2007). Hydrophobic groups (hate water) of the protein will interact with the compound β -carotene hydrophobic, while the hydrophilic groups (like water) of polysaccharides going out so it can be stable in the polar atmosphere, such as water.

This study aims to perform the binding of β -carotene pigment mold *Neurospora sitophila* and test the stability of the resulting binding reaction products. The binding process also conducted on β -carotene purely as a product comparison.

MATERIALS AND METHODS

Materials

Isolates of fungi *Neurospora sitophila*, pulp, maltodextrin, gelatin, β -carotene standard compounds, organic solvents and other chemicals, namely HCl, trypsin, cyclohexane, Sodium Sulfate.

Tools

Analytical balance, autoclave, plastic trays (plastic tray) incubator, vacuum evaporator, separating funnel, shaker bath, Chroma meter, HPLC, optical density, oven, and spray dryer.

Fermentation procedure *Neurospora sitophila*

Dried tofu that has been mashed, weighed as much as 200 grams of water and added with 600 ml (1: 3). Furthermore, the substrate is sterilized by autoclaving at 121 °C for 15 minutes. The substrates were autoclaved cooled, inoculated with 50 ml spore suspension of fungi *Neurospora sitophila* (10 million spores/ml) are then transferred into 2 pieces and perforated lidded tray with a size of 35 cm × 25 cm. Incubation is carried out at 30 °C for 3-5 days. Spores are yellow-orange harvest and store in the dark for further extracted.

Extraction of Carotenoids

Extract was filtered using filter paper of medium size. This extraction step is repeated until the entire pigment residues lifted and pale yellow spores. Further pigment extract was concentrated by means of a vacuum evaporator at 40 °C.

Extraction of carotenoids in the product GMB and GME follow the method Xiao Yun et al. (2007) were modified. The powder containing the carotenoid dispersed with 1 ml of HCl 0.1M and added trypsin (0.2 mg/ml). Fluid further incubated at 37 °C for 1 day.

Hydrolysis of protein molecules (trypsin) was stopped with 0.5 ml of acid solution (0.2M HCl). Cyclohexane is added at a certain ratio and shaken for 1 hour. The mixture is separated using a separating funnel, cyclohexane phase (top) is separated, added sodium sulfate dried and evaporated.

Carotenoids by HPLC analysis

Analysis of carotenoids was performed using HPLC following the method Sandman et al (2008) were modified, with a UV detector measured at a wavelength of 450 nm, mobile phase of acetonitrile: methanol: 2-propanol (85:

10: 5), a flow rate of 2 ml/minute, temperature 26 °C, type C18 column (Waters Symmetry size of 150 x 3.9 mm) and time analysis for 20 minutes. *B-carotene* Type II (Sigma-Aldrich) was used as the standard compound HPLC.

Making copolymer through Maillard Reaction

Making copolymer based on the principle of Maillard reaction, following the method of Pan et al, (2007) were modified. In making this copolymer used gelatin (Merck), maltodextrin with a DE value of 13 - 17. The copolymer is made from a mixture of gelatin-maltodextrin with a ratio of 4: 1. The Maillard reaction is carried out at a temperature of 50 °C, 100 rpm for 4 hours.

Carotenoids Binding process

Pulverizing carotenoids performed based on the method of Chen and Tang (1998) were modified. A total of 1.3 grams of carotenoid extract is diluted with a little technical ethanol. Gelatin-maltodextrin copolymer is prepared by mixing 40 grams of gelatin and 10 grams of maltodextrin, then dissolved in 1 liter of distilled water. Copolymerization reaction performed on a shaker apparatus with a temperature of 50 °C, 100 rpm, for 4 hours. Encapsulation of carotenoids GME (gelatin-maltodextrin-extract) is done by adding a carotenoid extract that has been dissolved in 1 liter of ethanol into the solution. Copolymer gelatin-maltodextrin that has been cooled. Furthermore, the solution is homogenized using a magnetic stirrer for 30 minutes. The powder is made by drying the carotenoid GME, GME suspension by means of spray-drier at an inlet temperature of 160 °C conditions, the outlet 70 °C, a pressure of 1 bar with an average flow rate of 8 ml/min. Samples are stored in a desiccator with a humidity of 20% - 30%, temperatures between 25 °C - 28 °C.

Results of the binding reaction (Binding yield / BY) calculated by the equation:

$$\% \text{ BY} = \text{MSC} \times 100 / \text{MSB}$$

MSC = Total mass of aggregate pigments after copolymerization

MSB = Total mass of aggregate pigments before copolymerization

GMB and GME powder and tested its stability against the solubility properties of carotenoids (OD.450), the intensity of the color by means of Chroma meter, and water content (Sudarmadji et al., 1997)

RESULTS AND DISCUSSIONS

Manufacture of gelatin-maltodextrin copolymers made through the Maillard reaction which is the reaction between the protein molecules with molecular saccharides. Copolymer casein-g-dextran has been made through the Maillard reaction is a reaction of transplant (grafting) dextran molecules with molecules of casein.

The copolymer is soluble in water at pH 7.0, but not soluble in ethanol. Meanwhile, β -carotene hydrophobic, solubility increases in ethanol (Pan, et al., 2007).

On this basis, the reaction binding carotenoid extract *Neurospora sitophila* with gelatin-maltodextrin copolymer made to improve the solubility of the carotenoid in the water.

The suspension of the carotenoid extract mixture with gelatin-copolymer maltodextrin, dried by means of a spray dryer to obtain powder carotenoid GME (gelatin-maltodextrin-extract). As a comparison, the same encapsulation process is carried out using pure β -carotene (Sigma), thus obtained GMB powders (gelatin-maltodextrin- β -carotene).

Drying results obtained in this study were obtained powder GMB value BY 60%, and powder GME with value BY (Binding Yield) 50%. The results of the spray drying process

depends on the configuration tool, while binding reaction efficiency is strongly associated with physio-chemical properties of the core material and a binder. The results were not much different from that reported by Nunez and Mercadante (2007) to the binding reaction lycopene compound with β -cyclodextrin, providing product acquisition by $51 \pm 1\%$. Chen and Tang (1998) reported a 67% by values for binding results carotenoids from carrot pulp with gelatin-sucrose.

Binding reaction products (Maillard Reaction) copolymer carotenoids with hydrophilic (water loving) compounds such as starch group can improve solubility in water.

The core of the particle hydrophobic (hates water) will act as storage space, while the outer shell (shell) that will stabilize hydrophilic particles in a water molecule dispersed water conditions (Pan et al., 2007).

In this study, solubility and stability of carotenoids and GME GMB powder in water determined value of absorbance at a wavelength of 450 nm (OD.450).

The powder stability testing performed until week 5. Analysis of the second powder, a decline in the absorbance began at week 3. The statistical test result, a decline in the average value OD.450 on GME powder 2.5% and amounted GMB powder 0.76%.

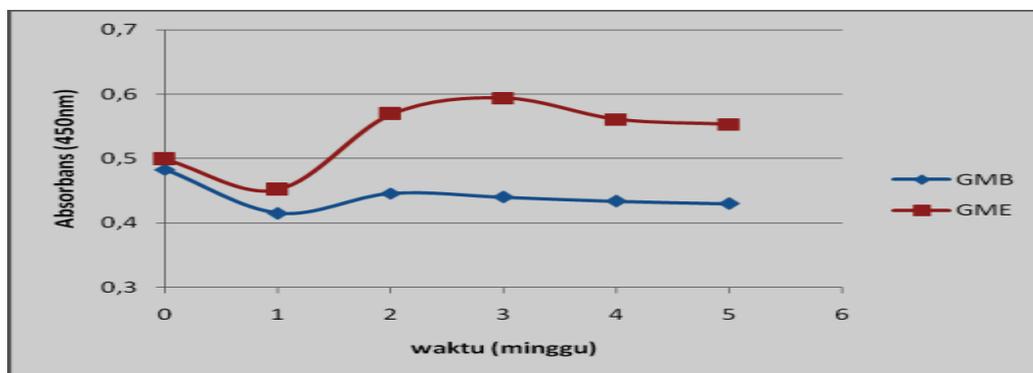


Figure 1. Stability Testing Results Carotenoids (OD.450) on Suspension GMB and GME

Increasing the value of OD occurred at weeks 1, especially on sample GME. This happens because a significant antioxidant response of *Neurospora sitophila* extract carotenoids which are a mixture of several carotenoids, and the response decreases after week 3. The study

conducted by Priatni et al. (2010) there are at least five carotenoid compounds that have antioxidant properties of extracts of *Neurospora sitophila*, among others: lycopene, neurospore, γ -carotene, β -carotene and phytoene.

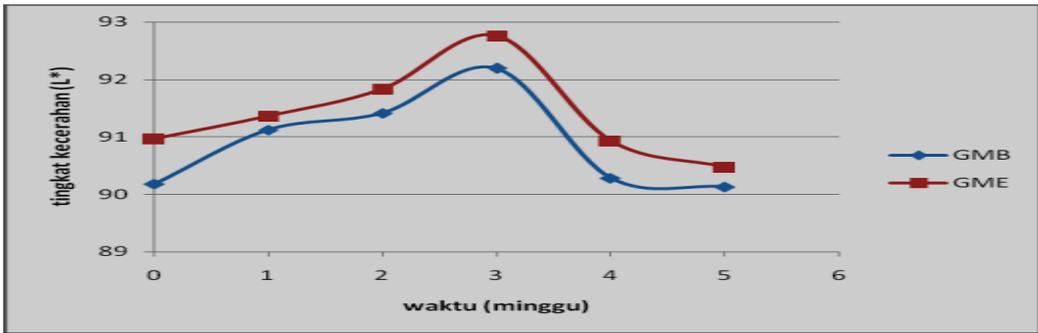


Figure 2. Stability Testing Results Color Brightness (L*) on the powder GMB and GME

Analysis of the powder GME showed a decline in the level of color brightness (L* value) average of 0.84% and a decrease in yellow (b* value) average of 8.64%. While the analysis of the powder GMB showed a decline in the level of color brightness (L* value) average of 0.76% and a decrease in yellow (b* value) average of 5.24%. Studies conducted Chen and Tang (1998) that the decline in the value of L*, a* and b* in powder carotenoid declines with increasing storage time.

Decrease yellow carotenoids during storage of the powder can be caused by the degradation of trans- β -carotene and formation to form the cis isomer. It has been widely reported that the formation of cis on a carotenoids may reduce the intensity of the color. As stated by Natalia et al. (2009) that were causing color changes in carotenoid compounds as a result of their defense mechanism by carotene due to the heat radiation so that the carotenoid isomers shaped-trans transformation changed into a form of transformation-cis.

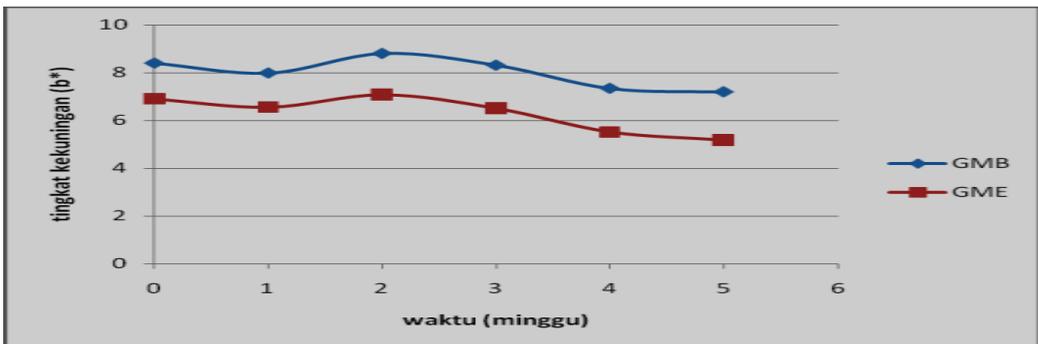


Figure 3. Stability Testing Results Level Yellowish (b*) on the powder GMB and GME

During stability testing, the GMB and GME powder placed in a eksikator with humidity (% RH) between 20% - 30% and temperatures between 25 °C - 28 °C. In the controlled

humidity and temperature, changes in the water content of the powder results copolymerization (Reaction Mailard) was observed during storage until week five (5).

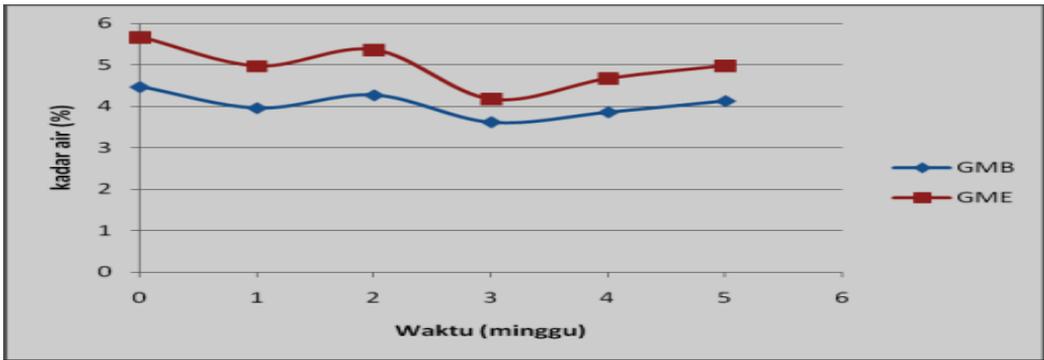


Figure 4. The results of the analysis of the water content of the powder GMB and GME

Results of statistical analysis of the powder showed elevated levels of water GME average of 5.48% and the powder GMB water levels increase an average of 4.09%. Increased levels of water, clearly occurred after week 3. According Onwulata, et al. (1998) have been used for some disaccharide binder. Various volatile substances have been successfully trapped in sucrose, maltose or lactose via freeze drying. The molecular structure of amorphous-shaped sugar molecules are transformed into crystalline (thin surface more

stable). Surface membrane of the disaccharide is watertight with high resistance against diffusion style at low water levels that are dispersed to form more complex molecules. The binding process can improve the ability of blocking duplicate water into the particles. The hitch can be formed through a combination of polymerization between lipids and disaccharide substance, so as to form a double wall. Reduction of water absorption is very important for the stability and effectiveness of the binding powders of carotenoids.

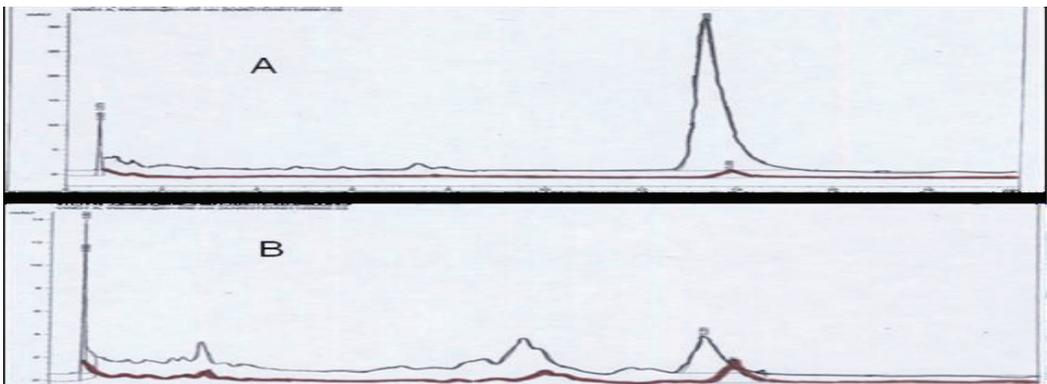


Figure 5. HPLC Analysis of Stability of Carotenoids in the GMB powder (A) and GME (B), 1: peak of β -carotene 0 Sunday, 1': β -carotene 5 Sunday

Results of HPLC analysis, showed a decrease in the content of β -carotene from 0.0090 ug be 0.0035 mg per gram powder and 0.178 g GME be 0.0075 mg per gram of powder GMB, after storage in eksikator in week 5. The degradation of the α and β -carotene follow first-order reaction kinetics. According to Robert (2003)

during storage powder carotenoid pigments of carrots with maltodextrin encapsulation yields, the inlet temperature to the spray dryer greatly affect the acquisition of pigment. Higher drying temperatures (>150 °C) may cause decomposition of carotenoid compounds.

CONCLUSIONS

The process of binding of β -carotene pigment *Neurospora sitophila* with gelatin-maltodextrin copolymer (GME) can increase the solubility in water and is stable at 20-30% RH storage for 3 weeks.

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INFLUENCE OF TECHNOLOGICAL FACTORS ON ACRYLAMIDE LEVEL FROM BISCUITS

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Abstract

The objective of this study was to investigate the influence of wheat flour extraction degree and baking parameters on acrylamide (AA) level in biscuits by using gas chromatography tandem mass spectrometry (GC-MS/MS). Color parameters were analyzed in an attempt to establish correlations with the investigated factors. Biscuits baked between 25 min ÷ 60 min at 200°C recorded values of AA level < LOD (4.63 µg/kg) to about 1.580 µg/kg. By baking biscuits at a temperature varying between 220°C ÷ 240°C for 20 min the AA level increased from 67.44 to 212.87 µg/kg. The highest AA level and the darkest color was obtained when using in the biscuits recipe the whole-wheat flour (F3) with an ash content of 2.37% d.m. (1580.33 µg/kg, ΔE^ = 35.66), followed by white flour F1 with 0.53% d.m. (387.82 µg/kg, ΔE^* = 61.79), respectively F2 with 0.44% d.m. (308.38 µg/kg, ΔE^* = 67.47). From the results obtained it can be said that the AA level is strongly influenced by biscuits baking parameters (temperature and baking time) and by the ash content of wheat flour used in the manufacture recipe.*

Key words: acrylamide, baking parameters, biscuits, GC-MS/MS, wheat flour.

INTRODUCTION

Acrylamide (AA) is a thermal chemical process contaminant that can be formed when foods are subjected to roasting, baking, frying processes at temperatures above 120°C (Ahrné et al., 2007, FDA, 2016). High temperature and long baking time may produce pleasant taste and specific color to food, and also can lead to the development of toxic substances such as AA (2-propenamide) (Mottram et al., 2002). In April 2002 a group of researchers from Sweden have announced that people consume AA through their daily diet by eating some common foods such as bread, crackers, chips, coffee etc. at much higher levels than the allowed dose in the drinking water (Tareke et al., 2002). The highest concentrations of AA were found to be formed in fried and baked potato products, followed by cereal products, coffee, and small amounts of AA were found in dairy, meat and fish products (Becalski et al., 2003; Ahrné et al., 2007; Alves et al., 2010). Since acrylamide is known for its neurotoxic effects and was classified by the International Agency for Research on Cancer as a carcinogen in animals and "probable carcinogenic to humans" (Group 2A) (IARC, 1994), there is a

permanent concern of experts from this field to decrease the concentration of AA from food at the lowest possible level.

There are several ways for AA formation in thermally treated foods, such as the Maillard reaction, involving a number of specific amino acids, for example asparagine, and compounds with a carbonyl group, for example, glucose, fructose (Mottram et al., 2002; Friedman, 2003; Claus et al., 2008; Hedegaard et al., 2008), and the second route for the formation of AA is the degradation of glycerol to acrolein, then to acrylic acid which reacts with ammonia and leads to the development of AA (IARC, 1994; Claus et al., 2008; Hedegaard et al., 2008).

The acrylamide content of food varies depending on the raw material, formulation recipe, processing conditions, production method etc. (Claus et al., 2008; Curtis et al., 2014; Przygodzka et al., 2015). Nguyen et al. (2016) showed that the acrylamide content of baked products is influenced by the type of sugar used in the manufacture recipe. By baking biscuits at 200°C, glucose produced a higher level of acrylamide than sucrose and fructose, the concentration of asparagine being higher in these samples. The lowest concentration of acrylamide was observed for

the recipe of biscuits obtained with sucrose. Besides the type and concentration of sugars, the formation of acrylamide in biscuits was influenced by the concentration of amino acids, temperature and baking time, pH, water activity, leavening agent. Food surface color is the first qualitative parameter evaluated by consumers and it is essential in product acceptance. The color can be easily considered an indication of Maillard reaction products. Food color formation is influenced by factors such as pH, water content, water activity, oxygen levels, relative humidity (Ahrné et al., 2007), but the most important are the parameters of the heat treatment, temperature and baking time.

The purpose of this research was to investigate the influence of the wheat flour extraction degree (expressed as ash content) and the influence of the cooking parameters (time, temperature) on the AA content of biscuits; also it were evaluated the CIELab chromatic characteristics (L^* , a^* , b^*) in order to establish the correlations between color parameters and investigated factors.

Investigation of technological factors on the AA level in biscuits, on the one hand is important for consumers to select the use of everyday products that have a lower level of AA, on the other hand it is important for producers of bakery products in order to know the factors leading to the formation of a higher level of AA in food matrices such as biscuits and similar products, and to determine optimal mitigation strategies to reduce this contaminant.

MATERIALS AND METHODS

Biscuits preparation

Wheat flour was the main raw material, which came in the highest proportion in the composition of biscuit samples, made in experimental variants. In Romania, based on the extraction degree it is defined the type of flour, which is presented as mineral content (ash), expressed in dry matter percent. The more the extraction degree is higher, the ash content of flour is higher.

In this study three types of wheat flours obtained by milling the same wheat variety were used: white flour with an ash content of

0.53% d.m. (F1), white flour with an ash content of 0.44% d.m. (F2), respectively whole-wheat flour with an ash content of 2.37% d.m. (F3).

It were made 18 experimental variants of biscuits, varying the:

- baking time (25, 30, 40, 50, 60 min) and maintaining a temperature of 200°C, using wheat flour type with a different ash content (F1, F2, F3)

- baking temperature (220°C, 230°C, 240°C) and maintaining the baking time at 20 min, using the white wheat flour (F1).

For the biscuits preparation of the 18 experimental variants were used: flour (F1, F2, F3) (750 g), margarine (250 g), sugar (250 g), eggs (4, around 200 g), baking soda (NaHCO_3 , 10 g), vinegar (3.5 mL), and salt (NaCl , 5 g). Margarine was mixed with sugar until a frothy masses was obtained, then the eggs, baking powder dissolved in vinegar, salt and finally flour were added. Kneading was done for 10-15 min, until getting a loose dough with proper consistency, which could be modeled easily. After kneading, the dough was packed in polyethylene bags and was allowed to rest in a refrigerator at 7°C for 60 - 120 min. The dough was shaped in the biscuits form, using a shaping machine, and then biscuits were placed in trays lined with parchment paper. Biscuit trays were placed in the oven, under the experimental conditions presented below. The experimental variants were realised in three separate batches, and for analyse were used average samples from 3 batches. Measurements were performed in triplicate, and the values were presented as mean \pm standard deviation.

To get the biscuit assortments in the experimental variants were used specific equipment for the manufacturing technology of bakery products, from the Pilot Experiments Plant for Cereals and Flours Processing from IBA Bucharest: Buhler laboratory automated flour mill, MLU-202 (10-12 kg wheat/h), dough mixer (Diosna, DM 08- 4/6), dough roller, shaped machine, rotary kiln oven with controlled temperature and baking time (ROTOR model) etc.

Reagents and Consumables

For the determination of AA content from biscuits were used a native AA standard, min.

99% purity, of concentration 1000 µg/mL in methanol (Ultra Scientific Analytical Solution, N. Kingstown, Rhode Island, USA), internal standard (IS) of labeled acrylamide with the carbon atoms marked (1,2,3-¹³C), min. 99% purity (+100 ppm hydroquinone), of concentration 1000 µg/mL in methanol (Cambridge Isotope Laboratories, Inc., Andover, MA, USA). All chemicals, solvents and reagents used to analyze the AA were of chromatographic purity and were purchased from Merck (Darmstadt, Germany), LGC Promochem GmbH (Wesel, Germany) and Scharlau (Sentmenat, Spain). The water used was obtained from a purification system, PURELAB Option- S7/15 and PURELAB Ultra Ionic system (Elga Labwater, High Wycombe, UK).

Moisture and ash content determination

Determination of wheat flour and biscuits moisture of the experimental variants was realised by the method AOAC (2000). The ash content of wheat flour was performed by gravimetric method by sample calcination at 550°C, according to method AOAC (2000).

Acrylamide analysis

Biscuits acrylamide was quantified by GC-MS/MS (Negoiță et al., 2014, Negoiță and Culețu, 2016). The calibration solutions and the derivatized extracts sample were analyzed using a gas chromatograph, type TRACE GC Ultra, coupled with triple quadrupole mass spectrometer (TSQ Quantum XLS) from Thermo Fisher Scientific (USA).

The analyses were performed in the electron impact ionization operation mode, positive (+EI); acquisition mode: selected reaction monitoring (SRM) and ion scanning mode - Product ("Product"). A capillary column based on polyethylene-glycol, TraceGOLD™ TG-WaxMS (Thermo Fisher Scientific, USA) with a length of 30 m x 0.25 mm inner diameter and film thickness of 0.25 µm was used as a stationary phase.

The column temperature in the oven was programmed at 65°C for 1 min, and then increased with 15°C/min up to 170°C (0 min), 5°C/min up to 200°C (0 min), respectively 40°C/min up to 240°C with maintaining at this temperature for 15 minutes. The flow of the

mobile phase, He, was 1.6 mL/min, transfer line temperature was 230°C. From the sample, 1 µL was injected into an injector PTV Right type, using an autosampler TriPlus AS (Thermo Fisher Scientific, USA), in split mode with a split ratio of 1:10 and the injector temperature 220°C. Under these conditions, the retention time of AA was 10.65 min ± 0.5 min AA and IS were identified by the appropriate ion fragments of ion derivatized 2-BPA (2 brompropenamida) and 2-BP (¹³C₃) A.

The fragmentation of the precursor ions *m/z* 151 and 154 was carried out with argon (1 mTorr), leading to the formation of product ions (daughter) with *m/z* 70 (2-BPA) and 73 (2-BP (¹³C₃) A), being used for quantification. The calculation of the AA concentration in the test samples were based on the appropriate peaks area ratios product ions with *m/z* 70 for 2-BPA and *m/z* 73 for 2-BP (¹³C₃)A.

AA concentration values of the experimental variants of biscuit samples were expressed both on dry matter (% d.m.) and as µg/kg AA of biscuits.

The analytical procedure to determinate the AA level from biscuits was characterized by a high degree of sensitivity (LOD = 4.63 µg/kg, LOQ = 13.89 µg/kg) and good precision (RSD (r) ≤ 5%, RSD (R) ≤ 6%) (Negoiță and Culețu, 2016).

Color analysis

The most common way of measuring food color was introduced by Commission Internationale de L'éclairage (CIE) in 1976, in which color is described in a three-dimensional scale, the amounts of *L**, *a**, *b** (CIE Colorimetric Committee, 1974, Francis and Clydesdale, 1975).

The color analysis was performed using a HunterLab colorimeter (Universal Software V4.01 Miniscan XE Plus). Instrument calibration was done with black and white pads supplied by the manufacturer. The color of the samples was measured using illuminate D65 with an angle of view of 10°.

It were calculated CIELAB'76 color parameters: *L**, *a**, *b**. According to CIE, *L** measured the object luminance intensity on a scale from 0 to 100, where 0 represents black and 100 white, *a** represents the color position of the object on a scale from pure red and pure

green, where pure green is -127, and pure red is +127, and b^* represents the position of the object on a scale color of pure blue and pure yellow, where pure blue is -127 and pure yellow is +127. For each sample, measurements were made on 10 different points, and the mean value was determined. Total color difference, ΔE^* was calculated using the following equation (1):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (1)$$

where $\Delta L^* = L_1^* - L_0^*$; $\Delta a^* = a_1^* - a_0^*$; $\Delta b^* = b_1^* - b_0^*$, L_0^* , a_0^* , b_0^* it refers to the standard color (black, where $L_0^* = 0$, $a_0^* = 0$, $b_0^* = 0$), L_1^* , a_1^* , b_1^* refers to the sample color.

Statistical analysis

AA content was expressed as mean \pm standard deviation.

The differences among sample groups were analyzed by one-way analysis of variance ANOVA followed by Tukey's test, $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSIONS

Since duration and temperature at which dough is thermally treated during baking highly influence the level of acrylamide and the color of the baked product, the objective of this study was to establish the influence of these parameters on the acrylamide content and color changes of biscuits.

Characterisation of flour types

The physico-chemical properties and color parameters of wheat flour used in the experimental variants are presented in Table 1.

Influence of the baking time and the flour type on the AA formation in biscuits

In this study 15 experimental variants of biscuits were made, varying the baking time (25, 30, 40, 50, 60 min) and the wheat flour used, with different extraction degrees, expressed in ash content (F1, F2, F3). The same baking temperature, 200°C, was used for all 15 experimental variants. The experimental variants when varying the baking time are presented in Table 2.

Table 1. Physico-chemical and sensorial properties of wheat flour used in the experimental variants

Physico-chemical and sensorial properties	Wheat type A		
	White flour		Whole-wheat flour
	F1	F2	F3
Moisture (%)	12.60	12.71	11.99
Ash (% d.m.)	0.53	0.44	2.37
Glucides (% d.m.)	73.2	74.1	70.21
Reducing sugars (% d.m.)	0.68	0.65	1.3
Celulose (% d.m.)	0.65	0.59	1.48
Color parameters, CIELab			
L^*	88.87	88.16	74.63
a^*	0.64	0.27	2.48
b^*	8.89	7.81	8.85
ΔE^*	89.31	88.51	75.19

From the data shown in Table 3 where the AA content of the experimental samples is presented as a function of baking time it can be noticed that for the three types of flour used in the manufacture recipe of biscuits, the AA level increases as increasing the baking time, respectively when decreasing the water content of biscuits. Also AA level increases with increasing ash content of the flour, as follows:

- AA level increased from $< \text{LOQ}$ (13.89 $\mu\text{g}/\text{kg}$) up to 387.82 $\mu\text{g}/\text{kg}$ for wheat flour F1 (0.53% d.m.),
- AA level increased from $< \text{LOD}$ (4.63 $\mu\text{g}/\text{kg}$) up to 308.38 $\mu\text{g}/\text{kg}$ for wheat flour F2 (0.44% d.m.),
- AA level increased from 14.34 $\mu\text{g}/\text{kg}$ up to 1580.33 $\mu\text{g}/\text{kg}$ for whole-wheat flour F3 (2.37% d.m.).

Table 2. Experimental variants to obtain biscuits using three different types of flour, varying the baking time

No.	Flour type	Variant	Cooking parameters, temp. (°C), time (min)	Experimental variants
1	White wheat flour (F1)	V21	200, 25	
2		V31	200, 30	
3		V41	200, 40	
4		V51	200, 50	
5		V61	200, 60	
6	White wheat flour (F2)	V22	200, 25	
7		V32	200, 30	
8		V42	200, 40	
9		V52	200, 50	
10		V62	200, 60	
11	Whole-wheat flour (F3)	V23	200, 25	
12		V33	200, 30	
13		V43	200, 40	
14		V53	200, 50	
15		V63	200, 60	

Table 3. Acrylamide and water content when varying the baking time of experimental variants

Time / min	Parameter ^a								
	AA (µg/kg)			AA (% d.m.)			Water content (%)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3
25	<LOD ^b	<LOD ^b	14.34 ^b ± 0.03	0.84 ^c ± 0.00	0.29 ^c ± 0.00	1.58 ^c ± 0.00	13.17 ^d ± 0.06	10.21 ^d ± 0.05	9.29 ^d ± 0.12
30	<LOQ ^b	<LOQ ^b	25.51 ^b ± 0.03	1.22 ^c ± 0.00	0.44 ^c ± 0.00	2.74 ^c ± 0.00	11.12 ^d ± 0.04	7.54 ± 0.10	7.00 ^d ± 0.21
40	55.12 ^b ± 0.0	45.70 ^b ± 0.18	533.73 ^b ± 0.02	5.89 ^c ± 0.00	4.85 ^c ± 0.00	54.35 ^c ± 0.04	6.41 ^d ± 0.08	5.73 ^d ± 0.05	1.80 ^d ± 0.07
50	108.87 ^b ± 0.04	59.53 ^b ± 0.26	1214.79 ^b ± 0.02	11.38 ^c ± 0.00	6.17 ^c ± 0.00	123.09 ^c ± 0.07	4.30 ^d ± 0.04	3.60 ^d ± 0.10	1.31 ^d ± 0.06
60	387.82 ^b ± 0.07	308.38 ^b ± 0.12	1580.33 ^b ± 0.03	39.46 ^c ± 0.03	31.38 ^c ± 0.02	159.81 ^c ± 0.12	1.70 ± 0.07	1.72 ± 0.06	1.11 ^d ± 0.08

^aThe results are expressed as mean ± standard deviation (n = 3); ^{b,c,d} data followed by different superscript letters within each column indicates a highly significant difference among each parameter according to Tukey's test (p < 0.001) (^b for AA level expressed in µg/kg, ^c for AA level expressed in % d.m., ^d for water content)

When varying the baking time of the experimental variants of biscuits, lower concentrations of AA were formed in biscuit samples obtained with white flour (F1, F2), compared with the biscuits samples obtained from whole-wheat flour (F3). Extraction degree of flour, expressed in ash content, is an important factor affecting the acrylamide level in the final product (Claus et al., 2008). Also, the acrylamide content of bakery products is influenced by the asparagine content of wheat flour used, products made from whole-wheat flour with an ash content higher than white flour producing a higher level of acrylamide in the equivalent model system (Claus et al., 2008; Przygodzka et al., 2015; FDA, 2016). In a study realized by Przygodzka et al. (2015) it was shown that the baking conditions and the

wheat flour used influence the acrylamide formation in white and dark breads. By increasing the baking temperature from 200°C to 240°C and decreasing the baking time from 35 min to 30 min, for both white bread and whole-wheat dark bread, the crust of bread baked at higher temperature produced a higher AA content. The AA content was higher in the bread crust than in bread sliced.

The results are in agreement with the findings of Capuano et al. (2009) who investigated the effect of flour type (wheat, rye, and whole-wheat flour), also the process conditions on a bread crisp model system and it was determined that the acrylamide amount was higher in the samples containing rye flour (301.0 µg/kg) and whole-wheat flours (291.0 µg/kg), than the ones with wheat flours (262.0 µg/kg). It can be

concluded that these results can be due to a higher content of free asparagine and ash content of rye and whole-wheat flours.

As acrylamide forms in foods through the Maillard reaction, the content of reducing sugars influence the level of acrylamide in the food system. The AA content of experimental variants of biscuits obtained in this study was in direct ratio with the content of reducing sugars presented in the flour used. A higher level of AA was obtained for the biscuits made with the flour F3 which presented the highest content of reducing sugars (1.3% d.m.), followed by the flour F1 (0.68% d.m.), respectively F2 (0.65% d.m.) (table 3). This result is probably due to the fact that sucrose began to hydrolyse, resulting a higher level of reducing sugars which produce asparagine degradation and acrylamide formation (Van Der Fels-Klerx et al., 2014).

Correlation between AA level and biscuits baking time

To establish a correlation between the AA level and the biscuits baking time (25 min, 30 min, 40 min, 50 min, 60 min) it were drawn the regression lines for each type of flour which led to the line equations and regression coefficient values. For each type of flour it were obtained positive linear correlation between the acrylamide level and the baking time:

- $y = 9.825x - 288.85$, $R = 0.8876$, for wheat flour F1 (0.53% d.m.),
- $y = 7.637x - 229.04$, $R = 0.8550$, for wheat flour F2 (0.44% d.m.),
- $y = 48.678x - 132.00$, $R = 0.9883$, for whole-wheat flour F3 (2.37% d.m.).

By using in the biscuits formulation recipe of a type of flour with a higher ash content (F3), a higher level of AA was observed compared to the biscuit samples obtained by using a flour with a lower ash content (F1, F2). Applying a higher baking time for biscuits, provide a higher level of AA in all cases of flour used.

The correlation between the AA level and the baking time of the biscuit is higher as the ash content of the wheat flour is higher.

Correlation between the AA level and the water content of biscuits

At baking time variation of the experimental samples of biscuits were obtained negative

linear correlations between the AA level and biscuits moisture for each type of flour used:

- $y = -2.8225x + 32.393$, $R = 0.8311$, for wheat flour F1 (0.53% d.m.),
- $y = -3.1228x + 26.668$, $R = 0.7992$, for wheat flour F2 (0.44% d.m.),
- $y = -16.398x + 135.04$, $R = 0.8636$, for whole-wheat flour F3 (2.37% d.m.).

When increasing the baking time of biscuits, it was produced the decrease of water content of biscuits and thus increased the AA level for all type of flour used. During baking, moisture loss progresses (Della Valle et al., 2012, Walker et al., 2012) and acrylamide formation enhance (Claus et al., 2008).

Same results were obtained by Acar and Gokmen (2009), who showed that the temperature and water content of baked products influence the acrylamide formation, higher levels of acrylamide being obtained at higher temperatures. Mulla et al. (2011) found that there is a correlation between the AA formation and water content of extruded snacks. An increase in the moisture content of the raw material produced a decreased of the AA level.

The correlation between the AA level and the water content of biscuits is higher as the ash content of the wheat flour is higher.

Correlation between the AA level and color parameters of biscuit samples

The effect of color development and increment of AA level in biscuits obtained at 5 different baking time, 25, 30, 40, 50, 60 min (200°C) and 3 types of wheat flour with different ash content was studied. Color is an important food attribute and can be an indicator of acrylamide formation during processing of food products (Lu and Zheng, 2012). The formation of color during baking is due to the Maillard reaction and caramelization of sugars (Purlis, 2010).

All experimental variants were analyzed in terms of CIELab chromatic characteristics represented by the color difference ΔE^* . Lu and Zheng (2012) evaluated the AA content in biscuits by analysing the color information using the principal component analysis (PCA) and least-squares support vector machine (LS-SVM) combined with fractal color. It was found that L^* , a^* , b^* have the best performance to identify the correlation between the AA

content and color changes of biscuits comparing with the prediction performance of fractal color.

In Figure 1 it is represented the AA level variation and color differences (ΔE^*) of the biscuits obtained in the experiment. It is shown that as the AA content increases, the color difference decreases.

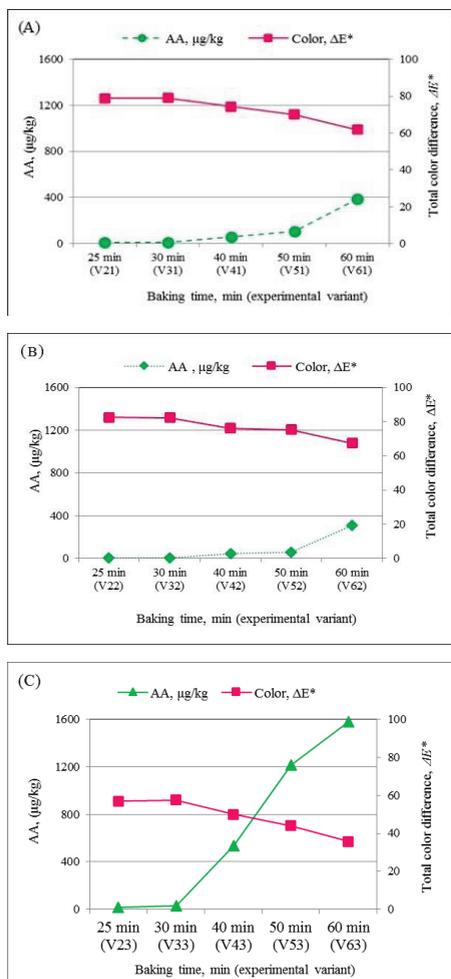


Figure 1. Variation of AA level and total color difference ΔE^* of biscuits obtained with flour F1 (A), flour F2 (B), and flour F3 (C) when varying the baking time

In order to establish the correlation between the AA level and total color difference (ΔE^*) of the experimental variants, the regression lines were plotted and the straight lines equations and the regression coefficients values between these parameters were determined.

A negative linear correlation was obtained between the AA level and the total color difference (ΔE^*) of the biscuit samples tested, the straight lines equations and correlation coefficients being as follows:

- $y = -21.272x + 1660.8$, $R = 0.9596$, for wheat flour F1 (0.53% d.m.),
- $y = -19.069x + 1547.7$, $R = 0.9264$, for wheat flour F2 (0.44% d.m.),
- $y = -75.809x + y = 4375.1$, $R = 0.9888$, for whole-wheat flour F3 (2.37% d.m.).

In all cases of flours used, when baking time increases, the total color difference, ΔE^* , decreases. The highest values of AA level and the lowest values of total color difference were recorded when using F3 flour type (2.37% d.m.), with the highest ash content obtaining the highest correlation between these parameters ($R = 0.9888$). The correlation is higher as the ash content of the flour used is higher.

Influence of the baking temperature on the AA formation in biscuits

In addition to the baking time, temperature is an important factor in the formation and reduction of AA in biscuits. It were realised 4 experimental variants (Table 4), to determine the baking temperature influence on the AA content formed in biscuits. It has been shown that, by keeping constant the baking time (20 min) and temperatures ranging from 220°C to 230°C, respectively 240°C, the AA level from biscuits increased approximately 3-fold (Figure 2).

Same results were obtained by Acar and Gokmen (2009) who showed that the temperature and moisture of baked products influence the acrylamide formation, higher levels of acrylamide being obtained at higher temperatures.

From Figure 3 it can be observed that the AA level increased as the baking temperature increases and the water content of biscuits decreases. The results are in accordance with the findings of Mulla et al. (2011) who showed that the AA formation is influenced mostly by the baking temperature and product moisture for extruded snacked. An increase of temperature determine a larger amount of AA. In 2016, US Food and Drug Administration (FDA) issued a guidance to provide

information regarding the reduction of AA levels in certain foods. For bakery products it is recommended to bake the products at lower temperatures and longer times.

The baking temperature should be increased in the first stage of the baking process and decreased in the last stage of baking.

Table 4. Experimental variants of biscuits obtained with flour F1 when varying the baking temperature

No.	Flour type	Variant	Baking parameters temp. (°C), time (min)	Experimental variants
1.	White	V200_20	200, 20	
2.	wheat	V220_20	220, 20	
3.	flour (F1)	V230_20	230, 20	
4.		V240_20	240, 20	

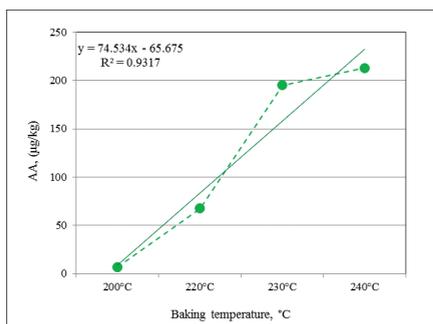


Figure 2. Variation and correlation between the AA level of biscuits and baking temperature

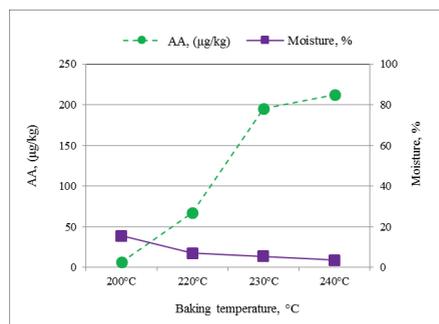


Figure 3. Variation of AA level and biscuits moisture of the experimental variants

Correlation between the AA level and the biscuits baking temperature

To establish the relationship between the AA content and the baking temperature of biscuits (200°C, 220°C, 230°C, 240°C) the regression line for the experimental variants was assigned and the equation and regression coefficient were determined (Figure 2).

From Figure 2 it can be noticed that there is a positive linear correlation between the AA level and baking temperature represented by a correlation coefficient of $R = 0.9652$. The AA content increased with baking temperature increase.

Correlation between color parameters and the AA level of biscuit samples when varying the baking temperature

The effect of color development and formation of AA in biscuits obtained at 4 different baking temperatures, 200°C, 220°C, 230°C, 240°C (20 min), with wheat flour with an ash content of 0.53% d.m. (F1) was studied.

Increasing the baking temperature leads to visible changes in both the biscuits crust color, viewed in the CIELab color parameters, L^* , a^* , b^* , and the level of AA formed. It is known that the crust color of products depends on the technological parameters, also the manufacture recipe and a higher level of acrylamide is located in the crust of bakery products (Ahrné et al., 2007; Claus et al., 2008; Della Valle et al., 2012; Przygodzka et al., 2015).

The variation of AA level and total color difference, ΔE^* , of biscuit assortments of the experimental variants obtained, represented in Figure 4, highlights that, as the AA content increases, the total color difference decreases.

To establish the relationship between the AA content and the total color difference, ΔE^* , the regression line for the experimental variants was assigned (Figure 5) and the equation and regression coefficient were determined. It was revealed that there is a negative linear correlation between the AA level and the total color difference, represented by a correlation coefficient of $R = 0.9989$.

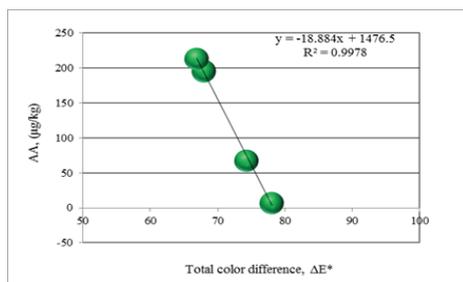


Figure 4. Variation of AA level and total color difference ΔE^* of biscuits experimental variants

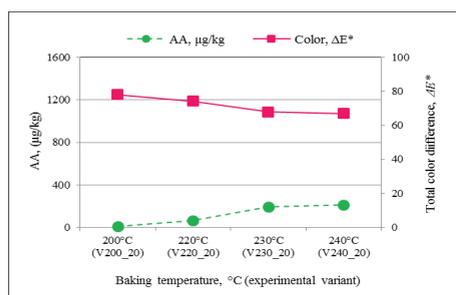


Figure 5. Correlation between AA level and total color difference ΔE^* of biscuits when varying the baking temperature

As shown in figure 4 and 5, the browning ratio is an important indicator of acrylamide concentration in biscuits, being influenced by the baking parameters.

Similar results were obtained by Gökmen et al. (2008) who shown that the surface browning and acrylamide formation in cookies followed the same kinetic pattern during baking at different temperatures (200 and 220°C) and times up to 25 min.

CONCLUSIONS

Investigating the influence of technological factors on the AA level of biscuits and similar products is important for manufacturers of bakery products in order to know the factors that lead to the formation of a high level of AA and establish the best ways to reduce this processing contaminant.

The use of white flours with a low extraction degree and a low ash content in order to obtain biscuits, caused a decrease of AA level, in regard to the use of whole-wheat flour with a high extraction degree, respectively high ash

content, resulted from the same variety of wheat.

Changing baking conditions showed that the AA level is strongly influenced by biscuits baking time and temperature. With the results obtained, it was found that the AA level is dependent of processing conditions (temperature and baking time), and increases with the increase of these parameters.

By measuring color parameters, L^* , a^* , b^* , of biscuits made of wheat flours, with different extraction degrees, it was shown that there is a linear correlation between the AA concentration and total color difference ΔE^* .

By increasing the time and baking temperature, the AA level increases and it were obtained darker products, producing low levels of total color difference ΔE^* .

In the case of biscuits obtained from flour with a high extraction degree, and high ash content, it was formed the highest AA level and the lowest values of the total color difference (dark colors of biscuits). For biscuits obtained from flours with a low extraction degree, respectively low ash content, it was formed the lowest level of AA and the highest values of the color difference (bright colors of biscuits).

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ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACTS MADE OF MUSHROOM MYCELIA DEVELOPED IN SUBMERGED CULTURE

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Abstract

The ethanolic extracts of dried biomass made of mushroom mycelium produced in the submerged cultures of eight edible/medicinal macromycete species *Ganoderma applanatum*, *Ganoderma lucidum*, *Laetiporus sulphureus*, *Flammulina velutipes*, *Trametes versicolor*, *Hericium coralloides*, *Pleurotus eryngii* 2600 and *Agaricus campestris* were evaluated for their antimicrobial activities. Pathogenic tested microorganisms were represented by five bacteria and two yeasts *B.subtilis* subsp. *spizizenii* ATCC 6633, *Staphylococcus aureus* ATCC6538, *Pseudomonas aeruginosa* ATCC9027, *Escherichia coli* ATCC 8739, *E.faecalis* ATCC 29212, *Candida albicans* ATCC 10231 and *Candida parapsilosis* CBS604. The mushroom biomass was obtained from segments detached from the mycelium culture grown on solid culture medium (PDA or MEA) placed in Erlenmeyer flasks with a liquid culture medium containing 2% malt extract (ME). After inoculation, the probes were incubated at the temperature of 25°C for 21 days. The obtained biomass was filtered and dried at the temperature of 70°C. The ethanolic extracts were prepared by adding 1 ml of 70% ethyl alcohol to 0.2 g of dried fungal biomass. Antimicrobial activities of the mushroom biomass extracts were evaluated by agar disk diffusion method. The results showed that *G. applanatum*, *L. sulphureus*, *F. velutipes*, *T. versicolor*, *H.coralloides* and *A. campestris* extracts had significant inhibitory activities especially against *B. subtilis* subsp. *spizizenii* ATCC 6633 bacterium while *G. lucidum* and *Pleurotus eryngii* 2600 extracts had no antimicrobial activity against any pathogenic microorganisms tested in this work. Further investigations will be conducted regarding the antimicrobial activity dependence on the fungal morphological part used in the extract (mycelium/fruiting body) and on the solvent type used for extracts' preparation.

Key words: Antimicrobial activity, ethanolic extracts, mushroom biomass, pathogenic microorganisms.

INTRODUCTION

The antibiotics represented a revolution in the field of medicinal sciences. The discovery and use of antibiotics during the 20th century have strongly decreased morbidity and mortality caused by bacterial infections (Chopra et al., 1997). Mathur and Singh, 2005 consider that the beginning of treating the bacterial infections using antimicrobial agents can be associated with the emerging of antimicrobial resistance in bacteria that cause diseases. This was a disadvantage because the antibiotics had to promise so much. Another study belonging to Lowy, F.D., 2003 show that in the early 1970's the idea that the large range of efficient antimicrobial agents can treat all bacterial infections faded among the physicians. This pessimistic attitude was adopted because the pathogens resistance to multiple antibiotics started to be present in the case of *S. aureus*, *P.*

aeruginosa and other bacteria (Lowy, F.D., 2003). Fischbach and Walsh, 2009 show that *S. aureus* bacteria is a violent pathogen. These authors sustain that the methicillin-resistant *S.aureus* (MRSA) causes a big mortality rate in the United States and also this bacteria is capable of enormous health care costs per year (Fischbach and Walsh, 2009). In the opinion of these authors the probability that the same deadly as MRSA vancomycin-resistant *S.aureus* (VRSA) can become a new powerful pathogen in hospitals rises depending on the MRSA increasing prevalence (Fischbach and Walsh, 2009; Linda M. Weigel et al., 2003). Fischbach and Walsh, 2009 and Falagas et al., 2005 also state that there are other pathogenic bacteria having smaller prevalence compared to methicillin-resistant *S.aureus* but being also dangerous such as: *E. coli* and *P. aeruginosa* which are resistant to penicillins, cephalosporins, carbapenems, monobactams,

quinolones, aminoglycosides, tetracyclines and polymyxins. The fungal pathogens possessing resistance to antifungal agents are also important. Different authors indicate that *C. parapsilosis* is placed on the second place after *C. albicans* in the blood samples (Trofa et al., 2008; Almirante et al., 2006; Brito et al., 2006; Colombo et al., 2007; Colombo et al., 2006; Costa-de-Oliveira et al., 2008; Fridkin et al., 2006; Krčméry et al., 2006; Messer et al., 2006; Pfaller et al., 2001; Pfaller et al., 1998; Rodero et al., 2004). *C. albicans* is a pathogenic yeast with resistance to antifungals such as miconazole and ketoconazole according to some authors (Casalnuovo et al., 2004). According to some authors the thoughtlessly use of antibiotics led to development of resistant pathogenic microorganisms (Andrade et al., 2006; Alves et al., 2014). Considering all the above studies about pathogenic bacteria and yeasts much hope is put on new antimicrobial agents. The macroscopic fungi known also as basidiomycetes represent potent sources on the fight against various pathogens antibiotic resistance. Alves et al., 2014 specify the possibility that mushroom extracts can be used both to lower the therapeutic doses of standard antibiotics and reduce microorganism's resistance to these drugs. The studies of Deepalakshmi and Mirunalini (2014) and Iwalokun et al. (2007) showed that oil extracted from *P. ostreatus* using petroleum ether and acetone inhibited the growth of Gram-positive and Gram-negative bacteria such as *E. coli*, *P. aeruginosa*, *B. subtilis*, and *S. aureus*. Moreover, methanol and chloroform extracts of *P. ostreatus* were found to have antimicrobial activity against Gram-positive bacteria (Karaman et al., 2010; Deepalakshmi and Mirunalini, 2014). Other studies revealed the antimicrobial activity of *G. lucidum* extracts against *E. coli*, *S. aureus* and *P. aeruginosa* attributed to bacteriolytic enzyme, lysozyme and acid protease. (Quereshi et al., 2010; Klaus and Miomir, 2007). Lindequist et al., 2005 and Smania et al., 1999 show that *G. applanatum* contains two steroids 5 α -ergosta-7,22-dien-3 β -ol and 5,8-epidoxy-5 α ,8 α -ergosta-6,22-dien-3 β -ol that have weak antimicrobial activity against both Gram-negative and Gram-positive pathogenic bacteria *E. coli*, *P. aeruginosa* and *S. aureus*. The study of Poucheret et al., 2006 and Wasser and Weis,

1999 also bring information about the antimicrobial activity of *G. lucidum* and *G. applanatum* mushrooms. On the other hand some authors consider that extracts of *Ganoderma* mushroom cannot be used as antibiotics because further research is needed (Gao et al., 2005). In the last years, more mushroom species are shown to have antimicrobial activity against pathogenic microorganisms. Mushrooms of *Trametes* genus contain coriolin which inhibit Gram-positive bacteria and *A. campestris* has a compound named campestrin which inhibit both Gram-positive and Gram-negative bacteria (Wasser and Weis, 1999). Other mushroom extracts, including *L. sulphureus* (Turkoglu et al., 2007) and *F. velutipes* (Poucheret et al., 2006) have already demonstrated their antimicrobial activity. Poucheret et al., 2006 states that *F. velutipes* possess antifungal activity. *L. sulphureus* was tested by Turkoglu et al., 2007 and it proved to have good antibacterial activity especially against Gram-positive bacteria such as *B. subtilis* and the ethanol extract had very good antifungal activity on *C. albicans*. In this context, the aim of our studies is to determine the antimicrobial activity of ethanolic extracts from the dry biomass (mycelia) of some mushroom species cultivated in submerged culture.

MATERIALS AND METHODS

Fungal material

The fungal material used in this experiment consisted of dry biomass of the mushroom species: *Ganoderma applanatum*, *Ganoderma lucidum*, *Laetiporus sulphureus*, *Flammulina velutipes*, *Trametes versicolor*, *Hericium coralloides*, *Pleurotus eryngii* 2600 and *Agaricus campestris*. Fungal material was provided from the collection of Faculty of Biotechnology (UASVM, Bucharest).

Microbial material

The tested microbial material was represented by: *B. subtilis* subsp. *spizizenii* ATCC 6633, *Staphylococcus aureus* ATCC6538, *Pseudomonas aeruginosa* ATCC9027, *Escherichia coli* ATCC 8739, *Enterococcus faecalis* ATCC 29212, *Candida albicans* ATCC 10231 and *Candida parapsilosis* CBS604. The

pathogenic microorganisms were provided from the Institute of Biology, Bucharest.

Mushroom biomass

Segments of 10 x 10 mm were detached from the mycelium culture grown on solid culture medium (PDA or MEA) and placed in Erlenmeyer flasks with a liquid culture medium containing 2% malt extract (ME). After inoculation, the probes were incubated at the temperature of 25°C under stirring conditions at 110 rpm for 21 days. After the incubation period, the obtained biomass was filtered and dried at the temperature of 70°C for 3 hours.

Preparation of ethanolic extracts

Extracts preparation was performed by using 1 ml of 70 % ethyl alcohol added to 0.2 g of dried fungal biomass. The alcoholic solutions were kept for 24 hours at the room temperature ($\pm 25^{\circ}\text{C}$) until use.

Determination of antimicrobial activity

Antimicrobial activities of the extracts were screened by the agar disk diffusion method. A

volume of 1 ml from each bacterial and yeasts suspensions were inoculated in Petri dishes on Luria Broth and YPG media respectively. After removing the excess suspension, sterile filter paper discs (5 mm diameter) soaked in ethyl alcohol extracts were placed on the surface of the inoculated medium. At 24 hours after the incubation at 37°C for bacteria and 30°C for yeasts, occurrence of inhibition halos around each disk was observed. Ethanol (70%) was used as negative control.

RESULTS AND DISCUSSIONS

Biomass extracts obtained from mycelia developed in submerged culture were tested against the mentioned bacterial and fungal pathogenic strains. The results showed a microbial activity in the case of *L. sulphureus* (P1), *A. campestris* (P3), *F. velutipes* (P4), *G. applanatum* (P6), *T. versicolor* (P7) and *H. coralloides* (P8) extracts (Table 1). The data relating to the antimicrobial activities of extract samples is summarized in Table 1.

Table 1. The antimicrobial activity of fungal dry biomass extracts against pathogenic microorganisms

Variant	Pathogen microorganism						
	<i>B. subtilis</i> subsp. <i>spizizenii</i> ATCC 6633	<i>S. aureus</i> ATCC6538	<i>P. aeruginosa</i> ATCC9027	<i>E.coli</i> ATCC 8739	<i>E. faecalis</i> ATCC 29212	<i>C. albicans</i> ATCC 10231	<i>C. parapsilosis</i> CBS604
P1	++	+	-	++	+	-	-
P2	-	-	-	-	-	-	-
P3	+	+	+	-	-	-	-
P4	++++	+++	+	-	-	-	-
P5	-	-	-	-	-	-	-
P6	++	-	-	-	-	-	-
P7	+	-	+	+	-	-	-
P8	+	-	+	-	-	-	-
M	-	-	-	-	-	+	+

P1. *L. sulphureus*; P2. *P. eryngii*; P3. *A. campestris*; P4. *F. velutipes*; P5. *G. lucidum*; P6. *G. applanatum*; P7. *Trametes versicolor*; P8. *H. coralloides*; M=control (70% ethyl alcohol).

Activities were classified according to the diameter of the inhibition zones around the disks containing 10µl/disk extract or control: +, <10 mm; ++, 10–15 mm; +++, 15–20 mm, +++++, >20 mm; -, without activity.

Data included in Table 1 shows that most tested mushroom extracts have antimicrobial activity against *B. subtilis* subsp. *spizizenii*. It can be noted that in the case of *L. sulphureus* the extract has medium to low inhibition on *B. subtilis* subsp. *spizizenii*, *S. aureus*, *E. coli* and *E. faecalis*. In the case of *A. campestris* extract small inhibition zone was visible in the case of *P. aeruginosa*, *B. subtilis* subsp. *spizizenii* and

S. aureus. *F. velutipes* extract had strongly inhibited the *B. subtilis* and *S. aureus* bacteria developing the largest inhibition halos. *G. applanatum* extract had moderate inhibitory effect against *B. subtilis* subsp. *spizizenii*, while *T. versicolor* and *H. coralloides* extracts had low inhibitory effect against *P. aeruginosa*. The extracts–pathogens inhibition effect is shown in Figure 1.

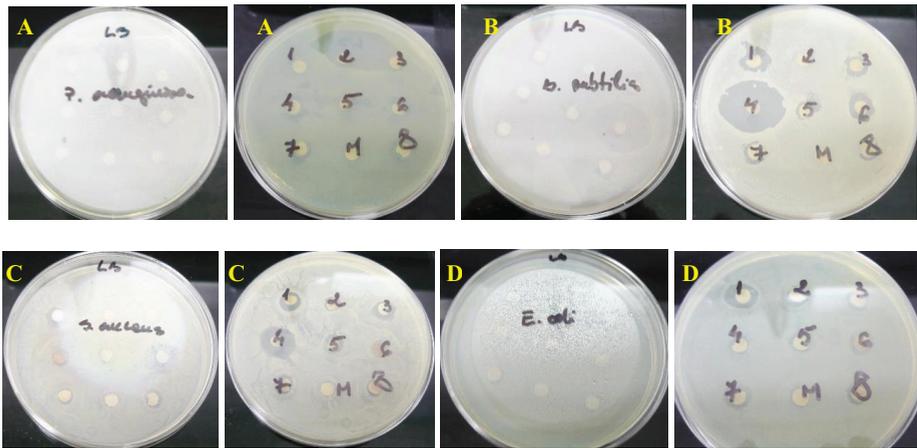


Figure 1. Antimicrobial activity of mushroom ethanolic extracts

A. *P. aeruginosa*; B. *B. subtilis*; C. *S. aureus*; D. *E. coli*

1. *L. sulphureus*; 2. *P. eryngii*; 3. *A. campestris*; 4. *F. velutipes*; 5. *G. lucidum*; 6. *G. applanatum*; 7. *Trametes versicolor*; 8. *H. coralloides*; M-martor (ethanol 70%)

The ethanolic extract of the *G. lucidum* and *P. eryngii* strains used in this study haven't shown any antimicrobial activity against the tested pathogen.

However, there are positive reports regarding the antimicrobial activity of *G. lucidum* mycelium tested by agar-well diffusion method.

For the preparation of dry biomass extracts methanol, acetone, chloroform and distilled water were used as solvents (Dijde et al., 2014; Kamble et al, 2011). Kamble et al, 2011 show that the extracts had good inhibitory effect at a concentration of 100 mg extract/ml distilled water on some pathogenic bacteria such as: *S. aureus*, *B. subtilis*, *E. coli*.

In the case of *P. eryngii* species the antimicrobial activity was tested on some pathogens such as: *S. aureus* COWAN 1, *E. coli* ATCC 25922, *C. albicans* FMC 17 by disk diffusion method with methanol extracts (Akyüz and Kirbag, 2009). Akyüz and Kirbag, 2009 show that the extracts of *P. eryngii* inhibited the growth of test microorganisms in various proportions. Meanwhile, in our study, none of the tested macromycete extracts showed any antimicrobial activity in interaction with pathogenic yeast species *C. albicans* ATCC 10231 and *C. parapsilosis* CBS604. In these cases, the results of extracts–pathogens interactions were not conclusive because the ethanol used as control had inhibitory effects.

CONCLUSIONS

The results showed that *G. applanatum*, *L. sulphureus*, *F. velutipes*, *T. versicolor*, *H. coralloides* and *A. campestris* extracts had medium to high inhibitory activities especially against *B. subtilis subsp. spizizenii* bacterium. In our study, none of the tested mushroom extracts had any antimicrobial activity in interaction with pathogenic yeast species *C. albicans* ATCC 10231 and *C. parapsilosis* CBS604. In this case, for better information about the mushroom antimicrobial activities more studies and experiments concerning the type of the fungal material (mycelium/fruited body) and the solvents used for extracts preparation are needed.

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NUTRIENT COMPOSITION OF PARTIALLY DEFATTED MILK THISTLE SEEDS

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Abstract

Milk thistle (Silybum marianum) is an annual or biennial plant of the Asteraceae family that and usually grows in dry, sunny areas in Romania, but throughout in the world. The milk thistle seeds have been used since ancient times to treat a large variety of liver and gallbladder disorders. Theophrastus (IV century B.D.) and Plinius (1st century A.D.) were the first to report the medicinal benefits of this plant. All parts of the plant can be used, but the milk thistle seeds are considered to be the most medicinally potent for therapeutic use. The seeds and extracts of the milk thistle plant are a well established herbal food for protecting, detoxifying and regenerating the liver, one of the most important organs of the human body.

We explored the physico-chemical properties as well the amino acids content of the milk thistle partially defatted seeds. The obtained results revealed that partially defatted milk thistle seeds are a good source of protein (20.35%), lipids (11.69%), total carbohydrates (38.16%) from which crude fiber (27.24%).

This by-product presents a high mineral content (mg/100g): calcium (912), magnesium (433), iron (80,5), zinc (7,38) and copper (2,69).

The partially defatted milk thistle seeds protein contained markedly amounts of essential amino acids such as arginine, leucine valine and lysine.

Key words: Milk thistle, Milk thistle seed oil, Protein, Amino Acids, Crude fiber,

INTRODUCTION

The milk thistle plant, *Silybum marianum* (family: *Astraceae*) is an annual or biennial plant, native to the Mediterranean area and some parts of the United States, which has now spread to other warm and dry regions (Hadolin M, et al. 2001).

The Asteraceae are one of the largest plant families, with more than 1600 genera and about 23,000 species, most of them existing in temperate regions (Jeffrey, 2007).

The herbalist John Gerard (1545–1612), author of the herbal *Generall Historie of Plantes* wrote, "My opinion is that milk thistle is the best remedy that grows against all melancholy diseases."

Several studies reveal the important role played by unconventional species, as excellent sources of macro and micronutrients in contributing to human dietary requirements.

Extracts from the mature milk thistle seeds are used as medical remedies for liver disease, liver cirrhosis and to prevent liver cancer (Angeles et al, 2005; Ramasamy K et al., 2008).



Fig. 1. Milk thistle flower

The milk thistle contains silymarin, which is composed of the flavanolignans silybin,

silydianin and silychristine, with silybin being the most biologically active. Silymarin is found in the highest concentrations in the fruit portion of the plant but is also found in the leaves and seeds. The seeds also contain betaine, trimethylglycine and essential fatty acids, which may contribute to silymarin's hepatoprotective and anti-inflammatory effects (Ramasamy K et al., 2008).

The seeds have numerous health beneficial components such as protein, with valuable content of essential amino acids, carbohydrates (especially crude fibres), minerals and some phytochemicals that have antioxidants or antimicrobial properties (Parry et al., 2008).

Therefore, the relevance of studying this traditionally consumed plant is an important strategy to improve the diversity of available foods, which today is receiving the focus of renewed attention.

The aim of the present study was to determine the nutritional composition of partially defatted milk thistle seeds, a byproduct during the manufacturing of the milk thistle seeds oil.

The milk thistle seeds' proteins have a good potential to be used as a valuable source of protein in nutrition.

In their study, Mahmoud A. El-haak et al. (2015) concluded that the whole milk thistle seeds contain high amounts of protein, lipids and total carbohydrates including crude fibers which could be used as a novel source of plant protein, oil and crude fibers. Also, it could be utilized as a suitable food ingredient in low fiber containing food.

Currently there are few works that reveal valuable potential of partially defatted milk thistle seed flour.

Therefore, the main objective of this study was to evaluate the content of valuable compounds from partially defatted milk thistle seeds flour, for use in food industry.

Adding partially defatted milk thistle seeds flour in food products improves the dietary intake of most micronutrients and fiber.

Daily consumption of these products is recommended in order to help preventing major non-communicable diseases such as cardiovascular diseases and certain cancers (OMS, 2003). Therefore, evaluation of the minerals and other chemical elements contained in food are important.

MATERIALS AND METHODS

Partially defatted milk thistle seeds, a byproduct during manufacture of the milk thistle seeds oil, was kindly supplied by SC Hofigal Export Import SA, (Bucharest, Romania).

This meal has been obtained from milk thistle (*Silybum marianum*) seeds on a large scale through dehulling, grinding and degreasing at low temperatures of less than 40 °C. The degree of damage to the components of this material may be considered to be low because all steps were performed at low temperature.

Moisture was determined at 103 °C (± 2 °C) until constant weight (ICC Standard No. 110/1).

Total fat was determined by extracting 10 g of sample with petroleum ether 40-65°C, using a Soxhlet apparatus.



Fig. 2. Milk thistle seeds

Total nitrogen was analysed following Kjeldahl method (official method no 950.36). Ash content (official method no 930.22) in muffle furnace at 450 - 500°C. Crude protein content was calculated by multiplying total nitrogen content by the factor 6.25. Crude fibers of samples were determined using a Fibretherm-Gerhardt apparatus.

Carbohydrate contents were calculated as the difference of 100 - (ash + protein + fat + moisture).

The extracted oil used for fatty acid profile evaluation, was determined according to Mahmoud A. El-haak et al. (2015). Ground seeds (approx. 10 g) were processed with 30 ml hexane/isopropanol (3:2, v/v) at room temperature under vigorous shaking for 1 hour in steel tubes containing four steel balls to facilitate homogenization. Ten tubes, each of them containing 10 g ground seeds, were used to extract oil from 100 g ground seeds. After 1

h shaking, the extracts were filtered through defatted filter papers with a Buchner funnel under vacuum, the remained defatted cake was also washed twice with 20 ml of the same solvent to extract all the possible residual oil content. Thereafter 35 ml of 6.7% sodium sulfate was added to the oil-containing solvent and thoroughly mixed. The upper organic solvent layers containing the oil were then separated and rotary-evaporated under reduced pressure at 35 °C. The extracted oil was stored at -20 °C for further analysis.

Fatty Acid profile

Using ¹H-NMR spectral technique, the fatty acids composition was determined, especially the concentrations of short-chain saturated fatty acids (C4-C8), di-unsaturated fatty acids, mono-unsaturated fatty acids and long-chain saturated fatty acids (>C8). ¹H-NMR spectra were recorded on a Bruker Ascend 400 MHz spectrometer, operating at 9.4 Tesla corresponding to the resonance frequency of 400.13 MHz for the ¹H nucleus. Samples were analyzed in 5 mm NMR tubes (Wilmad 507). The NMR samples were prepared by dissolving 0.2 mL oil in 0.8 mL CDCl₃. The chemical shifts are reported in ppm, using the TMS as internal standard.

The mineral contents were determined with inductively coupled plasma-mass spectrometer equipment (ICP-MS; Perkin Elmer NexION 300Q).

Quantity was performed using external standards (Merck, multi element standard solution) and all the standard curves were obtained at 6 different concentrations. Total mineral content was measured using their most abundant isotopes.

The dried samples were digested in a mixture of concentrated HCl. For the analysis of the amino acids content, samples were hydrolyzed at 100–120 °C in 6N hydrochloric acid for 22–24 hours under vacuum.

After evaporation to dryness of hydrochloric acid, dry residue was diluted using 4 mM stock solution of Norleucine (diluted 500 x with a deionized water solution containing 20 mg/L NaN₃). For the separation of amino acids by gradient anion exchange with pulsed electrochemical detection (PED) was used an ICS300 (Dionex-USA) equipment with the following eluents: deionized water, 0.250 M Sodium hydroxide and 1M Sodium Acetate.

Statistical analysis

All the measurements were performed at least in triplicate. The values of different parameters were expressed as the mean ± standard deviation (s_r).

RESULTS AND DISCUSSION

Chemical composition of partially defatted milk thistle seeds flour is shown in table 1 and display that this byproduct is a rich source of protein, lipids and crude fibers, since protein, lipids and crude fibers contents are 20.35%, 11.69%, and 27.24%, respectively. Ash is 10.79% and total carbohydrate is 57.17%.



Fig. 3. Partially defatted milk thistle seeds flour

These data confirm that partially defatted milk thistle seed flour is a good source of bio-compounds, especially crude fibers (27.24%, d.m.).

Table 1. Chemical composition of Partially defatted Milk thistle seeds (g/100 g based on dry weight bases)

Constitutes	Defatted milk thistle seed flour
Total lipids	11,69 ± 0.24
Ash	10.79 ± 0.11
Crude fibers	27.24 ± 0.45
Total protein (N x 6.25)	20.35 ± 0.24
Total carbohydrates	57,17 ± 0.05

M ± SD = means and standard division of triplicate trails.

Partially defatted milk thistle seed should be considered a source of interesting added value carbohydrate compounds with potential known prebiotic properties, useful to formulate functional foods as well as nutraceuticals.

In the present study, the contents of four biologically essential mineral elements were analysed: calcium (Ca), magnesium (Mg), iron (Fe) and manganese (Mg) and two essential trace elements: zinc (Zn) and copper (Cu).

Table 2. Minerals contents of defatted milk thistle seed flour (mg /100 g)

Constitutes	Defatted milk thistle seed flour	RDI (FDA 2011)
Calcium	912 ± 2.19	1000
Potassium	790 ± 1.65	4700
Magnesium	433 ± 2,20	400
Sodium	11.2 ± 2.6	2400
Iron	80.5 ± 1.85	18
Manganese	7.97 ± 1.91	400
Zinc	7.38 ± 1.70	15
Copper	2.69 ± 0.54	2

From performed analyses regarding minerals content it can be observed that partially defatted milk thistle seeds represent a material having important minerals content, 100 g assuring the daily intake for some of these elements according to The Reference Daily Intake (RDI) of macronutrients and micronutrients recommended by the FDA (2011).

The fatty acids profile of samples is presented in table 3.

Table 3. Fatty acids content of defatted milk thistle seed flour (g/100 g)

Constitutes	Defatted milk thistle seed flour
Short-chain saturated fatty acids	26,53
Mono-unsaturated fatty acids	33,47
Di-unsaturated	40,08
Poli-unsaturated	0

The amino acids composition of partially defatted milk thistle seeds is given in table 4.

The results show that partially defatted milk thistle seeds contained high amounts of all essential amino acids as arginine (12.59%), leucine (9.84%), valine (7.97%), lisine (7.38%). However, partially defatted milk thistle protein has a slightly smaller source of methionine (2.46%) and cysteine (2.16%).

Essential amino acids are very important for health since they are building blocks of

proteins, which carried functions of the human body (Zho, 2007).

Leucine has beneficial effects for skin, bone and tissue wound healing and promotes growth hormone synthesis.

Table 4. Amino acids composition of partially defatted milk thistle seeds flour (as g amino acid/100 g protein).

Type	Amino acids	Partially defatted milk thistle seed
Indispensable amino acids	Arginine	12.59
	Leucine	9.84
	Valine	7.97
	Lysine	7.38
	Phenylalanine	6.10
	Isoleucine	5.41
	Threonine	5.12
	Histidine	3.35
Dispensable amino acids	Glutamic	29.62
	Glycine	8.27
	Serine	7.67
	Proline	7.28
	Aspartic	6.91
	Alanine	6.69
	Tyrosin	5.41
	Methionine	2.46
Cysteine	2.16	

Lysine and valine are essential for muscle proteins. Tyrosine is of foremost importance for dopamine, norepinephrine and adrenalin synthesis. Isoleucine is necessary for the synthesis of hemoglobin in red blood cells. Phenylalanine may be useful against depression and suppressing appetite.

Regarding the nonessential amino acids content there are large amounts of glutamic acid

(29.62%) glycine (8.27%), serine (7.67%) and proline (7.28%).

In short, studies of the food potential of partially defatted milk thistle seeds are not yet available in the scientific literature. Taking into account that consumers are more and more aware about the food quality, especially from the nutritive point of view, the new food resources rich in bioactive compounds are necessary to be found. In this respect, partially defatted milk thistle seeds meet the expectations of such consumers.

CONCLUSIONS

The aim of this study was to evaluate the functional potential of partially defatted milk thistle seeds flour in order to be used for getting food rich in valuable biocompounds.

The chemical composition of the partially defatted milk thistle seeds flour using both classical and spectral methods was analyzed.

Increasing the content of partially defatted milk thistle flour in various food products lead to the increase in dietary fibers, minerals, protein, ash, amino acids and total fat content.

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INDUSTRIAL AND ENVIRONMENTAL BIOTECHNOLOGY

INFLUENCE OF CULTURE CONDITIONS ON GROWTH OF KERATINOPHILIC FUNGAL STRAINS

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Abstract

The aim of this study was to investigate the influence of culture conditions on growth of keratinophilic fungal strain of *Chrysosporium* sp. in the presence or absence of keratin substrate. The effect of pH, temperature, carbon and nitrogen sources on fungal growth and sporulation was evaluated. The pH values ranged from 4 to 9.5 and the incubation temperature ranged from 20°C to 35°C. Glucose, fructose, maltose, sucrose, starch and cellulose were used as carbon sources. As nitrogen source, yeast extract, ammonium salts, urea and vitamin B12 were tested. All tests were also performed with basal mineral culture medium supplemented with keratin powder from chicken feathers. The feathers were cleaned with ethylic alcohol, washed with distilled water, dried at 60°C and finally grounded several times with a Retsch ball mill until a fine powder was obtained. The influence of the culture conditions on growth was assessed by measuring the diameter of the colonies grown on the solid medium after 5 and 10 days of incubation. The colony sporulation degree was appreciated macroscopically and microscopically. The presence of keratin in the culture media stimulated distinctly the fungal growth as compared to the culture media without keratin. Alkaline pH and temperatures between 27 and 30°C are optimal for its growth. Certain C and N sources can stimulate the fungal growth, but this seems to be influenced by the incubation time.

Key words: fungal growth, keratin, keratinophilic fungi.

INTRODUCTION

Biodegradative properties are widespread in the living world, from bacteria (Kumar and Takagi, 1999; Korkmaz et al., 2004; Moniruzzaman et al., 2007), actinomycetes (Laba and Rodziewicz, 2010; Jayalakshni et al., 2011) to fungi (Mushin et al., 1997; Mushin and Aubaid, 2000; Riffel and Brandell, 2006; Singh, 2011). A number of fungi can use the hard biodegradable materials such as keratin as substrate, due to their enzymatic equipment. These fungi are able to use the keratinaceous substrate as unique source of carbon and nitrogen and play an important role on biodegradation of keratin waste in the environment (Kunert, 2000; Moallaei et al., 2006; Sharma et al., 2011). The main types of fungal genera with keratinolytic properties are: *Alternaria*, *Aspergillus* (Kim, 2003; Ali et al., 2011), *Chrysosporium* (Singh, 2002),

Cladosporium, *Curvularia*, *Fusarium*, *Myrothecium*, *Paecilomyces*, *Penicillium*, *Scopulariopsis*, *Sepedonium*, *Stachybotrys*, *Ulocladium*, dermatophyte fungi (Gupta and Ramnani, 2006; Monod, 2008; Saber et al., 2010). Members of the keratinolytic fungi group are found in soil (geophilic) as decomposers of the keratin materials (hair, claws, feathers, horns etc.) (Kanaahi and Ancy, 2012). The sources of keratin are numerous, such as feathers, wool, horns, hair etc. Keratin results in large amounts from the meat industry and accumulates in the environment due to its high stability, becoming a source of contamination and environmental pollution (Balakumar et al., 2013; Sharma and Gupta, 2016; Kumawat et al., 2016). Traditional methods of keratin degradation are expensive, consume large amounts of energy and can destroy some essential amino acids like methionine and lysine. Therefore

biodegradation of keratin waste and conversion of some wastes from food industry into accessible animal feed has a biotechnological importance (Lange et al., 2016).

Environmental factors have a significant role on the growth of keratinophilic fungi and influence the keratinase activity of keratinolytic fungi (Kadhim et al., 2015; Sharma and Sharma, 2009; Sharma et al., 2012).

Therefore, the aim of this study was to investigate the influence of culture conditions on growth of keratinophilic fungi in the presence or absence of keratin substrate.

MATERIALS AND METHODS

Fungal strains

The tests were carried out with *Chrysosporium* sp. isolated from farm soil (Figure 1) by Vanbreuseghem hair bait technique. The tested strain was grown and maintained on potato dextrose agar (PDA) slants at 4°C.



Soil rich in keratin materials

Collecting soil samples

Figure 1. Soil sampling

Vanbreuseghem hair bait technique. This technique consists in placing collected soil samples in sterile Petri plates. Soil samples rich in keratinic material were collected from several areas in sterile containers using sterile tools and tagged appropriately. The samples were stored at 4°C until their processing in the laboratory. Over the soil samples few sterile hair strands were added. To prevent the soil from drying out, the Petri plate was placed in other Petri plate and about 10 ml of sterile distilled water was added, forming a wet room. The Petri plates were incubated at room temperature for 4 weeks and observed daily until growth of fungal mycelium was observed on the surface of hair strands.

The hair strands covered with fungal mycelium were cultivated on culture media and mixed

cultures followed by pure culture were obtained (Figure 2).



Figure 2. Steps for fungal strain isolation by Vanbreuseghem hair bait method

Keratin substrate: keratin powder

The keratin powder was obtained as follows: chicken feathers were cleaned, sterilized with 3% ethanol, washed and dried at 60°C. They were then cut into small pieces and grounded several times with a Retsch ball mill until a fine powder was obtained.

Conditions of fungal cultivation

A mineral culture medium with a specific formula served as control in all experiments and was used as basal culture medium (B.C.M.) in the assays with different carbon (C) and nitrogen (N) sources. The basal culture medium had the following composition (g/L): 0.1, KH_2PO_4 ; 0.1, CaCl_2 ; 0.1, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 0.005, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 15, agar, pH 7.0, autoclaved at 121°C for 15 min. The effect of pH, temperature, carbon and nitrogen sources on fungal growth and sporulation was evaluated. The basal culture medium was supplement with 1% of each carbon and nitrogen source, respectively (Balakumar et al., 2013). The carbon sources used in the experiment were: glucose, fructose, maltose, sucrose, starch and cellulose.

The nitrogen sources used: ammonium salts (potassium nitrate, potassium sulphate,

potassium phosphate), yeast extract, urea and vitamin B12.

To test the influence of pH on fungal colony development culture media (basal culture medium with or without keratin) with the following pH values were used: pH 4; pH 4.5; pH 5; pH 5.5; pH 6; pH 6.5; pH 7; pH 7.5; pH 8; pH 8.5; pH 9 and pH 9.5.

The incubation temperatures for the plates with the basal culture medium ranged from 20°C to 35°C. Different variants of the culture medium were used, namely: basal culture medium with or without keratin powder, basal culture medium supplemented with C or N source and basal culture medium supplemented with C or N source and keratin powder. The medium was inoculated with 10 µl of fungal suspension. The plates were incubated at 27°C for 10 days. The influence of the culture conditions on growth was assessed by measuring the diameter of the colonies grown on the solid medium after 5 and 10 days of incubation. The colony sporulation degree was appreciated macroscopically and microscopically. Assays were performed in triplicate, using three-compartment Petri plates, with approximately 5 ml of culture medium in each compartment. All tests were performed in the ICECHIM laboratories.

RESULTS AND DISCUSSIONS

The isolated fungal strain was identified by macroscopic and microscopic examination as *Chrysosporium* sp. Macroscopically this strain showed a moderate growth, was flat, white to light beige in average colour, and a powdery surface texture. Microscopically it produced hyaline, smooth, one-celled pyriform to clavate conidia. These features are characteristic for *Chrysosporium* genus.

For *Chrysosporium* sp., carbon sources stimulated the fungal growth to a certain degree compared with the control, when measured after five days.

As can be seen in Figure 3, after 10 days of incubation on solid culture media on Petri plates, a better growth was observed in the presence of different C sources.

However, the presence of keratin powder stimulated the fungal growth (Figure 3 and 4) and sporulation compared to C sources which

did not stimulate. The average diameter of the fungal colony was 2.6 cm and 2 cm after 10 days of incubation, in the presence and absence of keratin powder, respectively (Figure 4).

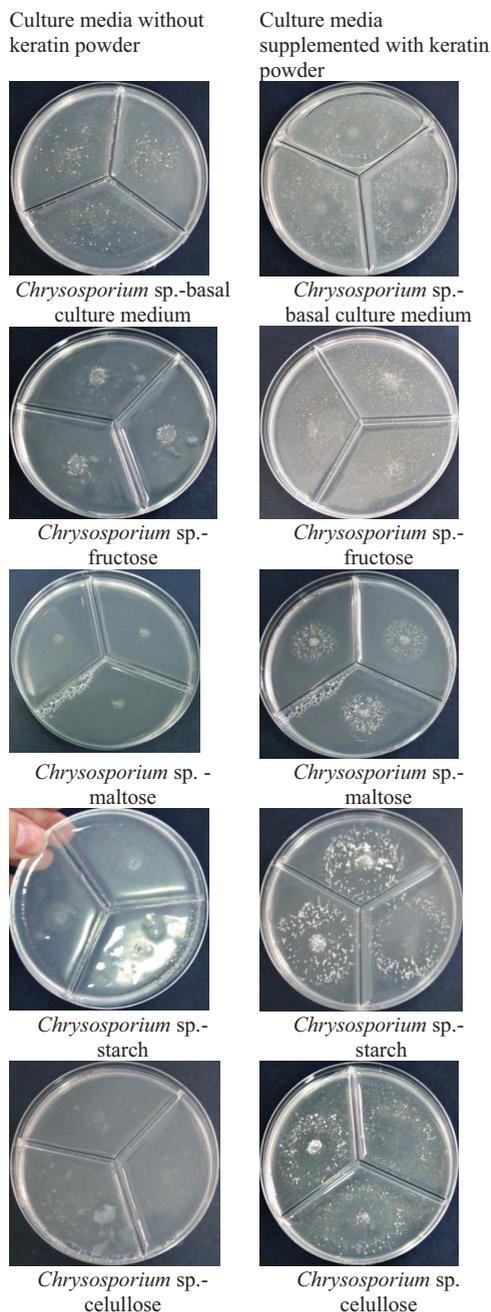


Figure 3. Carbon source (10 days of incubation)

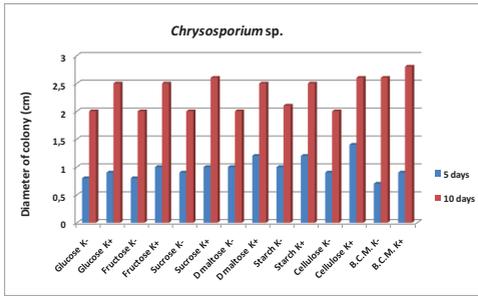


Figure 4. Effect of carbon source on fungal growth (10 days of incubation)

In the experiment with different sources of nitrogen, the addition of urea induced the largest growth after 5 days, while addition of yeast extract resulted in the largest growth after 10 days (Figures 5 and 6).

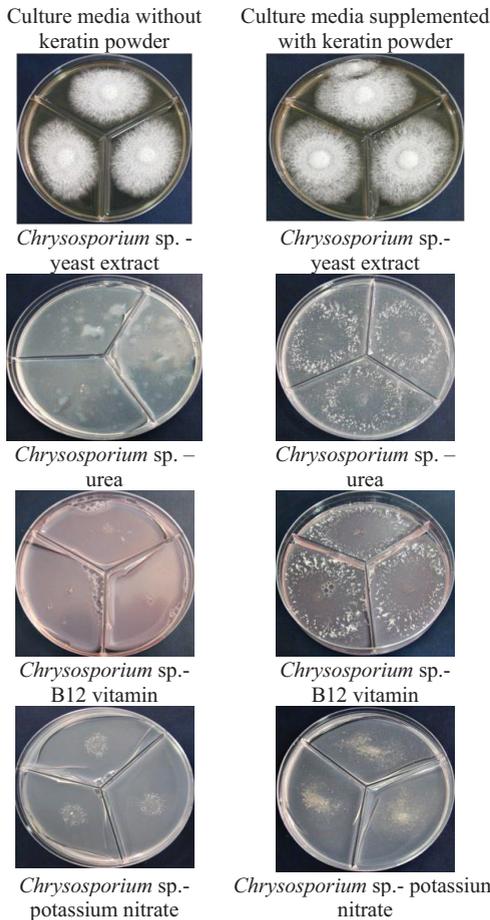


Figure 5. Nitrogen source (10 days of incubation)

Again, the presence of keratin had a positive effect on fungal growth. The average diameter of the fungal colony was 3.5 cm and 3 cm after 10 days of incubation, in the presence and absence of keratin powder, respectively (Figure 6).

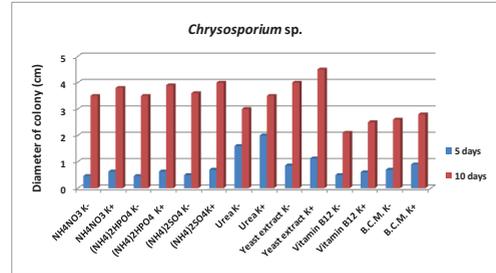
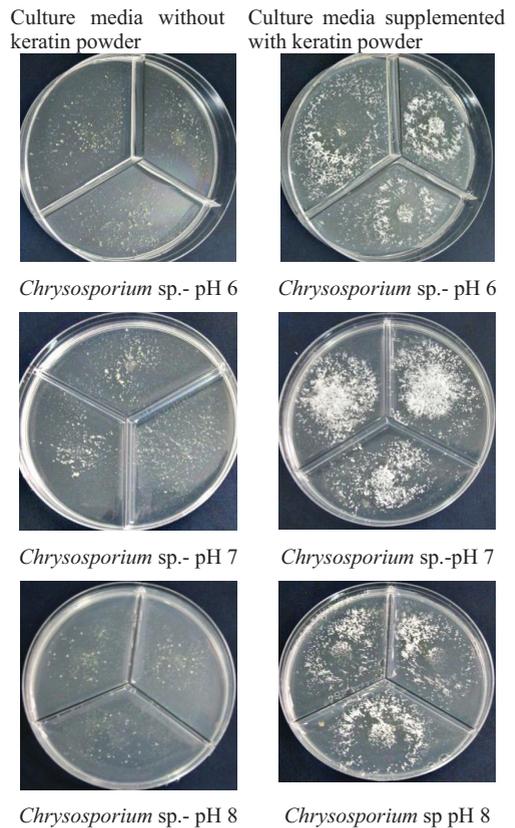


Figure 6. Effect of nitrogen source on fungal growth (10 days of incubation)

Chrysosporium sp. developed better at alkaline pH (Figure 7).





Chrysosporium sp.-pH 9 *Chrysosporium* sp. pH 9

Figure 7. Influence of pH value (10 days of incubation)

The presence of keratin powder in the culture medium positively influenced the growth of *Chrysosporium* sp. strain (Figure 8).

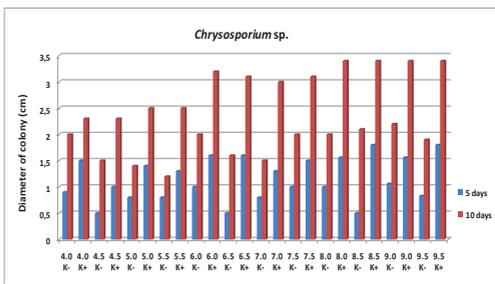
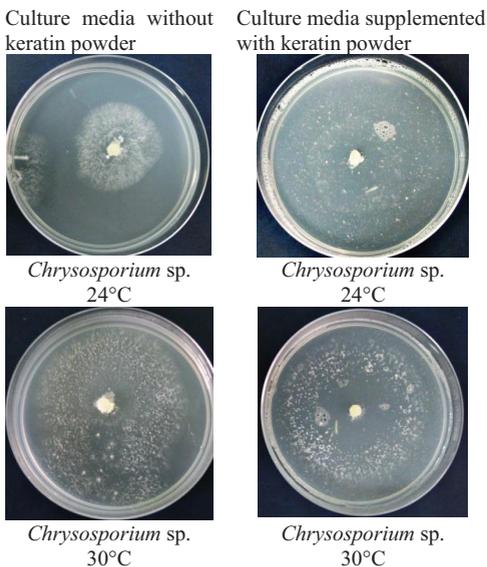


Figure 8. Effect of pH values on fungal growth (10 days of incubation)

Keratin had a positive effect, especially at 27°C-30°C (Figure 9 and 10).



Chrysosporium sp.
24°C

Chrysosporium sp.
24°C

Chrysosporium sp.
30°C

Chrysosporium sp.
30°C

Figure 9. Influence of incubation temperature value (10 days of incubation)

The optimum growth temperature was in the range 27-30°C (Figure 10). Our results are similar to those reported by other researchers (Sharma et. al, 2016).

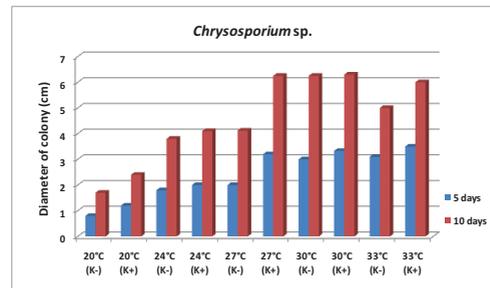


Figure 10. Effect of temperature on fungal growth

CONCLUSIONS

The presence of keratin in the culture media stimulates the growth of *Chrysosporium* sp. as compared to the culture media without keratin, but the degree of stimulation also depends on other factors, such as temperature, pH and the presence or absence of various C and N sources. Alkaline pH and temperatures between 27 and 30°C are optimal for its growth.

Certain C and N sources can stimulate the fungal growth, but this seems to be influenced by the incubation time. More studies are needed to understand this behaviour.

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EVALUATION OF *Trichoderma* spp. AS A BIOCONTROL AGENT AGAINST *Phytophthora parasitica*

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Abstract

The genus *Phytophthora* causes great damages to agricultural production, especially to potatoes and tomatoes cultures. To face these losses, it is of interest to reduce or inhibit the activity of this aggressive pathogen. Some species of *Trichoderma* have great potential for the biological control of several plant pathogens, including diseases caused by *Phytophthora parasitica*, *Rhizoctonia solani*, *Pythium ultimum*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum* etc. The purpose of this study was to assess the biocontrol efficacy of three *Trichoderma* strains (*T. asperellum* T36, *T. asperellum* T50, *T. harzianum* T78) against *Phytophthora parasitica*. In vitro tests were carried out using dual culture technique. In vivo tests were carried out with pepper seedlings (*Capsicum annuum* cv. Lamuyo) and conidial standard suspension of *Trichoderma* isolates as biocontrol agents. Of the three *Trichoderma* isolates tested for their effectiveness against mycelial growth of pathogen, *T. asperellum* T36 (81.2%) exhibited maximum inhibition of *P. parasitica*, compared with the control, followed by *T. asperellum* T50 (79.6%) and *T. harzianum* T78 (77.7%). Likewise for the in vivo test, the inoculation of the *Trichoderma* biocontrol agent showed that the percentage of dead plants seedlings was significant reduced. *T. asperellum* T36 is a useful biological alternative to pesticides for the control of *P. parasitica* in pepper seedlings.

Key words: biocontrol, *Phytophthora parasitica*, *Trichoderma*.

INTRODUCTION

The genus *Phytophthora* is classified as oomycetes and is an important plants pathogen. *Phytophthora* spp. has a severe economic impact on agriculture, the induced economic losses being approximately 170 billion US dollars (Haverkort et al., 2009; Wu et al., 2012; Fatima et al., 2015). *Phytophthora* spp. grows through the root and the stem system of plant, destroying it by, causing root and stem rot. The plants diseases were primarily managed with fungicide applications but the phytopathogens have developed resistance.

For the management of fungal crop diseases another effective way is the biological control. Some microorganisms have the ability to antagonize pathogens (Fatima et al., 2015). The genus *Trichoderma* is known as a biocontrol agent (BCAs) and can suppress diseases via several mechanisms including antibiosis, competence, mycoparasitism, enzyme activity,

induced plant defence, (Papavizas and Lumsden, 1980; Howell, 2003).

In this study we evaluated the biocontrol potential of three *Trichoderma* strains, *T. asperellum* T36, *T. asperellum* T50, *T. harzianum* T78 against *Phytophthora parasitica*.

MATERIALS AND METHODS

Fungal isolates

The fungal virulent pathogen *Phytophthora parasitica* belongs to Culture Collections of CEBAS-CSIC Institute, Murcia, Spain. The strain was isolated from pepper plants showing disease symptoms and was maintained on pea agar medium at 28°C for 7 days.

The biological control agents (BCAs), *Trichoderma asperellum* T36, *Trichoderma asperellum* T50 belong to Culture Collections of ICECHIM Institute, Romania. Both strains were isolated from soil. *Trichoderma harzianum* T78 was obtained from Culture Collections of CEBAS-CSIC Institute.

The antagonistic strains were grown and maintained on potato dextrose agar medium (PDA) at 26 to 28°C for 7 days.

Dual culture technique

The antagonistic activity of *Trichoderma* strains against *P. parasitica* was evaluated by dual culture method described by Edington et al., 1971.

Plates of rye agar medium were inoculated with a 5 mm disc from five-day-old cultures of each *Trichoderma* strains which was placed 2 cm away from the periphery of the plate. Same size agar disc of *P. parasitica* was placed at the opposite side of *Trichoderma* sample, 2 cm away from the periphery of the plate. The control contains only the fungal phytopathogen. The cultures were incubated at 28 °C in darkness. After 5 days of incubation the growth of the fungi was recorded by measuring the radial growth of the pathogens. The inhibition percentage was calculated in relation with the control by using the formula:

$$I (\%) = (C-T) / C \times 100$$

where,

I = percent of inhibition; C = diameter of radial growth of *P. parasitica* in control; T = diameter of radial growth of *P. parasitica* in the presence of antagonistic strains.

Greenhouse experiment

In this study four treatments were formulated: T1 - compost, black peat and *T. harzianum* T78 (10^6 ufc g⁻¹); T2 - compost, black peat and *T. asperellum* T36 (10^6 ufc g⁻¹); T3 - Compost and black peat; T4 - Black peat.

Seeds of pepper (*Capsicum annum* cv. Lamuyo) were sterilized in 10% NaClO for 3 minutes and rinsed for three times in sterile water. The pepper seeds were planted in 150 pots, with one seed per pot (Figure 1).



Figure 1. The pepper seedlings in growth chamber

For each treatment forty-eight pots were used: 18 pots were used as control (without pathogen) and thirty pots were inoculated with *P. parasitica*. All plants were incubated in growth chamber under daylight conditions. After 14 days of sowing the seedlings were inoculated with *P. parasitica* (10^5 ufc g⁻¹, in all treatments). The seedlings were harvested four weeks after sowing. The number of infected plants was recorded every day.

RESULTS AND DISCUSSIONS

In vitro growth inhibition of *P. parasitica* by *Trichoderma* strains

The antagonism of *Trichoderma* strains against *P. parasitica* was observed in dual culture.

Figure 2 and Table 1 show the inhibition of *P. parasitica* by the *Trichoderma* strains. The results of dual culture demonstrated that *T. asperellum* T36 determined the maximum growth inhibition of *P. parasitica* (81.2%), followed by *T. asperellum* T50 (79.6%) and *T. harzianum* T78 (77.7%) (Table 1).

Table 1. Inhibitory effects of *Trichoderma* isolates against *P. parasitica*

Antagonistic isolates	Inhibition in dual culture (%)
<i>T. asperellum</i> T36	81.2
<i>T. asperellum</i> T50	79.6
<i>T. harzianum</i> T78	77.7

Inhibition of *Phytophthora* growth by *Trichoderma* spp. was also reported previously by (Fatima et al., 2015; Jiang et al., 2016).

Greenhouse trial

The results of the greenhouse experiment showed that the inoculation of *Trichoderma* strains in the growing media was an effective treatment to control *P. parasitica* in pepper seedlings.

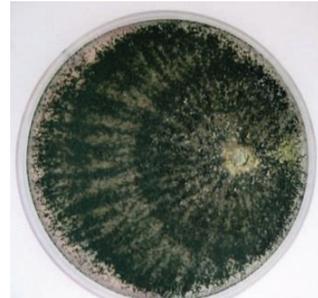
Figure 3 demonstrated that the treatment T2 (fortified with *T. asperellum* T36) showed the lowest percentage of dead pepper seedlings induced by *P. parasitica* (54%), followed by treatment T1, T3 and T4 (66%, 80% and 92.66%, respectively).



Control - *P. parasitica* alone



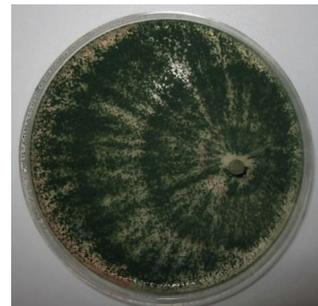
Dual plate culture of *T. asperellum* T36 and *P. parasitica*



T. asperellum T36 (control)



Dual plate culture of *T. asperellum* T50 and *P. parasitica*



T. asperellum T50 (control)



Dual plate culture of *T. harzianum* T78 and *P. parasitica*



T. harzianum T78 (control)

Figure 2. Antagonistic test between *Trichoderma* strains and *Phytophthora parasitica*

T. asperellum T36 (treatment T2) was more effective than *T. harzianum* T78 (treatment T1) in reducing the percentage of dead pepper seedlings.

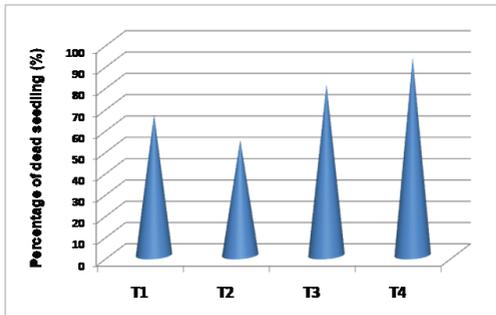


Figure 3. Percentage of dead pepper seedlings by *P. parasitica*

Similar results were reported previously by (Mpika et al., 2009; Segarra et al., 2013). Figure 4 shows the survival of a pepper seedling after harvesting (a) and a dead pepper seedling with stem rot (b).



a) Pepper seedling survival



b) Dead pepper seedling (stem destroyed)

Figure 4. Pepper seedling after harvesting

CONCLUSIONS

All three isolates of *Trichoderma* demonstrated biological control activity against *P. parasitica*. In the growth chamber experiment, the disease severity was reduced by *T. asperellum* T36 and

T. harzianum T78. Treatment T2 with *T. asperellum* T36 was the most effective treatment to control *P. parasitica* in pepper seedlings.

ACKNOWLEDGEMENTS

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PRELIMINARY STUDIES ON YEAST-PLANT SYSTEMS WITH APPLICATIONS IN PHYTOREMEDIATION

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Abstract

Phytoremediation represents an ecological and economic alternative for remediation of polluted environments. The combination of plants and xenodegrading microorganisms is often used for the improvement of the remediation process. Yeast strains *Yarrowia lipolytica* CMGB32, *Cryptococcus curvatus* YR-P2 and *Rhodotorula glutinis* RG5 were tested for assimilation of petroleum and *n*-hexadecane and biosurfactant synthesis. *Y. lipolytica* CMGB32 grew well on *n*-hexadecane over two weeks, *C. curvatus* YR-P2 degraded petroleum during the first ten days, while *R. glutinis* RG5 was more active at the beginning of incubation period. All strains produced good rates of biosurfactants. Bean (*Phaseolus vulgaris*) seeds were sown in pots with sterilized soil and a mixture of the three yeast strains in the presence of petroleum and *n*-hexadecane (2:1 v:v) and observed for a month. Similar experiments were performed using oil polluted soil from oil wells (Ploiesti area, Romania) and a mixture of polluted soil, sand and gravel. The plants grew slowly on sterilized soil, with visible results only after three weeks. The presence of oil well polluted soil allowed seed germination during the first week due probably to complex interaction between soil native microorganisms-yeast mixture-plant-pollutant which provided the necessary nutrients. Although *P. vulgaris* has the ability to convert contaminants in less toxic compounds and to fix atmospheric nitrogen, the yeast mixture seems to enhance the plant growth, fully developed plants being observed after two weeks compared to control plants. Soil aeration was also a determinant factor, since seeds planted in soil mixture showed the most rapid growth during first week. The results suggest a positive influence over long period of time of the yeast mixture on plant growth in presence of oil compounds. Further work aims optimization of the novel yeast-plant system as basis for phytoremediation studies.

Key words: *Yarrowia*, *Rhodotorula*, *Cryptococcus*, *Phaseolus vulgaris*, oil, phytoremediation.

INTRODUCTION

Phytoremediation represents a promising green technology aimed to restore the environment by using the ability of higher plants to convert a wide range of pollutants (petroleum, hydrocarbons from oil spills, heavy metals, household wastes) into less toxic compounds through various metabolic pathways. Phytoremediation involves different processes: phytoaccumulation, phytostabilization, phyto-degradation, phytovolatilization and rhizodegradation (Zhou et al., 2011). The possibility of applying phytoremediation *in situ*, without supplemental costs related, for example, to soil transportation and treatment *ex situ*, augmented the interest for improvement research studies. Thus, enhancement of phytoremediation by inoculation of plants with microorganisms is based on the multiple, synergic interactions between the plant root system and the microbial population (bacteria,

yeasts, fungi). Yeast species associated with the rhizosphere belong mainly to *Candida krusei*, *Candida maltosa*, *Cryptococcus curvatus*, *Cryptococcus laurentii*, *Debaryomyces hansenii*, *Metschnikowia pulcherrima*, *Pichia (Candida) guilliermondii*, *Rhodotorula glutinis*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica*. The yeast population is larger in the rhizosphere than in the rest of the bulk soil, due to the presence of chemical exudates (aminoacids, carboxylic and phenolic acids, carbohydrates, mucilage and plant cell lysates) resulted from plant and fungi metabolism (Botha, 2006). On the other hand, the yeasts contribute to the carbon and nitrogen cycle in soil, increase nodulation and stimulate mycorrhizal-root colonization helping to plant growth promotion (Singh et al., 1991). Many of the yeast species from the *Candida*, *Cryptococcus*, *Rhodotorula* and *Yarrowia* genera isolated from soil are also able to degrade hydrocarbons and to synthesize

biosurfactants that can be successfully used for bioremediation of the polluted soil, including for agriculture (Sachdev and Cameotra, 2013; Shekhar et al., 2015). The biosurfactants are low molecular weight surface-active amphiphilic compounds able to reduce the tension at hydrophobic /water interfaces, being thus able to enhance the availability of hydrocarbons for biodegradative processes.

Weed plants (Wei et al., 2010) are known to have a good potential for phytoremediation due to their extensive fibrous root system, which offers a large surface to establish a strong rhizosphere in the contaminated soil. However, legume plants have been studied intensively in many countries since, besides having an extensive root system, they are also nitrogen independent (Ndimele, 2010, Mathur et. al 2010). This fact is very important in oil-contaminated soils because they do not have to compete with the endogenous microorganisms for the limited supplies of available nitrogen. Thus, the plants can grow and produce sufficient biomass assuring an efficient bioremediation (Udom et al., 2015). For example, Yateem et al. (2000) studied the effect of three economically important plants, alfalfa (*Medicago sativa*), broad bean (*Vicia faba*) and rayegrass (*Lolium perenne*), on total petroleum hydrocarbon (TPH) degradation. The results obtained showed that, even if all three species presented normal growth at 1% TPH concentration, the degradation activity was higher in leguminous plants. Previous studies showed that these species, including *Phaseolus vulgaris*, were able to grow and reduce the concentration of pollutants from contaminated soil. Moreover, their root system present differences regarding the size and number of nodules, i.e. in plants grown on contaminated soil, the nodules were less numerous and larger than in control plants (Rosado et al., 2004; Nwoko et al., 2007; Udom et al., 2015).

In the present work three yeast strains *Yarrowia lipolytica* CMGB32, *Rhodotorula glutinis* RG5 and *Cryptococcus curvatus* YR-P2 are studied regarding their ability to degrade hydrocarbons (*n*-hexadecane and petroleum) and to produce biosurfactants using these substrates as sole carbon sources. Preliminary phytoremediation studies are performed comprising treatment of *Phaseolus vulgaris*

with the three yeast strains and evaluation of environmental conditions influence on plant growth in oil polluted soil.

MATERIALS AND METHODS

1. Biological materials

The yeast strains *Yarrowia lipolytica* CMGB32 (Csutak et al., 2015), *Rhodotorula glutinis* RG5 and *Cryptococcus curvatus* YR-P2 from oil-polluted environment (Csutak et al., 2012; Corbu et al., 2016) were maintained in the Collection of Microorganisms of the Department of Genetics, Faculty of Biology, University of Bucharest, Romania (CMGB) on Yeast Peptone Glucose Agar (YPGA) medium (0.5% yeast extract, 1% peptone, 0.2% glucose, 2% agar-agar).

Bean (*Phaseolus vulgaris*) seeds (20 g) were sterilized with 4% sodium hypochlorite solution, for 20 min. with permanent stirring, and after the hypochlorite was drain out, the seeds were washed four times with distilled water and placed on a sterile surface till fungal inoculation (Jones, 2017).

2. Assessment of hydrocarbon biodegradation

The yeast inoculum (0.3×10^6 cells/ml) was cultivated in Bushnell-Haas mineral medium (KH_2PO_4 1 g/l, K_2HPO_4 1 g/l, NH_4NO_3 1 g/l, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ 0.2 g/l, FeCl_3 0.05 g/l, $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ 0.02 g/l) supplemented with 1% *n*-hexadecane (Sigma), respectively, petroleum (Fluka). Yeast cultures were incubated for 14 days at 28°C, 120 rpm. Samples were collected at time 0 and after 2, 6, 10 and 14 days. The biodegrading abilities of the yeast strains were determined by monitoring cell counts on Thoma counting chamber and the pH (de Cassia Miranda et al. 2007; Piróllo et al., 2008).

3. Biosurfactants production. Determination of the emulsification activity

The yeast strains were cultivated for 72 h at 150 rpm, in 20 ml Yeast Peptone (YP) medium (1% yeast extract, 1% peptone) supplemented with 1% *n*-hexadecane (Sigma-Aldrich) for *Y. lipolytica* CMGB32 and *R. glutinis* RG5 and 1% petroleum (Fluka), respectively, 1% *n*-hexadecane (Sigma-Aldrich) for *C. curvatus* YR-P2.

After 72 hours, the production of biosurfactants was evaluated using the emulsification index ($E_{24}\%$) (Cooper and Goldenberg, 1987). Thus, cell-free broth from yeast cultures grown on biosurfactant production media were mixed (3:2 v:v) with *n*-hexadecane for the cultures grown on petroleum and with petroleum for the *n*-hexadecane grown cultures. The mixture was vortexed for 2 min at 2500 rpm and maintained at room temperature for 24 h before calculating the $E_{24}\%$.

4. Phytoremediation assays

Three parallel phytoremediation assays were performed in duplicates, in plastic pots, in lab conditions (at room temperature, and natural illumination) and observed over four weeks.

First, two bean seeds were sown in pots with sterilized soil as follows: MI Pp – reference pot with bean seeds and YNB medium (0.67% YNB with aminoacids and ammonium sulphate, Sigma) supplemented with 1% petroleum and *n*-hexadecane (2:1 v:v), SI Pp - sample pot with bean seeds, yeast inoculum (6×10^5 cells/g soil) represented by a mixture of *Y. lipolytica* CMGB32, *R. glutinis* RG5 and *C. curvatus* YR-P2 in YNB medium and the same proportion of hydrocarbons (after Boby et al., 2008). The first day, the plants were watered with YNB medium supplemented with hydrocarbonate substrate for allowing yeast growth, after which they were wetted uniformly with tap water.

In a second experiment, two bean seeds were sown in pots with oil polluted soil from oil wells (Ploiesti area, Romania): M - reference with bean seeds, P₃₊ - sample with bean seeds and yeast mixture (6×10^5 cells/g soil) in sterilized water.

Finally, we used a mixture of polluted soil, sand and gravel (2:1:1), where M Bis – the reference and P2 – the sample were prepared similar to the conditions from the second experiment. For both experiments the plants were wetted only with tap water.

RESULTS AND DISCUSSIONS

Hydrocarbon assimilation

The yeast strain *C. curvatus* YR-P2 showed similar growth profiles on both hydrocarbonate substrates (Figure 1A). The *n*-hexadecane was rapidly assimilated during the first two days of

incubation (1.7×10^6 cells/ml) after which the curve registered a descending profile until day ten. The ascending profile observed till the end of incubation period, might be due to the presence of secreted metabolites in the medium, which can be used as secondary substrates for cell growth. The petroleum, although stimulated the cell growth, was assimilated during a longer period of time, within a week (1.4×10^6 cells/ml), after which the cell multiplication decreased constantly.

This study is one of the few studies describing the assimilation of hydrocarbons by a *C. curvatus* strain. Thus, Sietmann et al. (2002) reported *C. curvatus* strains able to biodegrade biaryl compounds (biphenyl, dibenzofuran and diphenyl ether) producing mainly monohydroxylated intermediates as well as ring cleavage product. This might explain the good ability of *C. curvatus* YR-P2 to grow on petroleum containing approximately 80% aromatic hydrocarbons. Another explanation might reside in the fact that our strain was isolated from oil-polluted soil, the cells presenting adaptive metabolic pathways for consuming various classes of oil compounds.

Rhodotorula species, including *R. glutinis*, have been described as being able to degrade *n*-alkanes (Trama et al., 2014), aromatic compounds (Boşça and Sanin, 2015) and complex hydrocarbonate substrates such as petroleum and diesel (Shailubhai et al., 1984; de Cassia Miranda et al., 2007). The strain *R. glutinis* RG5 doubled the cell number (0.6×10^6 cells/ml) between the second and the sixth day of incubation on *n*-hexadecane, after which a drop was observed to the initial level and the plateau installed (Figure 1B). No growth was recorded when petroleum was used.

Y. lipolytica is a well known consumer of hydrocarbons, including *n*-alkanes (Fickers et al., 2005). The strain *Y. lipolytica* CMGB32 showed a high rate of *n*-hexadecane assimilate. The cell number tripled within only two days (1.0×10^7 cells/ml) and continued the accelerated growth until the end of the experiment reaching 1.1×10^8 cells/ml (Figure 1C).

As in the case of *R. glutinis* RG5, no growth was recorded on petroleum as sole carbon source. However, previous studies showed that *Y. lipolytica* CMGB32 produced biosurfactants in the presence of petroleum in YP medium

(Csutak et al., 2015). Since the assimilation tests were performed using a simple mineral medium (Bushnell-Haas), the results indicate the major impact of complex nitrogen,

aminoacids and vitamin resources from the YP medium (yeast extract, peptone), on the cell growth, biosurfactant synthesis and, therefore, on hydrocarbon assimilation.

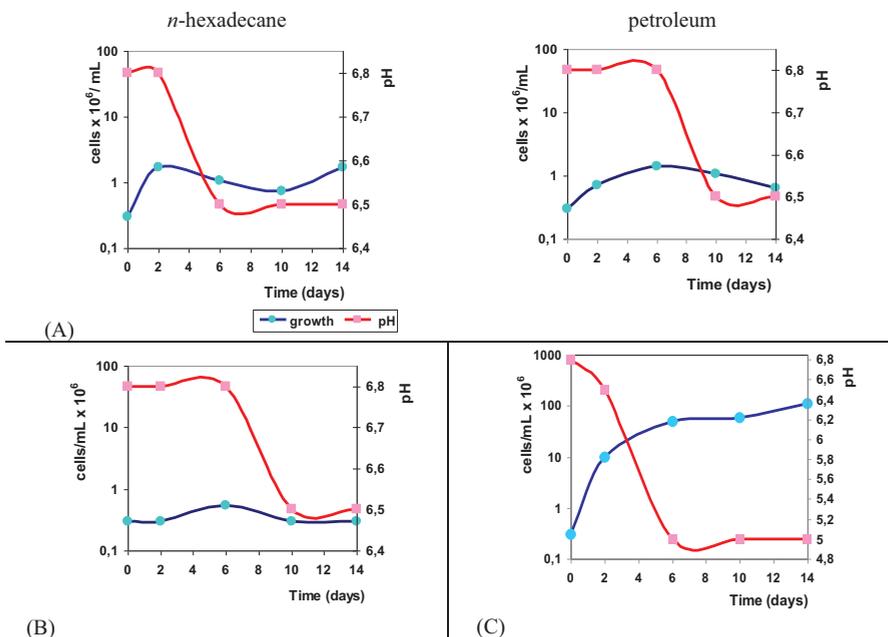


Figure 1. Growth curves and pH variation for *C. curvatus* YR-P2 (A), *R. glutinis* RG5 (B) and *Y. lipolytica* CMGB32 (C) over 14 days biodegradation of the hydrocarbonate substrates

Biosurfactant synthesis. Mechanism of hydrocarbon assimilation

Yeasts can assimilate hydrocarbons in pseudo-solubilized form due to production of biosurfactants. In the present study, we analyzed the synthesis of biosurfactants using as sole carbon source hydrocarbonate substrates for which we obtained the best growth rates for each of the three yeast strain tested.

Until present, there are few data on production of biosurfactants, respective sophorolipids, from *C. curvatus* (Daniel et al., 1999; Banat et al., 2014). The strain *C. curvatus* YR-P2 showed good growth rates both on *n*-hexadecane and petroleum. Therefore, the production of biosurfactants was tested using YP medium supplemented with each of the two hydrocarbonate substrates. The E₂₄ values were 44% when grown on petroleum and 39% when *n*-hexadecane was used as sole carbon source.

Previous studies mentioned the fact that low pH values could indicate an intensive metabolism of the hydrocarbons in the cells leading to excretion of fatty acids into the extracellular

medium (Oboh et al., 2006; Piróllo et al., 2008). Meanwhile, high pH values could be related to biosurfactant production activity (de Luna et al. 2009). By correlating our E₂₄ results with the data from Figure 1A, we can conclude that in the case of *C. curvatus* YR-P2, the hydrocarbonate substrates can be assimilated both throughout a passive process as well as in a pseudo-solubilized form. Thus, the *n*-hexadecane is internalized in the yeast cells rapidly within two days, using both mechanisms which results in high growth rates, a rapid production of fatty acids and low pH after only six days. On the contrary, during the same period of time, the petroleum seems to be assimilated more slowly, mainly due to biosurfactant synthesis.

After 72 hours on YP medium with *n*-hexadecane, *R. glutinis* RG5 produced biosurfactants with an E₂₄ of 33%, which represented the main mechanisms for assimilating the hydrocarbon in the cell (Figure 1B). *R. glutinis* strains were described as producing biosurfactants with high emulsification activity

when grown for 72 hours on cassava starch broth and yeast extract (Oloke and Glick, 2005) and on motor oil and burning waste vegetables (Yuri Max et al., 2012), while the same strain RG5 produced emulsification of petroleum (43%) on YP medium supplemented with *n*-decane (Csutak et al., 2012).

For the strain *Y. lipolytica* CMGB32 we obtained 52% emulsification activity in the presence of *n*-hexadecane after 72 hours, which is comparable to the results of Cirigliano and Carman (1985). In general, for *Y. lipolytica* are recommended longer incubation periods up to 144 hours in the presence of *n*-hexadecane and ground-nut oil refinery residue (Rufino et al., 2007; Amaral et al., 2008) while good biosurfactant rates were obtained after 72 hours using YP medium and petroleum (Csutak et al., 2015) or mineral medium with soybean oil refinery residue, glutamic acid and yeast extract (Rufino et al., 2008).

Observing the Figure 1C, the main mechanisms of *n*-hexadecane assimilation for *Y. lipolytica* CMGB32, seems to be based initially on biosurfactant synthesis, followed by a passive mechanisms, with a significant production of fatty acids and a drop of the pH.

Phytoremediation studies

The preliminary phytoremediation studies involved corroborated evaluations of plant growth in the presence of various hydrocarbonate substrates in the presence or absence (reference pots) of a yeast mixture. *P. vulgaris* seeds sown in sterilized soil grew slowly, even though we used a mineral medium (YNB) supplemented with hydrocabons as carbon source for allowing yeast multiplication at the beginning of the experiment. Visible plants were observed within three weeks and no further changes appeared afterwards (Figure 2).

Moreover, no significant difference could be noted between the reference (MI Pp) and the plants treated with the yeast mixture (SI Pp).

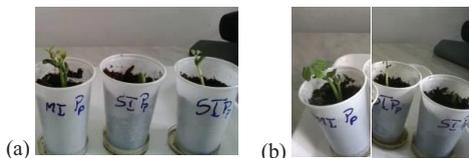


Figure 2. *P. vulgaris* seeds growth on sterilized soil with yeast mixture and added hydrocarbons after (a) three weeks and (b) one month

The situation changed when oil well polluted soil was used. After a week, the seeds already germinated and after only another six days, fully grown plants were observed (Figure 3a, b). The rapid evolution continued until the third week after which slowed down.

The influence of the yeast mixture on the plant development was obvious beginning with the third week. Thus, if within the first fourteen days, the reference plants (M) grew better than those treated with yeasts (samples P₃₊), during the third week the sample plants showed an accelerated growth surpassing the reference (Figure 3c). This could be explained by the adaptive response of the yeasts to the environmental conditions correlated with an intense metabolism.

On the other hand, the complex chemical composition of the oil well compared to the mixture of petroleum and *n*-hexadecane used on the sterilized soil, is also expected to contribute to the yeast metabolism during this second experiment. At last, but not at least, we must also consider the complex interaction between soil endogenous microorganisms - yeast mixture - plant - complex pollutant which provided the necessary nutrients for the rapid plant growth.

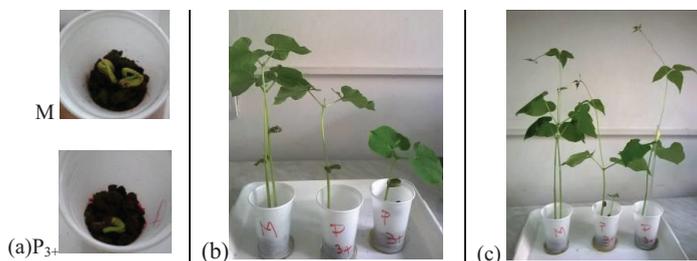


Figure 3. *P. vulgaris* seeds growth on oil well polluted soil and yeast mixture after (a) one week, (b) two weeks and (c) three weeks

Results even more spectacular were obtained when the oil well polluted soil was mixed with sand and gravel. In this case, a fast growth was observed within the first week (Figure 4).



Figure 4. *P. vulgaris* seeds growth after one week on oil well polluted soil mixed with sand and gravel in the presence of yeast mixture

This was most probably due a better aeration of the soil, an important factor both for microbial assimilation of hydrocarbons and for plant growth, since it seems that the porosity of the soil is related to changes in the diversity of the existing microbial communities (Crawford et al., 2012). However, by the end of the period of time, the development of the plants was rather similar to the previous experiment.

The results obtained during the preliminary tests, suggest a possible future modification of the yeast inoculum used for promoting *P. vulgaris* growth. Similar studies showed that a rate of 1×10^5 or 2×10^5 cells per gram of soil, could be more appropriate (Sampedro et al., 2004). Also, since all three yeast strains, *Y. lipolytica* CMGB32, *R. glutinis* RG5 and *C. curvatus* YR-P2, are good biosurfactant producers, a combination of yeast cultures and biosurfactants might assure a better hydrocarbon assimilation and plant development (Zhou et al., 2011).

CONCLUSIONS

All three yeast strains were able to assimilate *n*-hexadecane with highest rates for *Yarrowia lipolytica* CMGB32, while *Cryptococcus curvatus* YR-P2, isolated from oil-polluted soil, showed complex metabolic abilities and similar growth profiles on *n*-hexadecane and petroleum. Good results were also obtained for biosurfactant synthesis. The preliminary phytoremediation studies suggest that hydrocarbon degradation by the three yeast strains had an important positive influence on

P. vulgaris growth over long periods of time in presence of oil compounds. The newly established yeast-plant system will be further optimized for improvement of biodegradation process and plant growth, in order to enhance the phytoremediation process.

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PRELIMINARY RESEARCH ON ENERGETIC CAPITALIZATION OF LIGNOCELLULOSIC MATERIALS IN FORM OF BIOETHANOL

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Abstract

The huge amount of biomass composed of plant residues considered "waste" is a potential source of useful products. Works of this research were based on the study of the bioconversion potential in bioethanol of different types of wastes of lignocellulosic nature, obtained from the harvesting and processing of cereals (straw, cobs). Efficient capitalization of plant biomass is only possible when effective methods of delignification and decrystallization of the lignocellulosic complex are applied. Our research focused on the following general objectives: selection and characterization of lignocellulosic waste that can produce bioethanol, selection and adaptation of highly-productive microorganisms that ensures high conversion yields of bioethanol from the substrates obtained from lignocellulosic waste, experimentation of the technology at micropilot level. The raw materials used, wheat straw, barley straw and corn cobs, have been subjected to thermal pretreatment (autoclaving at 121⁰C for 30 minutes), enzymatic pretreatment (laccase), chemical pretreatment (NOH) and enzymatic hydrolysis (MethaPlus), in order to make available the polysaccharide substrates for the subsequent enzymatic hydrolysis. In case of alkaline pretreatment applied to all three types of lignocellulosic materials, best results were obtained when a solution of NaOH 4% was used. Anaerobic digestion of lignocellulosic materials led to ethanol concentrations of 4,9%, in case of corn cobs, 3,2% for wheat straw and 3,9, for barley straw.

Key words: bioethanol, biomass, lignocellulosic materials.

INTRODUCTION

Continues decrease of the amount of vegetation through natural desertification phenomena, fire or irrational exploitation of lignocellulosic waste, determines the production of excess CO₂, generating, along with the industrial activities, the well-known greenhouse effect, which heats slow but continuous the Earth, becoming an imminent threat to humanity (Malherbe et al., 2003; Levine et al., 1996). On the other hand, it is known that more than half of vegetal biomass from agriculture and forestry, consisting largely of lignocellulose compounds represents waste or by-products, that are thrown or partially used, thus losing a significant amount of raw materials used for the development of sustainable processes, which would help to conserve natural resources (Huang et al., 2011). Currently, worldwide, the recovery and reintroduction in the economic circuit of the

material resources are dealt as components of strategies for harmonizing the relationships between economic growth, consumption of natural resources and environmental protection (Huang et al., 2011).

On a global scale, are obvious a series of restrictions regarding material resources, which has made recycling to become an objective necessity. In this context, interest in capitalizing lignocellulosic biomass has increased considerably, knowing that they are the most abundant organic matter sources. The huge amount of biomass formed from plant residues considered "waste" represent a potential source of useful byproducts (Malherbe et al., 2003). Thus, many worldwide researches that are currently performing are justified for the use of lignocellulosic waste in developing sustainable processes and products. In many European countries, lignocellulosic plant waste mixed with animal manure is used, with promising results for bio fuel production (Sun et al., 2002). Therefore, one of the current directions

of international research is focused on improving the digestibility of lignocellulosic waste by applying combined physicochemical and enzymatic treatments in order to release the recalcitrant carbohydrate substrates by a controlled hydrolysis (Jorgensen et al., 2003; Arora et al., 2002).

Experiments performed in this paper were aimed at determining the influence of physical and chemical pretreatment of lignocellulosic materials on their susceptibility to further enzymatic hydrolysis, determining the degree of hydrolysis of pretreated lignocellulosic materials, depending on their nature and testing the ability to produce bioethanol from pretreated lignocellulosic materials, depending on their nature (Lisov et al., 2004; Ruggeri et al., 2003).

MATERIALS AND METHODS

Lignocellulosic materials and the enzymes used for their degradation are shown in the following tables.

Table 1. Lignocellulosic materials

Nº	Lignocellulosic material	Dry substance content (%)	Cellulose content (%)	Lignin content (%)
1.	Wheat straw	85	39,4	15
2.	Barley straw	87	37-42	18
3.	Corn cobs	85	50,2	20,4

Before use, straw and cobs were physically pretreatment by shredding to sizes between 1-10 mm.

Table 2. Enzymes

Nº	Enzyme	Producer	Composition
1.	Methaplus 100L	BIOPRACT GmbH, Ger.	β -glucanase, cellulase, xylanase
2.	Denilite 2S	Novozymes, Den.	laccase

Table 3. Pretreatments

Thermal pretreatment	Autoclaving, 121°C, 30 min
Enzymatic pretreatment	Laccase 10% reported to the weight of dried vegetal material. Samples were incubated at 55°C, 20 h, 200 rpm, pH 5.5.
Alkaline pretreatment	NaOH solutions: 0,5%, 1%, 2%, 4%. Samples were incubated at 50°C, 200 rpm, 2 h.

To determine the concentration of reducing sugars resulted from each step of the experimental protocol a spectrophotometric method was used based on the color reaction of reducing sugars with dinitrosalicylic acid.

Ethanol production was tested with *Sacharomyces cerevisiae*, at an inoculation ratio of 5% (Shin Sato et al., 2007).

RESULTS AND DISCUSSIONS

Experiments performed in this paper were aimed at: 1) determining the influence of physical and chemical pretreatment of lignocellulosic materials on their susceptibility to further enzymatic hydrolysis; 2) determining the degree of hydrolysis of pretreated lignocellulosic materials, depending on their nature; 3) testing the ability to produce ethanol from pretreated lignocellulosic materials, depending on their nature.

In a first set of experiments we focused on emphasizing the changes induced to the susceptibility of lignocellulosic materials to the enzymatic attack of cellulases and hemicellulases complex by performing thermal and chemical treatments combined with laccase treatment.

Table 4. The influence of thermal pretreatment on the enzymatic hydrolysis of lignocellulosic materials

Type of lignocellulosic material	Initial mg/ml	Hydrolysis 2 h	Hydrolysis 4 h	Hydrolysis 20 h
		mg/ml	mg/ml	mg/ml
Wheat straw	0,47	0,91	1,32	3,32
Blank				
Wheat straw	0,62	1,18	3,20	7,07
Barley straw	0,59	0,99	1,90	3,02
Blank				
Barley straw	0,65	1,28	3,31	7,28
Corn cobs	0,74	1,14	1,95	3,30
Martor				
Corn cobs	0,82	1,41	3,45	7,48

For each sample was carried out a blank that was incubated at room temperature for 30 minutes at 220 rpm, pretreated with laccase, and then hydrolyzed with Methaplus.

From the examination of the results shown in the table above it can be concluded that the application of thermal pretreatment combined to the laccase pretreatment, increases the amount of reducing sugars in the supernatant, compared with blanks, with the following

percentages: wheat straw, with 213%; barley straw, with 241%; corn cobs, with 226%.

The way which the concentration of NaOH solution influence the results of enzymatic attack further applied to lignocellulosic materials it was seen in the experiences carried out according to the following protocol:

Variant 1: 5 g vegetal material+80 ml DW

Variant 2: 5 g vegetal material+50 ml NaOH 0,5%

Variant 3: 5 g vegetal material+50 ml NaOH 1,0%

Variant 4: 5 g vegetal material+50 ml NaOH 2,0%

Variant 5: 5 g vegetal material+50 ml NaOH 4,0%

Each alkaline hydrolysis was preceded by a treatment with laccase.

The results are shown in the following table:

Table 5. The influence of alkaline pretreatment on the enzymatic hydrolysis of wheat straw

Variant	Initial mg/ml	Hydrolysis 2 h	Hydrolysis 4 h	Hydrolysis 20 h
		mg/ml	mg/ml	mg/ml
1.	0,47	1,83	3,47	9,41
2.	0,51	2,12	8,38	16,92
3.	0,83	2,57	8,88	21,04
4.	0,71	2,67	9,01	24,60
5.	0,65	2,73	9,07	26,35

After 20 hours of enzymatic hydrolysis, using a 4% NaOH solution (variant 5), was obtained an increase in the concentration of reducing sugars by 2.87 times, compared with Variant 1.

Table 6. The influence of alkaline pretreatment on the enzymatic hydrolysis of barley straw

Variant	Initial mg/ml	Hydrolysis 2 h	Hydrolysis 4 h	Hydrolysis 20 h
		mg/ml	mg/ml	mg/ml
1.	0,615	1,86	4,71	9,56
2.	0,665	2,25	8,64	18,20
3.	0,721	2,62	8,95	21,30
4.	0,651	2,65	9,09	24,99
5.	0,627	2,76	9,17	27,56

As shown from the data presented in Table 6, if after 2 hours of hydrolysis differences in the concentrations of reducing sugars accumulated to the untreated variant were between 27% and 49%, after 20 hours it was between 96% and 301%.

The greater the time of hydrolysis, become increasingly more and more obvious the differences induced by the use of different

concentrations of NaOH, reaching after 20 hours, between 180% and 280% in reducing sugars, compared to the control experiment (Table 6).

Table 7. The influence of alkaline pretreatment on the enzymatic hydrolysis of corn cobs

Variant	Initial mg/ml	Hydrolysis 2 h	Hydrolysis 4 h	Hydrolysis 20 h
		mg/ml	mg/ml	mg/ml
1.	0,74	2,28	5,50	11,02
2.	0,77	2,52	9,58	20,36
3.	0,82	2,92	10,18	22,50
4.	0,84	3,19	10,40	25,86
5.	0,89	3,44	10,94	28,33

In the case of corn cobs, it was also found the efficiency of alkaline pretreatment, which allowed after 2 hours of enzymatic hydrolysis, depending on the concentration of the alkaline solution, the release of reducing sugars of 10% to 50.9%, greater than the control experiment.

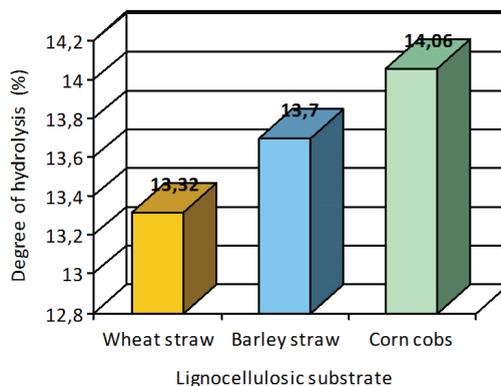


Figure 1. The degree of the enzymatic hydrolysis obtained after the thermal pretreatment of different lignocellulosic materials

The degree of the enzymatic hydrolysis of the three types of lignocellulosic substrates first subjected to autoclaving is expressed in percentage of reducing sugars on dry substance corresponding to the amount of processed lignocellulosic substrate.

The next figure illustrates the degree of enzymatic hydrolysis obtained after optimal alkaline pretreatment (4% NaOH solution) applied to the three types of lignocellulosic materials.

According to the data presented in Figure 3, enzymatically hydrolyzed and chemically pretreated corn cobs led to the best results regarding the content of bioethanol.

Anaerobic digestion of lignocellulosic materials led to ethanol concentrations of 3,2% in case of wheat straw, 3,9%, for barley straw and 4,9%, for corn cobs.

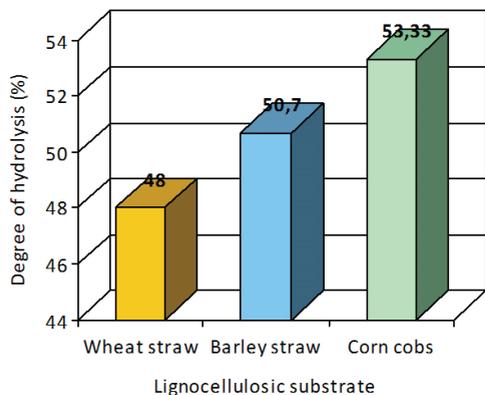


Figure 2. The degree of the enzymatic hydrolysis obtained after the alkaline pretreatment of different lignocellulosic materials

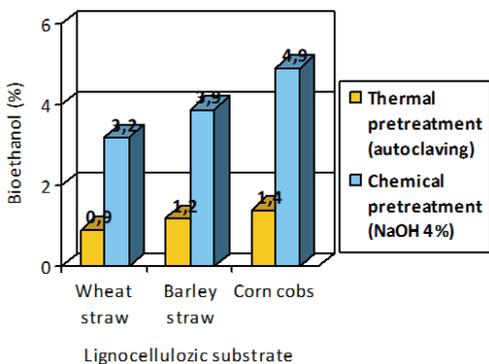


Figure 3. The productivity of processed lignocellulosic material in bioethanol

CONCLUSIONS

Thermal pretreatment consisting in autoclaving of substrates at 121⁰C, for de 30 minutes, allows the obtaining of enzymatic hydrolysis degree which varies depending on the nature of the lignocellulosic material, as follows: wheat straw (13,32%) < barley straw (13,7%) < corn cobs (14,06%).

Alkaline pretreatment is much more effective in terms of availability of polysaccharide substrates for further enzymatic hydrolysis, compared to the thermal pretreatment, allowing higher degree of hydrolysis: wheat straw (48%) < barley straw (50,7%) < corn cobs (53,33%).

Anaerobic digestion of lignocellulosic materials led to ethanol concentrations of 3,2% in case of wheat straw, 3,9%, for barley straw and 4,9%, for corn cobs.

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ISOLATION OF FUNGAL MICROBIAL STRAINS FROM GIURGIU NORD TECHNOLOGICAL PARK WASTEWATER TREATMENT PLANT

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Abstract

In the present study, several microbial strains were isolated in pure cultures from wastewater treatment plant of Giurgiu Nord Technological and Industrial Park, from five withdrawal points along the treatment process: point A, as the entry point of total water content after textile technological processes; point B, after colloidal particles removal stage with $Al_2(SO_4)_3$ coagulant; point C, after mechanical filtration, chemical treatment and sedimentation process stages, point D, from the water obtained after applying treatment technological stages, mixed with sewage water, plus point E, from the soil located in the vicinity of the treatment plant, characterized by high microbial load. Four semi-synthetic agarized nutritive media (PDA, MA, Czapek-Dox, Sabouroud), supplemented with 0.5% chloramphenicol for inhibition of certain bacterial species, were used for isolation of microbial load from the targeted samples. Highest microbial loads and variety were highlighted on plates isolated from soil (E samples), followed by A samples, B samples, and D samples, while C samples registered the lowest growth yield, possible due to inhibitory action of $Al_2(SO_4)_3$ coagulant. Morphological analysis of the obtained cultures revealed both filamentous fungi strains (specific growth) and bacterial growth. Isolated strains will be used in further tests, as both inactivated and viable microbial biosorbents, for removal of specific wastewater pollutants from aqueous solutions.

Key words: wastewater, fungi, bioremediation, textiles.

INTRODUCTION

Water pollution control has become a great concern due to the large number of pollutants, of various proveniences, as consequence of heavy industrialization (Shannon et al., 2008). Textile industry originated wastewaters contain a complex and diverse microbiota (Maza-Marquez et al., 2016), adapted to extreme physical-chemical conditions specific to their environment. Biological materials, especially bacteria, fungi (Singh and Singh, 2014) and algae are regarded as efficient biosorbents, possessing a wide variety of functional groups like hydroxyl, carboxyl, amino, phosphate, sulfhydryl, thioether, phenol, carbonyl, imidazole moieties, and complex enzymatic equipment. Microorganisms act by sequestration and binding of a wide range of industrial wastewaters specific pollutants (Wang and Chen, 2009), decolorisation and detoxification of coloured textile wastewaters (Ma et al., 2014), which are highly toxic and pose a real threat to the environment (Anjaneya et al., 2009). Microorganisms mediated wastewater remediation has received

increasing attention due to versatility and operating costs, but also stands out as environmental friendly treatment methods (Banat et al., 1996). Biosorption carried out by microorganisms has lately gained terrain as alternative efficient and cost effective treatment methods, when compared to conventional ones, like adsorption on organic resins or activated carbons (Hai et al., 2007). Fungal biomass can be used as efficient biosorbents, compared with bacterial biosorbents, due to their versatility regarding efficient use of alternative nutritive sources, with high yields of biomass (Svecova et al., 2006).

MATERIALS AND METHODS

Sample sources and isolation procedure

Water samples were collected from five withdrawal points from inside Giurgiu Nord Technological Park wastewater treatment plant: point A, as the entry point of total water content after textile technological processes; point B, after colloidal particles removal stage with $Al_2(SO_4)_3$ coagulant; point C, after mechanical filtration, chemical treatment and

sedimentation process stages, point D, from the water obtained after applying treatment technological stages, mixed with sewage water; point E, from the soil located in the vicinity of the treatment plant. Water sampling was carried out in polyethylene bottles (100mL), previously disinfected with 65% alcohol.

Nutritive media selection. Four synthetic and semi-synthetic nutritive media were selected for isolation and cultivation of microbial strains: 1) Czapek-Dox-Agar (CD) (Scharlau), semi-synthetic media with sodium nitrate as main source of nitrogen, recipe according to Thom and Raper; 2) Sabouraud-Dextrose-4% Agar (Sab) (Merck), synthetic media that allow growth inhibition of non-acidophilus microorganisms due to low pH value; 3) Malt-Extract-Agar (MA) (Scharlau), classical media for fungi growing, often used for isolation, maintenance and identification of fungal strains; 4) Potato-Dextrose-Agar (PDA) (Scharlau), selective nutritive media for fungi growing, with high content in sugars and low pH value, allow a good development of aerial mycelium. For inhibition of certain bacterial strains (both aerobic and anaerobic species), 0.5% chloramphenicol was used in the media, a thermostable antibiotic with wide spectrum.

Nutritive media plating. Both initial microbial isolations and subsequent cultivations were carried out on all four nutritive media (pH correction with 30% HCl and 5% NaOH), and poured into aseptic Petri dishes ($\varnothing 90\text{mm}$) in a layer of 0.5cm thickness (to ensure a corresponding humidity for the strains), and allowed to dry at room temperature (28°C for 2 hours). For initial microbial isolations, 1mL of each sample was inoculated in duplicates on each nutritive media (for sample E, 10g of soil were dissolved in 100mL of sterile distilled water), and spread uniformly on the surface of the solid media. Isolation in pure cultures involved depletion of loop biomass load (carefully collected in order to avoid contamination) on the surface of the nutritive media. Incubation was performed at room temperature ($30^{\circ}\text{C} \pm 2^{\circ}\text{C}$, for 10 days) for initial microbial isolations and in controlled conditions (28°C , for 14 days) for the two subsequent cultivation in pure culture.

RESULTS AND DISCUSSIONS

In the isolation step microbial growth was influenced by both samples types and selected nutritive media used. On post-incubation plates carried out from inoculation of sample A (Fig. 1), it can be highlighted both development of fungal structures (including yeasts) and bacteria.

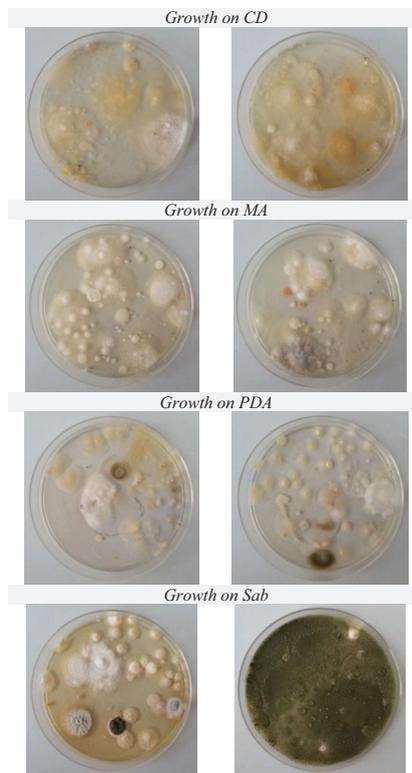


Figure 1. Sample A post incubation plates

Microbial development from sample A highlights development of both unsporulated mycelium and matured strains, but also presence of bacterial development.

The plates inoculated from sample B wastewater (Fig. 2) show lower microbial loads when compared to sample A plates, mainly due to presence of $\text{Al}_2(\text{SO}_4)_3$ as inhibitor agent in the aqueous inoculum. CD plates show both filamentous fungi specific strains but also bacterial ones.

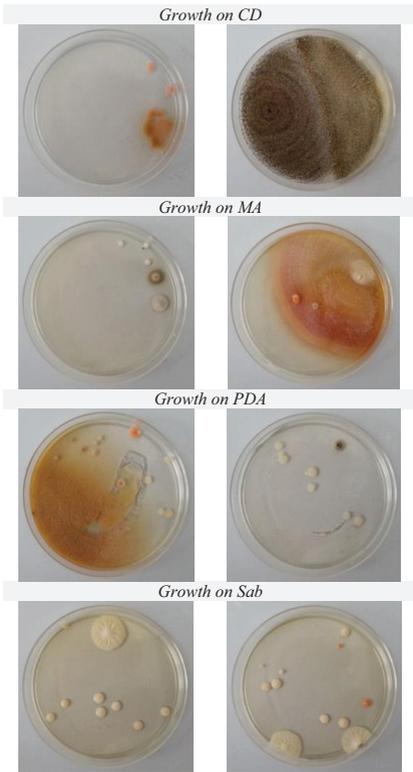


Figure 2. Sample B post incubation plates

Filamentous structures are also visible on MA and PDA plates, whilst on Sab plates both bacterial and yeast structures can be observed. Unlike plates from A and B samples, microbial load from sample C (Fig. 3) is greatly reduced, which may be caused by the action in time of the sulphate (as inhibitor) and of sedimentation process, which can mechanically bind microbial cells towards the lower part of the tank, as sampling was carried out from the upper part.

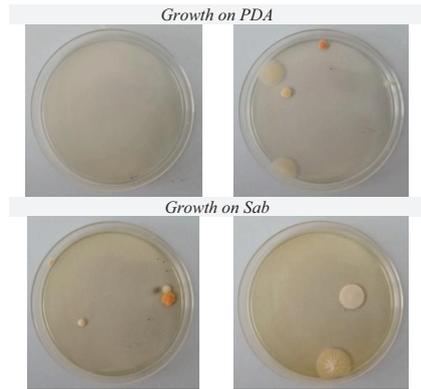
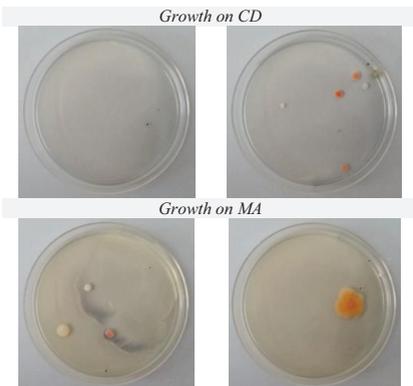


Figure 3. Sample C post incubation plates

Plates inoculated from samples D (Fig. 4), of heterogeneous composition, composed of post treatment water and sewage water, allowed the development of both bacterial and fungal species. The presence of the antibiotic in the media did not prevent the complete development of bacterial species, which may be caused either by the specificity of the antibiotic towards present strains or by high bacterial load of the samples.

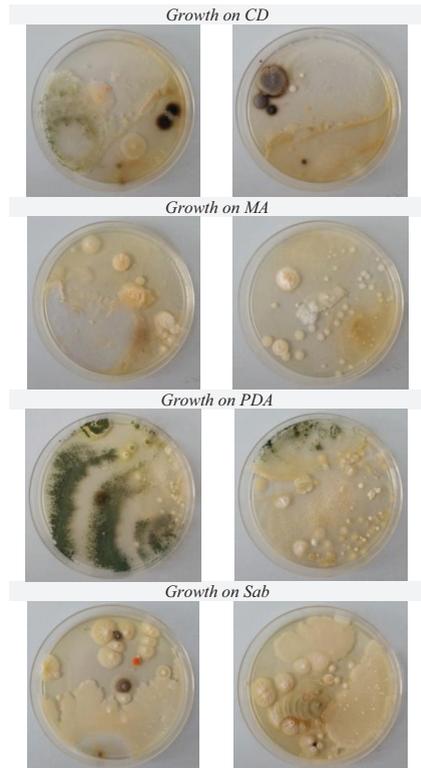


Figure 4. Sample D post incubation plates

Isolations carried out from soil sample showed strong growth of filamentous structures, these plates yielding the highest microbial load of all samples (Fig. 5). Also, it can be highlighted the development of aerial mycelium, due to significant nutritional value of used nutritive media.

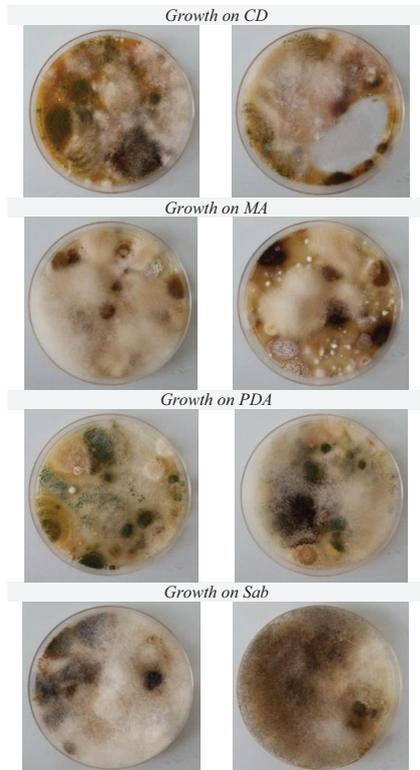


Figure 5. Sample E post incubation plates

Following isolations of main microbial species from four samples of textile industry originated wastewater and one soil sample, varying degrees of microbial growth can be observed, depending on both inoculated sample and the type of nutritive media used. The highest degree of microbial growth can be observed on soil plates, with the presence of at least four strains of filamentous fungi. From A, B and C residual water samples, the highest microbial load was present on plates from sample A and B, whilst sample C plates yielded lower microbial growth, possible due to action in time of $Al_2(SO_4)_3$, which lead to microbial inhibition, compared to sample II plates, where the sulphate was added and stirred in the water volume, with a shorter contact time. Sample D

isolates also show high microbial loads. Also, beside fungal specific morphological structures, bacterial development can also be observed, despite the presence of chloramphenicol, which can be caused by both low antibiotic specificity towards respective strains and high microbial concentrations, specific to industrial wastewaters.

Furthermore, two successive isolations in pure cultures were carried out from initial isolation plates that presented significant filamentous fungi specific structures growth, which lead to an uneven number of isolates per each media, due to affinity of each strain, specific to each sample, per each media used.

From sample A isolates, only one strain was targeted for cultivation in pure cultures (Fig. 6).

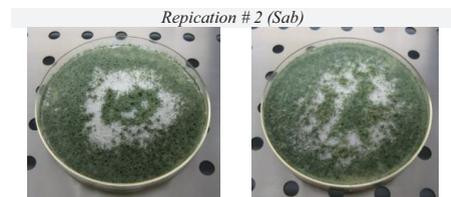
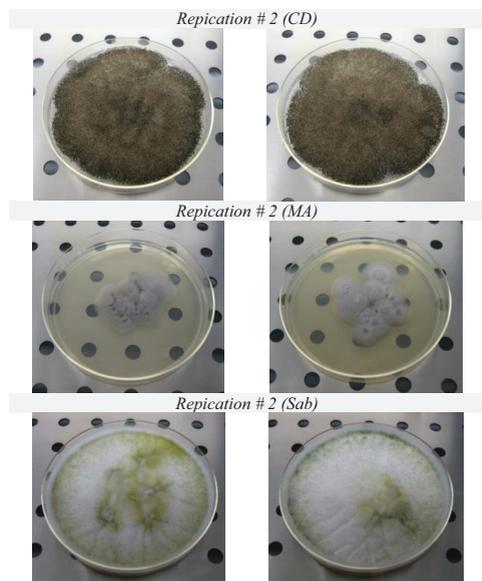


Figure 6. Sample A replication in pure cultures

Sample B replications targeted 7 strains on CD (1 strain), MA (1 strain), Sab (2 strains) and PDA (3 strains) (Fig. 7). Morphological characteristics of the obtained strains indicate filamentous fungi specific structures but also yeast specific structures (MA and PDA plates).



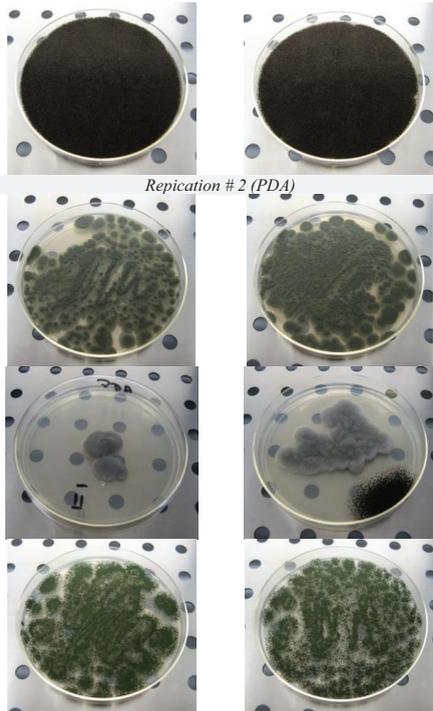


Figure 7. Sample B replication in pure cultures

Due to low bioburden of sample C plates, isolation in pure cultures targeted only one strain (grown on CD), which presents yeast like morphology (Fig. 8).

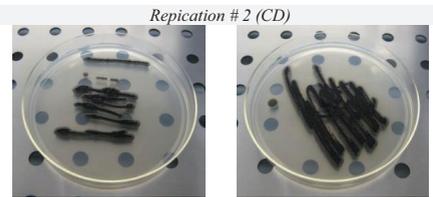


Figure 8. Sample C replication in pure cultures

Sample D platings targeted 3 strains, on CD, PDA and Sab media (Fig. 9), highlighting significant specific filamentous structures growth.

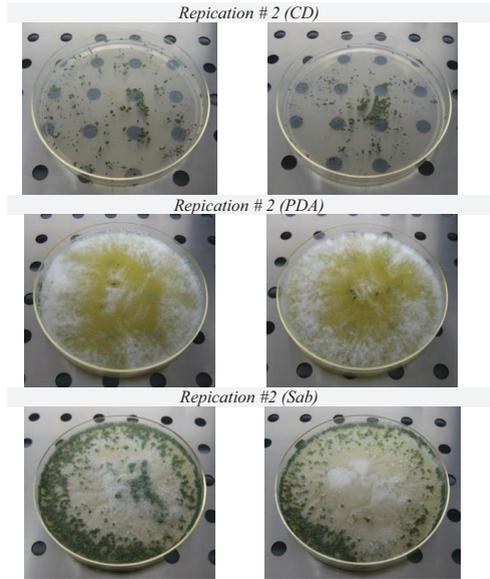
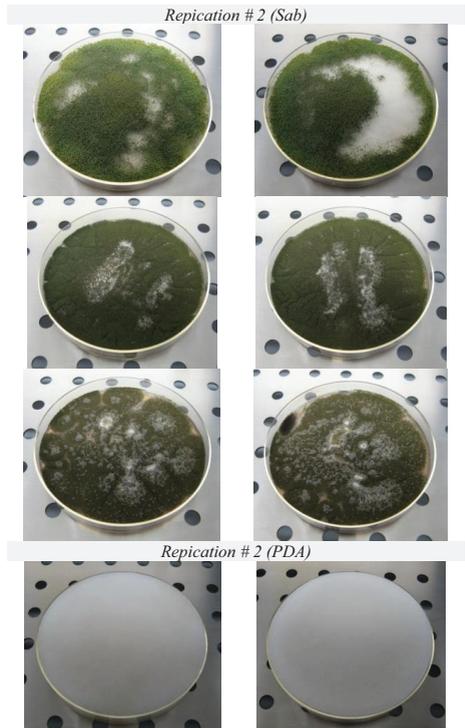


Figure 9. Sample D replication in pure cultures

Isolations in pure cultures from soil samples targeted 7 strains that yielded good aerial mycelium growth, with best growth on Sab media (3 strains) followed by PDA (2 strains), CD and MA (Fig. 10).



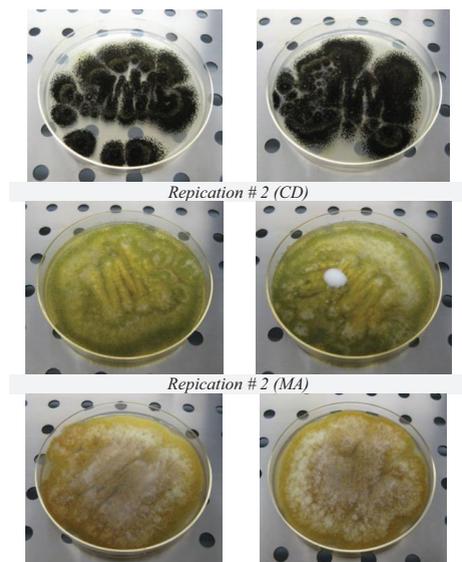


Figure 10. Sample E replication in pure cultures

CONCLUSIONS

Analysis of morphological characters of obtained microbial isolates revealed filamentous fungi and yeast specific structures, with strong development of aerial mycelium for some isolates. The strains isolated from textile processing originated wastewater will be further used for obtaining microbial biosorbents, for their active potential of bioremediation of main wastewater pollutants, backed up on their already native adaptability to presence of pollutants in the environment. Bioreactor scale biosorbents will be obtained for removal of specific wastewater pollutants from aqueous solutions: heavy metals, discoloration assays on textile dyes (Bemacid azo-dyes), BOD and COD reduction.

ACKNOWLEDGEMENTS

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POLYAROMATIC HYDROCARBONS UTILIZATION BY A *Pseudomonas* STRAIN

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Abstract

Polyaromatic hydrocarbons (PAHs) are major environmental pollutants, which are well known for their toxic effects on the organisms. However, different *Pseudomonas* strains are able to use these toxic compounds as growth substrate. *Pseudomonas aeruginosa* strain IBB_{Po16} was able to grow on nutrient-rich and minimal media in the presence of several toxic PAHs, such as naphthalene, methylnaphthalene, phenanthrene, fluorene. *P. aeruginosa* IBB_{Po16} cells exhibited a higher growth when they were inoculated on nutrient-rich medium in the presence of PAHs, as compared with growth on minimal medium. Naphthalene was less toxic for *P. aeruginosa* IBB_{Po16} cells, compared with methylnaphthalene, phenanthrene, and fluorene. *P. aeruginosa* IBB_{Po16} cells grown in the presence of PAHs produced specific extracellular secondary metabolites (i.e., surfactants, pigments). *P. aeruginosa* IBB_{Po16} cells grown in the presence of naphthalene possess *ndoM* (naphthalene dioxygenase) and *rhlAB* (rhamnolipin transferase 1) genes, whereas *C23DO* (catechol 2,3-dioxygenase) and *pahDO* (PAH dioxygenase) genes were not detected in this Gram-negative bacterium.

Key words: *Pseudomonas*, PAHs, secondary metabolites.

INTRODUCTION

Petroleum and petroleum products are important energy resources used in industry and in our daily life. Consequently, petroleum hydrocarbons are major pollutants of the environment (Al-Wasify and Hamed, 2014). Presence of polyaromatic hydrocarbons (PAHs) in soil and water is a major environmental problem, because most of them are toxic, mutagenic and/or carcinogenic for humans and other animals (Bugg et al., 2000; Al-Wasify and Hamed, 2014). PAHs are formed during pyrolysis of petroleum and petroleum products and they are components of petrogenic materials (e.g., crude oil, refined oil products, coal, tar, etc.) (Pies et al., 2008). Extensive research was carried out on the fate of these contaminants in the environment and in bioremediation systems (Foght and Westlake, 1988; Bugg et al., 2000; Al-Wasify and Hamed, 2014). Bacteria are considered as one of the dominant hydrocarbon-degrading organism found in the environment (Al-Wasify and Hamed, 2014).

PAHs which are compounds with two and more fused aromatic rings are rich sources of carbon and energy. Consequently, these compounds are used as growth substrate by

some bacteria (Bugg et al., 2000; Norman et al., 2004; Zhang et al., 2011; Al-Wasify and Hamed, 2014).

Different *Pseudomonas aeruginosa* strains were isolated from sites polluted with petroleum and petroleum products and these bacteria were able to produce extracellular secondary metabolites (i.e., surfactants, pigments) which enhance their competitiveness and survival.

It is well known that pyocyanin pigment has antimicrobial activity against a variety of microorganisms (Norman et al., 2004).

Furthermore, some *P. aeruginosa* strains produced rhamnolipid biosurfactant (mixtures of mono- and di-rhamnolipids) which enhance the biodegradation of numerous toxic hydrocarbons (Cameotra and Singh, 2009; Abdel-Mawgoud et al., 2009).

The aim of this study was to investigate the capability of *P. aeruginosa* IBB_{Po16} cells to grow on nutrient-rich and minimal media in the presence of several toxic PAHs, such as naphthalene, methylnaphthalene, phenanthrene and fluorene. The production of extracellular secondary metabolites (i.e., surfactants, pigments) by *P. aeruginosa* IBB_{Po16} cells grown in the presence of PAHs was also evaluated in this study.

MATERIALS AND METHODS

Polyaromatic hydrocarbons (PAHs) growth substrate experiments. *P. aeruginosa* strain IBB_{Po16} was inoculated into nutrient-rich LB medium (Heipieper et al., 1992). Flask was incubated 24 h at 30°C on a rotary shaker (200 rpm).

Overnight bacterial cultures were spotted (20 µl) on nutrient-rich (Heipieper et al., 1992) and minimal (Stancu and Grifoll, 2011) agar media. PAHs (i.e., naphthalene, methylnaphthalene, phenanthrene, fluorene) were supplied in the vapor phase. Controls were prepared in the same way but without addition of PAHs. Petri plates were sealed and incubated for 24-48 h at 30°C.

Overnight bacterial cultures were inoculated (100 µl) into nutrient-rich and minimal liquid media. Then, the tested PAHs (i.e., naphthalene, methylnaphthalene, phenanthrene, fluorene) were supplied in a concentration of 100 mg l⁻¹ (dissolved in dichloromethane) to the cell suspensions. Controls were prepared in the same way but without addition of PAHs. Flasks were sealed and incubated for 24-216 h at 30°C on a rotary shaker (200 rpm). The growth of the cells in the presence of PAHs was determined by measuring optical density at 660 nm (OD₆₆₀) using a SPECORD 200 UV-visible spectrophotometer (Analytik Jena, Jena, Germany). The cell growth in the presence of PAHs was investigated also by spot assay. The bacterial cultures (20 µl) were spotted on nutrient-rich agar. Petri plates were incubated for 24 h at 30°C.

Extracellular secondary metabolites. CTAB methylene blue agar (Siegmund and Wagner, 1991), King A and King B agar (King et al., 1954) were used to detect rhamnolipid surfactants, pyocyanin and pyoverdinin (fluorescein) pigments, respectively. The bacterial cultures (20 µl) were spotted on these selective media. Petri plates were incubated for 24-48 h at 30°C. The colonies which produced rhamnolipid surfactants were surrounded by dark blue halos on CTAB agar. The colonies which produced pyocyanin pigment were blue-green on King A and the colonies which produced pyoverdinin were yellow-green fluorescent on King B agar. Emulsification activity (*E*₂₄) assay was used to quantify the

surfactants in the cell-free culture broths (Abdel-Mawgoud et al., 2009).

Polymerase chain reaction (PCR). Genomic DNA was extracted from the cell pellets with Pure Link genomic kit (Invitrogen). For PCR amplification, 1 µl of DNA extract was added to a final volume of 25 µl reaction mixture, containing: 5 µl 5×GoTaq flexi buffer, 5 µl MgCl₂, 0.5 µl dNTP mix, 0.5 µl specific primers (23CAT-f/23CAT-r, Mesarch et al., 2000; ISPGRL1B/ISPGRR11B, Meyer et al., 1999; ndoM-f/ndoM-r, Márquez-Rocha et al., 2005; rhlA-f/rhlB-r, Medina et al., 2003), and 0,125 µl GoTaq G2 hot start polymerase (Promega). PCR was performed with a Mastercycler pro S (Eppendorf, Hamburg, Germany). The PCR program consisted in initial denaturation for 10 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 43°C, 50°C or 58°C for 30 sec, extension at 72°C for 2 min, and a final extension at 72°C for 10 min. After separation on 1.5% (w/v) TBE agarose gel (Sambrook et al., 1989) and staining with fast blast DNA stain (Bio-Rad) the PCR products were analyzed.

Reagents used during this study were procured from Merck (Darmstadt, Germany), Sigma-Aldrich (Saint-Quentin-Fallavier, France), Promega (Madison, WI, USA), Invitrogen (Carlsbad, CA, USA) or Bio-Rad Laboratories (Hercules, CA, USA). The PCR primers were purchased from Integrated DNA Technologies (Coralville, IA, USA) and Invitrogen (Carlsbad, CA, USA).

RESULTS AND DISCUSSIONS

The strain used in this study was *P. aeruginosa* IBB_{Po16} (GenBank accession number KT315654). This *P. aeruginosa* strain which produced some extracellular secondary metabolites (i.e., rhamnolipid surfactants, pyocyanin and pyoverdinin pigments) was formerly isolated by us from Poeni oily sludge (Stancu, 2017).

Polyaromatic hydrocarbons (PAHs) growth substrate experiments. *P. aeruginosa* IBB_{Po16} was able to grow on nutrient-rich and minimal agar media when several PAHs, such as naphthalene, methylnaphthalene, phenanthrene, and fluorene were supplied in vapor phase (Figure 1a, 1b). *P. aeruginosa* IBB_{Po16} cells

showed a higher growth when they were inoculated on nutrient-rich agar medium in the presence of PAHs (100%), as compared with growth on minimal agar (10%). *P. aeruginosa* IBB_{Po16} cells inoculated on nutrient-rich agar medium in the presence of PAHs produced the pyoverdinin (yellow-green fluorescent) pigment (Figure 1a) visible under UV light (366 nm),

while on minimal agar medium the pigment production was not observed. Was not surprising to observe such changes, because the bacteria which are exposed to toxic hydrocarbons under nutrient limited condition, generally exhibit different physiological changes (Sikkema et al., 1995; Bugg et al., 2000; Norman et al., 2004).

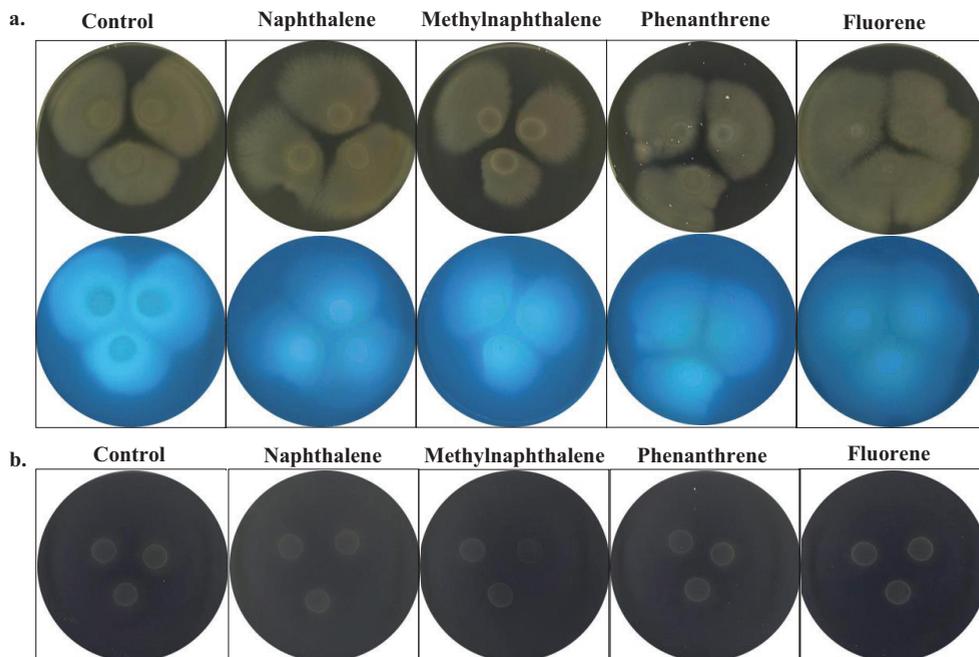


Figure 1. *P. aeruginosa* IBB_{Po16} grown in the presence of PAHs (in vapor phase). a. Nutrient-rich agar medium; plates were observed under visible white light (500 nm, upper side) and UV light (366 nm, down side). b. Minimal agar medium; plates were observed under visible white light (500 nm).

P. aeruginosa IBB_{Po16} cells were also able to grow on nutrient-rich and minimal media in the presence of 100 mg l⁻¹ PAHs (naphthalene, methylnaphthalene, phenanthrene, fluorene) (Table 1). As observed in previously assay, *P. aeruginosa* IBB_{Po16} cells showed a higher growth when they were inoculated on nutrient-rich medium in the presence of PAHs (OD increased by 0.21-1.55 times), as compared with growth on minimal medium (OD increased by 0.13-0.63 times). According with literature (Bugg et al., 2000), PAHs have high octanol-water partition coefficients (log P_{OW}) and partition readily into organic phases. The ordering of phospholipids in bacterial membranes creates a hydrophobic region in the interior of the membrane that can act as a

reservoir for accumulation of hydrophobic compounds, including of toxic PAHs (Bugg et al., 2000). As a result of accumulation of hydrophobic compounds in lipid membranes, loss of membrane integrity and alterations in enzyme activities have been described (Sikkema et al., 1995). It is well known that naphthalene (with log P_{OW} = 3.31), methylnaphthalene (log P_{OW} = 3.81), phenanthrene and fluorene (log P_{OW} = 4.49) are low molecular weight PAHs that contain two or three benzene rings. However, they belongs to the same group, naphthalene was less toxic for *P. aeruginosa* IBB_{Po16} (OD increased by 0.14-1.55 times), compared with methylnaphthalene, phenanthrene, and fluorene (OD increased by 0.13-1.24 times).

Table 1. Growth of *P. aeruginosa* IBB_{Po16} in the presence of PAHs (100 mg l⁻¹)

Variant	Cell growth									
	Nutrient-rich medium					Minimal medium				
	24 h	48 h	72 h	144 h	216 h	24 h	48 h	72 h	144 h	216 h
Control	1.12	1.34	1.80	2.22	2.18	0.19	0.20	0.21	0.81	0.39
Naphthalene	0.83	0.99	1.01	1.55	1.35	0.14	0.15	0.17	0.63	0.32
Methylnaphthalene	0.24	0.45	0.66	1.22	0.58	0.17	0.19	0.22	0.51	0.32
Phenanthrene	0.22	0.44	0.71	1.24	0.65	0.13	0.14	0.14	0.45	0.30
Fluorene	0.21	0.41	0.75	1.13	0.67	0.15	0.16	0.18	0.48	0.29

Cell growth, the OD at 660 nm was measured, and the values from the table represent the ratio between OD at 24, 48, 72, 144 or 216 h and OD at 0 h.

P. aeruginosa IBB_{Po16} cells inoculated on nutrient-rich and minimal media produced the pyoverdinin pigment visible under UV light (366 nm). As could be observed in Figure 2, the

colony fluorescence decreases when *P. aeruginosa* IBB_{Po16} cells were grown for 144 h in the presence of the PAHs (especially on minimal medium).

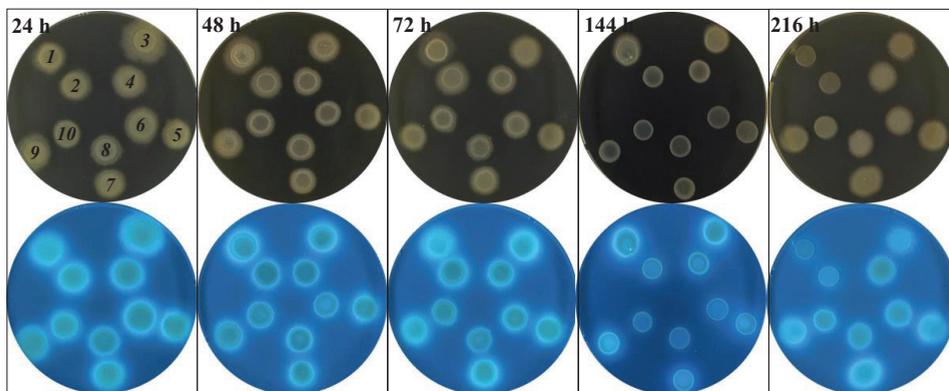


Figure 2. *P. aeruginosa* IBB_{Po16} grown in the presence of PAHs (100 mg l⁻¹). Nutrient-rich medium (1, 3, 5, 7, 9); minimal medium (2, 4, 6, 8, 10); control (1, 2), naphthalene (3, 4), methylnaphthalene (5, 6), phenanthrene (7, 8), fluorene (9, 10); plates were observed under visible white light (500 nm, upper side) and UV light (366 nm, down side).

Extracellular secondary metabolites. The production of extracellular secondary metabolites (i.e., surfactants, pigments) by *P. aeruginosa* IBB_{Po16} cells grown in the presence of PAHs (naphthalene, methylnaphthalene, phenanthrene, fluorene) was further investigated (Figure 3).

A dark blue halo was observed (under visible white light) on CTAB methylene blue agar for *P. aeruginosa* IBB_{Po16} cells grown in the presence of PAHs, as well as for the control cells. As expected, *P. aeruginosa* IBB_{Po16} produced rhamnolipid surfactants, and their emulsification activity was very good (data not shown). Like other PAHs with two or three benzene rings, naphthalene, methylnaphthalene, phenanthrene, and fluorene are

extremely resistant to nucleophilic attack and they are also recalcitrant to biodegradation due to very less solubility in water (Das et al., 2007). However, the biosurfactants produced by some bacteria, including by different *Pseudomonas* strains, increase the solubility of several PAHs, facilitating their assimilation and utilization by bacteria (Das et al., 2007).

P. aeruginosa IBB_{Po16} cells grown in the presence of PAHs produced pyoverdinin (yellow-green fluorescent) pigment on both King A and King B agar media (Figure 3). We observed that the pyoverdinin pigment production increased when *P. aeruginosa* IBB_{Po16} cells were grown on nutrient-rich and minimal media in the presence of the PAHs, as compared with respective controls.

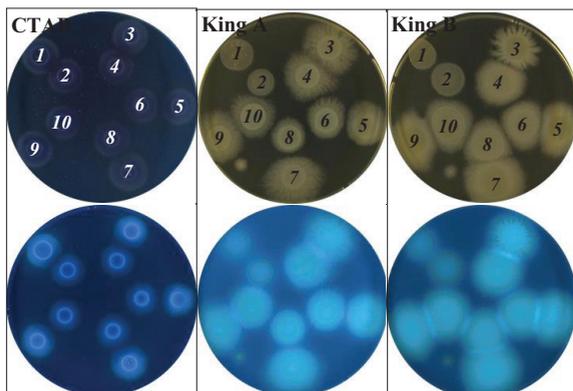


Figure 3. Production of extracellular secondary metabolites by *P. aeruginosa* IBB_{Po16} grown in the presence of PAHs (100 mg l⁻¹). Nutrient-rich medium (1, 3, 5, 7, 9); minimal medium (2, 4, 6, 8, 10); control (1, 2), naphthalene (3, 4), methylnaphthalene (5, 6), phenanthrene (7, 8), fluorene (9, 10); plates were observed under visible white light (500 nm, upper side) and UV light (366 nm, down side).

The production of pyocyanin (light blue-green) pigment by *P. aeruginosa* IBB_{Po16} cells grown in the presence of PAHs was acquired, when the plate with King A agar medium, was incubated for 24 h at 37°C.

Polymerase chain reaction (PCR). Genomic DNA extracted from *P. aeruginosa* IBB_{Po16} cells grown on nutrient-rich medium in the presence of naphthalene was used as template for PCR amplification of *C23DO* (catechol 2,3-dioxygenase), *pahDO* (PAH dioxygenase), and *ndoM* (naphthalene dioxygenase) catabolic genes, and *rhlAB* (rhamnosyltransferase 1) genes (Figure 4). According with literature, the *C23DO*, *pahDO* and *ndoM* genes are

responsible for aromatic hydrocarbon metabolism (Mesarch et al., 2000; Meyer et al., 1999; Márquez-Rocha et al., 2005), and *rhlAB* genes are involved in synthesis of rhamnolipid surfactants (Medina et al., 2003).

The annealing temperatures were 43°C, 50°C and 58°C when primers for these genes were used for PCR amplification. In the DNA extracted from *P. aeruginosa* IBB_{Po16} cells grown in the presence of naphthalene only *ndoM* (642 bp) and *rhlAB* (216 bp) genes were detected, whereas *C23DO* (238 bp) and *pahDO* (900 bp) catabolic genes were not detected in this bacterium.

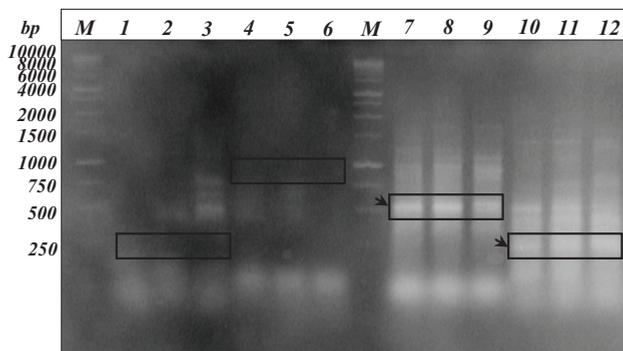


Figure 4. Detection of *C23DO*, *pahDO*, *ndoM*, and *rhlAB* genes in DNA extracted from *P. aeruginosa* IBB_{Po16} grown on nutrient-rich medium in the presence of naphthalene (100 mg l⁻¹). 1 kb DNA ladder, Promega (M); *C23DO* (expected fragment size 238 bp) gene (1-3); *pahDO* (900 bp) gene (4-6); *ndoM* (642 bp) gene (7-9); *rhlAB* (216 bp) gene (10-12); primers annealing of 43°C (1, 4, 7, 10), 50°C (2, 5, 8, 11), 58°C (3, 6, 9, 12).

CONCLUSIONS

P. aeruginosa strain IBB_{Po16} was able to grow on nutrient-rich and minimal media in the presence of tested PAHs (i.e., naphthalene,

methylnaphthalene, phenanthrene, fluorene). *P. aeruginosa* IBB_{Po16} cells exhibited a higher growth when they were inoculated on nutrient-rich medium in the presence of PAHs, as compared with growth on minimal medium.

Naphthalene was less toxic for *P. aeruginosa* IBB_{P016} cells, compared with methylnaphthalene, phenanthrene, and fluorene.

P. aeruginosa IBB_{P016} cells grown in the presence of naphthalene possess *ndoM* and *rhlAB* genes, which are involved in naphthalene degradation and rhamnolipid surfactants synthesis, respectively.

P. aeruginosa IBB_{P016} cells grown in the presence of PAHs produced some extracellular secondary metabolites, such as rhamnolipid surfactants, pyocyanin and pyoverdine pigments, which are well recognized for their multiple applications (e.g., bioremediation of petroleum polluted sites).

ACKNOWLEDGEMENTS

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RESEARCHES CONCERNING THE ENZYMATIC ACTION OF BYPRODUCT GRAPES

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Abstract

The objective of the present research was to convert the grape pomace as energy source, by controlling to increase hydrolysis of the complex sugars (celluloses, hemicelluloses) to fermentable sugars. Hydrolysis can be performed chemically or enzymatically following the appropriate pretreatment steps. The chemical pretreatment of the grape pomace substrat present many disadvantages such as the high costs, the special conditions and the formation of toxic by-products. For this reason, we used hydrolysis enzymatic process, as the most efficient method. The efficiency a commercial enzymatic product - MethaPlus L 100 (containing β -glucanase, cellulase, xylanase) was investigated to hydrolyse dry grape pomace, after physical pretreatment (ground 1mm sieve diameter) to fermentable sugar. The enzyme MethaPlus L 100 was added in different concentrations 0.1 – 1 % (w/w) referred to the substrate dry matter content. The reducing sugars concentration increased of 4.0 fold (when 0.01% MethaPlus L 100 was added) reported relatively to the untreated sample. However, an increase of enzymes concentrations not economically relevant. In the future, the efforts should be focused on the improving the enzymatic treatment of the grape pomace, to be used as nutritional component in animal feed.

Keywords: *enzymes, dry grape pomace, enzymatic hydrolysis, reducing sugars.*

INTRODUCTION

Continued growth in the consumption of animal products (meat, milk, eggs) livestock research oriented towards finding ways of meeting the requirements of the market. Also, it has been sought to solve problems relating to nutrition and health of animals, increase the bioavailability of nutrients present in the feed young animals, whose enzyme is not fully developed equipment, reducing the level of removal of nutrients by manures.

The addition of exogenous enzyme preparation in animal feed, presents the capacity to degrade various nutrients in the environment, carbohydrates, lipids, proteins, that turn them into simple compounds, absorbable, which penetrates the cell membrane (Beckers and Théwis, 2004).

Lignocellulosic biomass are mainly composed of cellulose (30-50%), hemicellulose (15-35%) and lignin (10-20%) (Badger et al., 2000; Girio et al., 2010; Mielenz, 2001; Pettersen, 1984).

Cellulose is a homopolysaccharide composed of β -D-pyranose units, linked by β -1, 4- glycosidic bonds. Cellobiose is the smallest repetitive unit and it is formed by two glucose monomers. The long-chain cellulose polymers are packed together into microfibrils by hydrogen and Van der Waals bonds (Vijai et al., 2014).

Hemicellulose is a mixture of polysaccharides, including pentoses, hexoses and uronic acids (McMillan, 1993). Hemicelluloses constitution enters into the plant cell membrane and that may not be degraded by endogenous enzymes, the animal is deprived of the use of plant cell intracellular constituents. For these reasons, hemicelluloses are classified as anti-nutritional factors for monogastric animals (Israel Roming et al., 2010).

The dried grape pomace (DGP) is a by-product of vine-making from grapes, which can be used in animal feeding mainly due to its properties of natural antioxidant and also to its valuable content of fatty acids, energy and protein (Habeanu et al., 2015).

Studies have been performed on the antioxidant effects of grape extracts due to their high content in polyphenols (Brenes et al. 2008, Chedea et al. 2010) the focus being on the evaluation of the effectiveness of these raw materials on animal products quality (Rojas and Brewer, 2008).

Actually, grape pomace is a blend of separated solids must (marc) or wine (fermented pomace) made of peels (55-65%), seeds (18.25%) and traces of grape clusters and wine extracted from pressing (Pomohaci et al., 2000).

Also, the grape pomace can be a potential source of fermentable sugars, to be used in animal feed.

The resulting product, after obtaining wine or other alcoholic fermentation of fruit, is a liquid condition and to be placed in animal nutrition requires preliminary drying (Voicu et al., 2014).

Grape pomace (GP) consists of the four major poly-saccharides in nature, namely cellulose (consisting of glucose subunits), hemicellulose (glucose, mannose, xylan and arabinose subunits), starch (glucose subunits) and pectin (D-galacturonic acid subunits) (Hulme, 1970, cited by Korkie et al., 2002).

The hydrolysis can be performed chemically or enzymatically, following the appropriate pretreatment steps.

The hydrolysis processes used in the past, were essentially chemical, but the costs and the formation of toxic by-products made them noncompetitive (Diguta et al., 2007; Israel Roming et al., 2005).

Enzymatic processes, which hold several advantages, are now substituting the chemical ones. The efficiency of enzymatic process is quite high and the mild process conditions require neither expensive materials nor high process energy (Diguta et al., 2007).

The use of enzymatic complex (β -glucanase, cellulase, xylanase) for hydrolysis of lignocellulosic substrate (grape pomace) into fermentable sugars gains more and more interest.

In this study, the objective was to examine the capacity of enzymatic hydrolysis of dry grape pomace, by using different concentrations of MethaPlus L 100 enzyme product. The optimum level of exogenous enzyme added on our substrate was done as well.

MATERIALS AND METHODS

Substrate

The by-product used in our study was grape pomace, provided by a Romanian distillery (SC Dionis Agrifood, in collaboration with the National Research Development Institute for Biology and Animal Nutrition (IBNA) - Balotești, Romania). The wet GP was dried at 90°C in a counter-flow hot air conveyor dryer (Habeanu et al., 2015).

This mixture was milled using 1 mm strainers, to increase the accessibility of hydrolytic enzymes at the complex polysaccharides (celluloses, hemicelluloses, starch).

Enzyme preparation

Enzymatic hydrolysis was performed with a commercial enzyme in different concentrations 0.1-1% w/w referred to the substrate dry matter content. The commercial enzyme preparation used is: MethaPlus L 100 (β -glucanase, cellulase, xylanase) produced by BIOPRACT GmbH, Germany.

Enzymatic hydrolysis

The enzymatic treatment was performed in one step process, by using MethaPlus L 100, for an additional hydrolysis period of 20 h. The hydrolysis incubation took place at 55°C, pH=5-5.5, on a rotary shaker at 200 rpm. The experiment were performed in duplicate and the results are presented as mean values. For each trial, were prepared a control sample (without enzyme additions).

Determination of reducing sugar concentration

After conducting the process of hydrolysis (20 hours), all the samples were centrifuged at 5000 rpm, time to 5 minutes.

Reducing sugars were determined in supernatant by using dinitrosalicylic acid reagent (DNS) at optical 640 nm, by the modified method described by Petterson and Porath (Iordăchescu and Dumitru, 1980). The degree of recalcitrant molecules (celluloses, hemicelluloses) was estimated by quantifying the amount of reducing sugars formed during enzymatic hydrolysis.

RESULTS AND DISCUSSIONS

The grape pomace can be used as nutritional component in animal feed due to the containing of

polysaccharides (Hulme, 1970, cited by Korkie et al., 2002) and high amounts of polyphenols with antioxidant properties (Alonso et al., 2002).

The present research was focused to convert the grape pomace as energy source, by enzymatic hydrolysis of the complex sugars (celluloses, hemicelluloses) to fermentable sugars (Figure 1). The enzymatic treatment was performed by using MethaPlus L 100 in different concentrations (Figure 1).

The reducing sugars concentration in the control (without enzymes addition) is lower,

approximately 5 times, compared with the last sample, where the concentration was high.

According to figure 1, the reducing sugars was 13% by using 0.01% MethaPlus L 100, representing an improving of 4 fold, reported relatively to the untreated sample. Additionally, an increase of enzymes concentration from 0.01 to 0.1% enhanced the reducing sugars concentration with approximately 24%. However, in economic terms, the increase of enzymes concentration is not relevant, because between the level concentrations of reducing sugars were not significant differences.

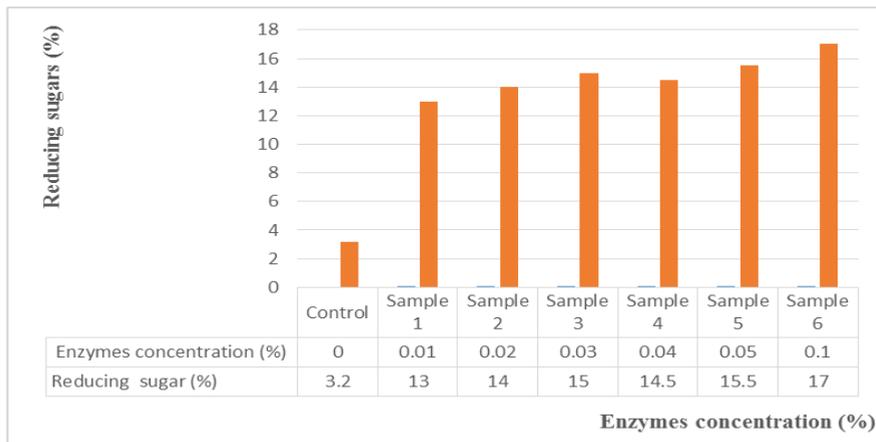


Figure 1. Reducing sugars of grape pomace after enzymatic hydrolysis

CONCLUSIONS

The object to this research was to investigate the influence a commercial enzymatic product at different concentrations, on the conversions of energy crops to fermentable sugars, in our case, MethaPlus L100 (enzymatic complex), by using as substrate dry grape pomace.

The optim level were obtained at the 0.01% concentration of enzyme product in the grape pomace substrate.

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In the future, the efforts should be focused on the improving the enzymatic treatment, by using other enzymes in association with/or not MethaPlus L100.

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SELECTION OF MICROALGAL STRAINS WITH LOW STARCH CONTENT AS POTENTIAL HIGH LIPID - CONTAINING ISOLATES

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Abstract

In the last forty years there is an increased scientific interest in deeper understanding lipid metabolism in photosynthetic microorganisms, aiming at using the most lipid-rich strains as source for biodiesel production. One of the many constraints is the selection of strains with high lipid content. This paper presents the isolation, purification and selection of three strains of photosynthetic microorganisms by using an already known method, iodine vapour method, (Work et al., 2010) which allows to rapidly and easily select those colonies which low starch content as potential high lipid- containing isolates. These results show that in the three selected strains, the fluorescence emission after Nile red addition is increased with different values: 7.597; 10.832 and 11.428 for strains 9.3.1, 9.8.2 and 12.9.0, respectively.

Key words: photosynthetic microorganisms, iodine method, Nile red, fluorescence.

INTRODUCTION

The ability of photosynthetic micro-organisms to accumulate lipids inside the cell received in the last four decades attention with respect to the use of photosynthetic microorganisms as sources of lipids for biodiesel (Sheehan et al., 1998; Chisti, 2007; Li et al., 2008; Liang et al., 2009; Demirbas, 2010; Huang, 2010; Mata et al., 2010; Amaro et al., 2011; Schuhmann et al., 2012; Borowitzka, 2013; Rawat et al., 2013; Velea et al., 2014; Ardelean and Manea, 2016).

This interest is based mainly on the their metabolic advantages over higher plants mainly with respect to shorter generation time (Demirbas, 2010; Amaro et al., 2011; Rawat et al., 2013; Ardelean and Manea, 2016). However, so far, there are some drawbacks (Demirbas, 2010; Amaro et al., 2011; Rawat et al., 2013; Ardelean and Manea, 2016).

In the last two decades there is an increased interest in understanding the competition between starch and lipids for intermediary metabolites (Libessart et al., 1995; Ball, 1998; Hu et al, 2008; Blaby, 2013; Davey et al, 2014;

Tamayo-Ordóñez et al., 2017), including the selection of clones with a low starch content.

There are many reports arguing that the clones with low starch content have a higher lipid content (Ramazanov and Ramazanov, 2006; Wang et al., 2009; Li et al 2010 a and b; Siaux et al., 2011; Work et al, 2010; de Jaeger et al., 2014; Sirikhachornkit et al., 2016).

However, there are also results showing that mutants with low starch content have the same lipid content as the wild type cells (Vonlanthen et al., 2015).

The aim of this paper is to present original results concerning the screening of naturally occurring photosynthetic microorganisms with low starch content, as possible potential high lipid containing strains, using the iodine vapour method (Work et al., 2010).

MATERIALS AND METHODS

Populations of photosynthetic micro-organisms relatively rich in lipids previously selected (Ardelean, 2015; Ardelean and Manea, 2016) were used as biological material in these experiments. Isolation and purification of

photosynthetic microorganisms from the consortia of populations was achieved by dilution method on classically solidified BG₁₁ (Ardelean, 2015).

Selection of colonies with low starch content was done using the qualitative iodine vapour method by placing solid I₂ pellets on the surfaces of agar plates to initiate sublimation (Work et al., 2010).

Estimation of lipid content was done both by microscopic method and by fluorescence quantification. The colonies of isolated and purified photosynthetic microorganisms were treated with Nile red (9-(Diethylamino)-5H benzo [∞] phenoxazin- 5) (Sigma Aldrich), one of the selective fluorescence markers for lipids (Greenspan et al., 1985; Chen et al., 2009).

The cells were incubated for 30 minutes in the presence of Nile red in order to allow as much as possible the penetration of cell wall and cell membrane; then, the microbiological samples were inspected using a fluorescence microscope, with respect to fluorescence signal in the red region as well as in the green region of the spectrum.

As with the microscopic method, for fluorescence quantification, the cells suspensions (OD_{730nm} 1,0 units) were incubated with Nile red for 30 minutes and then washed with fresh BG₁₁ medium.

The fluorescence emission of these cell suspensions was analyzed before Nile red addition (fluorescence emission of only photosynthetic pigments) and after Nile red addition (fluorescence emission of photosynthetic pigments and Nile red in the presence of cellular lipids).

The fluorescence spectra were recorded with spectrofluorometer FP-8300, excitation at 530nm and emission 600-750nm, PMT voltage 500 V, data interval 0.5nm and screen speed 100nm/min; the surface of the spectrum was calculated with Spectra analysis software associated to this instrument.

RESULTS AND DISCUSSIONS

In the figure 1 there are presented the macroscopic images of Petri dishes of three different populations, mixture of species, (9.3; 9.8 and 12.9 respectively) containing colonies (species) with different affinities for iodine vapour.

One can see that the majority of colonies are dark brown whereas few colonies are less coloured, as expression of lower starch content. It has to be said that there are wild type strains, naturally occurring in the mixed populations when grown in the so- called normal conditions, without the occurrence of any thermal, nutritional or osmotic stress which could increase the lipid content of the cells (Chisti, 2007; Li et al., 2008; Amaro et al., 2011).

The colonies with lower affinity for iodine vapours were further cultivated in liquid medium to increase their biomass, to microscopically check morphologic uniformity and to label the lipids with the lipid-specific marker Nile red.

In the figures 2, 3 and 4 there are presented the results concerning the microscopic images (both in bright field and in fluorescence microscopy) of the purified (but non axenic) strains (so called unialgal strains) 9.3.1., 9.8.2 and 12.9.0, respectively.

Each microscopic field has three images: one in bright field and two images of the fluorescent emission: red and green portion of the fluorescence emission spectrum of the Nile red. One can see that the red fluorescence signal is distributed distinctly within the cells of strain 9.8.2 and, especially, 12.9.0, suggesting the occurrence in these two strains of lipid droplets detectable by the use of classical optical microscopes.

Interestingly, these two strains gave higher enhancement of the fluorescence signal, after Nile red labelling (see table 1).

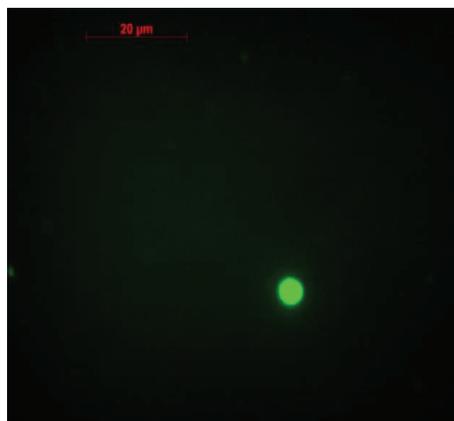
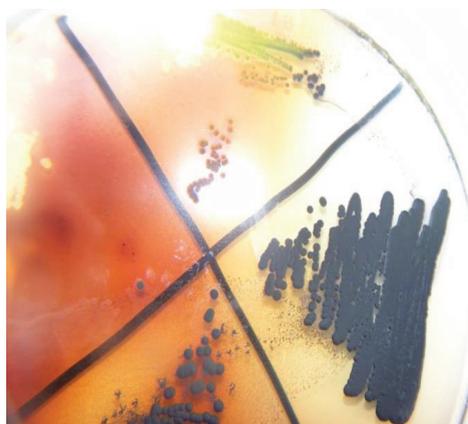
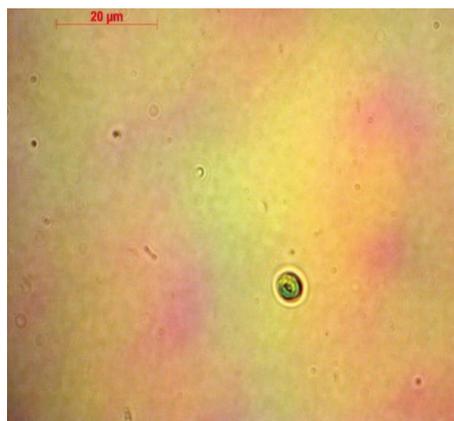


Figure 1. Colonies with different affinities for iodine vapour, after 14 days of autotrophic cultivation in normal conditions; from up to the bottom : mixed population 9.3; mixed population 9.8. and mixed population 12.9

Figure 2. Bright field and epifluorescence images (green filter and red filter) of cells belonging to the purified strain 9.3.1.

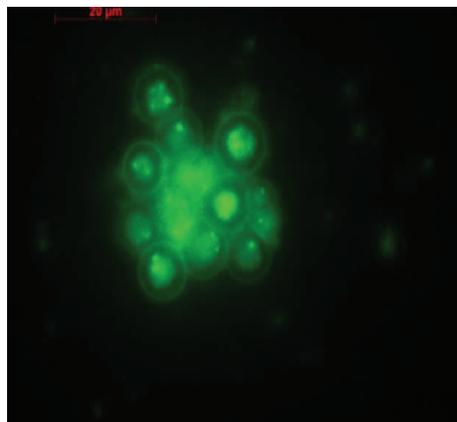
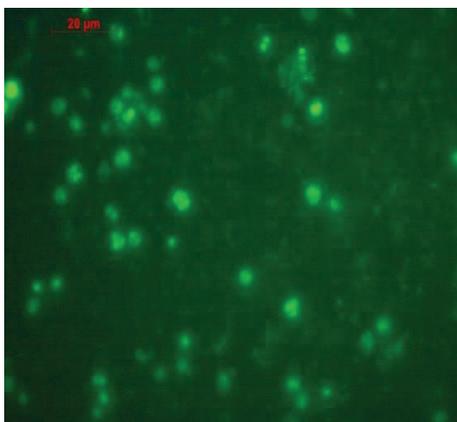
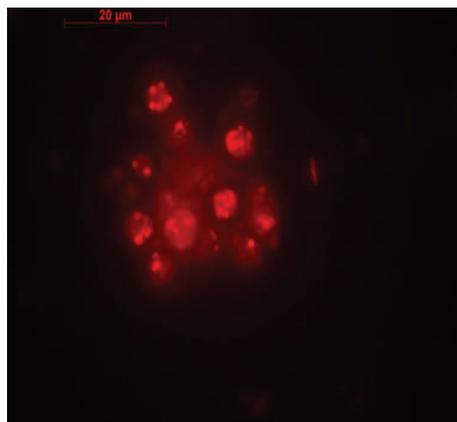
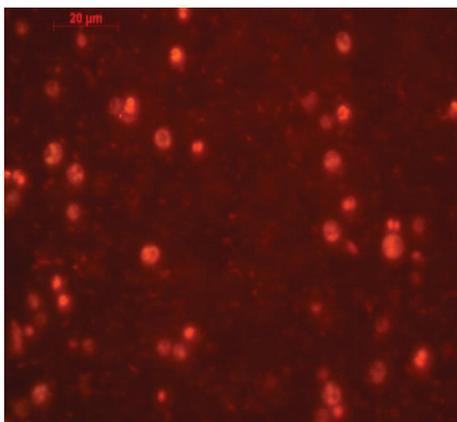
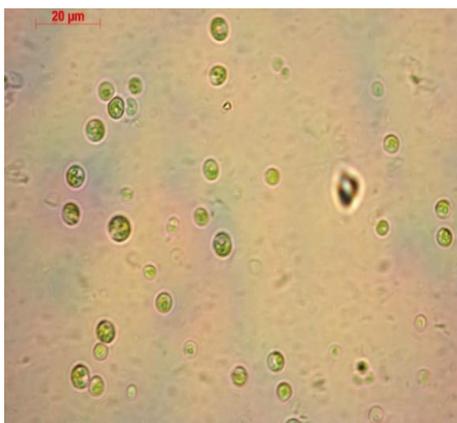


Figure 3. Bright field and epifluorescence images (green filter and red filter) of cells belonging to the purified strain 9.8.2

Figure 4. Bright field and epifluorescence images (green filter and red filter) of cells belonging to the purified strain 12.9.0

Further estimation of lipid content was done by measuring the emission fluorescence spectrum of isolates before and after Nile red labelling. In table 1 there are presented the results concerning the fluorescence emission intensity of the three isolates before- and after Nile red labelling.

Table 1. Fluorescence emission (arbitrary units) of purified strains (9.3.1; 9.8.2 and 12.9.0) in the absence and in the presence of Nile red (NR)

Strains	Fluorescence emission, without NR	Fluorescence emission, with NR
9.3.1	5.813	13.410
9.8.2	7.176	18.028
12.9.0	4.586	15.914

These results show that in the three selected strains, the fluorescence emission after Nile red addition is increased with different values: 7.597; 10.832 and 11.428 for strains 9.3.1, 9.8.2 and 12.9.0, respectively.

These differences represents the difference between fluorescence emission with Nile red and fluorescence emission without Nile red and are, probably, a expression of different lipid content of each strain.

CONCLUSION

Isolation, purification and selection of three clones of photosynthetic micro-organisms with estimated low starch content and, probably, different lipid content.

PERSPECTIVES

These isolates, as well as those to be obtained using the same method, are the biological material for further experiments concerning quantitative determination of biological parameters important for true candidates for lipid production.

These parameters concern lipid content and growth rate, both in the so-called normal conditions and under different type of stress (nitrogen or other nutrient limitation etc.) which, generally, increases the lipid content.

Furthermore, the selection method could be improved by the use of different strategies to decrease chlorophyll fluorescence signal which overlaps with red fluorescence of Nile red.

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EFFICIENCY OF POWDER INOCULUM AND MICROORGANISM ENCAPSULATION ON HYDROLYZATE SUGAR FERMENTATION OF NEWSPAPER CELLULOSE FOR BIOETHANOL PRODUCTION

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Abstract

*Immobilization of cells is a method for placing the microbial cells on a carrier material, wherein the catalytic activity of the cells is still high after storage for a long periode of time. The purpose of this study was to determine the optimum formula for the carrier material inoculum for bioethanol fermentation. The carrier material tested were based on different combination of wheat flour: rice flour, tapioca starch: corn starch and alginate. The study consisted of three phases: First phase is the preparation of the encapsulation of *Zymomonas mobilis*, *Kluyveromyces marxianus*, and *Saccharomyces cerevisiae*. The Second phase is the enzyme hydrolysis process and the third is fermentation of the hydrolysate into bioethanol by the consortium on the best carrier. The method used in this research is descriptive and experimental. Descriptive method carried out in the second phase. While the experimental method with completely randomized design is used in the research phase I and III. The consortium used were K1 (*Z.mobilis* and *S.cerevisiae*), K2 (*Z. mobilis* and *K marxianus*), and K3 (*Z. mobilis*, *S.cerevisiae* and *K. marxianus*). The data were statistically analyzed by ANOVA, and followed by Duncan Test in 5% significance level. The results showed that *S.cerevisiae* and *K.marxianus* on formula with a combination of wheat flour: rice flour is the best carrier material with cell density about $3,719 \times 10^{10}$ CFU / ml and $3,027 \times 10^{10}$ CFU / ml. Meanwhile, alginate is the best carrier for *Z.mobilis* with cell density about $3,576 \times 10^{10}$ CFU / ml. Best consortium in the process of bioethanol fermentation from cellulose waste is *Z.mobilis* and *S.cerevisiae* (K1), which have the highest ethanol concentration about 7.167%, the efficiency of fermentation about 61.2%, specific growth rate of 0,072 cells / hour, ethanol yield ($Y_{p/s}$) 0,23 g/g, microbial yield ($Y_{x/s}$) 0,33 g/g and a maximum ethanol productivity (q_p) of 0,58 g/g/h.*

Key words: Fermentation, Encapsulation, hydrolysis enzyme, carrier material, consortium.

INTRODUCTION

Bioethanol is one form of renewable energy, derived from biological sources, it is environmentally friendly because has a high oxygen content (35%) burning more completely and high value-octane, producing lower CO emissions (19-25%), (Kusumaningati et al., 2013). Waste paper with high cellulose contain can be used as raw material for bioethanol as it as a monomer constituent is glucose that can be fermented into ethanol. Based on the test results on the chemical composition of newspaper used which conducted in industrial engineering of agriculture faculty laboratories in Padjadjaran University, old newspaper used containing cellulose 43.17%, 27.18% hemicellulose, lignin 16.11%, 11.27% starch

content, and content of extractive 2,27%. In the manufacture of bioethanol, there are two processes that need to be considered, hydrolysis and fermentation. Hydrolysis is the process breaks down complex sugars into reducing sugars which classified as simple sugars. Fermentation is the process of breaking down sugars into alcohol and carbon dioxide which caused by the activity of microbial cells. Microorganisms that ferment bioethanol should be able to ferment the monosaccharide in the media. Microorganisms which most often used for bioethanol fermentation process is *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* and *Zymomonas mobilis*. However, the microrganisms that have relatively short lifetimes will be an obstacle in large-scale ethanol production, so we need a way to get catalytic activity of the microrganisms remains high in the long term. Immobilization of cells is a

method for placing of microbial cells in a certain space with a long period of time, in which the catalytic activity of the cells is still high. One of the cell immobilization technologies is dried culture. There are several techniques for obtaining dried cultures: spray-dried, freeze-dried and the powder culture. Methods powder culture is the most efficient method because it does not require elaborate preparation (Pumphan at al., 2013). Drying using vacuum drying is one method that can be applied for preservation of microorganisms in powder form. Method of drying by vacuum drying to maintain the viability of microorganisms when using the appropriate heating medium and performed under conditions appropriate to the nature of the dried microorganisms, (Goderska, 2012). Aside from the powder method of culture, there is also a method of encapsulation with alginate. The advantage of the alginate encapsulation process that alginate can form a semipermeable membrane that will protect the microorganisms from the environment is less supportive. The carrier is one of the success factors of immobilized cells (Devi, 2014), this study used a carrier material consists of a combination of wheat flour and rice flour, tapioca flour and a combination of corn flour and encapsulation with alginate. Three formulas of carrier material were considered to meet the requirements of the good carrier material nontoxic to the inoculant, the moisture capacity is relatively good, pH neutral, cheap, easily processed and available (El-Fattah et al., 2013). High starch content in the starch and corn flour will protect the microorganisms from high temperature during vacuum drying. The combination of different types of flours as a carrier material more effectively protect the microorganisms compared to one type of flour, because the nutrients in combination flour can support the growth of microbial cells more. Starch is composed of 70-80% amylopectin which has a high ability to bind water product, so that microbial cells are trapped and protected during the heating process will be higher (Alonso, 2016). Alginate has a high fiber and essential minerals that can be used as a carrier matrix of active compound (Goderska, 2012). The principles of encapsulation are that the nutrients and metabolites can be diffuse

through the membrane. The membrane serves as a barrier keep the contents of the cell and minimize contamination entering (Goderska, 2012). Until now, the uses of microorganism inoculum encapsulation is exclusive to the food products. It is expected from this study to be a significant step to help bioethanol entrepreneurs by providing ready-made microbial culture powder which can be stored longer and Its activities remains high.

MATERIALS AND METHODS

Materials

Materials used in this study are: alginate, CaCl₂ 2%; α -amylase; amyloglucosidase; hemicellulase; cellulase; Strains of *K. marxianus*, *S.cerevisiae*, and *Z. mobilis*, DNS reagent (3,5-dinitrosalicylic acid); potassium dichromate, Wheat flour, Rice flour, Tapioca flour, Cornstarch, YEPD (yeast extract peptone dextrose).

Methods

The method used in this research is descriptive and experimental methods carried out in a laboratory scale. This study includes three stages. Phase I is the stage of manufacture and encapsulation of microbial inoculant powder, III phase is the fermentation using immobilized microbial cells in the best carrier material. Research stages I and III were analyzed with experimentally. I phase is done with a completely randomized design (CRD), factorial design 3x3 with three replications. The first factor is microbial fermentation (M) and the second factor is the formulation of the carrier material (F). Phase III study conducted by completely randomized design (CRD) 3x7 factorial design with three replications. The first factor is a consortium of microbes in the carrier material (M) and the second factor is the sampling time (T) comprising of 11 degree. The results were then analyzed by analysis of variance (ANOVA). If they are different, we continue with Duncan Multiple Range Test at 5% significance level. This study includes three stages. The First Stage is manufacture and encapsulation of microbial inoculant powder, the second stage is the preparation of the substrate and hydrolysis process, and the third stage is a stage of fermentation using microbial

cells immobile in the best carrier material. Fermentation study conducted with a completely randomized design (CRD) 3x7 factorial design with three replications. The first factor is a consortium of microorganisms in the carrier material (M) and the second factor is the sampling time (T) comprising of 7 levels. The results were then analyzed by analysis of variance (ANOVA) and Duncan's Multiple Range Test at 5% significance level.

Preparation of inoculum Powders

Formula 1 is composed of rice flour and wheat flour and formula 2 consists of corn starch and tapioca flour with a ratio respectively of 50%: 50%. Carrier material is added sterile distilled water at a ratio of 1:10. Microbial suspension is added to the carrier material (1: 1). Cultures were then incubated for 6 days. Viability of microorganisms was performed every 2 days by counting the microbial population. Microbial cultures are then dried using vacuum drying at 45°C for 9 hours.

Encapsulation of Microbial cultures

The density of Microbial biomass suspension is 5 McFarland which has been calculated using total plate count (TPC) as the initial biomass. Furthermore, the microorganisms are suspended in 3 ml to 10 ml of sterile saline, then added 60 ml Alginate 3% (w / v). The mixture obtained is dripped into a solution of 0.1 M CaCl₂, thus forming a bead. The beads will harden within 15 minutes. Beads were washed with 0.85% NaCl solution and to reduce excess Ca ions and washed again with distilled water. Bead was stored at a temperature of 4 ° C and is ready for use as an inoculum.

Pretreatment of Newspapers used as cellulosic waste and Enzymatic Hydrolysis

Desizing process of newspaper used is done by soaking the paper in water, then dried in the sun and ground into powder paper with ± 40 mesh sizes. Then delignification process was made by adding 4% NaOH solution. The mixture is allowed to stand for 24 hours, and then rinsed with distilled water. Flour paper dried at 105°C for 6 hours was refined by grinding.

Enzymatic hydrolysis consists of liquefaction by α -amylase enzyme hydrolysis of

hemicellulose with hemicellulase, and saccharification with cellulase enzymes and amyloglucosidase combined. The paper flour was mixing with: α -amylase enzyme (0.52 mL/g), hemicellulase enzyme (0.001 g/g), cellulase enzyme (0.83 mL/g) amyloglucosidase doses (0, 56 mL/g) and a reducing sugar content set at 20% is used as a substrate for fermentation. Then into the substrate medium is added (NH₄)₂SO₄ 4% (w/v) and peptone 1% (w/v). A total of 200 ml of substrate is put into containers and sterilized.

Fermentation of hydrolyzate by Microbial Consortium in Materials Carrier Fermentation was conducted by SSF (Simultaneous Saccharification Fermentation) using microbial consortia in the best carrier at phase 1, which were a consortium I (*Z.mobilis* and *S. cerevisiae*), Consortium II (*Z. mobilis* and *K marxianus*), and consortium III (*Z. mobilis*, *S.cerevisiae* and *K. marxianus*). Inoculum in the form of powder is added as much as 10% (g/v) into the fermentation substrate. The mixture was fermented at a temperature of 28°C for 120 hours with agitation speed of 150 rpm. During the fermentation, samples were taken every 12 hours to measure parameters of a reducing sugar, ethanol and the number of microbial populations.

RESULTS AND DISCUSSIONS

Viability of the microorganism in the inoculum powder and encapsulated in alginate Formulation 1 was inoculum in a carrier material mixture of rice flour and wheat flour containing *S. cerevisiae*, *Z. mobilis*, and *K. marxianus*. Formula 2 was the inoculum in a carrier material mixture of cornstarch and tapioca starch containing *S. cerevisiae*, *Z. mobilis*, and *K. marxianus*.

Table 1. The viability of cells in culture inoculum powder in Formula 1 and Formula 2

Species of microorganism	Cell density (CFU/ml) of Microorganism in formula 1 (a mixture of rice flour and wheat flour)	Cell density (CFU / ml) of Microorganism in formula 2 (a mixture of corn flour and tapioca flour)
<i>S.cerevisiae</i>	3,36x10 ¹⁰	3,42 x10 ¹⁰
<i>Z.mobilis</i> ,	3,37x10 ¹⁰	3,17x10 ¹⁰
<i>K.marxianus</i>	3,63x10 ¹⁰	3,17x10 ¹⁰

Furthermore, each inoculum in the carrier material is incubated for 6 days, the incubation process aims to adapt and grow microorganisms in each formula. The results showed that all microorganisms can adapt well to the formula 1 and 2. It showed by the increasing number of microbial populations during the incubation process. According to Alonso (2016), a carrier material should also serve as a medium for microbial growth. After cultivated for 6 days, the inoculum powder was then dried using vacuum drying. High cell densities in the inoculum powder showed that microorganisms can adapt well in formula 1 and formula 2. After drying, the density of microbial cells decreased, this is caused by heating during the drying process, the water content in formula reduced carrier material and microorganisms that are not bound to the matrix formula death. Microorganisms with most decreased cell density, is *Z.mobilis* in formula 1 and 2, with the percentage of the decline reached respectively 73.9%, 82.6%. In formula 1 and 2 cell density for *K.marxianus* was decreased reached at respectively 36.9% and 38%. Meanwhile, *S.cerevisiae* in formula 1 had the lowest decrease in the cell density of about 20.6%, but in the formula 2, *S.cerevisiae* has decreased by about 50%. These results indicate that *Z.mobilis* is more vulnerable to heating process compared to *K.marxianus* and *S.cerevisiae*. Chosen alginate as the carrier, based on the preservation of microorganism cells in alginate can protect the microbial population and can be stored in a long time without reducing the microbial population (El-Fattah et al. 2013). In formula 3, initial Biomass (alginate) of *S. cerevisiae*, *Z.mobilis* and *K.marxianus* is 3.93×10^{10} CFU / ml; $3,903 \times 10^{10}$ CFU / ml; and $3,406 \times 10^{10}$ CFU / ml respectively. Once encapsulated, the cell density of *S.cerevisiae* was $1,81 \times 10^{10}$ CFU / ml; *K.marxianus* $1,934 \times 10^{10}$ CFU / ml; and *Z.mobilis* $3,674 \times 10^{10}$ CFU / ml. These results show that alginate can highly maintain the viability of microorganisms, especially in *Z.mobilis* with a cell density of $3,67 \times 10^{10}$ CFU / ml. This is the same conclusion with Yang et al. 2016. that the number *Z.mobilis* encapsulated in alginate able to maintain their viability. The results of cell viability in the carrier material are analyzed using Analysis of

Variance (ANOVA) and Duncan's Multiple Range Test (Table 2).

Table 2. Duncan's multiple range test results of cell viability in the carrier material in Formula 1, Formula 2 and alginate

Carrier Materials	Cell density of microorganisms		
	<i>S. cerevisiae</i>	<i>Z. mobilis</i>	<i>K. marxianus</i>
Wheat flour and rice flour (F1)	3.719 B c	1.673 A a	3.027 B b
Tapioca starch and corn flour (F2)	2.273 A b	0.995 A b	2.98 B a
Alginate (F3)	1.81 A a	3.576 B b	1.934 A b

Description: Capital letters are the same, read vertically, showed no significant difference ($p > 0.05$) and the same small letters, read to the horizontal direction indicates not significantly different ($p > 0.05$).

Table 2 shows that *S.cerevisiae* in the formulation of rice flour and wheat flour (F1) produces the highest cell viability which equal to 3.72×10^{10} CFU / ml, it also shows that the alginate *Z.mobilis* has a high viability is 3.57×10^{10} CFU / ml. While *K.marxianus* have high viability in all the flours but *K. Marxianus* not produce high viability in the alginate. From these results it can be seen that the combination of wheat flour and rice flour is the best carrier for *S. cerevisiae* and *K. marxianus* because it can maintain the viability of microbial cells during the process of preservation. Wheat flour and rice flour contains amylopectin amounted to respectively 83% and 75%. High content of amylopectin will help maintain the viability of microorganisms during the drying process using vacuum drying. Protein content in wheat flour also helps in the process of microbial protection. The existence of gliadin and glutenin proteins in the media can reduce heat conductivity. Interaction between flour and water to form gluten dough shaped matrix that is compact and has a strong structure that can trap and protect the cells during drying (Jobbehdar et al. 2013).

Ethanol Fermentation by Microbial Consortia in the Inoculum powder of Sugar hydrolyzate result of hydrolysis cellulose newspaper will be used as the substrate in the fermentation process. Hydrolysis and fermentation processes will be efficient and effective if will be implemented in on an ongoing basis without a long pause; it is often known as Simultaneous Saccharification and Fermentation (SSF). In this

study the fermentation of sugar hydrolyzate is fermented by *S.cerevisiae* and *K.marxianus* preserved in flour and rice, as well as *Z.mobilis* preserved in alginate. Levels of ethanol during the fermentation process of used paper can be seen in Fig. 1. Data were statistically analyzed using Analysis of Variance (ANOVA).

Figure 1. Levels of ethanol produced during fermentation.

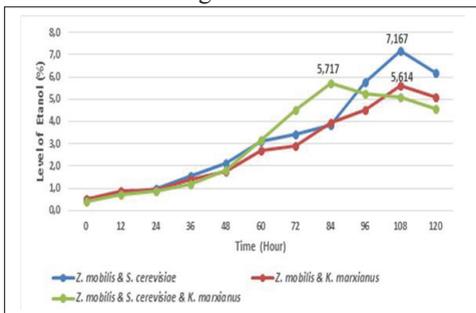


Table 3. Duncan's Multiple Range Test of the ethanol content (%) during the fermentation process of bioethanol from cellulose of a newspaper hydrolyzate.

Sampling Time	Consortium		
	<i>Z. mobilis</i> & <i>S. cerevisiae</i>	<i>Z. mobilis</i> & <i>K. marxianus</i>	<i>Z. mobilis</i> & <i>S. cerevisiae</i> & <i>K. marxianus</i>
0	0.517 A a	0.502 A a	0.415 A a
12	0.751 A a	0.854 A a	0.707 AB a
24	0.971 AB a	0.927 A a	0.883 AB a
36	1.572 BC b	1.411 AB b	1.176 AB a
48	2.143 C a	1.777 ABC a	1.791 B a
60	3.11 D a	2.685 BCD a	3.168 C a
72	3.447 DE a	2.89 CD a	4.501 D a
84	3.857 E a	3.959 DE ab	5.717 E b
96	5.746 F a	4.53 EF a	5.263 DE a
108	7.167 G b	5.614 F a	5.116 DE a
120	6.186 F a	5.102 EF a	4.589 DE a

Description: Capital letters are the same, read vertically, showed no significant difference ($p > 0.05$) and the same small letters, read to the horizontal direction indicates not significantly different ($p > 0.05$).

In the Table 3. The result showed that the longer the fermentation time, the concentration of ethanol produced is higher. This is because the number of cells and microbial activity is increasing as well, so the more the amount of sugar that is converted into ethanol. However, after 120 hours the ethanol content decreases, this is partly due to the sugar hydrolyzate as a nutrient is depleted. Ethanol concentration is highest at 108th hour, reaching 7.17%. Subsequently the ethanol concentration decreased after 120 hours which may result from ethanol began to be converted into organic acids and evaporation. According to Jayanti (2011) that ethanol fermentation results have a low level of concentration which is about 5-20%. If the ethanol concentration exceeds 15%, the ethanol will damage the cell wall and causing leakage of the plasma membrane, whereas according to Patterson and Ricke (2015) bacterial growth is inhibited by ethanol at a concentration of 10%. The use *Z.mobilis* and *Saccharomyces* sp. immobilized in agar-gelatin matrix will produce ethanol in the amount of 76 g / L in 48 hours of fermentation (Ryu, 1997). While according to Behera et al. (2012), the use of single-*Z.mobilis* cultured cells immobilized in alginate matrix will produce ethanol at 59 g / L. In addition, the fermentation using mixed cultures is considered more efficient than the fermentation using a single culture; this is due to the merger some enzyme activity to convert sugars to ethanol. Bioethanol fermentation by two cultures *Z.mobilis* and *Saccharomyces* sp. immobilized in agar-gelatin matrix, resulting in higher concentrations than ethanol fermentation by *Z.mobilis* and *Saccharomyces* sp. which is not immobilized. Reducing sugar content, cell density, and reducing the ability of microorganisms to ferment sugars during fermentation are the factors that determine the level of concentration of ethanol produced. The research showed that fermentation of reducing sugars of old newspapers cellulose by the consortium of *Z.mobilis* and *S.cerevisiae* produce microbial population, high ethanol concentration and low residual sugar. The results of the calculation of the efficiency of fermentation, showing that the consortium *Z.mobilis* and *S.cerevisiae* value fermentation efficiency 61.2%, with the value of the specific

growth rate (μ_{maks}) of 0072 cells / hour. This consortium also produces ethanol yield value results (Y_p / s) and biomass (Y_x / s) high of 0.23 g / g and 0.33 g / g. The maximum value of ethanol production (qp) was 0.58 g / g / j. This means that the fermentation of ethanol by a consortium *S.cerevisiae* and *Z.mobilis* is very efficient process. The results also showed that the microorganism population continues to rise, followed by a decrease in the concentration of reducing sugars proven that reducing sugar is used as a nutrient substrate for metabolic processes and cell formation. As the microbial population increases, then the ethanol content increased. *Z. mobilis*, produce enzymes including glucokinase and Fructokinase that convert sugar into ethanol via the Entner-Doudoroff (ED). ED pathway metabolizes glucose reduction via 2-keto-3-deoksi-6-phosphogluconate to form pyruvate; then pyruvate by pyruvate decarboxylase is converted into acetaldehyde which is then converted into ethanol (Eram and Ma, 2013). *Saccharomyces cerevisiae* it is capable to convert monosaccharides C-5 and C-6, such as glucose, fructose and galactose into ethanol. *Saccharomyces cerevisiae* has the enzymes as invertase and zymase that work together to transform sugars into ethanol. If sugar is available in the form of a disaccharide sugar, then the enzyme invertase will work and hydrolyze the disaccharide into monosaccharides. Furthermore, zymase enzyme will transform into alcohol and CO₂ monosaccharide (Azizah et al., 2012). Based on this research, it is known that the use of microorganisms encapsulated in a polysaccharide matrix and alginate capable of fermenting ethanol with quite high in the fermentation process for 5 days. Ethanol fermentation process using cell immobilization system gives better results comparing with the conventional because when using cell immobilization, product separation will be easier and cell stability can be maintained.

CONCLUSIONS

The combination of rice flour and wheat flour produces high viability in *S.cerevisiae* with cell density reaches $3,719 \times 10^{10}$ CFU / ml and in *K.marxianus* its cell density reaches $3,027 \times 10^{10}$

CFU / ml. While the 3% alginate is the carrier material for *Z.mobilis*, its produce high cell viability, reaching $3,624 \times 10^{10}$ CFU / ml. Consortium *Z.mobilis* and *S.cerevisiae* in the fermentation of bioethanol from waste paper produce the highest ethanol content is 7.17% with the value of fermentation efficiency reached 61.2%.

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A REVIEW ON THE ENZYMATIC INDICATORS FOR MONITORING SOIL QUALITY

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Abstract

Soil is a dynamic, living, natural system that is vital to the function of terrestrial ecosystems. Soil health is maintained by physical, chemical and biological factors. Physical factors include bulk density and soil porosity, indicators of soil compaction. Chemical factors (soil pH, inorganic nitrogen, available minerals and organic carbon content) provide information for the balance of soil solution and exchange sites. Biological and biochemical factors like microbial biomass, soil respiration, potentially mineralised nitrogen and activity of soil enzymes indicate the soil's ability to function or recover from disturbance such as climate change, pest infestation, pollution and human exploitation in agriculture. The enzymes play an important role in the decomposition and recycling of nutrients from dead plants and animal tissues, the fixation of nitrogen, the maintenance of soil structure and the inhibiting effects of pollutants. Therefore, the activity of soil enzymes can be used as an indicator of soil quality. This review is focused on the activity of some defining soil enzymes like β -glucosidases, cellulases, amylases, phosphatases, ureases, dehydrogenases, arylsulfatases and peroxidases, their importance in maintaining the soil health and their sources (plants, animals, organic compounds and especially microorganisms). It also offers information on a variety of methods developed to measure enzymes activity which can give relative information about the ecological status of soils.

Key words: biological indicators, enzymes, pollutants, soil quality.

INTRODUCTION

Soil is a dynamic, living, natural system which serves as a natural medium for growth of plants.

It can be defined by a selection of parameters according to their variability in time: stable (soil depth or granularity), relatively stable (salt content, the content of organic mass in soil, heavy metal contamination), relatively dynamic (pH, the content of nutrients), and dynamic (soil humidity, temperature, microbial activity and enzymatic activity) (Fazekašová, 2012).

One of the most important components of soil is soil organic matter due to its ability to maintain soil fertility and crop production, and to prevent soil degradation, erosion and desertification (Senesi et al., 2007). A key role in the decomposition of organic matter is played by the enzymes in soil. The main source of soils enzymes are microorganisms, but they can be found as well in plants and animals.

The purpose of this study is to evaluate the enzymatic activity of some enzymes in order to

describe and understand their role as a bioindicator of the soil health.

SOIL QUALITY

Soil quality is defined by its capacity to function, within land use and ecosystem boundaries, to sustain biological productivity, maintain environmental quality and promote plant, animal and human health (Doran et al. 1997). In order to assess the quality of soil, it must be used a unique balance of physical (texture, rooting depth, infiltration rate, bulk density, water retention capacity), chemical (pH, total C, electrical conductivity, nutrient level), biological and biochemical components (C and N microbial biomass, potentially mineralizable N, soil respiration, enzymatic activity) parameters (Gil-Sotres, 2005). The biological parameters are the primary indicators of soil health because they are sensitive to the changes that may occur in the presence of any degrading agent, while physical and chemical parameters alter only when the soil is subjected

to a drastic change in the environment (Filip, 2002).

Different agrochemicals are used in conventional agriculture, in last couple of decades, in order to help the farmer to minimize the economic losses caused by weeds, insects pests and pathogens. The extensive and improper use of this practices in crop production may lead to deterioration of soil quality, pollution of soil and water, loss of biodiversity and, the most important, increase incidence of human and ecosystem health problems (Baishya, 2015). Studies have shown that only 0.1 % of a pesticide is reaching the target organism, while the remaining bulk is reaching the soil, leading to disturbance of local metabolism, enzymatic activities and soil ecosystem (Sethi, 2016).

One of the consequences of conventional agriculture is soil degradation. The soil is subjected to a series of degradation processes which are linked to agriculture: erosion due to water, wind and tillage, compaction, declining soil organic carbon and soil biodiversity, salinisation and sodification and soil contamination (heavy metals and pesticides) (Baishya, 2015).

SOIL ENZYMES AS INDICATORS OF SOIL HEALTH

Activity of soil enzymes can be used as a soil quality indicator, due to the fact that are involved in energy transfer, release of inorganic nutrients for plant growth (C, N, P, S), organic matter decomposition, transformation of native soil organic matter, nitrogen fixation, detoxification of xenobiotics and the stabilization of soil structure (Utobo, 2014).

The health or quality of contaminated and remediated soils is evaluated by several representative enzymes such as: β -glucosidase, urease, phosphatase, dehydrogenase, peroxidase, arylsulphates, amylase, cellulose and peroxidase.

Cellulases

Cellulose is the most abundant structural polysaccharide of plant cell walls with β -1,4 -glucosidic linkages and represents almost 50% of the biomass synthesized by photosynthetic fixation of CO₂ (Eriksson et al., 1990). The breakdown of cellulose requires the synergic

action of a group of hydrolytic enzymes named cellulases. The degradation of soil organic matter from cellulose to glucose, is a result of the action of three important enzymes: endoglucanase (endo-1,4-D-glucanase EC 3.2.1.4), cellobiohydrolase (exo-1,4-D-glucanase, EC 3.2.1.91) and β -glucosidase (1,4-D-glucosidase, EC 3.2.1.21). Endoglucanase acts randomly, cleaving beta 1-4 glycosidic bonds within the cellulose molecule; the cellobiohydrolase removes cellobiose units from the ends of cellulose chains (Almeida et al, 2015). Meanwhile, the cellobiose molecules are cleaved by β -glucosidase, releasing glucose in the final cellulose breakdown process (Daroit, 2007), which provide a source of energy for decomposers (Gonnet et al, 2012). Cellulose from plant debris is degraded into glucose, cellobiose and high molecular weight oligosaccharides, releasing carbon as an energy source for use by the microorganisms (White 1982).

Cellulases are synthesized by a large diversity of microorganisms including both bacteria and fungi during their growth on cellulosic materials. The genera of *Clostridium*, *Cellulomonas*, *Thermomonospora*, *Bacillus*, *Trichoderma*, and *Aspergillus* are the most extensively studied cellulases producers (Kuhad et al., 2011).

Activities of cellulases in agricultural soils are affected by several factors like temperature, soil pH, water and oxygen contents (abiotic conditions), the chemical structure of organic matter (Deng and Tabatabai, 1994; Alef and Nannipieri, 1995), the addition of fungicides (Deng and Tabatabai 1994; Arinze and Yubedee 2000) and insecticides.

β -Glucosidase

Glucosidase (EC 3.2.1.21), a predominant enzyme in soils, plays an important role in catalysing the hydrolysis of various β -glucosides present in plant debris decomposing in the soil ecosystem. It is an important C energy source of life to microorganisms in soil (Chaea, 2017) and has the following soil functions: nutrient cycling (for plant growth), biodiversity and habitat, filtering and buffering (excess nutrients and toxic chemicals from the water), and soil structure.

β -Glucosidases are distributed among animals, plants, fungi, yeasts and bacteria (Veena et al.,

2011). Their activity has been observed in various plant species such as maize and sorghum (Verdoucq et al., 2003) and in microorganisms from roots of *Panax ginseng* plant (Zhang et al., 2001), *Penicillium purpurogenum* (Dhake and Patil, 2005), *Ceriporiopsis subvermispora* (Magalhaes et al., 2006), *Flavobacterium johnsonae* (Okamoto et al., 2000), *Trichoderma harzianum* type C-4 (Yun et al., 2001), *Lactobacillus plantarum* (Spano et al., 2005) and *Dyella koreensis* spp. (An et al., 2005).

Urease

Urease (EC 3.5.1.5) is one of the most efficient of all enzymes. This enzyme catalyses the hydrolysis of urea into carbonic acid and ammonia with a reaction mechanism based on the formation of carbamate as an intermediate (Tabatabai, 1982). In 1986, Burns has shown in his study that extracellular urease associated with soil organo-mineral complexes is more stable than urease in the soil solution. In addition he has discovered that urease extracted from plants or micro-organisms is rapidly degraded in soil by proteolytic enzymes.

Urease can be influenced by some soil factors such as cropping history, organic matter content, soil depth, management practices, heavy metals, temperature and pH (Yang et al., 2006).

They are synthesized by numerous organisms, including plants (*Canavalia ensiformis*, *Glycine max*, *Gossypium hirsutum*), fungi (*Aspergillus* sp, *Schizosaccharomyces pombe*), bacteria (*Bacillus pasteurii*, *Helicobacter pylori*, *Providencia* sp, *Staphylococcus* sp, *Klebsiella aerogenes*, *Aerobacter aerogenes*, *Proteus mirabilis*), algae (*Nitellopsis obtusa*) and invertebrates (Land snail *Otialactea*) (Krajewska, B., 2009).

Phosphatases

In soil ecosystems, these enzymes play critical roles in P cycles, possibly correlated to P stress and plant growth (Nannipieri et al, 2014).

Among the soil phosphatases, phosphomonoesterases (EC 3.1.3.1.) have been the most studied enzymes probably because they have activity both under acidic and alkaline conditions, according to its optimal pH, and because they act on low molecular P-compounds, including nucleotides, sugar

phosphates and polyphosphates (Makoi and Ndakidemi, 2008).

Microorganisms that produce phosphatases in soil includes soil fungi, particularly those belonging to the genera *Aspergillus* and *Penicillium*, along with *Pseudomonas* and *Bacillus* bacteria that produce mostly neutral phosphatase, while *Actinomycetes* produced only negligible quantities of phosphatases (Tarafdar and Chhonkar, 1979).

Dehydrogenases

Dehydrogenases (EC 1.1.1.) are a major group of the oxidoreductase enzymes class and they play a significant role in the biological oxidation of soil organic matter by transferring hydrogen from organic substrates to inorganic acceptors (Zhang et al., 2010). The soil dehydrogenases activity provides information on pesticide application, of oligoelements or of soil's processing management and on the biological activity and microbial populations in soil. These enzymes are produced by soil bacteria, genus *Pseudomonas*, with *Pseudomonas entomophila* as most abundant (Walls-Thumma, 2000).

Peroxidases

Peroxidases (EC 1.11.1) are involved in the breakdown of lignin, a component of the cell wall of plants and one of the most abundant organic polymers on Earth. The degradation of lignin contributes to soil pools of carbon and nitrogen and provides microorganisms with these essential nutrients (Sinsabaugh, 2010). Peroxidase can play a role in the detoxification of the soil as well, due to the fact they can help attenuate the toxicity of metal ions and phenolic molecules (Sinsabaugh, 2004). Peroxidase also is able to help decrease the harmful effects of reactive oxygen species that can accumulate in the soil (Sinsabaugh, 2010). Some white rot (*Basidiomycetes*) and soft rot fungi (*Ascomycetes*) produce extracellular peroxidases (Sinsabaugh, 2004).

Arylsulphatases

Arylsulphatases (3.1.6.1) are responsible for S cycling in soils because are involved in the mineralization of organic sulphur compounds to inorganic forms (SO_4^{2-}) for plant uptake (Tabatabai 1994). They are classified according to the type of the ester in: alkylsulphatases, steroid sulphatases, glucosulphatases, chondrosulphatases and myrosulphatases

(Tabatabai, 1982). Their presence in different soil systems is often correlated with the rate of microbial biomass, soil organic carbon content and rate of S immobilization (Mirleau et al, 2005). In soil they can be affected by various factors like pH changes, type and content of organic matter, heavy metal pollution and pollutants (Tyler 1981).

This group of enzymes are secreted by bacteria into the external environment (*Actinobacteria* sp., *Pseudomonas* sp., *Klebsiella* sp, *Aerobacter* sp. and *Raoultella* sp.), fungi (*Trichoderma* sp. and *Eupenicillium* sp.), plants and animals (Nicholls and Roy, 1971).

Amylases

Amylases (EC 3.2.1.) are classified in α -amylase (E.C. 3.2.1.1), β -amylase (E.C. 3.2.1.2) and glucoamylase (E.C. 3.2.1.3). Due to the fact that α -amylases are widely distributed in plants and soil, they play an important role in the breakdown of starch, converting starch like substrates to maltose, glucose and/or oligosaccharides. Instead, β -amylase converts starch limit dextrin and maltose (Thoma et al. 1971). In the end, glucoamylase hydrolyses maltose to glucose. The α -amylase can be found in microorganism such as bacteria from genus *Bacillus* (*Bacillus licheniformis*, *Bacillus stearothermophiles* and *Bacillus amyloliquefaciens*) (Padma, 2016) and fungal species such as *Aspergillusniger*, *A. oryzae*, *Thermomyces lanuginosu* and *Pencillium expansum*, plants and animals (Singh, 2016). β -amylase is synthesized mainly by plants (*Ipomea batatas*, *Arachis hypogaea* L.) (Hesam, 2015).

ENZYME ACTIVITY DETERMINATION

To give relative information about the ecological status of soil ecosystema variety of methods were developed to measure soil enzymatic activity.

The colorimetric determination of phosphatase activity involves the use of an artificial substrate, p-nitrophenyl phosphate (p-NPP). The p-nitrophenol is released by phosphatase activity after soil is incubated with buffered (pH 6.5) sodium p-nitrophenyl phosphate solution and toluene at 37° C for 1 hour (Tabatabai and Bremner, 1969).

In 1970, Tabatabai and Bremner have developed a colorimetric method for the determination of arylsulphatase. It is based on the determination of p-nitrophenol released after the incubation of soil with buffered (pH 5.8) potassium p-nitrophenyl sulfate solution and toluene at 37°C for 1 hour.

In order to determine cellulase activity in soils, it was developed a method that use 1 % carboxymethylcellulose (CMC) as a substrate, in acetate buffer (pH 5.9) and incubated for 24 hours at 37° C in order to determine the reducing sugar content in the filtrate (Deng and Tabatabai 1994).

Urease activity assay involves determination of the ammonium which is released by the enzyme after soil is incubated with tris (hydroxymethyl) aminomethane (THAM) buffer (pH 9.0), urea solution, and toluene at 37°C for 2 hour (Chae et al., 2017). The methods for the assay of amylase in soil was developed by Cole in 1977. They require the addition to the soil samples of toluene and acetate-phosphate buffer (pH - 5.5) containing 2% starch. The samples are incubated 24 hour at 30°C.

The activity of ligninolytic enzymes (peroxidase) was measured with the spectrophotometric assay, using L-dihydroxyphenylalanine (L-DOPA) as a substrat in the presence of hydrogen peroxide (Sinsabaugh et al., 1992).

The assay of β -glucosidase activity is based on the determination of the released p-nitrophenol after the incubation of soil with p-nitrophenyl- β -D-glucoside solution for 1 hour at 37° C. The colour intensity can be measured with a spectrophotometer at 400 nm (Tabatabai 1982). For the dehydrogenase activity, the most common laboratory procedure was the one developed by Casida in 1964. Dehydrogenase activity was determined with 2, 3, 5-triphenyltetrazolium chloride (TTC), after incubation at 30°C. The formed triphenylformazan has been extracted with an ethanol-acetic acid mixture and spectrophotometric evaluated at 540 nm (Wolińska and Stępniewska, 2012).

CONCLUSIONS

It is very essential to understand the activity of soil enzymes as a biochemical indicator of soil health.

Enzymes are very sensitive to major or minor changes in the environment and land management such as tillage, crop rotation, residue management, pollution. Thus they are closely related to ecological functions of soil such as biomass production, contaminated soil recovery, and ecosystem conservation.

The methods used in determination of soil enzyme activities are relatively simple, inexpensive and quick.

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SALT TOLERANCE OF BACTERIAL STRAINS ISOLATED FROM HYPERSALINE WATER LOCATED IN LOPATARI, ROMANIA

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Abstract

Saline waters are environments largely unexplored in which organisms are able to survive/withstand in the extreme conditions. The aim of this research was to explore saline water located in Lopătari, Buzău County, România. Several physico-chemical properties were assessed such as pH, density, concentration of cations and anions. The pH of water sample was slightly acidic (5.2). The ions of sodium, chloride and total dissolved solids were detected dominant $129.9 \text{ g}\cdot\text{L}^{-1}$, $209.83 \text{ g}\cdot\text{L}^{-1}$ and $113 \text{ g}\cdot\text{L}^{-1}$, respectively. A total of forty four (44) aerobic bacteria were isolated on culture media supplemented with 5%, 10%, and 15% NaCl, respectively. The isolates were screened by morphological criteria, Gram stain. All bacterial isolates were tested for their tolerance to different concentrations of salt in solid media. Two bacterial isolates (4.5%) has grown over a wide range of salt concentrations ranging from 0% to 12.5%, 17 bacterial isolates (38.6%) under salt concentration between 0% and 7.5%. Other 19 bacterial isolates (41%) demonstrated a large salt tolerance ranging from 5 to 25%. Halophiles can offer important opportunities in biotechnological applications such as food, pharmaceutical, detergents, environmental bioremediation and biosynthetic processes, being one of the main reasons for the future research of them.

Key words: bacterial isolates, hypersaline water, biochemical analysis, salt tolerance.

INTRODUCTION

In the last years, the research has been focused towards the detection of halophilic microorganisms with the ability to grow in saline environments and useful in different industrial applications (O'Brien et al., 2004; Enache et al., 2007; Enache et al., 2009; Oren, 2010; Neagu et al., 2014).

Hypersaline environments are typical extreme habitats distributed worldwide as hypersaline waters (natural or man-made salt lakes, the Dead Sea), salt mines, saltern pond brines or salted soils (Rodriguez-Valera, 1988; Ventosa, 2006; de la Haba et al., 2011).

Although, a lot of salted environments are located in Romania, only several works have been focused on the investigation of some halophilic microorganisms (especially bacteria and Archaea) isolated from few saline areas: Lake Telega (Enache et al., 2007), Salina Unirea (Cojoc et al., 2009), Balta Albă (Neagu et al., 2014) and Techirghiol (Enache et al., 2009).

The salt concentration of these ecosystems can influence the diversity of microbial communities. Non-halophiles grow optimally at less than 2% NaCl, slight halophiles grow optimally at 2–5% NaCl; moderate halophiles grow optimally at 5–20% NaCl; and extreme halophiles grow optimally above 20–30% NaCl (DasSarma and Arora, 2001).

The purpose of this research was to briefly explore the bacterial diversity from hypersaline water located in Lopătari, Buzău County, România and to assess salt tolerance of the bacterial isolates.

MATERIALS AND METHODS

Sample sources: Lopătari region is located in the Subcarpathians Curvature, in the Buzău County, România ($45^{\circ} 29'14.7'' \text{N}$ $26^{\circ} 37'24.8'' \text{E}$), in the basin of Slănic river. The study area is 56-60 km far away from Buzău and is part of the massive Sărule-Bisoca, which covers an area of about 57 hectares (Figure 1). About 1000 ml of surface water were collected during June 2016 in clean sterile bottles and stored at 4°C until use.

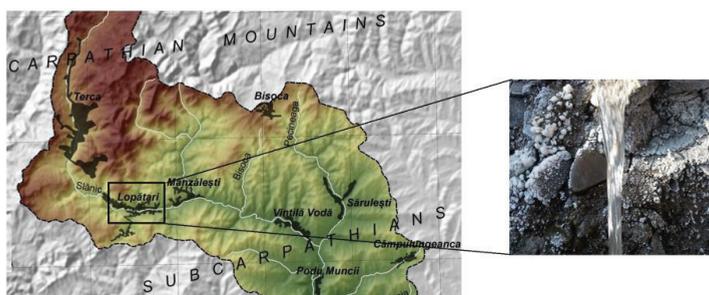


Figure 1 – Map site – hypersaline water streams located in the village Lopătari, Buzău County, România

Physico-chemical analysis of the samples included: pH using digital pH meter, Na^+ , K^+ by atomic absorption spectrometry (ISO 9964-1/1993), Ca^{2+} , Mg^{2+} by atomic absorption spectrometry (SR ISO 7980/1997), Cu^{2+} , Zn^{2+} by atomic absorption spectrometry (SR ISO 8288/2001), Fe^{2+} by atomic absorption spectrometry (SR 13315/1996) Mn^{2+} by atomic absorption spectrometry (SR8662-2/1997), TDS by electrochemical method (internal procedure) SO_4^{2-} by turbidimetry method (EPA 9038), Cl^- by volumetric method (SR ISO 9297/2001).

Isolation and identification procedure: To isolate **halophilic bacteria**, 50-100 mL were filtered using Millipore membrane filters (0.22- μm pore), and the membranes were placed on Nutrient Agar (Merck, Germany) supplemented with 5%, 10 %, and 15% NaCl, respectively. After incubation for several days at $36\pm 2^\circ\text{C}$, different colonies were selected and further purified on nutrient agar. The isolates were screened by classical methods (including cultural characteristics, microscopic morphology (cell shape and grouping, motility, Gram-stain reaction).

For the **halotolerance test**, a loop of bacterial cells from 24-48 h cultures was streaked in line

on the Nutrient Agar plate supplemented with different NaCl concentrations: 0 – 30%. After incubation at $36\pm 2^\circ\text{C}$ for 7 days, the ability of the bacterial isolates to grow in the presence of salt was monitored. All experiments were carried out independent, in duplicate.

RESULTS

Hypersaline water streams located in Lopatari, Buzău County, România were taken in account for this study This salt water is used frequently in home-preserved vegetables and fruits by local people.

As illustrated in Table 1, the pH of water sample was slightly acidic (5.2) in contrast to alkaline lakes, such as Balta Albă (pH 8.6) or Wadi Natrun Lake (pH 11).

The ions of sodium ($129.9 \text{ g}\cdot\text{L}^{-1}$) and chloride ($209.83 \text{ g}\cdot\text{L}^{-1}$) were detected at higher concentrations, with chloride value similar to the concentration of the Dead Sea. The total dissolved solids detected were $113 \text{ g}\cdot\text{L}^{-1}$.

Also, the potassium, magnesium and calcium ions were detected, but at low concentrations. Concentration of Fe^{2+} , Mn^{2+} , Cu^{2+} , Zn^{2+} ions were under LoQ (quantification limit= 0.1 mg/L).

Table 1 – Comparative physico-chemical properties of hypersaline waters

Source	pH	Density	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	Reference
Lopătari, România	5.2	1.19	129.9	0.17	1.7	0.28	209.83	3.89	In this study
Balta Albă, România	8.6	1.1	137	26	2	6	44	nd	Neagu et al., 2014
Great Salt Lake, SUA	7.7	nd	105	6.7	0.3	11.1	181	27	Grant, 2004
Dead Sea	6.3	nd	39.7	7.6	17.2	42.4	219	0.4	
Lake Magadi	11	nd	161	2.3	0	0	111.8	16.8	
Wadi Natrun, Zugm	11	nd	142	2.3	0	0	154.6	22.6	

Ions are represented as g per liter, density as cm^3 per g, n.d.- not determined

A total of 44 aerobic bacterial strains were isolated, from which 19 strains on culture media supplemented with 5% NaCl; 14 strains on culture media supplemented with 10 %, and 11 strains on culture media supplemented with

15% NaCl, respectively. 12 bacterial species were identified by classical methods. In addition, it was observed that the bacterial diversity decreases with the adaptability to high salt concentrations.

Table 2 – Salt tolerance of bacterial isolates

No.	Strains	NaCl concentrations									
		0%	2.5%	5%	7.5%	10%	12.5%	15%	20%	25%	30%
1.	10.5	+	+	+	+	-	-	-	-	-	-
2.	11.5	+	+	+	+	-	-	-	-	-	-
3.	13.5	+	+	+	+	-	-	-	-	-	-
4.	14.5	+	+	+	+	-	-	-	-	-	-
5.	24.5	+	+	+	+	-	-	-	-	-	-
6.	45.5	+	+	+	+	-	-	-	-	-	-
7.	46.5	+	+	+	+	-	-	-	-	-	-
8.	47.5	+	+	+	+	+	+	-	-	-	-
9.	48.5	+	+	+	+	+	+	-	-	-	-
10.	50.5	+	+	+	+	-	-	-	-	-	-
11.	51.5	+	+	+	+	-	-	-	-	-	-
12.	54.5	+	+	+	+	-	-	-	-	-	-
13.	55.5	+	+	+	+	-	-	-	-	-	-
14.	57.5	+	+	+	+	-	-	-	-	-	-
15.	58.5	+	+	+	+	-	-	-	-	-	-
16.	59.5	+	+	+	+	-	-	-	-	-	-
17.	60.5	+	+	+	+	-	-	-	-	-	-
18.	62.5	+	+	+	+	-	-	-	-	-	-
19.	63.5	+	+	+	+	-	-	-	-	-	-
20.	26.10	-	+	+	+	+	+	+	+	+	-
21.	27.10	-	-	+	+	+	+	+	+	+	-
22.	28.10	-	+	+	+	+	+	+	+	+	-
23.	29.10	-	-	+	+	+	+	+	+	+	-
24.	31.10	-	-	+	+	+	+	+	+	+	-
25.	32.10	-	+	+	+	+	+	+	+	+	+
26.	33.10	-	-	+	+	+	+	+	+	+	-
27.	35.10	-	+	+	+	+	+	+	+	+	+
28.	36.10	-	-	+	+	+	+	+	+	+	-
29.	37.10	-	-	+	+	+	+	+	+	+	-
30.	38.10	-	-	+	+	+	+	+	+	+	-
31.	39.10	-	+	+	+	+	+	+	+	+	+
32.	41.10	-	-	+	+	+	+	+	+	+	-
33.	65.10	-	-	+	+	+	+	+	+	+	-
34.	15.15	-	-	+	+	+	+	+	+	+	-
35.	16.15	-	-	+	+	+	+	+	+	+	-
36.	17.15	-	-	+	+	+	+	+	+	+	-
37.	18.15	-	-	+	+	+	+	+	+	+	-
38.	19.15	-	-	+	+	+	+	+	+	+	-
39.	20.15	-	-	+	+	+	+	+	+	+	-
40.	21.15	-	-	+	+	+	+	+	+	+	-
41.	22.15	-	-	+	+	+	+	+	+	+	-
42.	23.15	-	-	-	+	+	+	+	+	+	-
43.	25.15	-	+	+	+	+	+	+	+	+	-
44.	26.15	-	-	+	+	+	+	+	+	+	-

+/- (detection or not of bacterial growth on nutrient agar media supplemented with NaCl)

Salt tolerance of the bacterial isolates was tested on solid media with NaCl content ranging from 0 to 30%. All the bacterial

isolates were able to grow in the presence of 5-7.5% of salt (table 2). Among these strains, 17 bacterial isolates (38.6%) grow at salt

concentration up to 7.5% NaCl. In particular, 2 bacterial isolates (4.5%) showed tolerance up to 12.5% NaCl.



Figure 2 – Bacterial growth on culture media supplemented with 5%, and 25% NaCl

For 19 bacterial isolates (41%), the growth occurred over a wide range of salt concentrations ranging from 5 to 25% NaCl (Figure 2 and table 2). Others 3 bacterial isolates (approximate 7%) shown a large salt tolerance between 2.5% to 30% NaCl.

CONCLUSIONS

Extremes properties of the hypersaline waters located in Lopătari, România (e.g. pH low, high sodium and chloride concentrations) determined an especially interesting bacterial diversity. Of a total 44 bacterial strains isolated in this study, 4.5% demonstrated a salt large tolerance ranging from 0% to 12.5%; 7% ranging from 2.5% to 30%; 41% ranging from 5 to 25%, respectively. The ability of several bacterial isolates to grow in a very wide salt concentration can be of great importance for biotechnological future applications. Several bacterial isolates will be identified by molecular methods and tested for their antagonistic or enzymatic activity.

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CAMELINA SATIVA OIL-A REVIEW

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Abstract

Camelina sativa is an oil seeded plant belonging to the Brassicaceae family. It can be cultivated both in winter and spring season, having a remarkable capacity to adapt and resist to difficult climate conditions. Moreover, Camelina crop has shown resistance to pests and diseases which affect other crops from the same family. The synthesis of phytoalexins seems to be responsible for the unusual camelina defense system. Camelina oil is the main product resulted by extraction from seeds. The most common extraction methods are: mechanical extraction, solvent extraction and enzymatic extraction. Recently it has been considered also the supercritical-CO₂ extraction. The oil obtained contains an unsaponifiable fraction represented by tocopherols, sterols and a saponifiable fraction consisting in fatty acids. The fatty acids profile is mainly represented by unsaturated fatty acids- mono and mostly polyunsaturated (>55%) and saturated fatty acids (9.1-10.8%). The most frequent fatty acids from camelina oil are linolenic, linoleic, oleic and eicosenoic. In comparison with other Brassicaceae plants, camelina oil has a low content of erucic acid. Camelina oil, due to its composition, has multiple uses in various industries: feed technology for substitution or supplementation of other oils (fish, broilers) in animal diets, biodiesel production, jet fuel production, biopolymer industry (peel adhesion properties, paints, varnishes), cosmetic industry (skin-conditioning agent), in food products due to its high omega-3 fatty acid content and low erucic acid content and as milk fat substitution.

Key words: Camelina oil, extraction, fatty acids

INTRODUCTION

Camelina, scientifically named *Camelina sativa* is a dicotyledonous oil seeded plant belonging to Brassicaceae family. It is originated from Southern Europe and South-West of Asia (Dobre et al., 2014; Vollmann et al., 2015; Berti et al., 2016).

Morphologically, camelina has a smooth or hairy stem, whose height may be between 65 and 110 cm. The stem is ramified and as it reaches maturity, it becomes woody. The arrow-shaped leaves, with smooth to wavy edges, are 5-8 cm long. Most of the flowers, with 5-7 mm diameter, are autogamous. The very small seeds are kept in silicles (Berti et al., 2016).

Archeological evidences proving camelina existence in Europe show that its cultivation was performed since 4000 (BCE). In Romania, the camelina use is associated with the period of transition from Eneolithic to Bronze Age (Berti et al., 2016).

Camelina is an annual crop which can be cultivated both in spring and in winter seasons (Gesch and Archer, 2013, Dobre et al., 2014

Berti et al., 2016), winter varieties showing a high resistance to harsh climate conditions (Berti et al., 2016).

Camelina cultivation requires low water and fertilizers compared with other crops from the same family and the crop is more resistant to common pests and pathogens which usually affect similar crops (Kim et al., 2015). Its resistance may result due to synthesizing of phytoalexins (Vollmann et al., 2001). Moreover, it was noted the adaptability of camelina to different medium conditions (Berti et al., 2016) and its capacity to grow in marginal lands (Kim et al., 2015, Petre et al., 2015).

In Romania, both the climate and the soil are favorable for camelina cultivation (Imbrea et al., 2011).

CAMELINA OIL EXTRACTION

The principal camelina oil seed extraction methods are: mechanical extraction, solvent extraction and enzymatic extraction (Atabani, 2013).

The oldest method used for oil extraction from seeds is hydraulic pressing. It was first used in Europe, in 1795. This method was replaced, in time, by the extraction using screw presses (Rosenthal et al., 1996). Nowadays, manual ram press or engine driven screw are the most frequently used (Atabani et al., 2013).

The mechanical extraction doesn't require special resources, but the oil obtained must be subjected to additional processing like filtration and degumming (Atabani et al., 2013).

Another important aspect to be taken into consideration when choosing mechanical extraction is the fact that the equipment is adapted to certain types of seeds, which has an impact over the extraction yield. The seeds pre-treatment have also an impact on the extraction (Atabani et al., 2013).

The solvent extraction was first used in 1870, in Europe. In its beginnings the method was rarely used, but with time it has become a large scale process. The method can be used to complete the mechanical extraction (Rosenthal, 1996).

Using solvent extraction, a high yield may be obtained, making it a favorable option for oil production at large scale. Several factors, such as particle size, extraction solvent, temperature, stirring may influence the yields obtained with this method (Atabani et al., 2013). Moser et al. (2010) obtained a yield of 30.5 wt% oil from dried seeds with hexane extraction by Soxhlet method for 24 h.

The enzymatic extraction does not produce neither pollutant solvents, harmful for the environment, nor chemical substances dangerous for human health, which is considered a big advantage. On the other hand, one of the disadvantages of this method is the fact that it is time consuming (Atabani et al., 2013).

Several techniques can be used for the extraction of camelina oil such as: warm or cold mechanical pressing extraction, organic solvent extraction, supercritical CO₂ extraction (Berti et al., 2016).

Berti et al. (2016) have reported a higher yield when extracting camelina oil through Soxhlet method compared with supercritical-CO₂ extraction, respectively cold mechanical pressing.

Belayneh et al. (2015) compared the composition of oil obtained using 3 different extraction methods: classical extractions by Soxhlet and cold pressing and supercritical-CO₂ extraction. The extraction yields obtained were: 35.9%-Soxhlet extraction with hexane during 6 hours, 29.9%-cold pressing extraction, respectively 31.6%- supercritical-CO₂. The last mentioned method showed a high efficiency regarding oil recovery-88% of the quantity recovered normally with hexane extraction. There were no significant differences between the compositions of the three oils.

The supercritical-CO₂ extraction advantages are: supercritical-CO₂ is a cheap and easy to find (Moslavac et al., 2014) green solvent (Belayneh et al., 2015), it is easy to remove from products, it allows solvent recovery consecutive to other extraction methods. The main disadvantage of this method is the high price of the required equipment (Moslavac et al., 2014).

The main advantage of cold pressing is that it doesn't require organic solvents. The oil obtained retains the valuable bioactive compounds such as: fatty acids, phenols, flavonoids, tocopherols, minerals, fibers and many others (Moslavac et al., 2014). One of this method disadvantages is the low extraction efficiency compared to the classical solvent extraction.

The high quantity of antioxidants (tocopherols) in the raw oil is a major advantage regarding its time stability. Raw oil has a life span of 12-24 months compared with the one highly processed through refining, bleaching, deodorization, which has a life span of 6-9 months (Berti et al., 2016).

Cold pressing is affected by temperature, frequency and press nozzles size. Researchers have tried to optimize the camelina oil extraction by cold pressing. The best results were obtained when using a temperature of 52°C, a frequency of 20 Hz and nozzle size of 9 mm. In this optimal conditions, 289.5 ml of camelina oil with a temperature of 32.6 °C were obtained (Moslavac et al., 2014).

Mechanical extraction with a yield over 60% can be done with manual or engine mechanical presses (Atabani et al., 2013).

The unrefined camelina oil has a clear-yellow color and a broccoli like flavor, while refined oil has no smell and has a pale-yellow color (Atabani et al., 2013).

CAMELINA OIL COMPOSITION

In the camelina oil composition can be distinguished two fractions: one unsaponifiable (tocopherols, sterols) and another saponifiable (fatty acids).

Most of the camelina oil applications are possible due to its fatty acids content.

The fatty acids profile is mainly represented by unsaturated fatty acids- mono and principally polyunsaturated (>55%) and saturated fatty acids (9.1-10.8%). (Toncea et al., 2013)

Many researchers have found that the major component in the camelina oil composition is the omega-3 fatty acid- linolenic acid. Also almost the same percentages of linolenic, linoleic, oleic, eicosenoic and erucic acids were reported following studies conducted by different researchers, as it is shown in Table 1 (Shukla et al., 2002; Abramovič and Abram, 2005; Moser et al., 2010; Jurcoane et al. 2011; Toncea et al., 2013; Katar, 2013; Belayneh et al., 2015).

Table 1. Camelina oil fatty acids profile reported by different researchers

Compound	Content	Reference
C18:3-linolenic acid	30.5-50.3 %	Toncea et al., 2013
	32.6 %	Moser et al., 2010
	35.6%	Jurcoane et al., 2011
	28.0-33.4%	Katar, 2013
	32.8-33.0%	Belayneh et al., 2015
	38.1%	Shukla et al., 2002
	35.2%	Abramovič and Abram, 2005
C18:2-linoleic acid	16.6%-19.3%	Toncea et al., 2013
	19.6 %	Moser et al., 2010
	20.9%	Jurcoane et al., 2011
	18.5-22.4%	Katar, 2013
	18.3-18.5%	Belayneh et al., 2015
	16.0%	Shukla et al., 2002
	16.9%	Abramovič and Abram, 2005
C18:1-oleic acid	14.9-15.5%	Toncea et al., 2013
	18.6 %	Moser et al., 2010
	16.3%	Jurcoane et al., 2011
	15.1-17.0%	Katar, 2013
	15.7-15.9%	Belayneh et al., 2015
	18.7%	Shukla et al., 2002
	17.4%	Abramovič and Abram, 2005
C20:1-eicosenoic	15.2-17.5%	Toncea et al., 2013
	12.4 %	Moser et al., 2010
	No report	Jurcoane et al., 2011
	13.8-14.5%	Katar, 2013
	14.9-15.1%	Belayneh et al., 2015
	11.6%	Shukla et al., 2002
	14.9%	Abramovič and Abram, 2005
C22:1-erucic acid	1.6-4.2%	Toncea et al., 2013
	2.3 %	Moser et al., 2010
	1.6 %	Jurcoane et al., 2011
	2.9-3.9%	Katar, 2013
	3.3-3.5%	Belayneh et al., 2015
	2.5%	Shukla et al., 2002
	1.6%	Abramovič and Abram, 2005

Camelina oil has a high content of eicosenoic acid, which is rarely found in plants. This makes camelina a source of Medium Chain

Fatty Acid (MCFA), which in Europe, are now obtained only from palm and coconut oils (Righini et al., 2016). MCFA are known for

their positive action on metabolic disorders of lipids, by suppressing the fat depositions and oxidation in animals and humans (Nagao and Yanagita, 2010).

On the other hand, camelina oil has a low content of erucic acid, which may be an advantage considering that this acid is known to produce cardiac damage by its capacity to increase the triglycerides and free fatty acids (Pasini et al., 1992).

Tocopherols (vitamin E) and sterols are minor lipid components in camelina oil (Belayneh et al., 2015).

Regarding the sterol profile, the following compounds have been identified in the camelina oil: cholesterol, brassicasterol, campesterol, sitosterol, Δ^5 -avenasterol. Camelina oil has a high content of cholesterol compared with other commercial oils. The presence of brassicasterol is another particularity of camelina oil, since it can be found in few other sources (Shukla et al., 2002).

The sterols content reported by different authors was between 3600 mg/kg and 5900 mg/kg (Belayneh et al., 2015).

The total tocopherol content reported by different researchers was between 634 and 780 mg/kg. (Belayneh et al., 2015)

CAMELINA OIL USES

Camelina oil has many applications in different fields. The most explored application of camelina oil is the biofuel production (Berti et al., 2016, Goómez-Monedero et al., 2016). Nonetheless, the camelina by-products, such as camelina meal or cake are valorized especially for animal feed (Hixson și Parrish 2014, Hixson et al., 2014, Jaśkiewicz et al., 2014, Woyengo et al., 2016). The possibility of using camelina in its entirety makes it advantageous from an economical point of view (Righini et al., 2016). The main areas of research for camelina uses are: biofuel production, animal feed, biopolymer industry.

Biofuel production

Camelina oil is mainly used for biodiesel production. Also it is a viable choice for the jet fuel production. The jet fuel obtained from camelina oil tested versus the classical one

proved no degradation of the engines and its use resulted in lower soot and carbon monoxide emissions (Berti et al., 2016). For biofuel production, it was tested also the use of camelina meal, which by pyrolysis may lead to high energy liquid fuels with increased stability due to low oxygen content. (Goómez-Monedero et al., 2016)

Animal feed

Hixson and Parrish (2014) have tested the replacement of fish oil and fish meal used in aquaculture Atlantic cod diet with camelina oil and meal. The high content of omega-3 and omega-6 fatty acids, with a higher percentage of omega-3 acids, especially α -linolenic acid (30%) (Hixson și Parrish 2014, Hixson et al., 2014) from the camelina oil and the high level of raw protein from the meal make them appropriate to be used as replacements of fish oil and meal. The results of the research have shown, a decrease of weight of the cod fed with camelina oil, compared with the one fed with fish oil and even more the lowest weight was registered for those individuals for which together with the replacement of fish oil it was replaced a part of the fish meal used in their diet. Moreover, it was observed a change in the lipids profile (Hixson și Parrish 2014). Another study performed on Atlantic salmon showed an increase of total lipid content for the fishes fed with camelina oil as a replacement of the fish oil (Hixson et al., 2014).

A different reasearch whose aim was to compare the effects of supplementing the broilers' diet with three types of oils: camelina, soy and rape, showed also that camelina oil as a supplement in the diet leads to the increase of α -linolenic acid in the muscles and in the abdominal fat. Both, the content of fatty acids and the one of α -linolenic acid from the muscles were higher when the diet was supplemented with camelina oil compared to rape oil (Jaśkiewicz et al., 2014).

Camelina meal, a by-product of the seed pressing has a high content of oil rich in α -linolenic acid, which makes it appropriate for feed products. It may be a choice for poultry diet. (Woyengo et al., 2016) Due to its composition, camelina meal is a source of aminoacids and esential fatty acids oils. (Berti et al., 2016; Woyengo et al., 2016)

Use in biopolymer industry

Nowadays, there is a growing tendency for the use of vegetable oils in the petrochemical-based polymer industry, due to the fact that they are renewable environmentally friendly compounds. The epoxidized camelina oil showed peel adhesion properties, which makes it appropriate, after further formulation, for preparation of pressure sensitive adhesives. The adhesion properties of camelina oil may be increased when it is combined with other epoxidized oils such as soybean oil. In addition epoxidized camelina oil has the potential to be used as coating or resin (Kim et al., 2015).

Camelina oil may be used in combination with glycerol and epoxidized vegetable oil for the synthesizing of alkyd resins for manufacture of paints and varnishes (Nosal et al., 2016).

Camelina oil in cosmetics

The CIR (Cosmetic Ingredient Review) assessed the *Camelina sativa* seed oil and the hydrogenated *Camelina sativa* seed oil as safe to be used in cosmetics and personal care products, after conducting tests for skin irritation, allergenicity, phototoxicity, photoallergenicity and mutagenicity. These compounds, according with the EU Regulation, are allowed to be used in products marketed in Europe. In cosmetics, the above mentioned oils act as skin-conditioning agents-emollient or skin-conditioning agents-occlusive (Burnett and Fiume, 2011).

Human nutrition

In human nutrition camelina oil is valuable due to its omega-3 high content (Vollmann et al., 2001). The low content of erucic acid represents an advantage when using camelina oil in human diet, considering that the ingestion of high quantities of erucic acid are thought to be responsible of cardiac lipidosis (Vollmann et al., 2015).

Camelina is known to have the capacity to reduce serum triglycerides and cholesterol. Also, due to its fatty acids profile the camelina oil may be used in human diet as nutraceutical: in salads, for cooking, in margarine with enriched content of omega-3 fatty acids, in salad dressings, mayonnaise, ice cream (Abramovič and Abram, 2005).

Recently Faustino et al. (2016) have used camelina oil to obtain human milk fat replacement for babies. Due to its high polyunsaturated fatty acids content, with omega-3 fatty acid as the major compound, camelina oil can be used in food products.

CONCLUSIONS

Considering its high adaptability and its low requirements, camelina is a low input crop with multiple benefits.

Due to its fatty acids profile, camelina oil has an immense potential for various applications.

Until now, the main area of research for camelina oil applications is the biofuel production. The results obtained are promising. Other applications of camelina oil are: replacement or supplement of animal feed, applications in biopolymer industry, cosmetic uses, human milk fat replacement, and addition in food products.

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PRODUCTION AND OPTIMIZATION OF EXTRACELLULAR AMYLASE FROM A NEWLY ISOLATED STRAIN OF *Bacillus mycoides*

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Abstract

Microbial enzymes are known to be superior to enzymes obtained from other organisms, particularly for applications in industries on commercial scales. The species of the genus *Bacillus* are known to be producers of enzymes of industrial interest. Among them, amylolytic enzymes have got great biotechnological applications and economic exploitations. Amylases are known to be produced by a variety of bacteria and fungi and their applications at industrial level have stimulated interest to explore their amylolytic activity in several microbes to be used as bioresources.

A newly soil-isolate, identified as a *Bacillus mycoides* strain, was tested for its ability to produce extracellular amylase in liquid media, using multiple carbon sources and starchy substrates. Chip electrophoresis was used to obtain the electrophoretic profile of proteins derived from the bacterial isolate and a molecular weight of 60 kDa, characteristic for amylase produced by *Bacillus* genus, was obtained in two experimental media. Bioprocess optimization was designed using L9 and L16 Taguchi orthogonal arrays and analyzed by ANOVA statistical methods. A maximum enzymatic activity (10.44 U/ml) was determined when malt extract and ammonium sulphate, as starchy substrate and nitrogen source, were used. Optimum growth conditions were identified to be 32°C, 220 rpm and 48 hrs fermentation time, while the inoculation volume was 2%. A positive effect for amylase production was observed for citric acid and CaCl₂ interaction in the culture media.

Key words: *Bacillus mycoides*, amylase, biosynthesis, optimization, Taguchi.

INTRODUCTION

Enzymes are considered an indispensable component in biological reactions; these catalysts are highly specific, faster than chemical catalysts and environmentally safe. They can be produced using natural sources and this is a valuable fact in order to achieve a sustainable development (Sundarram et al., 2014). Microbial enzymes generally meet the industrial demands (Naidu and Saranraj, 2013); they are easy to obtain in high amounts with a low-cost and short time production (Gopinath

et al., 2017). Nowadays, amylases are one of the most studied groups of enzymes in biotechnology. A large number of amylases are commercially available and they have been industrially exploited for many decades, due to their biodegradability, which makes the processes cleaner for the environment. (Monteiro de Souza and Magalhaes, 2010, Naidu and Saranraj, 2013; Sundarram et al., 2014; Singh et al., 2015). The history of amylases dates since 1811, when Kirchoff discovered the first enzyme of this type. In 1930, Ohlsson made the first suggestion regarding the amylase classification and he

grouped them in α - and β -amylase, respectively according to the sugars type produced by the starch digestive enzyme reaction (Naidu and Saranraj, 2013).

Microbial amylases were predominantly used in scientific research and in industrial sectors. Apparently, the primary enzyme produced for industrial level (in 1984) was an amylase which had a fungal basis; its role was to treat digestive disorders (Shanmugasundaram et al., 2015). A wide range of bacterial and fungal species were isolated and used in various industrial applications. Terrestrial isolates, such as *Aspergillus* sp. (*A. oryzae*, *A. niger*, *A. awamori*, *A. fumigates*, *A. flavus*) and *Penicillium* sp. or species belonging to the *Bacillus* genus (*B. subtilis*, *B. coagulans*, *B. polymyxa*, *B. mesentericus*, *B. megaterium* and *B. cereus*) are very well known for their commercial uses in this field (Oyeleke et al., 2010; Gopinath et al., 2017).

Amylases constitute approx. 25% of the world enzyme market; the scientific world recognizes the great biotechnological significance of these starch-amylolytic degrading enzymes, which almost completely replaced the chemical hydrolysis of starch in industrial processes (Monteiro de Souza and Magalhaes, 2010; Naidu and Saranraj, 2013). Amylase applications have expanded in many other fields, such as: clinical, pharmaceutical, analytical chemistry, foods, detergents, textile, paper and distilling industries (Monteiro de Souza and Magalhaes, 2010; Gopinath et al., 2017). All amylases act on starch and yield small units of glucose and maltose (Gopinath et al., 2017), but the most widely used amylases are α - and β - subtypes, which act on α -1,4-glycosidic bonds. α -amylases catalyze starch hydrolysis through the cleavage of α -1,4-glycosidic bonds (they are able to cleave those bonds which are present in the inner part of amylose or in the amylopectin chain); the end products are oligosaccharides with variable length (mixtures of maltose, maltotriose and glucose units) (Monteiro de Souza and Magalhaes, 2010; Riaz et al., 2013). β -amylases act on the nonreducing end of a polysaccharide chain and yield successive maltose units (Sundarram et al., 2014).

Mesophilic microorganisms are the most popular producers used in industrial enzymes

application and the *Bacillus* genus is known as producer for amylase and protease with a significant industrial importance.

The production of bacterial amylases is usually carried out by submerged fermentation (SmF), in which natural and cheap substrates are preferred. Also, this type of process permits to optimize parameters like pH, temperature, incubation time, nutrient concentrations in an easier manner. Microbial amylases which are commercially available don't require complex downstream processing, because they can be used as crude preparation (except medical and pharmaceutical fields) (Monteiro de Souza and Magalhaes, 2010).

They are indispensable biological components in hydrolysis reactions with numerous advantages (especially for the starch industry). Therefore, new microorganisms with biotechnological potential regarding amylase production with new or improved properties are required.

The present study is focused on the *Bacillus mycoides* amylase production, starting from common methods for screening and continuing with process optimization. SmF is advantageous, because is an easily controlled process to control and also, depending on the strain and culture conditions, the enzyme can be inducible showing different producing patterns.

MATERIALS AND METHODS

Isolation of the bacterial strain

Bacterial isolate was obtained using standard pour plate method into nutrient agar (NA). 1 g of each freshly collected soil sample, from Dambovita County, was mixed with 9 ml of sterile distilled water for serial dilutions. The poured plates were incubated at 32°C for 48 hrs. Pure isolate was maintained on NA slants and stored at 4°C, for further studies.

Screening for amylase production

The bacterial strain was tested for amylase activity by employing zone clearing technique using nutrient agar medium supplemented with 1% soluble starch. The use of starch nutrient agar and iodine for detecting amylase producing microorganisms and the presence of clear zones surrounding microbial colonies as

evidence of starch hydrolysis have been reported since 1974 by Iverson and Fogarty, respectively (Singh et al., 2015). Inoculated plates were incubated at 32°C for 48 hrs and then they were checked for amylase production by addition of Lugol's solution (1% iodine in 2% potassium iodide, w/v). Clear zones of starch hydrolysis surrounding the colonies were measured (blue colour was evidence of negative results) (Lamabam et al., 2010; Sirohi and Prakash, 2015), two colonies showing large halo zones were selected for further studies.

Identification of the bacterial isolate

The microbial strain was identified using MALDI-TOF mass-spectrometry (MS) – a "shotgun" type proteomics technique for direct protein fingerprinting of bacteria. Based on the specificity of the mass spectrum for a large number of bacteria, fungi and yeasts, the dedicated MALDI Biotyper software identifies microorganisms by analysing the expression of the most abundant ribosomal proteins from the acquired mass spectra. The pattern of ribosomal protein expression is automatically compared by the software with a large number of reference patterns from its database. MALDI Biotyper analysis generates a characteristic mass and intensity distribution of those proteins and uses them to identify unknown samples by comparing their "fingerprint" with the patterns included in its open database (Tomulescu et al., 2015).

Amylase production using submerged fermentation (SmF)

Inoculum preparation – the isolate, 48 hrs actively growing culture, was used to inoculate 50 and 100 ml of IA medium (nutrient broth with 2% soluble starch), which were prepared in 500 ml Erlenmeyer flasks. IPS medium composition (g/L) was used for submerged fermentation: glucose 10.0, yeast extract 2.0, KH₂PO₄ 2.0, citric acid 1.0 and MgSO₄*7H₂O 0.5 and pH 7.5. After inoculation, the flasks, were incubated at 32°C for 24 hrs on a rotary shaker (220 rpm).

Fermentation media contained various sources of carbon (cornflour, starch, glucose, maltose, malt extract) and nitrogen (ammonium sulphate and peptone) in different concentrations. Also, calcium chloride was used as an enhancer for

amylase production. Inoculation volume was tested for 1, 2, 3, 10 and 20%. Cell growth and enzyme activity were studied taking into account the incubation period and the content of reducing sugars in production media. The classical method for medium optimization – changing one independent variable at a time and statistical experimental designs - L9 and L16 Taguchi orthogonal arrays, were applied to investigate the effects of different factors and interactions, on the amylase enzymatic activity; the significance of the effect of each factor and interaction was determined by ANOVA analysis. Trials were performed in duplicates or triplicates and the averages of amylase activity results were treated as responses.

Amylase assay. The extracellular enzyme solutions were obtained by centrifugation at 5000 rpm for 20 minutes at 4°C, using a high speed centrifuge. The cell free supernatants were collected and used to demonstrate the amylase activity, which was assayed as described by Bernfeld – the liberated reducing sugars were estimated by DNS method, with 3,5 – dinitrosalicylic acid. The reaction mixtures containing enzyme solutions and soluble starch as substrate (prepared in phosphate buffer, pH 6.9) were incubated at 30°C for 10 minutes. The reactions were stopped by adding 3,5 – dinitrosalicylic acid solution and then, these mixtures were heated again in a boiling water bath for 5 minutes and cooled at room temperature to develop brown colour. Absorbance of each sample containing the brown reduction product was measured at 546 nm in a UV-visible spectrophotometer. One unit of amylase activity was defined as the amount of the enzyme required to produce one μmole of maltose from starch, under the assay conditions.

Chip electrophoresis was used to obtain the electrophoretic profile of proteins derived from the bacterial isolate. The protein assay was performed on the chip in conjunction with Agilent 2100 Bioanalyzer, which allows software integration and multiple steps automate of electrophoresis (sample handling, separation, staining, discoloration and detection) combined with digital processing of data. Agilent Protein 80 Kit ladder was used to ensure the separation of the proteins (in the

range 5-80 kDa). The free supernatant was recovered by centrifugation of fermentation medium, at 6000 rpm for 20 min at 4°C. Samples were prepared with 4µl of supernatant and 2µl protein denaturing solution; the mixtures were incubated in a water bath at 95°C for 5 minutes and then diluted with 84µl double distilled water. Each sample was injected sequentially in the capillary, in which the protein separation was performed on the basis of their size; the protein detection was performed using a fluorescence detection system, at 630 nm.

RESULTS AND DISCUSSIONS

The soil environment is a good source for valuable microorganisms. Amylases are widely distributed and are ones of the most studied enzymes; they have a wide scale of applications, being capable to digest the glycosidic linkage found in starch or glycogen while, under aqueous conditions, liberating glucose, maltose and maltotriose. Nowadays, the renewed interest in the exploration of extracellular amylase production in bacteria and fungi is due to various industrial applications ranging from food to effluent treatment (Sankaralingam et al., 2012).

Identification of bacterial isolates

Based on the morphological, physiological and the most important, by the MS analysis, the bacterial isolate was identified as *Bacillus mycooides*. Fig. 1 shows the mass spectra of the identified isolate.

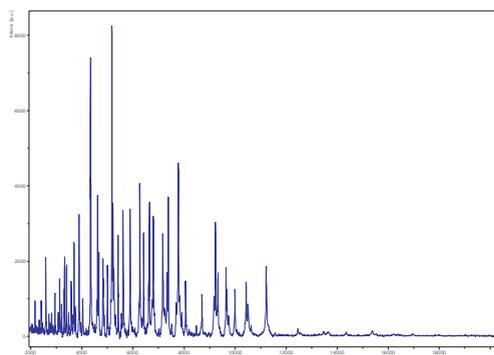


Fig. 1. The mass spectra of isolated bacterial strain identified as *Bacillus mycooides* (image generated with flexAnalysis v 3.3)

Enzyme production

Members of the genus *Bacillus* produce a large variety of extracellular enzymes of which amylases are of particularly significant industrial importance (Divakaran et al., 2011). The composition and concentration of media can greatly affect the growth and production of extracellular amylase in bacteria (Viswanathan et al., 2014). In this background, the production of amylase by submerged fermentation was investigated using over 30 biosynthesis media, in order to select the most suitable medium composition and to optimize it. To assess the optimal growth conditions, various sources of carbon (corn flour, wheat bran, rice flour, soluble starch, wheat starch, corn starch, malt extract, dextrin) and nitrogen (soy peptone, bacto peptone, yeast extract, soy flour, tryptone and NH_4SO_4), in different concentrations, were tested. Other factors, such as citric acid, CaCl_2 and inoculum volume, which could influence the enzyme production, were also investigated. The results of a first experiment showed a maximum amylase activity of 2.43 U/ml, using the *B. mycooides* as producing strain and medium F (corn flour - 10% as substrate, NH_4SO_4 1% as inorganic nitrogen source and 2% inoculum volume), under the following incubation conditions: 32°C, 220 rpm, and 48 hrs, fermentation time.

Chip electrophoresis

The bacterial amylase obtained in the experimental culture media, was subjected to an investigation with the Agilent 2100 Bioanalyzer to perform its electrophoretic profile (Fig. 2). For the assay, 5 variants of the medium composition were selected, in which enzyme activity was observed, namely: 4, 8, 15, 16 and 20, each consisting of two samples per chip. As a result of the electrophoretic profile of the 5 samples, the presence of amylase in the 15 and 16 medium was found, with 4% malt extract and NH_4SO_4 /soybean meal 1%. The use of the bioinformatics resources of the European Informatics' Institute - EBI, in particular the UniProtKnowledgeBase, allowed identification of the characteristic amylase for *Bacillus* sp. Based on the estimated molecular masses, the amylase from *B. mycooides* has shown a molecular weight of approx. 60 kDa.

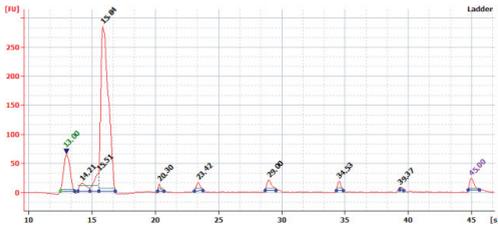


Fig. 2. a) Ladder of electrophoretic analysis

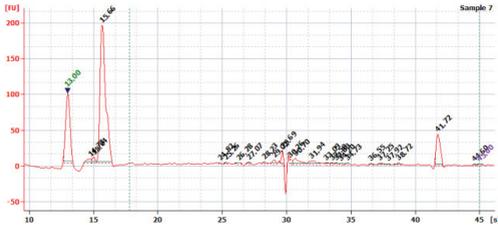


Fig. 2 b) Electrophoretic profile for sample 7: medium 15

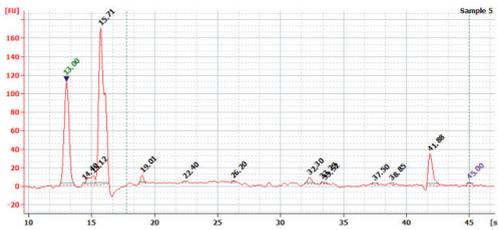


Fig. 2 c) Electrophoretic profile for sample 5: medium 16

Bioprocess optimization

Optimization of culture conditions is very important for maximum enzyme production. Due to the mesophilic nature of *B. mycoides*, 30-32°C was considered as optimum growth temperature, at pH 7.

It is known that high temperatures and pH values could inactivate the expression of the gene responsible for starch degrading enzyme (Nananganuru et al., 2012).

Amylase production depends on the characteristics of the culture and growth rate (Viswanathan et al., 2014).

Incubation time is an essential parameter to analyze. Fig. 3 shows a time growth experiment of *B. mycoides* using medium 15 (malt extract - 4%, NH₄SO₄ - 1%) and 2% level of inoculum, under optimum cultivation conditions.

A gradual increase was seen in cell growth from 4 to 36 h, after which a gradual decrease was observed.

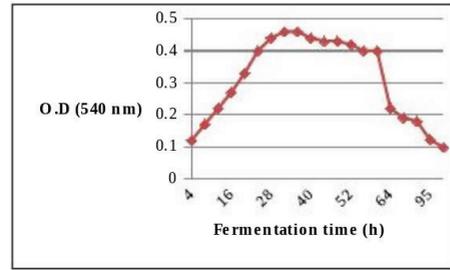


Fig. 3. Growth of *B. mycoides* with respect to time (hrs)

Thereby, incubation time is also a crucial factor for enzyme production and in all our experiments, 48 hrs was the best time for amylase activity, reaching its maximum (8.36 U/ml). Further increase in fermentation period did not have a positive effect on amylase production, rather it was decreased (4.84 U/ml); this fact could be happening due to the accumulation of other products in the cultivation medium (Hiteshi et al., 2014). Fig. 4 shows the amylase productivity, correlated with the biomass and fermentation period.

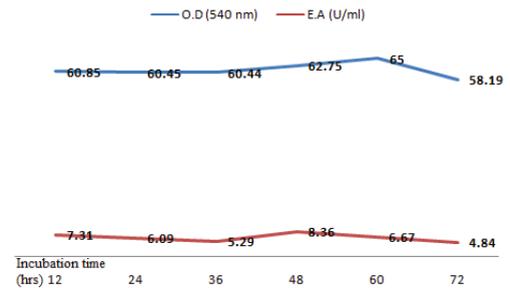


Fig. 4. Enzymatic activity in relation with incubation time and biomass

Unlike the conventional optimization method (changing one independent variable), using the Taguchi approach and ANOVA analysis as statistical optimization method offers a more balanced overview on factors influence and interactions, which also shows a targeted response (Sreenivas et al., 2004; Abel-Nabey and Farag, 2016).

In this paper, we describe the optimization of two selected fermentation media for alpha-amylase production, with the help of factorial design using Taguchi method; the experiments were planned to obtain a model consisting of 9, and 16 trials respectively.

The range and levels of the independent variables are presented in Table 1 and 2.

Table 1. Factors and levels for L9 Taguchi optimization model

Factor	Level I	Level II	Level III
A (malt extract)	2%	4%	6%
B ((NH ₄) ₂ SO ₄)	0.5%	1%	1.5%
C (inoculum volume)	1%	2%	3%
D (citric acid)	0.1%	0.2%	0.3%

Table 2. Factors and levels for L16 Taguchi optimization model

Level	Factor				
	A (1) Corn flour (%)	B (2) Ammonium sulphate (%)	C (3) CaCl ₂ (%)	D (4) Citric acid (%)	E (5) Inoculum Volume (%)
1	12	0.5	0.05	0.15	5
2	15	1	0.1	0.2	10
3	18	1.5	0.15	0.25	15
4	21	2	0.2	0.3	20

Fig. 5 shows the significance of the effect of each factor and interaction, determined by the ANOVA analysis. Experimental results for L9 orthogonal array trials suggest that the most significant influence on amylase activity is given by malt extract, followed by ammonium sulphate: 84.67% and 12.06%, respectively. The maximum activity (4.97 U/ml) was found for trial 7, which contained malt extract 6% and (NH₄)₂SO₄ 0.5%.

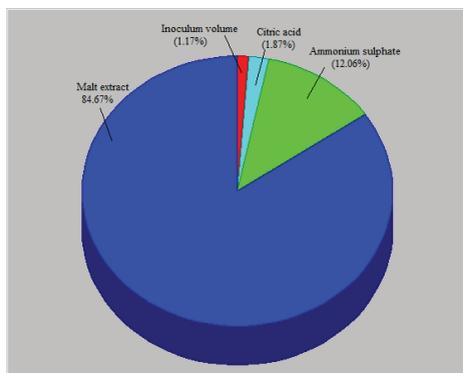


Fig. 5. ANOVA interpretation for L9 orthogonal array: main effects on amylase activity

Enzymatic activity was subjected to a multiple linear regression and the optimum conditions of the experiments were: malt extract 6 g/L,

ammonium sulphate 0.5 g/L, inoculum volume 2 % and citric acid 0.1 g/L.

Fig. 6 shows the average values and standard deviation, obtained in all of the 16 experimental trials. Higher amylase activities were observed in medium 9 and 16, with 10.87 U/ml and 10.44 U/ml, respectively. The lowest activities, 5.76 U/ml and 6.17 U/ml were registered for medium 11 and 13.

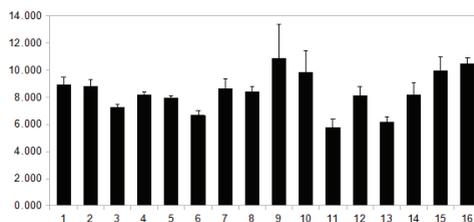


Fig. 6. L16 experimental design model: average values together with average standard deviation

Fig. 7 shows that the most significant contribution (55.54%) was observed for inoculation volume, followed by citric acid (22.83%) and ammonium sulphate (10.03%). An increased substrate concentration (corn flour) had not a relevant contribution to a higher enzyme activity. Following the ANOVA analysis, an interesting fact was observed: some interacting factor pairs, such as CaCl₂ - citric acid and NH₄SO₄ - citric acid, depending on their concentrations in fermentation medium, could influence the enzymatic activity. In our experiment, the influence was under 50% (43.64 and 36.91%), but a higher one could be obtained by using different factor levels, such as: 1 (0.05% CaCl₂) and 3 (0.25% citric acid) and 4 (2% NH₄SO₄) and 3 (0.25% citric acid).

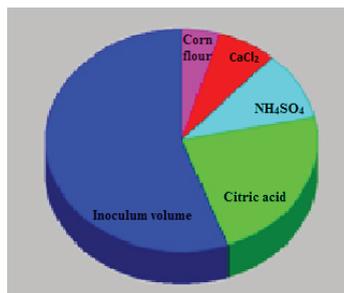


Fig. 7 L16 experimental design model: graphical representation of factors contribution to amylase activity

CONCLUSIONS

The production of economically-important amylases is essential for the conversion of starches into oligosaccharides; these enzymes are extensively used in the specific industry. Isolating and characterizing new strains, from different environments is a promising direction from the point of view of their biotechnological potential (Bozic et al., 2011).

The optimization of cultural conditions is very important to maximize the productivity and, in this regard, the present study focused on finding suitable conditions to optimal production of the required bio-product. The ability of the isolated strain to produce extracellular amylase was examined on solid and liquid media, with different inductors. Different carbon sources as starchy substrates, nitrogen sources and other medium components, such as citric acid, calcium chloride were adjusted in order to find the most suitable formula and to optimize it.

Further experiments will be done to purify the secreted enzyme, because the experimental results revealed an interesting perspective for this new soil isolated *Bacillus mycoides* strain, as amylase producer.

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FOOD SAFETY

CRITICAL REVIEW ON PROCESSING EFFECT ON NUTRITIONAL COMPOSITION OF FOOD PRODUCTS

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Abstract

An increase in the intake of food and constant changes in the formulation of food products has become evident in recent years. Therefore the efforts of processors together with academic circles in attending consumer demands for high quality food and dealing with raising economic standards, and nowadays particularly with ecological concerns, has triggered the development of emergent technological approaches for food processing. Recently, unconventional treatments technologies in food processing have gained increased industrial interest and have potential to replace, at least partially, the traditional well-established preservation processes (Pereira and Vicente, 2010). The objective of this work is to make a short review in respect to unconventional treatments of food, which are used nowadays in industry or are in the research and development phase. The paper presents the effects of novel techniques on the quality of food products, especially on the nutritional value.

Key words: *unconventional treatments, nutritional composition, food safety and preservation.*

INTRODUCTION

The food industry is one of the most important markets in the world, which struggles to get food from producer to consumer in the best possible conditions at the least expense (Cheng et al., 2010).

In recent years, attention has been increasingly paid to the nutritional characteristics of traditional foods and recipes, in order to accurately estimate dietary intake of the population, prevent diseases such as cardiovascular diseases, cancer, diabetes, etc., provide dietary information, and preserve some cultural elements. (Costa et al., 2013; Durazzo et al., 2017). The health-beneficial effects are thought to be related to macronutrients, micronutrients and bioactive compounds present in vegetables (Kim et al., 2016), meat, milk, eggs and other valuable products. In human nutrition food choices depend on a wide range of factors: culture, tradition, ethics, environment, consumption patterns, personal preferences, etc. (Durazzo et al., 2017).

A diversified diet is needed to meet daily micronutrient requirements (Uusiku et al., 2010).

Cooking is essential to achieve a palatable and safe product. Moreover, it may affect basic traits related to consumer preferences, such as flavor, tenderness, color and appearance (Dominguez et al., 2015). To maintain food products in conditions that are acceptable for consumption, the industry relies on different treatments and on the introduction of food additives, which are used especially to maintain the best conditions during the final steps of preparation, expedition and shelf-life (Carocho et al., 2015).

More than two decades ago, novel food processing technologies that were based on high-tech or cutting edge advances started to emerge to address productivity issues, extending product shelf life without affecting the nutritional content, organoleptic attributes and product specifications. Despite some of the technological advancements developing since the early 20th century, their applications for foods are still in a phase that needs a substantial amount of research to prove their pragmatic feasibility.

Current limitations related to high investment costs, incomplete control of variables associated with the process operation and lack of regulatory approval have been delaying a

wider implementation of these technologies on an industrial scale (Jermann et al., 2015). This study presents some unconventional treatments of food and how does or doesn't affect the nutritional composition of food products.

INFRARED TREATMENT (IR)

Infrared (IR) radiation has unique characteristics in its ability to transfer energy directly by radiation to a product, without heating the air. IR radiation is an electromagnetic radiation that falls between the region of visible light (0.38–0.78 μ m) and microwaves (1–1000 mm). It is transmitted as a wave and converted into heat when impinging on the food surface. Based on the wavelength, it can be divided into three regions – near- (0.78–1.4 μ m), mid- (1.4– 3.0 μ m) and far-IR (3.0–1000 μ m) IR technology has long been underestimated in the food field, despite its great potential.

It is generally applied to: the dehydration of vegetables, fish, pasta and rice; heating flour; frying meat; roasting cereals; roasting coffee and cocoa; and baking biscuits and bread. The technique has also been used for thawing, surface pasteurization of bread and packaging materials (Rastogi, 2015).

Uysal et al. (2009) demonstrated that the combination Microwave-IR oven for roasting of hazelnut resulted in a product of comparable quality with conventionally roasted ones with respect to colour, texture, moisture content and fatty acid composition.

Far-IR radiation resulted in higher antioxidative activity of extracts from peanut shells as compared to heat treated sample. The antioxidative activity was found to increase with an increase in exposure time (Rim et al., 2005).

IR heating of grass pea seeds resulted in a decreased TIA (trypsin inhibitor activity) compared with that of raw seeds. Reactive lysine proved to be relatively stable in the applied heating conditions. In addition, the process reduced the value of breaking load required for destructing a single seed. This may facilitate further processing, for example, flaking. Therefore, IR heating can be applied in processing of grass pea seeds (Sagan et al., 2016).

The effect of irradiation on nutritional or sensorial characteristic and physicochemical properties, as well as the interaction of food components under IR radiation, may further justify the use of IR radiation as a future food processing option (Rastogi, 2015).

PULSED ELECTRIC FIELD (PEF)

Pulsed electric field (PEF) processing is a non-thermal food-processing technology, which uses short bursts of electricity (Wang et al., 2014), which causes electroporation in the cell wall of microorganism and inactivating them (Li and Farid, 2016) providing fresh-like, safe foods and reduces loss of quality (Wang et al., 2014).

Pulsed electric field (PEF) has been used in active substances extracted (Lin et al., 2012), sterilization and destroy enzyme to prolong shelf life, and maintaining physical and chemical characteristics and nutritional value (Chen et al. 2009; Lin et al., 2017).

In general, PEF treatment systems are composed of PEF treatment chambers, a pulse generator, a fluid-handling system, and monitoring systems. The treatment chamber is used to house electrodes and deliver a high voltage to the food material. It is generally composed of two electrodes held in position by insulating material, thus forming an enclosure containing the food material. Therefore, the proper design of the treatment chamber is an essential component for the efficiency of the PEF technology (Elez-Martinez et al., 2012; Stefanoiu et al., 2015).

Zhao et al. (2008) showed that *E. coli* and *S. aureus* were inactivated by PEF treatment at 38.4 kV/cm for 160 and 200 ms reached 5.6 and 4.9 log reductions, respectively. PEF processing caused no considerable changes in color, GTP (green tea polyphenols) and total free amino acids.

In a study by Lin et al. (2017), pine nut protein hydrolysate was used to separate and identify antioxidant peptides. There were four peptides obtained including Gln-Cys-His-Lys-Pro (QCHKP), Gln-Cys-His-Gln-Pro (QCHQP), Lys-Cys-His-Gln-Pro (KCHQP), or Lys-Cys-His-Lys-Pro (KCHKP) QCHKP, QCHQP, KCHKP, and KCHQP. Among those peptides, KCHQP was with the strongest antioxidant

activity. In addition, the antioxidant activity of KCHQP can be improved by PEF treatment. With the optimal conditions of pulse frequency 1800 Hz and electric field intensity 15 kV/cm, the DPPH radical inhibition of KCHQP was increased from $89.10\% \pm 0.20\%$ to $93.22\% \pm 0.09\%$.

The combination of PEF and PAA(peracetic acid) was able to achieve up to 3 log reduction of *E. coli* and *Listeria* as well as 2 log/g reduction of native microbiota. PEF treatments did not cause any changes in colour and appearance of the blueberries. The concentration of anthocyanins and phenolic compounds in blueberries increased by 10 and 25% after PEF treatments (Jin et al., 2017).

The potential of pulsed electric technologies to improve the recovery of high-added value components such as proteins, total phenolic compounds and anthocyanin from berries was demonstrated by Barba et al. (2015). HVED (high voltage electrical discharges) technique was more efficient in terms of energy input compared to PEF and US (ultrasounds) treatments. Moreover, HVED + EE (supplementary extraction) (30%) showed at least two fold higher recovery yield of proteins and higher yield of polyphenols compared to other treatments. However, the maximum anthocyanin yield was found after applying PEF treatment and supplementary extraction with hot water at 50°C. The results obtained showed that PEF + HVED or PEF + supplementary extraction + HVED, can have good prospects for use in the food industry, i.e. by recovering in a first step sensitive compounds such as anthocyanins and subsequently more resistant compounds.

Processing of orange juice by pulsed electric fields (PEF) and thermal pasteurisation was carried out to compare changes in total phenolic concentration, hydroxybenzoic acid, hydroxycinnamic acids, flavonols, flavones and flavonones before and after being stored at 4°C for 180 days. Changes in the initial total phenolic concentration of the samples varied depending on the applied electric field intensity and thermal pasteurisation. Hesperidin and chlorogenic acids were detected as the most abundant flavonoid and phenolic acids in the orange juice, respectively. Except for syringic acid and neeroicitrin, the concentration of the

phenolic compounds identified in the orange juice samples enhanced after the PEF or thermal pasteurisation. The samples treated with PEF had more stable flavonoids and phenolic acids than those treated with the thermal pasteurisation. The PEF-treated samples had higher sensory scores than the heat-treated samples (Agcam et al., 2014).

In respect to the GSH(Glutathione) antioxidant activity the conditions for PEF treatment to maximize were: 8.86 mg/mL GSH, concentration, 9.74 kV/cm electric field intensity, and 2549.08 Hz pulse frequency. The change in structure and functional groups was analysed using NIR(Near InfraRed) and MIR(Mid InfraRed). There was no change following PEF treatment using MIR (Wang et al., 2014).

Applied on the kombucha beverages, there is an influence of PEF, especially at low feed flows, when it increases the bioactive contents, although there is no effect on the antioxidant capacity of treated samples (Vazquez-Cabral et al., 2016).

In the last years, there has been considerable interest in the adoption of PEF processing, and research into process scale-up. In other applications than foods, PEF processing can also improve the performance of industrial processes such as the removal of water from sludge, or the extraction of sugars and starches from plants, because the ruptured cells release their intracellular liquids more easily into their surroundings (Kempkes, 2010; Stefanioiu et al., 2015).

OHMIC TREATMENT (OH)

Ohmic heating technology is considered a major advance in the continuous processing of particulate food products. Ohmic heating is direct resistance heating by the flow of an electrical current through foods, so that heating is by internal heat generation (Goullieux and Pain, 2014). Heat is generated instantly inside the food, and its amount is directly related to the voltage gradient, and the electrical conductivity. The uniform heat generation results to uniform temperature distribution. The obvious advantage of ohmic treatments over conventional methods is the lack of high wall temperatures and limiting heat transfer

coefficients requirements. Its other advantages compared to conventional heating include maintaining the color and nutritional value of food, short processing time, and higher yield (Icier, 2012).

Ohmic treatment is used in a wide range of applications such as preheating, cooking, blanching, pasteurization, sterilization and extraction of food products (Yildiz-Turp et al., 2013).

A comparative study of the effects of ohmic and conventional pre-treatments used as blanching methods of artichoke heads prior to canning was investigated by Guida et al. (2013). The obtained data confirm that ohmic heating makes it possible to heat food products more quickly and uniformly than conventional methods, leading to a milder and efficient thermal treatment. Compared to conventional treatments, ohmic blanching is beneficial in terms of enzyme inactivation (for example POD-peroxidase and PPO-polyphenol oxidase) as well as preserving the colour of the fresh product, thus avoiding browning.

This treatment appears as a solution to reduce thermal damage because it heats materials in a rapid and homogeneous manner and may allow improved retention of vitamins, pigments and nutrients, resulting in less thermal damage to labile substances (Sarkis et al., 2012). Other examples for applications are the following: it's potential to increase dye diffusion in beet, its capability to extract sucrose from sugar beet, and its possibility to enhance the diffusion of soy milk from soybeans. Several past studies have shown an additional effect of electricity during the ohmic heating of plant tissues, vegetative microorganisms and bacterial spores (Somavat et al., 2013).

Ohmic heating is not only a useful thermal process in food stabilization, but also a pretreatment to prepare vegetal tissues before a mass transfer operation (e.g., diffusion, extraction, or dehydration) (Stefanoiu et al., 2015).

ULTRA HIGH PRESSURE (UHP)

High-pressure processing (HPP) is a method of food processing where food is subjected to elevated pressures (up to 87,000 pounds per square inch or approximately 600 MPa), with

or without the addition of heat, to achieve microbial inactivation or to alter the food attributes in order to achieve consumer-desired qualities. The technology is also referred as High Hydrostatic Pressure Processing (HPP) and Ultra High Pressure Processing (UHP) in the literature. HPP retains food quality, maintains natural freshness, and extends the microbiological shelf-life of the food (Gupta and Balasubramaniam, 2012; Ramaswamy et al., 2013; Stefanoiu et al., 2015).

The growing demand by consumers for more natural, minimally processed convenient food products that are safe, has stimulated food industry interest in high pressure processing (McArdle et al., 2011).

High pressure (HP) technology could maintain food quality attributes such as colour, flavour and nutritional values due to its limited effects on covalent bonds. Under pressure, (bio) chemical reactions can also be induced and it could affect those quality attributes, e.g., nutrition value.

The potentials of High Pressure processing (HP) have been pointed out for industrial food applications, allowing high retention of food quality attribute such as colour, flavour and nutritional values. The limited effect of HP (at moderate temperatures) on covalent bonds represents a unique characteristic of this technology. Therefore, in theory, most of the natural food quality aspects, for example nutritional values, can be maintained during HP treatment. Many studies on vitamin stability under HP (at moderate temperatures) have shown that HP does not significantly affect or affects only slightly the vitamin content of fruit and vegetable products, except at extreme pressure and temperature combinations (Oey et al., 2008).

Castanón-Rodríguez et al. (2013) studied the effect of high pressure processing on the lignocelluloses fraction of sugarcane bagasse and where was a function not only of pressure level and temperature, but also on the chemical used to pre-treat SCB (sugarcane bagasse). Results indicated that even though HPP, in the pressure range tested, induced changes on microstructure and crystalline structure of SCB, it did not enhance susceptibility of pre-treated SCB to the enzymatic hydrolysis and neither increased glucose yields.

HPP at the highest temperature (55 °C) had a positive effect on beef quality, by minimising meat toughening and water loss induced at high pressures. Moreover, no alteration of other quality parameters such as oxidative stability was detected after pressurising at higher temperatures. Comparison of pressure treated samples with conventionally cooked beef showed that even though HPP alters meat quality to some extent; it has a lower effect than conventional cooking on colour, pH, lipid oxidation and fatty acid composition parameters (McArdle et al., 2011). Also, HPP at ambient temperatures could be an appropriate method to produce tastier and more nutritive manuka honey (Akhmazillah et al., 2013).

High pressure processing at 400MPa induced the strongest alteration on swede quality. The results suggest that the strong structural modifications induced by HPP at 400 MPa would have played a role in the alteration of antioxidant properties of swede (Clariana et al., 2011).

Based on current knowledge, it can be concluded that in general HP treatment at moderate temperatures can maintain the vitamin content of fruit and vegetable based food products, however, mostly not at high temperatures. Vitamin stability is highly influenced by chemical reaction which can be enhanced by increasing pressure and temperature during HP treatment. As a consequence, HP treatment at extreme pressure and temperature combinations could result in vitamin degradation (Oey et al., 2008).

ULTRAVIOLET TREATMENT (UV)

Ultraviolet (UV) light is the part of the electromagnetic spectrum with wavelengths between 100 and 400 nm (Gomez- Lopez et al., 2012; Stefanoiu et al., 2015).

UV irradiation has been in use for decades for water disinfection and is an effective method for microbial decontamination of surfaces and packaging in the food industry (Bintsis et al., 2000).

The application of a combination of UV irradiation and PEF to freshly squeezed apple juice resulted in a similar total microbial reduction compared to the severe heat treatment. The relative antioxidant capacity of fresh apple juice decreased by 18.9% and the concentration

of total phenolic compounds changed from 22.74 to 15.98 mg gallic acid equivalents/l after 30 min of UV illumination. These results would indicate a potential for the use of these combined technologies for processing freshly squeezed apple juice (Noci et al., 2008).

In a study, Mendes de Souza et al. (2015) demonstrated that techno functional properties are not affected or are even improved by UV-C, foam ability was only improved on the egg white fraction and the foam stability was enhanced on all LEP. Emulsifying activity index was higher and more stable with the increasing UV dose. Nutritional value is largely maintained. The main UV-C radiation nutritional effect was on retinol content, which presented higher reductions as higher the applied doses, and on vitamin C content, which is less important since egg is not a common source of this vitamin. Under the studied conditions, a significant decay in the concentration of relevant secondary metabolites (lutein and zeaxanthin) has been stated.

No interference of UV-C light was observed on the mineral composition of liquid egg products. Foods rich in reducing sugars and proteins are susceptible to Maillard reaction, which reduces the nutritional value. No evidence of Maillard reaction in milk has been observed after 10 light pulses of 2,200 mJ/cm² each but the Vitamin A present in milk is degraded by UV treatment (Elmnasser et al., 2008).

It is common knowledge that preservation treatments degrade nutrients, a phenomenon that has to be assessed and minimized. UV-C illumination decreases ascorbic acid content of juices at a similar level to that caused by thermal treatments. Ascorbic acid present in orange juice is degraded by UV-C following first order kinetics at a rate of 0.175% per mJ/cm², with a reduction of 17.0% caused by 148 mJ/cm². Also for orange juice, a treatment of 299 mJ/cm² destroyed about 50% riboflavin and β-carotene, 17% vitamin C, 11% vitamin A, and did not affect folic acid or vitamin E. In apple juice, the reduction is reported to be from 5.4 to 4.0 mg/100 ml of juice (Gomez- Lopez et al., 2012).

However, UV light should not be considered as a harmful treatment to the nutritional and antioxidant quality of fruit juices if it is evaluated from a broader point of view. UV

treatment of fruits before juice extraction has been shown to yield excellent results to enhance phytochemical content. For example, UV-C illumination of grapes induces stilbene synthesis, especially that of trans-resveratrol, which will yield a phytochemical-enriched grape juice (Gonzalez-Barrio et al., 2009).

As far as the macro and micro-nutrient composition of milk is compared some different effects were noted between the treatments. The most noticeable change was a 35% reduction in cholesterol with UV treatment and 18% reduction in cholesterol with the UVP treatment, which indicates that UV does reduce the cholesterol and result in the possible conversion of cholesterol to COP's. Furthermore quantitative and qualitative measurements on enzyme activity also indicated no differences. The analysis of the fatty acid profile indicated difference in C18:1 cis n9 (Oleic acid) in the UV sample and C 18:0 (stearic acid) in the RM sample when compared to the other treatments (Falguera et al., 2011; Cilliers et al., 2014;).

Application on cold-stored shiitake, quality retention by the UV-C treatment of mushrooms following an additional shelf-life period at room temperature resulted in maintenance of tissue firmness, increase of flavonoid and ascorbic acid contents, and enhancement of antioxidant ability, which could be useful from the nutritional point of view. These results suggest that UV-C treatments may be a useful non-chemical way of maintaining shiitake mushroom quality and extending their postharvest life (Jiang et al., 2010).

Finally, the success of UV technology for low-UVT liquids depends on the correct alignment of the UV source parameters to the specific demands of the UV application (Gomez- Lopez et al., 2012).

OZONE TREATMENT

Ozone is a triatomic form of oxygen and is characterized by a high oxidation potential that conveys bactericidal and virucidal properties. Ozone inactivates microorganisms through oxidization, and residual ozone spontaneously decomposes to non-toxic products (oxygen), making it an environmentally friendly antimicrobial agent for use in the food industry (Patil and Bourke, 2012).

In this research, polyphenols were used as indicators to evaluate the degree of oxidation by ozone. It was apparent that the observed polyphenols and resveratrol contents showed no significant difference between treated and untreated groups ($P > 0.05$), which indicated that polyphenols and resveratrol were not affected by ozone treatment under the optimal conditions (Chen et al., 2014).

The effect of continuous exposure to ozone at 0.45, 0.9 and 2 $\mu\text{mol mol}^{-1}$ on quality changes during the storage of red and green chilli peppers at 10°C was investigated by Glowacz and Rees (2016). Total phenolic content was not affected by ozone but antioxidant activity was reduced in green chilli peppers exposed to ozone at 2 $\mu\text{mol mol}^{-1}$, due to lower ascorbic acid content in those samples. Ozone at 0.9 $\mu\text{mol mol}^{-1}$ extended the shelf-life of chilli peppers.

Fresh-cut papaya was treated with ozone (9.2 ± 0.2 l/L) at 10, 20 and 30 min to investigate its effect on phytochemicals and microbial load. Following a 20 min ozone treatment, the total phenolic content of fresh-cut papaya increased by 10.3% while the ascorbic acid content decreased by 2.3% compared to that of untreated control fruit (Yeoh et al., 2014).

Ozone, as a potential oxidant, is applied in various treatments including water treatment and equipment disinfection, as well as for the preservation of perishable items, namely fruits, vegetables, and meat (Seydim et al., 2004; Karaca and Velioglu, 2007; Priyanka et al., 2014). Ozone treatment was shown to be a promising technique for enhancing the antioxidant capacity of some fresh fruits such as banana, but at the same time a reduction in vitamin C content was also observed (Althman et al., 2010). Minas et al. (2012) demonstrated that the ozone treatment resulted in blocked ethylene production, delayed ripening, and stimulated antioxidant and antiradical activities of the fruit. The ripening induced carboxylation of kiwifruit protein, but it was reduced by ozone treatment, leading to improved postharvest behaviour.

Tiwari et al. (2009) demonstrated that ozone treatment (7.8% w/w, 10 min) resulted in significant reductions in anthocyanin content and ascorbic acid of the order of 98.2% and 85.8%, respectively in strawberries.

Ozone is an innovative athermal mode of disinfection with potential applications in the modern food industry. It is a safe way to oxidize contaminants while leaving no residues and without affecting the quality of food.

MICROWAVE TREATMENT (MW)

Microwaves are electromagnetic radiation with wavelengths from 1 mm to 1 m in length and with frequencies from about 300 MHz to 300 GHz (Scaman et al., 2014).

Microwave heating differs from conventional heating due to the fact that the microwave heating occurs via direct microwave material interaction, while the conventional heating requires the heat to be transferred from the external heat sources. Microwave causes the polarized or charged molecules to rotate back and forth in order to align to the periodically changing direction of the alternating electric field. The rotation leads to the inevitable frictions between the molecules and that causes volumetric heat generation within the material (Bhattacharya and TanmayBasak, 2017).

Microwave cooking is recommended for lentil preparation, not only for improving nutritional quality, but also for reducing cooking time (Hefnawy, 2011).

Carew et al. (2013) studied the microwave macerated must and assessed wine phenolic after two different hold times in a 70 °C water bath (1 and 8 h). As regards total phenolics and total and pigmented tannin, both long and short time wine was equivalent to control; conversely, for mean concentration of total pigment, free anthocyanin, and colour density, only the short hold time wine was significantly lower than control. Also, MW treatment has the possibility to eliminate laccase and better manage phenolic outcomes with evident benefit for the wine industry (Clodoveo et al., 2016).

The influence of simultaneous combination of microwave and steam cooking on contents of specific phytochemicals, carbohydrate and antioxidant activity of purple sweet potatoes (PSPs) was investigated by Xu et al. (2016) and compared to those of individual steaming and microwaving. Results showed that the contents of phytochemicals including total phenolics, flavonoids and anthocyanins and phenolic acids except caffeic acid increased after cooking to

different extent depending on cooking methods. The PSPs cooked by simultaneous combination of microwave (500 W) and steam (1700 W) for 12 min (M500-S1700-12) contained the highest of total phenolics, flavonoids, phenolic acids and anthocyanins. Simultaneous combination of microwave and steaming resulted in higher content of soluble sugar in PSPs. PSPs cooked by M500-S1700-12 showed the highest antioxidant activity, which was well accordance with higher contents of phytochemicals.

In a study, Conte et al. (2017) demonstrated that microwave treatment on honeybee had a damaging action on antioxidant compounds (i.e. reduction in the content of tocopherols).

Microwave-pretreated samples of pomelo retained higher amounts of pectin, naringin, and limonin compared with non-pretreated samples. No obvious change in the degree of pectin esterification was observed. Microwave pre-treatment process is a promising methodology for both preserving valuable compounds in pomeloflavedo during storage and acquiring essential oils (Liu et al., 2017).

Jouquand et al. (2015) showed in a study that beef burgundy cooked by microwave has the level of CML (carboxymethyllysine) product similar to that measured after traditional cooking. However, lysine degradation was more pronounced when traditional cooking was used compared to optimized microwave.

RADIOFREQUENCY TREATMENT

Radio frequency is another technique of dielectric heating that is quite similar to the principles of microwave technology. Heat is generated inside the product, resulting from the polarization of molecules and migration of ions that occurs at high frequency (Wang et al., 2003). The advantage of radio frequency over microwave energy is that penetration depth is deeper due to the frequency. The selected frequencies for industrial, domestic, scientific, and medical applications are 13.56, 27.12, and 40.68MHz (Awuah et al., 2005; Barbosa-Canovas and Bermudez-Aguirre, 2010).

A study by Orsat et al. (2001) was conducted to develop a processing method for the RF treatment of fresh-like carrot sticks to reduce their microbial load and their enzymatic activity while ensuring their quality. Results

showed that when compared with chlorinated water dipping and hot water dipping, RF-treated carrot sticks had better quality in terms of colour and taste.

Schuster-Gajzágó et al. (2006) exposed white mustard seed to RF with the intention of inactivating the endogenous enzyme myrosinase which was responsible for the development of pungent sharp flavour. In addition, these workers also assessed the impact of RF on compounds with health beneficial effects found in these seeds. They found RF could effectively inactivate myrosinase to a sufficient level to inhibit pungent flavour development while causing no damage to compounds of nutritional significance.

An atmospheric RF plasma treatment (20W and 40W) was used to improve the yield, expansion volume, and content of essential amino acids in the popped rice. The effects of atmospheric RF plasma treatment on the surfaces of popped rice were examined using a microscope. The results demonstrated that the percent yield ($42.2 \pm 6.6\%$) and expansion volume ($2.53 \pm 0.72 \text{ ml g}^{-1}$) of popped rice when using an electric frying pan were approximately 4 times higher than those observed when using a kitchen oven and approximately 3 times higher than those observed when using a microwave oven. However, the crude protein and elemental composition (P, K, Mg, Ca, Zn, Na, Mn, and Fe) of popped rice using a direct heat from an electric frying pan were found to be significantly lower than obtained using other methods. Atmospheric (RF) plasma at 40W could improve the quality of popped rice when it is popped in an electric frying pan. Higher percent yield ($53.2 \pm 1.6\%$), expansion volume ($3.59 \pm 0.06 \text{ ml g}^{-1}$), and essential amino acid content (5 mg amino acid g^{-1} protein of arginine, leucine, phenylalanine, threonine, and lysine) in the popped rice were observed after plasma treatment.

This research suggests that a combination of an electric frying pan and atmospheric RF plasma is highly effective for increasing the producing of popped rice (Puangjinda et al., 2016).

Nonetheless, the industrial potential of RF processing is interesting with its greater penetration depth than MW with well-designed applicators and heating/drying applications. The potential of RF is even greater when used

in hybrid systems that take the volumetric heating advantages of dielectric heating and couple them with conventional processing for efficient, rapid, and high quality results (Orsat and Raghavan, 2014).

CONCLUSIONS

In today's food market, consumers want healthy, biologically grown, preservative-free, high-quality produce.

Freezing, sterilizing, drying, refrigeration, and distribution of fresh product are used to replace the use of preservatives. Pasteurization can solve some shelf-life problems if a producer can distribute a refrigerated product. On the other hand, sterilization can offer greater shelf stability to foods. In some applications, dielectric sterilization can deliver quality products because the electromagnetic waves are able to heat the product 3-5 times faster than conventional sterilization systems. The sterilized product is not temperature abused; therefore, the food has better overall quality attributes than that processed by other available technologies (Orsat and Raghavan, 2014).

The conventional processing of products often requires a long heating time and those results in the degradation of food qualities, texture and nutritional values (Bhattacharya and Basak, 2017).

The unconventional treatments have been found to upgrade the processing by preserving the quality, texture and nutritional values.

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MARKET RESEARCH REGARDING THE DEMANDS OF THE BUSINESS OPERATORS ON THE SUPPLY CHAIN LOGISTICS

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Abstract

Berries are worldwide recognized as valuable source of polyphenols, especially anthocyanins, micronutrients, and fibers. They are mainly industrialized as frozen fruits but also in jams, or pies. Some berries are commercially important but there are some limitations such as: short shelf life of fresh fruit and soft texture which require special attention on the berry chain logistic. The berry industry varies from country to country as well as the types of berries cultivated or wild berries. Berries are one of the greatest assets of the Romanian forests and they are known to be products of a very high. Berries originating from non-polluted forests are very popular in the foreign markets. Unfortunately, most often they are sold in foreign markets without being locally processed and thus Romanian processors are losing valuable Romanian customers.

To analyze the requirements of the industry companies (production, harvesting and distribution of native berries) a questionnaire was designed and used as a research tool. This questionnaire represents a direct tool of collecting data and to communicate with the respondents. To investigate the value chain of berry fruits and its challenges, we opted for two ways: self-administered questionnaire which was either filled in on the spot or sent by electronic mail, then filled in by the person interviewed, and the questionnaire filled in by phone which was a faster and cheaper way to obtain the necessary answers. Over 200 questionnaires were sent by email to local branches of the National Forest Administration (ROMSILVA) but only 31 were filled in and sent to us. By phone, 20 persons of interest responded to our questionnaire.

Key words: survey industry, berries.

INTRODUCTION

Berries are becoming more and more sought after thanks to their flavor, nutritional values, and high contents of vitamins, minerals and fibers and not least their very good taste. At the same time, berries contain many secondary plant substances such as flavonoids and anthocyanins, which have antiviral, antioxidant and anti-inflammatory effects (Adams et al., 2009; Millogo et al., 2008). The harvesting period for berries varies with each species, climate conditions, from July to September and even October. Berries are picked by hand so they do not suffer any mechanical shocks. Picking berries for many represents an additional source of income. Market sales for berries are increasing from year to year, involving a large research effort to develop new techniques to offer the highest possible shelf-life and quality for their products. However, the conservation process of berries often leads to different kinds of damages, such as biochemical changes, loss of

texture and nutritive value and microbial cross contamination (Saltveit, 2003).

MATERIALS AND METHODS

The marketing research field uses different methods to investigate customers' opinions and preferences based on direct and indirect information and data on both qualitative methods and quantitative methods. While there are many ways to perform market research, most businesses use one or more of five basic methods: surveys, focus groups, personal interviews, observation, and field trials.

Surveys involve asking a series of questions to a sample of the target population that is large enough to be statistically valid. Surveys generally offer primarily closed-ended questions, although some open-ended questions may be included. Surveys can be administered by mail, telephone, email, Internet or in person. From the multiple choices of marketing research methods, taking into account our

objectives, the interview questionnaire-based method was chosen. In order to know and analyze the needs of the industry companies (production, harvesting and distribution of native berries) a questionnaire was made and used as a research tool. This questionnaire was a direct way of collecting data and to communicate with the respondent.

The questionnaire was made for the berries industry in 2016 and over 200 questionnaires were sent by email to local branches of the National Forest Administration (ROMSILVA) but only 31 were filled in and sent to us. By phone, 20 persons of interest responded to our questionnaire.

The data resulting from the questionnaires was analyzed and collected in Microsoft Office Excel Worksheet.

RESULTS AND DISCUSSIONS

The first question asks the respondents if they are either growers of berries, producers of berry products, pickers or retailers. Out of the 51 respondents, 39 of them cultivate and pick the fruits. 31 of the respondents are retailers and only 1 of the respondents is a company that is packaging fresh berries. Out of 51, 7 of them process fresh berries. The responses are represented in figure 1.

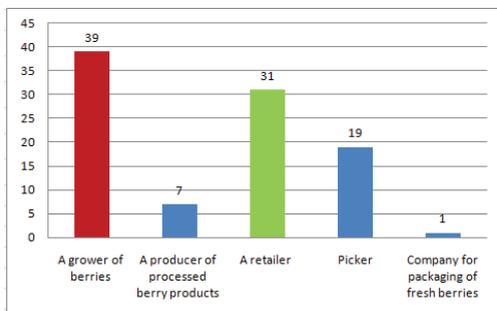


Figure 1. The part of the value chain of berries the respondents work

The second question refers to the type of berries the respondents work with. They had to choose between “conventional berries only”, “organic berries only” or “both conventional and organic”. Out of the 51 respondents, 39 work only with organic fruits, 4 of them with conventional fruits and 7 of them work with

both conventional and organic fruits. The responses are represented below in figure 2.

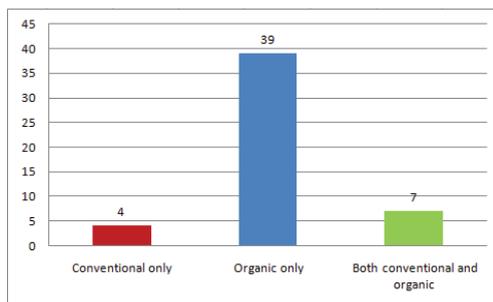


Figure 2. The type of berries that the respondents work with

In the next section of the questionnaire (Fresh berries category), the respondents are asked to choose in what part of the value chain they work. They were able to select one, two or all that apply.

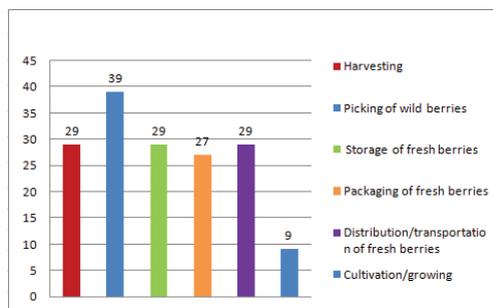


Figure 3. Fresh berries category – Value chain responses

After collecting all answers, out of 51 respondents, 39 stated that are cultivators and growers of berries, 29 are harvesting and storing them and 27 of them are handling the packaging of the berries. Only 9 of them are pickers. The results are presented in figure 3.

In the next question (figure 4) the respondents are kindly asked in what part of the value chain of the processed products of berries they work. They could choose from the four variants: Processing of berries, Storage of processed berry products, Packaging of processed berry products and Distribution/transportation of processed berry products.

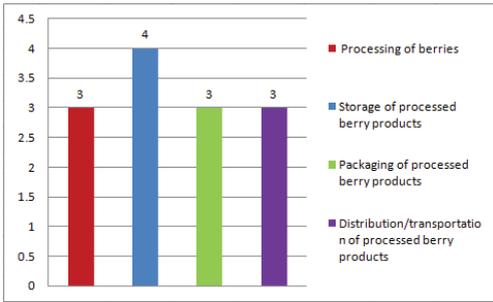


Figure 4. Processed berries – Value chain responses

At this question not all respondents provided us with an answer. Four of the respondents stated that they work in the storage of processed berry products value chain and the others stated that they work either in the processing of the berries, packaging of the processed berry products or in the distribution/transportation process.

In the next question the respondents are asked in what part of the value chain of reaching the consumer they are involved. They could choose from the following answers: Storage of fresh or processed berries and berry products, Packaging of fresh berries and berry products, Transportation, Assortment selection, Shelf display and Consumer relationship. The responses are shown in figure 5.

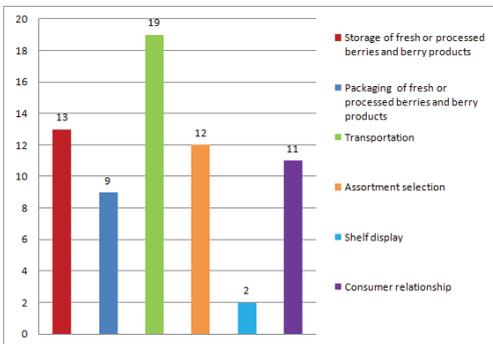


Figure 5. Reaching the customer

Out of the 51 respondents, 19 of them answered that they manage the transportation of the berries, 13 of them are working on storing the fresh or processed berries, 12 of them are working in the assortment selection and 11 of them are involved in the consumer relationship.

Only 2 answered that they work in the shelf display department.

In the next section, following the same three directions: *Fresh berries*, *Processed berries* and *Reaching the consumer*, it is shown the activities in which the respondents are encountering problems and want improvements.

In figure 6 are shown the answers of the respondents on the problems they encounter and want improvements in the fresh berry field of work.

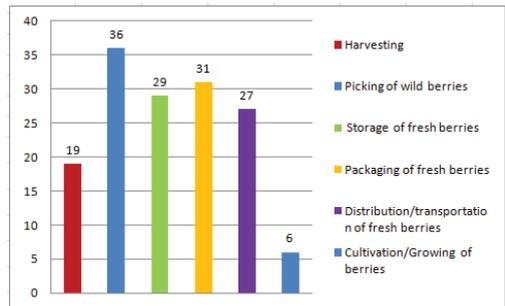


Figure 6. Problems – Fresh berries

Out of 51 respondents, 36 answered that they have problems and want improvements in the field of picking berries. Also, 31 of the respondents answered that they have problems in the packaging of fresh berries area.

In figure 7 are shown the answers of the respondents on the problems they encounter and want improvements in the processed berries field.

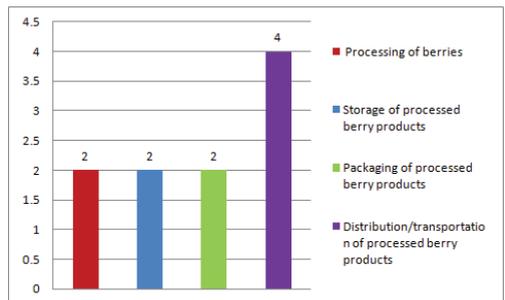


Figure 7. Problems – Fresh berries

Out of the total of the respondents who answered this part of the survey, 4 of them

encounter problems in the distribution and transportation of processed berry products.

In figure 8 are shown the answers of the respondents on the problems they encounter and want improvements in reaching the consumer.

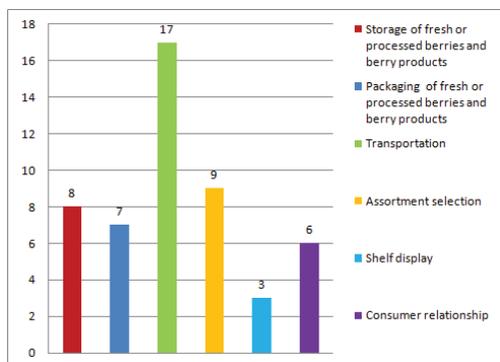


Figure 8. Problems – Fresh berries

Out of the total of the respondents who answered this part of the survey, 17 of them encounter problems in the transportation field. Following the same elements of the food chain *Fresh berries*, *Processed berry products* and *Reaching the consumer*, the respondents were asked their opinions on the following items: actual procedure, positive aspects, occurring problems and improvements needed.

In the topic concerning the cultivation and growth of fresh berries, our participants stated that the actual procedure in cultivating berries is the following: garden picking, fruits grown in the garden and greenhouse and using only forest berries. The positive aspects are high efficiency production, better quality and superior nutritional quality. The problems that our respondents answered are the lack of government subsidies and more structural programs to increase the productivity.

The actual harvesting procedure of fresh berries is manual picking and the main positive aspect is the possibility of collecting fruits during the harvest period. The problem is that it requires a lot of manpower at a high price.

The picking of wild berries is a manual process made by pickers. The positive aspects are that this brings jobs to the country side, the

selection of berries is at a high quality and for the casual pickers it's a very good source of income. The main problems stated by our respondents are that the pickers must be monitored, the presence of many illegal harvesting in an uncontrolled market is made and the manpower is insufficient.

The next question is regarding the condition of storage for the fresh berries. The respondents stated that the actual procedures that they use are the following: after picking the berries are sold in the markets, stored in the cooling chamber at 10-18°C, without controlled atmosphere or frozen at temperatures around – 30 ° to – 40° C. The positive aspects stated by our respondents were either that berries sell quickly and do not have the chance to spoil or the frozen berries retains overall integrity of tissues, nature, physicochemical and microbiological changes are reduced. The problems encountered are the lack of transportation means, the short shelf-life of the fruits and high energy consumption. Another problem is that the storage rooms need a high consumption of electricity.

The next questions are regarding the packaging procedure (respondents were asked to specify whether the packaging is done under modified atmosphere). The respondents told that the most common packaging techniques are glass jars, trays for fresh fruits and for processed foods. The main problems indicated are the short shelf-life of berries and the packaging price that make the berries more expensive.

In the processed berry products part, the respondents stated that the actual procedure consists in making jams, syrups, smoothies or nectars from the fresh berries. Also, some respondents stated that they use the berries to make yogurt with fresh berries. The positive aspects of these products are the high shelf-life and that the biological compounds are kept active in the products. Jams are stored at room temperature (24 – 25 C) and yogurts are kept at around 4 degrees. The distribution of these products is made either by normal trucks or refrigerated trucks. The shelf-life of jams is around 1 or 2 years. The positive aspects of these type of processed berry products is that they do not need refrigeration.

In the reaching of consumer part, most of the respondents stated that they sell the berries in open markets at ambient temperature or in supermarkets. The positive aspects of this are that the consumer can easily buy the fruits. The biggest problem brought up by our respondents is the short shelf-life of fresh berries, so they have to sell them very fast.

At the end of the survey we asked the participants to tell us other comments about the problems encountered in the berry value chain. The first comment stated that the price of the berries from the producer is high, so the price of berry jams is high. Another respondent said that they would like to increase the consumption of berry jams/syrups in the Romanian market, since the consumer prefers to produce such products in their own household (either from parents, grandparents, relatives, etc.), and thus ensuring the necessary product from this range this way, rather than purchasing the industrially processed products. Another interesting comment is stating that berries from the spontaneous flora are not affected by pollution, not treated with chemical fertilizers or with pest control substances. For these reasons, such berries are very sought after in the pharmaceutical industry.

CONCLUSIONS

These products of the forest hold a special importance from a medicinal point of view, but they can also be used as foodstuffs. Fruit processed by pressing are suitable for the production of juices which are rich in biologically active substances and their quantity is influenced by the ripening period of the fruit. In the first phase of the ripening, the fruits are rich in vitamin C and in the second phase of maturation they are rich in flavonoids, anthocyanins and carotenoids. On the value chain of the berries, the social component must be introduced. The existence of a quantitatively valuable cargo in a certain area represents opportunities for the people in that area to ensure income, even if they are seasonal and occasional.

The findings of this study can be grouped in three directions: fresh fruit, fruit processing and the relationship with the consumer. The main

conclusions of this study show that there are no government subsidies for fresh fruit harvesting and the harvesting process requires a large labor force and the workers demand high salaries. Another important conclusion is that there are no structural programs aimed at increasing productivity, so the industry cannot develop as fast as they would like. Fresh fruit harvesting is dependent on environmental and climate factors, which can sometimes be a problem in performing this operation. Regarding the storage of fresh berries, the main weak points are the lack of refrigerated means of transport, the short shelf-life of the fresh berries and the amount of energy needed to keep the berries refrigerated until they get to the customer. The main problem encountered by our respondents in the matter of packaging is the high price of the package itself, which increases the final cost of the berries. One of the main conclusions brought up by almost all of our respondents is the need to improve the shelf-life of the berries through processing.

ACKNOWLEDGEMENTS

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OPTIMIZATION AND EVALUATION OF ELISA IMMUNOASSAY FOR MYCOTOXIN DETECTION OF BREAKFAST CEREALS

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Abstract

Sixteen samples of breakfast cereals purchased from the Romanian market were studied in order to evaluate the possible occurrence of mycotoxins. The influence of microbiological and physical-chemical attributes that may determine the incidence of the toxins was studied. A BIOLOG system was used for the identification of the fungal strains. A sandwich-type enzyme-linked immunosorbent assay (ELISA) method was optimized and validated in-house for the quantification of two mycotoxins: deoxinivalenol (DON) and zearalenone (ZEA). The validation of the method was based on following performance parameters: accuracy (measured as percent error), precision (measured as coefficient of variance), reproducibility and repeatability (precision within- and between-day and analyst variability), limit of detection (LOD) and limit of quantification (LOQ). Recovery of the method was tested at low, medium and high levels of the working range (three concentration levels) for each mycotoxin in spiked breakfast cereal samples. According to the results, all samples presented levels far below their legal limits.

Key words: breakfast cereals, filamentous fungi, mycotoxins.

INTRODUCTION

Cereals are very susceptible to fungal attacks, both in the field and during storage. Depending on environmental conditions, a fungal infection, mainly produced by species of *Aspergillus*, *Fusarium* and *Penicillium*, may result in a mycotoxin contamination of the crop. Mycotoxins are a group of toxic compounds produced by spore-forming fungi. Mycotoxin contamination of cereals is a major issue in regard to public health, food safety and food security. Consequently, a regular contamination can be expected for cereal-based commodities (Montes et al., 2012).

Deoxinivalenol and zearalenone are two mycotoxins produced by members of the *Fusarium graminearum* species complex (Tralmazza et al., 2016). Both mycotoxins are the most found over the world and at acute doses they cause a wide range of toxic effects, while a constant intake of small amounts of mycotoxins leads to a weakened immune system. A wide range of food products could be contaminated with mycotoxins, both pre- and post-harvest (Zain, 2011). In this context, the purpose of this study was to investigate the incidence of deoxynivalenol and zearalenone

mycotoxins within breakfast cereals samples purchased from the Romanian market. The influence of microbiological and physical-chemical attributes that may determine the incidence of the toxins was studied, while a BIOLOG system was used for the identification of the fungal strains.

MATERIALS AND METHODS

Food samples

Sixteen samples of breakfast cereals were purchased from different supermarkets in Bucharest, Romania. Thus, there were selected four corn flakes samples, with (n=2) and without (n=2) added sugar, one of these last samples labelled as grains from ecologic farming. Müsli breakfast cereals with added dry fruits and sugar (n=2) and without sugar (n=1) were also the subject of these tests. Furthermore, whole grains breakfast cereals (n=6) were selected, as well as breakfast cereals with added sugar, vitamins and preservatives (n=3). All samples were kept in their original packages and stored at room temperature in a dark and dry place until analysis.

Physico-chemical and microbiological analysis

Humidity measurement was performed using a Mettler LJ16 infrared dryer (Mettler-Toledo Ltd., UK). The working method consists in drying 5 g of grounded and well homogenized sample at 130 °C. Water activity was measured using the Aquaspector AQS-31 (NAGY Messsysteme GmbH, Germany) at 24 °C. Grinded and ungrounded breakfast cereal samples were placed in the measurement cup and readings were noted. The pH of all samples was determined using an Inolab 730 WTW pH meter (WTW Wissenschaftlich-Technische Werkstätten GmbH, Germany). After grinding, 10 g of each sample were homogenized for 30 s in 90 mL distilled water using a stomacher (Seward Limited, UK) and then pH was determined. The acidity of each sample was determined. Thus, in a conical container 5 g sample were loaded and 50 mL water were added. The mix was stirred for 5-10 minutes. After homogenization, 3 drops of phenolphthalein solution were added to the aqueous extract and titrated with sodium hydroxide solution until the appearance of pink colour that persists for 1 minute.

Yeasts and moulds and *Enterobacteriaceae* were monitored. An amount of 10 g breakfast cereals was aseptically removed from the package using a sterile spatula and transferred to a sterile filter stomacher bag (Seward Limited, UK), containing 90 mL sterile homogenate solution (0.85% NaCl and 0.1% neutralized bacteriological peptone). The samples were homogenized using a stomacher (Seward Limited, UK) for 30 s at room temperature. Tenfold dilution series were made in sterile peptone saline solution as needed for plating. Dichloran Glycerol (DG 18) agar (Oxoid, UK) was spread-plated with 100 µL of the appropriate sample dilution and incubated at 25 °C for 7 days for yeasts and moulds. For members of *Enterobacteriaceae* family, 1 mL of the appropriate sample dilution was inoculated into 10 mL molten (45 °C) violet rose bile glucose agar (Oxoid, UK). After setting, 8 mL overlay of molten medium was added and incubated at 37 °C for 24 h. All plates were examined visually for typical colony types and morphological characteristics

associated with each growth medium. Microbial counts were expressed as log cfu g⁻¹.

Biolog OmniLog Identification system

Each isolated fungi was first grown on two plates of 2% malt extract agar, MEA (Scharlab SL, Spain), at 25 °C. After an incubation period of 5 days, conidia were collected with sterile cotton swabs. The swabs were dipped into screw-top culture tubes containing 16 mL Biolog FF inoculating fluid. The conidial suspension was adjusted at 75% transmittance by a turbidimeter, as recommended by the manufacturer. A volume of 100 µL was pipetted into each of the 96 wells of a single Biolog FF plate. The resulting plates were incubated at 25 °C and the biochemical reactions were recorded using a microplate reader with a 590 nm wavelength filter at 1, 2, 3, 4 and 7 days, as suggested in the Biolog product information.

ELISA analysis

ELISA type immunoenzymatic test was selected for mycotoxin detection, using Max Signal[®] ELISA test kits (Bioo Scientific Corporation, USA). All samples were first finely ground using a laboratory mill (MRC Ltd., Israel) and mixed thoroughly to achieve complete homogenization. Furthermore, for DON and ZEA detection, 5 grams of grinded sample were homogenized in 100 mL distilled water and 25 mL 70% methanol (v/v), respectively. Samples were homogenized vigorously using an orbital shaker (GFL Gesellschaft für Labortechnik mbH, Germany) and then centrifuged for 10 minutes at 4000 rpm and room temperature, using a high speed centrifuge, model 5804R (Eppendorf AG, Germany). Further, samples were diluted according to the test kit manual, using distilled water and sample extraction buffer, respectively. 100 µL (DON) or 50 µL (ZEA) standards and samples, in duplicate, were added into different wells. DON-HRP conjugate and zearalenone antibody mix were added to each well and further, the plates were incubated at room temperature for 45 minutes and 30 minutes, respectively. After washing the plates, 100 µL TMB substrate were added and

after incubating for 15 minutes at room temperature, 100 μL stop buffer were added. The plates were read on a Sunrise™ plate reader (Tecan Group Ltd., Switzerland). For each detection two repetitive samples have been used. The media of these samples has been employed in data analysis.

Method performance

Validation experiments established the performance characteristics of the ELISA method. The following performance parameters were investigated: accuracy (measured as percent error), precision (measured as coefficient of variance), reproducibility and repeatability (precision within- and between-day and analyst variability), limit of detection (LOD) and limit of quantification (LOQ). Recovery of the method was tested at low, medium and high levels of the working range (three concentration levels) for each mycotoxin in spiked breakfast cereal samples.

Data analysis

Microbiological and physicochemical experiments were replicated twice and analysis were run in triplicate for each replicate ($n = 2 \times 3$). Microbiological data were expressed as logarithms of the number of colony forming units (cfu g^{-1}). Means and standard deviations were calculated. Significance was defined at $P < 0.05$. LSD test which was applied only to microbiological data. Data were analysed with IBM® SPSS® Statistics 20 (IBM Corp., USA). Data analysis for metabolic profiling of fungi was conducted using BioTek Gen5 software (Biolog, Inc., USA).

RESULTS AND DISCUSSIONS

Effects of physico-chemical and microbiological attributes

Humidity and water activity are two physico-chemical attributes that have a great impact on the microbiological contamination of food products. These parameters were measured in order to assess the impact of the breakfast cereals type when samples were compared with each other.

According to figure 1, which indicates the humidity values, müsli and whole grains breakfast cereals noted the highest humidity values.

The pH measurement noted, as expected, the lowest levels for the müsli breakfast cereals with dry fruits added (values ranged from 4.77 to 5.76), while the highest values were registered by the breakfast cereals with added sugar and synthetic preservatives (values between 6.40 and 7.19).

When referring to the water activity measurement, the results registered between grinded and ungrounded samples were not significant different ($P > 0.05$).

However, the whole grains breakfast cereals noted the highest water activity values, while the corn flakes registered the lowest levels (data not shown).

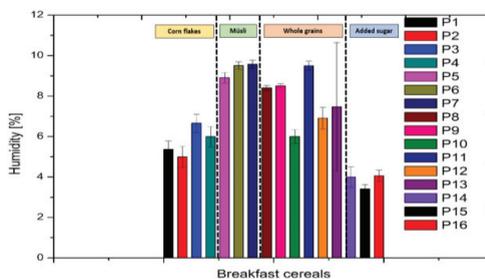


Figure 1. Humidity values for breakfast cereals

Furthermore, the microbiological results showed no *Enterobacteriaceae* contamination for any of the analysed samples.

Four samples, of which two müsli (P5 and P6) and two whole grains (P12 and P13) breakfast cereals samples noted yeasts and moulds levels of ca. 3 logs.

These microbiological results are closely related to the results on the incidence of mycotoxins, as showed further.

Biolog OmniLog identification system

The isolated fungi were confirmed using the Biolog OmniLog identification system.

The acceptable instrument readings (fig. 2) at the end of the incubation period noted a similarity index of 0.50-0.75 (good identification) for genus and species identification of *Aspergillus niger* (Singh, 2009; Wang et al., 2016).

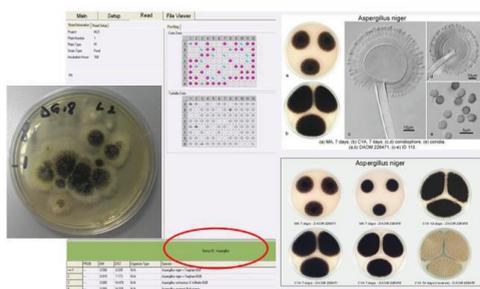


Figure 2. Identification of *Aspergillus niger* using Biolog OmniLog system

Aspergillus niger was identified in two samples, both müsli breakfast cereals with added dry fruits. The presence of added sugar in these food products did not influenced the presence of microorganisms, as *A. niger* was identified in both samples with and without sugar added commodities.

Occurrence of mycotoxins and analytical quality control

Concerning the occurrence of mycotoxins in cereal products, 4 samples noted no presence of deoxynivalenol or zearalenone, which represents a percentage of 25% of the analysed samples. Within these samples, most of them were whole grains breakfast cereals. Of all tested samples, only three registered levels of DON. The highest level of DON was 131.70 µg/kg, being noted by a breakfast cereal sample with a great percent of added sugar and synthetic preservatives (fig. 3). The corn flakes cereals noted no detectable level of DON. Regarding the ZEA concentrations, with the exception of the four samples mentioned before, all the breakfast cereals noted small levels of ZEA. The highest ZEA level was of 6,45 µg/kg and it was noted by a müsli breakfast cereal sample, with added dry fruits, results also noted by Mahnine et al. (2011). The same sample registered a DON level of 110.70 µg/kg and *A. niger* contamination, as this microorganism was isolated and further identified using the Biolog OmniLog system. Commercially available ELISA kits for detection of mycotoxins are normally based on a competitive assay format that uses either a primary antibody specific for the target molecule or a conjugate of an enzyme and the required target.

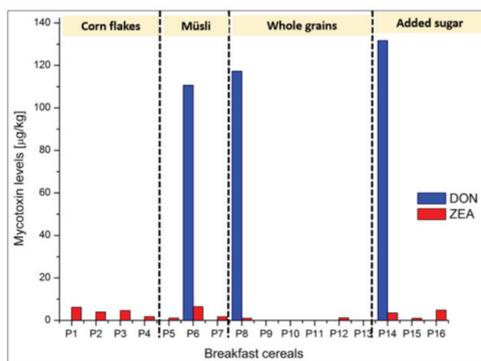


Figure 3. The occurrence of mycotoxins depending of the sample type

The recoveries and standard deviation of mycotoxins in the selected food matrix at different spiking levels are summarised in table 1.

Recovery rates at the lower spiking levels were between 117.67% for ZEA and 147.67% for DON, while for the highest spiking levels they were noted to be between 70.97% for ZEA and 147.67% for DON.

The lowest recovery corresponded to zearalenone at the 100 ppb spiking level (highest level).

Table 1. Recoveries and relative standard deviations (%) of mycotoxins in spiked breakfast cereals

Mycotoxin	Spiking levels (µg/kg)	Recoveries ± SD (%)
Deoxynivalenol	100	147.67 (±25.80)
	200	109.55 (±1.18)
	500	125.34 (±13.35)
Zearalenone	20	117.67 (±2.24)
	50	83.74 (±4.20)
	100	70.97 (±1.42)

The validation of the method for the selected food matrix was based on following performance parameters: accuracy (measured as percent error), precision (measured as coefficient of variance), reproducibility and repeatability (precision within- and between-day and analyst variability), limit of detection (LOD) and limit of quantification (LOQ).

The obtained results are given in table 2, along with other information regarding mycotoxin detection.

Table 2. Performance parameters for validation of mycotoxins for breakfast cereals food matrix

	Deoxynivalenol	Zearalenone
Maximum levels, µg/kg (EC Commission Regulation No. 1881/2006)	500	50 (maize based breakfast cereals) 100 (breakfast cereals excluding maize based breakfast cereals)
Precision, µg/kg	0.05	0.02
Reproducibility, %	4.98	1.83
Repeatability, %	3.31	4.92
Limit of detection, µg/kg	142.26	29.09
Limit of quantification, µg/kg	167.05	35.19

The accuracy of both methods was high. The difference between the taken value and the obtained results was very small, of 8,969 µg/kg for DON (-1.79%) and 4,735 µg/kg for ZEA (-4.74%), respectively. According to EU Commission Decision 2002/657/EC, the minimum accuracy of quantitative methods is between the range of -20% to +10%. Mean recoveries were respectively 98.21% for DON and 95.27% for ZEA. The stability was assessed during four weeks (n = 3 replicates) and the variation values were 446.48 µg/kg – 519.68 µg/kg for DON and 88.49 µg/kg – 105.64 µg/kg for ZEA. The LOD and LOQ of DON are 142.26 µg/kg and 167.05 µg/kg, while for ZEA they are 29.09 µg/kg and 35.19 µg/kg, respectively. Samples with a concentration higher than the LOQs were considered positive.

The advantage of the selected method is represented by the rapid sample preparation method and the short time requirement. The disadvantage of the method lies in the fact that they are for single use, which can increase costs of bulk screening. Additionally, competitive ELISA suffers from having a limited detection range due to the narrow sensitivity of the antibodies.

CONCLUSIONS

The occurrence of filamentous fungi and deoxynivalenol and zearalenone mycotoxins was determined in samples of breakfast cereals.

Aspergillus niger was isolated from two müsli breakfast cereals samples and one of this samples registered also the highest DON level, of 110.70 µg/kg and a ZEA concentration of 6.45 µg/kg.

It could be concluded from this study that müsli breakfast cereals seem to be more contaminated than the other cereal products that were analysed.

The registered mycotoxins level are consistent with the European legislation regarding these commodities, but the noted results show that a special attention to contamination levels in foods is necessary.

ACKNOWLEDGEMENTS

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CONTAMINATION OF POULTRY FEED BY POTENTIALLY TOXIGENIC FUNGI

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Abstract

This study examines the mycological quality of poultry feed in 69 samples (45 samples of chicken feed and 24 samples of feed for layers) in 2014 and in 44 samples of poultry feed (34 samples of chicken feed and 10 samples of feed for layers) in 2015. The total fungal count was determined using a dilution method, and standard mycological methods were used to identify potential toxigenic fungi genera.

*The chicken feed contained total fungal count above the allowed limit in 26.67% of samples in 2014, and in 14.71% of samples in 2015, while the total fungal count above the limit in the feed for layers was established only in year 2014, in 8.33% of the samples. Potentially toxigenic fungi species belonging to the genera *Aspergillus*, *Fusarium* and *Penicillium* were identified in both groups of studied poultry feed mixtures during both years. In chicken feed, the highest number of *Fusarium* positive samples in both years was recorded, 73.3% in 2014 and 64.71% of the samples in 2015. In the feed for layers, in the majority of samples (83.33%), *Fusarium* species were identified in 2014, while in year 2015, the species of the genera *Aspergillus* and *Fusarium* were identified in the majority of samples (90%).*

*Based on these results it can be concluded that the sanitary and hygiene conditions during the production of poultry feed must be determined by specific strategies for the reduction of the incidence of potentially toxigenic species of the genera *Aspergillus*, *Fusarium* and *Penicillium*. This strategy involves the use of complex and integrated measures to combat, especially during the growing of grain and other plants as the main raw materials, as well as during periods of storage and preservation of basic raw materials and finished mixtures used for poultry feeding.*

Key words: poultry feed, total fungal count, toxigenic fungi.

INTRODUCTION

Contamination of poultry feed by fungi secondary metabolites – mycotoxins is a major problem in poultry production causing harmful effects on the performance and health, and through consumption of poultry meat also poses danger to human health. The mixtures used in poultry nutrition include mostly grain as a source of carbohydrates, and potentially toxigenic fungi are the main contaminants of grain (Pleadin et al., 2015). In maize, which is the main component of poultry feed (component share of 50-60%; Jokić et al., 2004) about 19 genera of fungi are identified, of which the most common are species of the genera *Aspergillus*, *Fusarium* and *Penicillium* (Sivakumar et al., 2014). Mycotoxin producing fungal species are widespread in warm and humid environment, their control and decontamination of their metabolites is an important part of the poultry nutrition strategy. Reducing the moisture content (below 14%) in

the grain and feed mixtures is one of the basic preventive measures for the control of the fungi (moulds) growth in the process of production and storage of animal feed components (Osho et al., 2007). Considering the growing importance of food safety in the food chain, the adequate animal feed production management is essential because of the connection between feed contaminants levels, their presence in animal products (meat, eggs) and potential risk these contaminants pose to human health (Bhuyan et al., 2015).

Contamination of feed with mycotoxins is a major problem in the production of animal feed because it causes mycotoxicosis in animals. Aflatoxins are the most common mycotoxins in poultry production (Leggieri et al., 2015). Aflatoxin B₁ (AFB₁), produced by *Aspergillus flavus* and *A. parasiticus* (Pildain, 2008), is the most common mycotoxin in maize as a basic component of feed for poultry. The most common adverse effects of aflatoxin in the diet of chickens are reduced body weight and

increased liver and kidney weight in broilers (Zain, 2011). Another important mycotoxin in poultry nutrition is ochratoxin A (OTA) produced by the *Aspergillus* and *Penicillium* species. The prolonged feeding of poultry with feed contaminated with OTA causes reduced egg production, decrease in performance and body weight in poultry (Hassan et al., 2012). *Fusarium* mycotoxins, such as T-2 toxin and diacetoxyscirpenol (DAS) cause the weakening of the immune system and body resistance (Sokolović et al., 2008), necrosis and plaque formation (Zain, 2011). Since mycotoxigenic fungi are capable of producing more than one mycotoxin, and given that usually many different species of fungi develop in the feed at the same time, it is not uncommon that a wide spectrum of mycotoxins occur in feed, especially in feed prepared using multiple components (Streit et al., 2012).

The aim of this paper was to determine the total fungal count and to identify potentially toxigenic fungi in feed for poultry during the two-year period (2014-2015) and also to assess the potential danger of the presence of these contaminants in the food chain.

MATERIALS AND METHODS

The material for mycological analysis consisted of a total 69 samples of poultry feed mixtures in 2014 (45 samples of feed for chickens and 24 samples of feed for layers) and a total 44 samples in 2015 (34 samples of feed for chickens and 10 samples of feed for layers) originating from poultry farms in the vicinity of Belgrade in Serbia. Samples in the amount of 1 kg were collected successively (multiple times) in both study years. Mycological analysis was performed immediately after laboratory admission of the samples or the samples were stored for 2-3 days controlled temperature prior to the analysis. The moisture content of the tested samples was determined using a laboratory moisture meter (OHAUS MB35, USA), and mycological analysis was performed according to the method ISO 21527-2 (2008). Based on morphological characteristics, macroscopic (appearance of colonies) and microscopic (appearance of spores), potentially toxigenic fungi genera were identified in conformity with taxonomic criteria for genera

and species of fungi according to Watanabe (2002). The frequency of positive, i.e. samples contaminated by toxigenic fungi, was calculated according to the formula: Fr (%) = The number of samples were a fungal genus occurred/the total number of samples x 100.

Statistical analysis was performed with nonparametric test, using the SPSS software (IBM, Statistic 20). To determine the normality, the Shapiro-Wilk (SW) test was used, and to determine homogeneity of variance, the Levene's test. Because the Shapiro-Wilk test showed significant difference compared to the normal distribution, the significance of differences was tested using the Mann-Whitney U - test.

The correlation among individual values for moisture content, total fungal count and the frequency of fungal positive samples was determined using the Pearson correlation coefficient.

RESULTS AND DISCUSSIONS

The total fungal count and contamination of poultry feed samples with potentially toxigenic fungi of genera *Aspergillus*, *Fusarium* and *Penicillium* were examined in this paper.

Mycological analysis of samples of feed for chickens and layers established the total fungal count in the range from 1×10^1 to 2.41×10^5 cfu g⁻¹. In both study years, the highest number of samples of feed for layers was established with the total fungal count from 1.9×10^4 cfu g⁻¹, i.e. 58.30% of the samples in 2014, and 50% in 2015. Also, established is relatively high percentage of samples of feed for layers with the total fungal count of $1-2.41 \times 10^5$ cfu g⁻¹, i.e. 20% (2015) to 20.83% (2014) was established (Table 1).

The total fungal count is a parameter for hygiene and safety of animal feed, and, in the Republic of Serbia, according to the Regulation on the quality of animal feed (Službeni glasnik RS, 4/2010, 113/2012 and 27/2014), it should not exceed the value of 5×10^4 cfu g⁻¹ for younger categories of farm animals and 2×10^5 cfu g⁻¹ for adult animal categories. In the present study, in 26.67% of samples in 2014, and in 14.71% of samples in 2015 of chicken feed, and in 8.33% of samples of feed for layers in 2014 (Table 2), the total fungal counts above

the limit were established. Similar to these results, Dalcero et al. (1998) have found that the total fungal counts were above the permitted levels in samples of poultry feed in several months during the study period from May/1996 to May/1997 in Argentina. Likewise, in Iraq, Shareef (2010) has determined average total fungal count of 7×10^5 cfu g⁻¹ in samples of poultry feed collected during a two-year period (2005-2007). In contrast, according to Oliveira et al. (2006), the total fungal count in the tested samples of poultry feed originating in

Brazil did not exceed 1×10^4 cfu g⁻¹. Also, in Poland, the mean amount of moulds and yeasts in the analysed samples of feed for chickens was 7×10^2 cfu g⁻¹ (Cegielska-Radziejwska et al., 2013).

In the analysed samples of chicken feed, an average total fungal count was statistically significantly higher ($P \leq 0.05$) in 2014 compared to 2015, while in case of the samples of feed for layers, there was no statistically significant difference in the total fungal count between the examined years (Table 3).

Table 1. Level of fungal contamination of investigated poultry feed samples in 2014 and 2015

Fungal counts		Frequency (%)			
		Year 2014		Year 2015	
cfu g ⁻¹ *	log ₁₀ cfu	1	2	1	2
$1-2.41 \times 10^5$	5-5.38	13.33	20.83	11.76	20
$1-9 \times 10^4$	4-4.95	31.11	58.30	14.71	50
$1-9 \times 10^3$	3-3.95	20	4.17	2.94	0
$< 1 \times 10^3$	< 3	35.56	16.70	70.59	30

*Colony forming units per g of sample; 1- samples of feed for chickens; 2- samples of feed for layers

Table 2. Frequency of investigated poultry feed samples in 2014 and 2015 with total fungal count within limit values according to Regulation of Republic of Serbia

Fungal counts		Frequency (%)			
		Year 2014		Year 2015	
cfu g ⁻¹ *	log ₁₀ cfu	1	2	1	2
$> 50,000 (5 \times 10^4)$	> 4.7	26.67	33.33	14.71	50
$> 200,000 (2 \times 10^5)$	> 5.3	2.22	8.33	0	0

*Colony forming units per g of sample; 1- samples of feed for chickens; 2- samples of feed for layers

Table 3. Mean of total fungal counts (log₁₀cfu g⁻¹) in samples of feed for chickens and feed for layers in investigated years 2014 and 2015

Feed samples for chickens	cfu g ⁻¹ (log ₁₀) ± S.E.	Mediana
Year 2014	$3.59 \pm 0,18$	3.48
Year 2015	$2.99 \pm 0,22$	2.30
Level of significance	*	
Feed samples for layers	cfu g ⁻¹ (log ₁₀) ± S.E.	Mediana
Year 2014	$4.23 \pm 0,22$	4.58
Year 2015	$4.06 \pm 0,38$	4.69
Level of significance	ns	

cfu g⁻¹ - colony forming units per g of sample; * - significant - $P < 0.05$; ns - not significant - $P > 0.05$

Table 4. Frequency of contaminated samples with potentially toxigenic fungi from *Aspergillus*, *Fusarium* and *Penicillium* genera

Fungal genus	Frequency of fungal contaminated samples (%)			
	Year 2014		Year 2015	
	1	2	1	2
<i>Aspergillus</i>	44.44	79.17	55.88	90
<i>Fusarium</i>	73.33	83.33	64.71	90
<i>Penicillium</i>	53.33	54.17	17.65	30

1- samples of feed for chickens; 2- samples of feed for layers

High fungi colony counts in 2014 year can be explained through the influence of suitable climate conditions which were optimal for the development of toxigenic mould during the growing and harvesting phase of maize. An important prerequisite for the development of toxigenic fungi in the grain before and after harvest are suitable conditions of temperature and humidity. According to data from Paterson and Lima (2011), mild temperatures and wet weather during the growth of maize favour the development of *Fusarium* species. Likewise, Asselt et al. (2012) have found that high rainfall and wind speed during the silking of maize contribute to intensive *Fusarium* infection of the grains. Furthermore, one of the most important preconditions for the infection of grain during storage is the moisture content of the grain. Maize moisture content of $\leq 15\%$ is suitable for safe storage. According to Kana et al. (2013) moulds are capable to develop on dry surfaces and on feeds containing not more than 13% of moisture.

In both groups of investigated poultry feed samples, species of the genera *Aspergillus*, *Fusarium* and *Penicillium* were identified. In addition to these fungal genera, in a small number of samples genera *Mucor*, *Rhizopus* and *Alternaria* were identified. In both study years, in most samples (64.71-90%), *Fusarium* species were identified, and, in 2015, the same number (90%) of *Fusarium* and *Aspergillus* positive samples of feed for layers were identified (Table 4). Similarly, Dalcero et al. (1998) have found the highest incidence of the genera *Aspergillus* (85%) and *Fusarium* (70%) of the 130 samples of poultry feed, whereas according to Rosa et al. (2006), Shareef (2010) and Cegielska-Radziejwska et al. (2013), the most common in the investigated samples of poultry feed were identified species of the genus *Aspergillus*.

In samples of feed for chickens, the average moisture content was 11.57% in 2014 and 11.44% in 2015, while the average moisture content in samples of feed for layers was 10.96% in 2014 and 10.90% in 2015. Based on the data of the Pearson correlation analysis between the moisture content of the samples and the total fungal count, a negative correlation was established in the group of samples of feed for chickens in both study

years, 2014 ($r=-0.35$) and 2015 ($r=-0.18$), while the correlation in the group of samples of feed for layers was positive, in both years 2014 ($r=0.23$) and 2015 ($r=0.26$). These data are similar to the data of Rosa et al. (2006).

Considering the correlation between the most common fungi in contaminated samples in 2014, a positive correlation was established between *Aspergillus* and *Fusarium* positive samples of feed for chickens ($r=0.17$) and layers ($r=0.38$), while in 2015, this correlation was positive in samples of feed for layers ($r=0.06$), but not in samples of chicken feed ($r=-0.42$) (data not presented). Similar to these results, Scudamore et al. (1997) have found that aflatoxins produced by *Aspergillus* species and fumonisins produced by *Fusarium* species appear jointly mostly in the maize samples. Also, Oliveira et al. (2006) have found a simultaneous occurrence of AFB₁ and *Fusarium* mycotoxins in poultry feed mixtures.

CONCLUSIONS

Based on the obtained results of the total fungal count and contamination of samples by potentially toxigenic fungi, it can be concluded that the mycological analysis are justified and necessary for the assessment of health and hygienic correctness of poultry feed.

In regard to the two groups of samples of investigated poultry feed, 26.67% of samples of feed for chickens had total fungal count above allowed limit, in 2014, and 14.71% of the samples in 2015, while much smaller number of samples (8.33%) of the feed for layers had total fungal count above allowed limit, and only in 2014.

In both studied groups of poultry feed, potentially toxigenic fungi of the genera *Aspergillus*, *Fusarium* and *Penicillium* were identified, with the majority of the samples contaminated with *Fusarium* species, followed by species of the genus *Aspergillus*. In most of the studied poultry feed mixtures, a positive correlation between *Aspergillus* and *Fusarium* positive samples was established.

These results suggest the need for continuous, primarily, mycological but also mycotoxicological analysis of the quality of poultry feed, in order to provide health stability of poultry products for human consumption.

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FUNCTIONAL PROPERTIES OF CAMEL MILK AND THEIR INFLUENCES ON TECHNOLOGICAL APPLICATIONS

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Abstract

*According to the recent statistics by the Food and Agriculture Organization (FAO), the total population of camels in the world is estimated to be about 20 million, with Somalia having the largest herd worldwide. Camels are well adapted to harsh desert climates and can survive without drinking water for days. Therefore, camel (*Camelus dromedarius*) is of significant socio-economic importance in many arid and semi-arid parts of the world and its milk constitutes an important component of human diets in these regions.*

The amounts of lysozyme, lactoferrin, lactoperoxidase and immunoglobulins were found to be greater in dromedary camel milk than bovine or buffalo milk. This property has been shown to be a disadvantage in yoghurt production. As known like yoghurt, cheese is another fermented milk product, due to the activity of these compounds the enzymatic reaction is disturbed and the gelation process of milk is prolonged. These antimicrobial agents were reported to completely lose their activity in camel milk if heat-treated at 100°C for 30 min. But there are contradictory statements about the heating intensity. Therefore in this review on these studies are focussed. In addition the chemical composition of camel milk is compared with another ruminant milks. Camel milk has lots of functional properties. These are antioxidant activity, bioactivity, anti-cancer activity, hypoallergenicity.

Key words: camel milk, lactoferrin, immunoglobulins, heat treatment, functional properties

INTRODUCTION

There are about 18 million camels in the world (FAO, 1996) which support the survival of millions of people in arid and semi-arid areas. Meanwhile camel milk is considered one of the main components of the human diet in many parts of the world. Camels were domesticated and developed approximately 5000 years ago and throughout these years have played an integral role in the daily life of camel owners. They are distributed in Africa and Asia, where other livestock farming cannot be easily implemented (Gupta *et al.* 2015). Camels are very resistant animals of hunger and thirst. Variations in the contents of camel milk may be based on to several factors such as analytical methods, geographical area, nutrition conditions, breed, lactation stage, age and number of calvings (Khaskheli *et al.* 2005). The quality of camel milk and meat, since it contains both valuable essential nutrients, has acquired an important place in human nutrition (Adel *et al.* 2009). According to the recent statistics by the Food and Agriculture

Organization (FAO, 2008), the total population of camels in the world is estimated to be about 20 million, with Somalia having the largest herd worldwide (FAO, 2008).

Also according to FAO data the production of camel milk is 5.3 million/liter in the world. At the present time, depending on the camel cultivation camel milk production is also becoming increasingly common.

For this reason, the number of scientific research on camel milk have increased in recent years. They are mainly distributed in African and Asian arid and semi-arid areas, where other livestock farming cannot be easily applied (Gupta *et al.* 2015). Today, camels and their products have been using by humans for transport, traction power, milk, meat, fiber (wool and hair). At the same time, it is used as a raw material for textile industry.

Chemical Composition of Camel Milk

The camel has the ability to produce more milk for a longer period of time in arid zones and dry lands (an environment of extreme

temperature, drought, and lack of pasture) than in other domestic livestock species (Yagil and Etzion, 1980).

Geographical root and seasonal variations are factors which influence most changes in composition of camel milk. Camel milk contains 2.9 to 5.5% fat, 2.5 to 4.5% protein, 2.9 to 5.8% lactose, 0.35 to 0.90% ash, 86.3 to 88.5% water, and 8.9 to 14.3% solid-non-fat (SNF) (Khan and İkbal, 2001). Camel milk has similar protein content, lower lactose content (Elamin & Wilcox, 1992), and greater total cholesterol (Gorban and Izzeldin, 1999) compared with cow's milk. Camel milk has greater contents of vitamin C (Mehaia, 1994), ash, and sodium, potassium, phosphorus, zinc, iron and manganese (Gorban and Izzeldin, 1997) than cow's milk.

Seasonal variations also play a significant role in the composition of camel milk, also with camels of the same type and from the same district (Bakheit et al. 2008).

According to other research related to compositional, technological and nutritional aspects of dromedary camel milk the average values of camel milk composition reported from 1980 to 2009 are as follows: protein 3.1%; fat 3.5%; lactose 4.4%; ash 0.79% and total solids 11.9% (Adel et al. 2009). Rates of milk components are based on various types of animals.

Camel's milk is a good source of various vitamins and minerals and it has several medicinal and therapeutic effects and good antibacterial and antiviral properties (Yagil & Etzion, 1980; Balouiri et al., 2016). Some studies showed that camel's milk is an excellent source of components that are involved in some biological activities, such as defence against free radicals and reactive oxygen species. The world's total population of camels was reported to be twenty-two million in 2010 (FAO, 2012) that could produce about 300 million litres milk representing 0.2% of world's total produced milk in 2010 (IDF, 2010).

The amounts of lysozyme, lactoferrin and immunoglobulins were found to be greater in dromedary camel milk than bovine or buffalo milk (Benkerroum, 2008; El-Agamy et al., 2000; Kappeler et al., 1999; Konuspayeva et

al., 2007). This property has been shown to be a disadvantage in yoghurt production.

The growth of yoghurt culture in camel milk is delayed due to the presence of lysozyme (Abu-Tarboush, 1996; Jumah, Shaker, & Abu-Jadayil, 2001) which prolongs the gelation process (Jumah et al., 2001).

These antimicrobial agents were reported to completely lose their activity in camel milk if heat-treated at 100°C for 30 min (El-Agamy, 2000).

According to another observations and experiments unlike cow milk, it was found that camel milk can be preserved for a longer time at 30°C and most importantly the camel milk can be kept at 4°C for more than three months without any appearing change (Yagil, 1985).

The ability of camel milk to inhibit growth of pathogenic bacteria and its relation to whey lysozyme has been showed by Barbour et al. (1984).

At the same time, camel milk is higher in α -lactalbumin, as it is in human milk compared with cow milk.

Unpublished commercial data reported that some infant formula contains high level of α -lactalbumin in changing to breast feed milk.

Antimicrobial factors of camel and human milk

As shown in Table 1 camel milk is richer in immunoglobulins than human milk. However, its contents of lactoferrin and lysozyme were very low. El-Agamy and Nawar (2000) found that camel milk is contain 1.64 mg/ml of immunoglobulin G versus 0.67, 0.63, 0.70, 0.55 and 0.86 for cow, buffalo, goat, sheep and human milk, respectively.

A comparative study of lysozyme concentration in milk of different species (El-Agamy et al., 1997) showed that camel milk contained significantly higher content of lysozyme than cow, buffalo, sheep and goat but very low content as compared to lysozyme content of human, mare and donkey milks.

The same study showed that camel milk contained also significantly higher level of lactoferrin (0.22 mg/ml) than cow, buffalo, sheep and goat but very low compare with that of human milk.

Table 1. Antimicrobial factors in camel and human milks (El-Agamy et al., 1997)

Antimicrobial factor	Camel milk	Human milk
Mean values \pm SD		
Immunoglobulins (mg/ml)	1.54 \pm 0.032	1.14 \pm 0.055
Lactoferrin(mg/ml)	0.24 \pm 0.035	1.95 \pm 0.050
Lysozyme(mg/ml)	0.06 \pm 0.02	0.65 \pm 0.045

Nutritional properties of camel and human milks

Milk of all mammals contains the same principal components, namely water, proteins, fats, carbohydrates, vitamins and minerals, but their content varies widely between ruminant and nonruminant milk. Especially, camel milk contains all essential nutrients as cow milk (El-Agamy et al., 1998). Many components in bovine colostrum and milk exhibit specific biological activity in addition to their established nutritional values. During the past two decades, interest in these beneficial physiological effects and the possibility to utilise the components from milk have increased.

Even between various (non-) ruminants and within a same species the milk composition may differ considerably, given the influence of genetic factors (not only at species but also at breed level), physiological factors (e.g. lactation stage, milking interval), nutritional factors (e.g. feed energy value and composition) and environmental conditions (e.g. location, season). The values should therefore not be viewed as absolute but rather as indicative for the concentration range of milk components. Moreover, methodological differences regarding data collection between consulted papers may contribute to the spread of the presented values.

Therapeutic properties of Camel milk

According to studies, the production of camel milk has significantly increased during the last few years with now pasteurized fresh camel milk in the supermarket. Firstly, camel milk is supposed to have medicinal properties (El-Agamy et al., 1992). In studies camel milk is used jaundice, asthma, in the treatment of various diseases such as tuberculosis and it has been found to be helpful. In addition to this

column, cancer, diabetes, hypertension was identified that help to treat their patients (Hossam, 2015). Nowadays, there is a general need to start a number of camel milk based functional products to the commercial markets due to increasing demand in recent years (Al haj *et al.* 2010). These products have to be clinically proven and scientifically evident supported (Ghosh, 2009). Camel milk has lots of functional properties.

These are antioxidant activity, bioactivity, anti-cancer activity, hypoallergenicity activity (Habib et al., 2013). It is also known that the camel milk has a therapeutic potential against many diseases including cancer. In addition it has long been utilized for its benefit in broad range of diseases like Insulin Dependent Diabetes Mellitus (IDDM) (Agrawal et al., 2002; Agrawal et al., 2003; Agrawal et al., 2005), infant diarrhea (Yagil, 2013), hepatitis (El-Fakharany et al., 2008), allergy, lactose intolerance (El-Agamy et al., 2009; Konuspayeva et al., 2009; Cardoso et al. 2010). It contains extraordinarily high levels of insulin like molecule (Agrawal et al., 2002; 2003; 2005).

Camel milk is emerging as a potent therapeutic alternative which can help in reducing insulin doses in diabetic patients. It's well established role in management of Diabetes has rendered it the title of "white gold of desert". Epidemiological surveys strongly indicate low prevalence of diabetes in communities consuming camel milk. (Agrawal et al., 2013).

Composition of Camel milk colostrum

Colostrum is a complex fluid rich in nutrients and is also characterised by its high level of bioactive components, e.g. immunoglobulins (Igs), especially IgG1, growth factors, especially insulin-like growth factor-1 (IGF-1), transforming growth factor beta-2 (TGF-b2) and growth hormone (GH) as well as lactoferrin, lysozyme and lacto peroxidase (Butler, 1994; Pakkanen, 1998; Regester, Smithers, Mitchell, McIntosh, & Dionysius, 1997; Reiter, 1985). Camel colostrum differs in composition from regular milk in that it has a high content of whey proteins, mainly immunoglobulins G (IgG), providing the newborn with immunity.

Camel colostrum IgG consists of three main sub-classes, namely IgG1, IgG2, and IgG3 (Azwai

et al., 1996) the two latter sub-classes are devoid of light chains and have a molecular mass of 42 and 45 kDa, respectively (Hamers-Casterman et al., 1993). It has been reported that these heavy-chain antibodies interfere with several biological processes and may make it a good candidate for human therapy (Holt et al., 2003). To current knowledge, no information is available regarding the variation in IgG and other major whey proteins in camel colostrum and milk during the first week of lactation.

Antibacterial activity of Camel milk

Camel milk is reported to have an antimicrobial effect against Gram positive and Gram negative bacteria, including *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella typhimurium* (Benkerroum et al., 2004; El-Agamy and Khatib, 1992). This inhibitory activity was attributed to the presence of antimicrobial substances in camel milk, including lysozyme, hydrogen peroxide, lactoferrin, Lactoperoxidase and immunoglobulins (El-Agamy and Khatib, 1992). Lactoperoxidase (LPO) is a suitable enzymatic indicator of correct pasteurisation of camel milk and its products are heat-treated at 75 degrees C for 15 seconds (Wernery et al., 2013).

The inhibitory action of camel milk against *L. monocytogenes*, *S. aureus* and *E. coli* might be attributed to the presence of lactoperoxidase, hydrogen peroxide and lysozyme respectively (Benkerroum et al., 2004). The growth of *Salmonella Typhimurium* was inhibited by lactoferrin in camel milk through binding iron and making it unavailable for its growth (El-Agamy and Khatib, 1992; Ochoa & Cleary, 2009).

Antibacterial activity of Transferrin

Transferrin (TF) is a monomeric glycoprotein of 679 amino acids, with a relative molecular weight of approximately 80 kDa. Transferrin exists mainly in the serum and interstitial compartments of vertebrates and some invertebrates (Baker and Lindley, 1992; Retzer et al., 1996). Transferrin is found at a much lower concentration in human milk (< 50 µg/mL) in comparison with bovine milk (20 to 200 µg/mL) (Schanbacher et al., 1993). The principle physiological function of TF in

mammals is to transport ferric irons from sites of absorption to sites of utilization. Transferrin transports iron from the biological fluids into the cytoplasm via plasma membrane by receptor-mediated endocytosis (Pakdman and Chahine, 1997). Transferrin interacts with specific receptors present in variable amounts on target cells. Important target cells include the liver, bone marrow and muscle.

Antibacterial activity of Lactoferrin

Lactoferrin is a mammalian cationic iron-binding glycoprotein belonging to the transferrin family, which was discovered 70 years ago, and isolated simultaneously from human and bovine milks in 1960. Lactoferrin is present in the majority of external secretions and mucosal surfaces, milk being its main source. Lactoferrin binds two atoms of iron and due to this capacity several functions have been attributed to it, such as antibacterial, antioxidant, antitumoral and immunomodulatory (Sanchez et al., 1992).

It is widely distributed in all biological fluids and is also expressed by immune cells, which release it under stimulation by pathogens. Lactoferrin is a multi-functional protein with many beneficial properties, which makes it a functional food for a number of product, commercial and clinical applications (Adlerova et al., 2008). Lactoferrin is a glycoprotein with a molecular weight of about 80 kDa, which shows high affinity for iron. The molecular structure and amino acid sequence of human lactoferrin were discovered in 1984.

Almost all bacteria require iron for their growth; therefore LF devoid of iron is capable of preventing its utilization by some bacteria (Orsi, 2004). A large number of studies have demonstrated the bacteriostatic and bactericidal effect of LF, against a wide range of Gram-positive and Gram-negative bacteria (Farnaud and Evans, 2003). However, other mechanisms besides iron holding can be involved in the antibacterial activity of LF, such as blocking microbial metabolism of carbohydrates or destabilizing the bacterial cell wall (Sanchez et al., 1992).

Antibacterial activity of Lysozyme

Lysozyme (EC 3.2.1.17; muramidase) is a single polypeptide chain consisting of 129

amino acids, in which lysine is the N-end amino acid and leucine is the C-end one. It is a globular basic protein characterized by molecular weight of 14.3 kDa and cross-linked by four disulfide bonds (Masschalck et al., 2002; Cegielska et al., 2008). It is an important antimicrobial agent in milk, which kills bacteria by cleaving the β -1,4-glycosidic bond between C-1 of N-acetyl muramic acid and C-4 of N-acetyl glucosamine residues of the peptidoglycan in the bacterial cell wall (Zhao, et al., 2011; Li et al., 2011). Lysozyme appears to inhibit not only bacteria where the peptidoglycan layer is a major component of their cell-wall, but also viruses and eukaryotic microorganisms devoid of a typical peptidoglycan layer, suggesting that it acts by other mechanisms of action than the hydrolytic activity (Benkerroum, 2008)

Antibacterial activity of Immunoglobulins

Immunoglobulins in milk immediately brings to mind the relationship between mother's milk, transfer of passive immunity from mother to neonate, and the immature immune system of the neonate. Research in this field dates back to the late nineteenth century, however for many centuries herdsmen have capitalized on the linkage between maternal immune status and the immunological protection and development of the neonate (Butler, Kehrl, 2005; Wheeler et al., 2007). Immunoglobulins in mammary secretions come from several sources and represent a history of the antigen exposure of the mother and the response of her immune system. Immunoglobulins are transported through the mammary epithelial cells by receptor-mediated mechanisms and transferred out of the mammary gland by milk ejection during suckling. The immunoglobulins then enter the environment of the gastrointestinal tract of the neonate. Although that environment is primarily geared toward digestion to gain nutritional benefit, the immunoglobulins remain sufficiently stable to provide protective benefits for the neonate, either through uptake into the vascular system in the newborn of some species or through immunological function in the gastrointestinal tract. The immunoglobulins found in milk and the transfer of passive immunity from mother

to neonate have been reviewed by many authors.

Antifungal activity of camel milk components

Regarding the antifungal activity of lactoferrin, the first observation which can be made is that the great majority of research has been carried out on *Candida*, well known as one of the most dangerous opportunistic pathogens. As for bacteria, the anti-*Candida* activity of lactoferrin was initially considered as related to its ability to bind and sequester environmental iron. But in addition to the iron-chelating activity, a direct interaction between lactoferrin and *Candida* cells was demonstrated in our Department by Valenti et al. (1986).

Antiviral activity of camel milk components

In a few cases it is reported that lactoferrin failed to prevent virus infection. On the contrary, a long list of virus has been found to be sensitive to the inhibiting action of lactoferrin. This list includes several enveloped viruses such herpes simplex virus 1 and 2 (Hasegawa et al. 1994), human cytomegalovirus (Hasegawa et al. 1994), human immunodeficiency virus (Harmsen et al. 1995), hepatitis B virus (Hara et al. 2002), hepatitis C virus (Ikeda et al. 1998), respiratory syncytial virus (Grover et al. 1997), hanta virus (Murphy et al. 2000) and four naked viruses: rotavirus (Superti et al. 1997), poliovirus (Marchetti et al. 1999), adenovirus (Arnold et al. 2002) and enterovirus 71 (Lin et al. 2002).

Evaluation of camel's milk from technological aspects

The absence of β -LG might explain some of the differences observed between camel and cow milk regarding technological properties such as thermal stability during drying, heat induced aggregation and adherence to heating surfaces (fouling properties) as well as the thin consistency found in fermented camel milk (Merin et al. 2001; El-Agamy, 2007; El-Hatmi et al. 2007; Laleye et al. 2008).

A detrimental effect of heating on the beneficial health effects of milk, the most frequently cited arguments of raw milk advocates are a reduced susceptibility to

allergies, a higher nutritional quality and a better taste. However, the consumption of raw milk poses a realistic microbiological risk for the consumer. The presence of foodborne pathogens has been demonstrated in many surveys and foodborne infections have been repeatedly reported for *Campylobacter*, *Salmonella* spp. and human pathogenic verocytotoxin-producing *Escherichia coli* after raw milk consumption (Claeys et al., 2013; O'Mahony, Fanning and Whyte, 2009; Robinson, Scheftel, & Smith, 2014; Verraes et al., 2014).

Whey proteins of bovine milk are less resistant to heat denaturation compared to those of buffalo milk, which in turn are less heat resistant than camel whey proteins (El-Agamy, 2000). Even though camel whey proteins have a higher heat stability than bovine whey proteins at temperatures between 63 and 90°C (Farah, 1986), bovine milk coagulates much slower at higher temperatures. This could be related to the absence or very low levels of β -lg and κ -casein in camel milk (Farah & Atkins, 1992) as milk is more resistant to heat when it is characterized by a molar β -lg to κ -casein ratio close to 1 (Barłowska, Szwajowska, Litwinczuk, Król, 2011).

In another study is shown that the heat stability of camel milk was relatively lower at high temperature treatments. Heat coagulation time (HCT) in the range 100-130 degrees C was too short (< 2 min). Camel milk heat preservation can be done only by pasteurisation. After LTLT pasteurisation, counts of aerobic total and psychrotrophic bacteria were significantly ($p < 0.05$) reduced and coliforms were not detected (Kouniba et al., 2005).

There are some investigations applied the inactivity of enzymes, which have from the technological sides important. In this case the activity of alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), lactoperoxidase (LPO), lipase (LIP) and leucine arylamidase (LAP) in raw and pasteurised camel milk was studied, in order, to find a heat treatment indicator suitable to verify an effective pasteurisation. LAP activity in raw camel milk is too low and the data variation is too high for serving as a marker. The LPO results look promising. The enzyme activity in raw camel milk is high and the respective value

in pasteurised milk is predominantly below the detection limit of the method.

CONCLUSIONS

The production of camel milk is gradually increasing due to an increased interest by consumers in recent years. Camel milk was found to be different in some aspects from milk of other animal species, such as bovine milk. Use of camel milk is widespread not only during production of different kinds of milk products but also as cure material to heal different kinds of diseases such as cancer, diabetes, hypertension, autism dropsy, jaundice, tuberculosis, asthma.

Except from the therapeutic properties the use of camel milk is investigated in different area. Due to functional properties of some camel milk components such as lactoferrin, lysozyme and immunoglobulins the camel milk is longer storable than other kinds of ruminants milk. Some of functional properties are called such as antibacterial, antiviral, antifungal, antiallergic exc.

Camel milk is known as an alternative milk source and is widespread in many countries. The production of milk products such as yoghurt, chees, ice-cream, pasteurised milk especially in Somalia and Sudan.

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SIMULTANEOUS DETECTION OF THREE FORBIDDEN ANIMALS (PORCINE, CANINE, AND RAT) IN HALAL FOOD BY USING HIGH RESOLUTION MELTING ANALYSIS

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Abstract

Halal food approved for Muslim consumers is strictly noticed that avoiding from contaminations that do contradict from Islamic regulations, such as the contamination of animal forbidden by Halal standards. Importantly, High resolution melting analysis (HRMA) is a potential molecular technique, which is used for identifying the species of organisms. Therefore, the research objective is to use the HRMA technique for simultaneous detection of the three forbidden animals (pigs, dogs, and rats) that have high opportunity to be adulterated in Halal food. The HRMA, targeting a fragment of NADH dehydrogenase (ND5), ATPase subunit 6 (ATP6), and Cytochrome B (Cytb) genes, were developed in order to authenticate the forbidden animal and their mixtures. Species-specific primers were designed and combined in a multiplex HRMA resulting in different sequences and therefore different melting behaviours for each species. The multiplex HRMA was then evaluated the PCR specificity against the targeted DNAs of targeted and non-targeted. It's demonstrated that the method had no cross-reaction with DNA from the experimental animal species (pigs, dogs, rats, cats, monkeys, chickens, horses, sheep, goats, and donkeys). The HRMA profile of amplified amplicons from the targeted animals produced uniquely melting peaks that were easily distinguished for each species in this study. Taken together, all data indicates that this multiplex HRMA is a simple, fast, specific, and cost-effective detection method for pig, dog, and rat in halal food. In order to carry out the analysis of commercial food products, 150 commercial food products was used to screen by species-specific primers for pigs, dogs, and rat were combined in multiplex HRMA. This method revealed that one sample was contaminated with pigs' DNA in the examined products. Therefore, the HRMA could be used as a halal verification technique for detecting aforementioned forbidden animals contaminated in halal food products.

Key words: Halal food, Forbidden animal, Multiplex HRMA, species identification, Authentication.

INTRODUCTION

“Halal” in an Arabic word means “permit” or “lawful” according to Islamic regulations, not only for food but non-foods and services as well.

Therefore, Muslims or the believers in Islam shall consume only Halal products and services and avoid “Haram” or “Forbidden” products. Consuming Haram products would affect the faith and spirit of Muslims.

According to annually announced in coming 2050, the world population will continuously increase up to 9,000 million, which means resulting in increasing Muslim populations as well (Nakyinsige et al., 2012).

Besides, the world halal food industry exhibits high value of economics since Halal marketing is merely value of goods and services which have rapidly grown on demand and high consumer based that expanded gradually (Jafari and Scott, 2014).

Despite Thailand amongst the major food producers exporting varieties of food products to the world as well as to Muslim countries, almost all food manufacturing proprietors and producers are non-Muslims.

No matter how strict they comply with international food safety standards, the spiritual safety to protect faith and belief of Muslims has yet to be widely and clearly understood and often overlooked, which might result in

contaminating of forbidden composition regarding Islamic regulations from misunderstood or intended to produce halal products especially in meat processing industries (Dahlan, 2013).

From Islamic regulations, it indicates that various kinds of forbidden animals would not appear in Halal food such as pigs, dogs, rats, cats, reptiles, and others. The recently survey found that these animals have possibly mix and adulterated with qualified halal food to decrease the processing cost, which has occurred found in Thailand and other countries such as Vietnam, Indonesia and China (Dahlan, 2013; Ali et al., 2015).

Therefore, rapid and reliable methods is needed for these forbidden species identification. DNA-based PCR methods have become widely used to identify meat species authentication. As a result of high stability under high temperatures, pressures, and chemical treatments of DNA. It can be investigated on raw meat or cooked meat (You et al, 2013). High resolution melting analysis (HRMA) is one of DNA-based PCR method that allows genotyping and fingerprinting by discrimination separation DNA sequence variant such as single nucleotide polymorphisms (SNPs) and small insertion and deletions (indels) based on the shape of melting transitions (T_m) of real-time PCR. This method can also be applied for screening for the existence of unknown sequence variations without a sequencing process. However, simplex HRMA is laborious, expensive, and complex. To reduce cost and increase the speed of HRMA, a Multiplex HRMA was developed (Druml and Cichna, 2014; Iacumin et al, 2015). In this study we aim to apply the Multiplex HRMA for simultaneous detection of the three forbidden animals composed with pigs, dogs, and rats that have high opportunity to be adulterated in Halal food.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

Among the three target forbidden animals pigs ($n=4$), dogs ($n=4$), and rats ($n=4$). Porks were collected from Samyan market in Phatumwan district, Bangkok, Thailand. Dog meats were

collected from the Faculty of Veterinary Science, Chulalongkorn University, while rat meats were collected from Taladthai market Phatumthani province, Thailand. Meats and whole bloods from other species (cats, monkeys, donkeys, horses, chickens, sheep, and goats) were collected from various markets, zoological park organization of Thailand and the Faculty of Veterinary Science, Chulalongkorn University. Then, all samples were DNA extracted using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Finally, the extracted DNA concentrations were examined by a Nanodrop 2000 (ThermoScientific, USA). The quantified DNAs were stored at $-20\text{ }^{\circ}\text{C}$ until use.

Multiplex HRMA

All three of species-specific primer pairs used in this study are listed in (Table 1). Verification of the specificity of each primer pair was performed using NCBI BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

To develop technique, both simplex and multiplex HRMA were carried out by using a LightCycler®480 instrument (Roche, Germany).

In a total volume of 20 μL containing 20 ng of genomic DNA, 10 μL 2XQIAGEN Multiplex PCR (Qiagen, Hilden, Germany) 0.2 μM of forward and reverse primer, and 1x LightCycler ResoLight dye. The amplification condition was 1 cycle of 5 min at $94\text{ }^{\circ}\text{C}$; 29 cycles of 30 sec at $94\text{ }^{\circ}\text{C}$; 40 sec at $56\text{ }^{\circ}\text{C}$; 1 min at $72\text{ }^{\circ}\text{C}$ followed by HRMA ramping from $60\text{ }^{\circ}\text{C}$ to $99\text{ }^{\circ}\text{C}$ with 50 acquisitions. PCR products were analyzed by QIAxcel Capillary Electrophoresis system (Qiagen, USA).

The HRMA developed in this study was validated for its specificity and reliability. In terms of the specificity test, the assay was cross tested with all three target species and seven other animals (cats, monkeys, donkeys, horses, chickens, sheep, and goats).

Finally, for reliability (real-world performance testing), the developed assay was used to test 150 Halal food products, including 50 meat processing products, 30 dairy products, 20 seasonings, 20 snacks, and 30 bakery products.

Table 1. Details of primer sequence, PCR product size, and Tm (C°)

Species	Primer	Sequence (5' to 3')	Product size	Tm (C°)	References
Porcine	ND5-For	5'-GGCGCCATCCCAATTATAATATCCAACCTC-3'	144bp	81.50-81.57	Ali et al., 2015
	ND5-Rev	5'-TGATTATTCTTGGCCTGTGTGT-3'			
Dog	ATP6-For	5'GAGGTGCGGAAGCGGAGGGGCGGGGGCTCT	138bp	85.71-85.83	In this study
	ATP6-Rev	AGCCGTTTCGAT -3'			
Rat	Cytb-For	5'-GTGATAAAAAGCTGTGGTGC -3'	218bp	83.49-83.50	Yanita.I.W et al., 2015
	Cytb-Rev	5'-CCCCGTTGGCGGTGTAATA -3'			

Table 2. Cross reaction of the HRMA (*n=4), (+) amplification, (-) no amplification

Species*	Scientific name	Species specific primer		
		Pig-ND5	Dog-ATP6	Rat-Cytb
pig	<i>Sus scrofa</i>	+	-	-
Dog	<i>Canis lupus familiaris</i>	-	+	-
Rat	<i>Rattus argentiventer</i>	-	-	+
Cat	<i>Felis catus</i>	-	-	-
Monkey	<i>Macaca fascicularis</i>	-	-	-
Donkey	<i>Equus asinus</i>	-	-	-
Horse	<i>Equus caballus</i>	-	-	-
Chicken	<i>Gallus gallus</i>	-	-	-
Sheep	<i>Ovis aries</i>	-	-	-
Goat	<i>Capra aegagrus</i>	-	-	-

RESULTS AND DISCUSSIONS

Halal food safety has now become an important issue not only for food producers but also for consumers who desire and concern to quality food that obedience to health, religion, and fair prices (Rahman et al., 2014). Many researches have been reported that the halal food products collected from market surveillance were adulterated with forbidden meats such as pork, dogs, rats, monkeys, and others (Cai et al., 2012 ; Dahlan, 2014; Kitpipit et al., 2014 ; Ali et al., 2015 ; Yanita.I.W et al., 2015). HRMA is a molecular method that allows detecting and differentiating DNA amplicon by discriminating DNA sequence variants such as single nucleotide polymorphisms (SNPs) and small insertion and deletions (indels) based on the specific melting behaviour of the DNA amplicon (Sakaridis et al., 2013). This method can be applied to detect adulteration of forbidden animals DNA contaminated in food products by evaluating different melting behaviours of each forbidden animal species. To save cost and reduce the time, in this study, The multiplex HRMA was conducted by employing 40 DNAs from 10 animals (pigs, dogs, rats, cats, monkeys, donkeys, horses, chickens, sheep, and goats) (Table 2). Moreover, three pair of species-specific

primers targeting the intra-species conserved and inter-species hyper variable regions of mitochondrial ND5, ATP6 and Cytb gene were used to set up multiplex HRMA. Importantly, each set of primers was firstly evaluated the PCR specificity against the targeted DNAs of all animals. Each pair of primers was tested against its targeted forbidden animals with the other species. The results showed that only specific target was testily amplified (Table 2). The HRMA profiles of amplified amplicons from targeted animals were also assessed in this study, the raw melting curve data to form a normalized melting curve and melting peaks. In this study, the method highlighted uniquely melting curves and melting peaks that were easily distinguished for each species. To be illustrated, all species were distinguished by their species-specific melting curves and melting peaks. Generally, it can be seen that different genotypes have their own unique transitions that are merely shown by their HRMA profile (Figure 1). The accuracy of method was investigated in the testable conductivity in all assays were completely done in forty tested DNAs. The results significantly indicated that no cross amplification even on repetition in blind experiment and showed 100% accuracy (Figure 2).

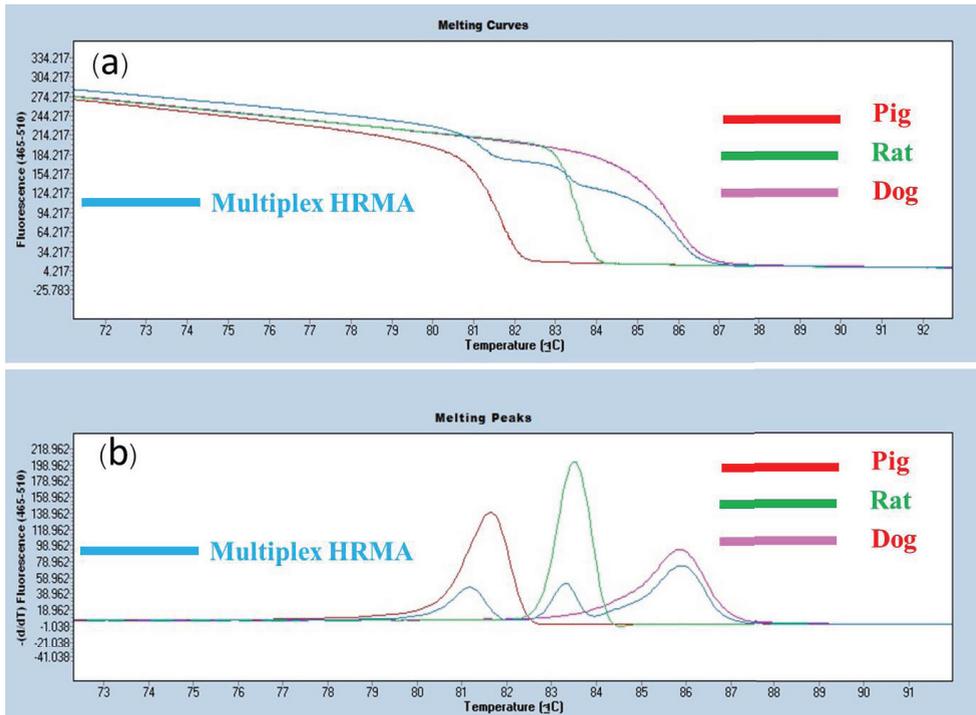


Figure 1. The HRMA profiles of amplified amplicons from species targeted animals: a normalized melting curve (a) and a melting peak (b)

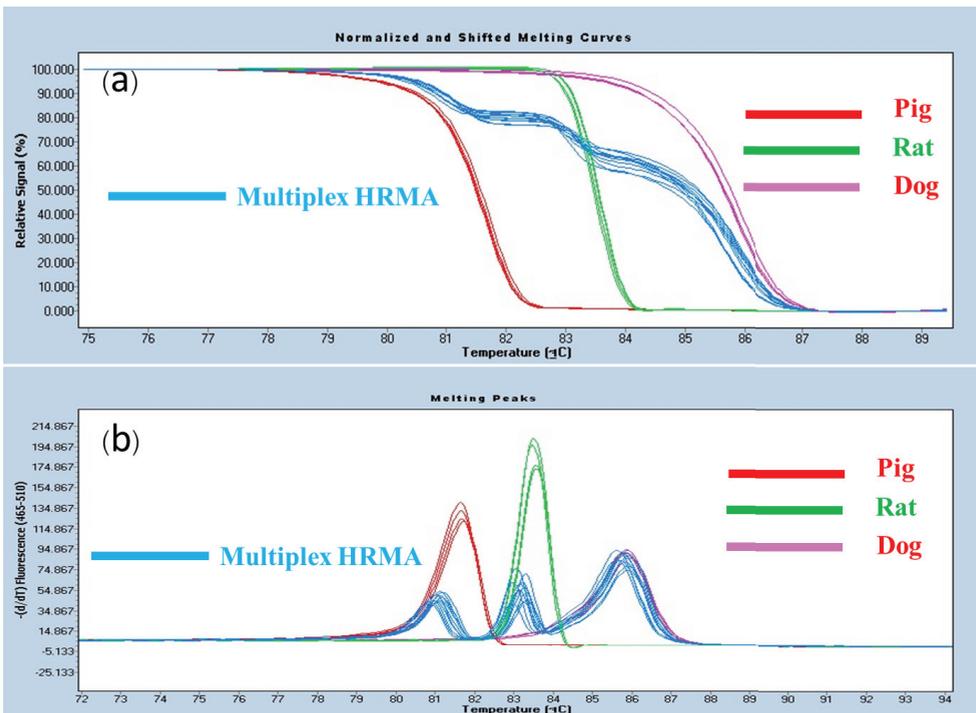


Figure 2. The HRMA profiles of forty blind experimental animals; a normalized melting curve (a) and a melting peak (b).

This revealed that there was high specificity of the established primers. In order to carry out the analysis of commercial food products, 150 commercial food products was used to screen by the developed multiplex HRMA.

The results indicated that one sample was contaminated with pigs' DNA, suggesting its usefulness for detection of pigs, dogs and rats. Therefore, the HRMA method could be used as a halal verification technique for detecting aforementioned forbidden animals contaminated in halal food products. Furthermore, the HRMA assay ultimately showed that it is simple, cheap, and rapid method, the cost per sample is comparatively lower than CE analysis (Ulca, 2015; Ali et al., 2015).

CONCLUSIONS

The HRMA method was scientifically developed in this study and it had been specifically proven in specificity and reliability for simultaneous detection of pig, dog, and rat DNA standards. The assay was also successfully validated to detect these three forbidden animal species in halal food products. Therefore, it could be potentially applied as a simple and rapid tool for halal verification technique to detect the forbidden contamination in halal food manufacturing.

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WHY HARMONIZE FOOD REGULATIONS AND WHAT IS NEEDED TO MAKE IT WORK?

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Abstract

Regrettably after decades of negotiations between countries and supranational organizations, there are still too many differences that hamper movement of safe food across borders and hamper innovations and it does not look like the differences will disappear soon. Therefore, where possible, serious scientists should continue to work together to provide scientifically correct evidence that may be used as tools by stakeholders to try influence negotiations and to try convince local authorities that harmonization is in the interest of everybody. To make it work in practice requires that those who need to know and that means most people, at all levels, understand the scientific evidence. Not only large companies are affected by unjustified differences in regulations, but also small companies and street vendors and ultimately all consumers, who in many countries have a democratic vote and thus are influential. In turn this makes it necessary that the science is translated in a language that those who need to know understand. The Global Harmonization Initiative therefore not only tries to find consensus on scientific issues, but also seeks means to make the findings understood by everybody, requiring simplification, but without losing the true scientific facts, and translation into local languages. Then having the results published in scientific journals, popular scientific magazines, newspapers and magazines aimed at the general public. Another crucial aspect is that those who do the negotiations understand what they are talking about, because expressions used in regulations and during negotiations tend to have – often vastly - different meanings in different countries or regions.

Key words: food safety regulations, food security, harmonization, science-based, toxicity.

INTRODUCTION

The fact that every year an estimated 600 million (almost 1 in 10 people in the world) fall ill after eating contaminated food and that 420,000 of them die (WHO, 2015), indicates that effective regulations are needed to protect consumers from safety hazards and misleading information. Regrettably, however, food regulations often differ between countries, often even between neighbouring countries, despite declarations by many countries and international organisations that harmonization is necessary. Moreover, the regulations often have no scientific basis and are factually wrong. The processes of harmonizing, however, seem to be extremely difficult and very slow, requiring persistence and willingness to accept changes in existing regulations. Many meetings between representatives of participating countries are needed to make progress and meetings often take place just once annually. It therefore looks like the differences will persist for a long time – if not forever. That these differences exist is the reason for many food safety and food security problems in the world, especially in low-income countries. The differences hamper trade, because of difficulties at the border between countries. In

particular small trading companies often are unaware of the differences and discover them when their food products reach the border. Much food and many food products are perishable and delays at the border for inspection and negotiation with authorities are costly. A selection of examples will be discussed that illustrates the consequence of the regulatory differences. In the worst case authorities seize and destroy food and food products that are in reality healthy, only because the law requires so and challenging the correctness of the law is virtually impossible under such circumstances.

TOXICITY AND CARCINOGENICITY

Differences in regulations may be used to hide protectionism as seems to be the case with the maximum residual level (MRL) of carbendazim in orange juice in the USA. Carbendazim is one of the few pesticides that are allowed to protect oranges (and other produce) from spoilage by moulds. Because carbendazim is safe in the concentrations needed, it is allowed in most countries. The maximum residual level (MRL) allowed in the European Union is between 100 and 700 parts per billion (ppb; parts per 10⁹) and in Canada between 500 and 6000 ppb. The MRL in the USA is 10 ppb

and the FDA decided that “to ensure the continued safety of orange juice FDA is also sampling import shipments of orange juice and will deny entry to shipments that test positive for carbendazim.” (FDA, 2012a). Interestingly the US Environmental Protection Agency stated “There is no public health concern from drinking orange juice containing carbendazim at reported levels” (FDA, 2012b).

In 2005 the UK government ordered the destruction of £100,000,000 worth of food products because these products contained trace amounts of Sudan Red 1, originating from chilli powder used as an ingredient. At the time, Dr. Julie Sharp representing Cancer Research UK declared "The risk of cancer in humans from Sudan Red I has not been proven and any risk from these foods is likely to be very small indeed." And indeed, a person who drinks 800 litres per day of contaminated Worcester sauce for the rest of his life has a chance to suffer damage from the chemical; based on animal testing. Be aware that there are no known cases of cancer caused by Sudan Red 1 in humans.

Nature is a producer of chemicals that are suspected to be only man made (exogenous chemicals). Chemicals that are not allowed but nevertheless present in food, however, are not necessarily additives. Most man-made chemicals also occur in nature in concentrations that due to much better techniques can be detected now, but not previously. They are produced by animals, microbes (bacteria, fungi, parasites), plants and geochemical processes (e.g. volcanoes). They include e.g., chlorinated organic compounds. More than 5000 different natural organic halogens have been identified in nature (Gribble, 2003; Gribble, 2011).

All food and food products originate from nature, being exposed to soil and air. Soil is produced by microbes and microbes produce antibiotics, causing the presence of low concentrations of antibiotics in soil. Consequently produce growing on or in the soil, will contain low concentrations of antibiotics and so does meat, because the feed cattle consume grows on the soil. In 2006 the European Court of Justice ruled that food containing trace amounts of antibiotics, no matter how minute the amount, must be seized and destroyed (Court of Justice, 2006). In this particular case the antibiotics were furazolidone and chloramphenicol in meat. These antibiotics have frequently been administered in million times higher quantities than found in the food for several successive days to treat infections in babies in developed countries. In the case reference is made to a similar dispute in 2002 about frozen duck breasts and rabbit meat from China, consignments

that were accompanied by health and export certificates issued by the Chinese authorities for dispatch purposes. In these products residues of chloramphenicol (1.4 ppb) and furazolidone (49 ppb) were detected in the duck breast and residues of furazolidone (2.7 ppb) in the rabbit meat. Regulations about the use of antibiotics in animal husbandry and fishery differ between countries. This applies to what antibiotics are allowed and to the concentrations considered acceptable in the product (Collignon and Voss, 2015). The EU law requires absence, “zero tolerance”. The meaning of “absence” and thus the meaning of the law have become dependent on sensitivity of analytical techniques and often have little to do with safety of the product. In China, the concentrations reported are considered safe. Developing countries often export the best quality of their products to developed countries, leaving lower quality for the local population. The EU position is that the food must be destroyed and is not allowed to be returned to the country of origin. It is distressing that the best food from a country is destroyed for scientifically unjustifiable reasons, leaving a population suffering from poorer quality of food.

In June 2014 in The Netherlands furazolidone was found in meat making the government order the destruction of 2474 calves to protect consumers, despite the statement of the Netherlands Food and Consumer Product Safety Authority that the meat was safe to eat. The average exposure to humans eating meat would be only 1.2 µg per meal (and worst case 8 µg per meal). The internationally recognized amount to cause potential harm is 3 µg per day during a life time (i.e. 50 or 70 years). There are no reports of harmful effects of therapeutic doses of 200 mg per day during 21 days (WHO, 1993), which is 25,000 times more than the worst case amount.

One of the reasons why the general public is concerned about chemicals is the conviction that chemicals are carcinogenic. This is because anytime a chemical is mentioned in the press as being carcinogenic, only the chemical is mentioned and not the amount needed to make the chemical carcinogenic. While for some chemicals like aflatoxin, very small amounts may cause cancer, other chemicals must be present for a long time in a high concentration. Moreover, it depends on whether the chemical is administered as a pure chemical or in a food matrix, the way it usually enters the body. Our body consists for more than 0.3 % of sodium chloride (NaCl). Nevertheless, even NaCl is carcinogenic (WCRF/AICR, 2007). Globally many people drink coffee every day.

Coffee contains many confirmed carcinogens, including acetaldehyde, benzaldehyde, benzene, benzofuran, benzo(a)pyrene, caffeic acid, catechol, 1,2,5,6-dibenzanthracene, formaldehyde, etc. These compounds are present in relevant concentrations. Nevertheless, a thorough review of 1277 studies done in the period 1970-2015 shows that the consumption of moderate amounts of coffee does not increase the risk of developing cancer (Pourshahidi, 2016). It is time that publicists will publish in such a way that also the general public understands the meaning of studies of carcinogenicity. It should be made perfectly clear that the research is done using animals and giving these animals high doses in a short time to approach small doses over a long period of time. Moreover, the chemicals tested are not administered in a natural food matrix as in the normal way of consumption. Finally, it ought to be mentioned that if animals develop cancer from a chemical this does not prove that humans would do so too. There is a vast amount of evidence that this often is not the case (Bracken, 2008). There are in-vitro methods that are more accurate, fully relevant to humans, cheaper, and provide results in a single day. These in-vitro methods are 100% relevant to humans because they make use of competent human liver cells (Darroudi, 2010).

Lessons

Firstly, there is an almost complete lack of understanding by politicians, the general public and authorities that toxicity is **never** a matter of a substance alone but always a matter of a certain amount of a substance. For every substance there is an amount where it does not do harm and there is an amount that is deadly. In between, the substance can be healthy, mildly unhealthy or sickening. Absence may also be unhealthy and may also cause death. Laws or regulations that require total absence are absurd, because virtually nothing is totally absent in the environment and hence also in food. By requiring absence, the methods of analysis determine what is considered safe. A few decades ago “absence” meant for many products less than a few milligrams (mg), 10^{-3} g, because of the detection limits of the methods of analysis that were available. Since then methods of analysis have improved tremendously and most substances can be measured in quantities of picograms (10^{-12} g), femtograms (10^{-15} g) or even less. The presence of chemicals in such small amounts is often natural and hence, unavoidable. The presence of picograms or femtograms of substances in food, however, means that the substance is not absent and legally such food is not in compliance with regulations that require absence.

The second issue is that regrettably there are (pseudo)scientists who make money by scaring people and publishing about food safety what since the presidential election in the USA in 2017 have become known as “alternative facts”. If something has a chemical name, it probably is toxic or carcinogenic and will harm your health. If in the European Union it has an E-number, it is because they want to hide that it is something with a chemical name. Thirdly, regulations that are based on “alternative facts” leave no room for common sense and judges who in most cases are not food scientists or toxicologists, have no freedom to reach sensible conclusions. Fourthly, there are companies that are led by greedy people who care about shareholder value and bonuses, not about the health of people.

MICROBIOLOGICAL FOOD SAFETY

Although the general public tend to be much more interested in chemical food safety, most food safety incidents are caused by microbes. Contrary to chemicals, microbes can multiply and that usually is the reason for safety incidents. Healthy people rarely get ill from eating food that has a low number of microbes, but if the number increases the situation changes. There can be two main reasons for illness, firstly the microorganisms themselves may cause illness by destroying the cells that constitute human organs (e.g., Salmonella and Listeria strains) others produce toxic substances (e.g., Staphylococci and Bacillus strains). To present a serious risk, a certain number is required, the “infectious dose”. For a few types of microbes the infectious dose can be just a few while for others it may be several millions. It depends on the individual’s health and also the type of food. Some food effectively neutralizes the acidity in the stomach and thereby makes an important barrier ineffective. To produce enough toxin to make a healthy person ill, in most cases many millions of bacteria are needed and therefore, the cause usually is the growth of toxigenic bacteria in the food before it is consumed. In most instances food poisoning happens during preparation when not enough attention is paid to hygiene. The number of people per incident is then relatively low, although in cases where restaurants are the source of the incident, still hundreds of people may be affected. Matters, however, can be very bad when a food processing company is ignoring hygiene or is fumbling with the processing conditions (such as pasteurization, cooking at the required temperature or drying) that are intended to make the food products safe. In such cases thousands or more people may be affected.

In 1985 a Mexican company produced Queso Fresco, a Mexican-style soft cheese, from unpasteurized milk that contained *Listeria monocytogenes*. This resulted in 62 deaths (CDC, 1985). It took authorities a month to find the source of this outbreak of listeriosis. In 1993 there was an outbreak of *Escherichia coli* O157:H7 in Jack in the Box restaurants in the USA. The outbreak was traced back to undercooked meat contaminated with faecal matter. The company had ten months earlier been warned about using undercooked burgers and contaminated beef, but went on with their practices. It caused 4 deaths and up to 700 people getting ill (CDC, 1993). In 2011 there was an outbreak of a foodborne disease in Germany caused by *Escherichia coli* O104:H4 (EHEC) causing Haemolytic Uremic Syndrome (HUS). Because it was very difficult to find the source, this became one of the world's most widespread outbreaks of foodborne illnesses with 3950 people getting ill and 53 died. First cucumbers from Spain and the Netherlands were suspected as source of infection. Follow up studies failed to confirm that cucumber was the source. Finally the European Food and Safety Authority (EFSA) found the source to be fenugreek seeds imported from Egypt. The distributor had sold the seeds to 70 companies, 50 of them in Germany who used them to grow sprouts. (CDC, 2011). In 2008-2009 there was an outbreak of foodborne illness in the US, where more than 700 people got ill and 9 people died. The source was found to be products with ingredients from the Peanut Corporation America (PCA) that were contaminated with *Salmonella thyphimurium*. Albeit being aware of the contamination of their ingredients, they still sold them leading to an enormous foodborne infection scandal involving 46 states, 360 companies and 3,900 infected products with PCA product ingredients. The owner of the Peanut Company America received the largest criminal sentence of all food safety cases; 28 years of prison (CDC, 2009).

Lessons

There are three major causes of incidents that cause severe damage to the health of many people and even deaths. Firstly there are professionals in the companies who know but do not want or dare to speak out. If they report to their superiors, they are put under pressure to deny and hide the facts and go on with their job or lose it. Secondly, companies do not have the knowledge, expertise and/or capacity to ensure that the food or food products they make are safe. If this is the case, the company should not be allowed to produce food before they meet these requirements. Thirdly, of course there can be a new, unknown cause that could not be predicted or

in any case had not been predicted. For everything there is a first case. As soon as the incident has been reported and the incident is serious, i.e. may lead to severe health problems, information available should carefully but also quickly be checked for correctness and then be shared with authorities and made public in an understandable way.

MYCOTOXINS

As mentioned earlier, all food and food products originate from nature, and thus have been exposed to soil and air. Under certain but in many countries often prevailing circumstances nature supports the growth of moulds that produce harmful substances that are toxic in very low concentrations: mycotoxins. In some countries this problem causes half of the harvest of staple food to become unfit for consumption. Because of the scarcity of food, in many cases people still consume such food with devastating consequences – no time for developing tumours, but acute poisoning leading to a very painful process of dying. Much is known about controlling the growth of the harmful moulds, during the growth phase (e.g., methods to keep the top of the soil dry), during transport (by better protection of the products, avoiding moulds passing the skin of the products) and during storage (e.g., controlling the humidity by thermal insulation of silos) (Aldred *et al.*, 2004; Shapira and Paster, 2004). Where the soil is contaminated with too high concentrations of mycotoxins the roots of the crop will absorb the toxin (Hariprasad, 2013). Growing crops on such soil should not be allowed. Methods to abate these problems, however, are applied only in some regions, regrettably not everywhere. This is partly because they are not known everywhere and partly because they require investments.

GENETICALLY MODIFIED ORGANISMS

Crops can be enhanced and losses be limited by using genetically modified crops. The use of GMO crops is an important method to alleviate hunger on the southern hemisphere (Herrera-Estrella and Alvarez-Morales, 2001). In the developed world, it is easy to find support for abandoning genetically modified food. There is no shortage of food and is very lucrative to be against it as a (pseudo)scientist. Writing books that are negative about GMOs may make them bestsellers. Many NGOs have been founded to fight against GMOs, researchers received and receive horrible threats. People are encouraged to destroy GMO test crops by these anti-GMO NGOs, such as happened in the Philippines in 2013 (Alberts *et al.*, 2013). Fake

scientists get attention on television and in the press. In some cases they even become professors (be it extra-ordinary, paid by NGOs who easily collect money to fight against the technology). Personally, I am convinced that these “scientists” know better, but fame is easier to obtain in the negative way than by doing decent research. It is also much easier to make money as an anti. Serious, decent scientists are accused of being corrupt and paid by industry. Industry-supporting governments are accused of being ruthless and against nature. Everything is twisted such that it looks like decent genetic research cannot be right. That populations starve and many die because of anti-GMO activities, such as e.g., in 2001, when during the massive famine in Southern Africa several governments in the region objected to genetically modified (GM) grain, citing health and environmental concerns, Zimbabwe blocked the GM food aid from entering the country. In Zambia, where some GM grain had already arrived, the government placed it under lock and key, banned its distribution and then blocked another 40,000 tonnes that were on the way (Africa Renewal, 2003). This is the result of overwhelming activities of antis, in particular in Europe, who claim with no evidence that GM food is dangerous. The local governments choose to let their citizens starve to death rather than giving them GM food. The reality is that, because of the negativism by antis, both in the USA and in Europe very thorough research has been done to find out about negative aspects or any harmful incidents resulting from the use of GM crops. The EU alone spent more than € 300 million on the safety of GMOs. The main conclusion to be drawn from the efforts of more than 130 research projects, covering a period of more than 25 years of research, and involving more than 500 independent research groups, is that biotechnology, and in particular GMOs, are not per se more risky than e.g. conventional plant breeding technologies (European Commission, 2010). Another reality is that hundreds of millions of people consume GM food daily and there is not a single report of a health incident related to GM food.

Lessons

That genetically modified (GM) crops have been proven to be as safe as non-modified crops is not sufficient to use GM crops to fight against hunger. Reasons are that since the development of GM technology there have been antis, people who feel that GM products are dangerous, supported by scientists at the time that not much research on the safety of GMOs had been done. To maintain this opinion after decades of research is only understandable because some scientists do not

understand the research or do not want to know for other reasons. Antis always get attention and therefore have much influence on the public opinion and equally on politicians and law makers, regulators. It is easy to mislead the general public because the technology is new and therefore not familiar. What is lacking is scientifically correct but nevertheless clearly understandable explanations, what GM means, how it is done and why it is safe. That may not be easy, but it is essential to overcome the problems and thereby not missing an important way to beat hunger in the world. Instead of scientists talking to each other, they need to find a language that non-scientists understand.

FOOD FRAUD

Food fraud is the illegal tampering with food for economic gain. There are many examples of food fraud, such as the addition of substances to enhance the colour or products. In Hungary, in 1994, lead oxide was added to dried paprika to enhance the colour and that way made low-quality product look better (Williams, 1994). This caused 46 people hospitalised and 59 people arrested. Similarly in China the non-food colorant Sudan Red 1 was added to chilli powder. The chilli powder had been used in many products all over the world but in this case resulted only in financial consequences because some governments demanded the destruction of food containing trace amounts of the colorant. Also in China, in 2008, and again for financial gain, irresponsible and greedy companies had diluted milk and masked this by adding melamine, making the protein content looking the same as in unadulterated milk, because the melamine enhanced the nitrogen value in the Kjeldahl analysis that at the time was still used. This was a case with severe health consequences because of infant formula made with the melamine-adulterated milk. According to a report from the Chinese Ministry of Health, 294 000 infants had been affected by melamine-contaminated infant formula by the end of November 2008. More than 50 000 infants have been hospitalized, and six deaths had been confirmed (FAO/WHO, 2009). Although China had by far the most victims, contaminated products found their way to all continents. These are just a few examples of the many cases of adulteration of food and food products. From scientists who have been involved in the investigation of the incidents we know that the magnitude of the consequences of these cases of food fraud could have been limited. In some companies employees who knew about a food safety issue were instructed not to make the issue known to anyone, to avoid damage to the

reputation of the company. Being scared to lose their jobs – having families to support – they indeed did not report to the authorities (e.g., Motarjemi, 2014).

Lessons

By adding unauthorised additives to food, the food may become unhealthy to consume and may lead to severe damage to consumers and even death. In some cases unauthorised substances may be added out of ignorance. Regrettably, however, there are scrupulous people and organisations with as sole objective to earn money for themselves and/or their shareholders. In both cases, employees who know about such issues and report it to their superiors usually do not dare to raise their concerns to authorities. Their jobs are at stake. Thus there is a need for a way to make it possible to report such malpractices anonymously and from everywhere, so that measures can be taken to limit the damage, while at the same time having a possibility to rapidly check if such reports are honest and not a way of revenge of an unhappy employee.

INNOVATION

National regulations require evidence of safety for every new product, ingredient or process. The objective, to ensure that food put on the market is safe, self-evidently is correct. However, there are many differences in requirements between countries, firstly in what substances are allowed and in what concentrations and secondly in the methods to be used to provide the evidence of safety. For international trade this means that for every country to which a company wants to export and for every country from which a company wants to import high costs are involved in finding out what the differences are and then in doing the additional work to be able to provide the data required. Not only is this costly, it also takes considerable time and hence will cause delays in marketing of new products. To justify investments in research and development of new products and processes, the target market must be large enough. With so much uncertainty about national approvals, justification becomes hard, it makes companies to refrain from investments in innovations. Globally harmonized requirements and test methods would make innovations more attractive.

ORGANISATIONS THAT ATTEMPT TO HARMONIZE REGULATIONS

That harmonization of laws and regulations for food and food products are highly desirable has been recognised for a very long time. Several

organisations are active, be it to various degrees, with such harmonization. The Codex Alimentarius Commission (CAC), established by the FAO in 1961 and supported by the World Health Organisation (WHO) a year later, is trying to harmonize food safety requirements since its first meeting in 1963. CAC is recognised by the World Trade Organisation (WTO) as the international authority on food safety. The commission, however, suffers from a number of drawback. Firstly, as an intergovernmental organisation, representatives are inclined or probably often instructed to protect the interest of their country. Many times the representatives are not scientists but non-scientific governmental officials. Moreover, by far not all countries are represented in all committees (meetings usually are attended by representatives of 50-60 countries) and the frequency of meetings of these committees is low, usually once yearly, causing progress to be slow, despite the work done between these meetings. Lastly, there are many countries for which harmonization is extremely important, that simply cannot afford to participate in all meetings. Nevertheless, CAC standards are important because of their recognition by the WTO.

The Global Food Safety initiative (GFSI) was founded in 2000 by food retailers and manufacturers with the objective to make the supply chain safer, through the harmonization of food safety standards. It is a non-profit organisation that is managed by the Consumer Goods Forum (<http://www.theconsumergoodsforum.com/about-the-forum/our-mission>). GFSI is charged with providing continuous improvement in food safety management systems to ensure confidence in the delivery of safe food to consumers worldwide (Global Food Safety Initiative, 2011).

The Global Harmonization Initiative (GHI) is the single international organisation that is impartial: non-governmental and non-industrial. Its membership consists of individual food scientists from all over the world and thus members are not representing a country or company. The goal of the organisation is to promote harmonization of global food safety regulations and legislation, based on solid scientific evidence. Since its inception in 2004, it gradually became clear that the differences in regulations between countries are not related to the lack of consensus between scientific experts. Rather scientists fail to communicate with non-scientists: the general public, politicians, and editors of magazines, newspapers and other mass communication media. GHI is addressing a number of crucial issues to resolve the issues discussed.

Firstly, before countries may usefully discuss food safety, they need to understand the terminology used, they need to realise that words often have different meanings between countries. This is the case even if countries use the same language, like the UK and the USA. There is reason why there are dictionaries UK-English/USA-English. The differences can be dramatic as for instance in discussing concentrations, as discussed above: A billion in the USA is thousand times less than a billion in the UK; in the case of a trillion the difference is a million (fortunately million means the same in both countries). Countries disagree what many relevant words mean, e.g. in the case of a very important subject: The definitions of Food Additives differ between e.g., Canada, Japan, USA and the EU and they all differ from the Codex Alimentarius definition. That is the reason why GHI has a Working Group Nomenclature on Food Safety and Quality. The mission of this WG is to harmonise definitions in these areas. The WG has started with Russian and English, and will then move to Spanish, French and Italian. These “basic” languages could then be used for translations into other languages.

Secondly, it is imperative that those who decide on regulations, politicians, understand at least the basics of food safety. Because politicians are strongly influenced by the electorate, it is also important that the general public understands the basics of food safety. Regrettably, with possibly few exceptions, they do not. Understanding the consequences of lack of hygiene and hygienic measures is essential to reduce food safety incidents as well as premature spoilage of food. By far most of the hygiene related food safety incidents are due to handling of food by housewives or -men, caterers and street vendors. In addition, regrettably there are not enough capable food inspectors, they often have not had sufficient education and training to recognise wrongdoings related to food safety and hygiene. To reduce the number of demands for absurd regulations, a large enough part of the population needs to understand that any chemical is only harmful if there is too much of it and that often not enough of the same chemical may cause illness. Similarly, it must be understood that to become ill from microbes too requires a certain number and also many microbes are essential for people’s health. The WG Education of GHI therefore is working on the production of material for the education and training at all levels, suitable for translation in local languages but also using a pictorial language because according to the United Nations almost 800 million people in the world cannot read (D’Ameida, 2015); in developing

countries the illiteracy often is >60% but even in the USA it is >14% (Statistic Brain, 2014). Further GHI is cooperating with IUFOST and the World bank in the Global Food Safety Curriculum Initiative (GFSCI). The aim of the initiative is to provide food safety students globally with a science-based education in food safety so that, as professionals, they can fulfil expressed needs by governments, industry and academia in their countries and regions (<http://foodsafety.iufost.org/global-food-safety-curricula-initiative>).

Thirdly, it is unacceptable that reporting of the contamination of food that may seriously affect people’s health are suppressed by e.g., the management of companies, to avoid financial consequences or by authorities, to avoid damage to the reputation of a country. Governments should protect those who - for good reasons and after not being heard by their management - in good faith warn authorities to prevent harm to consumers. This requires harmonized regulations based on ethics, self-evidently taking into account that there could be false reports. Incidents reported must be checked and undue harm to companies and countries should be avoided. The WG Ethics of GHI is developing proposals for such global regulations. In addition GHI is developing a global incident alert network for unauthorized food additives (GIANUFA). Anytime it is found that an unauthorised (illegal) substance is added to food, wherever on the globe, that may harm consumers, the individual who made the discovery should alert a dedicated committee. If needed, this may be done anonymously and such that the individual cannot be traced or identified. A committee then should have the means and a protocol to verify the issue in a very short time, using a global network. In the same time, experts should decide the potential harm that may be caused. Depending on the severity, an alert should be spread globally by a press release, initially in English. Then the essential information should be sent to all relevant authorities. The press release should then also be translated – as soon as possible, but carefully because correct translation is also essential - into local languages, this should be done by local food scientists, if needed supported by professional translators. That way the entire world could be alarmed in just a few hours, preventing undue damage (sickness, death) of consumers.

CONCLUSIONS

By far not everything that is relevant has been discussed in this article. Harmonization of test methods, viruses and prions for instance have not got the attention these topics deserve, but more

information on these topics can be found in the recommended literature. The intention of this article is to demonstrate that there is a need for regulations, but also that prevailing regulations often are flawed and there are many unjustifiable differences in regulations between countries. To remove barriers to trade and to avoid undue destruction of healthy food, regulations must be based on sound science and be globally harmonized. To make it work, education, training and communication related to food safety must be improved so that the general public is armed against misinformation. Then of course, governments must be made to want to improve laws and regulations for which an impartial global group of food scientists is willing to deliver the tools.

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MISCELLANEOUS

PROFILE OF HIGH RISK WASTING FOOD CONSUMER IN ROMANIA

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Abstract

The paper aimed to present the profile of Romanian consumer with high risk food waste behaviour, in terms of weighted impacts. It is based on data provided by National Research and Development Institute for Food Bioresources-IBA Bucharest, the ADER 15.1.1. project "Socio-economic impact of food waste on the food safety and climate change crisis context". The target group was national wide representative for over 18 y.o. urban population. Data have been processed into the following indicators: age, revenue, education, number of family members, geographical location, cities size. Over 900 persons responded, from 153 cities. The questionnaire was aiming for both individual and all family scale answers. Although the urban national percentile average of food waste is 10,4%, there is a wide scale of values, according to individual and social profile of the respondents. 1/5 of the population is wasting almost 50% from the total food waste. The study details the consumers behaviour, based on the previously mentioned indicators. The results indicated as high risk food waste consumer components: age under 35 y.o., living in big cities, university degree, medium to high income, shopping from retail network.

As a conclusion, the consumers' behaviour it is a major indicator of food waste risks, offering improvement resources to the entire food industry sector, in formulating new valuable targeted products.

Key words: food waste, biotechnology, food security, climate change, food risk management.

INTRODUCTION

Food waste became one of the most relevant topics related to food security and climate change, as well as economic crisis cycles, on public debates, media and scientific environment (Ortiz-Mirandal et al., 2016).

After 1997 a total of 2340 research articles were published in 801 journals were published as well as in 161 Web of Science subject categories (Chen et al., 2016).

On global level, the waste/loss of food is estimated over 35 percent, strongly depending on the country development level, most developed countries generating more waste (Chalak et al., 2016).

The tendency is present also on EU level, together with increased environmental aspects, affecting the natural resources control, loss of natural habitats and the well-being of the population (Lucifero, 2016).

On individual level, people living in big towns are producing more waste (Secondi et al., 2015).

In order to understand the mechanism of food waste generation, studies on food waste/loss composition suggested that 97% of Danish households have avoidable food waste products in their bin; the multi persons households are more likely to generate losses than one member households (Edjabou et al., 2016).

Important studies in Italy and Spain indicate that age is an important factor of variation in food waste generation; youth population proved to be most inclined to waste segment of population (Mondejar et al., 2016).

An intervention study on 217 German households, to promote food-waste prevention behaviour identified perceptual, behavioural and motivational predictors to develop an action strategy (Karolin, 2016).

Stefan et al. studied the drivers of food waste in Romanian households, focused on influence of intention not to waste food, importance of shopping and planning routines. Their conclusions indicates that changing the food waste behaviour implies efforts towards providing

skills and tools to take the food related activities (Stefan et al., 2013).

In this context, the paper presents an analysis of the Romanian consumer profile from urban area, aiming to identify the variation of the food waste behaviour based on geographical and individual parameters.

MATERIALS AND METHODS

In order to determine the profile of high risk food waste consumer, the following indicators were used: geographical indicators – type of city, county, historical region; personal indicators – gender, age, education, income.

Also households' membership and number of children under 16 y.o were considered as relevant indicators.

A focus group with 15 respondents and pilot test of 65 consumers were conducted in order to develop the questionnaire.

The data were collected in 2016 by Computer Assisted Telephone Interview (CATI) method. The testing group consisted in a randomised stratified sample of national wide 960 respondents from 153 cities, of which were validated 902, according to National Statistics Institute data, from urban area, age >18 years old, reachable by phone or cell phone. The confidence interval is 95%.

Interpretation and analysis of the data was made using SPSS Statistics Data Editor Program. The variability was determined with ANOVA method.

Dependent values considered are level of food waste/loss mass per person and food waste/loss yearly tendency.

RESULTS AND DISCUSSIONS

Distributions and histograms of the sample group

The factors and variables analysed in the present study are structured following personal and group characteristics. They are structured upon ANOVA method, as following:

Individual factors: A1 – Gender; A2 – Age group; A3 – Educational background

Group factors: B1 - Number of family members; B2 - Number of children under 16 y.o/ family; B3 - Monthly family income; B3 – Computed factor income/pers.

Geographical factors: C1 – Historical region; C2 – City type

Variables: V1 – Weekly quantity of food waste/loss/ family; V2 - Food waste/loss level, comparing to last year; V3 – Computed variable weekly quantity of food waste/loss/ pers. Although the age data were collected individually, it was consolidated on significant groups corresponding to young adults, adults (2 groups) and seniors. Authors valued as big the municipalities.

Over 100000 inhabitants, considering that Bucharest influence is studied on Historical region factor. The income scales are correlated to average income in Romania.

Table 1. Variable view matrix

Factor/ Variable		Valid values
<i>Individual factor</i>	A1 Gender	1 – Female
		2 - Male
	A2 Age group	2 - < 35 y.o.; 3 – 36 – 50 y.o.
		4 – 51 – 65 y.o.; 5 - > 65 y.o.
	A3 Educational degree	2 – professional schools degree or less; 3 – college degree; 4 – university degree
<i>Group factor</i>	B1 Number of family members	1 to 7
	B2 Number of children < 16 y.o.	0 to 4
	B3 Monthly family income	3 - < 1500 lei; 4 – 1500-2500 lei; 5 – 2500-3500 lei; 6 - >3500 lei
<i>Geographical factor</i>	C1 Historical region	1 – Bucharest; 2 – Transilvania; 3 – Muntenia, Dobrogea & Oltenia; 4 – Moldova & Bucovina
		C2 City type
<i>Variable</i>	V1 Weekly quantity of food waste/loss/ family	1 – none/< 500 gr; 3 – 500-1000 gr; 4 – 1000-2000 gr; 5 - >2000 gr
		V2 Food waste/loss level, comparing to last year

Group structure statistics indicate normal distributions of the sample for original data sheet. Also the mean value of V2 Food waste/loss level, comparing to last year,

indicates very low time variance of the quantity of the food waste/loss (mean 2.04).

Table 2. Descriptive statistics of Individual factors

	A1 Gender	A2 Age group	A3 Educational background:
Mean	1.47	3.36	3.00
Std. Deviation	.499	1.043	.769
Variance	.249	1.088	.592
Skewness	.129	.150	-.008
Std. Error of Skewness	.081	.082	.082
Kurtosis	-1.988	-1.162	-1.309
Std. Error of Kurtosis	.163	.163	.164
Range	1	3	2

Table 3. Descriptive statistics of Group factors

	B1 Number of family members	B2 Number of children under 16 y.o./family	B3 Monthly family income	B4 Computed income/pers
Mean	2.73	.36	4.52	1.9776
Std. Error of Mean	.040	.024	.039	.03150
Std. Deviation	1.204	.705	1.127	.91840
Variance	1.450	.497	1.270	.843
Skewness	.550	1.921	.021	1.378
Std. Error of Skewness	.082	.082	.084	.084
Range	6	4	3	5.40

Table 4. Descriptive statistics of Geographical factors:

	C1 Historical region	C2 City type
Mean	2.4634	1.88
Std. Error of Mean	.03529	.030
Std. Deviation	1.05996	.902
Variance	1.124	.813
Skewness	.175	.235
Std. Error of Skewness	.081	.081
Range	3.00	2

Table 5. Descriptive statistics of variables

	V1 Weekly quantity of food waste/loss/family	V2 Food waste/loss level, comparing to last year	V3 Computed weekly quantity of food waste/loss/ pers.
Mean	2.04	2.04	.8724
Std. Error of Mean	.048	.019	.02494
Std. Deviation	1.424	.541	.74056
Variance	2.027	.292	.548
Skewness	.888	.031	2.592
Std. Error of Skewness	.082	.085	.082
Range	4	2	4.86

Two computed variable were included: monthly income per person, computed from dividing family monthly incomes to the family members' number and food waste/loss per person, computed from dividing the family

food waste/loss to the family members' number. They are analysed as separate data and related to the family level data.

The computed variables (income/pers. and weekly food waste/loss/pers.) indicate more likely Cauchy, respectively Weibull distributions.

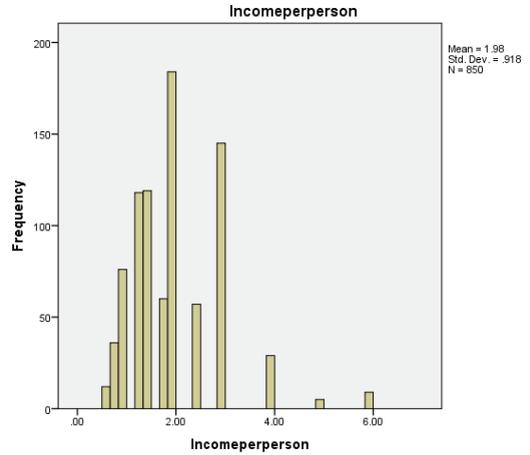


Figure 1. Histogram of B4 Computed income/pers

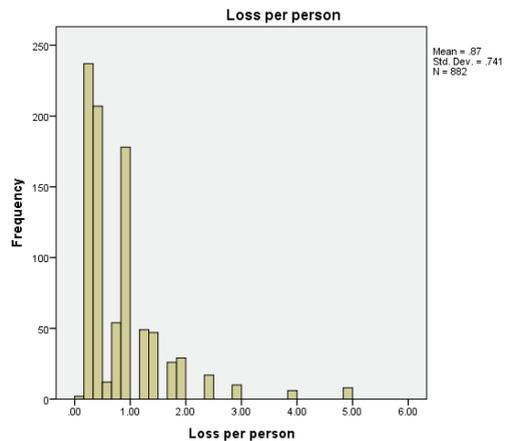


Figure 2. Histogram of V3 Computed weekly quantity of food waste/loss/ pers.

Determination of variance of food waste/loss level and in time evolution

There were no statistically significant differences between group means as determined by one-way ANOVA one way for V2 variable - Food waste/loss level, comparing to last year related to considered individual, group and geographical factors.

Neither there were no statistically significant differences between group means as determined by one-way ANOVA one way for all variable related to geographical factors (C1 Historical regions and C2 City type):

Table 6. Influence of geographical factors on food waste/loss and on in time behaviour evolution

	C1 factor		C2 factor	
	F	Sig.	F	Sig.
V1 variable	2.177	.089	1.102	.333
V3 computed variable	.693	.557	.465	.628

There were statistically significant differences between groups as determined by one-way ANOVA related to V1 variable - Weekly quantity of food waste/loss/family and computed V3 variable - Weekly quantity of food waste/loss/pers., and the individual factors (A1 – A3).

Table 7. Influence of individual factors on food waste/loss and on in time behaviour evolution

	A1 factor		A2 factor		A3 factor	
	F	Sig.	F	Sig.	F	Sig.
V1 variable	5.851	.016	18.477	.000	14.971	.000
V3 computed variable	4.990	.026	3.607	.013	6.270	.002

Males are more inclined to waste than women. Related to age, the young adults group, under 35 y.o. is more likely to waste/ loss food than the rest of the population.

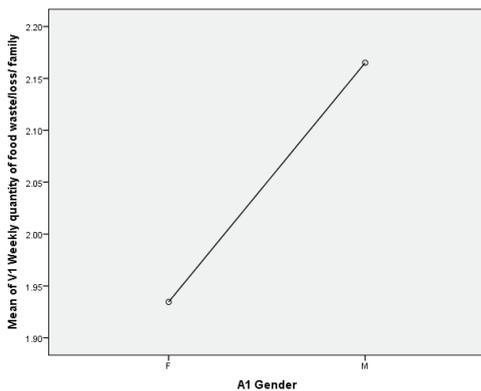


Figure 3. Weekly family food waste/loss – gender correlation

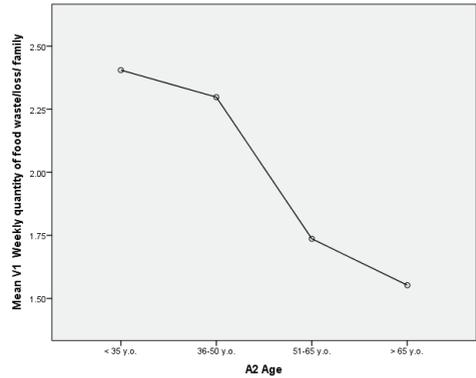


Figure 4. Weekly family food waste/loss – age correlation

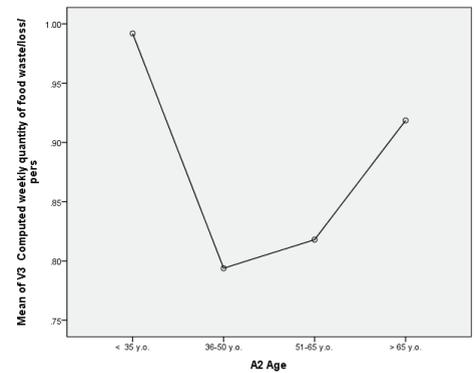


Figure 5. Weekly computed individual food waste/loss – age correlation

Level of education (A3 factor) indicates that highly educated individuals are more likely to have high risk behaviour on food waste/loss, both on family as an individual weight.

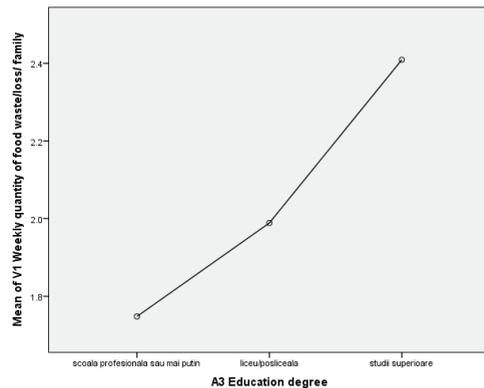


Figure 6. Weekly family food waste/loss – educational degree correlation

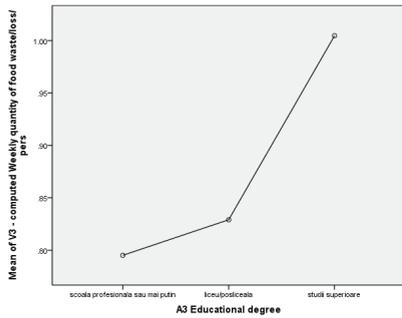


Figure 7. Weekly computed individual food waste/loss – educational degree correlation

The fact is strongly related to the level of income, rising with the education. One way ANOVA indicates statistically significant differences between these groups: $F(29.274, .495)=59.157, p<.001$

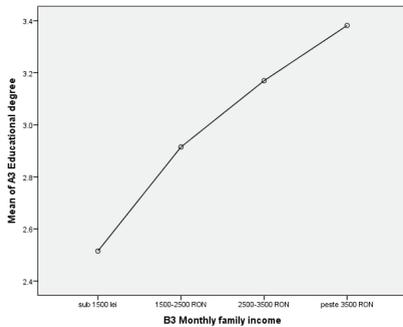


Figure 8. Income – educational degree correlation.

Related to group factors (family dimension, children under 16 y.o., monthly income) ANOVA revealed a significant statistical difference between groups for V1 and computed V3 variable, apart from the variance of individual food waste/loss (computed V3) with the monthly family income (B3 factor).

Table 8. Influence of group factors on food waste/loss and on in time behavior evolution

	B1 factor		B2 factor	
	F	Sig.	F	Sig.
V1 variable	7.469	0	4.438	0.001
V3 computed variable	32.326	0	6.352	0
	B3 factor		B4 factor	
	F	F	F	Sig.
V1 variable	15.816	15.816	3.031	0
V3 computed variable	2.021	2.021	11.588	0

The means plots indicate more likely the for 4 members families to have a high risk family food wasting behaviour (V1 variable). On computed individual food waste/loss means (V3 computed variable), single persons statistically seems to have the highest risk behaviour.

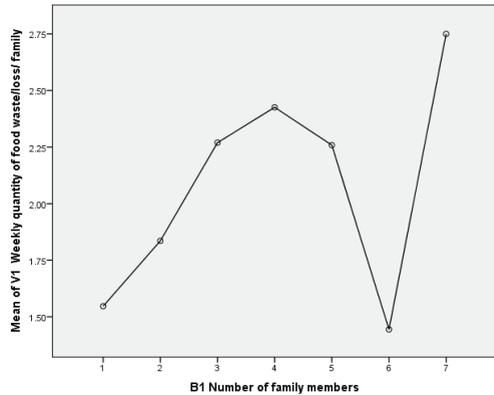


Figure 9. Weekly family food waste/loss – number of family members' correlation

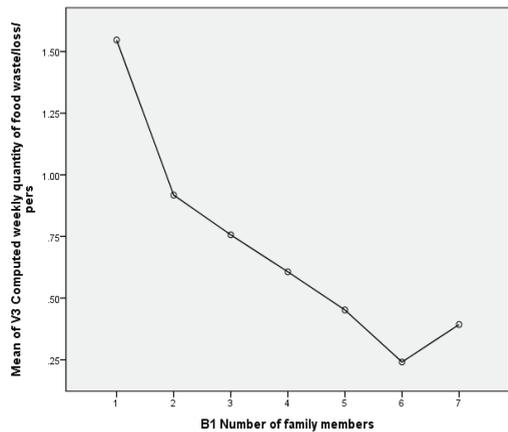


Figure 10. Weekly computed individual food waste/loss - number of family members' correlation

Number of children under 16 y.o. in a family (B2 factor) suggest a similar tendency. Families with 3 or more children are most likely to form a high risk group for household level food waste/loss behaviour (V1 variable). However, no children families are more likely to waste food, on individual level (V3 variable):

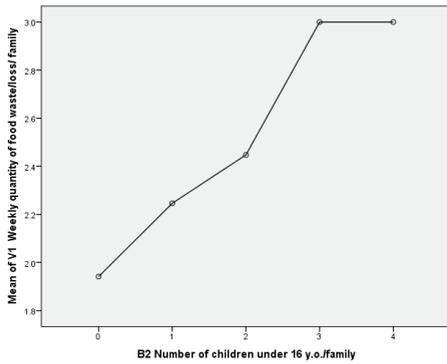


Figure 11. Weekly family food waste/loss – number of children correlation

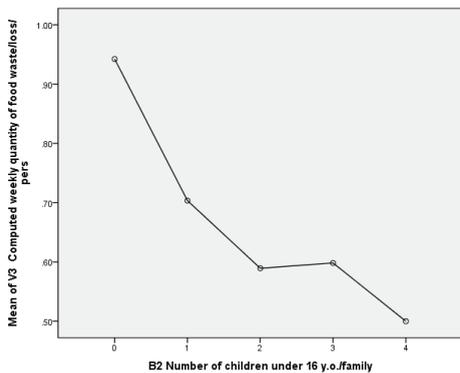


Figure 12. Weekly computed individual food waste/loss – number of children correlation

The family monthly income factor (B3 factor) indicates the higher the income, family or computed per person weight, the more likely to generate a high risk food waste/loss behaviour:

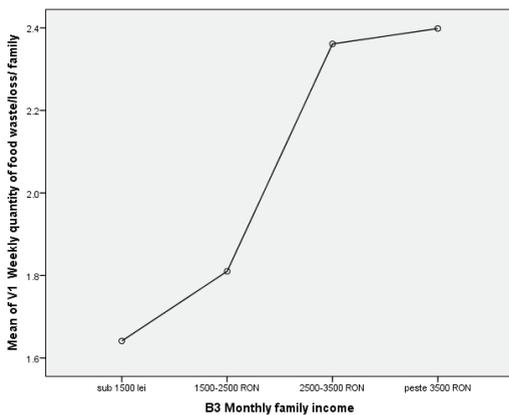


Figure 13. Weekly family food waste/loss – monthly family income correlation

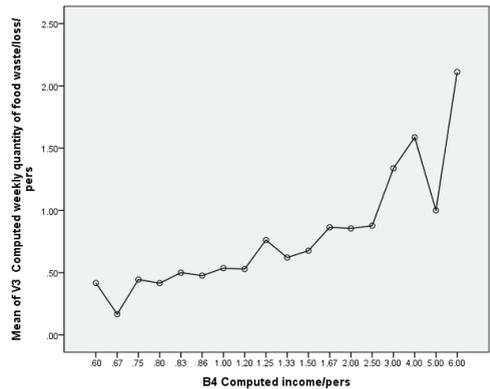


Figure 14. Weekly computed individual food waste/loss – computed income/pers correlation

CONCLUSIONS

The high risk profile on food waste/loss behaviour indicates multiple groups' population. Based on individual factors, under 35 y.o. single male adults, with higher education, are more likely to have a risking food consumption behaviour. Presumed lack of interest towards food resources management, time management deficit and influence of modern consumer society marketing instruments are some of possible causes.

The group factors indicate the monthly income as most relevant in generating food wasting behaviour. On households scale, 4 members' families seem to be most vulnerable to food waste risks. Also the households with up to 3 children under 16 y.o.

There's not relevant proof of the geographical influence on food waste/loss behaviour.

The data recommend further studies in consuming patterns of the above population groups, in order to identify the specific impact of each influencing factor.

ACKNOWLEDGEMENTS

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GM PLANTS AS BIOFACTORIES OF PHARMACEUTICAL PROTEINS: PRESENT STATE AND FUTURE DEVELOPMENTS

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Abstract

The use of genetically modified organisms (GMOs) as biofactories for the production of recombinant proteins of commercial interest is at present one of the major applications of 'molecular' biotechnology, and the business basis of many modern biotech companies. Since marketing in the early 1980s of recombinant human insulin, synthesised in Escherichia coli, hundreds of proteins with pharmacological activity, used for the diagnosis, treatment or prevention of human (and animal) diseases, have been produced in different platforms. The intrinsic limitations of bacterial cell cultures – especially the lack of the machinery for post-translational modifications of proteins, which are required for the synthesis of pharmacologically active proteins – have made mammalian cell cultures the system of choice for the industrial production of biopharmaceuticals. These are robust, reliable and highly controlled production systems, optimised over the years and for which GMP ('good manufacturing practice') procedures have been established and approved by the competent authorities. Mammalian cell cultures, however, have also important limitations and drawbacks, mostly regarding their high costs, relatively low productivity and lack of flexibility to scale-up or -down the production, in response to market demands. Many of these limitations could be overcome with the use of plant biofactories, the so-called '3rd generation' of genetically modified plants used for the commercial production of recombinant proteins including, more specifically, pharmaceutical proteins: 'molecular pharming' or 'pharma crops. However, despite the important advantages – at least theoretically – of GM plants, development of this kind of production platforms has been slow and the first plant-made biopharmaceuticals have been approved for human use only recently. This has been due mostly to regulatory issues rather than to scientific or technical problems, but recent developments indicate a rapid growth of this technology, even if it is limited to niche markets for specific plant-made protein drugs.

Key words: biopharmaceuticals, cell cultures, molecular 'pharming', recombinant proteins, transgenic plants.

INTRODUCTION

The *in vitro* construction, for the first time, of biologically functional bacterial plasmids conferring antibiotic resistance when transformed into *Escherichia coli* (Cohen et al., 1973) marked the birth of the 'recombinant DNA technology' or 'genetic engineering', starting a revolution in the experimental strategies used to investigate biological processes. In addition, from a practical point of view, this new biotechnology opened the possibility to produce large amounts of any protein, by expression of the corresponding gene in a suitable 'genetically modified organism' (GMO), once that gene had been cloned in an appropriate vector. In 1976, Herbert Boyer – one of the authors of the 1973

seminal paper – and venture capitalist Robert Swanson, founded Genetech, the first modern biotech company, devoted to the commercial production of pharmaceutical products using recombinant DNA technology. Human insulin synthesised in *E. coli* was the first recombinant therapeutic protein to reach the market, in 1982 (produced under licence by Eli Lilly & Co.), followed by recombinant human growth hormone, in this case made directly by Genetech, which was approved by the FDA in 1985.

Since then, different GMO systems have been used as 'biofactories' for the synthesis of a wide range of proteins with pharmacological activities, such as hormones, growth factors, blood clotting factors, antibodies, enzymes or vaccines, and many other proteins with

application in the diagnosis, prevention or treatment of human (and animal) diseases. Bacterial cultures are the simplest platforms for commercial production of recombinant proteins, but their intrinsic characteristics, regarding the capacity to synthesise pharmaceutically active products, have limited their use to the synthesis of a reduced subset of small and simple proteins that do not require post-translational modifications for their biological activity. At present, *in vitro* cultures of mammalian cells are the system of choice for the industrial production of commercial biopharmaceuticals, despite their technical complexity and high costs. The use of the so-called '3rd generation' of transgenic plants as biofactories for the production of recombinant proteins for the pharmaceutical industry has been proposed a long time ago, after efficient methods for plant genetic transformation were established in the late 1980s. Despite a number of theoretical advantages of plant-based platforms, as compared to bacterial and mammalian cell cultures – and also in relation to the production of recombinant proteins in the milk of transgenic mammals – development of commercial 'pharma crops' is lagging far behind fermenter-based systems. In this short review, we will briefly describe the main characteristics of the different production platforms, with their pros and cons, the advantages of GM plants-based systems and the reasons why their potential has not yet been realised. We will also discuss some recent developments which point to a wider use of transgenic plants for the production of biopharmaceuticals in the near future, at least for specific applications.

BACTERIA AND YEAST CULTURES

In vitro cultures of bacterial cells – of *E. coli*, in most cases – show several advantages for the production of recombinant proteins, being simple, reliable and relatively cheap systems, easy to establish and maintain, since there is a vast knowledge on the procedures for bacterial genetic manipulation. Bacteria grow very fast, to a high cell density in the cultures, which present high protein production levels. These systems are well-established, have been used for many years, and do not pose regulatory problems.

Bacterial cultures have also serious drawbacks, especially for the expression of complex and multimeric proteins, which in many cases are not produced in an active form in the cells, since they do not fold or are not assembled properly. However, their most important limitation is that the bacteria do not possess the machinery responsible for post-translational modification of proteins. Most human proteins are modified by phosphorylation, acetylation, glycosylation, etc., and the presence of these groups, especially a correct glycosylation, is essential for their biological (and consequently pharmacological) activity. Therefore, this platform can only be used in practice for the synthesis of simple, generally small proteins that do not require glycosylation or other post-translational modifications for their activity (e.g. some polypeptide hormones such as insulin, growth hormone, parathyroid hormone or calcitonin).

Yeast cell cultures, mostly of *Sacharomyces cerevisiae* and more recently of *Pichia pastoris*, are also used for the production of recombinant proteins. They have characteristics similar to those of bacterial cells, both regarding advantages and limitations. Although they are eukaryotic cells, in which proteins can be post-translationally modified, the patterns of protein glycosylation, are different from those of mammalian cells. Therefore, the range of recombinant human proteins that can be synthesised in yeast in a pharmacologically active form is also similar to bacteria.

MAMMALIAN CELL CULTURES

In vitro cultures of mammalian cells are also very robust, highly controlled and reliable systems. There is a long experience in their industrial use as biofactories, and a whole body of 'good manufacturing practices' (GMP) has been developed over the years, with approval by the competent regulatory authorities. Unlike bacteria, however, they ensure (in general) the synthesis of pharmacologically active products, since post-translational modifications of the recombinant protein are the same or very similar than that of the native human protein *in vivo*.

That is why mammalian cell cultures – for example, of Chinese Hamster Ovary (CHO)

cells, the 'golden standard' in the industry – are currently the system of choice for the production of biopharmaceuticals, despite having several drawbacks and limitations:

- i)* very complex systems, with high costs of both, up-front investment and maintenance (high-tech, high-cost);
- ii)* slow growth of the cells and limited productivity;
- iii)* the difficulty (technical and economic) to scale up or down production;
- iv)* the risk of contamination by human pathogens (e.g., viruses or prions).

GENETICALLY MODIFIED (GM) PLANTS: MOLECULAR 'PHARMING'

Production of recombinant proteins in the so-called '3rd generation' of transgenic plants provides an alternative, or rather a complement, to fermenter-based systems of genetically modified microorganisms or mammalian cells in *in vitro* cultures. The term 'molecular farming' was coined to describe the use of GM plants as biofactories of recombinant proteins, in general, and 'molecular *pharming*' or 'pharma crops' if we refer specifically to pharmacological proteins. These plant-based systems have – at least theoretically – a number of important advantages over other commercial production platforms:

- i)* the methods of plant genetic transformation – mediated by *Agrobacterium tumefaciens* (Herrera-Estrella et al., 1983) or by microprojectile bombardment (Klein et al., 1987) – and regeneration of transgenic plants are relatively cheap and simple, as compared for example to the generation of transgenic animals.
- ii)* the production systems can be established with low up-front investment and maintained cheaply, since they are based on common techniques used for centuries in agriculture (low-tech, low-cost).
- iii)* the production can be scaled (up or down) easily and cheaply, to adapt to market demands (in principle, simply by increasing or decreasing the cultivation area).
- iv)* in general, proteins are synthesised in a pharmacologically active form, since the systems of post-translational modification (e.g. glycosylation) in plants are similar to those of mammalian cells.

- v)* the synthesis of the recombinant protein can be directed to specific organs (and organelles) by using tissue-specific promoters and proper subcellular localization signals. Thus, the protein can be 'encapsulated' in natural plant structures, for example in the endosperm of seeds, facilitating in this way the storage of the protein in an active form, without requiring special conditions such as refrigeration
- vi)* there is the possibility of developing simple and efficient purification methods.
- vii)* there is no risk of contamination with human pathogens.

TRANSIENT EXPRESSION SYSTEMS

Besides generation of stably transformed GM plants, protocols have been established for the transient expression of the recombinant proteins in plant tissues, tobacco and alfalfa leaves being the commonest targets. Different vectors can be used to deliver cloned DNA: *Agrobacterium tumefaciens* (by vacuum-infiltration in the leaves), recombinant plant viruses or hybrid vectors containing viral sequences delivered by *Agrobacterium*. These transient expression systems allow the rapid production of large quantities of biopharmaceuticals, which would be needed to treat large numbers of individuals in a short period of time in case, for example, of epidemics or bioterrorist attacks.

EDIBLE VACCINES

Another specific application of 'pharma-crops' is the low-cost oral delivery of protein drugs bioencapsulated in plant cells (Kwon and Daniell, 2015). Among all these plant-derived recombinant proteins, especial attention has been given to the production of edible vaccines, to replace 'traditional' vaccines (consisting in live attenuated viruses or bacteria, killed or inactivated bacteria, or specific surface antigens of pathogens). Ingesting plant material containing a suitable recombinant antigen will induce an immune response at the level of the intestinal mucosa, via gut-associated lymphoid tissues. The natural 'encapsulation' of the recombinant vaccine in plant cells or subcellular structures (plastids, protein bodies, etc.), apart from protecting the protein from

acid and enzymes in the stomach, may help activate the immune system without the need of an adjuvant. Accordingly, a variety of vaccine targets – surface antigens of different viruses, *E. coli* and cholera toxins, etc. – have been expressed in edible transgenic plants, such as maize, tomato, lettuce, spinach or rice, and some of these products are undergoing clinical trials, previous to approval and commercialisation.

This approach would reduce many of the problems associated with the production and application of traditional vaccines, such as the high cost of vaccine purification, as well as transport and distribution issues (most important in developing countries): no need to maintain the 'cold chain', avoiding risk of transmitting infections by the use of non-sterile syringes, etc.

SOME EXAMPLES OF RECOMBINANT PROTEINS EXPRESSED IN GM PLANTS

Since the early 1990s, hundreds of recombinant proteins, many of them with pharmacological or therapeutical activities, have been expressed in transgenic plants. Old field trials included, for example, the production of: *i*) cholera toxin in alfalfa (Noble Foundation, 1992); *ii*) serum albumin, α - and β -globin, and procollagen in maize (Limagrain, 1998); *iii*) lactoferrin, antitrypsin, lysozyme, antithrombin and serum albumin, in barley (Washington State University, 2001); *iv*) aprotinin and trypsinogen from cow, glycoprotein gp 120 from HIV, and enterotoxin subunit B from *E. coli*, in maize (ProdiGene, 2002).

More recently, SemBioSys Genetics Inc., a Canadian biotech company, developed a production platform in safflower (*Carthamus tinctorius L*) seeds: the recombinant proteins were fused to oleosins and stored in oil bodies, which allowed very simple purification methods. Two proteins produced in this system, Apo AI (the lipoprotein associated with HDL, 'good cholesterol') for prevention and treatment of cardiovascular disease, and human insulin, were undergoing clinical trials when the company went bankrupt in 2012, before they could be marketed.

Another example of a 'pharma crop' is Ventria Bioscience's transgenic rice, expressing two

recombinant human milk proteins (lactoferrin and lysozyme) with anti-diarrhoea effects. The rice grains were processed into a powder to make oral rehydration solutions, which were planned to be the first commercialised transgenic over-the-counter 'medical food'. This product, however, never reached the market and at present several variants are in clinical trials for hospital treatment of more serious conditions, such as diarrhoea associated to antibiotic treatments, chemotherapy-induced diarrhoea, HIV-associated chronic inflammation or inflammatory bowel disease.

PRESENT SITUATION OF PLANT-BASED BIOPHARMACEUTICALS

For over 20 years, a large number of recombinant proteins have been produced in transgenic plants, many with potential clinical applications, proving the economic and technical advantages of 'molecular farming', over conventional production platforms using mammalian cell cultures. When referring specifically to plant-made biopharmaceuticals, however, in most cases this work has been limited to academic or 'proof-of-concept' studies; until recently, no plant-produced pharmacological protein has been approved to be used in humans. Therefore, the recombinant proteins which are currently produced in plants are marketed as reagents for research or used in various industries: cosmetics, detergents, food, etc., but production of pharmaceutical proteins in GM plants is lagging far behind the fermenter-based systems of cultured mammalian cells.

This is due, in part, to the long and expensive procedures (including clinical trials) required to bring to the market a new pharmaceutical protein for use in humans. Yet, regulatory and technical issues are in general more relevant than purely scientific advances. There are no clear specific rules applicable to the production of pharmaceuticals in plants, and it is very difficult to adapt those existing at present, established for such different biological systems as cells in *in vitro* cultures (similar regulatory problems exist in the case of the production of therapeutic proteins in transgenic animals). In addition, worldwide, there are only a few facilities authorised for the production of

recombinant proteins in plants according to 'good manufacturing practice' (GMP) for clinical development.

Nevertheless, in recent years, there has been a substantial boost in commercial development and potential applications of molecular pharming, and several specific products are currently undergoing clinical trials, at different phases (Stöger et al., 2015). Some of the specific milestones that mark this development are:

i) The approval by the FDA, in May 2012, of recombinant glucocerebrosidase (commercial name: 'Elelyso'), enzyme used to treat Gaucher's disease, a lysosomal storage disorder. The protein is produced by an Israeli company, Protalix Biotherapeutics, in a carrot cell suspension culture (Tekoah et al., 2015). This was the first pharmaceutical recombinant protein produced in a GM plant system approved for human use... although the plant cell culture was not so different technically from an animal cell culture.

ii) Production of the experimental drug ZMapp by transient expression in leaves of *Nicotiana benthamiana*. ZMapp contains a combination of three humanised monoclonal antibodies that recognise a surface glycoprotein of the Ebola virus, and was proven to be effective against the virus in infected primates (Qiu et al., 2014).

iii) Production in stably transformed GM tobacco of mAb 2G12, a neutralizing anti-HIV monoclonal antibody. The antibody is used as a topical prophylactic to prevent virus infection (by vaginal application prior to sexual intercourse). The relevant authorities approved a phase I clinical trial, which demonstrated the safety of its use (Ma et al., 2015). This project, 'Pharma-Planta', funded by the EU Sixth Framework Programme, was used to establish and develop an approved manufacturing process for a recombinant plant-made pharmaceutical protein, according to 'good manufacturing practice'. The complete procedure, from gene to harvest, included the design of expression constructs, plant transformation, the generation of production lines, master and working seed banks and the detailed investigation of cultivation and harvesting parameters and their impact on biomass, product yield and intra/interbatch variability (Sack et al., 2015).

PERSPECTIVES

'Molecular pharming' will probably never replace the systems of animal cell cultures, much more developed, and where the industry has made major investments. Yet the advantages of using transgenic plants as biofactories for the production of biopharmaceuticals provide a very interesting market niche for specific products. GM plants may be the best choice to produce recombinant proteins with particular properties or for specific uses, which cannot be synthesised by the current platforms due to low efficiency or very high costs. Some of these specific applications could be:

i) the synthesis of products of higher pharmacological activity or better quality, for example by engineering in the GM plants specific glycosylation patterns

ii) the production of oral vaccines, encapsulated in a plant matrix

iii) the production of proteins that must be rapidly obtained, in response to an emergency – such as vaccines for pandemic diseases or a bioterrorist attack – using transient expression systems

iv) development of simplified purification steps, to reduce the cost of downstream processing

v) synthesis of proteins required in massive amounts... (e.g., human serum albumin, insulin, topical microbicides)

vi) ... or at a very low scale (e.g. for 'personalised medicine')

vii) for products that cannot be produced in cell cultures, for example toxic proteins

CONCLUSIONS

The use of plant biofactories for the commercial production of biopharmaceuticals cannot compete, in general, with the well-established, fermenter-based mammalian cell culture systems. Yet plant-based platforms represent an attractive alternative for the production of specific proteins that cannot be synthesised in mammalian cells, or when the particular characteristics of the desired product make the use of cell culture unsuitable or unprofitable. The scientific basis for production of recombinant proteins in transgenic plants are well established and, as described in the

previous paragraphs, ‘molecular pharming’ has several economic and technical advantages over cell culture systems. Further development of the technology is to be expected in the near future, once GMP (‘good manufacturing practice’) procedures for biopharmaceuticals production are more widely established for plant systems and approved by the competent authorities, so that the present regulatory limitations are overcome.

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EUROPEAN FOOD LAW, YOUNG GOVERNANCE

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Abstract

Together with the growth and development of the European Union, the European Food Law has experienced fundamental modifications. If before the Bovine Spongiform Encephalopathy scandal, the food safety legislation was market oriented, nowadays its direction converges to the protection of the consumer. As a result, the actual food safety set of laws bases itself on scientific opinion, together with other factors that the risk manager has to consider when legislating.

This paper is depicting the steps taken by the European food legislation until reaching its actual form. It starts with describing the frame in which the European Food Law was approached before its changes, and it continues with presenting the drafts needed to build its present shape. In addition, because the current legislation is founded on the concept of risk analysis, the term will be elaborated both from a general perspective but also with respect to its applications in the food industry field.

Key words: food law, food safety, GFL, Codex Alimentarius, risk analysis.

INTRODUCTION

The legislation governing food industry at European Union level can be considered a new type of governance, due to the fact that it is the resultant of fundamental and sharp, but recent transformations.

If before 1997 the law was structured in such a way to assure and promote the good functioning of the Single European Market, after this time limit, the safety of the consumer becomes of first importance, and it grows to be a decisive unit in drafting the legislative acts.

In this way, in the new basic act (*Reg. (EC) 178/2002*) in its 6(1)th article, it is stipulated that '*food law shall be based on risk analysis*'. Food legislation becomes par excellence science-based, and for increasing the confidence in this new perspective, the EU recognized body responsible for the scientific research and makes the results available to the European Commission is an independent authority (European Food Safety Authority – EFSA).

The next section of this paper will treat details with regards to the history and evolution of the risk analysis both from a general perspective as

well as its role within the international and European food legislation.

THE CONCEPT OF RISK ANALYSIS

Historically, the first mentions about risk analysis are dating back in the year 3200 before Christ, when the Asipu group, (scholars from the old Mesopotamia) was in charge to provide advice and recommendation when there was the case of a chancy situation (Proske 2008, referring to Convello 1985). Such type of case assessment, can be considered as an incipient kind of risk analysis (Grier, 1981). However, the resolutions proposed by the Asipu experts from the Tigris-Euphrates valley were based on the signs they received from the gods. A closer meaning of the risk analysis term to the one used nowadays, dates from the XIV century when the most cost-effective way of transportation for commerce, was by ships. In that period, the term risk was used to describe the hazardous situation in which a shipment could fall into (Proske, 2008).

Besides the food and pharmaceutical field, risk analysis is a term that in present is used in many other areas, such as environmental

studies, banks and insurance covers, economy, aeronautics or in agriculture (Szajkowska, 2012).

Risks can be both analysed from a qualitative perspective as well as from a quantitative point of view. The qualitative approach is known to be less transparent than the quantitative ones, which are based on numbers, statistics and modelling.

Risk analysis and the food field

Similar to other dynamic systems (Jongen, Meulenberg, 2005), foodstuffs are subject to the laws of chemistry and of physics, through the fact that they undergo changes in time. Both the possible changes, as well as the initial status of the food can lead to hazards (physical, biological or chemical). As a result, food can represent a risk for the human beings as well as for the animals and the environment (Lunning, Marcelis, 2008). Recently, WHO has shown that foodborne diseases cause 230 000 deaths each year. The most common reason for these deaths are the diarrhoeal diseases. Alarmingly, 125 000 of children under 5 years old died due illnesses caused by contaminated food (WHO, 2015).

In the past, people performed risk analysis by selecting the types of food they consumed, but also by acquiring information about what can be eaten and how to prepare certain products. Such knowledge was handed down to the next generations, became habit and was enriched with the lapse of time (The Nordic Group of Ministers, 2002). Nonetheless, the high number of foodborne illnesses, the variety of food products as well as the internationalisation of commerce, have led to the necessity of a general guide to be used in analysing risks. As a result, both the national and international levels were expected to release such guidelines. This request, of presenting a harmonised way to cope with risks, was handled by the World Health Organization in Codex Alimentarius (FAO, WHO, 2005). Several definitions were provided, and the definition of risk analysis states: 'Risk analysis – a process consisting of three components: risk assessment, risk management and risk communication.' (Codex Alimentarius Procedural Manual, 25th edition, 2016).

A similar definition was provided by the European Union in the basic law, *Regulation (EC) 178/2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety*. Additionally, this act stipulates that risk analysis is fundamental in obtaining 'a high level of protection of human life and health' (Art. 6(1), Reg. (EC) 178/2002).

The differentiating element between the definition introduced by Codex Alimentarius and the one proposed by the European Parliament and the Council, is that, at European law level, the three components of risk analysis are *interconnected*, while the Codex Alimentarius Commission only lists them. However, in general the concept or risk analysis is illustrated as following:

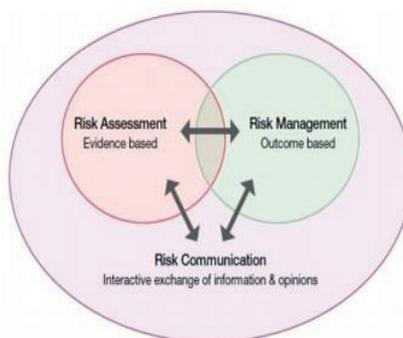


Figure 1. The concept of risk analysis (Source: <http://apvma.gov.au/node/15506>)

The illustration depicted in *Figure 1* shows that the components of risk analysis are interconnected, so they can influence each other.

HISTORY OF THE EUROPEAN FOOD LAW

The Bovine Spongiform Encephalopathy Crisis

The Bovine Spongiform Encephalopathy (Hereinafter: BSE, BSE Crisis) Crisis started when the British Ministry of Health declared that the possibility of the disease being transmissible to people exists. The corresponding period in which this scandal

evolved, coincided with the installation of the European Single Market. Together, these two events have challenged the population's trust in the food industry and in the way the European Single Market functioned. This unusual framework has led to the food safety code being described as 'contested governance' and it raised major questions about the functioning of the legislation of the food field : 'who, where, how and in what basis the decisions are taken' (Ansel, Vogel, 2006).

In the report that resulted from the investigations regarding the BSE scandal, (hereinafter: the BSE report), a tone of revolt can be distinguished, which reflects the lack of trust the population had towards the system that governed food safety.

According to the BSE report, communication (in the sense of informing the public and in the sense of cooperation) was considered to be poor during the fight with the disease. The dissemination of knowledge was defective and the possible channels to be used for communication were not known by the possible participants to the consultations. This reasoning is based on the text of the BSE report, which describes how the director of the Sanitary Services himself, Mr. Meldum, was not aware of the risks that the disease could imply. In this context, Mr. Meldum declared that there are no risks for human-beings, but the main problem was the population being concerned of the emergence of a new illness. In addition, the Sanitary Services director considered it was not a matter of consumer safety, but a problem targeting their trust (BSE Crisis Report, 1997).

Green Paper and White Paper

In the same year as when the BSE Crisis Report was drafted, the institutional scene of the European Food Law started to be adjusted. The worries of the consumers have been listed and presented in May 1997 when the European Commission published *The Green Paper on European Food Law*, which served as a cornerstone for the White Paper, published in January 2000 (Goodburn, 2001).

The White Paper on Food Safety is the response of the European Commission on the consequences of the BSE crisis over the general condition of the population. Policy makers had

to reflect on the answers they gave, in such a way to regain the consumer trust.

The revolution is that, in 2000, on the base of the newly-published White Paper, the European Commission is moving its attention from the well-functioning of the Single Market to the welfare of the consumers. The simple consumer is now able to express its opinions on food safety matters; this is an important step, as it can be seen as an attempt of the European Commission to involve more parties at risk management level, when decision are taken. In addition, The White Paper brings the new perspective for food law that of a food safety policy based on risk analysis and on the precautionary principle. As a result of the BSE scandal, traceability is to be considered for securing a 'successful food policy'.

Last but foremost, together with The White Paper, Europe takes a big step in the restructuration of the food legislation by setting up the European Food Safety Authority (EFSA). This is the way to convert food law into a science-based governance and to perform the risk assessment in an independent manner, excluding the other factors (such as: consumer perception, political or social issues, environmental related aspects, etc.) that may be taken into account at risk management level. EFSA only delivers scientific opinions and, according to the description made in the White Paper, it is characterized by the following major qualities: independence, transparency and eminency.

General Food Law

Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety (General Food Law or GFL) was published in January 2002 and sets the actual European principles and legal expectations in the food safety field, as well as for the feed safety. Additionally, GFL provides with definitions for the most important terms used in food law. In this sense, the general act defines 'food' (Art. 2, GFL) and with *article 14(1)*, it sets the fundamental obligation '*food shall not be placed on the market if it is unsafe*'.

Independently from the general objectives setup as stipulated in article 5 (to assure a high level of life protection and human health and to protect the interests of the consumers, good practices in the food trade and animal health and welfare protection), Regulation (EC) 178/2002 describes the European Food Safety Authority, how it is installed, the mission of EFSA as well as its obligations. It is believed that this regulation makes European food legislation to be considered a respected body within the general laws at European Union level (Berends, 2006).

Noteworthy, GFL makes it very clear that the consumer protection is the major objective of food law, while the functioning of the Single Market (as established in the Treaty on The Functioning of the European Union at article 28) only has the back seat.

GFL is the legislative act that is in vigour in present and it serves with the basic principles for the policy-makers in elaborating specific legislation.

CONCLUSION

European Food Law has known abrupt changes in the last 30 years, after the BSE crisis acted as a trigger for the total shift in the governance. Before 1997 the free movement of goods was promoted, while nowadays the focus is on the high level of consumer protection.

The actual legislation becomes harmonized to the principles stipulated in Regulation (EC) 178/2002 and the evolution of laws brings all the wings of food law on the same ground, that of protecting the consumers.

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TOTAL PHENOLIC ANALYSIS, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF SOME MUSHROOM TINCTURES FROM MEDICINAL AND EDIBLE SPECIES, BY *IN VITRO* AND *IN VIVO* TESTS

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Abstract

Wild mushrooms are commonly used in various pathologies. However, there are few studies concerning species characteristics from different geographical areas. The aim of the study was to determine the antioxidant and antimicrobial effects of 17 triple tinctures prepared from 9 species of mushrooms with medicinal potential, harvested from the region of Moldova, Romania. The study was conducted in parallel with seven commercial species by *in vitro* studies (DPPH and ABTS scavenging and chelating activities) and *in vivo* (antioxidant activity by using *Kluyveromyces marxianus* yeast strain). A direct correlation of *in vitro* antioxidant activity to that expressed *in vivo* was determined in the case of a high content of phenolic compounds. Tincture prepared from *Hericium coralloides* presented the lowest antiradical capacity. A correlation between the different *in vitro* antioxidant activities was determined for the *Tuber indicum* and *Piptoporus betulinus* species. Tinctures which have a high degree of protection against oxidative action of H_2O_2 had a medium value of $EC_{50} < 1$ mg/mL. The results obtained proved that certain compounds identified in trace amounts may significantly increase the biological effect, as the protocol for tinctures obtaining directly influenced the response and content expressed *in vitro* and *in vivo*.

Key words: extract, dried fruit bodies, scavenging activity, *Candida*.

INTRODUCTION

Tincture is a water-alcohol solution rich in biologically active substances of plant origin, obtained from one or more species with medicinal properties. The biological activity *in vitro* and / or *in vivo* is due to the presence of the complex of biologically active substances. The main active molecules are phenolic compounds, which are expressing the therapeutic activity of the final product (tincture) (Gird et al., 2005). From the classical to the modern methods of extraction (ultrasound assisted extraction) all aim to increase the efficiency of active principles extraction, which depends on the species of medicinal herbs or mushrooms used as raw materials (Valachovic et al., 2001).

In the case of fungi, tinctures utilization is less widespread. In Romania, administration of extracts from medicinal mushrooms is made in dry form, as lyophilized or atomized powder. Traditional medicinal species are used: *Lentinula edodes*, *Ganoderma lucidum*, *Agaricus brasiliensis*, *Heridium erinaceus*.

Using these species and many others as well, that is known by traditional medicine, has been driven by the need to find new biologically active compounds from natural sources, which will not cause side effects (Lindequist et al., 2005). The most frequent use of these extracts is the antitumor one, as a supplement to the chemotherapeutic medication (Safarzadeh et al., 2014). In this study, it was determined the effect of antioxidant and antimicrobial triple tinctures obtained from 13 species of mushrooms with medicinal potential, harvested in Moldova region, Romania, plus seven commercial species and a control of plant origin (*Juniperus communis* L.). All results have been correlated with the amount of phenolic compounds ascertained in each tincture.

MATERIALS AND METHODS

Samples. Nine of wild medicinal species (*Ganoderma applanatum*, *Trametes hirsuta*, *Perenniporia fraxinea*, *Fomitopsis pinicola*, *Ganoderma australe*, *Trametes ghibosa*,

Lenzites betulina, *Piptoporis betulinus*, *Schizophyllum commune*) was harvested from Moldova region, Romania in autumn of 2013 and spring 2014. *Craterellus tubaeformis*, *Hypomyces lactifluorum*, *Volvariella volvacea* was buy from Oregon Mushroom, USA. *Tuber indicum* was by from Metro Graz, Austria. Also, three dried edible species was used (*Auricularia judae*, *Boletus edulis* and *Cantharellus cibarius*) bought from supermarket. *A Juniperus communis L. (SC Stef Mar SRL Râmnicu Vâlcea, Romania)* bush fruit was used as a control.

Preparation of triple tinctures. Fifty grams of dried mushroom species were extracted with 250 mL ethanol 50% for fifteen days, at room temperatures with a manual daily agitation. The obtained extract was supplemented with the same solvent and mixed with new dried samples (same quantities), repeating the same protocol twice. Each extracts was filtered with Whatman No. 4 filter paper (Essaidi et al., 2013).

Determination of antioxidant activity, *in vitro*. Chelating activity, DPPH and ABTS scavenging activities were determined spectrophotometrically (517 nm, respectively 415 nm), according following formula: scavenging activity (%) = $[(A_C - A_S)/A_C] \times 100$, where A_C is the absorbance of the control and A_S is the absorbance of the sample (Liu et al., 2013).

Determination of antioxidant activity, *in vivo*. Antioxidant activity *in vivo* was presented in a previous study with slightly modification. Yeast strain *Kluyveromyces marxianus* was used and the viability was read to Colon Quant, by comparison with a sample of untreated cells (Vamanu & Nita, 2014a).

Determination of antimicrobial capacity. The antimicrobial capacity was tested *in vitro* against certain strains of bacteria and yeasts obtained from the Collection of the Faculty of Biology, University of Bucharest (Table 2, Vamanu, 2017). For this protocol, it was used a Bioscreen C MBR and a serial dilution was made in a proper media. After 48 hours the optical density was read at 600 nm and expressed as the minimum inhibitory concentration (MIC) of tinctures which means the minimum concentration that inhibited the tested strains growth.

RESULTS AND DISCUSSIONS

1. *In vitro* determination of the antioxidant activity.

In order to assess the antioxidant capacity, *in vitro* and *in vivo* tests was conducted, leading to point out the specific properties of each tincture. Even if an thorough evaluation requires the use of several solvents in parallel, in the case of tinctures for human use other alcohols cannot be used to demonstrate the effects of the composition in biologically active substances. The quantification of the antioxidant effect, demonstrated by free radical scavenging and chelating activity, was achieved in all tested tinctures and was presented in **Table 1**. The tincture prepared from *H. coraloides* presented the lowest ($p < 0.04$) antiradical capacity (EC_{50} values were 16.07 and 21.77 mg/mL), whereas *P. betulinus* and *T. indicum* displayed the lowest chelating capacity ($p < 0.05$). For this parameter, the EC_{50} value of the two species was about the same, and was not correlated with the antiradical one. Although a study about the activity of such medicinal mushroom extracts is lacking, the results could be appreciated as appropriate to the studies demonstrating that a hydro-alcoholic solvent will result in the presence of a high concentration of antioxidant compounds (Martins et al., 2015; Vamanu, 2013a). Compared with another widely used solvent (methanol), the triple tincture of *P. betulinus* presented a similar EC_{50} value for the scavenging activity against DPPH radicals (Reis et al., 2011). Only in 7 species of fungi were determined EC_{50} values which were lower than 1 mg/mL for DPPH scavenging activity.

Table 1 presents the values for the power reduction expressed by tested tinctures. A correlation was observed with previous values for species *Tuber indicum*, *Piptoporis betulinus* and *Hericium coraloides* because they had the lowest reducing power and DPPH scavenging capacity. Tinctures that determined a maximum donation capacity of an electron were *Ganoderma australe* and *Craterellus tubaeformis*. The EC_{50} index showed values of up to 1 mg/mL.

2. Determination *in vivo* of the antioxidant activity.

The protective action exerted *in vivo* is performed by inhibiting the reactions resulting in formation of free radicals, by means of phenolic component (in particular). The absorption of these biologically active compounds will cause an increase in the reaction capacity of the organism at the cellular level (Silva et al., 2009). By using the tinctures of mushroom dried fruit bodies an increase was recorded in the resistance of eukaryotic cells, *K. marxianus*, to the stress caused by reactive oxygen species generated by the decomposition of hydrogen peroxide. As shown in **Table 1**, the protective action was correlated with the decreased pro-oxidant action of metals in transition, by their transformation in more stable complexes (Koncic et al., 2011). These results are directly influenced by the membrane absorption of some flavonoids (e.g., catechin), to which may be added the total amount of sugars present in the samples, which could directly influence cell viability (Stefenon et al., 2010).

Thus, was ascertained the ability of tinctures to protect the cells that are in the exponential growth phase to oxidative stress induced in the presence of H₂O₂. As expected, the cells of *K. marxianus* displayed different degrees of sensitivity to the oxidative treatment. Depending on the exerted effect (**Table 1**), only the tinctures T4 - T10 showed a high degree of protection against the oxidative action of H₂O₂, with an average EC₅₀ value of <1 mg/mL. Instead, tinctures T11 - T17 had a reduced protection level to the oxidative stress. In this case, cell viability presented low values, of below 30%. The first three tinctures had a mean degree of protection, of around 46 ± 3.47%. T4 - T6 determined an increase of the average viability of about 25-30%.

Compared to previous *in vivo* studies, increasing cell viability of *K. marxianus* was correlated with an increase in glutathione peroxidase and reductase enzyme activity, if the studies are related to an untreated control. The protective effects are correlated to the polyphenolic content. Thus, certain phenolic compounds had a stimulating effect on the metabolism of yeast cells, which would be translated in increasing the viability after the

oxidative stress. Among these compounds, the stimulating cause of this behaviour would be anthocyanins, compounds found in plants and in fungi as well, having a direct role in the vascular protection and in stimulation of the cognitive processes (Baroni et al., 2012; Cho et al., 2003).

In any event, the antioxidant effects *in vitro* and *in vivo* are never the result of only one single class of bioactive compounds. They are the result of complex interaction between several classes of compounds that exert their pharmacological effects in certain situations. As regards the *in vivo* effect, the digestive activity should not be ignored, as it has a direct influence on the activation of the phenolic component. Facilitating the absorption of valuable components will be the result of a correct functioning of various physiological functions that are related to the structure and fermentation activity of human colonic microbiota.

3. Determination of antimicrobial capacity.

Evaluation of antimicrobial activity expressed as MIC demonstrated an inhibitory capacity of all tinctures. **Table 2** shows that from all edible species only *Auricularia judae* and *Cantharellus cibarius* had a significant inhibitory activity against *Candida* strains (p<0.05). Otherwise, from the wild species of mushrooms, only with the tincture of *Phellinus pomaceus* were obtained low MIC values, of about 8 mg/mL. For most species modest MIC values were obtained, requiring high concentrations, of at least 16 mg/mL.

The inhibitory activity against Gram - positive strains was especially expressed in the case of *Staphylococcus aureus*, which is known for its resistance and high infectivity. Species *Trametes versicolor*, *Trametes hirsuta* and *Perenniporia fraxinea* showed inhibitory activity against *B. cereus* and *L. innocua*, widespread in nature, causing food poisoning. The antimicrobial activity against these strains is determined, according to previous studies (Vamanu, 2017), by the flavonoid component. The researches have shown the effect caused by the presence of myricetin and the mentioned tinctures may contain the compound that has also been identified in extracts from the fungus mycelium (Vamanu, 2014). In addition, Gram-

negative strains are known to present a significant resistance to the alcoholic or aqueous plant extracts (Oh et al., 2013). This aspect was also described in the case of these tinctures obtained from edible and/or medicinal mushrooms, for example against *E. coli*. A larger number of tinctures have been identified, having a MIC value of 64 $\mu\text{L}/\text{mL}$.

To all this is added the presence of polysaccharides, which can in turn bind phenolic compounds, thus participating in the

expression of the biological value. Even the alcohol will favour the presence of such polysaccharides, which often can be seen on the bottom of the brown bottles in which the tinctures are stored. Tinctures of mushrooms (eg *Hericium coraloides*, *Trametes versicolor*, *Piptoporis betulinus*) display, after stored overnight in a refrigerator, a white deposit of polysaccharides. At the moment when the solutions reach room temperature, the polysaccharides are re-dissolved.

Table 1. Antioxidant activity (EC_{50} values, mg/mL) of mushroom tinctures

Tinctures (mushroom species)	DPPH scavenging activity	ABTS scavenging activity	Chelating activity	<i>In vivo</i> antioxidant activity
T1 (<i>Auricularia judae</i>)	5.04	0.56	5.67	3.23
T2 (<i>Cantharellus cibarius</i>)	8.97	7.67	4.07	4.62
T3 (<i>Boletus edulis</i>)	4.67	8	2.87	6.69
T4 (<i>Tuber indicum</i>)	1.2	7	12.56	0.57
T5 (<i>Craterellus tubaeformis</i>)	0.6	0.27	1.33	0.50
T6 (<i>Hericium coraloides</i>)	16.07	21.77	5.98	0.55
T7 (<i>Fonies fomentaris</i>)	0.6	0.14	5.80	0.70
T8 (<i>Trametes versicolor</i>)	1.2	0.2	2.54	0.73
T9 (<i>Phellinus pomaceus</i>)	0.7	0.1	3.61	0.63
T10 (<i>Trametes hirsuta</i>)	1.3	4.34	6.87	0.97
T11 (<i>Perenniporia fraxinea</i>)	1.5	0.04	4.63	> 50
T12 (<i>Fomitopsis pinicola</i>)	0.7	0.24	1.03	> 50
T13 (<i>Ganoderma australe</i>)	0.5	0.1	0.71	> 50
T14 (<i>Trametes ghibosa</i>)	0.9	0.14	1.48	> 50
T15 (<i>Lenzites betulina</i>)	1.2	1.07	4.76	> 50
T16 (<i>Piptoporis betulinus</i>)	4.57	7.02	13.76	> 50
T17 (<i>Schizophillum commune</i>)	0.5	0.3	3.02	> 50

Table 2. Antimicrobial activity ($\mu\text{L}/\text{mL}$) of mushroom tinctures

Tinctures (mushroom species)	<i>Listeria innocua</i>	<i>Bacillus cereus</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>	<i>Candida sp.</i>	<i>Candida albicans</i>
T1 (<i>Auricularia judae</i>)	32	16	64	16	32	32	8
T2 (<i>Cantharellus cibarius</i>)	32	16	32	16	16	16	8
T3 (<i>Boletus edulis</i>)	64	64	64	32	32	8	8
T4 (<i>Tuber indicum</i>)	64	64	32	16	32	32	32
T5 (<i>Craterellus tubaeformis</i>)	16	64	64	32	32	32	32
T6 (<i>Hericium coraloides</i>)	16	8	16	16	8	32	16
T7 (<i>Fonies fomentaris</i>)	32	32	32	32	8	32	32
T8 (<i>Trametes versicolor</i>)	8	8	16	32	8	32	32
T9 (<i>Phellinus pomaceus</i>)	32	16	16	32	8	16	8
T10 (<i>Trametes hirsuta</i>)	8	8	8	32	8	16	16
T11 (<i>Perenniporia fraxinea</i>)	8	8	8	16	8	32	32
T12 (<i>Fomitopsis pinicola</i>)	16	32	8	16	16	32	16
T13 (<i>Ganoderma australe</i>)	16	8	16	32	16	32	16
T14 (<i>Trametes ghibosa</i>)	8	32	8	8	16	16	16
T15 (<i>Lenzites betulina</i>)	32	16	16	32	8	32	32
T16 (<i>Piptoporis betulinus</i>)	32	32	16	16	8	16	32
T17 (<i>Schizophillum commune</i>)	16	16	16	16	8	32	16

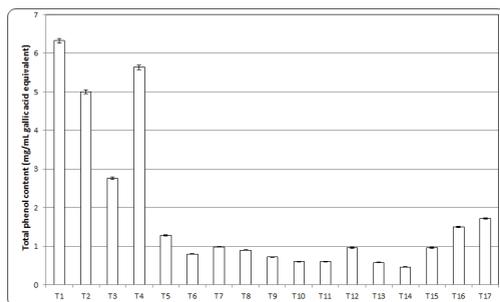


Figure 1. The total phenolic content in mushroom tinctures

4. Determination of total phenolic and flavonoidic compounds

The total composition in phenolic compounds is shown in **Figure 1**. The highest content in phenolic compounds was determined in the tinctures of *Tuber indicum*, *Cantharellus cibarius* and *Auricularia judae*, with a content exceeding 5 mg/mL gallic acid equivalent. Instead, the tinctures T6 - T15 had the lowest content of maximum 1 mg/mL gallic acid equivalent. These results demonstrate that the assessed biological effects, *in vitro* and *in vivo*, were primarily due to the content in flavonoids, whose maximum values were correlated with the tinctures whose biological activity was maximal. These findings do not mean that the phenolic compounds, that most frequently are represented by gallic acid (Vamanu & Nita, 2014a), protocatechuic acid or phydroxybenzoic acid (Leal et al., 2013) do not participate in the expression of antioxidant effect, for example, but have only a secondary role (**Figure 2**).

In this situation, a direct correlation was revealed of these compounds with the antioxidant activity *in vitro*. Tinctures derived from species *Craterellus tubaeformis* and *Piptoporis betulinus*, had values that exceeded 100 µg / mL catechin equivalent. An exception was obtained with the tincture of the species *Perenniporia fraxinea*, which exceeded by approximately 10% the previously mentioned value, but this was only slightly correlated with the antioxidant activities *in vitro* and *in vivo*. It is better correlated with the antimicrobial activity, which shows that in the same species there is not a direct correlation between the antioxidant and the antimicrobial activity. These studies disagree with other previous

results corresponding to the mycelia of the species *Pleurotus ostreatus* and/or *Coprinus comatus* (Vamanu, 2013a).

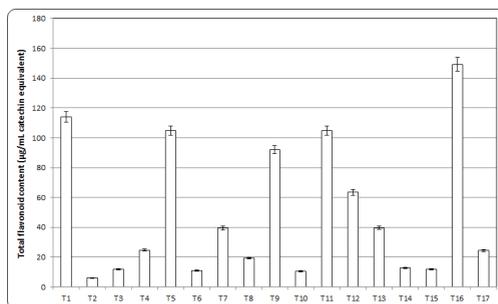


Figure 2. The total flavonoid content in mushroom tinctures

Due to the various biological processes by which acts, a direct correlation between the biological activities has not been found. The highest R^2 values were determined for *Craterellus tubaeformis*; for example a value of approximately 0.745 was obtained between the scavenging activities and the reduction power of the Fe ion. This value was as well appropriate in the case of a correlation with *in vivo* antioxidant activity.

The chelating activities the values decreased by about 30%. Instead, the antimicrobial activity was primarily correlated to the total phenolic content. According to some previous studies it depends on the combination of various phenolic compounds and on the chemical structure (Vamanu, 2013b; Sandigawad & Patil, 2010). If we refer to recently published studies, a significant part of the antioxidant answer, expressed at least *in vitro*, is due to the polysaccharide component, which would correspond to the non-correlation between the decreased phenolic content and the EC_{50} value expressed by some species (eg, *Hericium coraloides*). Thus, a direct correlation of the *in vitro* antioxidant activity to that expressed *in vivo* was determined in the case of a high content in phenolic compounds. These results were consistent to a content that exceeded 6 mg/g gallic acid equivalents for *Agaricus bisporus* (Liu et al., 2013). Also, the research results have shown the presence among the phenolic compounds not only of caffeic acid and homogentisic acid but also of the

flavonoids miricitine and routine (Liu et al., 2013; Vamanu & Nita, 2014b).

CONCLUSIONS

The results do not directly certify the relationships between the content of biologically active compounds and the biological activities *in vitro* and/or *in vivo*, but indicate that certain compounds identified in trace amounts may significantly increase the expressed effect. The same situation was registered in the case of identification of some specific phenolic compounds in medicinal plants (Vamanu & Nita, 2013). Thus, the various technological processes of extraction directly influenced the content and the biological response expressed *in vitro* and *in vivo*. Optimization of obtaining the tinctures from the fructification body of edible and medicinal mushrooms is underway, aiming at increasing the content of these bioactive compounds, and also at finding other possible effects. Tests are under consideration regarding the effect of absorption and digestion processes on the stability of the chemical composition.

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SELECTIVE FRACTIONS OBTAINED FROM PLANT SPECIES CULTIVATED IN ROMANIA WITH POTENTIAL EFFECT ON COUNTERACTING DISEASES ASSOCIATED WITH AGING PROCESSES

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Abstract

The aim of these studies was to obtain *Rosmarini folium* selective fractions with beneficial effect in counteracting diseases associated with aging processes.

By four different methods 9 selective fractions were obtained. HPLC analysis and quantitative determination of active principles from selective fraction show that the values obtained from individual assessment by HPLC were well correlated with the values obtained by the spectrophotometrically methods. The selective fractions have a total flavonoid content expressed as rutin from 2.004 to 66.970% and respectively 0.651 to 10.284% polyphenolcarboxylic acids expressed as rosmarinic acid.

Antioxidant activity evaluation showed that polyphenolcarboxylic acids rich fractions such as RIII: 10.284%, RIIC: 10.257%; had 89.44%; 88.12% antioxidant activity in 1% dilution and 87.52%; 89.07% antioxidant activity in 0.1% dilution, similar to rosmarinic acid who has antioxidant activity of 88.99% respectively 89.84% in dilution of 1% respectively 0.1%. The fractions with 2.642% - 3.950% polyphenolcarboxylic acid content exhibited an antioxidant activity of 45.73% - 87.5% in 1% dilution and of 8.75 to 87.500% activity in 0.1% dilution.

Comparing the antioxidant activity of selective fractions and the polyphenolcarboxylic acids content expressed as rosmarinic acid it can be concluded that when the concentration of polyphenolcarboxylic acids increases the antioxidant activity also increases, though not an exact correlation can be made.

A correlation between the flavones content of the selective fractions and antioxidant activity can not be made by this method.

Key words: *Rosmarinus officinalis*, selective fractions, antioxidant.

INTRODUCTION

Some chemical compounds derived from plant species, such as rosmarinic acid, caffeic acid or other compounds from the polyphenolcarboxylic acids class; diosmin, diosmetin or other flavonoidic compounds are known for their antioxidant properties.

Since free radicals are considered the main responsible agents of premature aging and also of diseases associated to aging status, compounds with antioxidant activity are regarded as basic elements of the anti-aging strategy (Ashok et al., 1999).

Among the plant species with antioxidant properties is *Rosmarinus officinalis* L. (Lamiaceae family) (Yeset-Celiktas et al., 2007; Babovic et al., 2010).

Rosmarinus officinalis L. has been used since ancient times in traditional medicine. Due to its

special therapeutic properties, this species was widely cultivated.

The most important active components of this species are: polyphenolcarboxylic acids, flavonoids, di- and triterpenoids and volatile oil (Begum et al., 2013).

Recent studies have confirmed the pharmacological activity of *Rosmarinus officinalis* leaf extract, based on active substances such as acids polyphenolcarboxylic including rosmarinic and caffeic acid or flavones including diosmin (Begum et al., 2013; Hernadez-Hernadez et al., 2009; Cosio et al., 2006).

For example, *Rosmarinus officinalis* L. leaves (*Rosmarini folium*) stimulates cerebral circulation and microcirculation due to rosmarinic acid composition (Aruoma et al., 1994) and exhibits an anti-stress action based on antioxidant properties against free radicals and peroxides (Frankel et al., 2000). Due to

polyphenolcarboxylic acids content, this species show hypocholesterolemic and choleretic-cholagogue action and due to flavones (including diosmin) exhibit antiseptic and healing action (Istudor et al., 2001).

MATERIALS AND METHODS

The vegetal material consisting of leaves of *Rosmarinus officinalis* L. (Rosmarini folium) was obtained from culture, dried and ground as a fine powder (sieve VII).

Selective fractions obtainment:

Method I consisted of repeated extraction - two times of the active substances from 200 g Rosmarini folium, with 50% ethyl alcohol v/v (vegetal material / solvent ratio = 1/10 m/v for the first extraction and 1/5 m/v for the second extraction) at boiling temperature of the solvent for 1 hour per extraction with continuous mechanical stirring, followed by cooling and filtration of the extracts.

The reunited solutions were rota-evaporated for alcohol removal. The resulting aqueous solutions were spray-dried and selective fractions RI were obtained.

Method II consisted of active principles extraction from 300 g Rosmarini folium with 50% alcohol (plant / solvent = 1 / 10 m/v ratio) at boiling temperature for 1 hour with continuous mechanical stirring, followed by cooling and filtration of the extracts. Hydroalcoholic extract solution was evaporated to a volume of 1/1 m/v plant/solvent mixture and centrifuged. A precipitate (which was labeled as RII0 after drying) and an aqueous solution were obtained. In order to obtain selective fractions, aqueous solution was further processed by:

- Three successive liquid-liquid extractions with ethyl ether, followed by solvent removal from the reunited etheric extracts resulting RIIA;
- Three successive liquid-liquid extractions with chloroform, followed by solvent removal from the reunited chloroformic extracts resulting RII B;
- Three successive liquid-liquid extractions with ethyl acetate, followed by solvent removal from the reunited ethyl acetate extracts resulting RII C;
- Three successive liquid-liquid extractions with 1-butanol followed by solvent removal

from the reunited butanolic extracts resulting RII D;

- Adding acetone in a 2/1 v/v acetone/ aqueous extract ratio resting for 24 hours at 4-6⁰C, filtration, and drying the precipitate resulting RII E (Figure 1).

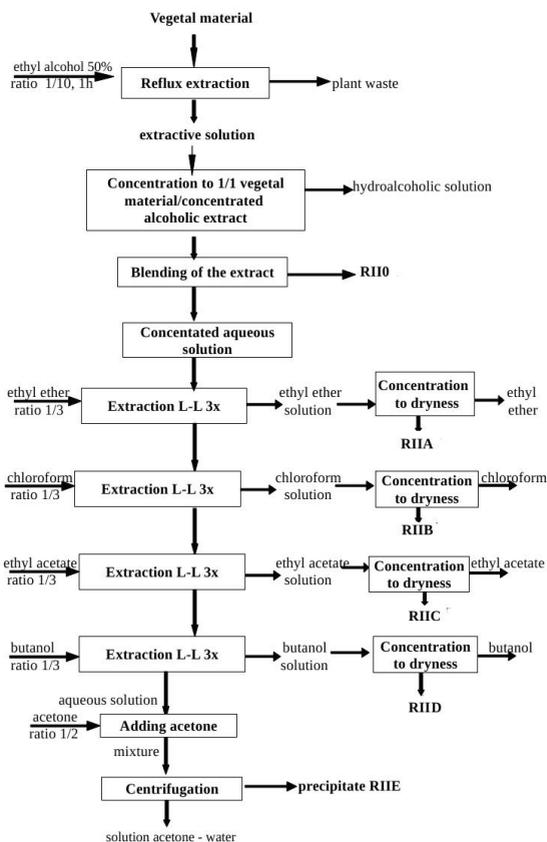


Figure 1. Selective fractions obtainment - method II

Method III consisted of repeated extractions times of the active substances from 200 g Rosmarini folium with methylic alcohol (plant / solvent ratio = 1 / 10 m / v for the first extraction and 1/5 m/v for the second extraction) at boiling temperature of the solvent for one hour per extraction with continuous mechanical stirring, followed by cooling and filtration of the extracts. Methanolic solutions were reunited, the solvent were removed by rotaevaporation resulting RIII selective fractions.

Method IV consisted of macerating 200g Rosmarini folium in acetone (plant/solvent ratio = 1/7 m/v), removing the solvent from acetone solution and re-extracting the residue

in methanol. The active substances were extracted from moist plant material with 20% ethanol (plant/solvent ratio = 1/10 m/v) at boiling temperature of the mixture for 2 hours, followed by hydroalcoholic solution evaporation to an aqueous extract. Methanolic and aqueous extract were reunited and filtered. The resulting precipitate was dried and selective fractions RIV were obtained.

HPTLC analysis of selective fractions was performed using Silica Gel 60F₂₅₄ as stationary phase and a mixture of ethyl acetate - acetic acid - formic acid - water (100:11:11:27v/v/v/v) for chromatographic elution. The plates were scanned under 360 nm after the derivatization with NP/PEG. The reference compounds for HPTLC analysis were from Sigma-Aldrich: caffeic acid, rosmarinic acid, chlorogenic acid, rutin, hyperoside and diosmin. (Wagner et al., 1996; Reikh et al., 2008)

HPLC analysis of selective fraction consisted in chromatographic separation on a Purospher ODS column (250x4,6 mm, 5 μ) at 40^oC, using a gradient elution (both mobile phase and flow). The mobile phase was a binary gradient: water with orthophosphoric acid (pH=2,5) and methanol. The eluent absorbance was monitored at 330 nm. The reference substances were from Sigma-Aldrich: caffeic acid, rosmarinic acid, rutin, diosmin and luteolin.

Quantitative determination of active principles from selective fractions consisted of determination of flavones by a colorimetric method based on their property to form intensely yellow complex with Al₃⁺ and of determination on polyphenolcarboxylic acids by a colorimetric method based on the property of phenols to form nitrocompounds or nitro oxime with nitrous acid which give red stain when dissolve in alkaline solutions due to their weak acid character. For the quantification of flavones, rutin was used as reference substance and for polyphenolcarboxylic acids quantification rosmarinic acid was used as reference substance. (Roumanian Pharmacopoeia the Xth Edition, 1993)

Analysis of antioxidant action

DPPH assay: In each reaction tube 100 μ L vegetal extract of different concentrations was mixed with 3900 μ L of 0.0025 g/L DPPH at

room temperature for 30 min. 50% methanol solution was used as control.

The reduction of the DPPH free radical was measured by reading the absorbance at the wavelength 515 nm. Rosmarinic acid (from Sigma-Aldrich) was used as reference substance. Inhibition ratio (percent) was calculated from the following equation:

$$\% \text{ inhibition} = [(\text{absorbance of control} - \text{absorbance of sample}) / \text{absorbance of control}] \times 100$$

DPPH radicals react with suitable reducing is measured spectrophotometrically at 515 nm. (Sanchez Moreno, 1998).

For determination of antioxidant activity, the selective fractions were chosen according to the yield obtained from 100 g plant and depending on the flavones and polyphenolcarboxylic acids content.

RESULTS AND DISCUSSIONS

Nine Rosmarini folium selective fractions selective fractions were obtained by experimental methods mentioned above. The quantities of product obtained from 100g plant are shown in table 1.

Flavonoids (rutin, hyperoside, diosmin) and polyphenolcarboxylic acids (rosmarinic acid, caffeic acid, chlorogenic acid) were identified by HPTLC in most of selective fractions .

The content of caffeic acid, rosmarinic acid, diosmine, rutin and luteolin in each fraction was determined by HPLC method. The values obtained from individual assessment by HPLC were well correlated with the values obtained by the spectrophotometrically methods mentioned above. For example, rosmarinic acid content from the selective fractions, determined by HPLC, correlates with the polyphenolic acids content expressed in rosmarinic acid, determined by the headline method.

The flavonoid content expressed as rutin and polyphenolcarboxylic acids expressed as rosmarinic acid of Rosmarini folium selective fraction selective fraction are shown in table 1.

The selective fractions obtained by processing the leaf of *Rosmarinus officinalis* contain 0651-10.284% polyphenolcarboxylic acids expressed as rosmarinic acid and 2.004 – 66.970% flavonoids expressed as rutin.

Table 1. The content of active principles of *Rosmarini folium* selective fractions

Active principles/ Bioactive product	Quantity of product from 100g plant	Polyphenolcarboxylic acids expressed as rosmarinic acid % g/g	Flavonoids expressed as rutin % g/g
RI	16.45g	3.950	7.856
RII0	4.43g	2.642	2.078
RIIA	0.31g	3.792	13.634
RIIB	0.13g	0.651	4.800
RIIC	0.63g	10.257	66.970
RIID	1.06g	3.041	16.653
RIIE	3.10g	2.380	2.004
RIII	18.73g	10.284	2.929
RIV	5.94g	3.016	2.745

The most affluent fractions in polyphenolcarboxylic acids expressed as rosmarinic acid are RIII (10.284%) and RIIC (10.257%) followed by RI (3,950%), RIIA (3,792%), RIID (3,041%) and RIV(3,016%), RII0 respectively RIIE contains the 2,642% respectively 2,380% and the fraction with most low content in polyphenolcarboxylic acids expressed as rosmarinic acid are RIIB (0,651%). The most affluent fractions in flavonoids expressed as rutin it is RIIC (66,970%) followed by RIID (16,653%), RIIA (13,634%), RI (7,856%) and RIIB (4,800%). The other factions contain 2,929% (RIII), 2.745 % (RIV), 2,078% (RII0) and 2,004 % (RIIE). Antioxidant activity of selective fractions is shown in figure 2.

Using the method for the analysis of the antioxidant activity described, it can be noted that rosmarinic acid in a percentage of 1; 0,1; 0,01 and 0,001 exhibits an antioxidant activity of 88,99% ; 89,84; 66,15% and 9,14% .

The selective fractions RIII respectively RIIC containing 10.284%, respectively 10.257% polyphenolcarboxylic acids in dilution of 1% and 0.1% had a similar antioxidant activity with the reference substance - rosmarinic acid in the same dilution. Thereby RIII shows an antioxidant activity of 89,44% and 87,52% respectively RIIC an antioxidant activity of 88,12% and 89,07%.

Some selective fractions with a lower polyphenolcarboxylic acids content such as RI (3.950%), RII0 (2.642%) exhibit over 80%

antioxidant activity in dilutions of 1% and 0.1%. Thereby RI shows an antioxidant activity of 87,50% and 80,00% and RII0 manifest an antioxidant activity of 84,32% and 87,88%.

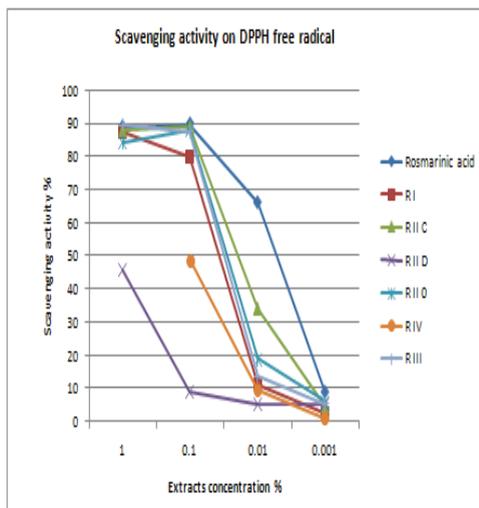


Figure 2. The antioxidant activity of the selective fractions from *Rosmarinus officinalis*

Even the polyphenolcarboxylic acids content of the selective fraction RIID (3,041%) and RIV (3,016%) is similar to the content of RI (which exhibits a good inhibitory potential), these fractions show a weaker antioxidant activity. Thereby RII0 in a dilution of 1% and 0,1% exhibits an antioxidant activity of 45,73% and 8,75% and RIV in a dilution of 0,1 % exhibits an antioxidant activity of 48,69%.

Comparing the antioxidant activity of selective fractions and the polyphenolcarboxylic acids expressed as rosmarinic acid and flavones expressed as rutin content it can be concluded that when the concentration of polyphenolcarboxylic acids increases, the antioxidant activity also increases, though not an exact correlation can be made (Colceru-Mihul S., et al.,2016).

A correlation between the flavones content of the selective fractions and antioxidant activity can not be made by this method. Thus, the selective fraction RIIC with 66.970% flavones content expressed as rutin shows antioxidant activity similar to RIII with 2.292% flavones content expressed as rutin.

CONCLUSIONS

From *Rosmarinus officinalis* L. leaves (*Rosmarini folium*) 9 selective fractions enriched in flavones and polyphenolic acids were obtained by different methods.

Six selective fractions were tested for antioxidant activity resulting that the two selective fractions exhibited an antioxidant activity comparable with the rosmarinic acid.

It can be concluded that a high content of polyphenolcarboxylic acids expressed as rosmarinic acid lead to a higher antioxidant activity but an exact correlation can not be made.

The antioxidant activity of the flavones was not highlighted by the method used in this study for the antioxidant activity evaluation.

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EFFECTS OF ROSEHIP SEED FLOUR ON THE RHEOLOGICAL PROPERTIES OF BREAD DOUGH

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Abstract

The effects of rosehip seed flour (RSF) on the rheological properties of bread dough were investigated. RSF was substituted with bread wheat flour at the proportions of 5, 7.5 and 10%. RSF was found to have 7.4 % protein, 4.6% lipid, 79.4% dietary fiber, 97.60 g/kg GAE total phenolic, and 759.48 IC50=µg/ml antiradical activity. Among farinograph parameters: water absorption, development time and softening value of RSF added doughs were found lower than the control dough while stability was showed slightly increase as the RSF level increases from 5% to %7.5. However it began to decrease after this level. Textural measurements by using Kieffer dough and gluten extensibility rig studies showed that inclusion of RSP did not cause any significant change at the maximum resistance to extension and extensibility of doughs when 5% of RSP was added. As the level of RSF increased from 5 to 7.5 and 10% respectively, a decrease in extensibility of doughs was observed. The addition of RSF resulted in significant effects on pressure, extensibility and energy of doughs measured with Dobraszczyk/Roberts dough inflation system of the texture analyser. A significant increase was observed in pressure and energy of doughs who were positively correlated with increased level of RSF. On the other hand a weakening was noted at higher levels (from 5% to 10%) when compared with control dough. As a result; substitution at 5 % of RSF gives rheological parameter values at least as good as the control sample and it can be used as valuable ingredient to enhance the functional properties of bakery products.

Key words: Rosehip, farinograph, Kieffer, dough inflation system, extensibility.

INTRODUCTION

Rosehips (*Rosa spp.*) are members of the genus *Rosa*. Approximately up to 200 species are grown in the world, 25% of them are found in Turkey (Murathan et al., 2016a). Members of the *Rosaceae* family have long been used for food and medicinal purposes. Rosehip fruits are good sources of bioactive compounds, phytonutrients and minerals (Murathan et al., 2016b). Due to presence of these compounds rosehip fruits and seeds have prophylactic and therapeutic actions against the infectious and inflammatory diseases, diabetes, gastrointestinal disorders, common cold, diarrhea and urinary tract diseases (İlyasoğlu, 2014).

Rosehip fruits are generally consumed in the form of tea, wine, jam, jellies, and marmalade. Strugała et al., (2016) reported that extracts of rosehip fruit with broad biological activity could potentially be useful as a functional food ingredient. The inside of the rosehip fruit is hairy and contains a large number of seeds which rate between 20-44% (Yıldız and Nergiz,

1996). These seeds, by products of rosehip industry, are used generally as an animal feed, whereas they contain even higher amounts of specific nutritionally valuable and biologically active components. For example the lipid fraction of the rosehip seed contains more than 50% polyunsaturated fatty acids. And also they are a valuable source of minerals, are quite rich in phosphorus and potassium. Hence, these by-products rich in bioactive compounds and dietary fiber could be used as a functional ingredient in bakery foods. To the best of our knowledge, there are no reports on the effects of rosehip seed powder (RSF) on the rheological properties of bread dough. Thus the objective of this study was to determine nutritional and phytochemical composition of the rosehip seed and its effects on the rheological characteristics of bread dough.

MATERIALS AND METHODS

Materials

Commercial bread wheat flour was supplied from Berberoğlu Milling Factory (Burdur,

Turkey). Farinographic properties of the flour were as follows: 58.7% of water absorption, 7.9 min stability and 2.2 min of dough development time. The proximate compositions of wheat flour, analysed by the AACC (2001) methods, were 14.5±0.01% moisture, 0.61±0.03% ash, 10.47±0.03% protein, 29.9±0.12 % wet gluten, 96.2±0.20 % gluten index, sedimentation 31±1 ml and falling number 362±2 s. Total dietary fibre assay kit was purchased from Megazyme Company (Wicklow, Ireland). Other chemicals were purchased from Merck (Darmstadt, Germany) and were of analytical grade.

Preparation of RSF

Rosehip seeds were provided by manual separation of the seeds from pomace which was the by product of rosehip marmalade plant. They were dried in a cabinet drier at 55±2C then ground in a hammer mill (Tekpa, Turkey). The ground material was passed through 300 µm sieve. The fraction which passed through sieve was collected and vacuumed in packages with barrier property and used for the study.

Chemical analysis of RSF

RSF was analysed for moisture (Method 44-01.01;AACC, 2001), ash (Method 08-01.01; AACC, 2001), total lipid (Method 30-25.01; AACC, 2001), protein-N×6.25 (Method 46-12.01;AACC, 2001), total dietary fibre (Method 32-05.01;AACC, 2001), total phenolic (TP) (Singleton and Rossi, 1965) and antiradical activity by using α-diphenyl-β-picrylhydrazyl (DPPH) (Dorman et al., 2003). Colours of dried and ground PSF samples were determined with Minolta CR 400 (Minolta Co Ltd., Tokyo, Japan) and was expressed as brightness, L*, a*, and b* values. The lightness value (L*) represents the black-white colours; a* represents the green-red colours; and b* represents the blue-yellow colours.

Farinograph measurements

PSF blends at 0%, 5%, 7.5% and 10% levels were prepared by replacing wheat flour. The effect of PSF on the mixing profile of the dough was studied using farinograph (Brabender, Duisburg, Germany) according to the standard AACC Method-54-21.02 (AACC, 2001). Water absorption, development time, stability and degree of softening (12 minute after maximum) of control dough and PSF

containing doughs were determined. The 300 g mixing bowl was used.

SMS/Kieffer Dough and gluten extensibility ring measurements

Texturometer (TA-XT2, Stable Micro Systems, England) equipped with SMS/ Dough and gluten extensibility ring was utilized to measure the elastic properties of the dough samples according to the methods of Kieffer et. al. (1998). Water was added according to farinograph water absorption for each formula and then mixed until optimum dough development. The test was carried out in ten replicates under the following conditions: pre-test speed 2.0 mm/sec, test speed 3.3 mm/sec, post- test speed 10.00 mm/sec, distance 75 mm, trigger force auto-5 g, data acquisition rate: 200 pps. The resistance to extension (force in g), extensibility (distance in mm) and area under the force vs. distance curve (g.s) values of dough samples were determined.

Dobraszczyk / Roberts (D/R) dough inflation system measurements

Large strain biaxial extensional rheological properties of dough were measured during bubble inflation using the D/R dough inflation method (Dobraszczyk, 1997). Doughs for the dough inflation test were first prepared in a farinograph, using 300 g flour, 2% salt and water (according to farinograph water absorption for each formula) addition and mixed to peak time. Then dough was rolled to a thickness of 8 mm by using roller mechanism. 55mm circular pieces were cut from this sheets by using cookie cutter. Then circular pieces were pressed for 30 sec. The samples and apparatus surfaces were coated with paraffin oil to prevent moisture loss and dough surface drying. The five discs were compressed in turn, then stacked up in holders to prevent moisture loss and rested for 30 min at 25±5 C and tested at TA-XT2 texture analyser (Stable Micro Systems, England) in five replicates under the following conditions: pre-test speed 8.63 cm³/sec, test speed 26.70 cm³/sec, volume: 2.000.000 mm³, Trigger volume: 30.000 mm³. When the break or rupture of dough bubble was occurred the test stopped automatically. As a result, the following parameters were obtained: bi-axial extensibility, L (mm); tenacity, P (mm) and deformation energy, W (J) at bubble failure.

Statistical analysis

All measurements were performed three times. Statistical analyses were carried out SPSS 16.0 procedures using one-way analysis of variance (ANOVA). $P < 0.01$ was considered to be significant using Duncan's test.

RESULTS AND DISCUSSIONS

Some characteristics of rosehip seed flour

The results of the proximate analysis and colour values of the RSF were presented in Table 1. The ash and crude protein content of RSF were found higher while crude fat was found lower than the report of İlyasoğlu (2014) who was characterized the rosehip (*Rosa canina L.*) seed and seed oil.

Dietary fibers, were the most abundant macronutrients with 79.4 %. This value is higher than the dietary fiber contents of grape seed flour (Gül et al., 2013), *Moringa oleifera* seeds (Anudeep et al., 2016) and soybean seeds and its by-product okara (Cuenca et al., 2008). Dietary fiber is currently considered a critical ingredient in food products because of its health benefits, among them promoting healthier bowel function, decreasing cholesterol levels in the body, controlling blood glucose levels, preventing constipation, lowering the risk of obesity, certain kinds of cancer and heart disease (Kurek and Wyrwicz, 2015). Thus the RSF used in present study with higher dietary fiber content can be used as an ingredient with specific functions in food production.

Table 1. Proximate composition and colour values of the RSF

Constituent	Value
Moisture %	6.3 ± 0.05
Ash %	2.3 ± 0.01
Crude protein	7.4 ± 0.58
Crude fat	4.6 ± 0.36
Total dietary fiber	79.4 ± 0.42
Colours	
L	65.2 ± 0.01
a	6.8 ± 0.02
b	14.4 ± 0.01

Total Phenolic Compounds and Antioxidant Activity of RSF

Total phenolic content and antiradical activity of RSF was determined as 97.60 g/kg GAE, and 759.48 IC₅₀=µg/mL respectively. This is rather higher than the findings of İlyasoğlu

(2014), who reported that the total phenolics of *Rosa canina L.* seeds as 2554 ± 15 µg/g seed and seed oils as 215.4 ± 28.0 µg/g seed. However, the rosehip seed had a higher content of TPC than the four different rosehip species (*Rosa L.*), growing in the East Anatolia region of Turkey (Murathan et al., 2016b). The differences might be explained by different growing conditions, genotypes, and extraction conditions. The data suggested that these rosehip seeds might potentially serve as natural sources for dietary phenolic compounds.

The antioxidant activity of the studied seed was lower than the value of petals, fertilized flowers, ripening, and overripe hips, except unripe hips, of *Rosa micrantha* species reported by Quimaraes et al. 2010 and rosehip (*Rosa canina L.*) reported by Strugala et al., (2016). The TEAC (TEAC stands for Trolox Equivalent Antioxidant Capacity) values of the rosehip seeds were found by İlyasoğlu, (2014) to range from 7.29 to 10.71 to µmol TE/g for ethanol and acetone extracts respectively.

Effects of farinograph parameters

The effects of increasing levels (0, 5, 7.5 and 10%) of RSF on wheat flour dough properties is shown in Table 2. There were significant differences ($P < 0.01$) in water absorption, development time, stability and softening degree between the control flour (no PSF) and the flour groups with added PSF. With PSF addition, the percentage of water absorption decreased by about one percent. When the PSF percentage increased did not affect this value. By replacing gluten containing wheat flour with different ratios of RSF, the interaction between water and fiber in the dough was restricted.

Stronger flour typically has longer development time and smaller softening degree. But PSF which have a significant level of dietary fiber has caused the weakening of the dough. Fiber in RSF fortified doughs competed for water and delayed gluten development during mixing. Thus a decrease at development times and softening degrees of RSF containing doughs were observed.

Dough stability, was found to increase with the addition of the RSF. These results are in agreement studies on brewer's spent grain and apple pomace (Ktenioudaki, 2013).

Table 2. The effect of rosehip seed flour on the farinograph properties of dough

Sampl es	Level of RSF (%)	Water absorption (n)	Development time (min)	Stability (min)	Softening degree (12 min)
Control	0	58.7a (1)	2.2a	7.9d	73.0a
RSF	5	57.5b	1.9b	8.3c	63.0b
	7.5	57.4b	1.9b	8.5b	63.0b
	10	57.4b	1.7c	9.0a	57.0c

⁽¹⁾=Mean values in same column followed by different letters are significantly different ($P < 0.01$).

Effects of Kieffer dough and gluten extensibility rig parameters

Table 3 listed the resistance to extension (R value), extensibility (E value) and area (A value) of doughs enriched with various substitution levels of RSF. There was no significant difference observed as far as R and E value of control dough and 5% RSF added dough were concerned. The R value of the dough was increased as the substitution level increased over 5%. Whereas the E value was shown opposite effect. Hence, the addition of excessive RSF (more than 5%) resulted in a stiffer and less extensible dough.

However A value was higher in the 10% RSF enriched dough than in the dough enriched with 5% and 7.5 RSF%. The effect of RSF on the rheological properties of dough, less flexible and more rigid, was consistent with previous research using different dietary fiber sources such as Malva aegyptiaca L. leaves powder (Fakhfakh et al., 2017), brewer's spent grain and apple pomace (Ktenioudaki, 2013).

Table 3. The effect of rosehip seed flour on the Kieffer dough and gluten extensibility values of dough

Samples	Level of RSF (%)	Resistance to extension, R (g)	Extensibility, E (mm)	Area (g.s)
Control	0	19.7 ^c	22.6 ^a	239.5 ^a
RSF	5	19.3 ^c	22.1 ^a	218.1 ^c
	7.5	24.8 ^b	20.1 ^b	218.7 ^c
	10	30.1 ^a	16.9 ^c	230.5 ^b

⁽¹⁾=Mean values in same column followed by different letters are significantly different ($P < 0.01$)

Effects of Dobrasczyk/Roberts dough inflation system parameters

Figure 1 showed that a partial substitution of wheat flour by the RSF on the rheological

characteristics of the dough (P, L and W). Biaxial extensibility was measured by Dobrasczyk/Roberts dough inflation system which determines dough rheology under conditions of strain similar to those of baking expansion (Dobrasczyk, 1997; Dobrasczyk and Morgenstern, 2003) was significantly affected from the addition of RSF in to bread wheat flour.

When the substitution level increased, the tenacity of the dough (P) was significantly ($p < 0.01$) higher from 90.5 mm (control sample) to 206.4 mm and 222.9 mm at 5% and 10% substitution level respectively. However, a significant ($p < 0.01$) decrease in the extensibility (L) was observed.

By comparing the control L values they decreased from 32.10 mm to 21.6 mm, 20.5 mm and 15.7 mm for 5, 7.5 and 10% addition levels of RSF respectively. W value was increased in doughs which contain RSF.

Higher W value was obtained at 7.5% RSF doughs with 176.3 J. But at 10% PSF W value began to decrease.

Although almost similar results (for P, L and W values) were obtained between 5% and 7.5% RSF addition level, the difference at the 10% additional level was even more pronounced.

The main lines of the results Kieffer dough and gluten extensibility rig and Dobrasczyk/Roberts inflation system are in agreement with each other. Rheological behaviour of doughs after addition of RSF were changed at same trend at both measurements.

Their results showed that by incorporating higher levels of RSF will increase the force and decrease the extensibility.

The effects observed in the reduction of the extensibility and increase of the resistance to extension of the dough is related to gluten network changes due to fiber rich RSF addition. Higher levels of addition of RSF in the flour caused more dilution of the gluten matrix, weakening its extensible characteristics. In terms of manufacturing of products, these findings mean lower retention of gas and lower volume and higher firmness.

The negative effect on the formation of the gluten network by excess amounts of fiber were also reported from Gül et al. (2009).

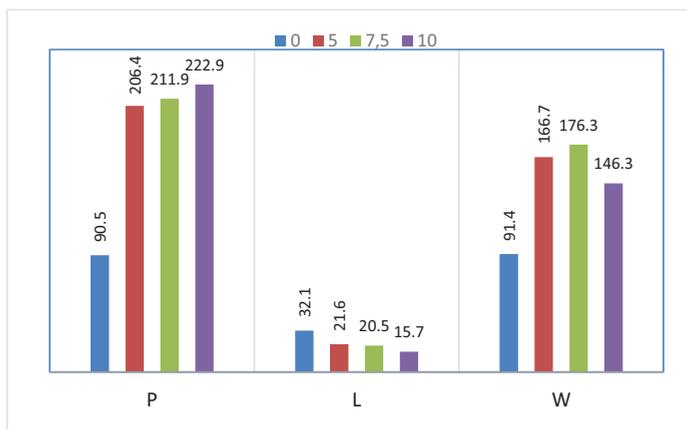


Figure 1. Dobraszcyk/Roberts dough inflation system values of doughs substituted with 0, 5, 7.5 and 10% PSF. P (mm): tenacity, L (mm) extensibility, W (J): deformation energy

CONCLUSIONS

In the preparation and processing procedures of rosehip to various food products can lead to discard its seeds. The findings of this study showed that rosehip seed was a valuable by product with its high concentration of dietary fiber, ash, protein, total phenolics and antioxidant activity. Antioxidative dietary fibers are important class of food ingredients that can be added to introduce extra health benefits to various bakery products.

Rheological properties of dough are closely related to quality of baked products because the changes in dough rheology during development affect both the incorporation of gas bubbles and their ability to hold gas and volume of loaf bread. The addition of RSF in dough formulations had varying effects on the dough properties. Incorporating RSF to wheat flour was lead to decrease of water absorption, development time, softening degree, extensibility and area of Kieffer measurement, while it was lead to increase stability, resistance to extension, tenacity and deformation energy of dough. Marked weakening was noted at higher levels (7.5% and 10%) of supplementation as compared with control dough.

Based on all results, substitution at 5% of RSF gives rheological parameter values at least as good as the control sample. On the other hand if producers want to produce non aerated bakery products at which no need to higher volumes, RSF can be used much more concentrations than 5%. As a result; we can

suggested, that rosehip seeds could be used as a food ingredient due to their high nutritional content and functional abilities.

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THE INFLUENCE OF ROSEHIP SEED FLOUR ON BREAD QUALITY

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Abstract

Effects of dried and ground rosehip seed flour (RSF) on bread quality and in particular on loaf volume, length, height, crumb and crust colour, texture and sensory properties were determined. RSF was added in levels of 0, 5, 7.5 and 10% to wheat flour sample. There was a considerable decrease detected at both loaf volume and loaf height whereas a sharp increase was measured at firmness and chewiness of breads as the concentration of RSF were increased. Brightness (L value) of crumb went down while redness (a value) went up as the ratio of RSF in bread formulation was increased. When 10% RSF was added total dietary fiber content of breads were doubled as compared to control. Sensory evaluation by a consumer panel resulted in a less rated scores at loaf volume, shape-symmetry, colour and structure of crust, crumb colour, grain structure, flavour, taste and aroma of RSF added breads than control. However all these parameters were affected significantly by the level of RSF. Beside general sensory attributes, acceptability and purchasing intent of 5% RSF containing breads were found as near as the control breads. According to the obtained results, RSF can be used up to 5% level without negatively affecting the technological and sensory quality of breads. Also it can be used above 10% to enhance the dietary fiber content of breads but in this case further studies should be needed to improve technological quality of breads.

Key words: rosehip, bread, texture, sensory, acceptability, purchasing intent.

INTRODUCTION

Rosehip fruits from the family of *Rosaceae* are an important source of proteins, carbohydrates, energy, sugars, particularly the reducing sugars, ascorbic acid, antioxidants, carotenoid pigments, minerals, organic and fatty acids (Demir and Özcan, 2001; Böhm, et al., 2003; Ercisli, 2007; Guimaraes, et al., 2010; Murathan et al., 2016). Rose hips are well known for their efficiency in strengthening the body's defence against infection, and particularly the common cold (Demir and Özcan, 2001).

During the last years, the use of rosehip fruits as raw materials for a lot of products such as jams, juices, etc. has started. During production of some rosehip products, seeds are discharged and they generally used as animal feed. Whereas seeds are also have high nutritional and bioactive compounds.

İlyasoğlu (2014) reported that the rosehip seed contains valuable phytochemicals such as phenolic compounds, carotenoids, ascorbic acid, polyunsaturated fatty acids, antioxidant activity. Beside rosehip seeds are important

source of dietary fibers (Gül and Şen, 2017). High fiber intakes are associated with lower serum cholesterol concentrations, lower risk of coronary heart disease, reduced blood pressure, enhanced weight control, better glycemic control, reduced risk of certain forms of cancer, and improved gastrointestinal function (Anderson et al., 1994).

High fiber breads can be produced by incorporating nutritional and functional rosehip seeds.

Consumption may be increased by giving above mentioned information about the health benefits of these fibre enriched breads (Gül and Gül, 2011).

There is a great interest in utilising the valuable by products of food industry as functional food ingredients instead of discarding them. The present study was thus undertaken with an objective of utilizing rosehip seeds for human consumption as a source of functional ingredient in breads.

This paper reports the effects of powdered rosehip seed on the physical, technological, textural and sensory properties of bread enriched with rosehip seed flour.

MATERIALS AND METHODS

Materials

Commercial bread wheat flour was supplied from Berberoğlu Milling Factory (Burdur, Turkey). Farinographic properties of the flour were 58.7% of water absorption, 7.9 min stability and 2.2 min of dough development time. The proximate compositions of wheat flour, analysed by the AACC (2001) methods, were 14.5±0.01% moisture, 0.61±0.03% ash, 10.47±0.03% protein, 29.9±0.12 % wet gluten, 96.2±0.20 % gluten index, sedimentation 31±1 ml and falling number 362±2 s. Salt and bread yeast were provided from the local market. Other chemicals were purchased from Merck (Darmstadt, Germany) and were of analytical grade.

Preparation of rosehip seed flour

Rosehip seeds were provided by manual separation of the seeds from pomace which was the by product of rosehip marmalade plant. They were dried in a cabinet drier at 55±2C before grinding the dried samples with a grinder mill and sieved to obtain a flour particle size of less than 300 µm.

Bread making with RSF substitution

Bread was prepared by AACC Method 10-10.03 (AACC, 2001) with some modifications. RSF was replaced the bread wheat flour at 0, %, 5%, 7.5% and 10% (w/w) levels. The other ingredients were yeast (3 g/100 g), salt (1.5 g/100 g) and water (variable depending on the farinograph absorption, it was determined in our previous research (Gül and Şen, 2017).

Dough was optimally mixed until dough development by a mixer (Günsa, Industrial Kitchen Equipment, İzmir, Turkey), rested for 30 min at 25±2C and 75±5% relative humidity. After first fermentation dough was scaled into pieces according to 100 g flour weight basis, hand-rounded, molded and placed into baking pans for the second fermentation at 25±2C and 75±5% relative humidity for 90 min.

Baking was carried out at 275±2C for 15 min in a stone flour electrical oven (Enkomak, Antalya, Turkey). Breads were cooled at room temperature, and packed in plastic bags until further analysis.

Evaluation of bread quality

Volume of the bread was determined one hour after the end of baking process by the method of displacement of rapeseed (AACC, 2001). Width, length and height of breads from each batch were measured by digital calliper.

Crumb colour of breads

Colours of crumb were measured with a colorimeter (Minolta CR-300, Minolta Co Ltd., Tokyo, Japan). Minolta L indicates brightness, -a to +a indicates green to red, and -b to +b indicates blue to yellow. Each loaf of bread was cut in to slices, each of 2.00 cm in thickness. Five readings were taken from the middle of each crumb for colour measurement. Average of five measurements for L, a and b values were recorded.

Total dietary fiber content of breads

Total dietary fiber content of RSF and breads were measured by AACC Method 32-05.01 (AACC, 2001). Total dietary fiber assay kit was purchased from Megazyme Company (Wicklow, Ireland).

Textural profile analysis (TPA) of breads

The TPA test consists of compressing a 25 mm thickness bread slice two times in a reciprocating motion that imitates the action of jaw. After cooling for 4 h breads were cut in to slices of 25 mm thickness with a bread knife. The central two slices were used to perform textural analysis on a texture analyser (TA-XT2, Stable Micro Systems, Surrey, UK) equipped with a cylindrical probe of 36 mm in diameter. The bread slice was placed on the heavy duty platform and the pre test speed: 1 mm/s, test speed: 1.7 mm/s, post test speed: 10 mm/s and strain: 40% were achieved. Based on the force deformation curves, parameters like hardness, adhesiveness, springiness, and cohesiveness and chewiness values were calculated.

Bread sensorial evaluation

For determining consumer acceptability of the breads, the loaves were evaluated for appearance characteristics (loaf volume, symmetry, crust colour and crust structure), internal properties (colour of crumb, grain structure, texture), taste, flavour and overall quality

characteristics. The 15 trained panellists were members of the Department of Food Engineering at Süleyman Demirel University in Turkey, ranging in age from 25 to 40, with 7 being female, non-smokers. Panellists were asked to evaluate the above attributes of the samples and to rate each attribute on a scale from 1 (dislike extremely) to 5 (like extremely) using five point hedonic scale (Meilgaard et al., 1999).

Statistical analysis

All measurements were carried out in three replicates. Analysis of variance (ANOVA) was conducted by using the SPSS 16.0 General Linear Model procedure. The calculated mean values were compared using Duncan's multiple range test with significance defined at $P < 0.01$.

RESULTS AND DISCUSSIONS

Effect of RSF substitution on loaf volume, height, width and length values of the breads

Loaf volume, height, width and length values of bread samples are presented in Table 1. The loaf volume of the breads prepared from RSF showed lesser volume in comparison to the control (100% wheat flour bread). Compared to the 0 % RSF control, the replacement of 5, 7.5

and 10 % RSF resulted in 22, 25 and 28 % reduced volume, respectively. Similar results were obtained by Boubaker et al. (2016) and Wu and Shiao (2015) they revealed that the reduction of the bread volume as consequence addition of fiber concentrate from artichoke stem by-products and pine apple peel fiber, respectively. Gül et al. (2009) also was emphasized that addition of wheat bran or corn bran into wheat flour was led to progressive decrease of loaf volumes of fibre enriched breads. A possible reason is that probably due to the dilution of gluten and physicochemical reactions among fiber components, water and gluten (Kurek et al., 2016). There is a confirmed competition for water between gluten proteins and fiber polysaccharides that part of the water molecules interact with the fiber polysaccharides rather than protein molecules (Nawrocka et al., 2017).

Regarding height of breads, as in the bread volume, Table 1 was showed a significant decrease of this parameter as consequence of increasing levels of RSF addition. A slight decrease were found between RSF added breads and control breads. However, length was not significantly different ($P < 0.01$) from control and enriched breads (Table 1).

Table 1. The effect of rosehip seed flour on the physical parameters and total dietary fiber content of the breads

Samples	Substitution level of RSF (%)	Loaf volume (cm ³ /100g)	Height (mm)	Width (mm)	Length (mm)	L	a	b	Total dietary fiber (%)
Control	0	561.7a*	66.6a	75.9a	131.4a	65.60a	-0.23a	7.53c	5.43c
RSF	5	434.2b	59.3b	72.9b	131.3a	56.07b	2.46c	7.75b	9.17b
	7.5	420.6c	57.7c	73.2b	131.0a	53.91bc	3.40b	8.53ab	9.40b
	10	404.4d	55.0d	73.1b	131.3a	51.68d	4.06a	8.84a	10.87c

Values in the same column with different superscripts are significantly different ($P < 0.01$). RSF: Rosehip seed flour.

Effect of RSF substitution on crumb colour of the breads

The effects of RSF addition on the bread colour are summarized in Table 1. The crumb colour of RSF-enriched breads were different from that of the control. Incorporation of RSF decreased the lightness (L) value and increased the a and b value of breads parallel to increasing levels of RSF as compared with the control breads. Darker colour of RSF enriched breads could be due to the original colour of rosehip seed fiber. However, as mentioned by Gomez et al. (2003) the crumb bread colour is

usually similar to the colour of the ingredients because the crumb does not reach as high temperatures as the crust. Therefore, it is reasonable to perform measurements only for crumbs, the colour of which directly correlates with ingredients used in the dough production. Colour change of the breads with high content of dietary fiber is mainly associated to Maillard and caramelization reactions (Kurek et al., 2016). Wu and Shiao (2015) reported that baked bread with increasing (0–15%) pineapple peel fiber substitution had darker, redder and less yellow colour than control bread. Fakhfakh

et al. (2017) verified that the addition of mallow powder produced breads with darker colour than the control bread, similar to what was found in the present study.

Effect of RSF substitution on total dietary fiber content of the breads

The total dietary fiber content (TDF) of the breads supplemented with different percentages of RSF is presented in Table 1. As expected, the incorporation of RSF to the formulation, gradually and significantly increased TDF content compared to the control sample. At the 10% RSF substitution level, TDF content of breads were increased to double (from 5.43% to 10.87%) of the content of control bread. Significant increase TDF content in wheat bread rolls with 0, 4, 8, 12, 16 and 20 % of flour replaced with oat fiber powder was also reported by Kurek et al. (2016). Roth et al. (2016) was stated that the 10 % of dried distiller's grains, by-product from ethanol production, can provide a valuable amount of dietary fiber to bakery products.

A claim that a food is a "source of fiber", and any claim likely to have the same meaning for the consumer, may only be made where the product contains at least 3 g of fiber per 100 g or a claim that a food is "high in fiber", and any claim likely to have the same meaning for the consumer, may only be made where the product contains at least 6 g of fiber per 100 g (Eur-lex, 2006). Therefore breads containing RSF at all substitution levels can be labelled "high in fiber".

Effect of RSF substitution on texture profile of the breads

The effect of RSF on bread texture was shown at Table 2. It can be seen that the addition of RSF resulted in significant ($p < 0.01$) increased hardness as compared to control bread. The hardness value of the breads were gradually increased from 2190 g to 3998.8 g when RSF was elevated from 0 to 10%. Chewiness of breads also increased significantly with RSF addition, but there was no difference between chewiness of 7.5% and 10% RSF containing breads. Our results coincides with the findings

of Frutos et al. (2008) and Boz (2015) they observed increase in bread hardness and chewiness with substitution of artichoke fiber and whole barley flour, respectively.

The aggregation of the gluten proteins, observed as a result of the fiber supplementation, is probably connected with partial dehydration of the gluten network. After fiber addition competition for water starts among gluten proteins and fiber polysaccharides. Thus part of the the water molecules interact with the fiber polysaccharides rather than protein molecules. The rest of the water molecules form strong hydrogen bonds with the gluten proteins which resulted in more firm and stiff dough (as shown in our previous research) and breads with lower volume and higher firmness (Nawrocka, 2017).

Adhesiveness is a measure of the tendency to adhere to contacting surfaces (especially the palate, teeth, tongue) during mastication (Chen, 2007). It is worthwhile to note here that adhesiveness was significantly influenced both by the addition of RSF and its increasing percentage. The adhesiveness was decreased with increase in RSF level, and the extent of decrease was greater for bread containing higher amount of RSF. Addition of fiber increased the stiffness of the dough and bread thus adhesiveness value gradually decreased from -5.14 g.sec to -26.87 g. sec on addition of increasing amounts (0–10%) of RSF.

No statistically significant differences were noted in the springiness of the control and RSF breads. An addition of 5% RSF in the bread formulations was not lead to any significant changes in the crumb cohesiveness and gumminess (Table 2). There was, however, a slight decrease in crumb cohesiveness between bread made with 7.5 and 10% RSF and the control. On the other hand gumminess of breads were significantly increased over 5% of RSF. The highest gumminess value (2201.6) was obtained at levels of 10% RSF. The results for the 5% of RSF added were coincident with those of Frutos et al. (2008) that did not found significant differences for the cohesiveness in breads supplemented with a 3%, 6% and 9% artichoke fiber.

Table 2. The effect of rosehip seed flour on the texture profile parameters of the breads*

Samples	Substitution level of RSF (%)	Hardness (g)	Adhesiveness (g.sec)	Springiness	Cohesiveness	Gumminess	Chewiness
Control	0	2190.9d	-5.14d	0.97a	0.65a	1533.5b	1386.3c
RSF	5	2495.4c	-8.9c	0.98a	0.61a	1541.2b	1596.5b
	7.5	3645.7b	-16.91b	0.89a	0.55b	2195.9a	1894.4a
	10	3998.8a	-26.87a	0.96a	0.53b	2201.6a	1860.8a

Values in the same column with different superscripts are significantly different ($P < 0.01$). RSF: Rosehip seed flour

Effect of RSF substitution on sensory parameters

Taste panel tests and consumers' questionnaire are the most commonly used for consumers' preference of a bakery products. Sensory analysis was carried out by checking loaf volume, crust colour, crust appearance, crumb colour, chewiness, taste and aroma of fresh prepared breads. The current study highlighted significant effect ($p < 0.01$) of RSF supplementation on different sensory attributes (Figure 1). There was a relationship between sensory and instrumental measurements. Loaf volume values, given by the panel to the control and RSF substituted breads were correlated with the values found for the instrumental volume measurements (Table 1), with decreasing values for loaf volume, as the proportion of PSF was increased in the formulation.

The colour of bread crust is an important parameter to determine its acceptability. Supplementation of wheat bread with RSF decreased both crust colour and crust appearance scores. Crust colour and crust appearance of 5% RSF added breads was found slightly lesser than the control, whereas their scores were decreased significantly ($p < 0.01$) over 5% replacement level of RSF.

The highest chewiness scores were observed in control and then they decreased when the incorporation of PSF increased. Likewise, the taste and aroma results also showed that there was a decreasing trend of the averages of scores when the incorporation of PSF increased (Figure 1). These results are in agreement with the findings of Peighambardoust and Aghamirzaei (2014). Chewiness was correlated with the values found for the instrumental texture determinations (Table 2), with increasing values for hardness, chewiness, and gumminess decreasing for cohesiveness and adhesiveness, as the level of PSF was increased in the breads.

Breads prepared from blends with 5% RSF were judged better than those prepared from other blends in respect of all tested sensory parameters. Reduced acceptability of breads prepared from blends in comparison to control can be attributed to the familiarity of panellists with the former. The scores obtained for the sensory parameters were similar to those obtained by Feili et al. (2013) in breads formulated with jackfruit rind flour which added to bread formula at 5%, 10% and 15% levels.

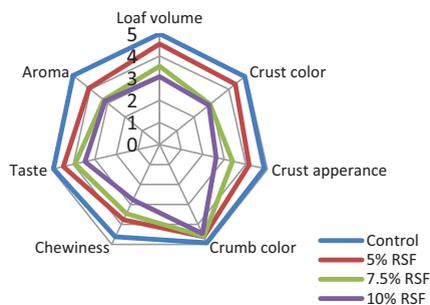


Figure. 1. Sensory evaluation of breads prepared with wheat flour fortified with 5, 7.5 and 10% RSF. The control represented the product without enrichment

Effect of RSF substitution on overall acceptability and purchasing intent

Overall acceptability and purchasing intent scores of the control and RSF enriched breads were presented in Figure 2. The results revealed that the scores for both of these two parameters were decreased significantly as the replacement level increased from 0 to 10%. At the higher levels, the acceptability and purchasing intent decline mainly due to the lower loaf volume, harder texture and lesser chewiness of the breads. The addition of a 5% of RSF to bread did not affect the acceptability of the bread in a great extent. However, in case

of purchasing intent, the decrease was significant by the addition of RSF but there was no great difference between the purchasing intent of 5% and 7.5% RSF containing breads.

Results showed that increasing RSF in the bread samples decreased the overall acceptability and purchasing intent scores especially at addition levels of 7.5% and 10% (Figure 2). Therefore, we suggest that the fiber-enriched bread can be prepared with 5% RSF in order to increase functional properties of bread and maintain the sensory acceptability for the consumers. A survey of the literature showed that bread enrichment up to 5% jackfruit rind flour (Feili et al., 2013), 3% lemon fiber (Chang et al., 2015) and 3% of mallow powder (Fakhfakh et al., 2017), gave satisfactory consumer acceptability.

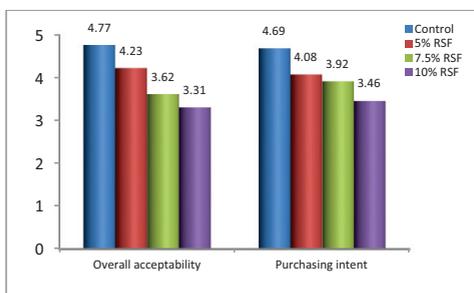


Figure 2. Overall acceptability and purchasing intent of breads prepared with wheat flour fortified with 5, 7.5 and 10% RSF. The control represented the product without enrichment.

CONCLUSIONS

We have shown that bread parameters such as loaf volume, loaf height, hardness, colour and chewiness were affected by the incorporation of rosehip seed flour, especially over 5% addition, whereas the dietary fiber content was increased. A high dietary fiber content in bread is a very important characteristic for consumer who demand bread with high functional properties and high nutritional content.

The sensory quality, overall acceptability and purchasing intent of bread with 5% PSF was almost similar to that of the control bread. These results are important technologically to improve new bread formulations. Therefore, rosehip seeds (a valuable by product of the

rosehip processing industries) may be used as a nutritional, healthy, functional, economical and novel ingredient in high fiber bread formulations to produce a bread of acceptable baking properties. Beside these due to very low price of rosehip seeds those findings can be considered significant from industrial and economic points of view.

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HPTLC IDENTIFICATION OF BIOACTIVE COMPOUNDS AND ANTIOXIDANT ACTIVITY OF *Pleurotus ostreatus* AND *Lentinus edodes* EXTRACTS

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Abstract

Because of the special flavour and of the therapeutic properties, *Pleurotus ostreatus* and *Lentinus edodes* are among the most cultivated and consumed mushrooms species. This work aimed to evaluate the antioxidant properties and free radical scavenger activities of *Pleurotus ostreatus* and *Lentinus edodes* methanol extracts by phosphomolibdenum and DPPH- assays respectively, total phenolic content (Folin – Ciocalteu) and identification of bioactive compounds by HPTLC (high-performance thin layer chromatography). The obtained fingerprints of extracts, in tree systems (for phenols, coumarins and triterpenoid saponins) have shown the presence of ferulic acid as main compounds in *Pleurotus ostreatus* extract and ergosterol (provitamin D2) in both of them. Total phenol content was 2.03 g/100g for *Lentinus edodes* extract and 1.24 g/100g for *Pleurotus ostreatus* extract. Radical scavenger activity and total antioxidant capacity, was higher for *Lentinus edodes* extract (90.14%, 203.05 mg AA) in comparison with the results obtained for *Pleurotus ostreatus* extract (85.32%, 168.34mg AA). The mushrooms examined in this work could represent important and accessible sources of natural antioxidants for food, food supplement and cosmetic industry.

Key words: mushrooms, *Pleurotus ostreatus*, *Lentinus edodes*, HPTLC, antioxidant.

INTRODUCTION

The use of natural remedies is one of the most exciting areas of interest that is supported by a long history of traditional use and of scientific research results. Mushrooms are part of natural ecosystems having an important role in the health maintaining process. Also, for centuries, mushrooms are used in food industry and in traditional medicine. Edible and/or medicinal mushrooms are either collected from nature or cultivated. Mushrooms cultivation is important for nutritional security, for recycling solutions of agro-waste and also for providing quality controlled products. The nutritional and chemical composition of mushrooms is responsible for their therapeutic properties. *Pleurotus ostreatus* - oyster mushroom (*Pleurotaceae*) was cultivated for the first time during the World War I, as a subsistence measure (Eger et al., 1976). Now is the third most cultivated mushroom around the world, having a high nutritional value because of the

content in essential amino-acids as alanine, arginine, glutamine and glutamic acid, carbohydrates—specially glucans, water, proteins, vitamins B,C,D,K and minerals Ca, P, Fe, K, Mn, Cu, Zn, Mg and Se, ascorbic and folic acids (Fernandes et al., 2015; Fontes Vieira et al., 2013; Xia et al., 2011). The mushroom also, contain essentials unsaturated fatty acids palmitic, stearic, oleic and linoleic acids (Hadar and Cohen-Arzi 1986) and phenolic compounds as ferulic, p-coumaric, galic, gentisic and homogentisic acids (Palacios et al., 2011) and ergothioneine (Dubost et al. 2007; Bhattacharya et al., 2014). Correlated with the chemical composition, *Pleurotus ostreatus* extracts have antioxidant, immunomodulatory, anti-inflammatory properties and lowering cholesterol and triglycerides levels activity (Fengguo et al. 2011; Fontes Vieira et al, 2013; Facchini et al. 2014; Sanjana et al. 2013; Tong et al. 2009). *Lentinus edodes* (*Pleurotaceae*) has excellent nutritional values. The Japanese name of this

mushroom is Shiitake derived from the association with Shii tree (*Castanopsis cuspidate* Schottky) and – take the Japanese word for mushroom. Fructification body contains 88-92% water, proteins, lipids, carbohydrates, vitamins and minerals. Dry mushroom has higher nutritional values as of most of the vegetable consumed every day. Dry mushroom is rich in carbohydrates and protein, containing 58-60% carbohydrates, 20-30% protein (which are digested at a rate of 80-87%) 9-10% fibre, 3-4% fat and 4-5% ash (Rahman and Choudhury, 2012).

Shiitake is a considerable source of vitamins, particularly provitamin D₂ (ergosterol) - 325mg%, which under ultraviolet light (UV) and heat turn into calciferol (vitamin D₂), containing also B vitamins, thiamine (B₁), riboflavin (B₂), niacin (B₁₂) and pantothenic acid (Hobbs 1995; Przybylowicz et al., 1990; 199; Mizuno 1995, 11, 7-21; Hobbs 2000; Wasser and Weis, 1997). Minerals identified in dry mycelium are Fe, Mn, Ca, Mg, Cd, Cu, P, Zn, Ge, Br and Sr (Casaril et al., 2011). Shiitake contains water-soluble ((1-4)-, (1-6)- α -D-glucans) and antitumor polysaccharides (lentinan) and fatty acids (linoleic, palmitic, oleic, stearic, tetradecenoic and myristic acids). Being one of the most studied mushrooms, with a large utilization in traditional medicine shiitake has proved pharmacological effects as: immunomodulatory, anticarcinogenic, antitumor, cardiovascular, hepatoprotective, antiviral, antibacterial and antiparasitic (Wasser 2005).

This paper aims to present the HPTLC profiles of bioactive compounds – phenolic – of the methanolic extracts obtained from the two mushroom species *Pleurotus ostreatus* and *Lentinus edodes* cultivated in Romania, as well as total polyphenolic content and antioxidant activity by DPPH and phosphomolibdenum assays.

MATERIALS AND METHODS

Raw material – *Pleurotus ostreatus* (fruiting body) sample and *Lentinus edodes* sample (fruiting body) were obtained from local mushroom producers. Voucher specimens are deposited in INCDCF-ICCF Plant Material Storing Room.

Sample preparation: the samples were prepared by extraction with methanol - vegetal material/ solvent rate -1/10m/v for 1h at boiling temperature of the solvent. The solutions were filtered and frozen until analysis.

HPTLC Analysis - The analysis was made according to TLC Atlas - Plant Drug Analyses (Wagner and Bladt, 1996) for the determination of characteristic fingerprint for chemical compounds. 3-7 μ l of the samples and 1-5 μ l of references substances (10⁻³M ferulic acid T1, chlorogenic acid –T2 and 1mg/ml ergosterol T3-Sigma-Aldrich) were loaded as 10mm band length in the 20 x 10 Silica gel 60F254 TLC plate using Hamilton- Bonaduz, Schweiz syringe and CAMAG LINOMAT 5 instrument. Polyphenolic compounds: the mobile phases (A) consisted in 100:11:11:27 (v/v/v/v) ethyl acetate-acetic acid-formic acid-water and (B) consisted in 1:1 (v/v) toluene-diethyl ether. The TLC twin chamber was pre-saturated with mobile phase for 30 min at ~20°C. The plate was developed in the mobile phase up to 90mm. After development, plates were dried and derivatized in for A system in Natural Product followed by PEG4000 reagent and for B in KOH 10%. The fingerprints were evaluated at UV with a WinCats and VideoScan software. Triterpenoid saponins according to American Herbal Pharmacopoeia®, Reishi Mushroom, 2006: 3-12 μ l of the samples were loaded as 10 mm on band length in the 20 x 10 Silica gel 60F254 TLC. The mobile phase (C) consisted in 9:1 (v/v) dichloromethane: methanol. The plate was developed in the mobile phase up to 70mm. The plate was dried and derivatized in vanillin–sulphuric acid reagent. The fingerprint was evaluated in visible light.

Total phenol content - Total phenol content (TPC) determined according to Folin – Ciocalteu method (Ph Eur.6). Briefly, 1ml of the extract was transferred to a 25ml volumetric flask, 10ml of water and 1ml of Folin Ciocalteu reagent was added. The volume was made to 25ml with 5% sodium carbonate (w/v). The blend was left at room temperature for 30 minutes. Then the absorbance of the samples was read at 760nm with a UV/VIS spectrophotometer (Helios λ , Thermo Electron Corporation). Distilled water was used as a blank solution.

Free radical scavenging assay- was evaluated using the Sanchez-Moreno et al. (1998) assay. The extracts concentration were 0.1% in methanol. 50µl aliquots of the extract were mixed with 2950µl of the DPPH methanolic solution (0.0025g/l). The radical scavenging activity of the extracts against 2,2-diphenyl-1-picryl hydrazyl radical (Sigma-Aldrich) was determined by measuring UV absorbance at 517nm. A blank solution was prepared containing the same amount of methanol and DPPH and measured after standing at room temperature 30 minutes. The radical scavenging activity (RSA) was calculated using the following formula:

$$\% \text{ inhibition} = \{(AB - AA)/AB\} \times 100.$$

Where AB is the absorption of blank sample and AA is the absorption of tested extract solution.

Total antioxidant capacity (TAC) assay

Was assessed by phosphomolybdenum method, according to Prieto et al. 1999. To 0.3ml ethanolic solution of the sample (concentration 0.1mg/ml) was added 2.7ml of reagent solution (0.6M sulphuric acid, 28mM sodium molybdate, and 4mM ammonium phosphate). The mixtures were incubated at 95°C for 90

minutes. After cooling the samples to room temperature, their extinction was measured at 695nm with UV-VIS spectrophotometer. Ethanol was used as negative control. The antioxidant capacity was expressed as ascorbic acid equivalent (AA) to 1mg of active substance. The calibration curve is linear for ascorbic acid in the range of 0.001 to 1mg/ml, n= 6, r²=0.999.

RESULTS AND DISCUSSIONS

The HPTLC fingerprint profile is utilized as identification method of a species. This is a technique that can be use either in research or in production processes.

In figure 1, the polyphenolic fingerprint of *Pleurotus ostreatus* extract (Figure 1- Track 1) is characterized by two major blue fluorescent zones, one of them – ferulic acid (Rf~0.9) in system A. C system is characteristic for coumarins according to Wagner and Bladt, 1996. In the extract were identified ferulic acid (Rf~0.55) and coumarins aglycones as tree bright blue prominent fluorescent spots at Rf~0.1, 0.6 and 0.8.

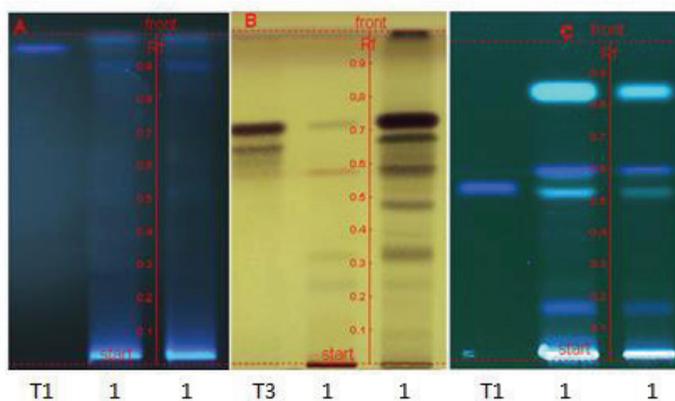


Figure 1. HPTLC fingerprint profile of *Pleurotus ostreatus* mushroom
1 - *Pleurotus ostreatus* extract; T1- ferulic acid (ref.); T3 - ergosterol (ref.)

System A - 100:11:11:27 (v/v/v/v) ethyl acetate-acetic acid-formic acid-water;

B - 1:1 (v/v) toluene-diethyl ether;

C - 9:1 (v/v) dichloromethane: methanol.

B system is used (American Herbal Pharmacopoeia®, Reishi Mushroom, 2006) for identification of triterpenoid saponins in *Ganoderma lucidum* mushroom. *Pleurotus*

ostreatus fingerprint is characterized by the presence of ergosterol (T3) and of four major purple - brown spots between Rf ~0.3-0.9.

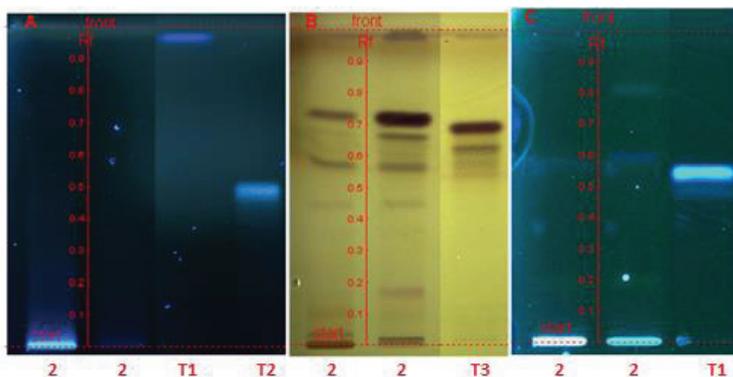


Figure 2. HPTLC fingerprint profile of *Lentinus edodes* mushroom
1 – *Lentinus edodes* extract; T1- ferulic acid (ref.); T2 –chlorogenic acid ; T3 - ergosterol (ref.)
 System A - 100:11:11:27 (v/v/v/v) ethyl acetate-acetic acid-formic acid-water;
 B - 1:1 (v/v) toluene-diethyl ether;
 C - 9:1 (v/v) dichloromethane: methanol.

In figure 2, in A system for *Lentinus edodes* extract there are not specific spots/zones for polyphenolic compounds. In C system, in UV-365 nm, two prominent blue fluorescent zones are found in the Rf ranges 0.6 and 0.8, specific to simple coumarins and one yellow-green zone (non-substituted coumarin) (Rf~0.2) with KOH reagent.

In C system ergosterol was identified at Rf~0.7. Also, there are three purple-brown zones at Rf ranges 0.2-0.9.

Total phenol content was determined from the extrapolation of the calibration curve ($y=0.0525x-0.020$, $R^2 = 0.992$), which was obtained for gallic acid reference substance (Sigma Chemical Co., St. Louis, USA). The results were expressed as grams of gallic acid equivalents (GAE) per 100g extract. The results obtained for methanolic extract mushrooms was 2.03 g/100g for *Lentinus edodes* extract and 1.24 g/100g for *Pleurotus ostreatus* extract. Redox properties of phenolic compounds allow them to act as antioxidants agents (Soobrattee et al., 2005). Determination of free radical scavenging activities by DPPH assay is routinely used for plant extracts (Aksoy Lacine, et al., 2013).

DPPH is a stable free radical, that accepts an electron or hydrogen radical to become a stable molecule.

For evaluation of total antioxidant capacity was used phosphormolybdenum method that is based on the reduction of Mo (VI) to Mo (V). This reduction is based on the antioxidant

compound and the formation of green phosphate /Mo (V) complex at acidic pH.

Table 1 presents the antioxidant activity of the extracts obtained by the two methods DPPH and TAC.

Table 1 Antioxidant activity

No	Extract	DPPH radical scavenging activity (%)	TAC (mg ascorbic acid equivalents)
1	<i>Pleurotus ostreatus</i>	85.32 ±0.35	168.34 ±0.84
2	<i>Lentinus edodes</i>	90.14±1.21	203.05±2.35

The results obtained in our research showed that *Lentinus edodes* methanolic extract have a higher content in TPC, comparative with *Pleurotus ostreatus* methanolic extract. Also, radical scavenging activity and TAC are in a dose- dependent manner with the total phenol content. Research results showed that polyphenols are responsible for the antioxidant activity of fruits, vegetables and mushrooms (Ferreira et al., 2007). Also, ergosterol compound identified in both extract was found to have antioxidant activity correlated with the content, in a study conducted by Shao S et al, 2010.

CONCLUSIONS

Mushrooms are important sources of antioxidant compounds that are capable of reducing the effects of free radicals. Our results show that *Lentinus edodes* and *Pleurotus*

ostreatus mushroom species could play a protective role in diseases related to oxidative stress and also can be important sources of phenolic acids and ergosterol.

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DRY AND FRESH HERBA OF *Satureja montana* L.: A COMPARATIVE STUDY REGARDING CHEMICAL COMPOSITION AND ANTIOXIDANT CAPACITY OF VOLATILE OILS

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Abstract

Mountain or winter savory *Satureja montana* L. is one of the most cultivated aromatic plant from *Lamiaceae* family. As most of the species from their genus, *Satureja montana* L. contains essential oils which differ in chemical composition as plants are from different origin or has been dried in different regime. This study has been shown that chemical composition and antioxidant capacity are different for dry and fresh herba and even has been varying if the herba was dried in air or in the oven. It has been demonstrated that the major compound, carvacrol, of volatile oils from dry herba was found to be in higher percent than in volatile oils extracted from fresh herba. In contrast, different sesquiterpenes concentrations were higher for fresh herba. The antioxidant capacity was two times bigger for the oil extracted from dry herba. This surprising feature could be explained by the higher carvacrol concentration and lower thymol concentration determined in volatile oil extracted from dry herba.

Key words: *Satureja montana* L., volatile oils, carvacrol, fresh and dry herba.

INTRODUCTION

Since ancient times, aromatic herbs and spices, rich in volatile oils, have been used as additives because of their ability to improve flavor and food-preservation properties. Today, they are not only valued for improvement of organoleptic properties, but also for their nutritive and medicinal benefits (Cavar et al., 2008; Vidovic et al., 2014) as well as for their potential in commercial exploitation in various fields such as aroma and flavor enhancers, cosmetics and pharmaceutical products (Mirjana and Nada, 2004).

One of the most important families of medicinal plants is *Lamiaceae*, which consists of over 3000 species (Vladic et al., 2016). The genus *Satureja* belongs to the *Lamiaceae* family, and comprises over 30 herbs, subshrubs, and shrubs (Cavar et al., 2008; Garcia-Rellan et al., 2015; Kremer et al., 2015).

Satureja montana L., commonly called winter or mountain savory, is a bushy perennial subshrub with woody stems at the base, small

linear leaves, pale pink and white flowers (Chizzola, 2003; Wesolowska et al., 2014). *Satureja* species are used widely as a flavoring agent of food products, and as traditional herbal medicine for the treatment of gastrointestinal disorders (antispasmodic and antidiarrhoeal) (Marin et al., 2012).

Because of its preference for dry climatic conditions, *S. montana* has developed several morphological and physiological adaptations, effecting oil yields and composition (Mirjana and Nada, 2004).

Most of the *Satureja* species contain essential oil in all their aerial botanical parts. The yield of volatile oils obtained by hydro-distillation of aerial parts of those plants species have been found between 0.3 - 5%. Volatile oils are rich in aromatic monoterpenes such as carvacrol, thymol, p-cymene, β -caryophyllene, γ -terpinene and linalool, which are responsible for the characteristic smell and taste (Chizzola, 2003; Ciulei, 1993; Jafari et al., 2016) and give the oil certain biological properties such as antibacterial, fungicidal, antiviral, antioxidant,

antispasmodic and antidiarrhoeal (Mirjana and Nada, 2004). Flavonoids, tannins, acids and exudates are other known compounds of *Satureja* species (Jafari et al., 2016).

The aim of this study was to determine the difference between chemical composition and antioxidant capacity of fresh and dry herba of *S. montana* L. harvested in 2016: summer (July) and late autumn (November).

MATERIALS AND METHODS

Plant material

Plants of *Satureja montana* L. was gathered in July and November 2016, from the Young Naturalists Station 21°13' E longitude, 45°45' N latitude, from Timisoara county.

The aerial parts harvested in July were left to air-dry in a well-ventilated room for at least 15 days before packaging in paper bags, and stored until extraction at room temperature.

Aerial parts harvested in November were divided in two parts: one was processed fresh and the other one was dried at 35°C for 7 days using a drying oven (Model FD23, Binder, Germany).

Essential oil extraction

For the oil extraction, dried leaves and flowers were subjected to hydro-distillation using a 1L Clevenger apparatus. The extraction yields have been 2% for dry leaves and 0.5% for fresh herba.

Gas Chromatography/Mass Spectrometry (GC/MS) analyses of essential oils

The separation and identification of different compounds had been done using a GC-MS system Shimadzu 2010 Plus gas chromatography apparatus (Shimadzu, Kyoto, Japan) and triple quadrupole mass spectrometer (TQ 8040). The column used was a capillary column 1MS Accent (30 m length; 0.25 mm i.d.; 0.25 µm film thickness, Macherey-Nagel, Duren, Germania) with helium as the carrier gas at 0.83 L min⁻¹. The oven temperature was programmed at 70 °C for 11 min, then 5 °C/min to 190 and 20 °C/min to 240 °C, and then left at

240 °C for 5 min. The injector temperature and MS source were maintained at a temperature of 250 °C and 200 °C, respectively. Identification of different compounds has been done based on their mass spectra using NIST 14 library and Willy 09 library. Retention indices (RI) were calculated for separate compounds relative to C4-C26 *n*-alkanes mixture.

Antioxidant assays

For this assay a spectrophotometric analysis was performed using as reference a relatively stable organic radical DPPH (2,2-diphenyl-1-picrylhydrazyl). This assay is based on the ability of the antioxidant to scavenge the radical cation DPPH. Results were expressed as Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC) using a Trolox calibration curve (Tuberoso et al., 2007).

Antioxidant capacity was determined for the essential oils. Different quantities of oils were mixed with 0.5 mL of 0.4 mM DPPH solution in methanol as presented in (Arsenijevic et al., 2016).

The spectrophotometric readings were carried out after a 1-hour period of incubation, in the dark, at room temperature, with a ScanDrop Nano-volume Spectrophotometer from AnalytikJena (Germany) at 517 nm using a 10 mm quartz cuvette. A Trolox calibration curve in the range 0.02 – 4.00 mM was prepared, and data were expressed in Trolox equivalent antioxidant capacity (TEAC, mmol/l).

RESULTS AND DISCUSSIONS

Essential oil composition

The essential oil extraction yields differ between fresh herba and dry herba. For fresh herba the yield has been 0.5%, while for dry herba has been 2%.

The chemical composition of volatile oils has been determined using gas-chromatography coupled with mass spectrometry techniques. The chromatogram for volatile oils extracted from dry herba is shown in Figure 1.

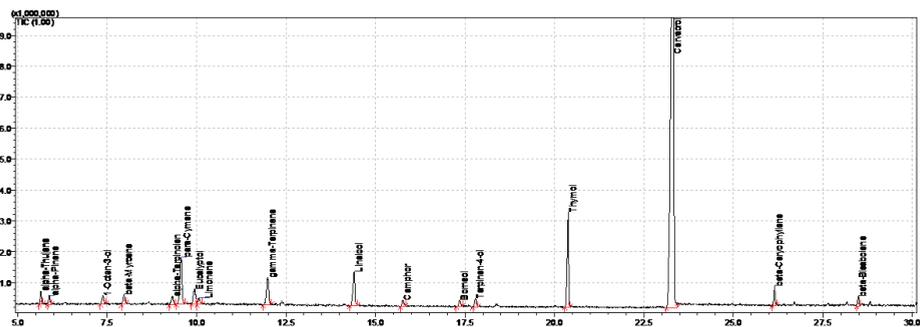


Figure 1. The composition of essential oil from dry herba of *Satureja montana* L.

It has been shown that in the composition of essential oils extracted from dry herba of *Satureja montana* L. the main component is carvacrol (an oxygenated phenolic monoterpenoid) at a level of 72% from all identified compounds (Table 1 for all chemical composition). Usually, the amount of carvacrol in *Satureja montana* L. essential oil varied from 15.19% to 69.99% (Mihajilov-Krstev et al., 2014; Mirjana and Nada, 2004; Skocibusic and Bezic, 2003; Wesolowska et al., 2014).

Table 1. Chemical composition of *Satureja montana* L. volatile oils

Compound	Retenti on time (s)	Dry herba	Dried in oven herba	Fresh herba
α -Thujene	5.666	0.8	1.32	1.58
α -Pinene	5.899	0.63	0.97	0.63
1-Octen-3-ol	7.391	0.71	1.42	0.78
β -Myrcene	7.994	0.84	0.61	0.94
α -Terpinolen	9.34	1.05	1.04	0.5
<i>para</i> -Cymene	9.6	5.12	15.17	10.57
Eucalyptol	9.923	1.72	0.44	0.04
Limonene	10.006	0.67	0.11	0.17
γ -Terpinene	12.016	3.03	1.68	2.19
Linalool	14.387	3.01	1.05	0.45
Camphor	15.726	0.62	0.07	0.16
Borneol	17.332	0.5	0.33	0.19
Terpinen-4-ol	17.795	0.81	1.13	0.94
Thymol	20.407	6.68	10.23	18.92
Carvacrol	23.286	71.95	61.87	59.46
β -Caryophyllene	26.163	1.24	2.27	2.12
β -Bisabolene	28.501	0.62	0.29	0.36

Such high content of carvacrol has been found in plants harvested in temperate climate. Interesting, the composition of carvacrol was higher in volatile oils obtained from dry herba

compared with that obtained from fresh or dried in the oven herba (Table 1). In contrast, other minor components as beta-caryophyllene, thujene or terpinen-4-ol were higher for fresh herba.

Furthermore, the concentration of thymol was 3 times higher in fresh herba than dry herba (Table 1, Figure 2).

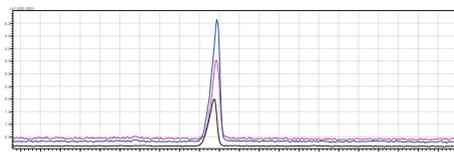


Figure 2. The chromatographic peak for Thymol from essential oils of dry (black), dry in the oven (pink) and fresh (blue) herba of *Satureja montana* L.

Antioxidant capacity

The antioxidant capacity of volatile oils extracted from different herba has been determined as Trolox equivalent antioxidant capacity (TEAC, mmol/l) and relative inhibition to sample without plant extract (Table 2).

Table 2. Antioxidant capacity of volatile oils extracted from *Satureja montana* L.

Antioxidant capacity	Dry herba	Dry herba in oven	Fresh herba
TEAC, mmol/l	0.65	0.78	1.27
% inhibition	18.18	21.82	35.27

All type of oils have quite high antioxidant capacity. The higher antioxidant capacity of all volatile oils has been determined for the oil extracted from dry herba. This surprising effect could be explained by higher concentration of carvacrol which has been shown to have such properties in other plants volatile oils (Ang et

al., 2015). The tymol concentration was lower for dry herba which could be another reason of a better antioxidant capacity exhibited by this oil.

CONCLUSIONS

The present research had shown the difference in chemical composition and antioxidant capacity of volatile oils extracted from dry and fresh herba. The chemical composition of volatile oils is similar but the concentration of the major compound, carvacrol, is higher in dry herba, which determine higher antioxidant capacity.

More studies are needed to understand if really just one of the compound contained in the essential oil is responsible for the antioxidant capacity and which other compound/s could prime or inhibit its activity.

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