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SUMMARY

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AGRICULTURAL BIOTECHNOLOGY

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THE GENETIC VARIABILITY EVALUATED WITH MOLECULAR MARKERS ON THE BILBERRY TISSUE LINES

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Abstract

The tissue lines derived from the Arieşeni, Retezat and Valea Sebeşului bilberry genotypes grown on a WPM medium supplemented with 40, 60 and 80 mg/l AS, were subject to molecular analysis through amplification of DNA samples with ISSR and RAPD primers. The results of agarose gel electrophoresis of the amplification products show the existence of significant differences in the model of polymorphic bands obtained from tissue lines, compared to the mother plant.
Based on the obtained results, the presence of somatic variability, induced in the tissue lines under the influence of AS, is emphasized. It consists in the absence of binding sites of the HB-12 ISSR marker compared to the mother plant. Considering the statements in the specialty literature, concerning the mutagenic effect of growth, we can state that AS caused changes within the DNA level in the bilberry calluses selected by us under the experimental conditions.

Keywords*: molecular analysis, bilberry tissue lines, variability.*

INTRODUCTION

Vaccinium myrtillus L. species provide raw materials to obtain a wide range of natural medicines (phytotherapeutic products), dietary supplements, natural dyes and preservatives. Worldwide there is a strong preference for the use of herbals detrimental drugs produced by chemical synthesis.
Techniques for in vitro cultivation of plant tissues and cells allow the obtaining and selection of proliferative tissue lines producing secondary metabolites. In order to establish a selection technology of cell lines producing biologically active substances (anthocyanins) by *in vitro* culture, cells response is tested on different tissue culture media with proper hormonal balance. The results demonstrate the appropriateness of such researches and open the possibility of developing a system for production of these secondary metabolites in greater quantities (Botau, 2009; Botău, 2009).
Analysis of variability induced by artificial culture of cells and tissues is quick and easy using DNA amplification techniques with different molecular markers. Koonjul et al. (1999), Iandolino et al. (2004), cited by Pop R. (2008) show that amplification of DNA fragments, especially RAPD method, is better by the inclusion of PVP in the mixture reaction of PCR. It will be appreciated that the polyphenols remaining in DNA template solution can inhibit DNA amplification, but by adding PVP, the polyphenols are absorbed.
In our experiments, we used two techniques based on PCR: RAPD and ISSR technics, in order to obtain a model of polymorphic DNA bands specific for the study of genetic variability in spontaneous blueberries tissue lines.

MATERIALS AND METHODS

Biologic material is represented by blueberry callus originated genotypes (local populations) Arieşeni, Valea Sebeşului and Retezat. Tissue lines (callus) with leaf and stem origin were subcultured on woody plant medium (WPM) supplemented with 40, 60 şi 80 mg/l AS and their growth was assessed by statistical calculation of variance (Ciulca, 2006).
Tissue lines were analyzed using molecular technics: DNA samples were amplified with ISSR primer HB 12 and RAPD one OPA 05,

chosen based on literature. Depending on the presence (1) or absence (0) of the polymorphic bands at blueberry tissue lines we appreciated their genetic variability compared with mother plant.

DNA isolation and purification was done using the modified CTAB method and the samples were amplified with the specific primers. For the amplification the Green Taq master mix (Promega) was used following the next programs: cycle profile for ISSR primer: 1 cycle at 94°C for 3 min followed by 45 cycles at 94 °C for 30 sec, 54°C for 45 sec and 72 °C for 2 min and for RAPD: 1 cycle at 95°C for 5 min followed by 45 cycles at 95 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min.

The mention DNA extraction method was selected, even if it is a very complex one, because it was very important to remove the polysaccharides, the polyphenols and other components which interfered with the amplification reactions. Besides, 1% Polyvinylpyrrolidone (PVP) was added in the amplification mixtures to improve the reactions.

The samples amplification was done in three repetitions for each primer. The same primers were used for the genotyping of the three blueberries ecotypes, obtaining the same fingerprint each time.

RESULTS AND DISCUTIONS

1. **Results regarding the callus growth in subculture**

The results presented in table 1 show that the Valea Sebeşului population registered the highest values of callus growth in subculture on WPM medium supplemented with 60 mg/l AS, with a mean of 2.83 g, followed by population Arieşeni on the same hormonal balance, with values of 2.78 g for the stem callus.

The Retezat population shows a mean value of 2.70 g callus weight.

Considering the combined effect of the three factors (table 1) on blueberry callus grown in subculture, at Arieşeni population it was observed an amplitude of variation of 0.57 g with 2.21 g boundaries for leaf callus under the influence of hormonal balance 1.5 mg / l NAA 1.5 mg / l BAP + 40 mg / l AS and 2,78 g boundaries for stem callus subcultured on medium WPM supplemented with 1,5 mg/l ANA + 1,5 mg/l BAP + 60 mg/l AS.

Table 1. *The effect of the genotype, callus origin and AS concentration on the growth of bilberry callus subcultured on medium WPM (1,5 mg/l ANA+1,5 mg/l BAP)*

Genotype	**Arieşeni**	
Concentration (AS)	Callus origin	
	Leaf	**Stem**
40 AS	y2,21 b	x2,54 b
60 AS	y2,50 a	x2,78 a
80 AS	x2,26 b	x2,38 b
Genotype	**Retezat**	
Concentration (AS)	Callus origin	
	Leaf	**Stem**
40 AS	y1,92 b	x2,35 b
60 AS	y2,33 a	x2,70 a
80 AS	y2,20 ab	x2,51 ab
Genotype	**Valea Sebeşului**	
Concentration (AS)	Callus origin	
	Leaf	**Stem**
40 AS	y2,35 a	x2,62 a
60 AS	y2,56 a	x2,83 a
80 AS	x2,34 a	x2,34 b

$DL_{5\%}$=0,23g $DL_{1\%}$=0,31g $DL_{0,1\%}$=0,40g

The concentration of 60 mg/l AS positive significantly influenced the blueberry callus growth in subculture, regardless of its origin, with significant differences compared to the other AS concentrations tested. Under influence of this concentration (60 mg/l AS) were registered too significantly differences between calli with different origins, stem callus producing the best values.

Callus growth at Retezat population was strongly influenced by AS concentration and callus origin, there is significant differences between leaf and stem calli. Stem callus grew better under concentration 60 mg/lAS influence.

Valea Sebeşului blueberry population registered the best growth values in stem callus subculture under concentration of 60 mg/l AS influence, recording significant differences between 80 mg/l AS (where lower values were obtained) and the others two concentrations (40, 60 mg/l AS). The highest values were observed at variants with content of 60 mg/l AS by stem callus cultivation at populations: Valea Sebeşului (2,8 g), Arieşeni (2,7 g) and Retezat (2,6 g) (Figure. 1).

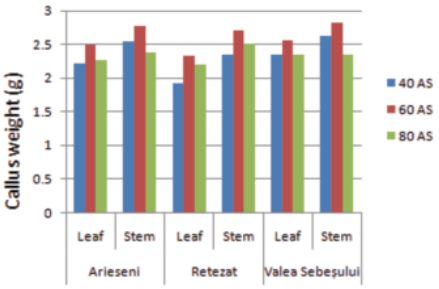

Figure 1. *The callus growth in subculture (30 days), on solid WPM medium.*

2. Results regarding the genetic variability on the tissue lines level evaluated with molecular markers

The tissue lines derived from the Arieşeni, Retezat and Valea Sebeşului genotypes grown on a media supplemented with 40, 60 and 80 mg/l AS, were subject to molecular analysis through amplification of DNA samples with the ISSR HB 12 primer.

The results of agarose gel electrophoresis of the amplification products showed the existence of significant differences in the model of polymorphic bands obtained from tissue lines, compared to the mother plant (Figure 2).

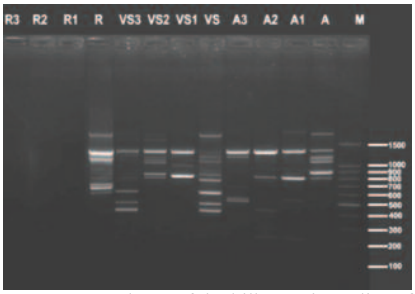

Figure 2. PCR analyses of the bilberry tissue lines from Arieşeni, Valea Sebeşului and Retezat populations with the ISSR marker HB12

M – molecular marker (100-1500 pb), A- mother plant Arieseni, A1 tissue line 40 mg/l AS, A2 tissue line 60 mg/l AS, A3 tissue line 80 mg/l AS, VS Mother plant Valea Sebesului, VS1 tissue line 40 mg/l AS, VS2 tissue line 60 mg/l AS, VS3 tissue line 80 mg/l AS, R- Mother plant Retezat, R1 tissue line 40 mg/l AS, R 2 tissue line 60 mg/l AS, R 3 tissue line 80 mg/l AS

A lack of bands can be observed, corresponding to DNA fragments with the length between 800 -1300bp and those with the length of 600-100 bp, at tissue lines grown in concentrations of 40 and 60 mg/l AS, from both the Arieseni population and the Valea Sebeşului population. The tissue lines grown in the presence of 80 mg/l AS have a pattern of bands different from the other lines and from the mother plants, at the Valea Sebeşului and Arieseni genotypes. The tissue lines derived from the Retezat genotype showed no pattern of polymorphic bands. The lack of bands suggests that the DNA lost the binding sites for the ISSR HB-12 marker.

Based on the obtained results, the presence of somatic variability, induced in the tissue lines under the influence of AS, is emphasized. It consists in the absence of binding sites of the HB-12 ISSR marker compared to the mother plant. Considering the statements in the literature, concerning the mutagenic effect of growth regulators (Marele, 2009; Vicas, 2009) we can state that AS caused changes within the DNA level in the bilberry calluses selected by us under the experimental conditions.

A total number of 6 tissue lines derived from the Retezat genotype, grown under the presence of three concentrations of AS (40, 60, 80 mg/l), were subject to molecular analysis using RAPD OPA 05 marker. The pattern of polymorphic bands (Figure 3.) indicates the existence of somatic variability at the level of tissue lines compared with the mother plant. It can be observed that at the same concentration of AS (40 mg/l), tissue lines are different: one reveals four bands and the other has none. At the concentration of 60 mg/l AS no polymorphic bands have been registered.

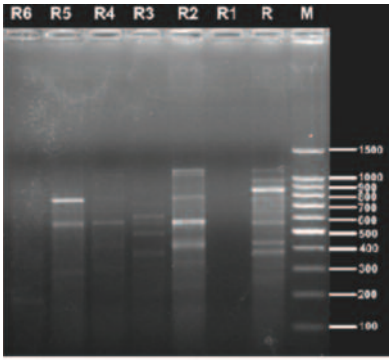

Figure 3. PCR analyses of the bilberry tissue lines from Retezat population with RAPD OPA 05 marker

M - Molecular marker (100-1500 pb), R- Mother plant Retezat, R1 tissue line 40 mg/l AS, R 2 tissue line 60 mg/l AS, R 3 tissue line 80 mg/l AS.

The tissue lines grown under the presence of 80 mg/l AS are also different: one has two bands

and the other has none. Unlike the mother plant, the lack of bands in some tissue lines indicates the lack of DNA binding sites for the marker OPA 05. These results demonstrate that AS cause changes at the DNA level in the selected tissue lines, cultivated *in vitro*.

CONCLUSIONS

To obtain an abundant growing callus tissue, at spontaneous bilberry, *we recommend* the utilization of Retezat, Arieşeni and Valea Sebeşului genotypes, from stem explants grown on solid WPM media, supplemented with a uniform ratio between auxinic ANA and BAP citokinine and a concentration of 60 mg /l AS.

The origin of callus influences the growth ability of callus in subculture. Stem callus grows better than the leaf callus, and there are significant differences between them.

The obtained results revealed a relatively high genetic polymorphism at the level of the studied spontaneous bilberry genotypes and good discriminatory power of the RAPD and ISSR techniques. These analyses can be used to determine genetic differences at spontaneous bilberry, allowing the determination of genetic differences between populations.

For carrying out the molecular analyses *we recommend* the utilization of the primers **HB-12, HB-15** and **UBC 818**, which revealed the highest rates of polymorphism (100%).

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DETECTION OF MOLECULAR POLYMORPHISM OF *PUCCINIA TRITICINA* FROM WHEAT IN ROMANIA

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Abstract

Leaf rust of wheat (Triticum aestivum L.) is caused by Puccinia triticina Ericks and is a common disease of this plant species in northwest, southern, southeast, and eastern Europe. It has been shown that the severity of leaf rust increased in the last years in many regions of Europe and highlighted the need for selection of wheat cultivars with race specific resistance genes. In order to select wheat resistant lines and develop efficient management programs for disease control, the characterization (phenotypic and molecular) of leaf rust races isolated from specific area is an important step of researches. To date, several molecular markers have been used to describe variation in P. triticina in Europe and worldwide.

The aim of this work was to study the molecular diversity of P.triticina collected from Romanian fields (Fundulea, Livada, Pitesti). Single-uredinial isolates were obtained from several populations of P.triticina and used for inoculation of detached wheat leaves cultivar F133. Genomic DNA was isolated from 20 fresh single-uredinial samples (2-10 mg spores) and from eight populations of P. triticina urediniospores (10-20 mg spores) using OmniPrep for Fungus kit. The molecular polymorphism was evaluated through RAPD (with eight decameric primers) and by using 15 SSR primers and two ISSR primers.

Results showed a reduced polymorphism at population level, but not identity, as indicated the RAPD analysis. No more than two alleles were detected in the leaf rust populations with the SSR markers used in experiments according to our separation protocol. Differences in the SSR loci were detected in the single-uredinial isolates. Similar results were obtained with ISSR primers: ISSR 17898A primer allowed the detection of the highest polymorphism among the individual isolates. Evidence for molecular variability between single urediniospores isolates will be correlated with the virulence phenotypic analysis in further studies.

Keywords*: Puccinia triticina, RAPD analysis, SSR markers*
*first and second authors have a similar contribution

INTRODUCTION

Puccinia triticina Eriks is the causal agent of wheat leaf rust, a disease that have a significant economic impact on wheat (*Triticum aestivum* L) production in Romania and worldwide. Intensive studies have been performed in order to evaluate the level of resistance in wheat cultivars and to select the most resistant ones, but the results showed that the resistance is correlated with the virulence phenotypes of the specific (local) fungal population (Kolmer et al., 2001). Nowadays, more than 70 leaf rust resistance genes in wheat have been analysed and their distribution in specific geographic areas is associated with the local *P. triticina* populations (Singh et al., 2013). The isolation and characterization of local fungal populations could offer information to understand the genetic mechanisms underlying variations in *P. triticina* and to development effective strategies to control the disease (Wang et al., 2010). Several methods have been used to identify the molecular markers in order to evaluate the genetic variations in *P. triticina* populations: amplified fragment length polymorphism - AFLP) (Kolmer et al., 2001), random amplified polymorphic DNA - RAPD (Kolmer et al., 1995; Kolmer et al., 2000) and simple sequence repeat SSR markers (Duan et al., 2003; Szabo et al., 2007; Mantovani et al., 2010; Visser et al., 2012). Most of the studies showed the genetic diversity in *P. triticina* populations from central Asia, North America and Europe

(Kolmer et al., 2000; Ordonez et al., 2007; Mantovani et al., 2010; Kolmer et al., 2011; Kolmer et al., 2012).

MATERIALS AND METHODS

1. ***P. triticina*** **isolates**: The eight *P. triticina* populations isolated from common wheat from Romanian fields (Fundulea, Livada, Albota Pitesti), as well as 20 single-uredinial isolates were used in the experiments (*Table 1*).

Table 1. Samples of *P. triticina* used in experiments

Population	*Fungal origin (location/year/source)*	*Single-uredinial isolates*
RB2012	Fundulea/2012/ population from field	
DuRes 14	Fundulea/2012/ population obtained by *in vitro* multiplication (on plantlet)	MP14/1-14/10; MP26
DuRes 17/18/25/26/27	Fundulea/2013 /population from field	MP17/1-17/10
DuRes 19	Fundulea/2012/ population from field	

Single-uredinial isolates were obtained from several populations of *P. triticina* using the susceptible wheat leaves, cultivar F133, according to detached primary leaf technique (Goyeau et al., 2012) with slight modifications (Ittu, 2013, personal communication): approximately 10-12 ml of water agar (10 g. L^{-1}) containing benzimidazole (100 mg.L^{-1}) (*Fluka*), used to retard senescence, were distributed in Petri dishes. Eight or ten of leaf sections (size 3-5 cm) were placed on agar surface and the cut ends were inserted in agar. Strips of agar were overlaid the cut edges of the leaf section, thereby delaying senescence, and to prevent contamination by other microorganisms, too. Each plate was sprayed with urediniospores suspensions in Tween 20 (0.001%) (*Calbiochem*, Germany). Then, inoculated leaves in the Petri-dish were placed into a dew chamber at 80-90% relative humidity and 15°C for 24 hours. The temperature was increased to 25°C for 16 hours photoperiod and 18°C for 8 hours in dark. Evaluation of leaf rust development was carried out based on appearance and the rate of pustule development.

2. **DNA isolation** from *P.triticina* spores was performed using two methods, with the commercial kit *OmniPrep for Fungus* (*Geno Tech*, USA) and with the CTAB method, as described by Wang et al., 2012.

2.1. Genomic DNA was isolated from fresh single-uredinial samples according to Wang et al. (2012) with slight modifications. An amount of 2-10 mg fresh uredospores were crushed in a 1.5 ml microcentrifuge tube with 300 µl of extraction buffer [100 mM Tris–HCl, pH 9.0; 20 mM EDTA, pH 8.0; 1.4 mM NaCl; 2% cetyltrimethylammonium bromide (CTAB)]. The protocol was improved by the addition of 0.1 g sterile glass bead (diameter 0.45-0.50 µm) in lysis buffer, and ground by vigorous stirring spores (2 times for 1 minute) using Minibead beater equipment (*Biospec Products*, UK). Then, 300 µl of extraction buffer was added to each tube and the mixture was supplemented with 60 µl SDS (20% sodyumdeodecylsulphate) and incubated for 2 hours at 65°C. The tubes were mixed gently every 20 minutes. Proteins were denature and removed by repeated extractions with 660µl Tris saturated phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v). Phases were separated by centrifugation, the aqueous phase removed and DNA precipitated with 700 µl of isopropanol. After 1 hour at -20°C, the phases were separated by centrifugation at 14000 rpm for 7 minutes. DNA was resuspended in 100 µl of TE buffer (10 mM TrisHCl, pH 7.6, 1 mM EDTA) and RNase 10 mg /ml (*Fermentas*, France) was added, then the mixture was incubated for 30 minutes at 37°C. DNA was pelleted by alcohol precipitation, dried and resuspended in 25 µl of Tris–EDTA buffer (10 mM Tris–HCl, pH 7.6; 1 mM EDTA).

2.2. Genomic DNA from eight populations of *P. triticina* urediniospores (10-25 mg spores) was isolated using *OmniPrep for Fungus* kit (*Geno Tech*, USA) according to the manufacturer's instructions, with some modifications: the spores were incubated with 2 µl lyticase (*Sigma*) solution (200 U final concentration), at 37^0C, for 45 minutes and mechanical disintegrated using Minibead beater equipment (*Biospec Products*, UK) for 90 seconds.

DNA concentration was determined using using NanoDrop ND-1000 Spectrophotometer (*ThermoScientific*, USA).

3. **Molecular analysis with RAPD**. RAPD-polymerase chain reaction conditions, running and scoring of gels were previously described, as well as the 10-base primers UBC 402, 450,

489, 517, 519, 521, 538, 556 (Kolmer et al., 1995). Briefly, 10 – 100 ng/µl DNA was amplified in 25 µl reaction volume using AmpliTaq mixture (*Applied Biosystems*, USA) and 2.5 µl primer (10 µM). The thermocycler was programmed at 94^0C for 3 min; followed by 40 cycles of 1 min at 94^0C, 2 min at 36^0C and 2 min at 72^0C; then 10 min at 72^0C.

4. **Molecular analysis with SSR markers**. *P. triticina* isolates were profiled with 15 SSR primer pairs (*Table 2*). The PCR reactions were performed in a volume of 50 µl, contained 1 µl ADN (10 – 100 ng µl), 2.5µl of each primer (10 µM), 4 µl dNTP Mix (200 µM dATP, dCTP, dGTP, dTTP), 4 µl of $MgCl_2$ (25mM), 1 µl of 360 GC Enhancer, AmpliTaq360 DNA Polymerase (1.25U/reaction) and tampon AmpliTaq (10X) (*Applied Biosystems*, USA). Amplification conditions were similar, the differences being the annealing temperatures (59^0C, 57^0C, and 52^0C).

Table 2. The primer sequences and characteristics of 15 *Puccinia triticina* SSR primers used in this study (Duan et al., 2003; Szabo et al., 2007; Visser et al., 2012)

Locus	*Primer sequence (5' – 3')*	*Product (pb)*	*Allele*
PtSSR 3	F: TTCAATTTGCCCCTTGACTC R: AGGTAGCATTGCCAGTGGCA	271-301	4
PtSSR 68	F: GACTCAGCCCACTGCTAA R: GATGGCGACGTATTTGGTCT	305–327/337, 347,356, 360,362	9/5
PtSSR 76	F: GGCGTCGTATTTCTCGTAGC R: TTCGGACTACTGGGTAAGCA	393402/404, 409	3/2
PtSSR92	F: CCAAGGAACAGTCCACCAAG R: GAGTCGGGTAAGCCATCTGA	242-252/234, 237,246, 249	4/4
PtSSR 151A	F: TCATCGCACTCCACTCAGAC R: ATGCTGCCCAACCTGCTC	456 –476/495	4/1
PtSSR 152	F: CTCCGTTCCTCTTTCTGTCG R: CCATCGCAACCAACAAACA	384-388/411, 416	2/2
PtSSR 154	F: ACGGTCAACAGCCAACTACC R: CCTCGTCATCCTGGTTGAGT	242,272/260, 276,279, 281,287	7/5
PtSSR 158	F: GACGACTTCGTCACTGCTGA R: GAGGAGAAGCCGTTCTGTTG	227-232/236, 241	3/2
PtSSR 164	F: GTGGAAGTGAGCGGAAGAAG R: GGAGATGGGCAGATGAGGTA	214-222/210, 218,220	3/3
PtSSR 173	F: CTCAGCGACCTCAAAGAACC R: GAGACGACGGATGTCAACAA	211-219/210, 121,217, 220	4/4
PtSSR 184	F: GGTCTGGCGAATCTTTCCTT R:CATTTTTAGTTGTGAGCCCTTG	373-569/331, 338,480, 491	5/4
PtSSR 186	F: GCCACGAGAAATACATAGAA ATAAAA R: GGTTGTTGATGGGCTTGAGT	335-347/367, 372, 376, 381	6/4
RB4	F: CAGTATTGTGGTGGTTGGATG R: ACTCAAGAATAATGGGGAACA	244, 230	2
RB10	F: TAAGATTGGTGGTATGTGGTG GA R: TTGTCTTTCATCTCATCCAGCC	218	1
RB29	F: CTCACCAAACATCAAGCACC R: GAGCCTAGCATCAGCATC	118-180	9

5**. Molecular analysis with ISSR markers**: Two ISSR primers were used in the experiments: primer 17898A that amplified the repetitive sequence $(CA)_6$ AC, and primer HB15 specific for the repetive sequence $(GTG)_3$ GC. PCR was performed into 0,2 ml tubes containing 25 µl final volume of a reaction mixture consisting of 1X buffer, 0,2mM dNTPs, 1µl of 10µM primer, 1.3U Taq (AmpliTaq 360), 2mM $MgCl_2$, 2 µl DNA and 0.5 µl enhancer solution. The thermocycler was programmed 2 min at 94°C, followed of 35 cycles of: 30 sec at 94°C, 45 sec at 44°C, 1.30 min at 72º C and 10 min at 72°C.

The PCR products have been visualized by gel electrophoresis in agarose 0.8-2%. Sizes were estimated by comparison with DNA size markers, 100 bp DNA Ladder (*Promega*, USA).

RESULTS AND DISCUSSIONS

Detached primary leaf technique has been used to obtain single-uredinial isolates of *P. triticina*. Artificial laboratory conditions (temperature, humidity) allowed germination of urediniospores from the populations and at the end of experiments 21 single-uredinial isolates were retained from *P. triticina* populations DuRes 14 and DuRes 17 (*Figure 1*).

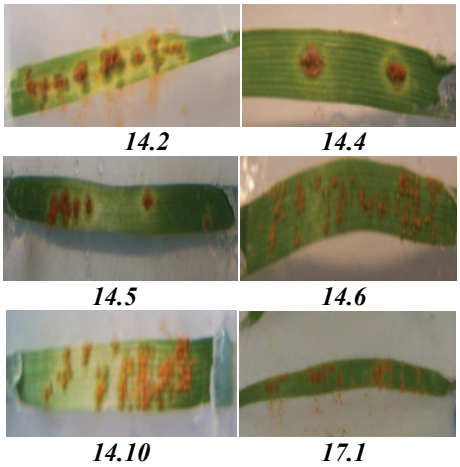

Figure 1. Morphological aspect of single-uredinial isolates of *Puccinia triticina* on F133 wheat cultivar

The urediniospores multiplied *in vitro* by detached primary leaf technique were used for DNA isolation with CTAB method, while the DNA from fungus populations was isolated with a commercial kit. The CTAB method was efficient in DNA isolation of samples with small amounts of spores (under 10 mg), while the *OmniPrep for Fungus* kit was used for samples with 10 - 20 mg of urediniospores. For the later, the extraction protocol was optimize using both mechanical and chemical treatments for spore lyses and a supplementary DNA

cleaning step (*Table 3*). The concentration and purity of DNA obtained with the commercial kit was higher than the quality of DNA obtained by CTAB method. For both methods, the grinding of the spores improved the amount of nucleic acid isolated.

Table 3. Results obtained using *OmniPrep for Fungus* kit for DNA isolation of *Puccinia triticina*

Sample	*Spores (mg)*	*DNA concentration (ng/ul)*	*DNA purity*	
			(A_{260}/ A_{280})	*(A_{260}/ A_{230})*
RB2012	18.4	238.6	2.1	2.3
DuRes14	18.4	503.4	1.73	1.88
DuRes17	16.8	50.0	1.73	0.77
DuRes 18	18.9	507.0	1.84	1.75
DuRes19	17.6	221.7	1.73	0.95
DuRes25	17.1	61.0	1.53	1.52
DuRes 26	16.7	121.5	1.86	2.08
DuRes 27	17.7	74.5	1.58	1.57

Molecular markers have been proved to be valuable tools in the evaluation of genetic diversity within and between species and populations. It has been showed that different markers might reveal different variations, their distribution throughout the genome and the extent of the DNA target in plant or fungal populations and species. The optimization of the polymerase chain reaction (PCR) favoured the development of different molecular techniques such as RAPD, AFLP, simple sequence repeats (SSR or microsatellite), sequence-tagged sites (STS), or intersimple sequence repeat polymorphic DNA (ISSR) (Le Maitre et al., 2013). The molecular analysis using ISSR markers was poorly documented for *P. triticina*, although these molecular markers have been extensively used for other *Puccinia* species (Spakman et al., 2010).

In the first step of the study we compared the RAPD profile of three populations of *P. triticina* RB2012, DuRes 14 and DuRes 18 and of the single-uredinial isolate MP 26, using 8 UBC primers. The molecular phenotypes distinguished by each primer generally varied by one to three bands. However, the RAPD profiles were identical for *P. triticina* populations DuRes 14 and DuRes 18 for the primer UBC 521. The initial results showed a reduced polymorphism at the population level. Moreover, the molecular analysis of population DuRes 14 and the single-uredinial isolate MP 26 (developed from population DuRes 14) with primers UBC 519 and UBC 538 showed a clear intrapopulation molecular differentiation (*Figure 2*). Further comparative examination of the RAPD profiles of the higher number of populations and isolates will provide information necessary for a reliable genetic analysis of the *P. triticina* isolated from Romanian fields.

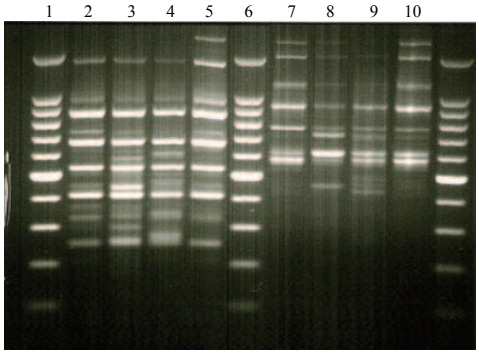

Figure 2. The RAPD analysis with UBC 519 (left) and UBC 538 (right) primers for *Puccinia triticina* DuRes 14 (lane 2, 7), DuRes 18 (lane 3, 8), RB2012 (lanes 4,9) and MP26 (lanes 5,10). Lane 1,6 - Benchop 100bp DNA Ladder (Promega)

Two types of primers were used to analyse the molecular polymorphism of *P. triticina* populations and isolates: primers for SSR and primers for ISSR markers. The selected 15 SSR markers were tested on three fungal populations (DuRes 14, DuRes 18 and RB2012) and on single-uredinial isolate MP26 (*Table* 4*)*. The results obtained with the primer PtSSR 184 showed the genetic differentiation among the populations and isolates. Thus, for this microsatellite locus the SSR profile of the populations DuRes 14 and DuRes 18 showed only one allele (~590 bp), while in the population RB 2012 were detected two alleles (~590bp an 430bp). Moreover, in single-uredinial isolate MP 26, obtained from population DuRes 14, there are two alleles, showing a clear evidence of the intrapopulation molecular polymorphism. Similar results were obtained during genetic analysis of isolates from DuRes 14 and DuRes 17 populations with ISSR primers, ISSR 17898A primer allowed

the detection of the highest polymorphism among the individual isolates.

Table 4. The results obtained with 15 SSR primers selected for genetic analysis of *Puccinia triticina*

Primer	T_a (^0C)	*Amplicon lenght on agarose gel (bp)*			
		DuRes 14	*DuRes 18*	*RB2012*	*MP 26*
PtSSR 3	57/52	-	-	-	-
PtSSR 68	57	-	-	350	350
PtSSR 76	57	400	400	390	390
PtSSR 92	57	240	240	250	240
PtSSR 151A	52	510	510	490	490
PtSSR 152	57	400	400	400	400
PtSSR 154	59	270	270	290	290
PtSSR 158	59	220	220	240	240
PtSSR 164	59	210	220	210	210
PtSSR 173	59/57	210	210	220	220
PtSSR 184	**59**	**590**	**590**	**430, 590**	**430, 590**
PtSSR 186	59/57	380	380	380	380
RB4	57/52	240	240	240	240
RB10	52	-	-	-	-
RB29	52	-	180	180	180

Additional research on the virulence and molecular polymorphism will be needed in order to better characterize the *P. triticina* populations and isolates from Romanian fields.

CONCLUSIONS

1.The detached primary leaf technique was optimized and improved to obtain single-uredinial isolates of *P. triticina*.
2.The CTAB method was efficient in DNA isolation of *P. triticina* samples with small amounts of spores, while *OmniPrep for Fungus* kit was used to obtain better quality DNA.
3.The molecular polymorphism of the *P. triticina* isolated from Romanian fields was detected using RAPD, SSR and ISSR markers and clear evidenced among the single-uredinial isolates. Results showed a reduced polymorphism at the population level, but not identity, as indicated the RAPD analysis. No more than two alleles were detected in the leaf rust populations with the SSR markers used in experiments according to our separation protocol.

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EXTRACELLULAR HYDROLASES OF HALOPHILIC MICROORGANISMS ISOLATED FROM HYPERSALINE ENVIRONMENTS (SALT MINE AND SALT LAKES)

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Abstract

This work aims to reveal the ability of halophilic microorganisms, both bacteria and archaea, isolated from salted lakes and salt crystals from the salt mine, to produce a wide range of extracellular hydrolytic enzymes able to degrade several macromolecular substrates, such as sugar based polymers or proteins. A relatively wide positive spectrum of extracellular hydrolases for tested substrates was recorded from archaeal and bacterial strains isolated from investigated salted area. The number appears to be higher if comparing isolates from salt lakes with isolates from salt crystal. In the case of tested halophilic bacteria were found enzymes capable of hydrolyzing starch, casein, Tween80 and carboxymethylcellulose. In the case of microorganisms belonging to the Archaea domain, identified on the surface of the crystal and belonging to Halorubrum genus was detected the ability to degrade starch and seldom Tween80. In some cases, a combined hydrolytic activity has been observed. One halophilic bacterial strain combined cellulase and esterase activities and other strains combined two or more hydrolytic activities. The enzymes degrading starch appear to have a wide distribution and when compared the 16S rRNA phylogeny distribution of investigated strains with the absence or presence of amylase activity, the data showed a very high correlation degree.

Keywords: *extracellular hydrolases, halophilic archaea, halophilic microorganisms, salt lake, salt mine.*

INTRODUCTION

Halophilic microorganisms are widely distributed in environments characterized by salinity (mainly sodium chloride concentration) from negligible content until to saturation. Such kind of environments are largely spread in the entire world, the most frequently associated with hypersaline attribute being the Dead Sea and Great Salt Lake from United States (Minegishi, 2013; Oren, 2002; 2013). Excepting these areas, saline environments are also represented by salterns, salt mines, salted soil and many neutral or alkaline salted lakes like Wadi Natrum in Egypt (Oremland, 2013; Oren, 2013).

In Romania, several saline and hypersaline environments are represented by various anthropocentric and natural salt lakes, like Techirghiol, Balta Albă, Movila Miresei, Amara (Enache et al., 2012; Neagu et al., 2014) and several salt mines located mainly in the proximity of Carpathian Mountains or in sub-Carpathian hills. The present study has been focused on the salted area in Slănic Prahova where the exploitation of salt has been conducted from 1685 (Enache et al., 2008; 2009) until today following various technologies (Drăgănescu and Drăgănescu, 2001). The major consequence of salt exploitation for the environments in this area is represented by the formation of several salt lakes in the opening of former salt exploitations which are known as Green, Red and Shepherd Bath and Bride Cave. The previous studies (Enache et al., 2007; 2008) have indicated that the investigated areas were populated by halophilic microorganisms inhabiting both the surface and the inside of salt crystals (Enache and Kamekura, 2013). These organisms were both bacteria and archea belonging to the genera *Halobacterium*, *Halorubrum, Haloarcula,* predominantly identified within the crystal.

Generally, the hypersaline environments (including salt mine) represent an important source for isolating microorganisms that produce enzymes which could be regarded as

having industrial relevance (Moreno et al., 2013). Enzymes showing the activity over a wide spectrum of salinities have been isolated from various halophilic archaeal and bacterial microorganisms (Sanchez-Poro et al., 2003). These enzymes showed in many cases thermo- and alkalitolerant properties and those polyextremophilic features could be a tremendous advantage for their potential in various biotechnologies (Enache and Kamekura, 2010; Margesin and Schinner, 2001; Oren, 2010; Ventosa and Nieto, 1995) or in agriculture fields (Shivanad et al., 2012).
Extracellular hydrolases of halophilic microorganisms play a key role for these microbial strains to use organic compounds in salted environments and support their use in obtaining several products of commercial interest (Enache and Kamekura, 2010; Kamekura et al., 1982). This work aims to reveal the ability of halophilic microorganisms, both bacteria and archaea, isolated from salted lakes and salt crystals from the salt mine to produce a wide range of extracellular hydrolytic enzymes able to degrade several macromolecular substrates, such as sugar based polymers or proteins.

MATERIALS AND METHODS

The sample collection

The rock salt samples were taken from the wall of subterranean salt mine, Unirea, located in Slanic Prahova. Samples were taken from several points of the mine by courtesy of the mine staff. Crystals apparently (visually) considered to have biological material inclusion were used in further experiments as described below. Being estimated at the debut of the experiments that the probability to isolate halobacterial strains was very low, there were not tailored any statistical analysis. Accordingly, one gram of salt crystal with no apparent contamination by clay or soil was immersed and shaken in sterile 10% NaCl solution to wash the outside and was then dissolved in 50 ml of sterile 10% NaCl. In order to isolate halophilic bacteria, one ml of this solution was mixed with 20 ml of autoclaved molten agar medium (around 55^0C) MH with the following composition (g/l): NaCl - 100, $MgCl_2x6H_2O$ - 7, $MgSO_4x7H_2O$ - 9.6, $CaCl_2x2H_2O$ - 0.36, KCl - 2, $NaHCO_3$ - 0.06, NaBr - 0.026, glucose - 1, proteose peptone - 5, yeast extract - 10 (Ventosa et al.,1989). The halophilic archaeal strains were isolated from the crystal sample following previously described protocol (Enache and Kamekura, 2013).

The water samples were taken from the surface of salted lakes Shepherd Bath, Green Bath, Red Bath and Bride Cave located in Slănic, Prahova county, at about 100 km north from Bucharest, in the summer period. Approximately one liter of water from each site was taken in a sterile bottle, closed after filling and maintained in appropriate conditions until to laboratory where the samples were transferred to 4^0C before microbiological analysis. The archaeal strains were isolated in a medium (I) with the following composition (g/l): NaCl (125), $MgCl_2x6H_2O$ (160), K_2SO_4 (5), $CaCl_2x2H_2O$ (0.1), yeast extract (1), peptone (1), soluble starch (2), agar (20). The medium pH was 7.0-7.2. For enzyme detection experiments the strains were grown in JCM medium No. 168 which contained (g/l): Bacto casamino acids (5), Bacto yeast extract (5), sodium glutamate (1), trisodium citrate (3), $MgSO_4x7H_2O$ (29.5), KCl (2), NaCl (175.5), $FeCl_2x4H_2O$ (0.036), $MnCl_2x4H_2O$ (0.36 mg). The medium pH was 7.0 – 7.2 before autoclaving.

Differentiation of archaea and bacteria isolates

The susceptibility of the novel isolates to antibiotics or bile salts, namely to chloramphenicol and sodium-deoxycholate was tested in order to confirm the belonging of the isolated strains to *Bacteria* domain and to differentiate them by halophilic archaea. For this purpose, there were used two solidified medium variants, with 0,004% deoxycholic acid sodium salt and with 0,002% chloramphenicol. Strains that were able to grow in the presence of Na deoxycholate were considered as halophilic bacteria (Kamekura et al., 1988; Kamekura and Seno, 1991).

Detection of extracellular hydrolytic activities

Biochemical test for starch hydrolysis was performed according to standard procedures at optimum concentration of NaCl for growth of

all the investigated strains. Hydrolysis of Tween 80 were detected using the method of Gutierrez and Gonzalez (1972). Casein and carboxymethyl cellulose hydrolysis was tested on solidified appropriate medium (JCM medium no. 168 for archaea and MH medium for bacteria) as previously described (Cojoc et al., 2009; Enache et al., 2008). All the investigation was performed in a medium supplemented with the appropriate substrate and using the optimum NaCl concentration for growth.

16S rRNA gene sequence analysis

Total DNA was extracted and purified using the method of Tamaoka adapted for halophilic archaea (Enache et al., 2007; 2008). The 16S rRNA genes were amplified by PCR, using the archaeal specific forward and reverse primers 5'-TCCGGTTGATCCTGCCG (position 8 – 24) and 5'-GGAGGTGATCCAGCCG (position 1540 – 1525), respectively. The resulted DNA fragments were sequenced using BigDye Terminator Cycle Sequencing Kit (Pharmacia Biotech) and ABI Prism DNA genetic analyzer (Applied Biosystems). The sequences obtained were analyzed using BLAST and aligned with other reported haloarchaeal 16S rRNA gene sequences using CLUSTAL W 1.7 software. A phylogenetic tree was reconstructed by the neighbor-joining method.

RESULTS AND DISCUSSIONS

The results obtained in this study and in previous works (Enache et al., 2008; Enache and Kamekura, 2013) revealed that investigated area hosts microbial strains belonging both to bacteria or archaea. In order to estimate the spectrum of extracellular hydrolases were investigated 16 archaeal strains and 13 bacterial strains isolated from salt crystal and 12 archaeal strains isolated from salted lakes. From the archeal strains originating from rock salt, six were isolated from the surface of the crystal and the remaining ten from the inside of the crystal (Enache and Kamekura, 2013).

The data showed in Table 1 and Figure 1 revealed that archaeal strains either from salt mine or salted lakes are devoid of capacity to degrade carboxymethyl-cellulose (CMC). Also, in the case of archaeal strains isolated from salt mine, the caseinolytic activity could not be detected (Table 1). The amylase activity was detected at 56% for archaeal strains isolated from salt mine and 83% at archaeal strains isolated from salted lakes. A different percentage was recorded for Tween 80 hydrolysis, namely 92% of the strains isolated from salted lakes harbored this activity by comparison with only 12% in the group of strains isolated from salt mine. This difference could be attributed to different anthropic factors which affected the investigated sites, the salted lakes and surface of the salt crystals from salt mine being exposed to high potential polluting factors which could generate substrates for esterase and lipases. This conclusion is sustained also by the high percent (85%) of bacterial strains isolated from salt mine (located at the surface of the crystal), which show the ability to degrade Tween80 (Figure 1, Table 1).

Table 1. Detection of extracellular enzymatic activities (positive/total tested strains) in cultivable halophilic archaea and bacteria isolated from Unirea salt mine and hypersaline lakes (Green Bath, Shepherd Bath, Red Bath and Bride Cave) in Slanic Prahova, Romania; CMC = carboxymethyl-cellulose

	Salt mine		Salted lakes
Tested substrates	Archaea	Bacteria	Archaea
Starch	9/16	3/13	10/12
Casein	0/16	6/13	2/12
Tween 80	2/16	11/13	11/12
CMC	0/16	2/13	0/12

Table 2. Distribution of tested cultivable halophilic archaea between genera of family *Halobacteriaceae*. Salted lakes: Green Bath, Shepherd Bath, Red Bath and Bride Cave. Salt mine: Unirea. All sampling sites are located in Slanic, Prahova, Romania

	Archaea	
	Salt mine	Salted lakes
Haloarcula	1	1
Halobacterium	2	0
Haloferax	0	11
Halorubrum	13	0
Total tested strains	16	12

This high percentage together with the facts that archaeal strains from salt mine which presented the capacity to degrade Tween 80 were located at the surface of the salt crystal and 92% of archaeal strains from salted lakes

were able of degrading Tween 80 support the hypothesis that positive strains from the salt mine, located either inside or on the surface of salt crystal, are transferred from other sites and should not be considered as native population of salt crystal. Around 15% of the bacterial strains isolated from salt mine showed ability to degrade carboxymethyl-cellulose (CMC) and 46% casein, in opposite with archaeal strains, where CMC degrading activity is absent and only 16% of strains isolated from salted lakes were able of hydrolyzing casein (Figure 1, Table 1). On the other hand, the amylase activity was less represented in the case of bacterial strains where only 23% showed this capacity (Table 1, Figure 1).

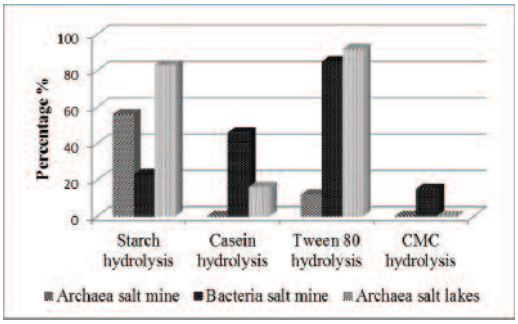

Figure 1. Distribution of extracellular hydrolytic activities in investigated sites

The 16S rDNA gene sequencing showed that investigated archaeal strains belonged to the genera *Haloarcula* (*Har.*), *Halobacteria* (*Hbt.*), *Haloferax* (*Hfx.*) and *Halorubrum* (*Hrr.*) (Figure 2, Table 2). The strains of *Har* genus could be isolated both from the salt mine and salted lakes but strains belonging to *Hbt* and *Hrr* genera could be isolated only from the salt mine. On the other hand, the strains belonging to *Hfx* genus were distributed only in salted lakes and could not be detected in salt mine (Tables 2 and 3).

Table 3. The distribution of tested cultivable halophilic archaea accordingly with sampling sites; the salted lakes and salt mines are described at previously tables

Lakes	Crystal of salt mine	
	Surface	Inside
Haloferax (predominant) *Halorubrum*	*Halorubrum*	*Halorubrum* (predominant) *Haloarcula* *Halobacterium noricense*

Based on this data could be concluded that in salted lakes are predominant *Hfx* genus and in salt mine *Hrr* genus. The phylogenetic tree reconstructed by the neighbor-joining method derived from sequences of 16S rDNA (Figure 2) revealed the same distance 0.041 which appears at the two nodal points on which are based the group of strains isolated from salt lakes (in blue color in Figure 2) and the group of strains isolated from salt crystal (red color marked strains isolated from the surface of the crystal and black color strains isolated from inside of the crystal).

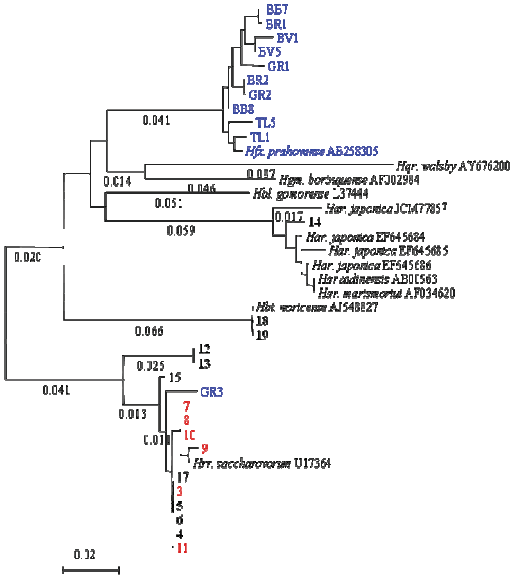

Figure 2. Phylogenetic tree reconstructed by the neighbor-joining method derived from sequences of 16S rDNA. The same distance 0.041 appears at the two nodal points on which are based the group of strains isolated from salt lakes (in blue) and the group of strains isolated from salt crystal (red color marked strains isolated from the surface of the crystal and black color strains isolated from inside of the crystal)

The clade of haloarchaea isolated from salt lakes is consituted by strains showing amylase and esterase activities (Figure 2, blue color clade). The clade of haloarchaea isolated from salt mine consists of strains characterized also by the presence of amylase activity (Figure 2, clade with red color). Some strains grouping separately, like GR3, 12, 13, 14, 18 and 19 are characterized by the absence of both amylase and esteraze activity. Based on the data showed in Figure 2 and Table 1 resulted that the presence of amylase activity is supported well

by phylogenetic classification, in spite of some incongruences recorded in the case of some strains isolated from salt mine. On the other hand the data showed in Table 1 revealed that some strains harbor more than one extracellular hydrolytic activity. The presence of such combined extracellular hydrolytic activities can offer a good potential for halophilic organisms to be applied as an economic alternative to some current biotechnological processes.

CONCLUDING REMARKS

A relatively wide positive spectrum of extracellular hydrolases for tested substrates was recorded from archaeal and bacterial strains isolated from investigated salted area. The number appears to be higher if comparing isolates from salt lakes with isolates from salt crystal and the enzymes degrading starch appear to have a wide distribution. When compared the phylogenetic distribution of investigated strains and the absence or presence of amylase activity, the data showed a very high correlation degree.

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EFFECT OF SOME BACTERIAL ANTAGONISTS ON GROWTH AND MYCOTOXIN PRODUCTION OF *FUSARIUM GRAMINEARUM* AND *F.CULMORUM* ISOLATES

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Abstract

The impact of mycotoxin contamination of foodstuffs and feedstuffs on human and animal health is well documented. Trichothecene mycotoxins are metabolic compounds produced by various Fusarium species such as F.graminearum, F. culmorum, F. sporotrichioides, F. poae and F. equiseti on different grains like wheat, oats or maize. The most significant thrichothecenes produced by fusaria are T-2 toxin, HT-2 toxin, diacetoxyscirpenol (type A) and nivalenol, deoxynivalenol, 3- and 15-acetyldeoxynivalenol (type B). Deoxynivalenol (DON), mainly synthesized by F. graminearum and F. culmorum (that produce Fusarium Head Blight disease = FHB), is the most common trichothecene contaminant of wheat, and appears to play an important role in the aggressiveness of both species toward wheat. In order to prevent the contamination of the cereals with mycotoxigenic fungi, various strategies were developed (cultural practices: tillage, crop rotation; use of fungicides, resistant cultivars or biological control agents = BCAs). Because of the low efficacy of fungicides to control members of the Fusarium head blight Complex, the selection of highly efficient antagonists is of great interest for the specialists all over the world. Environmental friendly, the use of BCA is an important additional strategy that can be used as part of an integrated management of FHB.
For this reason, the aim of our work was to select potential microbial antagonists that are able to inhibit the growth of Fusarium graminearum and F.culmorum, and to prevent the presence of DON on wheat. 37 Fusarium spp. strains were used in experiments and the in vitro aggressiveness of these isolates was determined. Twelve fungal strains with differences in the aggressiveness potential and DON biosynthesis were selected and used for interactions with eight bacterial strains isolated from compost or soil. Four out of the eight bacterial strains used were selected based on their high inhibitory activity against all the fungal isolates. Specific microscopically modifications of hyphae were observed at the edge of fungal colonies near the antagonist. The effect of the bacterial antagonists on DON accumulation in specific culture medium was also checked by TLC method. The results showed the possibility to use one or more bacterial strains as a tool for the biological control of FHB agents that survives in crop residues.

Keywords: *biological control agents, mycotoxins, Fusarium.*

INTRODUCTION

Fusarium head blight is the most important disease of wheat (*Triticum aestivum* L.) being found in large regions of the world. Intensive studies were performed for the identification and characterization of the pathogen, and up to 19 species have been associated with the disease (Parry et al., 1995). Among these species, the most prevalent pathogens are *Fusarium graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schweinitz) Petch] and *Fusarium culmorum* (W. G. Smith) Saccardo (Walter et al., 2010). Moreover, in a recent study, Dean et al. (2012) presented a list of the Top 10 most important fungal plant pathogen, where in fourth place is *Fusarium graminearum,* recognized by the specialists as one of the most significant pathogen for cereals. The importance of *Fusarium* species is related not only to the diseases produced to infected plants but also to mycotoxin biosynthesis (trichothecenes, for example) and accumulation in infected plant material. The

impact of mycotoxin contamination of foodstuffs and feedstuffs on human and animal health is well documented (Cano et al., 2012; Iqbal et al., 2014). Trichothecene mycotoxins are metabolic compounds produced by *Fusarium* species such as *F.graminearum, F.culmorum, F.poae, F.sporotrichioides,* and *F.equiseti* on wheat, oats or maize. It was shown that the most significant thrichothecenes produced by fusaria are: T-2 toxin, HT-2 toxin, diacetoxyscirpenol (type A), deoxynivalenol (DON), nivalenol (NIV), 3- and 15-acetyl deoxynivalenol (type B), mainly synthesized by *F. graminearum* and *F. culmorum*. Recent studies (Audenaert et al., 2013) summarizes the arguments related to the significance of mycotoxins like DON in the competition for niches on crop residues and organic matter: DON is an antimicrobial metabolite effective against various eukaryotic soil organisms (Sorocco et al., 2012), and DON can affect the metabolite production of other soil-residing fungi, such as *Trichoderma* sp., that are known for their strong outcompeting capacity by mycoparasitism (Matarese et al., 2012).

Several strategies have been developed for the control of *Fusarium spp.* infections of cereals (cultural practices: tillage, crop rotation; use of fungicides, resistant cultivars or biological control agents = BCAs) (Mesterházy et al., 2012; Leplat et al., 2013; Schrader et al., 2013). Because of the low efficacy of fungicides to control members of the Fusarium head blight Complex (Mesterházy et al., 2011) the selection of highly efficient antagonists is of great interest for the specialists all over the world (Wagacha and Muthomi, 2007; Vujanovic and Goh, 2011). Environmental friendly, the use of BCA is an important additional strategy that can be used as part of an integrated management of FHB (directed against fungal growth and/or mycotoxins biosynthesis/ accumulation).

The aim of this work was to select potential microbial antagonists isolated from natural sources (soil or compost) that are able to inhibit the growth of *Fusarium graminearum* and *F.culmorum*, and to prevent the accumulation of DON.

MATERIALS AND METHODS

Culture media

The media used in the present study were potato dextrose agar (PDA) for fusaria cultivation and Luria-Bertani medium for maintaining bacterial strains. For mycotoxin production, potato sucrose medium (PSA) was used. The potato sucrose agar media consisted of 200 g sucrose and 20 g agar for a liter of potato extract prepared in laboratory from 200 g peeled potato boiled in water. The reason behind choosing this media for the culture of *Fusarium* strains relies in the findings of Jiao et al. (2008) according to whom the expression of important trichothecene biosynthesis genes (*tri4* and *tri5*) were up-regulated in a high sucrose containing media but not in glucose. Also, Vujanovic and Mansour (2011) reported that 15-ADON chemotype of *Fusarium graminearum* produced DON and ZEA mycotoxins only in the presence of high sucrose concentrations (20% and above).

Biological material

Three strains of phytopathogenic *Fusarium* genus used in experiments are presented in table1.

Table 1. Fungi strains used in experiments

Strain designation	Fungal species	Source
Fc46	*Fusarium culmorum*	NARDI Fundulea (Ittu et al, 2010)
G28	*Fusarium culmorum*	NARDI Fundulea (Ittu et al., 2010)
F183	*Fusarium graminearum* DSMZ 4527	RDIPP, Bucharest
Fs	*Fusarium solani*	Isolated from potato tuber, FB Collection, Bucharest*
FORL	*Fusarium oxysporum* f.sp. *radicis lycopersici* ZUM 2407	RDIPP, Bucharest
F54	*Fusarium spp.*	Isolated from potato tuber, FB Collection, Bucharest*

*Collection of the Faculty of Biotechnology Bucharest

Eight bacterial strains were used in experiments: B1-B6 strains were isolated from compost tea and selected according to their inhibitory action against different fungi (data not shown); *Bacillus amyloliquefaciens* BW (Sicuia et al., 2011) and *Bacillus pumilus* BIR isolated from soil.

Antagonism evaluation

Dual-culture assay was used to examine the degree of fungal inhibition by bacterial strains. Mycelia plugs (5mm diameter) cut from the margin of 6 days old culture of each *Fusarium* strain were placed in the middle of the PDA Petri plates. 10 µl of each bacterial culture were placed at 20 mm from the edge of fungal colony situated at the centre of the plate. The plates were incubated at 26ºC. The mycelial growth of *Fusarium* strains, both in control and dual-culture plates, were linearly measured and recorded daily for 5 days. The antagonistic activity of the bacterial strains was calculated according to Cornea et al. (2008).

In order to examine both the fungal growth inhibition and the effect on mycotoxin production of the selected bacterial strains, the interactions were conducted on PSA medium. Four individual bacterial strains (B1, B5, BW and BIR) and a mixture of these strains (equal volumes of each bacterial strain were combined) were used in a dual-culture manner, as previously described. The plates were incubated for seven days at 26^{o}C and examined in comparison with the control (fungal strains grown on PSA medium without bacteria).

Extraction and analysis of mycotoxins

Mycotoxins (trichotecenes) extraction was performed according to Mačkinaitë (2006) with some adaptations. Sample zones in the shape of a disc of 0.5cm diameter located approximately 0.2 cm behind the contact zone between *F.graminearum/ F.culmorum* and selected bacteria were excised and subjected to mycotoxins extraction. Control samples were extracted from the edges of the *F.graminearum / F.culmorum* growth area following the same procedure. The sample plugs were left overnight in a 5ml of chloroform/methanol mixture (2:1) extraction solution and evaporated under vacuum. The sediment was resuspended in chloroform and 20 µl of each sample was spotted onto TLC plates. Deoxynivalenol standard was used at a concentration of 100 µg mL^{-1} (5µl/spot). The extracts were separated on silica gel 60 plates (10x10 cm, 0.20 mm thick; Roth) with toluene/ethyl acetate/formic acid (5:4:1) as mobile phase. The chromatograms were air dried and the fluorescent spots were revealed under UV light (365 nm) after spraying with 20% aluminium chloride in absolute ethanol and drying at 110ºC for 10 minutes (Vujanovic and Mansour, 2011).

RESULTS AND DISCUSSIONS

Evaluation of the inhibitory effect of bacteria on the Fusarium strains

It was reported by several authors that crop residues, like wheat straw, could be the major sources of the fungal inoculum for fusaria due to the ability of pathogenic fungi to survive in infested debris (as saprotrophic mycelium or as resting chlamydospores). However, the surviving of fungi in such residues can be affected by other microorganisms, via competition, parasitism, and predation or by influencing the rate of plant debris decomposition (Sarroco et al, 2012). For these reasons, the identification of new antagonists from soil is of great interest for preventing plant contamination with fungal pathogens.

Therefore, *in vitro* screening was conducted to determine the ability of various bacterial strains previously isolated from soil or compost (data not shown) to suppress strains of *Fusarium spp.* isolated from different plant host (seeds of *Triticum aestivum* or potato tubers)(table 2).

Table 2. Results of preliminary inhibitory assay

Bacterial strains	Inhibition of fungal growth					
	F183	G28	FC46	FORL	F. solani	F54
B1	+++	+++	+++	+++	+++	+++
B2	+	+	+	++	++	+++
B3	++	+	++	++	++	+
B4	-	-	-	-	-	-
B5	+++	+++	+++	+++	+++	+++
B6	+	+	+	++	++	+
BW	++	+++	++	+++	+++	++
BIR	+++	+++	+++	+++	+++	+++

Where: + + + = strong inhibition of the fungal growth; + + = moderate inhibition of the fungal growth; + = slight inhibition of the fungal growth; – = no inhibition of the fungal growth.

Best results, i.e. inhibition against almost all tested fungi were obtained with the new isolates B1 and B5, as well as with BW and BIR strains (Figure 1).

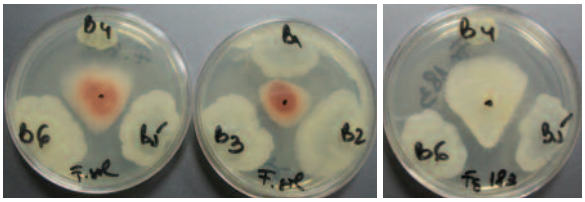

Figure 1. Inhibition of *Fusarium solani* and *F.graminearum F183* by the selected bacterial strains on PDA medium

Based on these results, the selected four strains were used for the inhibition assays on PSA medium, against *F. graminearum* and *F. culmorum* strains, and their inhibitory efficacy was evaluated.

It was shown that the aspect of bacterial cultures was different due to the high sucrose content of the culture medium, but the inhibitory activity was not affected. No clear areas of inhibition between bacteria and fungi were observed on PSA, comparing with the results on PDA; moreover, bacteria tended to cover the edges of fungal colonies (figure 2). The highest inhibition efficacy was detected for B1 strain, and the most reduced, for BW strain (table 3).

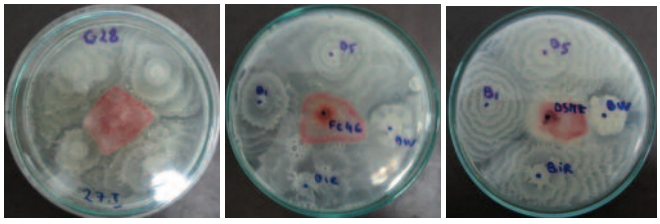

Figure 2. Inhibitory effect of four selected bacterial strains against *F.culmorum* G28*, F.culmorum* Fc46 and *F.graminearum* F183 (DSMZ) on PSA medium

It has been suggested by some authors (Kloepper et al., 2004) that mixing different strains of BCAs can increase efficacy against pathogens, the effect being superior compared to individual strain. In our experiments, when a mixture of all selected bacterial strains (B1, B5, BW and BIR) was tested, the inhibition area was different from that detected for each of the strains when were tested alone (table 3). The highest inhibitory action of the bacterial mixture was observed against *F.graminearum* F183 strain which is highly pathogenic and toxigenic.

Table 3. *In vitro* inhibitory efficacy of the selected bacterial strains against phytopathogenic *Fusarium* spp. strains

Fungal strain	Antifungal efficacy (% of inhibition)				
	B1	B5	BW	BIR	Mixture
FC46	75.6	78.04	53.65	65.85	69
F183	70.1	68.29	43.9	70.7	78
G28	80.24	80.48	58.5	60.9	70.85

The inhibition efficacy of the bacterial strains mixture against the other two fungal strains, Fc46 and G28 (figure 3), was comparable with that of individual bacteria, except BW. These results suggested that the efficacy of the inhibition could be influenced not only by the specific interactions between bacteria but by target fungal pathogen (the natural variability of pathogenic fungi could be an important factor of limiting the *in vivo* efficacy of bacteria based biopreparates). In order to improve the effectiveness of selected bacteria, the optimizing the proportion between bacterial strains in the mixtures is necessary.

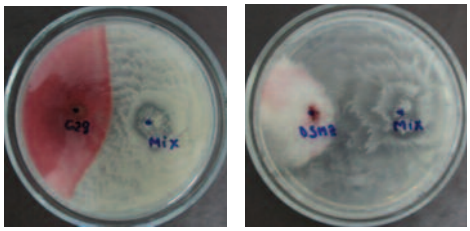

Figure 3. Inhibition of fungal growth (G28 and F183 – DSMZ) by bacterial mixture (B1, B5, BW and BIR)

These findings suggest that the selected bacterial strains could be efficient for the

inhibition of some fusaria. Moreover, previous studies with *B.amyloliquefaciens BW* proved that this strain is able to produce various enzymes associated to plant growth promotion and antifungal activity and could be considered as a competitive rhizobacteria which can be successfully used to bio-activate the vegetal mulch and to control *F.oxysporum* f.sp. *radicis lycopersici* FoRL (Sicuia et al., 2012).
Experiments with bacteria able to suppress various *Fusarium* species development have been reported over time (Sadfi et al. 2001, Johansson et al., 2003, Omar et al., 2006); their results proved the efficiency of natural rhizobacteria as biocontrol agents. The results obtained in this study confirm these findings and the possibility of isolation of new highly antagonistic bacterial strains from natural sources.

Trichothenece production in selected fusaria and influence of bacterial treatment

The genetic ability of *F.graminearum F183, F.culmorum Fc46 and F.culmorum G28* to synthesize DON was previously described, all of them contain *tri 5* gene and *tri7* gene and are able to produce DON at increased level, and to induce severe effects on wheat seeds (no germination was obtained after inoculation) (Cornea et al., 2013). It is well documented that trichothecenes contaminating food and feed are harmful for human and animal health and the prevention of mycotoxins accumulation or their biodegradation is of great interest for scientist. As it was presented above, biological control using bacteria has been explored as an additional or alternative possibility of managing fungal infection and/or mycotoxin accumulation.
In our study, the reduction of DON accumulation in media inoculated with mycotoxigenic fusaria by interaction with natural bacterial isolates was examined. In this respect, antagonistic bacterial strains selected for their significant *in vitro* inhibitory activity against *Fusarium* isolates were co-cultivated with their target on solid PSA medium. After seven days of incubation, the DON was extracted in chloroform and the extracts were examined by TLC methods (figure 4).

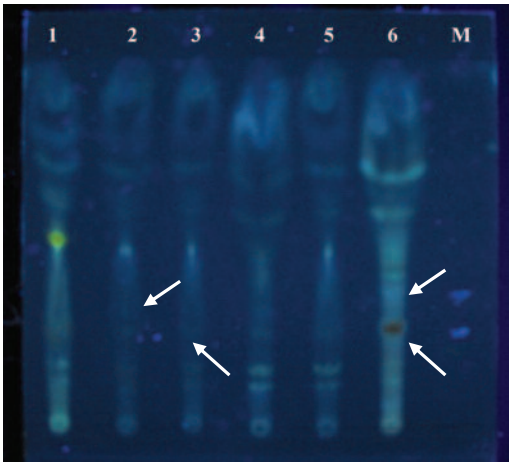

Figure 4. Thin layer chromatography of *F.culmorun* Fc46 extracts: 1 – Fc46 + bacterial mixture; 2 – Fc46 + B1; 3 - Fc46 + B5; 4 – Fc46 + BW; 5 – Fc46 + BIR; 6 – Fc46 control (no bacteria); M – DON standard. The arrows indicate the position of DON

The results proved that in the presence of B1 and B5 bacterial strains, at least in the case of Fc46, no spots corresponding to DON were observed on the TLC plates, most probably due to the biosynthesis inhibition rather than bacterial degradation of mycotoxins, but this conclusion need to be confirmed.
This is, to our knowledge, the first report where inhibition of *Fusarium* species by bacterial biocontrol agents was coupled with chromatographic methods to determine the mycotoxin levels in the fungi-bacteria contact areas. The inhibition areas were observed between the fungi and bacteria but the specific mechanism involved in fungal growth inhibition will be clarified in further experiments.

CONCLUSIONS

The results of this study proved that properly selected bacterial isolates have the potential to significantly suppress the growth of *F.graminearum* and *F.culmorum*.
The efficiency of the inhibition could be influenced by the specific interactions between bacterial strains (competition, antagonism) and by the particularities of target fungal pathogens belonging to the same species. For this reason, the inhibitory efficiency of antagonistic bacteria could be improved by mixing them in specific ration.

The TLC method revealed the effect of biocontrol bacteria on DON biosynthesis/accumulation in tested fusaria, and proved that at least two bacterial strains (B1 and B5, isolated from compost) were able to reduce the level of DON in the extracts of *F.culmorum* Fc46.

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ESTABLISHMENT OF TESTS FOR FACILITATING SCREENING OF DROUGHT TOLERANCE IN SOYBEAN

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Abstract

Abiotic stress due to environmental changes causing water deficit, extreme temperatures and low atmospheric humidity decreases plant productivity. One of the elements of overcoming the stress is to develop new varieties. However, drought tolerance has a complex nature which makes this problem difficult to solve and involves different approaches and methods. The process of creating new varieties could be facilitated by screening of genotypes with higher resistance. In this respect laboratory tests in in vitro and ex vitro conditions could complement in vivo experiments. To make stress models availability of a factor causing or imitating the stress is required. In addition, choosing appropriate criteria of correspondence between responses in field and in laboratory conditions is crucial.
The high molecular weight substance polyethylene glycol (PEG) causes osmotic shock which is one of the components of the drought. Different concentrations (2-10 %) of PEG were used in our experiments. PEG was added into the media for germination of seeds and its effect on water absorption and seed development was followed. Soybean lines obtained previously and varieties (used as standard) were studied. Positive correlation between the level of drought tolerance of the genotypes in the field and in laboratory experiments was identified. The results are interesting for establishment of quick screening tests for identification of lines appropriate for breeding programs of soybean, commonly used grain legume with multiple product application.

Keywords*: drought tolerance, Glycine max, PEG, soybean, screening.*

INTRODUCTION

Soybean (Glycine max (L.) Merrill.), an oil and protein rich plant is the largest cultivated pulse crop in the world, providing more than half of the world's phytoprotein. Soybean oil is the basic one for cocking in the countries of Asia and is the preferred one in other countries like USA where its consumption has the biggest share of 61 % of the market of edible vegetable oil [US DAERS 2011]. Soybean is attracting greater interest with its nutraceutical value and as a component of wholesome food and health based nutrition programs. Soya foods reduce the risk of heart disease, osteoporosis and certain forms of cancer. [Messina and Messina 2010; Vij et al. 2011]. Compared to other grain legumes soybeans have higher fat and protein content, and lower carbohydrate content. The grain differs not only in macronutrients but in micronutrients, too, being rich in mineral elements, vitamins and specific constituents promoting health benefits. It contains phytochemicals such as isoflavones which have been a subject of intensive research during the last years due to their phytoestrogen activity [Kosturkova and Mehandjiev, 2002; Sakthivelu et al., 2008a]. Soybean global importance is growing up, too, due to its multipurpose applications not only as a food for humans and feed for animals but as a source of more than 200 industrial products like environmentally friendly solvents, lubricants, cleaners and paints.

Despite great success in soybean breeding and improvement of cultivation this crop still faces the *problems* caused by biotic and abiotic stress which reduces production and grain quality. [Szilagyi, 2003; Hirt and Shinozaki, 2004; Todorova and Kosturkova 2010]. In soybeans, drought causes 50% yield losses. Plants have developed a number of molecular, cellular, and

physiological mechanisms to cope with environmental stress. Due to the complex nature of the tolerance improvement of plant performance include both traditional and modern breeding studies. Success in creating better adapted varieties depends upon the efforts by various research domains [Landjeva et al., 2008; Amudha and Balasubramani, 2011, Ji Huang et al., 2013]. Classical breeding have been complemented with new methods of plant biotechnology and molecular biology allowing simulation of the desired stress *in vitro*, selection on cell level and exploitation of genes for resistance [Raia et al, 2011, Arumingtyas et al., 2012].

The objectives of the present study were to find out appropriate criteria for simple and quick screening of soybean genotypes with higher tolerance to drought using different approaches. Response of plants to drought conditions in the field and seed germination *in vitro* under osmotic stress were studied to find out a parallel between stress response at different stages of development.

MATERIALS AND METHODS

Plant material. Bulgarian soybean (*Glycine max* L.) lines, obtained by induced mutagenesis and/or hybridization were object of the present investigations. The American variety Hodson and the Bulgarian Variety Daniela were used as a world and a national standards.

Field trials. Soybean was grown in the experimental field of Pavlikeni under irrigated and non-irrigated conditions. Field experiments were carried out in four repeats with 14 m^2 sowing and 5 m^2 yield plot size. Seeds from each genotype are sown in four rows at 70 cm distance. Depending on the soil humidity during the critical phase of flowering - pod setting irrigation was applied once or twice in a norm of 70 - 90 m^3/da.

Laboratory experiments. Water stress was simulated by polyethelene glycol (PEG) with molecular weight (MW) 6000 (Duchefa Biochemie, The Netherlands). Seeds were plated for 24 hours on filter paper in Petri dishes containing distilled water as control and water solution of PEG in two concentrations 5 % w/v and 10 % w/v for evaluation the very first response to the osmotic stress. Water absorption was measured by the difference of initial seed weight and imbibed seed.
In vitro experiments. Seeds were washed with liquid soup surface disinfected by dipping in 70 % v/v ethanol for 1 min, followed by 30% v/v commercial bleach and rinsed three times in autoclaved distilled water. Seeds were plated *in vitro* on solidified basal Murashige and Skoog (MS) medium [Murashige and Skoog, 1962] Osmotic stress *in vitro* was simulated by adding PEG in two concentrations 5 % w/v and 8 % w/v to MS medium before autoclaving. Seeds were grown in culture rooms at temperature of $24^o \pm 2^o$ C. After 10 days seed germination percentage, seedling initial root length and shoot size were recorded. Twenty seeds in two replicates were used for each variant.

RESULTS AND DISCUSSIONS

The present study revealed differences in plant performance between the physiological and agronomical characteristics of the tested selected soybean lines. Vegetation period of plants in drought conditions is with two days shorter (102-117 days) than that of plants being irrigated (104-119 days). Four of the tested lines (L1, L2, L3, L5) did not show great differences between the vegetation period under irrigation and non-irrigation conditions like the Bulgarian standard variety Daniela. This may suppose higher tolerance to drought. Comparing the lines with the international standard Hodson, six of the lines (L1, L4, L5, L7, L8, L10) had longer vegetation period in simulated drought conditions which supposes that these lines have higher tolerance than cv Hodson. All the lines under water deficiency were more productive than the international standard Hodson (60 kg/da) but less yielded (with exception of Line 1) compared to the Bulgarian standard Daniela (155 kg/da). Comparing yield of tested lines under favourable and stress conditions less reduction of yield (by 59 kg/da, 80 kg/da and 80 kg/da) was recorded for Lines 1, 6 and 7, respectively (Figure 2). Reduction of yield for the both standards and the other lines was 110-120

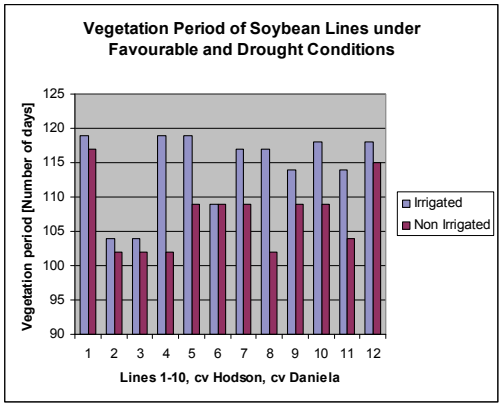

Figure 1. Vegetation period of soybean lines under favourable and drought conditions reps. with irrigation and without irrigation.

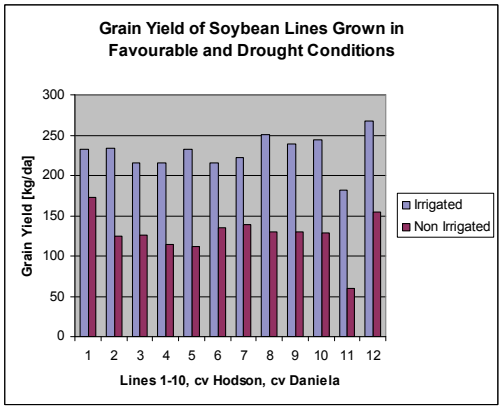

Figure 2. Yield of soybean lines grown in favourable and drought conditions resp. with irrigation and without irrigation.

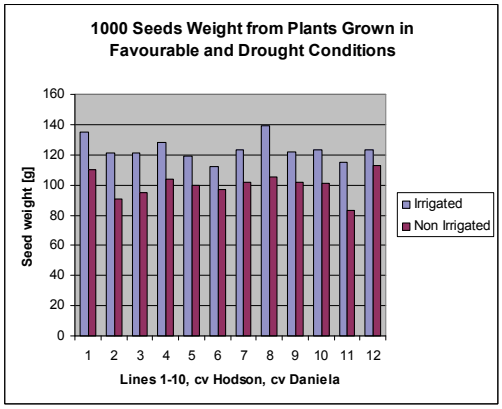

Figure 3. Seed yield of soybean lines grown in favourable and drought conditions resp. with irrigation and without irrigation.

kg/da. Concerning seed weight – the largest seeds were observed in line L8, and the smallest – in lines L2 and L3. The weight of 1000 seeds is around 20 g less in plants under drought stress (Figure 3).

Rate of water absorption by seeds during the first day differs among the genotypes in the range of 25 % (Figure 4). There is a tendency of slower absorption under osmotic stress caused by PEG presence in the soaking solution. Suppression of imbibing under stress is less profound in lines L1, L6, L7, L8, L9, and L10 which are ranked as more productive in the field. These lines had relatively bigger grains, too.

Seed germination under control conditions in the field varied among the lines in the range of 23 % marked by the lowest and the highest values of the both varieties, Daniela (59 %) and Hodson (82 %) (Figure 5). These margins were wider for germination *in vitro* on MS medium for the varieties Daniela (50 %) and Hodson (90 %) and even bigger for two lines with lowest values for L8 (30 %) and L1 (40 %). A tendency for positive correlation in two thirds of the cases between *in vivo* and *in vitro* germination was observed. Addition of PEG (both concentrations) to the MS medium inhibited germination of the both varieties, particularly Hodson. Lower number of germinated seeds was observed in half of the selected lines. Suppression caused by PEG (both concentrations) was not profound for most of the lines and even stimulation of germination was observed for some of them (L1, L5, L8, L9) but strict parallel with their yield in the field was not drawn.

In control conditions on MS medium root length of seedlings of the both varieties Daniela and Hodson was similar being 25 mm and 28 mm, respectively (Figure 6). Most of the lines had longer roots than the national and international standards. On MS medium containing 5 % PEG roots were longer in half of the lines (L2, L3, L7, L9, L10), shorter in two lines and without significant difference in the rest ones. Stimulation of root growth under the mild osmotic stress was observed for lines which had better performance in the field under drought conditions (L1, L6, L7). All seedlings developing on medium with the higher

concentration of PEG 8 % had shorter roots. However, suppression of root elongation was stronger in lines with lower tolerance to water deficiency in the filed experiments.

Shoot development was more diverse in both varieties and the lines as well. In the control size of Daniela shoots (17 mm) was nearly twice less than that of Hodson (40 mm) (Figure 7). Most of the tested lines had bigger initial stem ranging from 60 mm to 90 mm. Addition of PEG 5 % in the medium caused slight

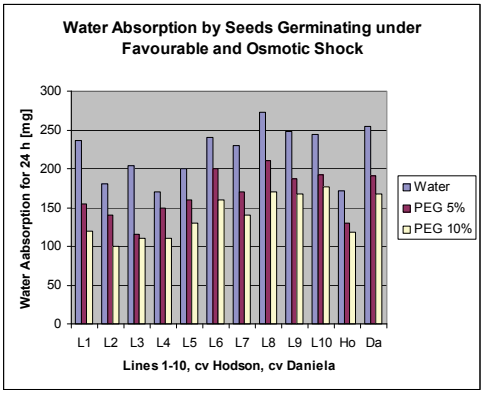

Figure 4. Water absorption by soybean seeds soaked for 24h in water (favourable conditions) and water solution of PEG (MW 6000) causing osmotic stress

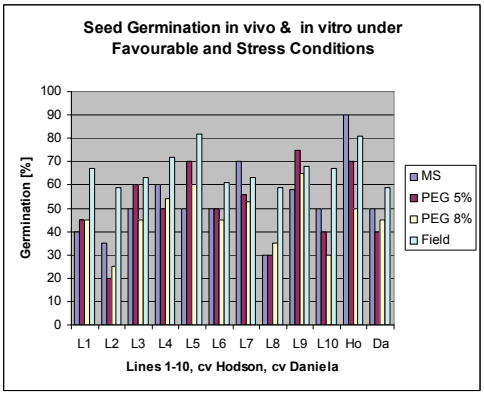

Figure 5. Seed germination *in vivo* in the field and *in vitro* under favourable conditions on MS medium and under osmotic stress on MS medium supplemented with PEG.

decrease of the growth in the both standards and different levels of depression in the lines.

Stronger osmotic shock caused greater suppression but stimulation in two lines as well (L2 and L10). It was difficult to draw any parallel between the shoot size and the productivity of lines in the field. However, concerning weight of 1000 seeds, some observations were made that growth of seedlings on media containing PEG was suppressed more in lines with smaller seeds (L7, L10, L3, L5).

Several authors reported the use of PEG for *in vitro* drought screening in crop plants [Maan and Punia 2006, Gopal et al, 2007; Kosturkova et al, 2008, Sakthivelu et al 2008b, Govindaraj et al. 2010]. Seed germination and seedling

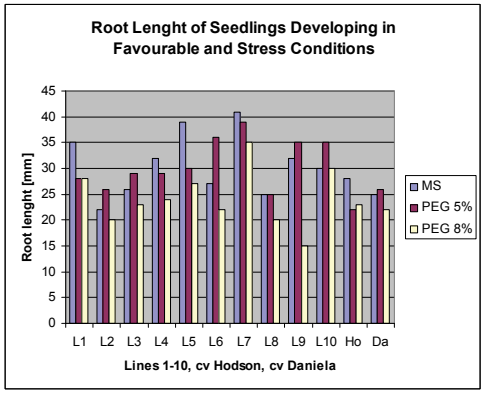

Figure 6. Root length of seedlings after 10 days of *in vitro* cultivation on MS medium (control) and under osmotic stress caused by PEG added to the MS medium

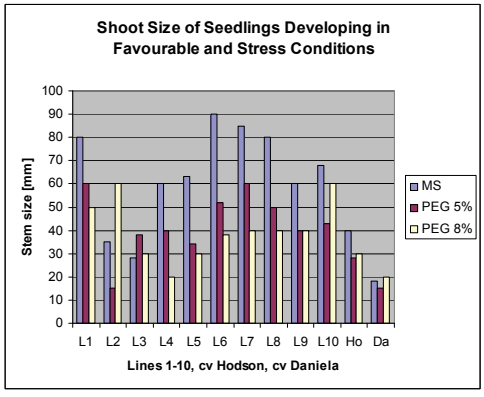

Figure 7. Shoot size of seedlings after 10 days of *in vitro* cultivation on MS medium (control) and under osmotic stress caused by PEG added to the MS medium

growth characters under different water stress regimes was studies for screening the drought tolerant genotypes under *in vitro* conditions.

Only 11 % seed germination with no seedling and shoot development was observed on MS liquid medium supplemented with 20 % PEG [Maan and Punia 2006]. Significant genotype

differences were recorded studying response of various traits (germination percentage, root length, shoot length, root / shoot ratio) to five different moisture stress levels by using PEG-6000 [Govindaraj et al. 2010].

This study is an extension to our previous experiments [Kosturkova et al, 2008] for comparing parameters of seedling development and plant performance in the field. Different criteria like seed germination, root length and stem size in water solution of PEG in different concentrations (2-15 %) were followed. Clear tendency of positive correlation between seed yield from one hand and seed germination and seedling growth stages from the other was observed allowing predicting of plant performance in field conditions under abiotic stress, and evaluation of germplasm for higher drought tolerance as a quick test for screening of desired genotypes.

CONCLUSIONS

The present study revealed that the selected lines obtained after induced mutation and hybridization were less effected by osmotic shock in field conditions. Valuable information was obtained about their yield. It was observed in the laboratory experiments that more productive lines absorb bigger amount of water during the first 24 h of germination under osmotic stress caused by different concentrations of PEG added to the water. This could be a criterion for preliminary quick screening for drought tolerance. Development of the selected lines in *in vitro* conditions gave valuable information about the effect of osmotic shock caused by presence of PEG in the media. Suppression and stimulation of seed germination *in vitro* caused by PEG was observed but strict parallel with the yield in the field could not be drawn and could not be used as a criterion. Similar is the situation concerning the diverse results concerning shoot size. However, seedling growth on media containing PEG was less suppressed in lines with larger seeds. Suppression of root elongation was stronger in lines with lower tolerance to water deficiency in the filed experiments. These two characteristics of *in vitro* development are possible candidates for criteria in developing models and quick tests for preliminary screening of drought tolerance. This study examines not only the effects of the drought conditions and the water access deficiency *in vitro* simulated by the high osmotic substance PEG on the development and productivity of new soybean genotypes. Here an attempt is made to find correlation between field performance and *in vitro* growth parameters what could not been seen in many of the publications examining possibilities for establishment of tests for quick screening.

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BIOLOGICAL CONTROL OF GROWN GALL IN HORTICULTURE

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Abstract

Crown gall is a widespread disease of cultivated culture all over the world. The bacterial pathogen Agrobacterium tumefacience colonize the xylem vessels of the plants.The tumors form in stems and roots of fruits culture. Large tumoursmay death of new plants in the nursery and in the plantation. Tumouregenesis causing root decay may be factor involved in the complex <replant disease> syndrome. Development of measures to controle crown-gall is carried out in direction for obtaining tumorless plants at the expens of preventive treatment of woundings so as to deteriorate the interaction of pathogen with the cell of host plant. Utilization the strain of soil inhibiting Pseudomonas fluorescens CR-330 D which synthesize the substances (bacteriocins) with preventing activity to nopaline and octopine strains of pathogenic agrobacteria. Liquid concentrate suspension of this bacteria - biological preparat < Paurin> -were applicated for preplanting treatment of rooted saplings. Using <Paurin> to treat apple MM-106 wilding prior to planting into fruit nursery allowed to decreases the outcome of apple saplings affected by crown gall down to 2 – 2.5 %. He is capable to compete with soil microflora and get reliably fixed in rhisosphere without phytotoxicity, which is rather advantageous for using biological control against repeated inoculation with crown gall pathogen.

Keywords: grown gall, bacterial strain, biological control.

INTRODUCTION

Microbial diseases affect numerous plant crops, resulting in huge loss and decrease in quality and safety of agricultural products. Crop disease control relies mainly on chemical pesticides that are currently subject to strong restrictions and regulatory requirements.
Microorganisms continue to play a highly significant role in the discovery of antiinfectives by producing a wide range of antimicrobials. Biocontrol of plant pathogens through bacterization technique has gained momentum, and *Pseudomonas*, one of the most diverse Gram-negative bacterial genera, had been selected for the study. *Pseudomonas fluorescens*, a plant growth-promoting rhizobacteria (PGPR) widely distributed in soil, has the ability to colonize the rhizosphere of host plants and produce a wide range of compounds inhibitory to a number of serious plant pathogens
Grown gall is diseases that are large number of plants-hostes. The causative agent of disease is gram-negative bacteria *Agrobacterium tumefaciens* (Smith and Towns) – the heterogene, presented in the nature by various strains with specific properties depending on a plant. In fruit nurseries of Moldova the tumors of a grown gall develop on roots of vegetative multiple stocks of an apple-tree, seed stocks of stone cultures and in places of graft (Figure 1).

Figure 1. Young apple plant with symptoms of grown gall

Their growth breaks of nutrients intended for plants. The quantity of saplings of an apple-tree on a stock of M-4 reaches 65.4%, on M- 9 –

24.3%, on MM-106 – 14.7%. At an grafting on seed stocks defeat of saplings of a pear makes 42.4%, a peach on almonds-3.2 %, an apricot on zherdele-5.8%, plums on a cherry plum-5 %, sweet cherry-5%. For this reason annual saplings in nursery often don't conform to the standard and after plant to a garden trees lag behind in growth and often perish, without having reached fructification. Many yellow leaves, troncs with less volume are visually observed. The attacks of plants in gardens with weak and average damaged roots make to 18% from total number of plants on one hectare. In some gardens of plum and a peach about 94% of trees are struck. Tumors are result of genetic interaction of agent parasite in plants cell, the consequence control of disease can't be solved by the standard chemical methods. Problem of science is searching of alternative systems of the prevention and phytopathogens a grown gall at the expense of application of ecologically safe biological preparations of a microbiological origin. The particular interest in this sense is representing the bacteria pick from rizosphere of plants.

Team of researchers from Institute of molecular genetics of the Russian Academy of Sciences on the basis of studying of numerous isolates of bacteria, allocated around a radical zone of plants, selected the strain of *Pseudomonas fluorescens* CR-330D producing the bacteriocins - the low-molecular substances of not proteinaceous nature suppressing activity of *Agrobacterium tumefaciens* (Khmel et al.,1995).

MATERIALS AND METHODS

Two-daily suspension of living bacterial cells *of Pseudomonas fluorescens* CR-330D, with the dilution 10^7 CFU/mL was applied to spraying plants before a sowing and landing material. Bacterial suspension of a strain antagonist of the causative agent of grown gall use for treatment the stouns of cherry, plum, peach, and also the layers of vegetative increased scrops of an apple rootstocks before plant in nursery, sprayed the places of woundings on mother bushes of rootstocks. Before planting of fruit trees in the garden the suspension of *Ps. fluorescens* used for bacterization of root system of a landing material year-old sapling fruit trees and added them with irrigation water. The biological preparation on the basis of strain *Pseudomonas fluorescens CR-330D*-"PAURIN" is registered by the State center for certification of phytosanitary production in the Republic of Moldova.

RESULTS AND DISCUSSIONS

In time of vegetative multiplication the rooting stem of rootstocks of apple MM 106, M 26, and the bacterization the roots of scions before landing of the first field of nursery was carried out. In time of dig ap the saplings from nursery the essential decrease galled roots on landing material of an apple-tree after treatment by PAURIN in relation to control was observed. The received results are presented in the Table 1.

Table 1. Influence of prelanding treatment of rootstocks with "PAURIN" on gall inhibition of roots the saplings in nursery (2011-2012 years)

Breeds	Rootstook	Variants	Quantity of plants	Galle-daplings piece	% of disease	Biological effect %
APPLE	MM-106	Control	1500	246	16,4	-
	M-26	Control	1420	112	7,9	-
	MM-106	Paurin	1450	37	2,6	84,1
	M - 26	Paurin	1210	16	1,3	83,5
Sweet cherry	Bitter cherry	Control	1620	68	4,2	-
		Paurin	1540	12	0,8	80,9
Plam	Wild plam	Control	2300	73	3,2	-
		Paurin	2300	9	0,4	87,5

Treatment the layers of vegetative stock of an apple-tree (M106 and M26) before landing in the first field of nursery by biopreparat "PAURIN" suspension promoted down to infection by grown gall to 2.6% -1.3% at 16.4 – 7.9% in control where landing of layers was made without biological product. Thus, biological efficiency of a biological product of PAURIN if compared to control against grown gall in time of raise saplings of an apple in nursery made 84.1% and 83.5% depending on a stock clone. After bacterization of stones of sweet cherry and a wild plum by preparation PAURIN suspension the yeield of healthy saplings of these breeds increased by 5-8 times in comparison with control. Biological efficiency of biofungicide in react against a grown gall at cultivation of saplings of sweet cherry and a cherry plum made 80.9% and 87.5%. Before landing of gardens with rooting apple saplings take bacterisation of root system

of saplings by PAURIN biological product on the total area of 86 hectares. Submitted data testify to considerable decrease in galled roots of apple trees in comparison with control (Table 2).

Table 2. Influence of prelanding treatment the roots of apple saplings by "PAURIN"on gall inhibition of roots the saplings in garden (2011-2012 years)

Breeds	Rootstook	Variants	Quantity of plants	Galled (piece)	% of disease	Biological effect %
Apple	MM-106	Control Control	1000	143	14.3	-
	M-26		1020	97	9.7	-
	MM-106	Paurin	1000	24	2.4	83.2
	M - 26	Paurin	1000	12	1.2	87.6

On materials of the table show that in control apple saplings on a rootstock of MM106 are infected with grown gall more (14.3%), than on M26–9.7% ,while after PAURIN treatment the number of plants with tumors decreased by 6-9 times and made 2.4%-1.2% at 16.4%-9% in control. Biological efficiency of biofungicide Paurin to controle against a grown gall when landing young saplings of an apple made 83.2% and 87.6%.

Pseudomonas fluorescens is plant growth promoting rhizobacteria (PGRB) inhabit the soil around the root surface and involved in promoting plant growth and development via production and secretion of varios regulatory in the vicinity of rhizosphere. PGRB facilitate the plant growth directly by either assisting resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant by controlling or inhibiting them and development in the forms of biocontrol agents (Munees and Kibret, 2014). This strain *Pseudomonas fluorescens* 330-D may be utilise as bio-inoculants to promote plant grow, improve the development of root system of plants. The use the suspension liquid of *Pseudomonas fluorescens* with titre 10^7 ucf/mL before the planting the samplings of vine in soil (50ml/kg soil), their application in time vegetation period by foliar pulverization has been studied (Table 3).

The dates of table show significantly increased plant dry weight, radicle and shoot length, shoot weight of own root samplings of vine.

Table 3. Influence the treatment of bacterial suspension *Ps. fluorescens* (titre10^7ufc/ml) on development the own root samplings of vine sort "Codrinskii"

Variants	Weight of roots (g) 1 plant (M±m)	Length (cm) of roots increase1 plant (M±m)	Length (cm) of shoot increase 1 plant	% to control
1	2	3	4	5
Control	7.58± 2.78	16.09± 2.32	41.04± 3,71	100
Ps. fluoresc. bring in soil	5.10± 0.63	16.30± 1,37	30.83± 3.18	75.12
Ps. fluorescens pulverisation of leavs	9.04± 2.26	23.80± 3.92	51.91± 8.85	126.5
Ps. fluorescens in soil + pulverization of leavs	10.68±0, 89	28.75±2.64	48.34± 4.19	117.7

It may be explained by properties of metabolits secreted by bacterial cells: phytohormone auxin (IAA/indol-3-acetic acid). This substance interferes with the many plant development processes because the endogenous pool of plant IAA may be altered by the acquisition of IAA that has been secreted by soil bacteria. IAA increases the rate of xylem and root development, controls processes of vegetative growth, initiated lateral and adventitious root formation, affects photosynthesis, pigment formation, increases root surface area. Interaction of *Ps. fluorescens* with the plant root or shoot can result in plant resistance to phytopathogenic bacteria. This phenomenon is called induced systemic resistance (Lugtenberg and Kamilova, 2009).

Pseudomonas fluorescens have been reported to produce antibiotic compounds like phenazines, pyrrole derivatives, indole derivatives, 2.4-diacetylphloroglucinol etc. (Ahil Sajeli Begum et al., 2014).

When laying a new garden apple trees (varieties Champion, Florina, Idared, Jonagold, grafted on rootstock M26), the roots of seedlings were treated with biologic PAURIN to prevent bacterial infection of plants root cancer (*Agrobacterium tumefaciens*).

To improve rooting and growth of young trees start to the working solution (5 L of the bacterial suspension per 500 liters of water) was added 0.02% trace elements. Exposure soaking the roots of seedlings was 30 minutes. After planting, watering the plants was carried out with an aqueous solution of a biological

product rate of 0,5 L Paurin one tone of water. Survival of plants in the garden was 100%. Counts and parametric measurements were performed in the middle of summer and the end of the growing season by 30 experienced trees. In early August in the embodiments of application of the bacterial strain showed a significant increase in the length of the young compared to the growth control. Area of the leaf treated plants were larger and differed by more intense color.

At the end of the growing season on apple varieties Florina, the total growth of shoots treated before planting trees ranged from 7.4 to 9.2 m on the majority of the trees in the garden evolved 16-18 shoots length reaches 40-76 cm. Seedling stem diameter spray averaged 3.4 cm, which is 47.8% more than in the controls (2,3 cm). In the control, all indicators are significantly inferior variant of the experiment is presented in Table 4.

Table 4. Growth-stimulating effect of a biological product Paurin in combination with trace elements at preplant roots apple varietie Florina

Parameters	Version control	Experimental variant
The average area of the leaf, cm^2	76,4	92,1
Average number of shoots, pieces	12,5	16,7
The average length of the shoot, (cm)	29,8	43,2
The average diameter of the trunk, (cm)	2,3	3,4
The average length of the total growth, (cm)	372,4	726,2

CONCLUSIONS

Preplanting treatment of vegetative stocks of an apple-tree and seeds of stoun-fruit cultures with "PAURIN" (titre 10^7 cfu/mL) reduced level of a disease of fruit plants in comparison with control (biological efficiency of 80.9% to 87.5%) depending on breed.

Biological product PAURIN application in complex with micronutrients at planting apple seedlings strongly stimulated plant growth.

Bacterisation by biofungicide "PAURIN" the roots of saplings of an apple before landing in a garden reduced number of no galled plants to 2.4-1.2% in comparison with control of 14.3-9.7%. The received results testify that the biological product PAURIN can be applied successfully to prelanding bacte-rization of root system vegetative sets of apple rootstocks and seedlings of stoun fruit crops in nursery and saplings at a laying of young gardens to control against a grown gall.

Use of these properties, along with primary development of the microorganism on surfaces of stones, roots, shanks, a layer, tronc plants and in a rizosfer creates a physical and biochemical obstacle to development of pathogenic strains of the agent of a root cancer, improve the development of root system and shoots of plants.

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ANTIFUNGAL ACTIVITY OF SOME LACTIC ACID BACTERIA ISOLATED FROM MATERIALS OF VEGETAL ORIGIN

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Abstract

Lactic acid bacteria have been considered to be promising natural biological antagonists for mycotoxigenic fungi that contaminate various food commodities. Apart from important economic losses, mycotoxin producing fungi have harmful effects on human and animal health. The public necessity for high quality food, without addition of chemical preservatives, with extended shelf life, determined the search of new strains of bacteria able to produce lactic acid and their use to control the fungal growth of plant pathogenic and mycotoxigenic species. The effect of 27 strains of lactic acid bacteria have been assayed on the growth of Alternaria solani plant pathogenic fungi isolated from heavily infected tomatoes as well as on potential mycotoxin producing fungi Aspergillus ochraceus isolated from tomato roots grown in greenhouse and Penicillium digitatum isolated from infected oranges. Interaction between lactic acid bacteria and selected mycotoxigenic fungi was tested by overlay assay method. Discrete spots of liquid cultures of tested lactic acid bacterial strains were placed on MRS agar and after incubation, overlaid with soft PDA containing propagules of tested fungi. Data were collected after 48 hours concerning the diameters of clear visible inhibition zones of fungal growth around the lactic acid bacterial strain spots. Preliminary tests have shown that selected lactic acid bacteria could inhibit the development of test fungi. A number of 11 lactic acid bacterial strains have shown antifungal activity on both Aspergillus ochraceus and Penicillium digitatum. Another 5 strains of lactic acid bacteria did not inhibit any of the two mycotoxigenic fungal species. The effect of lactic acid bacteria on plant pathogenic species Alternaria solani did not provide conclusive results and need further study. The results could be used in future experiments for obtaining performing biological agents with application in food safety.

Keywords: *antifungal activity, lactic acid bacteria, mycotoxigenic fungi.*

INTRODUCTION

Various food commodities can be contaminated by a wide spectrum of filamentous fungi, leading to important economic losses.

Fungal infection causes food spoilage with high impact on organoleptical properties. The contamination of various food commodities with mycotoxigenic fungi has harmful effects on human and animal health (Bryden, 2012).

The major species of mycotoxigenic fungi responsible for food spoilage belong to genera *Aspergillus, Fusarium* and *Penicillium* (Gerez et al., 2009). Literature cites various effects of mycotoxins that are highly toxic metabolites synthetsised by various fungal species on human health such as carcinogenic, teratogenic, immunotoxic, neurotoxic, hepatotoxic and nephrotoxic (Bryden, 2007; Wild and Gong, 2009).

Prevention of fungal growth on various food commodities is the best method of protection from the harmful effects on human and animal health. Several physical and chemical methods have been developed to control fungal growth, but some moulds acquired the ability to resist chemical treatment and some preservatives.

Due to general public demands for preservatives free food and feed, biopreservation, the control of one organism by another, has become an alternative that was subject to further investigation (Schnurer and Magnusson, 2005).

Lactic acid bacteria have been considered to be promising natural biological antagonists for

mycotoxigenic fungi that contaminate various commodities (Magnusson et al., 2003; Trias et al., 2008). Dalie et al. (2010) reviewed the main mechanism involved in antimicrobial efficiency of lactic acid bacteria: the yield of organic acids, competition for nutrients and production of antagonistic compounds. Representatives belonging to genera *Lactococcus* and *Lactobacillus* and, to a lesser extent, to *Pediococcus* and *Leuconostoc* are recognized for their ability to prevent and limit the growth of mycotoxigenic fungi by the synthesis of antimicrobial proteins called bacteriocins (Savadogo et al., 2006).

The public necessity for high quality food, without addition of chemical preservatives, with extended shelf life, determined the search for new strains of lactic acid bacteria that are able to control the fungal growth of plant pathogenic and mycotoxigenic species.

The aim of this work was to assess the effect of different lactic acid bacterial strains on growth of mycotoxigenic fungal species and to select performant strains as biopreservatives for fruit and vegetable commodities.

MATERIALS AND METHODS

The 27 strains of lactic acid bacteria were obtained from dr.Medana Zamfir, Institute for Biology Bucharest and have been assayed on the growth of three fungal isolates from fruits and vegetables: *Alternaria solani*, a plant pathogenic fungus isolated from heavily infected tomatoes, and two potential mycotoxin producing fungi – *Aspergillus ochraceus* isolated from tomato roots grown in greenhouse, and *Penicillium digitatum* isolated from infected oranges. Interaction between lactic acid bacteria and selected mycotoxigenic fungi was tested by overlay assay method (Magnusson et al., 2003). Discrete spots of liquid cultures of tested lactic acid bacterial strains were placed on MRS agar and after 24 h incubation at 30^{o}C, overlaid with soft PDA containing propagules of tested fungi. Data were collected after 48 hours incubation at 25^{o}C concerning the diameters of clear visible inhibition zones of fungal growth around the lactic acid bacterial strain spots. Values obtained represented the mean of three replicates per assay.

RESULTS AND DISCUSSIONS

Interaction between the 27 lactic acid bacterial strains and potential mycotoxigenic *Aspergillus ochraceus* isolated from greenhouse tomato roots (Figure 1) ranged between no visible inhibition and clear visible inhibition zone as shown in the aspect of petri plates (Figure 2). The assay results (Figure 3) showed that 4 lactic acid bacterial strains (15, 61, 122 and 113) had inhibition zone diameter over 25 mm and 18 strains below this value. Five strains had no inhibition effect on mycotoxigenic fungus *Aspergillus ochraceus*.

Figure 1. *Aspergillus ochraceus* - pure culture

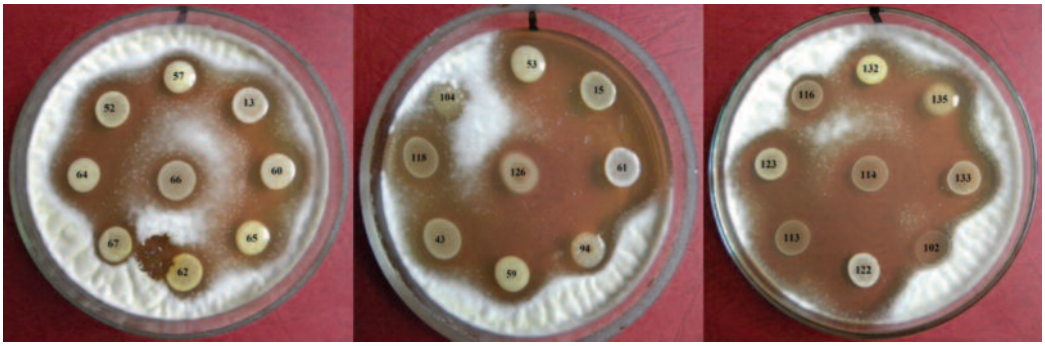

Figure 2. Petri plates with 27 lactic acid bacteria tested against *Aspergillus ochraceus*

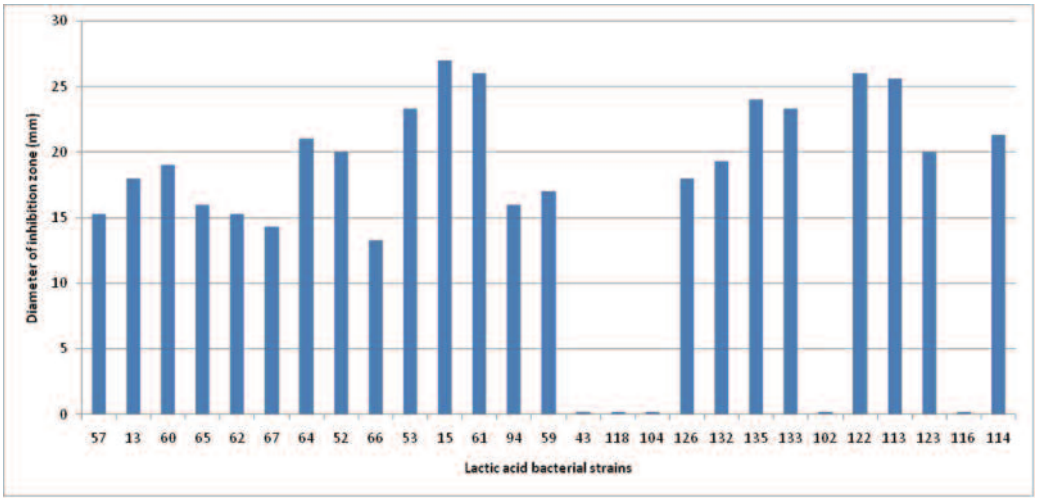

Figure 3. Comparative inhibition zone diameter of 27 lactic acid bacteria tested against *Aspergillus ochraceus*

Our research is in concordance with similar assay carried out by Munoz et al. (2010) when the growth of mycotoxin-producing *Aspergillus nomius* VSC 23 was inhibited by *Lactobacillus fermentum* and *Lactobacillus rhamnonus* in both consecutive and simultaneous inoculation. Djossou et al. (2011) showed that ten lactic acid bacterial strains isolated from silage coffee pulp demonstrated antifungal effect against ochratoxigenic mould *Aspergillus carbonarius*. Two of them belonging to *Lb.plantarum* showed an inhibition with clear zone area between 20-30 mm diameter for three assays against two isolates of test mould.

The inhibitory effect of lactic acid bacterial strains was stronger when they assayed against mycotoxigenic *Penicillium digitatum* isolated from orange (Figure 4). Petri plates showed large inhibition zones around 17 lactic acid bacterial strains (Figure 5). These strains presented inhibition zone diameters over 25 mm and 4 strains had lower values (Figure 6). No inhibitory effect was registered for 6 lactic acid bacterial strains. Research carried out by Trias et al. (2008) showed that 4 lactic acid bacterial strains isolated from fresh fruits and vegetables were able to inhibit the development of infection produced by *Penicillium expansum* on apples by 20%. Organic acids were the

preferred mediators of inhibition. Inhibitory effect of lactic acid bacteria on development of another *Penicillium* isolate (*P. nordicum* BFE 487) was demonstrated by modified agar spot assay (Blagojev et al., 2012). Concentrated culture filtrate of *Lactobacillus plantarum* 21B isolated sourdough presented efficient antifungal activity against *P. corylophilum*, *P. roqueforty*, *P. expansum*, *Aspergillus niger*, *A.flavus* and *Fusarium graminearum* due to the capacity to produce phenyllactic and 4-hydoxy-phenyllactic (Lavermicocca et al., 2000).

Figure 4. Orange infected with *Penicillium digitatum*

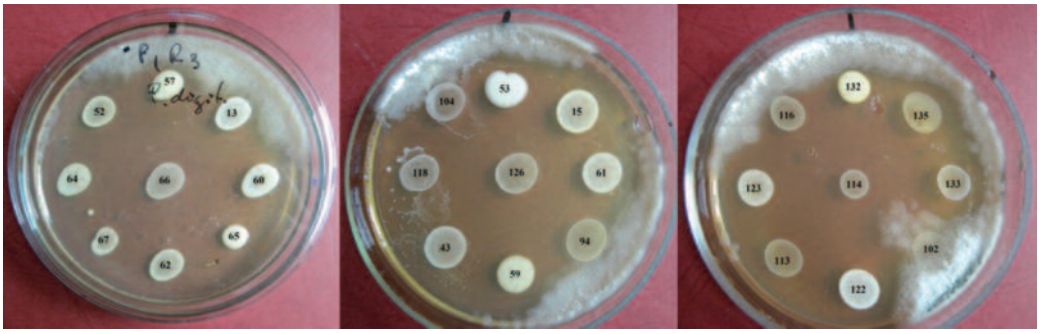

Figure 5. The inhibitory action of 27 lactic acid bacteria tested against *Penicillium digitatum*

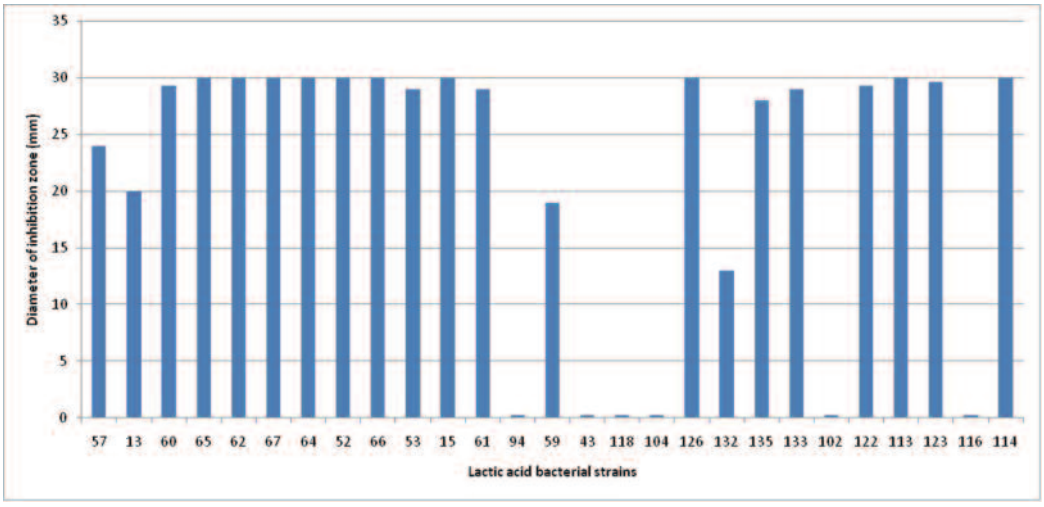

Figure 6. Comparative inhibition zone diameter of 27 lactic acid bacteria tested against *Penicillium digitatum*

The results of the assay against *Alternaria solani* isolated from tomatoes (Figure 7) was not conclusive enough because the fungus

sporulation needs a longer incubation period than the 48 hours until the reading of inhibition diameters was done in the present assay.

Figure 7. Tomato infected with *Alternaria solani*

As a general aspect in the assay carried out, a number of 11 lactic acid bacterial strains have shown antifungal activity on both *Aspergillus ochraceus* and *Penicillium digitatum* (strains 15, 52, 53, 60, 64, 113, 114, 122, 123, 133, 135). Four of these strains (15, 61, 113 and 122) presented inhibitory zone diameters over 25 mm when tested against both potential mycotoxigenic fungal species. Another 5 strains of lactic acid bacteria (43, 102, 104, 116 and 118) did not inhibit any of the two mycotoxigenic fungal species. Literature also cites data from agar well diffusion assay where one isolate *Lactobacillus lactis* subsp. *lactis* CHD 28.3 showed a broad spectrum of antifungal activity against *Aspergillus flavus* IARI, *Aspergillus flavus* NCIM 555, *Aspergillus parasiticus* NCM 898 and *Fusarium* spp., six strains showed inhibitory effect against one fungal species and other lactic acid bacterial strains didn't present the capacity to inhibit the growth of any mycotoxigenic fungal species (Dalie et al., 2010).

CONCLUSIONS

A number of 11 lactic acid bacterial strains have shown antifungal activity on both *Aspergillus ochraceus* and *Penicillium digitatum.*

The lactic acid bacterial strains 15, 61, 113 and 122 presented the highest antifungal activity against both potential mycotoxigenic fungi.

Another 5 strains of lactic acid bacteria did not inhibit any of the two mycotoxigenic fungal species.

The effect of lactic acid bacteria on plant pathogenic species *Alternaria solani* did not provide conclusive results and need further study.

The results could be used in future experiments for obtaining performing biological agents with application in food safety.

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ECOTECHNOLOGY FOR FULLY RECOVERY OF FRUIT TREE WASTES THROUGH CONTROLLED CULTIVATION OF EATABLE MUSHROOMS

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Abstract

Year by year, large amounts of fruit tree wastes, such as etiolated leaves, woody wastes (old branches, dried trunks, unproductive shoots) resulting from annual pruning or cleaning of fruit trees are produced in many orchards becoming in the end huge sources of pollution. The main objective of this research work was focused on the development, implementation and testing of experimental model for ecological recycling of fruit tree wastes through controlled cultivation of the eatable mushroom species Lentinus edodes, Pleurotus ostreatus and P. eryngii on substrates made of leaves, branches and trunks of apple, plum and apricot trees. All three mushroom species were used as pure culture to be grown on different variants of substrates made of fruit tree wastes, namely etiolated dried leaves, shoots and trunks as well as distilled marc of fruits after their use as raw matter for alcohol fermentation and distillation. All substrates were steam sterilized at 123 °C, 50 min, and after that they were inoculated with the pure mushroom cultures. After 30-50 days of incubation at 23 °C, depending on the mushroom species, the first buttons emerged and after one or two days they developed the mature fruit bodies. After a period of 120 days mushroom growing on the substrates made of fruit tree wastes the results showed a fast development of Pleurotus species, respectively P. ostreatus was faster than P. eryngii, and had registered a better productivity than L. edodes. The implementation of such an experimental model of green technology will determine the full recovery of all fruit tree wastes produced in orchards (leaves, branches, wood stems) and their fast recycling through the natural food chains of the whole organic matter. Inside the newly formed food chains, the fruit tree wastes were the basic link on which the eatable mushroom species, such as L. edodes, P. ostreatus and P. eryngii that decompose lignin, hemicellulose and cellulose have synthesized natural organic compounds through the carpophores that were finally used as food products by the human consumers.

Keywords: *ecotechnology, fruit tree wastes, mushroom cultivation, L. edodes, P. ostreatus, P. eryngii*

INTRODUCTION

Agricultural works related to the fruit tree growing as well as fruit processing have generally been matched by a huge formation of wide range of cellulosic and woody wastes that accumulate every year in the most orchards all over the world (Beguin and Aubert, 1994; Verstraete and Top, 1992).

Many of these lignocellulose wastes cause serious environmental pollution effects, if they are allowed to accumulate in the orchard or much worse to be burned on the soil. So far, the most recent approaches on lignocellulose-degrading procedures have been directed to the applications of environmental protection, with emphasize on the bioremediation technology of lignin and cellulose decomposition, including their biodegradation and bioconversion through the controlled cultivation of eatable mushrooms (Carlile and Watkinson, 1996; Smith, 1998).

In this respect, the main aim of this work was to find the best eco-technology of recycling the fruit tree wastes by using them as growing sources for eatable mushrooms in order to extend the food chain in orchard ecosystems (Moser, 1994).

MATERIALS AND METHODS

Eatable mushroom species and culture media

According to the main purpose of this work, three fungal species from Basidiomycetes, namely *Lentinus edodes*, *Pleurotus eryngii* and *P. ostreatus* from University of Pitesti culture

collection were used as pure cultures in experiments. The stock cultures were maintained on malt-extract agar (MEA) slants at 25 °C for 5-7 days and after that they were stored at 4 °C. The fungal cultures were grown in 250-mL flasks containing 100 mL of MEB medium (20% malt extract, 2% yeast extract and 20% peptone solution in pure water up to 100%) at 23 °C on rotary shaker incubators at 110 rev min^{-1} for 5-7 days (Petre and Petre, 2008).

Methods used in experiments

Preparation of liquid fungal inoculum

The mushroom cultures for experiments were prepared by inoculating 100 mL of culture medium with 3-5% (v/v) of the seed culture and then cultivated at 23-25 °C in rotary shake flasks of 250 mL. The experiments were conducted under the following conditions: temperature, 25 °C; agitation speed, 90-120 rev min^{-1}; initial pH, 4.5–5.5. The seed cultures were transferred on culture medium and grown for 7–12 days (Petre and Petre, 2013a).

Incubation of mushroom cultures

The experiments of this research project were achieved by growing all the mushroom species in special growing chambers, where all the culture parameters were kept at optimal levels in order to get the highest production of fruit bodies (Raaska, 1990; Chahal and Hachey, 1990). During the experiments, the influence temperature, pH level, inoculum size and volume and incubation time on mycelia net formation and especially, on fruit body induction were investigated (Petre et al, 2012; Stamets, 1993; Chahal, 1994).
All the culture substrates for mushroom growing were inoculated using liquid inoculum with the age of 5–7 days and the volume size ranging between 3-7% (v/w). During the period of time of 18–20 days after this inoculation, all the mushroom cultures had developed a significant biomass on the culture substrates made of fruit wastes (Petre and Petre, 2012).
The optimal temperatures during the incubation for mycelia growing were maintained between 23–25 °C. The whole period of mushroom growing from the inoculation to the fruit body formation lasted between 30–60 days, depending on each fungal species used in experiments (Petre et al, 2012).

Preparation of mushroom culture substrates

After inoculum preparation, the seed cultures were cultivated on special growing media made of lignocellulosic materials resulted from apple, plum and apricot wastes that were used as substrates for mushroom development (Petre and Petre, 2013b). These fruit tree wastes were mechanical pre-treated to breakdown the lignin and cellulose structures to be more susceptible to the enzyme actions (Leahy and Colwell, 1990; Glazebrook et al., 1992). All these pre-treated wastes were disinfected by steam sterilization at 123^{o} C for 50 min. The final composition of culture substrates used in experiments is presented in Table 1.

Table 1. Composition of substrate variants (%) made of fruit tree wastes

Substrate ingredients (w/v)	Composition of substrate variants (%)		
	Apple wastes (S1)	Plum wastes (S2)	Apricot wastes (S3)
Fruit marc collected after alcohol distillation	35	25	30
Fruit tree sawdust of milled branches	20	15	17
Etiolated dried leaves of fruit trees	10	17	15
Wheat bran	4.5	3.5	3
Chalk	3	3	3.5
Gypsum	2.5	1.5	1.5
Pure Water	25	35	30

As control samples for each variant of culture substrates, used for the experimental growing of all mushroom species were used only woody chops of oak kept in pure water three days before the experiments and then sterilized by steam at 123 °C, 50 min. in order to be disinfected, inoculated with liquid mycelia of the same mushroom species and incubated up to 60 days at 23 °C.

Preparation of Mushroom Spawn

A significant example to describe the mushroom spawn preparation is the following:

3,000 g of fresh apple marc collected after alcohol distillation, 2,000 g of apple tree sawdust made of milled branches and 1,000 g of etiolated dried leaves belonging to apple trees were mixed in 3,000 L of pure water by adding the following supplements: 500 g wheat bran, 300 g of chalk, 200 g of gypsum, in order to obtain three variants of natural substrates for mushroom spawn growing. The mixed ingredients were loaded in 1,000 mL glass jars and 10 kg heat resistant polyethylene bags, being then steam-sterilized at 123 °C, for 50 min. After cooling, when the temperature decreased below 35°C, all recipients containing sterilized substrate were inoculated with different amount of inoculum developed in liquid medium (Petre and Petre, 2013).

Then, all glass jars and heat resistant polyethylene bags filled with sterilized and inoculated substrates were incubated at 23-25 °C, until the spawn fully colonized the whole growing contents, prepared as it was previously shown (Figures 1 and 2).

Figure 1. Glass jars filled with *P. ostreatus* mycelia grown on apple wastes

Figure 2. Polyethylene bags filled with *L. edodes* mycelia grown on apple wastes

RESULTS AND DISCUSSION

During the whole processes of fruit body formation and development, the culture parameters were set up and maintained at the following levels depending on each mushroom species: air temperature 15–17 ^{o}C, the air flow volume 5–6 m^3/h, air flow speed 0.2–0.3 m/s, the relative moisture content 80–85%, light intensity 500–1,000 luces for 8–10 h/d.

According to the main results of this research work, the eco-technological procedure of recycling the fruit tree wastes by using them as growing sources for eatable mushrooms *L. edodes, P. ostreatus* and *P. eryngii* was established as it can be seen in Figure 3.

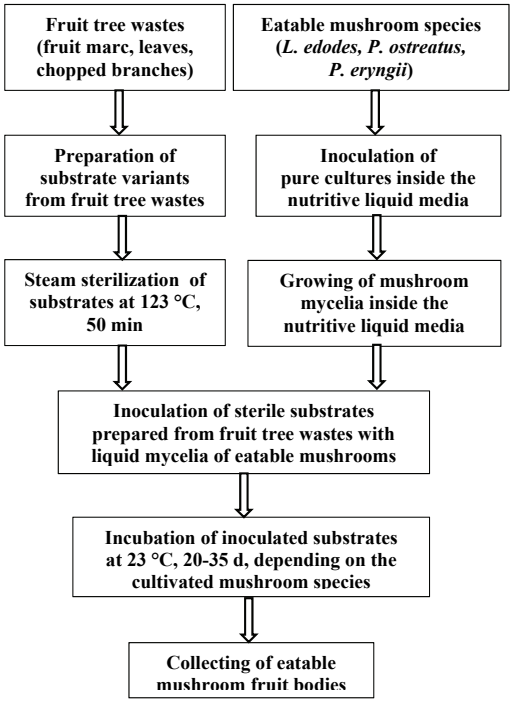

Figure 3. The ecotechnology of recycling the fruit tree wastes by using eatable mushrooms

Amongst certain physiological properties, the incubation temperature correlated with the pH value, as well as the age and volume of mycelia inoculum may play an important role in mushroom hyphae development as well as in fruit body formation (Stamets, 1993; Ropars et al., 1992; Carlile and Watkinson, 1992; Wainwright, 1992).

In order to study the effects of initial pH correlated with the incubation temperature upon fruit body formation, *P. ostreatus*, *P. eryngii* and *L. edodes* were cultivated on substrates made of fruit tree wastes at different initial pH values (4.5–6.0). To find the optimal incubation temperature for mycelia growth, these eatable mushrooms were cultivated at different temperatures ranging from 20-25 °C (Table 2).

To test the influence of inoculum age as well as inoculum volume during cultivation cycles, the same eatable mushroom species *P. ostreatus*, *P. eryngii* and *L. edodes* were grown on substrates made of fruit tree wastes during different time periods between 30 and 60 days, varying the inoculum volume (5-7 v/w), as it is shown in Table 3 and Table 4.

Table 2. The influence of initial pH and temperature upon fruit body formation of *P. ostreatus*, *P. eryngii and L. edodes*

pH (pH units)	Temperature (^{o} T)	Final Weight of the Fresh Fruit Body (g / kg substrate)		
		P. ostreatus	*P. eryngii*	*L. edodes*
4.5	18	175±0.23	191±0.10	180±0.02
5.0	21	193±0.15	203±0.05	297±0.14
5.5	23	198±0.10	195±0.15	351±0.23
6.0	26	181±0.12	179±0.12	280±0.03
6.5	29	173±0.09	105±0.23	257±0.15

Table 3. The effect of inoculum age upon fruit body formation of *P. ostreatus*, *P. eryngii and L. edodes*

Inoculum Age (h)	Final Weight of the Fresh Fruit Body (g /kg substrate)		
	P. ostreatus	*P. eryngii*	*L. edodes*
264	123±0.14	128±0.05	135±0.23
240	141±0.10	150±0.28	157±0.17
216	154±0.12	195±0.90	193±0.15
192	155±0.23	221±0.25	215±0.05
168	169±0.37	235±0.78	241±0.07
144	210±0.20	248±0.03	259±0.12
120	230±0.15	253±0.05	264±0.21
96	215±0.09	230±0.15	253±0.10
72	183±0.05	205±0.23	210±0.05

Table 4. The effect of inoculum volume upon fruit body formation of *P. ostreatus*, *P. eryngii and L. edodes*

Inoculum Volume (v/w)	Final Weight of the Fresh Fruit Body (g /kg substrate)		
	P. ostreatus	*P. eryngii*	*L. edodes*
7.0	234±0.12	215±0.20	220±0.05
6.5	245±0.15	248±0.23	251±0.20
6.0	253±0.15	257±0.07	280±0.15
5.5	243±0.12	235±0.03	247±0.07
5.0	255±0.23	215±0.15	235±0.03

All data are the means ± S.D. of triple determinations.

Analysing the registered results of the initial pH and temperature upon the mushroom fruit body formation, the optimal pH levels were determined as being 5.0–5.5. It can be noticed that the highest values of mushroom production was obtained for *L. edodes* species, when it was grown on the fruit waste substrates having the initial pH 5.5 at 23 °C.

The inoculum age of 120 h as well as an inoculum volume of 6.0 (v/w) had beneficial effects on the fungal biomass production. The whole period of mushroom growing from the inoculation to the fruit body formation lasted between 20–35 days depending on each mushroom species used in experiments. The most significant influence of the volume as well as age inoculum upon the final weight of mushroom fruit bodies was registered for the cultures of *L. edodes*, on the next places being *P. ostreatus* and *P. eryngii* species.

Regarding the whole duration of the mushroom cultivation cycles from all the tested species during present experiments, *P. ostreatus* was registered as being the fastest mushroom culture (20–25 days), followed by *P. eryngii* (25–30 days) and finally, *L. edodes* as the longest mushroom culture (30–35 days).

CONCLUSIONS

1. From all eatable mushroom species which were tested in our experiments, *P. ostreatus* was registered as the fastest mushroom culture (25–30 days), then *P. eryingii* (35–45 days) and finally, *L. edodes* as the longest mushroom culture (45–60 days).
2. The inoculum age of 120 h as well as an inoculum volume of 6.0 (v/w) have beneficial effects on the fungal biomass production.
3. The optimal pH values were between 5.0–5.5 correlated with the best temperature levels of 21-23° C for higher mushroom fruit body production.
4. After a period of 120 days mushroom growing on the substrates made of fruit tree wastes the results showed a fast development of *Pleurotus* species, respectively *P. ostreatus* was faster than *P. eryngii*, and had registered a better productivity than *L. edodes*.
5. The implementation of such an experimental model of green technology will determine the fully recovery of all fruit tree wastes produced in orchards (marc, leaves, branches, wood stems) and their fast recycling through the natural food chains of all organic matter.

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STUDIES CONCERNING THE *IN VITRO* CULTIVATION OF SOME INDIGENOUS MACROMYCETE SPECIES

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Abstract

For long time mushrooms presented interest for consumption as food, as traditional medicine or in bioremediation, due to their nutritional, antioxidant, antimicrobial and therapeutic values. The valorisation of indigenous species of mushrooms both for research or practical applications is a goal for researchers all over the world and requires well characterized collections. Despite the importance of macromycetes, few collections are developed in Europe and they contain mainly standard strains. The existence of extremely rich mycoflora in the forests and grasslands of Romania creates favourable conditions for initiating a scientific approach with definite applicability, in order to diversify the cultivated assortment by introducing of some new indigenous mushroom strains and species. For example, several species from spontaneous mycoflora of Romania have nutritional value or could be used as therapeutic agents: Agaricus campestris, Clytocibe geotropa, Boletus edulis, Hericium coraloides, Pleurotus ostreatus, Armillaria mellea, Flammulina velutipes, Coprinus comatus, Laetiporus sulphureus, Ganoderma applanatum, Ganoderma lucidum etc. Exploitation of such fungal species by introducing into controlled culture may diversify the range of mushrooms for domestic market or for obtaining of innovative products. For this reason the aim of our work was the realization of a fungal germplasm collection based on indigenous isolates obtained from natural ecosystems. 40 mushroom varieties belonging to at least 35 species were isolated and in vitro propagated on MEA or PDA media. The possibility of submerged cultivation of mycelium for some species as well as antimicrobial potential was also examined. Using specific biotechnologies the new mushroom strains included in our collection will be tested for genetic variability and for other important characteristics (producing of enzymes, degradation abilities, antimicrobial and/or antitumor properties etc).

Keywords: *fungal germplasm collections, in vitro cultivation, Romanian mycoflora, wild edible medicinal mushrooms*

INTRODUCTION

Edible mushrooms are considered food with a high nutritional value and some of them therapeutic. Spread all over the world, fungi (kingdom Fungi / Mycota) have almost 200 thousand species, subspecies and varieties. It is estimated that there are about 140 000 mushroom species on earth and only 22 000 of these are known (Alves et al., 2012). Until now a number of 2000 mushroom species are reported in Romania (Popescu, 2006). In fungi, besides the composition rich in high in fiber, quality protein, vitamins and minerals, there are biologically active compounds such as: b-glucan, polysaccharides, phenolic compounds, which are involved in antitumor, antiviral, immunomodulatory, hypoglycemia, cholesterol lowering, antioxidant, anti-inflammatory and antimicrobial activities(Kalac et al., 2012.). Mushrooms are not only found to have medicinally properties but also found to have significant antioxidant capacity (Aggarwal et al., 2012). Therefore, mushrooms can be used both as a food ingredient and in pharmaceutical industry. In spontaneous flora of Romania, among of edible mushrooms with therapeutic value are: *Agaricus campestris, Cantharelus cibarius, Pleurotus ostreatus, Armillaria mellea, Flammulina velutipes, Coprinus comatus, Laetiporus sulphureus, Ganoderma lucidum, Ganoderma applanatum, Laetiporus sulphureus* etc. In Asian countries some of these mushrooms are cultivated widely. An example is *Flammulina velutipes*, a fungus species which in China, according to Yang (1986) and Wang (1995) has been cultivated since the 8th century. Experience in the field

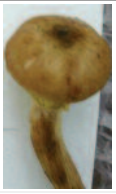
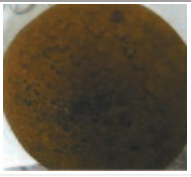
and the existence of extremely rich mycoflora in the forests and grasslands of Romania creates favourable conditions for initiating a scientific approach with definite applicability, in order to diversify the cultivated assortment by introducing of some new indigenous mushroom strains and species and to valorise the large quantities of the existing recyclable agro-forestry materials. Using specific biotechnologies the new mushroom strains obtained and the genetic variability of this precious biological material will be stored / preserved through the establishment of a mycelium strains collection, which can be developed further. In this context, the goal of our work was to obtain the fungal inocula (mycelium and young fruiting bodies) from native mushroom species and initiation of a fungal germplasm collection originated in Romanian mycoflora.

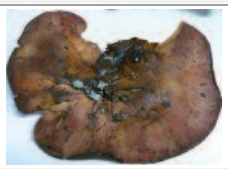
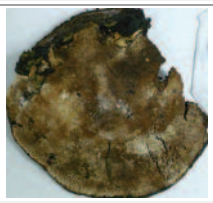
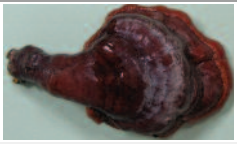
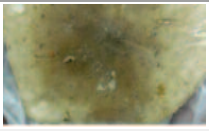
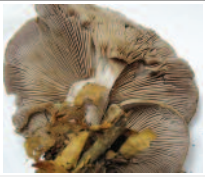
MATERIALS AND METHODS

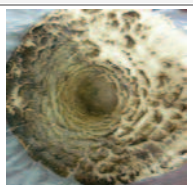
Materials. 18 wild edible and/or medicinal mushroom species were harvested from the different regions of Romania (Sinaia, Chitila, Teleorman, Voinesti and Bucharest) (Table 1) and were authenticated by specialists (Dr. ing. Ioana Tudor and Dr. ing. Paul Covic).

***In vitro* cultivation**.Initially, fruiting bodies of the selected mushrooms were surface washed with sterile distillate water. The mycelia were isolated by aseptically removing a small piece of mycelium from inside. The pieces were transferred in to Petri dishes on 2% malt extract agar or PDA (potato-dextrose-agar) media. The plates were incubated at 25^0C in the dark. The mycelia growing on the medium surface were picked and transferred to PDA or malt extract media. The pure cultures were kept on these media for further use.

Table 1.Wild edible medicinal mushroom species from Romanian mycoflora and their properties

Species	Location	Properties	Morphological aspect
Agaricus campestris	Voinesti forest	Edible Medicinal - enhance the secretion of insulin, treatment of ulcers .	
Armillaria mellea	Fundata forest Chitila forest	Edible Medicinal (Meniere's Syndrome, vertigo, epilepsy, neurasthenia and hypertension), antioxidant, antimicrobial (Jiangsu Journal of TCM. 1980; Donnelly et al, 1985 Obuchi et al., 1990; Kalyoncu et al., 2010)	
Boletus edulis	Fudata forest	Edible Medicinal (antiviral, anti-inflammatory, antimicrobial, antioxidant) (Chang and Miles, 2004; Shu-Yao T, 2007)	
Bovista plumbea	Fundata forest	Edible Medicinal (head affections, diabetes, ovarian cysts, acne) (Jain, 1997)	
Coprinus sp.	USAMV-Bucharest campus	Edible with caution - poisonous if consumed with alcohol Medicinal – anti-tumor (Ohtsukaet al., 1973), anti-fungal (Florianowicz, 2000)	

Fistulina hepatica	Chitila forest	Edible Medicinal- antibacterial, antioxidant (Ribeiro et al., 2007)	
Flammulina velutipes (Enokitake)	Fundata forest	Edible Medicinal- regulation of the immune system, cancer immunotherapy, antioxidant (Bao et al., 2009)	
Ganoderma applanatum	Chitila forest	Medicinal - antibacterial (including *E. coli* and *Staphylococcus aureus),* anti-inflammatory, and anti- tumor, tumor inhibition, immune stimulation, homeostasis, esophageal carcinoma, rheumatic TB and as an anti-viral for HIV, etc. (Acharya et al., 2005).	
Ganoderma lucidum	Teleorman forest	Medicinal –antioxidant, cardiovascular and immune systems, hepatoprotective, anticancer, antibacterial (Clinical Biochemistry and Nutrition, 2007)	
Hericium coralloides	Fundata forest	Edible Medicinal-antibacterial and nematicidal activities, anti-inflammatory properties, anti- tumours, nerve regenerator in Muscular Dystrophy, Parkinson's Disease, Alzheimer's and Dementia (Mori et al., 2008; Mizuno et al, 1992; Mizuno and Takashi, 1995).	
Lepista nebularis (*Clitocybe nebularis*)	Fundata forest	Edible Medicinal- stops leukemia T cells from proliferating, antimicrobial activity (Kim et al, 2008)	
Lepista nuda (*Clitocybe nuda*)	Fundata forest	Edible Medicinal- antioxidant and antimicrobial properties (Dulger et al., 2002), immunologic effects (Lyn et al., 2011)	
Laetiporus sulphureus	Carol Park - Buharest	Edible Medicinal - hemolytic and hemaglutination activities (Mancheño et al., 2005), antimicrobial and antioxidant activities (Turkoglu et al., 2007)	

Macrolepiota procera	Chitila forest	Edible Medicinal-antioxidant properties	
Pleurotus ostreatus	Chitila forest Voinesti forest	Edible Medicinal- cardiovascular, hypertensive, hypercholesterolemia (Wasser, 2002), antioxidant and antimicrobial activities (Vamanu, E., 2013)	
Trametes versicolor	Sinaia forest	Medicinal – in a variety of cancers, including Gastric, Lung, Breast and Colorectal, Hepatoprotective, herpes, Chronic Fatigue Syndrome (Medicinal Mushrooms - A Clinical Guide by Martin Powell)	
Stropharia sp.	Fundata forest	Edible, but undesirable due to mildly spicy taste Medicinal- antitumor (Ohtsuka et al., 1973), neuromodulatory effects (Moldavan et al., 2001)	
Xylaria polymorpha (Dead Man's Fingers)	Fundata forest	Medicinal- cytotoxicty against human cancer cell lines (Shiono et al., 2009), antibacterial activity (Ramesh, 2012).	

RESULTS AND DISCUSSIONS

18 wild mushroom species were harvested from different indigenous regions (Sinaia, Voinesti, Chitila, Fundata, Teleorman forests and Bucharest parks), in order to analyze their ability to grow under *in vitro* conditions and to initiate a germplasm collection for further investigation. Two types of culture media, 2% malt extract agar (MEA) and potato-dextrose-agar (PDA), were chosen for *in vitro* cultivation of the samples. Depending on the species, after 1-3 weeks of incubation the mycelia have been well developed on both types of culture media (fig. 1). For some wild mushroom species, primordia of young fruiting bodies have been emerged from the mycelium grown on the medium surface (e.g. *Trametes versicolor*, fig.1T). In addition, fungal mycelia samples exhibited different colours and morphological characteristics (fig.1). The pure cultures obtained in this way were kept in collection on PDA or MEA slants for further use. Thus, over 40 mushroom varieties belonging to at least 35 mushroom species were isolated and *in vitro* propagated on MEA or PDA media.

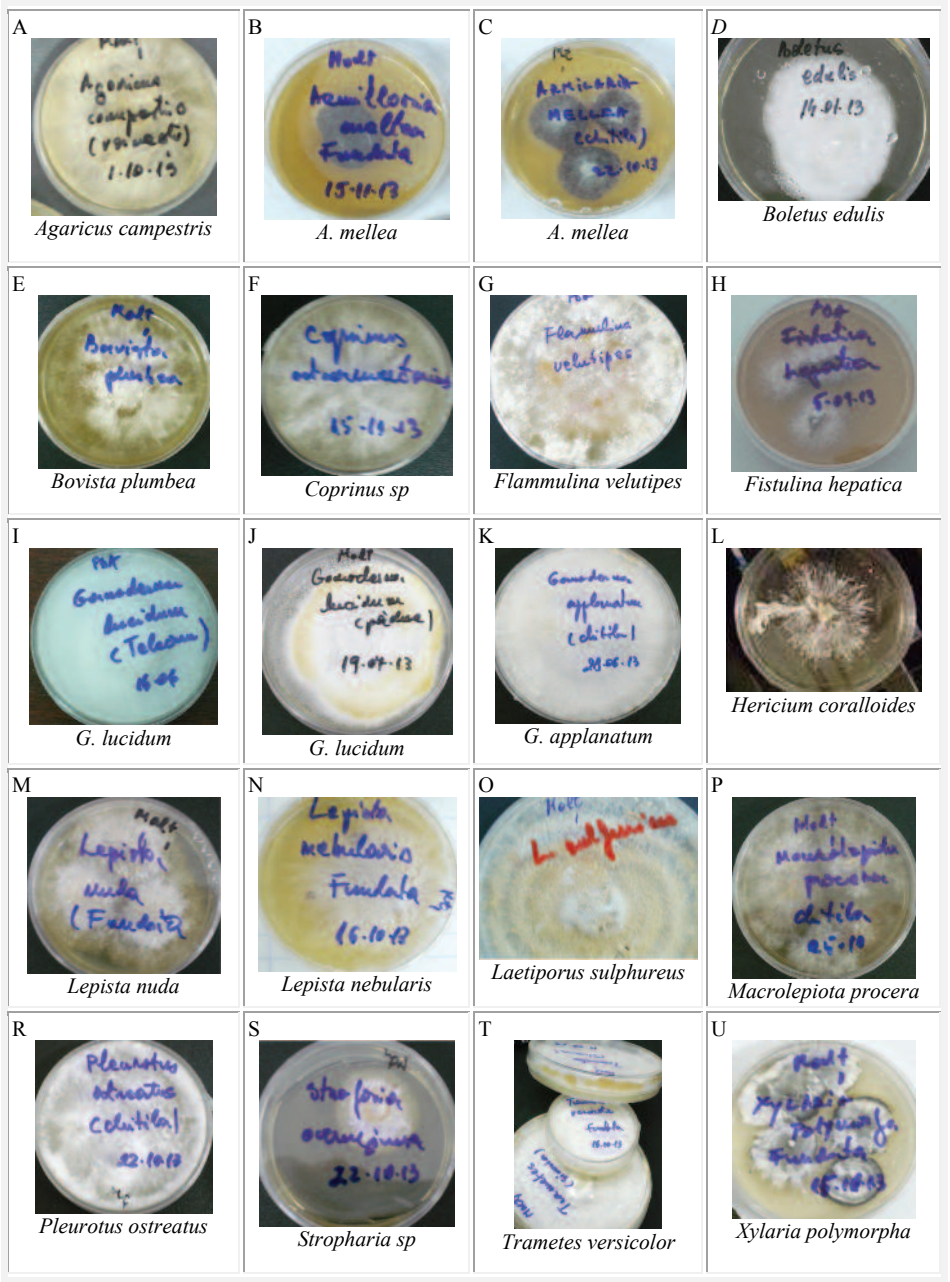


Figure1. Fungal mycelia of various wild edible and/or medicinal mushroom species isolated from indigenous mycoflora

CONCLUSIONS

The i*n vitro* wild edible medicinal mushrooms cultivation (in Petri dishes or other culture vessels) could have many advantages:

- It is much faster than growing mushrooms in a natural way;
- This method may have industrial benefits;
- Mycelium grown on agar is therefore used to inoculate logs (or other substrates) in plantations;

- The methods offer the possibility of cloning or genetic manipulation mycelium.

In addition, wild edible medicinal mushrooms can be a source of nutraceuticals with valuable antioxidant properties, which can positively influence the oxidative stress in cells and related diseases (Ferreira, 2009). Using specific biotechnologies the new mushroom strains included in our collection will be tested for genetic variability and for other important characteristics (producing of enzymes, degradation abilities, antimicrobial and/or antitumor properties etc).

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ANTIFUNGAL ACTIVITY OF FOUR PLANTS AGAINST ALTERNARIA ALTERNATA

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Abstract

Natural fungicides are gaining increased attention because of their environmentally friendly properties, being easily accessible and relatively cost effective, in the perspective of sustainable methods of plant disease control The aim of the present work was the investigation of the in vitro antifungal activity of the ethanolic and aqueous extracts obtained from four locally available traditional medicinal plants from Romania, collected from different regions of Southern part of the country. The four plants taken into consideration were absinth (Artemisia absinthium), rosemary (Rosmarinus officinalis), jimson weed (Datura stramonium) and cocklebur (Xanthium strumarium), and their antifungal properties were tested against the plant pathogenic fungus Alternaria alternata. All extracts obtained from the selected plants presented antifungal potential, demonstrated by the inhibition of the mycelial growth. Generally, the ethanolic extracts showed a higher antifungal activity than the aqueous extracts, for all the tested variants. The results of this study confirm that the ethanolic and aqueous extracts of the selected plants can be used as an alternative in control of the tested phytopathogenic fungus.

Keywords*: Alternaria alternata, antifungal activity, plant extracts.*

INTRODUCTION

Fungal plant diseases represent an important cause of increased annual crop losses. More than 70% of all major crop diseases are caused by fungi (Agrios, 2005).

Alternaria alternata causes leaf spots and blight on a large variety of agricultural and horticultural crops such as: tomato *(Lycopersicon esculentum)*, potato (*Solanum tuberosum*), carrot (Daucus carota), cauliflower (*Brassica oleracea var. botrytis)*, broccoli (*Brassica oleracea -Botrytis Group*), cabbage (*Brassica oleracea var. capitata*), peppers (*Capsicum annuum*.), beans (*Pisum sativum)*, apple (*Malus domestica*), peach (Prunus persica) and citrus species. Moreover, *A. alternata* can also attack a several weeds and ornamental plants.

There is also little doubt that sensitivity to *Alternaria* is an important factor in the induction of allergic rhinitis and asthma on immunodepressed patients, especially in children (Kuna et al., 2011)

Generally, the control of plant diseases and pests is well established with synthetic fungicides and other agricultural practices such as crop rotation inter-cropping and sanitation (Pretty, 2008). However, in the recent years the farmers all over the world have reported an efficacy decrease of the treatments with traditionally used fungicides to control early blight and other plant diseases (Fairchild et al., 2013). Furthermore, the inappropriate use of fungicides, such as applying increased and more frequent dosage units (Genet et al., 2006) has resulted on the one hand in the occurrence of fungal resistance (Brent and Hollomon, 2006; Mcgrath, 2001; Haouala, 2008) and on the other hand in hazardous effects in human and animal health and on the environment resulting in ecological imbalances (Pramila and Dubey, 2004).

Products based on natural components used in controlling and combating phytopathogenic agents have gained increased attention lately, in search of eco-friendly methods that can be used either for mass production or for some the organic and bio farming niche production.
Moreover, the natural fungicides are easily accessible and relatively cost effective, in the perspective of sustainable methods of plant
The research in this area started initially in a more likely confirmation direction of the antimicrobial potential, with medicinal plants used empirically in folk's medicine to treat various diseases. Subsequently, the scope of work expanded to applications of the use of products obtained from these plants in the phytopathogens area.
Various reports on medicinal plants extracts have shown inhibitory effects against phytopathogenic fungi *in vitro* (Rodino et al. 2013a; Haouala, 2008; Rodino et al. 2013b; Singh et al. 1998; Yasmin et al., 2008; Harlapur et al., 2007; Prashith et al 2010)
Currently, there are also conducted studies on some species of plants such as weeds (Srivastava and Singh, 2011) or trees (Mahlo et al., 2010) with less known medicinal value, that could be used to control plant diseases and pests.
In view of this, in the present paper was investigated the antifungal activity of extracts obtained from locally available plants, namely absinth or wormwood (*Artemisia absinthium*), rosemary (*Rosmarinus officinalis*), jimson weed or thornapple (*Datura stramonium*) and cocklebur (*Xanthium strumarium*) against the fungus *Alternaria alternata.*

MATERIALS AND METHODS

Plant material collection

The plant materials and plant parts used in this study were as follows:
1- absinth (*A. absinthium*) aerial part,
2 - rosemary (*R. officinalis*) aerial part,
3 (a) - jimson weed (*D. stramonium*) leaves.
3 (b) - jimson weed (*D. stramonium*) fruits.
4 - cocklebur (*X. strumarium)* fruits.
The plants were collected from different agricultural lands in Southern part of the country. The healthy vegetal material was shade dried and minced to a fine powder in order to be used for the extraction.

Preparation of plant extract

The *alcoholic extracts* used in the antifungal assay were obtained by maceration. For this process was used a quantity of 5g of dried, fine powdered plant to 50 ml of 70 % ethanol. The mixture was left in sealed glass recipients for 96 hours, at room temperature, in darkness, with occasionally stirring. The obtained extract was filtrated through filter paper (Whatman no.1) under vacuum.
The *aqueous extracts* were prepared by infusion using the same ratio of 1:10 (w:v) of powdered dried plant material to sterile distilled water. The solution thus obtained was kept at 4°C in sealed recipients and used within the next eight hours.

Fungal culture

The fungal culture belongs to the collection of the Faculty of Biotechnologies from the University of Agronomic Sciences and Veterinary Medicine of Bucharest.
The present experiments were carried out using a 7 day old culture.

Antifungal activity testing

The effect of the plant extracts taken into study against the mycelial growth of *A. alternata* was tested by poisoned food technique.
An appropriate quantity of each extract was incorporated in sterilized PDA medium to reach desired concentrations for each plant solution treatment. Three concentrations of ethanolic extracts at 10%, 5% and 2.5% were used in the assessment. Regarding the aqueous extracts a concentration of 10% was evaluated.
Mycelial discs of 6 mm diameter, taken from the margins of an actively growing culture of the fungal pathogen were aseptically placed in the centre of the 70 mm plates containing solidified poisoned PDA. The experiment was run in triplicate. The Petri-plates were incubated at 25 ± 2 °C. The measurement of the mycelial growth dynamics of the fungus were recorded on a daily basis, beginning with 24 hours after inoculation. The whole experiment was carried out for 9 days until the control colony reached the margins of the Petri plate.

The control was considered the plate containing the organic solvent (sterile double distilled water or ethanol) incorporated and the control with non-poisoned PDA.
The percentage of the inhibition of the mycelial growth (I) due to different treatments was calculated on the formula:

$$I\ (\%) = (1-d_t/d_c)*100\ (\%)$$

Where,

- d_c is the average fungal colony diameter measured in control plate, with no treatment,
- d_t is the average fungal colony diameter measured in treated plates (Ogbebor et al., 2008)

RESULTS AND DISCUSSIONS

All the extracts obtained from the selected plants presented antifungal potential against the tested fungus, demonstrated by the inhibition of the fungal mycelial growth. In general, the ethanolic plant extracts showed more intense antifungal activity than the aqueous extracts for all the tested variants.
The results revealed that the antifungal activity of the extracts was dose dependent, being negatively influenced by decreasing the concentration of the extracts in the growth media.
When using the concentrations of 10% ethanolic plant extract in PDA medium resulted in a fungistatic effect demonstrated by the 100% inhibition of mycelial growth in all variants studied.
Regarding the growth inhibition dynamics of the variant using 5% extract concentration in growth medium it could be observed a very slow development of the fungus compared to the not treated control.
The beginning of growth was delayed with 3 days in the case of rosemary and four days for cocklebur, and after that, a slow growth was seen. The final measurements revealed that the extract obtained from *X. strumarium* had the highest inhibitory activity, equal to a percentage of 86.7%, (mycelium growth was 4 mm), while the one obtained from *D. stramonium* fruits presented the lowest inhibition activity against *A. alternata,* mycelium growth reaching 15 mm on the 9th day since inoculation. At that moment the on the control plate the tested fungus reached the margins.
The daily measurements of the radial growth are given in *Figure 1*.

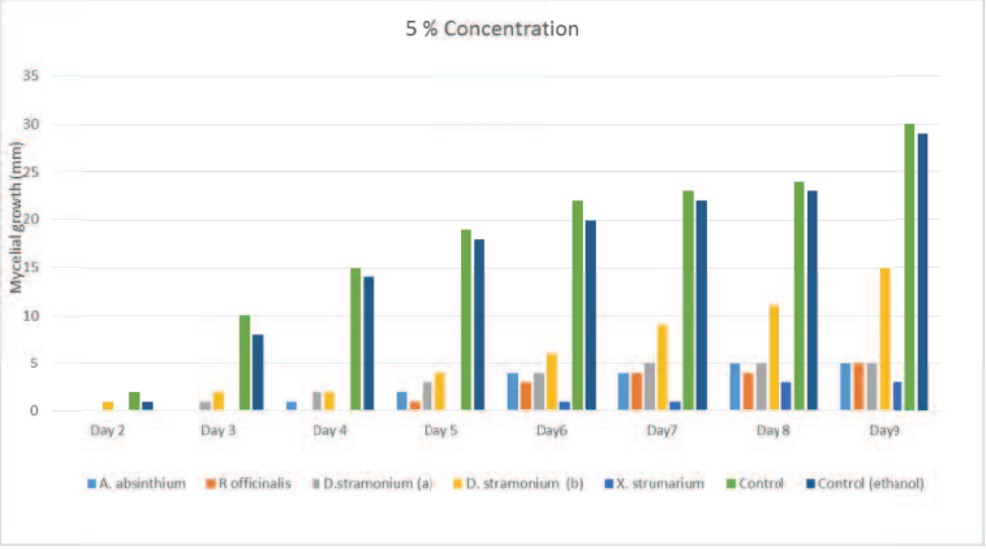

Figure 1. The mycelial growth of A. alternata under the treatment with selected ethanolic plant extracts

In the next phase of experiments, when incorporating the appropriate ethanolic plant extracts quantity to reach 2.5% concentration in the inoculated growth media, the antimicrobial activity decreased in intensity, the fungus developing more rapidly than in previous set of variants, its dynamics being closer to the one of the control.

The *X. strumarium* extract reduced the radial growth of the fungus to 15 mm, while the jimson weed fruits extract treatment resulted in a mycelium growth of 24 mm, this value being close to the development of the control colony with no treatment included, as can be observed in Figure 2.

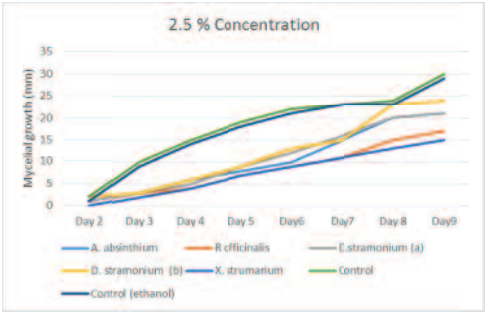

Figure 2. The mycelial growth of A. alternata under the treatment with selected ethanolic plant extracts

The absinth and jimson weed leaves extracts respectively, showed similar results, of 30% inhibition of growth (Table 1), calculated after the formula mentioned in the previous section.

The ethanolic extract of *X. strumarium* was found to be most effective for the control of the test fungus.

All of the aqueous plant extracts showed a lower effect than the ethanolic ones. Their activity at 10% concentration against the fungal colony growth only reached a maximum inhibition percentage of 60% for the *D. stramonium* (a) extract. The lowest results were obtained for the fruit extract of the same plant, approximately 16.7%. The daily measurements effectuated on radial growth are graphically reflected in Figure 3.

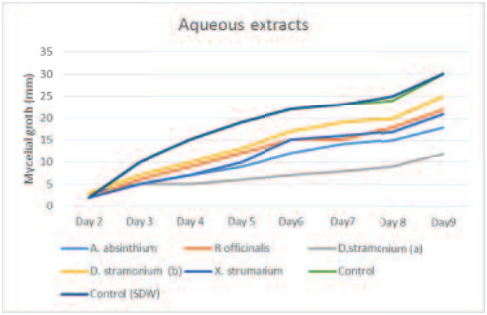

Figure 3. The mycelial growth of A. alternata under the treatment with selected aqueous plant extracts

Therefore, among the aqueous extracts, the one obtained from *D. stramonium* was found to possess the most effective fungitoxic potential for the control of *A. alternata.*

Table 1. The effect of the plant extracts of the inhibition of *A. alternata* growth

Plant extracted	**Percentage of inhibition % of the mycelial growth**		
	5% Ethanolic Extract	**2.5% Ethanolic Extract**	**10% Aqueous Extract**
A. absinthium	83.33	30.00	40.00
R. officinalis	83.33	43.33	26.67
D. stramonium (a)	83.33	30.00	60.00
D. stramonium (b)	50.00	20.00	16.67
X. strumarium	86.67	50.00	30.00

The vegetal material taken into study in the present paper, two medicinal plants and two weed species, is easily available belonging to spontaneous flora of our country. Simple methods of extraction were chosen, in order to make them to be easily exploited in the practical *in vivo* control of plant diseases.

Choosing as raw material the weed species for plant derived fungicides can lead both to an ecofriendly method of disease control and

deliver a solution for weed management of agricultural crops, creating economic uses for these unwanted species (Srivastava et al., 2011).

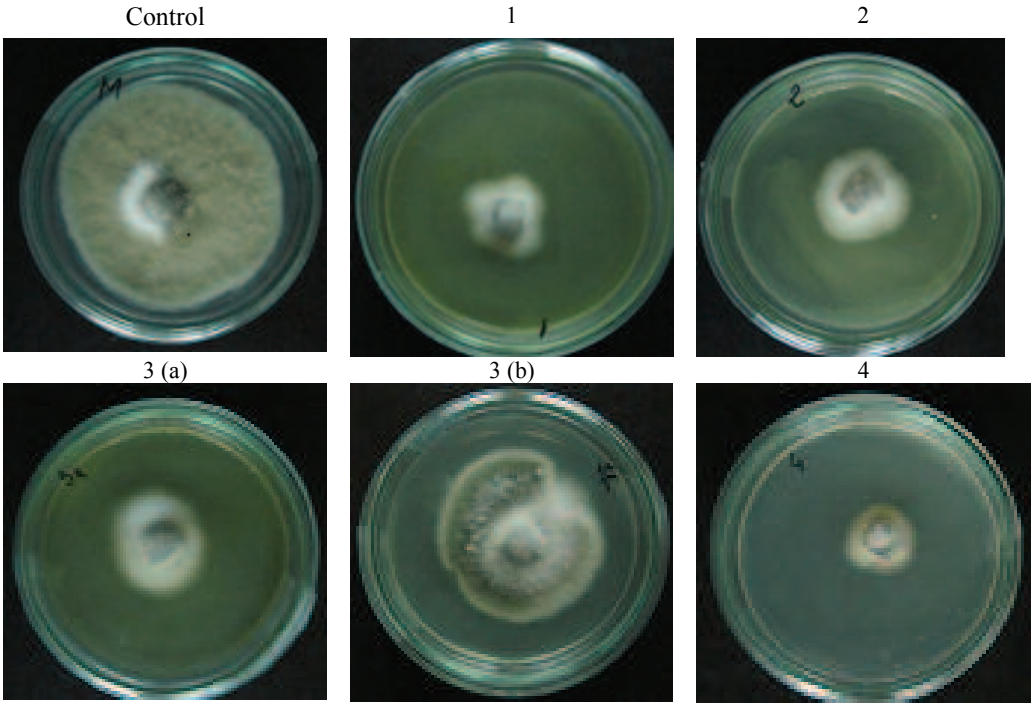

Figure 4. Antifungal activity of the selected plant extracts against *A alternata*. 1. *A absinthium,* 2. *R. oficinallis*, 3. (a). *D. stramonium* leaves, 3. (b). *D. stramonium fruits*, 4. *X. strumarium*,

CONCLUSIONS

Given the fact that there are a limited number of studies presenting evidence of the antimicrobial activity of the medicinal plants against phytopathogen fungi, investigation on this direction still needs to be done.

Further studies should also be done on phytochemical characterization of the extracts obtained.

The results obtained support and confirm the ethnopharmacological uses of the tested plants and provide preliminary information that can be used for further evaluation.

This study shows optimistic results regarding the potential of plant species as sources of plant based products with activity against plant pathogenic fungi.

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PESTS AND DISEASES MANAGEMENT USING COMPATIBLE BIOCONTROL BACTERIA AND ENTOMOPATHOGENIC FUNGAL STRAINS

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Abstract

Pest and disease management using biocontrol microbial strains is a request of the organic agriculture or a phytosanitary alternative that can decrease chemical inputs in the integrated agricultural systems. Biocontrol bacteria of Bacillus spp. proved to suppress soil-borne phytopathogenic fungi. RDIPP selected strains of Bacillus amyloliquefaciens, B. licheniformis and B. subtilis provided to be useful in plant protection and formulated them as bioproducts for seed and soil treatments. For pest biological control, entomopathogenic fungi such as Beauveria bassiana, B. brongniartii, Isaria farinosa, Metarhizium anisopliae and Verticillium lecanii are known as efficient.
For this reason, the aim of our work was to select compatible microbial strains of biocontrol bacteria and entomopathogenic fungi that could be applied together, as simultaneously treatments, for suppressing diseases and pests attack. Results revealed in vitro compatibility of Bacillus licheniformis 77.1s biocontrol strain with Beauveria spp. entomopathogenic fungi. These biological control microorganisms could be used in combination to prevent in the same time pests and diseases.
As a requirement for environmental safety, the selected microbial strains were ecotoxicologicaly tested according to the GLP principles (Good Laboratory Practices) and OECD guidelines. Results proved that the selected strains were non-toxic for non-target species of the aquatic and soil macrofauna: Daphnia magna (crustacean) and Eisenia foetida (earth worm) respectively.

Keywords: *biocontrol bacteria, entomopathogenic fungi, microbial compatibility*

INTRODUCTION

Pest and disease management using biological control methods could be sustained with microbiological techniques. The bacterial and fungal biocontrol agents possess different complex and efficient mechanisms of action useful enough to suppress pest and disease negative impact on crops (Mishra et al., 2013). Biocontrol bacterial strains have a wide range of beneficial traits such as antagonism by antibiotics production, lytic enzymes or bioactive volatile compounds, competition for nutrients and niches, induction of systemic resistance in plants or increase plant growth promotion (Ownley and Windham, 2007; Constantinescu et al., 2010). The entomopathogenic fungi could manifest pest parasitism or produce different lytic enzymes (such as proteases and peptidases, chitinases and lipases), certain metabolic acids, or it could secret toxins with inhibitory action against insects (Khan et al., 2012).

Naturally occurring biocontrol results mostly from a mixture of antagonists (as in suppressive soils) rather than from high population of a single antagonist (Mishra et al., 2011; Mishra et al., 2013). Previous studies on combining different biocontrol agents for pests and diseases management included fungal antagonist mixtures (Datnoff et al., 1995, Núñez del Prado et al., 2008), bacteria and fungi mixtures (Koppenhöfer and Kaya, 1997; Koppenhöfer et al., 1999; Hassan et al., 1997; Mishra et al., 2013) and bacteria mixtures (Raupach and Kloepper, 1998; Stockwell et al., 2011). However, most of the studies refer to combining microorganism with same target activity either for pest control (Koppenhöfer and Kaya, 1997; Koppenhöfer et al., 1999; Thurston et al., 1993, 1994), or for disease

suppression (Guetsky et al., 2002; Stockwell et al., 2011). Co-inoculation of biocontrol microbial agents can lead to an incompatibility between the microorganisms by inhibiting each other (Mishra et al., 2013). Therefore, it is necessary and important to analyze microbial compatibility prior to apply or formulate biocontrol microbial mixtures for plant protection. Considering these, we analysed *in vitro* compatibility between different strains of biocontrol *Bacillus* spp. with several entomopathogenic fungi in order to reveal a compatible microbial mixture for biological pest and disease control.

Since biological control methods aim to reduce negative impacts on the environment, we subjected the microbial strains studied to different ecotoxicological evaluations.

MATERIALS AND METHODS

Microbial strains

Six bacterial biocontrol strains, *Bacillus subtilis* B49b, 98a and Us.a2, *Bacillus licheniformis* 77.1s, *Bacillus pumilus* OS15 and *Bacillus amyloliquefaciens* OS17 were used in this study. These strains were previously selected in the frame of PN-09-40-02-01 project as bacterial biocontrol agents (BCA), for their beneficial characteristic in plant protection. Routinely, these strains were grown on Luria Bertani agar medium at 28ºC, for 48 h.

The enthomopahogenic fungi (EPF) used in the study were *Beauveria bassiana* DSM62075 (IMB 7389), *B. brongniartii* DSM6651 (E 1246/91), *Metarhizium anisopliae* DSM1490 (OSU Ma Re 4SS), *Verticillium lecanii* (sin. *V. hemileiae*) DSM63098 (IMB 11471) and *Isaria farinosa* (sin. *Paecilomyces farinosus*) IHEM 2526, purchased from DSMZ (Germany) and BCCM/IHEM (Belgium) international collections. These fungal strains were routinely grown on potato-dextrose-agar (PDA) medium, for 14 days at 26ºC.

Microbial compatibility assessment

Microbial compatibility between the BCA and EPF strains was performed *in vitro*, using the dual culture technique. Tests were carried out on PDA plates. The fungal inoculum consisted in mycelia plugs of 1 cm in diameter, placed in the middle of the plate. The bacterial strains were streaked at 2 cm from the fungal colony, on both sides. Plates were than incubated at 28^{o}C for 14 days.

After co-cultivation, the fungal growth was evaluated using a modified version of Islam *et al.* (2009) calculation, to reveal the bacterial influence on the mycelia development:

$$Fungal\_inhibition(\%) = \frac{R_{control} - R_{interaction}}{R_{control}} \times 100$$

where: $R_{control}$ = the radius of mycelial development in control plate, $R_{interaction}$= radius of mycelial development in co-cultivation with BCA.

Bacterial growth inhibition was evaluated according to Manka and Manka algorithm (1992).

Ecotoxicological evaluation of entomopathogenic fungi

All five EPF strains were subjected to ecotoxicological evaluation on *Daphnia magna* crustacean and *Eisenia foetida* earthworm. The studies were performed according to the GLP principles, using the C2 and C8 method for acute toxicity on Daphnia and earthworm, respectively; both included in the Regulation (EC) no.440/2008. The entomopatogenic fungi were tested in different concentration, depending on the species: *Beauveria bassiana* at $3.6x10^{7}$cfu/ml, *Beauveria brongniartii* at $1.9x10^{8}$ cfu/ml, *Metarhizium anisopliae* at $7.0x10^{7}$cfu/ml, *Verticillium lecanii* at $1.7x10^{9}$cfu/ml and *Isaria farinosa* at $1.4x10^{8}$cfu/ml. These concentrations are the one recommended and used for pest biocontrol.

The acute toxicity test with daphnid crustaceans was performed according to "immobility" test using the static method, certified as Good Laboratory Practice (GLP). The "immobility" criterion is approved by several international organizations, such as ISO and OECD, and represents the inability of the test organisms to resume swimming within 15 seconds after gentle agitation (Persoone et al., 2009). During the trial, the *Daphnia* were not fed, except of 2 ml testing solution administered to each of them. The experiment included a control group exposed to the same experimental conditions, such as period of analysis, water quality, oxygen concentration, pH of the tested solutions, parameters that are

compulsory by the European Standard. The *Daphnia* were examined every 24 and 48 hours, according with the OECD guidelines (2004), in order to establish the degree of *Daphnia* immobilization. Experimental parameters of the water, as the concentration of dissolved oxygen and water temperature and pH, were also registered at the beginning and in the end of the test.

For the toxicological evaluation on *Eisenia foetida*, the fungal strains were prepared as aqueous suspensions at the mentioned concentrations, using fungal spores from PDA cultures. The chloracetamide was used as a toxic reference standard. Suspensions to be tested were applied to an artificial soil in which adult *Eisenia foetida* earthworms were placed. For the ecotoxicological studies, the *Eisenia foetida* must be at least two months old, with fully developed clitellum and individual body weight between 300-600 mg. The artificial soil used as substrate was prepared according to the standard procedure, and consisted in peat (10%), kaolin (20%) and industrial quartz sand (70%). For each experimental variant, 2500g of artificial soil with 20% humidity was moisturized to 35% humidity by adding 375ml fungal spores suspension. Each experimental variant had for replicates. Tests were performed in glass vessels of 1L capacity, covered with a fine mesh for ventilation. The test conditions were 20±2°C temperature and continuous light of 400 to 800 lux in intensity; requirements provided according to the standard procedure. All operations performed were recorded in data tables according to the Annexes from the technical procedure. Results were recorded after 7 and 14 days, were the ecotoxicological effect of the tested fungi on *Eisenia foetida* was examined by counting the adult earthworms that survived to the tested concentrations.

The BCA strains of *Bacillus subtilis* Us.a2, *B. pumilus* OS15 and *B. amyloliquefaciens* OS17 were also ecotoxicologicaly evaluated on *Eisenia foetida* earthworms. Tests were performed as mentioned for EPF. The bacterial strains were prepared as aqueous suspensions of 10^8 cfu/ml.

RESULTS AND DISCUSSIONS

In vitro compatibility between biocontrol bacteria and entomopathogenic fungal strains

In vitro co-inoculation assay between the EPF *Beauveria bassiana* and tested *Bacillus* biocontrol strains revealed a strong mycelia growth inhibition. Likewise, *Bacillus subtilis* 98a and Us.a2, *B. pumilus* OS15 and *B. amyloliquefaciens* OS17 were influenced by the presence of *B.bassiana*. This fungal strain inhibited bacterial ability to colonize the surface of the PDA medium in the proximity of the fungi, therefore the bacterial growth avoided to near the *B.bassiana* mycelia. The bacterial strains whose growth was not influenced by *B. bassiana* were *Bacillus subtilis* B49b and *B.licheniformis* 77.1s.

The compatibility studies between the biocontrol bacteria and *Beauveria brongniartii* revealed a slight growth inhibition to each interaction partner. Results obtained after 7 days of co-inoculation are included in table 1.

Table 1. Co-cultivation of *Beauveria brongniartii* DSM6651 with different bacterial bio-control strains (after 7 days of incubation)

Experimental variants	Radial growth of the EPF (cm)	Fungal inhibition
Beauveria brongniartii DSM6651 fungal control	1.2	-
Bacillus subtilis B49b	1	16.7%
B. subtilis 98a	0.9	25%
B. subtilis Us.a2	0.9	25%
B. pumilus OS15	1.1	8.3%
B. amyloliquefaciens OS17	0.9	25%
Bacillus licheniformis 77.1s	1.1	8.3%

The growth inhibition of *Beauveria brongniartii* was inhibited with 25% by *B. subtilis* Us.a2, 98a and *B. amyloliquefaciens* OS17 bacterial strains. *Bacillus subtilis* B49b inhibited the mycelial growth of *B.brongniartii* with 16,7%, while *B.licheniformis* 77.1s and B. pumilus OS15 inhibited fungal growth with 8,3% after 7 days of cultivation. *Beauveria brongniartii* manifested also a slight limitation of growth on *Bacillus subtilis* Us.a2 and 98a strains. Therefore, these two bacterial trains could not colonize the surface of the PDA

medium, as would normally do, and maintained a distance of 5mm (figure 1) and 1mm respectively, from the fungus. Results revealed that *Bacillus licheniformis* 77.1s, *Bacillus subtilis* B49b, *Bacillus pumilus* OS15 and *Bacillus amyloliquefaciens* OS17 strains were not visibly affected by this entomopathogen (figures 1 and 2).

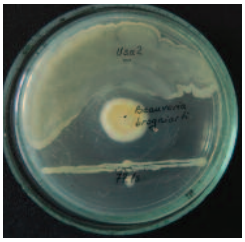

Figure 1. Co-cultivation of *Beauveria brongniartii* with *Bacillus subtilis* Us.a2 and *B.licheniformis* 77.1s

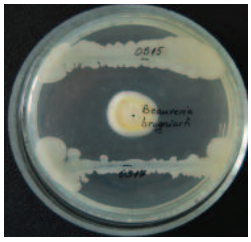

Figure 2. Co-cultivation of *Beauveria brongniartii* with *Bacillus pumilus* OS15 and *B.amyloliquefaciens* OS17

Regarding *Metarhizium anisopliae*, by co-cultivation with biocontrol bacteria, the fungal growth was inhibited with 28.6 to 57.1% after 7 days of incubation (table 2). Likewise, *Bacillus subtilis* Us.a2 and 98a and *B.amyloliquefaciens* OS17 were also inhibited by this fungus. Their colonising ability was limited in the proximity of *M.anisopliae* (figures 3 and 4). *Bacillus licheniformis* 77.1s growth was not influenced by this fungus, and *Bacillus subtilis* B49b (figure 3) and *Bacillus pumilus* OS15 (figure 4) showed only a slight inhibition after 14 days of incubation with this *Metarhizium anisopliae* strain.

Table 2. Co-cultivation of *Metarhizium anisopliae* DSM1490 with different bacterial bio-control strains (after 7 days of incubation)

Experimental variants	Radial growth of the EPF (cm)	Fungal inhibition
M.anisopliae DSM1490 fungal control	1.4	-
Bacillus subtilis B49b	1	28.6%
B. subtilis 98a	0.8	42.8%
B. subtilis Us.a2	0.8	42.8%
B. pumilus OS15	0.8	42.8%
B. amyloliquefaciens OS17	0.6	57.1%
Bacillus licheniformis 77.1s	0.8	42.8%

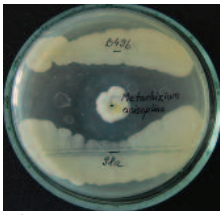

Figure 3. Co-cultivation of *M.anisopliae* with *Bacillus subtilis* B49b and 98a

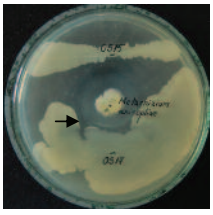

Figure 4. Co-cultivation of *M.anisopliae* with *B.pumilus* OS15 and *B.amylolique-faciens* OS17

The EPF inhibition by some BCA strains is more obvious after 14 days of incubation (table 3).

Table 3. Mycelia inhibition of some entomopathogenic fungi in co-cultivation with different bio-control bacterial strains (after 14 days of incubation)

EPF strains	Cultural characteristics	Fungal control	Biocontrol bacterial strains					
			B49b	98a	Us.a2	OS15	OS17	77.1s
Beauveria bassiana DSM62075	Mycelia radius (cm)	2.3	0.5	0.7	0.5	0.6	0.5	1.3
	Fungal growth inhibition	-	78.3%	69.6%	78.3%	73.9%	78.3%	43.5%
Beauveria brongniartii DSM6651	Mycelia radius (cm)	3.7	0.5	0.7	0,1	0.6	0.5	n.a.
	Fungal growth inhibition	-	78.3%	69.6%	66.7%	73.9%	78.3%	11.1%
Metarhizium anisopliae DSM1490	Mycelia radius (cm)	3.8	0.7	0.8	0.9	0.9	0.8	2.3
	Fungal growth inhibition	-	81.6%	78.9%	76.3%	76.3%	78.9%	39.5%
Verticillium lecanii DSM63098	Mycelia radius (cm)	1.5	0.5	0.5	0.5	0.8	0.8	0.8
	Fungal growth inhibition	-	66.7%	66.7%	66.7%	46.7%	46.7%	46.7%
Isaria farinose IHEM 2526	Mycelia radius (cm)	1.9	0.5	0.5	n.a.	0.5	0.5	n.a.
	Fungal growth inhibition	-	73.7%	73.7%	n.a.	73.7%	73.7%	n.a.

Regarding BCA co-cultivation with *Verticillium lecanii*, the bacterial strains *B.licheniformis* 77.1s, *B.pumilus* OS15 and *B.amyloliquefaciens* OS17 inhibited fungal growth with 46.7%, and *B.subtilis* Us.a2, B49b and 98a strains inhibited this fungus with 66.7% after 2 weeks of co-cultivation, comparing with the control fungal growth. No inhibition was shown on the fungal growth in co-cultivation of *Isaria farinosa* with *B. subtilis*

Us.a2 and *B. licheniformis* 77.1s strains. Only *B. pumilus* OS15, *B.amyloliquefaciens* OS17 ans *B. subtilis* B49b and 98a strains inhibited *I. farinosa* growth.

During the co-inoculation interactions between tested biocontrol *Bacillus* strains and some of the EPF strains, a precipitation compound was observed in the area of fungal growth inhibition. Similar aspects were also described by Cornea et al. (2009), Machado et al. (2010) and Sicuia et al. (2013) on lectin producing filamentous fungi. Kossowska et al (1999) mentioned that *B.bassiana* is capable to produce lectins. Other lectin producing EPF, described in the literature, are *Conidiobolus obscurus* (Latgé et al., 1988) and *Paecilomyces japonica* (Park et al., 2004). In our studies, we noticed this characteristic at *Beuveria bassiana* co-inoculated with *Bacillus subtilis* and *B.amyloliquefaciens* strains, at *Beuveria brongniartii* co-inoculated with *Bacillus subtilis* B49b and Us.a2 strains, and to *Metarhizium anisopliae* alone or co-inoculated with bacteria. An interesting phenomenon was noticed in co-inoculation of *M .anisopliae* with *B.amyloliquefaciens* OS17, the EPF precipitation halo was diminish and a more intense precipitation line was seen when bacterial colony became closer. In addition, the OS17 bacterial colony created a slight line of precipitation at its proximity due to *M.anisopliae* presence (figure 4).

As Sicuia et al. (2013) previously reported, the presence of precipitation line is strictly dependent on both fungal and bacterial strains interactions. These aspects are observed only in co-cultivation of lectin producing fungi with certain bacterial strains. Same authors attributed these aspects to the specific sugar binding of bacterial glycoconjugates by the lectin or lectin-like compounds secreted by the fungi.

Regarding the influence of the EPF tested on the growth of BCA strains, only B. licheniformis 77.1s strain was not influenced by any of the fungi tested (table 4).

Table 4. Bacterial growth inhibition of different bio-control bacterial strains in co-cultivation with some entomopathogenic fungi (after 14 days of incubation)

Bacterial strains	Entomopathogenic fungi				
	Beauveria bassiana DSM62075	*Beauveria brongniartii* DSM6651	*Metarhizium anisopliae* DSM1490	*Verticillium lecanii* DSM63098	*Isaria farinosa* IHEM 2526
Bacillus subtilis B49b	–	+ / –	+	–	–
Bacillus subtilis 98a	+ +	+	+ +	+ / –	–
Bacillus subtilis Us.a2	+ +	+	+ +	+ / –	–
Bacillus pumilus OS15	+ +	+ +	+	–	–
B. amyloliquefaciens OS17	+ +	+ +	+ +	+ / –	–
Bacillus licheniformis 77.1s	–	–	–	–	–

Legend: + + + + = very strong inhibition of the fungal growth; + + + = strong inhibition of the fungal growth; + + = moderate inhibition of the fungal growth; + = slight inhibition of the fungal growth; – = no inhibition of the fungal growth.

The ecotoxicological evaluation of the entomopathogenic fungi

The ecotoxicological evaluation of the EPF *Beauveria bassiana*, *B. brongniartii*, *Metarhizium anisopliae*, *Verticillium lecanii* and *Isaria farinosa* at the standard concentration used for pest biocontrol revealed no toxic effects on *Daphnia magna*. There were no immobilization or adverse reaction recorded. However, *Verticillium lecanii, Metarhizium anisopliae* and *Isaria farinosa* revealed a stimulatory action on the reproduction cycle of *Daphnia*. The study has been validated, since the quality criteria of the C2 method from the Regulation (EC) no. 440/2008 were fulfilled. There was no mortality registered in the control, and the concentration of oxygen in water was between 6.0 to 6.5 mg/L (≥ 3 mg/L) at the end of the test, in both control and test variants.

The ecotoxicological evaluation of the EPF towards *Eisenia foetida* earthworms revealed no toxicity after 14 days observation. All five EPF strains tested at the standard concentration used for pest biocontrol showed no mortality within the earthworm populations. In the untreated control, all 40 earthworms survived, as well.

The BCA strains *Bacillus subtilis* Us.a2, *B. pumilus* OS15 and *B. amyloliquefaciens* OS17 were also ecotoxicologicaly evaluated towards *Eisenia foetida* earthworms. After 7 days of incubation, no mortality was registered to any of the testing variants. However, after 14 days of incubation, a slight mortality percent was registered in each experimental variant, including the control (table 5).

Table 5. Ecotoxicological evaluation of some bio-control bacterial strains towards *Eisenia foetida earthworms* (after 14 days of incubation)

Experimental variants	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Total earthworms / experiment		Mortality average (%)
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	
Bacillus subtilis Us.a2	8	2	8	2	8	2	9	1	**33**	**7**	**17.5**
B. pumilus OS15	9	1	9	1	9	1	10	0	**37**	**3**	**7.5**
B. amyloliquefaciens OS17	10	0	10	0	8	2	8	2	**36**	**4**	**10.0**
Control	9	1	10	0	10	0	9	1	**38**	**2**	**5.0**

According to the standard procedure, since the mortality in the control was less than 10% at the end of the test, the ecotoxicological studies towards earthworms were considered valid.

CONCLUSIONS

The compatibility between the beneficial microorganisms such as biocontrol bacteria with the entomopathogenic fungi could allow simultaneous treatments for suppressing diseases and pests attack.

The lowest inhibitory activity was registered between *Beauveria brongniartii* DSM6651 and *Bacillus licheniformis* 77.1s, where the fungal inhibition was only 11.1%, and the bacterial growth was not influenced by the fungi.

The highest inhibitory activity was registered between *Metarhizium anisopliae* DSM1490 and *Bacillus subtilis* B49b, where the biocontrol bacteria suppressed the fungal growth to 81.6%, and the expansion of the bacterial growth was inhibited in the presence of the fungi.

The inhibitory activity of the biocontrol bacterial strains against some of the entomopathogenic fungi could be influenced by the slow growth of the fungi comparing with the high ability of some bacteria to colonize the culture media.

None of the EPF strains tested influenced the growth of *Bacillus licheniformis* 77.1s.

No toxic or negative effects towards *Daphnia magna* crustacean and *Eisenia foetida* earthworms were found in the ecotoxicological studies, when testing the entomopathogenic fungal strains at the standard concentration used for pest biocontrol.

The biocontrol bacterial strains, tested at 10^8cfu/ml towards *Eisenia foetida* earthworms showed reduced mortality percents of 7.5 to 17.5% in the ecotoxicological studies.

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IN VITRO APPROACHES ON THE DEVELOPMENT AND PROLIFERATIVE GROWTH OF INDUCING CALLUS FROM SOMATIC EXPLANTS OF HOT CHILI PEPPER (*C. ANNUUM* L. CV. PINTEA AND THE CV. DE CAYENNE)

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Abstract

Starting from the fact that synthesis and accumulation of secondary metabolites can be stimulated by in vitro cell culture from somatic tissues, the purpose of this study was to obtain biologically active substances from callus cell proliferation of varieties of hot chili pepper, like Pintea and De Cayenne genotype (C. annuum L.).
Somatic explants taken after 21 days from the regenerated plantlets from germinated seeds in aseptic in vitro conditions, like hypocotyls, cotyledons, young leaves and apex were inoculated on several variants of hormonal combinations, added to the recipe of basal Murashige and Skoog (1962) culture medium.
For the tested variants we used phytohormones, like auxins (NAA and 2.4-D) and cytokinins (kinetin) in concentrations ranging from 0.5 mg/L for kinetin and 0.3-1.0 mg/L for NAA and 2.4 D. The best results on the active growth of callus, were obtained for Pintea variety when there were utilized the cotyledons and apex (100%) and in the case of young leaves, the result was 58% on media supplemented with kinetin 0.5 mg/L .
Comparing with this genotype, for explants of De Cayenne variety cultivated on the same combination of tested culture medium (MS supplemented with 0.5 mg/L kinetin), the results of 100% were obtained only at the apex level and for the other types of tested somatic explants, the values recorded was 94% in the case of young leaves and only 55% for cotyledons.

Keywords*: pepper, somatic explants*, *callus induction, direct organogenesis.*

INTRODUCTION

Capsicum is a genus of the flowering plant family *Solanaceae*. Its species have been cultivated in America since thousands of years, and are now cultivated worldwide. *Capsicum* consists of approximately 20-27 species from which five are domesticated - *Capsicum annuum, Capsicum baccatum, Capsicum chinense, Capsicum frutescens* and *Capsicum pubescens* (Walsh et al., 2001; Heiser et al., 1969; Bosland, 1994).

The fruits of capsicum have a variety of names like chili pepper, red or green pepper, sweet pepper, bell pepper, miniature paprika, among others.

The various colours exhibited in *Capsicum* are due to mixture of esters of capsorubin, zeaxanthine, crytoxanthine, capsanthin and other carotenoids. These various and extractable colours of *Capsicum* fruits is extensively used in the food processing industry in wide range of products.

Capsicum is an excellent source of vitamins A, B, C and E and also rich in minerals like molybdenum, potassium, manganese and thiamine. β Carotenoids and vitamins C and A are powerful antioxidants that destroy free radicals. The total antioxidants is completed by phenolic compounds, which occur in peppers in connection with sugars.

Even chilli contains seven times more vitamin C than orange. It also contains bioactive nutrients, such as violaxanthin, lutein, β-cryptoxanthin and β-carotene.

The therapeutic properties and pungency exhibited in *Capsicum* contain capsaicinoids ($C_{18}H_{27}NO_3$) alkaloids specific for *Capsicum* genus, which show many pharmacological properties.

As medicine, it is used for neuralgia, rheumatic disorders, non-allergic rhinitis, among others,

thus their importance is widely known as a wellbeing food (Khomendra et al., 2013).
Plant regeneration system by organogenesis in *Capsicum* has been reported from diverse explants (Swamy et al., 2014).
Direct somatic embryogenesis was first described in chilli pepper by Harini and Sita (1993) and in sweet pepper by Binzel et al. (1996). Number of chemical and physical factors like media components, phytohormones, pH, temperature has been extensively studied also for a large number of plant species (Fett-Neto et al., 1995).
The type and concentration of auxin or cytokinin or the auxin/cytokinin ratio alters dramatically both the growth and the product formation in cultured plant cells (Mantell and Smith, 1984).
In this report, an efficient system for inducing of proliferating growth callus from *Capsicum* somatic explants (Pintea and De Cayenne genotype) starting from young leaf, apex, hypocotyls and cotyledons explants using auxins and cytokinins is reported.

MATERIALS AND METHODS

Explant sources: experiments were carried out using as explants sources plantlets having approx. 2cm in height, obtained by germinating chilli pepper seeds in „*in vitro*" culture conditions.
Seeds of two varieties of *C. annuum* L. (cv. Pintea and the cv. De Cayenne) were properly washed in running tap water and sterilized in ethanol (70%) for 5 minutes and sodium hypochlorite (10%) for 10 minutes and then, a final rinse for three times with sterile distilled water. Seeds were inoculated on full-strength Murashige and Skoog (1962) basal medium (MS), with the addition of 3% sucrose, 0.8% agar and with different concentrations and combinations of plant growth regulators, like auxins (NAA and 2.4-D) and cytokinins (kinetin) in concentrations ranging from 0.5 mg/L for kinetin and 0.3-1.0 mg/L for NAA and 2.4 D. Plant growth regulator supplements were added prior to the media autoclaving.
The pH of the culture media was adjusted to 5.5 using NaOH 1N and solidified with 0.8% (v/v) agar before autoclaving at 121°C for 15 min. After inoculation, seeds were maintained in dark for 10 to 12 days for germination, at 25°C and later they were exposed to 16 hours of light followed by 8 hours of darkness photoperiod.
Explants were collected after 21 days from healthy plantlets grown in the culture bottle in the laboratory. Explants used for the experiment included hypocotyls fragments, cotyledons, young leaves and apex. They were excised from seedling and cut into 1cm long segments and explants were inoculated immediately on MS medium, supplemented with 0.5 mg/L NAA and 1 mg/L 2.4 D (V1 variants) and with 0.5 mg/L Kinetin (V2 variants) for each variety.
Morphogenetic culture establishment: the explants were placed on the surface of the culture media variants (Table 1), distributed in 12 cm ø Petri plates (containing 7-10 mL of sterile autoclaved culture medium solidified with 8 g/L agar) and the incubation was performed in the growth chamber, at 25± 2°C, under a 16/8 h photoperiod, with a light intensity of 3000 lux. The periodical transfers on fresh culture media were performed at 3 week intervals.

Table 1. Culture media variants used for inducing of proliferating growth callus from somatic explants of *Capsicum*

Variant	Auxins (mg/L)		Cytokinins (mg/L)	Somatic explant type used
	NAA	2.4D	Kin	
V1	0.5	1	-	hypocotyls, apex, young leaves
V2	-	-	0.5	cotyledons, young-leaves, apex

Legend: NAA = α –naphthalene-acetic acid; 2.4-D = 2.4-dichlorophenoxyacetic acid; Kin = Kinetin.

RESULTS AND DISCUSSIONS

Plant cell culture offers a promising approach for a large scale production of phytochemicals and has several advantages over whole plant production. Callus initiation involves three major considerations: selection of explants, medium and culture conditions (Hall et al., 1988).
In this experiment, explants were collected from the plantlets which were contamination of free „*in vitro*" healthy plants for direct regeneration after 21 days of seed inoculation.

Two different combinations of auxins and cytochinins (V1 and V2) were used in the first stage of callus initiation and for callus culture establishment.
The beginning of callus development was observed within 2 weeks after explants inoculation on the inductive media.
The calli developed on these two variants were yellowish–green, partly friable, with a pronounced propensity to develop roots (Fig. 1, Fig. 2).

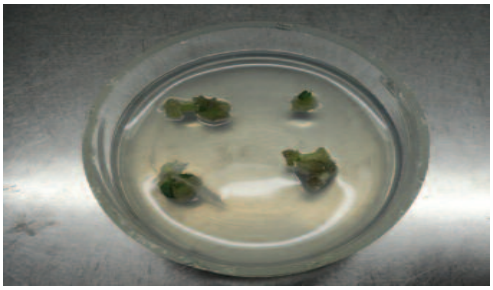


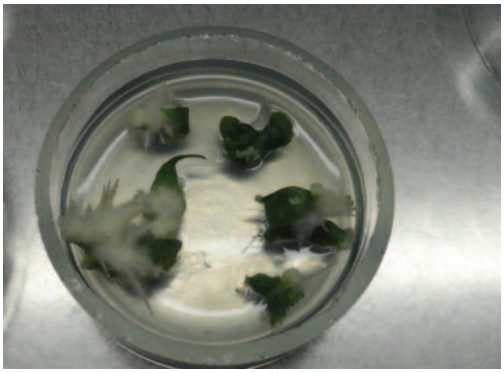
Figure 1. Developing calli induced from apex and explants after 3 weeks since inoculation on 0.5 mg/L NAA and 1 mg/L 2.4 D (Variant 1) of *C. annuum* L. (cv. Pintea)

The transfers were performed on fresh culture media at 3 weeks intervals, and the increase of the callus biomass on every culture media variant was recorded, by weighing their callus biomass from every culture vessel compared with the initial weight of the transferred callus piece.
The average increase of callus biomass /culture dish (4-6 explants inoculated/Petri plate) evaluated after 15 weeks of initiating the experiment with somatic chilli pepper explants cv. Pintea are shown in Table 2 and those for cv. De Cayenne in Table 3.

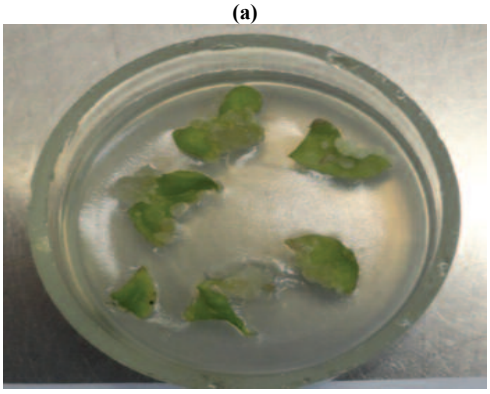
Table 2 Average of callus biomass increase/ culture vessel during a 15 weeks of initiation of the experiment in somatic explants of chilli pepper cv. Pintea. (g)

Variant	Type of used explant	Average of callus biomass increase/culture vessel (g)
V1	hypocotyl	2.74
V1	apex	1.29
V2	cotyledon	3.10
V2	young leaves	3.05
V2	apex	2.92

Legend: V1 = 0.5 mg/L NAA and 1 mg/L 2.4 D; V2 = 0.5 mg/L Kinetin



(a)



(b).

Figure 2. Developing calli induced from cotyledons and young leaves explants after 3 weeks since inoculation on Variant 2 of *C. annuum* L. cv. Pintea(a) and the cv. De Cayenne (b).

Table 3 Average of callus biomass increase/ culture vessel during a 15 weeks of initiation of the experiment in somatic explants of chilli pepper cv. De Cayenne (g)

Variant	Type of used explant	Average of callus biomass increase/culture vessel (g)
V1	hypocotyl	0.86
V1	young leaves	1.63
V2	cotyledon	1.99

Legend: V1 = 0.5 mg/L NAA and 1 mg/L 2.4 D; V2 = 0.5 mg/L Kinetin

The best results on the active growth of callus, were obtained for Pintea variety when it was utilized the hypocotyls fragment explants inoculated on V1 variant supplemented with 0.5 mg/L NAA and 1 mg/L 2.4 D and when utilized the cotyledons cultivated on media supplemented with kinetin 0.5 mg/L.
Comparing with this genotype, for explants of De Cayenne variety cultivated on the same combination of tested culture medium (MS supplemented with 0.5 mg/L kinetin), the results of 64% were obtained only at the

cotyledons level and for the other types of tested somatic explants, also the values recorded was lower than the cv. Pintea explants.

The calli developed from chilli pepper cv. De Cayenne young leaves cultivated on V1 variant supplemented with 0.5 mg/L NAA and 1 mg/L 2.4 D, were yellowish – green, compact with an activ proliferation activities (Fig. 3).

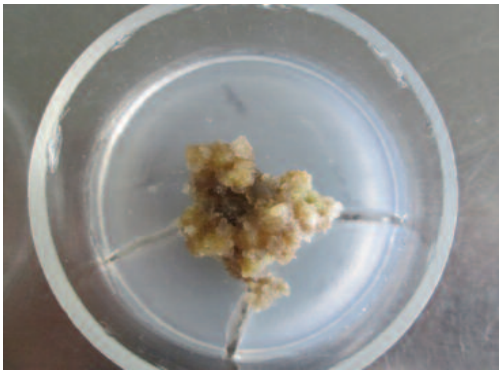


Figure 3. Developing calli induced from young leaves and explants after 15 weeks since inoculation on 0.5 mg/L NAA and 1 mg/L 2.4 D (Variant 1) of *C. annuum* L. (cv.De Cayenne)

Comparing the results for proliferative growth of inducing callus, the most successful explants are often cotyledons for both tested genotype varieties of hot chili pepper, Pintea and De Cayenne.

CONCLUSIONS

Through repeated subculturing at intervals of 3 weeks of various somatic explants for callus proliferation, on the hormonal recipes Variant 1 and Variant 2, it was found after approximately 4 months to the experiments initiation, an increase in biomass of callus culture vessels, that registered average values of 2.62 g / Petri plate culture for Pintea variety, respectively 1.49 g / Petri plate for De Cayenne variety chilli pepper explants.

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THE EFFECT OF SUCROSE CONCENTRATION ON *IN VITRO* CALLOGENESIS OF GOLDEN ROOT – ENDANGERED MEDICINAL PLANT

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Abstract

Rhodiola rosea is a medicinal plant, containing a range of antioxidant compounds, including p-tyrosol, organic acids (gallic acid, caffeic acid, and chlorogenic acid), and flavonoids (catechins and proanthocyanidins). The stimulating and adaptogenic properties of Rhodiola rosea are attributed to p-tyrosol, salidroside, rhodioniside, rhodiolin, rosin, rosavin, rosarin, and rosiridin. The recent investigations showed that sucrose influences secondary metabolism in cell, tissue and organogenic cultures. Higher sucrose levels in certain limits lead to increased production of alkaloids in in vitro cultures of various plants. The aim of this work was to study the influence of different sucrose concentrations on the process of callus formation. The nutrient medium containing different percentage of sucrose (20, 30 and 40 %) were used. Leaves excised from in vitro propagated golden root plants were plated for callus induction and maintenance. The results showed that the callogenesis of golden root plants was more effective (2 - 3 folds) when sucrose was 20 g/l compared to higher concentrations of sucrose at the background of the same combinations of plant growth regulators. The obtained calli differed in structure and color. HPLC analysis of the calli was performed.

Keywords: *endangered plant, Rhodiola rosea, golden root, in vitro, calli, sucrose, HPLC*

Abbreviations
BAP – N6-benzylaminopurine;
IAA – Indolyl-3-acetic acid;
Kin – Kinetin;
MS – Murashige and Skoog medium, 1962;
NAA – a- naphthyl acetic acid;
2-iP – 6-(y,y-dimethylallyl amino) purine;
2,4-D – 2,4- dichlorophenoxyacetic acid.
CAS – casein hydrolysate
HPLC – High-performance liquid chromatography

INTRODUCTION

Rhodiola rosea L. (Golden root, Rose root, *Sedum roseum* (L.) Scop., *S. rhodiola* DC.) is a valuable species for Bulgarian medicinal plants genfund. The restoration abilities of the wild plants are defined as relatively limited due to both, low germination and very low coefficient of vegetative propagation, traditionally used (Platikanov and Evstatieva 2008). The extracts obtained from the plants have antidepressant, anticancer, cardio protective and central nervous system stimulation effects (Kelly, 2001; Brown et al., 2002). The plant root, rhizomes and calli produce pharmaceutically important compounds referring to 6 different groups:(1) phenylpropanoids; (2) phenylethanol derivaties (3) flavonoids; (4) monoterpenes; (5) triterpenes; (6) phenolic acid (Ganzera et al., 2000; Linh et al., 2002). The antioxidant activity in different tissues and organs in wild and in *in vitro* cultivated plants and calli of *Rh. rosea* 4 due to four phenolic component, including flavonoids (salidroside, rosarin, rosavin and rosin), identified by HPLC (Furmanowa et al., 1998).

Plant *in vitro* cultures offer possibilities for the production of secondary metabolites in bioreactors, manipulation of metabolic pathways and metabolic engineering. Studies showed that the sucrose can influence secondary metabolites production in cell and organ cultures (Fowler, 1983; Paiva and Janick, 1983). The results of many investigations have concluded that the increase of sucrose within certain limits leads to increased production of alkaloids in tissue cultures of various plants

(Wijnsma et al., 1986; Duraz et al., 1994 Vázquez-Flota et al., 1994). Less data are available for the effect of other carbon sources. According to Paiva and Janick (1983) increasing the concentration of sucrose but not that of the glucose influences the content of lipids, alkaloids and anthocyanins in the zygotic and somatic embryos of *Theobroma cacao* cultivated *in vitro*. There are data showing that the presence of maltose in nutrient medium also increases accumulation of secondary metabolites (Kinnersle and Henderson, 1988; Rao and Narasu, 1999).

There is not a great number of experiments investigating the possibilities for callus formation in different species of *Rhodiola* and following by the chemical analysis of the biologically active substances and determination of the parameters for their optimal synthesis in *in vitro* condition.

Sheng et al. (2005) investigated the induction and maintenance of callus cultures in *Rhodiola quadrifida* (plant used in traditional Chinese medicine over 1000 years). Callus cultures were induced using Murashige and Skoog (1962) MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (1.0 mg/l) , α- naphthyl acetic acid (NAA) (2.0 mg/l), N^6-benzylaminopurine (BA) (0.5 mg/l) and kinetin (0.1 mg/l). For maintaining the calli MS medium was supplemented with 2,4-D (1.0 mg/l), BA (0.1 mg/l) and kinetin (0.5 mg/l). After 30 days of cultivation the content of salidroside in calli cultures was measured and it was found out that callus tissue was able to produce biologically active substances. Calli induced from the stem and leaves of *Rhodiola kirilowii* had potential to produce salidroside. The latter amount is influenced from the type of the explant, temperature and period of cultivation (Li Wei et al., 2005). Soo Jung Kim et al. (2004) found that calli obtained from *Rhodiola sachalinensis* cultivated on Gamborg's B5 nutreint media enriched with 0.5 mg/l NAA, 1 mg/l BA and 5 % sucrose can produce high level of salidroside (0.41 % on the basis of dry weight) compared to intact root (0.17 %). György et al. (2004) and György (2006) obtained and cultivated for a long time callus from *Rh. rosea* in liquid medium supplemented with precursors of biologically active substances with the aim to increase the synthesis of substances of the basic biologically active complex. In these cultures, rosin content was elevated to 1.25% dry weight while rosavin was 0.083% dry weight.

The aim of this investigation was to study the possibility of callus induction from *Rhodiola rosea* Bulgarian ecotype and secondary metabolites production by calli using different concentrations of sucrose.

MATERIALS AND METHODS

Callus cultures. Leaves excised from *in vitro* propagated *Rhodiola rosea* plants were used for callus induction. Leaves were plated on solidified culture media containing different combinations and concentrations of phytoregulators (Tasheva and Kosturkova, 2012a; Tasheva and Kosturkova, 2012b, Tasheva and Kosturkova, 2013c).

Composition of culture media for callus induction in vitro. Different variants of nutritious media containing Murashige and Skoog (1962) basic medium (MS) supplement with phytoregulators in various combinations and concentrations were used (Table 1). However, content of sucrose was different (20 g/l, 30 g/l and 40 g/l) while the content of agar-agar was constant (6.0 g/l). Culture media were autoclaved at 1.1 $kg.cm^{-2}$, 121° C for 20 min. The experiments were in two replicates.

In vitro cultivation condition. Callus cultures were induced and maintained in cultivation room at temperature of 23-24° C and dim light (20 $\mu Mm^{-2}s^{-1}$). Response of 30 to 50 explants was examined for each medium variant and Sigma Plot 11.1 applied for statistics.

Table 1. Culture media composition [mg/l] for induction and maintaining of callus cultures of *Rhodiola rosea*.

Media variant	BAP	2,4-D	IAA	NAA	CAS*
1. C-MS Control					
C-1	1.0	1.0			
C-2	1.0	0.5			
C-3	0.5	1.0			
C-4	1.0	1.0			1000
C-5	1.0	0.5			1000
C-6		0.1	0.2		1000
C-7	1.0			0.5	1000

*Casein hydrolysate

Phytochemical analysis. For quantity determination of the main polyphenols: salidroside, rosavin, rosin and rosarin in *in vitro* obtained calli was used HPLC.

Extract Preparation. The calli from nutrient media C-1, C-2, C-3, C-4, C-5, C-6 and C-7 were dried at room temperature.

Callus extraction: Hundred milligrams of the finally powdered calli was extracted three times with 1 ml methanol. After centrifugation at 3000 rpm for 10 min, the supernatants were combined and adjusted to the final volume of 1 ml with methanol. The solution was injected in triplicate.

Analytical methods (HPLC): HPLC analyses were performed on an Agilent 1100 Series HPLC system, equipped with a MWD UV-Vis detector. The column used was Chromsep SS (250 x 4.6 mm ID), precolumn Intertsil 5 ODS 2. The mobile phase was water (A) and acetonitrile (B), applied in the following gradient: from 92.5A:7.5B (v/v) in 45 min to 80A:20B (5 min isocratic elution). Each run was followed by a 15 min equilibration period. The flow rate was 1 ml/min, 10.0 µl of sample was injected. The effluent was monitored at wave length of 205 nm and 254 nm.

RESULTS AND DISSCUSIONS

Rhodiola rosea leaf explants placed on MS medium supplemented with various concentrations of auxins and cytokinins formed callus within 4 weeks.

In our previous experiments induction of callogenesis was successful from leaf explants, isolated from *in vitro* propagated plants, on MS media enriched with BAP in concentration from 0.5 mg/l to 2.0 mg/l; 6-(y,y-dimethylallyl amino) purine (2-iP) - 0.3 and 3.0 mg/l; 2,4-D - from 0.1 to 2.0 mg/l; IAA - 0.2, 0.3 and 1.0 mg/l; NAA - 0.5, 1.0, 1.5 mg/l and casein hydrolysate 1000 mg/l (Tasheva and Kosturkova, 2012a). The highest response to formation of callus (62.85% and 73.17%) was observed on two media-containing 1 mg/l BAP and either 1 mg/l or 0.5 mg/l 2,4-D (Table 2) . Calli formation was less effective or calli was not formed when sucrose was 30 g/l and 40 g/l in the culture medium (0.5 mg/l BAP as compared to 1 mg/l 2,4-D in variant C-3 for instance).

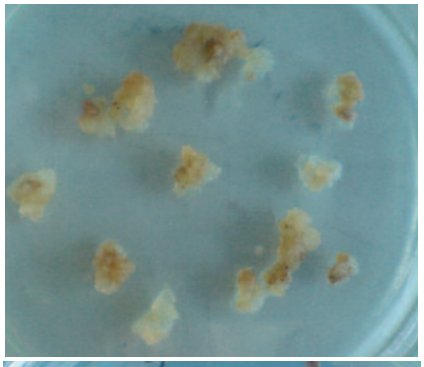
Table 2. Callogenesis induction and characteristics of the calli tissues on 30-th day of cultivation.

Nutrient Medium №	Callus induction [%] on different concentrations of sucrose			Characteristics of the callus tissue growth on different concentration sucrose		
	20 g/l	30 g/l	40 g/l	20 g/l	30 g/l	40 g/l
MS.	0	0		Explants whitened and died		
C-1.	62.85	33.00	4.00	½ loosy calli with beige color; 12 % brownish and loosy calli	33 % compact calli like structures	Calli like structures
C-2.	73.17	26.00	-	Compact, grainy calli, pale yellow color	Pale yellow color	Necrotic yellowish explants
C-3.	43.90	8.00	-	Loosy, liquidy calli, pale, yellow color	Pale yellow color	Necrotic yellowish explants
C-4.	54.17	53.00	12.00	Compact, solid calli, grainy, yellow-beige color	53% liquidy, loosy calli like structures; 47 %-brown, necrotic	liqued calli like formation
C-5.	79.2	36.5	8.2	Soft, grain calli, beige in color	Soft calli, yellow-beige in color	Yellow calli like structures, necrotic
C-6	48	10.2	-	liquidy calli; pale beige in color	liquidy calli;	Necrosis
C-7.	51.78	11.00	-	Fresh calli, yellow-green color	20 % bud induction	Necrotic yellowish explants

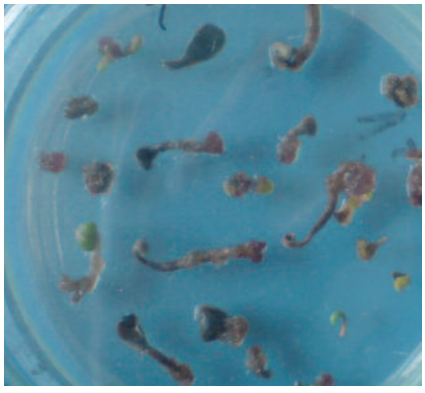
Induction of callogenesis on the same variants of phytoregulators but with a higher concentration of sucrose (3% or 4%) was twice, three times lower percentage. For example the percentage of induced calli was 62.85% and 33%, respectively in culture medium variant C-1 containing 1.0 mg/l BAP and 1.0 mg/l 2 4-D and 2 % or 3 % sucrose (Figure 1) . Callus induction rate was 73.17 % and 26 %, respectively, in variant C-2 containing 1.0 mg/l BAP, 0.5 mg/l 2,4-D and 2 % or 3 % sucrose. Variant C-4 containing BAP (1.0 mg/l), 2,4-D (1.0 mg/l) and casein hydrolysate (1000 mg/l), and 2 % or 3 % of sucrose stimulated formation of callus by 54.17% and 53.00 %, respectively. Variant C-7 (BAP – 1.0 mg/l, NAA –0.5mg/l and casein hydrolysate – 1000 mg/l), and 2 % and 3% of sucrose induced calli up to 51.78% and 11.00 %, respectively.

Obtained calli had different characteristics – morphology, color etc.

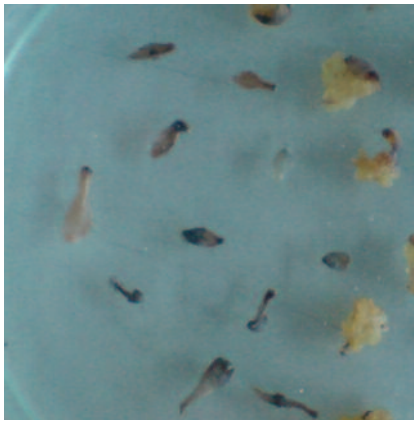
Growth and Characteristics of the Callus. Callus growth rate and tissue characteristics varied depending on the culture media composition.



A.



B.



C.

Figure 1. Callus cultures on variant C-1containing different concentrations of sucrose A: 2 %, B: 3%, C: 4%

Culture medium composition was important for callus structure and texture which varied among variants and within one medium variant. BAP in concentration of 1 mg/l favored fast growing compact tissue with grain structure (var. C-2, C-4, C-5 and C-7) which was easy to be maintained. Its color varied from greenish to yellowish pale. Calli in other variants were predominantly liquidy and loosy with beige-brownish color that was not suitable for further use.

HPLC analysis. The HPLC analyses of the callus cultures induced and grown at various concentrations of sucrose in the medium (samples C-1, C-2, C-3, C-4, C-5, C-6 and C-7 passage II) did not show presence of basic compounds responsible for the pharmacological effect of the species or the produced biologically active substances were in negligible quantities.

Soo Jung Kim et al., (2004) found that *Rhodiola sachalinensis* calli cultivated on B5 nutrient medium enriched whit 5% sucrose can produced high level of salidroside (0.41% on the basis of dry wt) compared to the intact root (0.17%). In the other valuable species, like *Leucojum aestivum* Georgieva (2012) observed negative correlation between the levels of carbon source in the medium and the accumulation of galantamine in organogenic cultures, what was in contradiction to the results obtained by Sellés et al. (1997) in a similar experiment using *in vitro* cultures of narcissus. The greatest amount of galantamine is measured in explants grown without carbon source. In all other cases the

amount is much less. In variants with glucose, quantity of the galantamine decreases when increasing glucose concentration. In variants with maltose and with sucrose were observed some increase in their highest concentrations

CONCLUSIONS

The present study contributed to determine the proper concentrations of sucrose in culture media for the development of callus cultures of Bulgarian Golden Root. The optimal concentration of sucrose (2 %) was determined for efficient induction and maintaining of callus with grain structure suitable for long-term cultivation. The callogenesis efficiency expressed by the percentage of explants forming undifferentiated tissue, their growth and characteristics depended significantly on the type and concentrations of the phytoregulators.

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BIOMASS PRODUCTION AND WASTEWATER TREATMENT FROM AQUACULTURE WITH *CHLORELLA VULGARIS* UNDER DIFFERENT CARBON SOURCES

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Abstract

Algae are sustainable sources of biomass for fuel, food, feed and essential for their growth are light, CO2, and inorganic nutrients like nitrogen and phosphorous. The aim of our study was to explore the effect of different carbon sources on biomass accumulation in microalgae Chlorella vulgaris and its ability to remove N and P compounds during its cultivation in aquaculture wastewater. Microalgae cultivation was initiated in bioreactor from 500ml Erlenmeyer flask containing 250ml wastewater from semi closed recirculation aquaculture system. The cultures were maintained at room temperature (25-27ºC) on a fluorescent light with a light:dark photoperiod of 12 h: 12 h. The microalgae were cultivated in wastewater with two different carbon sources: carbon dioxide (2%, v/v), and sodium bicarbonate ($NaHCO_3$) (1.125g.l^{-1}). The growth of strain was checked for 96 hours period. In our study C. vulgaris showed better growth in wastewater from aquaculture with bicarbonate utilization as carbon source during the experiment.

Keywords*:aquaculture, biomass, carbon sources,Chlorella vulgaris, wastewater.*

INTRODUCTION

Algae are sustainable sources of biomass for fuel, food, feed and essential for their growth are light, CO_2, and inorganic nutrients like nitrogen and phosphorous. Algae are fast growing and can be cultivated in systems that don't require agricultural land and thereby it doesn't compete with another culture (Mercer and Armenta, 2011). Wastewater treatment is another possible use of algae since they grow well on the nutrients present in the water (Larsdotter, 2006). The uptake of dissolved nutrients by microalgae is the primary way to remove nitrogen in aquaculture systems (Attasat et al., 2013; Sirakov et al., 2013). Many authors have studied the use of microalgae to treat wastewater from aquaculture. Aquaculture systems involving microalgae production and wastewater treatment seems to be quite promising for microalgae growth combined with biological cleaning (Mata et al., 2010).

Chlorella vulgaris Beyerinck (Beijerinck) is a robust and fast growing microalgae species commonly cultivated and interesting regarding the production of secondary metabolites (Mansson, 2012).*C. vulgaris* is highly valued for its protein content, as it can be used for is potential biomass. The factors like CO_2, intensity of light, wavelength affects the growth rate of the *C. vulgaris* (Sankar and Ramasubramanian, 2012).

Often carbon and nitrogen are the most important nutrients contributing to the biomass produced (Prabakaran and Ravindran, 2012). Carbon is the most important element found in algal biomass and it constitutes over 50% in typical algal biomass (Becker, 1994). Using captured CO_2 for microalgae growth is limited by the high cost of CO_2 capture and transportation, as well as significant CO_2 loss during algae culture. The algae grow poorly at night, but CO_2 cannot be temporarily stored until sunrise. For these reasons, it is necessary a more efficient and a cheap source of carbon such as sodium bicarbonate. Microalgae have the ability to use organic carbon as an energy source and this is important because it can minimize the inhibitory effects of seasonal and diurnal light limitation on growth in outdoor cultures.

The aim of our study was to explore the effect of different carbon sources on biomass accumulation in microalgae *C. vulgaris* and

itsability to remove N and P compounds during its cultivation in aquaculture wastewater.

MATERIALS AND METHODS

Microalgae strain, medium and cultivation

C. vulgaris (SKU: 100-CVC00-50) which is a green alga belonging to the division *Chlorophyt*a and class *Trebouxiophyceae* was purchased from Algae depot – USA (www.algaedepot.com). The wastewater used like a media for tested algae cultivation originate from semi closed recirculation aquaculture system (semi – closed RAS), before it cleaning at mechanical and biological filters.

Algae cultivation was initiated in bioreactor from 500ml Erlenmeyer flask containing 250ml wastewater. The experiment was conducted in variants with carbon dioxide (2%, v/v) and sodium bicarbonate ($1.125 g.l^{-1}$).Three luminescent lamps Sylvania Aqua Star – 18w, 10 000 K were placed at a distance of 30 mm from flasks. Light regime was adjusted at 12:12 h light:dark cycle in an illumination incubator until the end of experiment. The temperature was kept between 25 and 27°C. The strains were checked for 96 hours growth period. In the laboratory, the samples of wastewater were filtered through a 25mm, 3µm glass microfiber filters (GF/C) mounted on a Millipore filtration unit. The cells in exponential period were inoculated (10%, v/v) in a liquid medium.

Optical density, chlorophyll and carotenoid content of microalgae culture

Optical densities of microalgae cultures were measured at 0, 24, 48, 72 and 96 hours after the start of the experiment in three replicates. The sample with volume one ml was appropriately diluted with deionised water and the average value was recorded by absorbance at 450 nm with the help of spectrophotometer DR 2800 (Hach Lange).

The isolation of pigments from algae cells included the following procedures: harvesting 2 ml of microalgae cells by centrifugation at 10000 rpm, two times for 3 min and discarding the supernatant, suspension of cells in 2 ml methanol/water 90:10 v/v and mixing of Vortex for 1 min., heating of the suspension for half an hour in a water bath at 60ºC, cooling of the samples at room temperature, centrifugation the suspension (10000 rpm for 3 min) and discarding the supernatant with dissolved pigments. The absorbance of the pigments extract (665, 652 nm for chlorophyll content (a+b) and 470, 666nm for carotenoids content) was recorded by using spectrophotometer. The chlorophyll content was computed ($mg.l^{-1}$) according Porra et al. (1989) and carotenoid content was computed ($mg.\ l^{-1}$) according Lichtenthaler (1987).

Hydrochemical analysis

Samples for hydro chemical analysis were taken at the beginning of the trial, 24, 48, 72 and 96 hours after the start of the experiment in three replicates. The samples were centrifuged at 300 rpm for 10 min, for freeing them from algal cells (Lee and Lee, 2002).

The measurement of pH was made with a portable combined meter and with a pH probe (Hach Lange).

Other analyzed hydro chemical parameters were measured spectrophotometrically with spectrophotometer DR 2800 (Hach Lange). The methods and range of tests which were used during the experiment are shown it table 1.

Table 1. Methods and range of tests used to monitor the water quality parameters during experiment

Quality parameters	Determination method	Measuring range ($mg.l^{-1}$)
Ammonia	Indophenol blue	0.015 – 2
Nitrate - nitrogen	2.6 dimethylphenol	5 - 35
Total nitrogen	Koroleff digestion + 2.6 dimethylphenol	5 - 40
Phosporus (ortho + total)	Phosphormolybdenum blue	0.15 – 4.5

Data analyses were conducted by using one-way Analysis of Variance ANOVA (MS Office, 2010).

RESULTS AND DISCUSSIONS

The algae need the carbon for the photosynthesis. About 50% of the alga biomass is made up of carbon and is therefore needed to a large extent for good growth (Becker, 1994). The natural CO_2 present in the air is not sufficient to maintain optimal growth. In our study the optical density of *C. vulgaris* was 1.25 in wastewater with carbon source – $NaHCO_3$ used like a growing media for 96hour (Figure 1). It was with 12% higher optical

density compared to wastewater with carbon dioxide for the same strain. Jeong et al. (2003) receive a similar result in the culture of *C. vulgaris* that showed the highest growth rate on 5 day (1.17 optical densities). Elvira-Antonio et al. (2013) was reached higher productivity in *C. vulgaris* cultures with 1g.l^{-1} sodium carbonate. Goswami et al. (2012) observed that CO_2 gas in algal culture has a poor dissolving capacity and most of it tend to lost in the air, so it is convenient to use bicarbonate form instead of CO_2 gas.

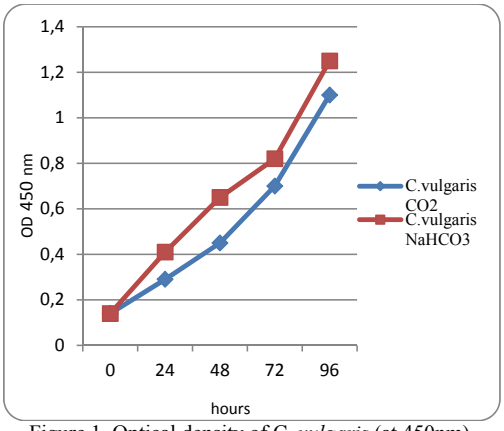

Figure 1. Optical density of C. *vulgaris* (at 450nm) cultivated in wastewater from aquaculture with different carbon source

Ahmad et al. (2013) established that *Chlorella* grow well in wastewater as well as in the nutrient medium. In our experiment the highest chlorophyll content was determinate in *C.vulgaris* cultivated in wastewater with carbon source – NaHCO3 (9.6 mg.l^{-1}) (Figure 2). Our results correspond with Chinnasamy et al. (2009), which growth response of *C. vulgaris* in terms of biomass and total chlorophyll showed a similar pattern. Šoštarič et al. (2009) cultivate *C.vulgaris* in bicarbonate concentration of 1.05 g.l^{-1} in the pure solution from the modified Solvay process and determined 5.2 mg.l^{-1} chlorophyll.

The microalgae *C. vulgaris* is known to produce pharmaceutically important carotenoids: canthaxanthin and astaxanthin (Mendes et al. 2003). Increased production of carotenoids in presence of higher amounts CO_2 might add to the economic utility of this algal strain.

In our study the quantity of carotenoids in *C. vulgaris* were higher (2.1 mg.l^{-1}) in cultures grown in wastewater with $NaHCO_3$ carbon source, compared with the carotenoids of wastewater with carbon dioxide - 1.89 mg.l^{-1} (Figure 3). Chinnasamy et al. (2009) cultivated *C.vulgaris* in 6% $CO_2$and established 2.0 mg.l^{-1} carotenoids. We achieve the same results with at less 2% CO_2 added in wastewater from aquaculture.

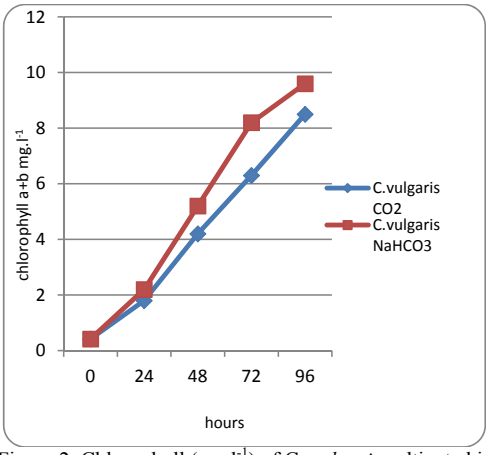

Figure 2. Chlorophyll (mg.l^{-1}) of C. *vulgaris* cultivated in wastewater from aquaculture with different carbon source

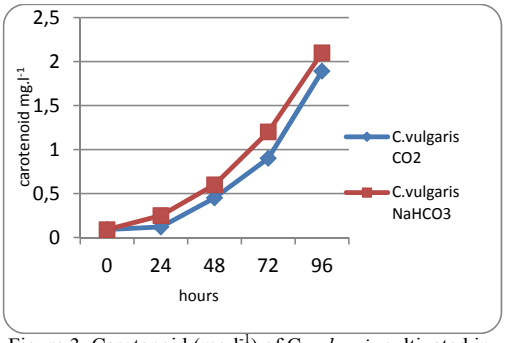

Figure 3. Carotenoid (mg.l^{-1}) of C.*vulgaris* cultivated in wastewater from aquaculture with different carbon source

The pH will rise as the algae grow and consume the carbon dissolved in the water. This indicates that *Chlorella* can be flocculated efficiently by increasing the pH of the culture to 11 (Mansson, 2012). During our trial the measured pH varied from 6.08 to 9.94 in tested algae strain and the pH value increased mostly in cultivation with $NaHCO_3$ (Figure 4). This is in compliance with Sorensen et al.(1996), who

maintained that, increase of pH is due to the use of CO_2(aq) from bicarbonate to compensate the lack of CO_2 from gas supply. It is important to maintain pH within an adequate range to avoid the loss of carbon dioxide existing in the media.

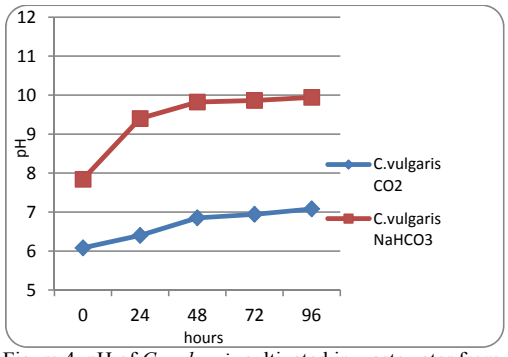

Figure 4. pH of *C. vulgaris* cultivated in wastewater from aquaculture with different carbon source

The concentration of nitrate at the beginning of the experiment was 4.43 mg.l^{-1}. In the end the most effective reduce of nitrate was in *C. vulgaris* cultivating in wastewater from RAS with $NaHCO_3$ carbon source (0.1mg.l^{-1}) and the differences were statistically proven ($P \leq 0.05$) (Table 2). This result obtained for *C. vulgaris* with $NaHCO_3$was with 13.5% better compared with the same of *C. vulgaris* with 2% CO_2. Elvira-Antonio et al. (2013) obtained 85.13% nitrogen removable growing *C. vulgaris* in the presence of 1 g.l^{-1} $NaHCO_3$. Shi et al. (2007) reported that the nitrate was removed from synthetic wastewater by the *C. vulgaris* after 4 days, like in our experiment.

At the beginning the ammonium was 14.3 mg.l^{-1}, and after 96 hour their quantity reduced up to 0.42 mg.l^{-1} for *C. vulgaris* with $NaHCO_3$ carbon source and 0.43 mg.l^{-1} for *C. vulgaris* with 2% CO_2. In our experiment, after 96 hours the ammonium decreased with 97% in wastewater from RAS with $NaHCO_3$ carbon source. Our results about ammonium removal efficiency was comparable to studies conducted by Martínez et al. (2000), who described elimination of NH4+ (between 79% and 100%) after188.25 h, and with González et al. (1997), who reported ammonium removal efficiencies of 90% from agro-industrial wastewater after 216 h. Shi et al. (2007) established that ammonium removal efficiency by *C. vulgaris* in wastewater was 78% in the first 3 days.

The better uptake total nitrogen (7.69%) was observed again in *C. vulgaris* with $NaHCO_3$ carbon source compared with 2% CO_2. At the end of the trial after the cultivation of our strain in wastewater with sodium bicarbonate the total nitrogen decreased with 71.2%.

Table 2. Hydrochemical parameters during the experiment (Data are expressed as mean ± standard error).

Parameters	*C. vulgaris* CO_2	*C. vulgaris* $NaHCO_3$
Nitrate	1.5±0.04*	1.33±0.04*
Amonium	4.81±1.55ns	4.38±1.51ns
Total nitrogen	7.03±0.91ns	6.51±0.96ns
Total phosphorus	1.51±0.03*	1.36±0.04*

*p≤0,05

The efficiency in total phosphorus removal from wastewater was better in wastewater with $NaHCO_3$ used as carbon source used for *C. vulgaris* cultivation, compared to 2% CO_2. In the beginning was measured a level of phosphorus compounds 3.6 mg.l^{-1}. At the end of the experiment for *C. vulgaris* cultivation in wastewater with $NaHCO_3$ carbon source phosphorus decreased with 91.1%, while with 2% CO_2 – 90.2%. Shi et al. (2007) demonstrated result that about 90% of the phosphate was removed from synthetic secondary wastewater within 2 days by *C. vulgaris* microalgal strains.

Aslan and Kapdan (2006) used *C. vulgaris* for nitrogen and phosphorus removal from wastewater with an average removal efficiency of 72% for nitrogen and 28% for phosphorus. Shi et al. (2007) performed experiments with *Chlorella* to remove nitrate from municipal wastewater and reduce levels of phosphate, ammonium and nitrate in synthetic secondary wastewater. In their experiment ammonium was removed less rapidly by the algae than phosphate. The specifics depend on the type of wastewater, the type of algae and their growth conditions, and most importantly on the relationship between the amount of biomass applied and the hydraulic loading of the wastewater (Shi et al., 2007).

CONCLUSIONS

Our results showed that wastewater from aquaculture with carbon source sodium bicarbonate promote better algal growth of *C. vulgaris* compared in wastewater with carbon source 2% CO_2.

Higher purification effect for tested hydrochemical parameters was observed in the wastewater with added sodium bicarbonate like carbon source for cultivation of the algae species.

C. vulgaris can be used for biological treatment of wastewater originate in aquaculture.

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ANTIOXIDANT CAPACITY AND THIN LAYER CHROMATOGRAPHY OF ETHANOL EXTRACTS OF *Allium ursinum* L. AND *Allium bulgaricum* L.

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Abstract

The radical scavenging capacity of 70 % ethanol extracts obtained from Allium ursinum L. and Allium bulgaricum L. was investigated in the present paper. The antioxidant capacity of the extracts was estimated with the use of ABTS, DPPH, FRAP, and CUPRAC assays and the total phenolic content was evaluated as well. The Allium bulgaricum extract appeared to possess a better antioxidant activity compared to the Allium ursinum extract, which was in accordance with the established higher content of total polyphenols for A. bulgaricum extract was 0.41 ± 0.09 mg GAE/g fresh plant weight. In comparison the polyphenols in the A. ursinum extract were found to be 0.40 ± 0.03 mg GAE/g fresh plant weight. The conducted simple TLC method for rapid determination of allicin and alliin in Allium spp. suggested a presence of those substances in both extracts considering the distinctive spots.

Keywords*: Allium spp., antioxidant capacity, TLC.*

INTRODUCTION

Garlic is well known across the centuries. It was used as a medicine by early civilizations (Rivlin, 2006; Thomson and Ali 2003). Moreover, garlic was mentioned as a medicine in some religions (Green and Polydoris 1993; Kahn 1996; Moyers 1996, Bergner 1996). Old Indians believed that it has a good effect for the treatment of joint infections, heart and digestive diseases which is well known nowadays (Woodward, 1996; Rivlin, 1998). As attention has been made more for the usage of plants in the early Renaissance, garlic has taken some importance as it was chosen to be grown for medical purposes (Moyers, 1996). Wild garlic (bear's garlic, wood garlic) grows in fens and river woods of Central Europe. The fresh leaves or dried herb is used in local cuisines of Europe. Since it has not been cultivated yet, it didn't gain any importance until several years ago where people started to look for this as it is natural.

Allium bulgaricum (samardala) is a glabrous plant, 50-100 (150) cm high. The leaves are 30-50 cm long and 10-20 mm wide, thin, with a proeminent central nervure on the back, making it look triangular in section. The plant is found only in limited areas. It is more famous for the flowers in the gardens, than for its healing or flavouring properties. It is poorly known in the other countries as a medical plant or as a culinary spice. Data is missing even in the specialized guides for aromatic and medical plants (Cheshmedjiev I., 2002). There are studies, connecting phyto-nutrients in *Allium* spp. plants with the possibility to reduce the risk of a number of illnesses (Lanzotti, V., 2006): coronary heart disease (Gorinstein et al., 2007; Siegel et al., 2004), cancer (Sengupta et al., 2004), obesity, diabetes, disturbances of the gastrointestinal tract, hyper-cholesterolemia, and inflammatory diseases (Kalayarasan et al., 2009; Takahashi et al., 2008).

Many researchers studied traditional used plants (Alexieva, 2010) and evaluated their antioxidant effect of various plant extracts (Alexieva, 2012 a; Alexieva, 2013) but mainly in terms of essential oils (Alexieva, 2012 b).

Allium ursinum is a wild relative of Europe and Asia. The Latin name is due to the brown bear's taste for the bulbs and its habit of digging up the ground to get at them. Ramsons leaves are edible; they can be used as salad, spice, boiled. The bulbs and flowers are also very tasty. Ramsons leaves are easily mistaken for lily of the valley, sometimes also those of

Colchicum autumnale and *Arum maculatum*. All three are poisonous and possibly deadly. A good means of positively identifying ramsons is grinding the leaves between one's fingers, which should produce a garlic-like smell.

Alliin and isoalliin were the main cysteine sulfoxides found (Schmitt et al. 2005). If the plants are dried, many of the compounds are degraded so that the use of the fresh plant is recommended or alternatively it has to be lyophilised. In the fresh leaves of *A. ursinum* 0.005 % allicin and 0.07 % methyl-L-cysteinsulfoxid as well as E-glutamylpeptides such as E-glutamylallylcysteinsulfoxid have been found (Wagner and Sendl, 1990; Matsuura et al. 1996), E-glutamylallylcysteinsulfoxid reported to inhibit angiotensin-converting enzyme (Sendl et al. 1992; Rietz et al. 1993). Other components such as lectins and flavonoids have been found (Carotenuto et al. 1996; Smeets et al. 1997a, b). Flavonoids were described to be responsible for inhibition of platelets aggregation in humans (Carotenuto et al. 1996). As a property similar to other *Allium* species, it has a marked antioxidant activity because of the high content of carotenoids, chlorophylls, flavonoids and low toxic oxygen radicals (Stajner et al. 2003).

Many volatile compounds such as sulfides and disulfides have been identified in *Allium ursinum* (Schmitt et al., 2005).

The strong antioxidant properties of representatives of *Allium* spp. have caused considerable food technologist interest.

Moreover, there are phenolic compounds in *A. ursinum*. The bulbs and the leaves were found to contain 2.3 mg/g and 3.24 mg/g (dry weight) of total free phenolics, respectively, and the same amount of bound phenol forms (1.0 mg/g) (Djurdjevic et al., 2004). These phenolic compounds could be important because the phenolic compounds have antioxidant effects that are effective in prevention and treatment of different diseases (Stajner et al., 2003). The phenolic compounds in *A. ursinum* may be flavonoids. There are five flavonoids separated from *A. ursinum* (Carotenuto et al., 1996).

Thin layer chromatography (TLC) is a sensitive and effective analytical method, which can be performed easily and quickly. TLC enables to achieve precise separations of mixtures of high complexity, while only very small sample amounts in ranges of some milligrams are needed. This method is highly suitable for separations of plant extracts based on their complex constituents. Today, a large assortment exists enabling to detect nearly all natural products structures individually. Therefore, TLC is still a popular method widely used in research (Petkova & Denev, 2013; Petkova et al., 2013).

The aim of the present study was to determine the total phenolic content as well as the free radical scavenging activity of *Allium ursinum* and *Allium bulgaricum*. This study tried to clarify the existence of alliin/allicin in the tested samples. In addition, the objective of this exploration was also to try to fill in the blank in the research and apply these results not only in culinary technology but also in therapy treatment.

MATERIALS AND METHODS

Extract preparation

Allium bulgaricum and *Allium ursinum* plant material was subjected to a heat reflux extraction with 70 % ethanol (v/v) for 30 min. The extracts were then filtered and stored at 4 ºC without adding any preservatives.

Thin layer chromatography

In the present study thin layer chromatography was used to identify the chemical compounds of the *A. bulgaricum* and *A. ursinum* extracts. TLC was carried out on TLC sheets silica gel 60 with fluorescence indicator F254 20x20 cm (MERCK). The procedure was performed according to (Kanaki and Rajani 2005). For analysis of the alliins and allicins n-butanol: acetic acid: water 60:40:20 was chosen as solvent system. The amino acid Alanine was used as reference showing similar Rf according to alliin. Therefore 5 mg of alanine were dissolved in 1.5 ml methanol.

Detection was made by spraying with ninhydrine reagent. Spray reagent for detection of amino (-NH_2) groups: 200 mg of ninhydrine was dissolved in 100 ml water. After heating at 100 ºC for 5 min alanine and the alliins could be detected as red or pink spots, while allicins were visualized as orange spots in VIS.

Determination of total phenolics

A modified Kujala et al. (2010) method with Folin - Ciocalteu's reagent was used for the

determination of the total polyphenolic content (TPC). Gallic acid was employed as a calibration standard and the results were expressed as mg gallic acid equivalents (mg GAE) per gram of plant fresh weight.

Determination of antioxidant activity

ABTS radical scavenging assay

The radicals scavenging activity of the ethanol extract against radical caption ($ABTS^{\bullet+}$) was estimated according to Re et al. (1999) with some modifications. $ABTS^{\bullet+}$ was produced by reacting 7 mM of $ABTS^{\bullet+}$ solution with 2.45 mM of potassium persulphate, and the mixture was kept in the dark at room temperature (20 - 22 °C) for 12-16 h. At the moment of use, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.7 ± 0.02 at 734 nm and equilibrated at 30 °C. Each sample (0.01 ml) was added to 1 ml of $ABTS^{\bullet+}$ solution and mixed vigorously. After reaction at 30 °C for 6 min, the absorbance at 734 nm was measured. The percentage of inhibition of $ABTS^{\bullet+}$ by the obtained extracts was calculated for each sample using the following formula:

% Inhibition = [(AB-AE)/AB] x100,

Where: A_B = absorbance of the control without sample; A_E = absorbance of the test sample with $ABTS^{\bullet+}$.

The TEAC value was defined as the concentration of Trolox having equivalent antioxidant activity expressed as μM TE per gram fresh weight (μM TE/g FW).

DPPH radical scavenging activity

The ability of the extracts to donate an electron and scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined by the slightly modified method of Brand-Williams, Cuvelier, and Berset (1995). Freshly prepared $4x10^{-4}$ M methanolic solution of DPPH was mixed with the samples and a standard solution in a ratio of 2:0.5 (v/v). The light absorption was measured at 515 nm and the percentage of inhibition of $DPPH^{\bullet}$ by the obtained extracts was calculated for each sample using the following formula:

$$\% \text{ Inhibition} = [(A_B-A_E)/A_B] \times 100$$

Where: A_B = absorbance of the control without sample; A_E = absorbance of the test sample with $DPPH^{\bullet}$.

The DPPH radical scavenging activity was presented as a function of the concentration of Trolox. The unit of Trolox equivalent antioxidant capacity (TEAC) was defined by the concentration of Trolox having equivalent antioxidant activity expressed as μM TE/g FW.

Ferric-reducing antioxidant power assay (FRAP)

The FRAP assay was carried out according to the procedure of Benzie & Strain (1996) with slight modification. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe (II)-tripyridyltriazine compound from colorless oxidized Fe (III) form by the action of electron donating antioxidants. Briefly, the FRAP reagent was prepared from 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37 °C in a water bath prior to use. One hundred and fifty microliters of plant extracts were allowed to react with 2850 μl of the FRAP reagent solution for 4 min at 37 °C. The absorbance of the reaction mixture was recorded at 593 nm. The results were expressed as μM TE/g FW.

CUPRAC assay

The CUPRAC assay was carried out according to the procedure of Ak and Gulcin, 2008. To a test tube were added 1 mL of $CuCl_2$ solution (1.0×10^{-2}M), 1 mL of neocuproine methanolic solution (7.5×10^{-3}M), and 1 mL NH_4Ac buffer solution (pH 7.0), and mixed; 0.1 mL of herbal extract (sample) followed by 1 mL of water were added (total volume = 4.1 mL), and mixed well. Absorbance against a reagent blank was measured at 450 nm after 30 min. Trolox was used as standard and total antioxidant capacity of extracts was expressed as μM TE/g FW.

Statistical analysis

All measurements were carried out in triplicates. The results were expressed as mean ± SD and statistically analysed using MS-Excel software.

RESULTS AND DISCUSSIONS

Thin layer chromatography

In the present study TLC for Bulgarian Allium and wild garlic was done. The aim of the experiment was to compare the leaf extracts of both plants in respect of their chemical composition in particular of alliins and allicins and their by-products (Figure 1.).

By analyzing the TLC plate it seems to be difficult to establish any visible difference between the two samples. However, the presence of alliin was conducted based on the reddish pink alliin spots which show a similar Rf range like the red spot of alanine. Other alliins are shown as red and pink spots above the alliin spot. The orange spots below alliin are considered to be allicin. The already mentioned results are in accordance with those reported by Sabha (2011). Based on the results we can assume lower concentration of alliin in wild garlic in comparison to Bulgarian Allium.

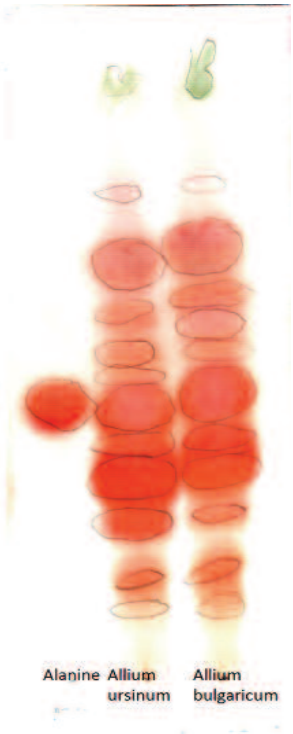

Figure. 1 TLC of Alanine and 20 µl extract of *A. ursinum* and *A. bulgaricum*, respectively.

Antioxidant activity

Total polyphenolic content

The total phenolic content was determined using Folin-Ciocalteu method, reported as gallic acid equivalents by reference to a standard curve. The total phenolics ranged from 0.40 ± 0.02 to 0.41 ± 0.08 mg GAE/g FW (Table 1). The values of polyphenolic content in the *A. bulgaricum* extract were established to be 0.41 mg GAE/g FW and those in the *A. ursinum* - 0.40 mg GAE/g FW. The results concerning the *Allium ursinum* correspond to several studies conducted by Stajner et al. in years 2003 and 2008.

Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities (Van Acker et al., 1996). Due to the presence of those compounds in the studied extracts the antioxidant activity was also studied.

Table 1. Total phenol content (mg GAE/g FW) and *in vitro* antioxidant activity (µM TE/g FW) of *Allium spp.* ethanol extracts

Plant/Method	*Allium ursinum*	*Allium bulgaricum*
TPC	0.40 ± 0.02	0.41 ± 0.08
$TEAC_{DPPH}$	1.86 ± 0.22	4.77 ± 0.88
$TEAC_{ABTS}$	11.37 ± 1.96	5.79 ± 0.25
$TEAC_{FRAP}$	4.56 ± 0.04	7.16 ± 0.06
$TEAC_{CUPRAC}$	4.65 ± 0.08	7.69 ± 0.14

Antioxidant activity

The results from the DPPH, ABTS, FRAP and CUPRAC assays are presented in Table 1.

The DPPH assay is commonly used for fast evaluation of the antioxidant capacity due to the simplicity of the assay. Higher TEAC value indicates that a sample has stronger antioxidant activity. In accordance with the results of the TPC study the DPPH assay confirmed the higher values established by the *A. bulgaricum* ethanol extract – 4.77 ± 0.88 µM TE/g FW. Other previous conducted studies have stated the alcoholic extracts to possess better antioxidant activity compared to the aqueous ones (Sapundjieva et al, 2012).

The scavenging activity of the extracts toward ABTS radical was in favour of the *A. ursinum* extract – 11.37 ± 1.96 µM TE/g fresh plant weight. These results were contrary to the results in all other conducted assays – TPC, DPPH, FRAP, and CUPRAC. This is probably due to the different mechanism of contribution of each individual component to the total radical scavenging activity of the studied samples. The authors therefore strongly suggested that, when analyzing the AOA of samples, it is better to use at least two methods

due to the differences between the test systems (Ou et al., 2002).

The FRAP values of the *A. bulgaricum* extract were also higher than those of the *A. ursunum* – 7.16 ± 0.06 μM TE/g FW. The cupric ion (Cu^{2+}) reducing ability of ethanol extracts of *Allium spp.* leaves is shown in Table 1. Among the two investigated extracts the *Allium bulgaricum* leaves extract showed the higher CUPRAC value – 7.69 ± 0.14 μM TE/g FW. The results of this assay correspond well to the already mentioned results pursuant to the other methods.

CONCLUSIONS

The results obtained confirmed *A. ursinum* and *A. bulgaricum* phenolic compounds to be a contributor of the established antioxidant capacity of the ethanol leaves extracts. Furthermore, the simple TLC assay resulted in identifying the important antibacterial component- allicin in the investigated *Allium* spp. samples.

The outcomes of this study showed that there is a great potential of both *Allium ursinum* and *Allium bulgaricum* for the development of foods rich in compounds with antioxidant and antimicrobial properties.

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METHOD FOR DETERMINATION OF PROVITAMIN A IN MEAT BASED SAMPLES

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Abstract

The method described in this paper was adapted for determination of provitamin A from meat based products that were supplemented with plant oils contain carotenes. The first step consisted in grease saponification with alcoholic KOH solution and antioxidant, for 30 minutes. Acetone and hexane were used for the extraction of carotenes several times. Finally the absorbance was determined at 450 nm. Quantification was realised with a calibration curve prepared using 5 µg/ml β-carotene solution. The method was tested for determination of β-carotene in three samples of meat products supplemented with sea-buckthorn (Hippophae rhamnoides) oil. For comparison non supplemented meat products was considered too. The provitamin A content ranged between 0.109 and 0,218 mg/100g and after one week between 0.079 and 0.195 mg/100g in the product tested. Different values were obtained according to the preparation technology.

Keywords*: meat products, provitamin A, sea-buckthorn oil.*

INTRODUCTION

Vitamin A is the generic name applied to a group of fat soluble compounds with biological activity that includes: retinol (alcohol), retinal (aldehyde), retinoic acid (carboxylic acid) and pro-vitamin A carotenoids such as ß-carotene. Vitamin A is an essential compound needed in small amounts by humans for the normal functioning of the visual system; for growth and development; for maintenance of epithelial cellular integrity, immune function, and reproduction (Hatchcock, 1997).

Human body necessary of vitamin A is provided by animal food as preformed retinol (mainly as retinyl ester) and by fruits and vegetables as pro-vitamin A carotenes. Preformed vitamin A in animal foods occurs as retinyl esters of fatty acids in association with membrane-bound cellular lipid and fat-containing storage cells (Castermiller, 1998).

Preformed vitamin A is found almost exclusively in animal products, such as milk and dairy products, liver and fish liver oils (especially), egg yolk, while pro-vitamin A carotenes are found in green leafy vegetables, yellow vegetables and yellow and orange noncitrus fruits (Booth et al., 1992).

Preformed vitamin A is also used to fortify processed foods, which may include sugar, cereals, condiments and oils (Rodriguez-Amaya, 1997). Food fortification is one of the nutritional interventions used to improve the dietetic intake of the population and reduce the consequences of micronutrient deficiencies.

Meat and meat products are poor in vitamin A, except for liver and other organ meats, but are rich in saturated fats, that are cholesterol suppliers. Most of the processed meat products contain high amounts of fat, which are related to chronic diseases such as obesity and cardiovascular heart diseases. For this reason meat industry is interested in reduction of saturated fatty acids and cholesterol in processed meat products, so that a partial substitution of animal fat with vegetable oils is taking into account. Vegetable oils are characterized by a high level of mono- and polyunsaturated fatty acids, therefore some of these, such as olive oil, may be considered as a substitute for animal fat in processed meat products (Jiménez-Colmenero, 2007).

Our research considering the enrichment of some meat based products with sea-buckthorn oil, which has a high biological value due to a favorable mix of predominantly MUFA and PUFA and naturally occurring antioxidants including carotenes and vitamin E. The amount of provitamin A expressed as β-carotene was analysed in four samples of such meat products obtained by different preparation technologies using added sea-buckthorn oil.

The aim of the reported research was to adapt an experimental model easy to apply in laboratory for determination of provitamin A, a method suitable for such complex matrices as meat products, which contain varied proportions of water, meat and fats.

For more complicated matrices (presence of fats or proteins), sample preparation can be performed using various procedures, such as direct extraction, enzymatic hydrolysis or saponification followed by extraction with organic solvents. Traditionally, fat-soluble vitamin analysis in complex matrices is performed by alkaline saponification, in the presence of antioxidants, followed by liquid extraction with organic solvents such as hexane, petroleum ether, diisopropyl ether, chloroform (Mendoza et al., 2003; Jedlička et al., 2005). Saponification is used to facilitate extraction by releasing carotenoids from the sample matrix (Salo-Väänänen et al., 2000). Retinoids are thermo labile, photosensitive, and easily attacked by oxidants: oxygen, trace metals, daylight, excessive heat, therefore both the presence of an antioxidant and working in dark colour containers are necessary.

The absorbance of the obtained carotene extract is finally measured at 450 nm then quantification is realized with a calibration curve prepared with β-carotene solution.

MATERIALS AND METHODS

Three samples of meat based products containing sea-buckthorn oil (S1, S2, S3) and one sample with no supplement added (C), used as control, were analysed. The supplemented samples were obtained by different preparation technologies using the same proportion of sea-buckthorn oil.

The samples were also analysed after seven days of chilling storage at 4-6°C.

The determination method is based on Romanian standard 13058-91 which was adapted to a complex matrix such as meat products. The amount of carotene was spectrophotometrically determined and quantified as β-carotene.

Samples preparation. Weighted samples (1 to 5 g) were finely grounded in the presence of 1 g of ascorbic acid as antioxidant. After homogenization, the samples were transferred in tubes with 20 ml alcoholic KOH solution in order to perform a grease saponification. For this purpose the tubes were placed into 80˚C water bath for 30 minutes with 200 rpm agitation.

Extraction of β-carotene. After cooling, the saponified samples were transferred in separatory funnels and the extraction of β-carotene was performed several times with portions of 5 ml acetone each, till the last extract became colorless. The acetonic extracts were collected in another separatory funnel, and then 15 ml hexan were added and moderate stirred. Two layers are separated after a couple of minutes: the inferior layer, consists in acetone and water, was removed. The clear extract of β-carotene was collected in a volumetric flask and the volume was adjusted to 20 ml with hexane.

Quantification. Finally the absorbance was measured at 450 nm. Quantification was realized with a calibration curve prepared with 5 μg/ml β-carotene solution. The limit of quantification was 0.2 μg/ml β-carotene.

Statistical analysis was performed using statistical package ANOVA.

RESULTS AND DISCUSSIONS

Characterization of the analytical method

The amount of provitamin A was determined according to the described method in sample S2, which registered 0.211 mg/100g fresh weight β-carotene.

To ensure that the developed method is suitable for provitamin A analysis, some performance characteristics were determined: the precision expressed as repeatability and reproducibility within-laboratory, sensitivity.

The precision (variability) of an analytical procedure is usually expressed as the standard deviation (S) or coefficient of variation (=

relative standard deviation, RSD) of a series of measurements.

Repeatability expresses the precision under the same operating conditions over a short interval of time.

In order to establish these parameters five determinations were performed by the same analyst, on portions of 3g each of the same sample (S2) in the same conditions, at short time intervals.

The results (Table 1) allowed us to calculate the standard deviation, so the determined amount of β-carotene is 0.2186 ± 0.0079 mg/100 g F.W.

Table 1. Determination of repeatability as expression of precision of analytical procedure

Repetition	Provitamin A (β-carotene) mg /100g	Standard deviation	RSD %
1	0.211	0.00792	3.6251
2	0.225		
3	0.215		
4	0.213		
5	0.229		

Reproducibility (within-laboratory) expresses the precision obtained with the same method on identical test material under different conditions (execution by different analysts, with the same or different equipment, in the same laboratory, at different times).

For determination of this parameter the analysis were performed by three analysts using the same sample (S2), the same protocol, the same equipment (table 2), in the same day. In this case, the standard deviation was higher, so that the calculated amount of β-carotene was 0.2216 ± 0.0115 mg/100 g F.W.

Table 2. Reproducibility determination

Analyst	Provitamin A (β-carotene) mg/100g	Standard deviation	RSD %
A1	0.234	0.01159	5.2287
A2	0.211		
A3	0.220		

Sensitivity is a measure for the response of the instrument or of a whole method to the concentration of the analyte or property, e.g. the slope of the analytical calibration graph. We plotted the calibration graph in 10 points of the reference standard (β-carotene) (Figure 1). The slope must be constant on the working range and the value of correlation coefficient R^2 must be close to 1.

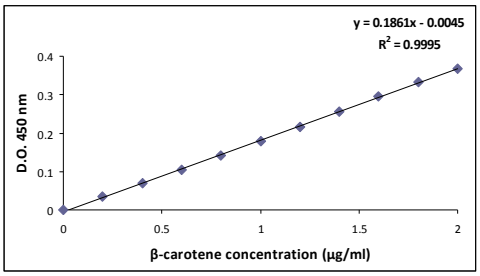

Figure 1. The calibration graph for provitamin A (β-carotene) determination

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected. The calibration graph reveals value 0.2 µg/ml β-carotene as limit of detection for our determination method.

Determination of provitamin A in the samples of meat based products supplemented with sea-buckthorn oil

The adapted method was tested on three samples of meat products enriched with provitamin A by adding sea-buckthorn oil. The control consisted in the same type of meat product without oil supplementation.

Table 3. Provitamin A (β-carotene) content in the samples of meat products supplemented with sea-buckthorn oil

Sample type	Provitamin A (β-carotene) mg/100g	
	Fresh sample	After 7 days chilling storage
C	0.003	0.007
S1	0.146	0.127
S2	0.218	0.195
S3	0.109	0.079

The obtained results (Table 3) revealed that the control sample (C) registered only 0.003 mg β-carotene/100 g F.W., value which indicates that such meat products contain almost no provitamin A. The amounts of provitamin A determined in the samples supplemented with sea-buckthorn ranged between 0.109 and 0.218 mg β-carotene/100 g F.W., depending on the preparation technology used.

As result of preserving the meat products by chilling storage at 4-6°C temperature, a decreasing of the amount of provitamin A registered in all the analysed samples after seven days. However, a differentiation was observed regarding the decreasing of the β-carotene content: sample S3 sample registered 27.52% loss of β-carotene, while in the sample S2 the loss was only 12.84% of β-carotene.

CONCLUSIONS

The results of the tests indicate that the performance characteristics of the method are according to the requirements for its practical application, therefore this method can be a practical procedure suitable for determination of provitamin A in complex matrices such as meat products.

Usually the meat based products contain no provitamin A (β-carotene). However, as result of supplementation of meat based products with sea-buckthorn oil, varied amounts of provitamin A (β-carotene) were determined in the sample analysed. The provitamin A content ranged between 0.109 and 0,218 mg/100g according to different preparation technologies applied.

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LOSS OF THE INTEGRITY IN BEER ALUMINIUM CANS

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Abstract

Canned beer samples obtained from Romania factories were analyzed for the double sealed parameters like body hook and seam gap. Sampling location was Bucharest (Romania). Deviant variation from standard was found in 12 from 18 samples analyzed. Also several measurements showed how standard cans look like and were compared with the other ones. The results of previous studies showed that the defective values of double seam may conduct to leakage of liquids or vapors into or out of the can or to the bacteria contaminations. The double seam, then, is an interlocked metal-to-metal joint that provides the hermetic seal. The small voids that naturally occur in this metal-to-metal joint are filled by the sealing compound when it is under proper compression (seam tightness). The experiments revealed that voids can become larger and more apt to cause leakage through the double seams when these common conditions exist.

Keywords: *body hook, beer, ending, chemical contamination, seam*

INTRODUCTION

Double seamer problems are very common in the process of filling and ending Aluminum cans for beer and beverages. Several parts of the tin plate can are influential in the canning process, but most relevant discuss is about seam and its most common defects. The development of double seam is the result of two separate operations precisely related in timing.

In the first part of the seaming process, the end curl is clinched to the body flange. The end is made of three thickness, while the can is made of two thickness. The purpose of the second operation during the process is to complete the closing by pressing this thickness tightly. The sealing compound, previously applied to the end, will form an elastic gasket to compensate the imperfections and ensure an hermetic closure can. Measurements, visual inspections and tests can be used in order to note variations in the finished seam (Bill Armsden, 1999).

Part of researches tries to underline some of the visual defects that can be evident in the general aspect of the can. The visual aspect of the closing is very important for the quality and it may conduct to serious defect that can affect the innocuity of the filled beverage (Seam control, IMETA, 2014).

Many common seamer problems are not handled accurately due to the inadequacy of operators to find the correct cause. This can be time consuming as fabricating is shut while the specialist efforts to adjust the difficulty on the seamer. In some cases, a corner of the chuck or roll can be damaged in one area, which can cause scuffing, wrinkle (tightness issue), sharp seam or various other problems. A cracked chuck can cause the double seam thickness to vary and even cause a bump that can compromise the integrity of the can's vacuum hermetic seal (BCME, 2005).

There are abundant systems of controlling the seam but one method is the use of a special projector. This system only serves to check one cut section of the seam. In case a incomplete seam takes place in one head, it is compulsory to control a second one from the same head before conducting to the adjustment (Bill Armsden, 1999). In case of a sizable defect the seamer has to be carefully examined before additionally controls. On high speed lines hundreds or thousands of scraps can pass if the machine is not instantly inspected and an action is not taken (Ferrum Can Seaming Machine, 2007).

In order to obtain good results from the control tests it is important to take note of all the measurements taken and to consider the results as a definite rule.

MATERIALS AND METHODS

All the measurements were taken in more than fifty aluminum cans with beer bottled in.

METOP Seam System GT is a portable double seam control system for beverage cans. For measuring of all internal properties of the seam METOP has built in video measuring abilities into a powerful computer. Combine the Seam Computer, the Mini Desk and the Winseam software results safe seam quality inspection (Metop Manual, 2014).

Figure 1: can section
Source: Beverage Can Makers Europe Double Seam Reference Manual

Measurement process is starts with typical seam section saw who sectioned seamed area that includes the can flange and the ending combined. External measurements are to be recorded before cans are sectioned. These measurements are countersink depth and seam thickness. Can sectioned at two diametrically opposed points (Seam control, IMETA, 2014).

There are some limitations of this method that does not allow for tightness rating evaluation and therefore should always be used in conjunction with the full teardown method. When using the section method a second set of numbered cans are required to evaluate the tightness rating. To maintain seam integrity the critical parameters must be achieved at all times. The Double Seam parameters are measured on the screen shown using the cursor lines.

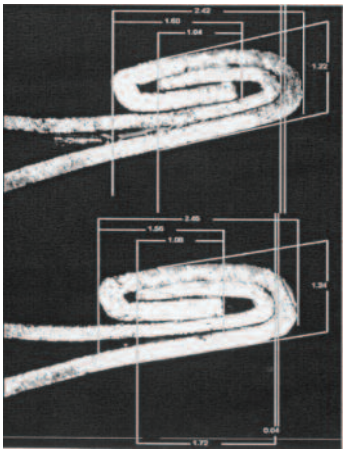


Figure 2: Cursor lines limits

Source: Metop portable Seam System report

Preprogrammed software enables calculations to be made to complete the Double Seam measurement.

Teardown Method

Measurements are to be taken (internal) for end hook (operating parameter). The End Hook is measured using a micrometer ensuring the Hook is perpendicular to the micrometer anvils.

Another operating parameter is body hook. The Body Hook is measured as shown in the photo.

Figure 3: Body Hook measure

Source: Beverage Can Makers Europe Double Seam Reference Manual

Actual Overlap

The actual overlap refers to the amount by which the body Hook and the end Hook

overlap each other within the double seam. The Actual Overlap is calculated from the following formula.

Actual Overlap = EH + BH + (1.1 x te) – SL

EH = End Hook

BH = Body Hook

SL = Seam Length

te = End Thickness

Critical Parameters:

Body Hook Butting (BHB)

The length of the Body Hook in relation to the internal length of the Seam must be sufficient to ensure that it is embedded into the lining compound. This is calculated by the formula shown and is the primary sealing area.

Body Hook Butting = BH – 1.1 tb x100

SL – 1.1(2 te+tb)

BH = Body Hook

SL = Seam Length

tb = Body Thickness

te = End Thickness

Sample preparation and measurements

Samples were taken from the production beer line as the line worked in good parameters. Also when the line had issues that conduct to defective sealing of cans multiple batches were taken for measurement. All the samples were double cut with the saw and manually, also Metop machine, measured for all causeless parameters.

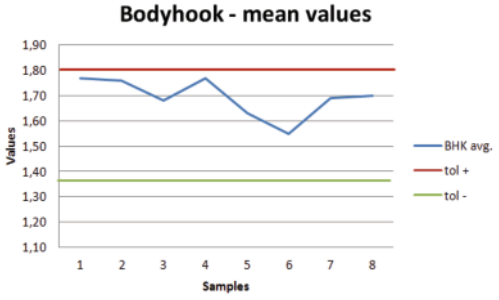
RESULTS AND DISCUSSIONS

Below are presented the results for a set of analysis conducted on cans, which had the results within the normal specifications, together with a set of the used terminology.

Table 1: Double seam control analysis – batch 1

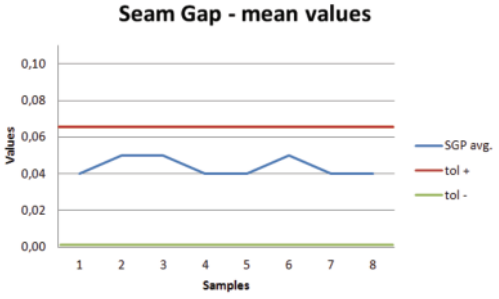
Head no.	CHT mean	CSK mean	SHT mean	STH mean	BHK mean	OL mean	CHK mean	SGP mean	BHB min	Tightness
1	168,09	6,78	2,46	1,22	1,77	1,28	1,67	0,04	90	99
2	167,91	6,79	2,43	1,24	1,76	1,27	1,66	0,05	92	99
3	168,31	6,91	2,41	1,19	1,68	1,23	1,64	0,05	90	99
4	168,12	6,73	2,46	1,23	1,77	1,29	1,72	0,04	88	99
5	168,22	7	2,48	1,18	1,63	0,96	1,55	0,04	81	99
6	168,29	6,89	2,42	1,19	1,55	1,06	1,64	0,05	80	99
7	168,14	6,81	2,46	1,22	1,69	1,18	1,66	0,04	85	99
8	168,12	6,79	2,46	1,24	1,7	1,08	1,58	0,04	85	99
tol +	168,4	7	2,7	1,24	1,8	1,35	1,8	0,07	92	100
tol -	167,6	6,7	2,4	1,12	1,4	0,75	1,4	0	72	99

Out of the 8 samples, all registered values within the tolerance limits for all the parameters.



Graph 1: Body Hook mean values, batch 1

The body hook values were close to the upper limit of 1.8, but did not exceed it, while the seam gap values registered median values.

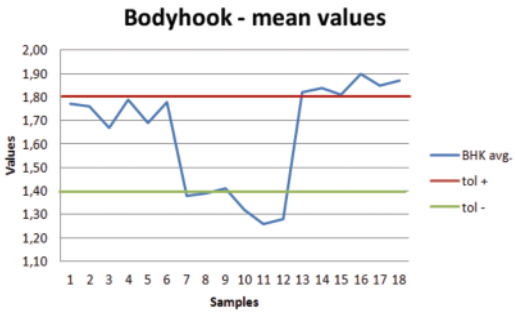


Graph 2: Seam Gap mean values, batch 1

Next was performed the analysis of a batch which implied more damaged cans and were presented the values of two main parameters.

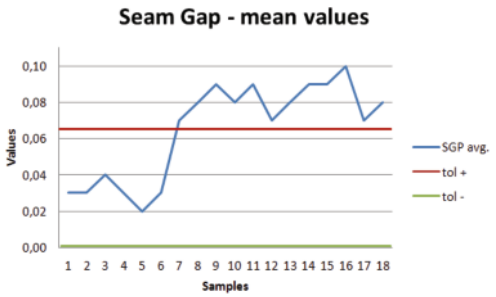
Head no.	BHK avg.	SGP avg.
1	1,77	0,03
2	1,76	0,03
3	1,67	0,04
4	1,79	0,03
5	1,69	0,02
6	1,78	0,03
7	1,38	0,07
8	1,39	0,08
9	1,41	0,09
10	1,32	0,08
11	1,26	0,09
12	1,28	0,07
13	1,82	0,08
14	1,84	0,09
15	1,81	0,09
16	1,9	0,1
17	1,85	0,07
18	1,87	0,08
tol +	1,8	0,07
tol -	1,4	0

Table 2: Double seam control analysis – batch 2



Graph 3: Body Hook mean values, batch 2

From the 18 cans analyzed, 12 had values of the body hook outside the tolerance levels (1.40-1.80). the lowest value was 1.26, the highest was 1.9, while the average reached 1.64.



Graph 4: Seam Gap mean values, batch 2

Regarding the Seam Gap analysis, 12 cans out of 18 registered values outside the tolerance levels (0.00-0.07), with a maximum measured value of 0.10, a minimum of 0.02 and an average of 0.65 .

CONCLUSIONS

It is important to follows a number of factors which give constant attention to double seams of high quality. The seam dimensions, the degree of wrinkle in the cover hook, and the position and shape of the body and cover hooks must satisfy pre-determined specifications.

Metal pick-up is where metal from the can or end has dissolved into the product after this could be iron or aluminum depending on the substrate used for the can and end production. metal pick-up will occur at significant sites of exposed metal which are in contact with the product. If good seam quality is not maintained, metal pick-up can occur anywhere on the can or end. Experience and test pack monitoring (under variable double seam conditions) shows that the most likely source is from the internal body hook radius of the double seamed can.

The metal pick-up is caused by the product reacting with the metal substrate at a site of exposed metal. In particular the forming of the body hook during seaming can cause a degree of lacquer cracking in this region, which results in potential exposure of the metal substrate.

The quantity of dissolved metal will determine if flavour issues become detectable and

subsequent consumer complaints arise. There are industry guidelines for levels of iron and aluminum which are considered acceptable. many cases customers have created their own specifications which may be more stringent, particularly if their products are sensitive to taints. Dissolved iron is much more likely to give a taint as this is detected by the average consumer at much lower threshold levels.

If the product is in contact with exposed metal then dissolution of the metal will start immediately. The levels of dissolved metal will increase the longer the storage period. The levels achieved in a given time will depend on the amount of exposed metal and the temperature storage conditions. Cans which are stored inverted or horizontally will be affected more so than those stored upright for a similar time period. It is essential that good seam control is maintained and the seams are centered within the industry specification. The primary objective of the compound within the double seam is to provide a hermetic seal at all points of the seam .

The compound will effectively protect any exposed metal on the body hook radius created during seaming if the seam thickness values are centered on nominal and seam gap is minimized. When the seam thickness is centered on nominal then the compound will prevent product access to the body hook radius area. If the seam gap is high and/or in combination with a loose double seam then there is a potential path for the product to gain access to the exposed metal. Good seam control (in particular seam gap and seam thickness) will prevent product ingress to the body hook radius which is the most common source of metal pick-up. Additionally good control of filling conditions, in particular the headspace air content, will help to reduce the risk of metallic of flavor issues. The corrosion rates will be accelerated if there is more oxygen present. Undercover gassing at the point of seaming is used by most fillers to reduce the level of air/oxygen in the headspace.

The hermetic seal performs several functions. It keeps bacteria out of the can, probably its most important function. It prevents seepage of the pack from the can, leakage of liquids or vapors into or out of the can, and maintains the desired vacuum or pressure in the can.

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EXOPOLYSACCHARIDE PRODUCTION BY SELECTED LACTIC ACID BACTERIA ISOLATED FROM FERMENTED VEGETABLES

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Abstract

Lactic acid bacteria (LAB) play a key role in the food fermentation process since they contribute to the texture, flavor, quality and conservation of the fermented products. Several LAB strains have been shown to produce exopolysaccharides (EPS), with potential applications in food industry, since they can act as natural thickeners that improve the texture of the final product, decrease syneresis and reduce the fat levels in fermented foods. In situ production of EPS by LAB to get a desired texture and mouthfeel of some fermented products is being explored, in order to replace polysaccharides from plants or animals, currently in use. Moreover, it has been suggested that some EPS produced by LAB have prebiotic activity, contributing to the promotion of human gastrointestinal health. During this study, five new EPS-producing LAB strains have been selected from 21 strains isolated from fermented vegetables. The mucoidness/ropiness of the colonies developed on MRS agar media containing different carbon sources (glucose, sucrose, fructose, galactose, lactose, or xylose) was firstly observed. EPS presence in the culture supernatant was detected through gel permeation chromatography (GPC). The EPS material was isolated from these strains by acetone precipitation, then dialysed, dried and weighted. The molecular mass was estimated by the same GPC method, while the monomer composition was determined by automated thin layer chromatography (TLC), after hydrolysis with 8N HCl. One of the positive strain, Leuconostoc mesenteroides/pseudomesenteroides 406 has been shown to produce large amounts of EPS, of about 15 g/L and two strains, Leuconostoc citreum/lactis/garlicum 167 and Leuconostoc sp. 208 were able to produce around 6 g/L of EPS. All isolated EPS have a high molecular mass, of above 1400 KDa, and a monomer composition dominated by the presence of glucose. The influence of the growth medium composition and incubation temperature on the EPS biosynthesis was also investigated. Three LAB strains, that were shown to produce high amounts of EPS, have been selected to be used in this study. It was shown that some mild stress conditions might stimulate, in some cases, the EPS-production.

Keywords: *exopolysaccharides, fermented vegetables, lactic acid bacteria, salinity stress.*

INTRODUCTION

Lactic acid bacteria (LAB) have been used around the world to improve the preservation, sensorial characteristics and nutritional value of a large variety of products, such as milk, meat and vegetables (Doyle and Beuchat, 2007; Wood and Holzapfel, 1995; Wood, 1997). Several LAB strains can also contribute to the improvement of the texture and viscosity of fermented products by means of the synthesis of exopolysaccharydes (EPS). EPS can be classified into two groups: homopolysaccharides (HoPS) and hetero-polysaccharides (HePS) (De Vuyst and Vaningelgem, 2003; Hassan, 2008). HoPS are composed of one type of monosaccharide subunits, while HePS are formed from a backbone of repeated subunits of different monosaccharides, e.g. D-galactose, D-glucose or L-rhamnose, in different ratios. Well-known examples of HoPS include the dextrans and fructans produced by *Leuconostoc mesenteroides* and *Streptococcus salivarius*, respectively (De Vuyst and Degeest, 1999), while HePS are synthetized by many LAB including strains of *Streptococcus thermophilus, Lactococcus lactis* and a number of lactobacilli (De Vuyst el at. 2001).

Although their function and relevance for the bacteria are not completely understood, it has been suggested that EPS may play a significant role in the protection of cells against dehydration, phagocytosis, phage attacks or toxic compounds (De Vuyst et al., 2001). EPS may also contribute to the adhesion of microorganisms onto solid surfaces and to intercellular communications (De Vuyst and Degeest, 1999). EPS may also alter the techno functional properties (e.g. viscosity or water binding capacity) of fermented foods such as

yogurt, cheese or sourdough (Hassan and Awad, 2005; Costa et al., 2010) and, consequently, the sensory properties of these products (Mozzi et al., 2006). Therefore, LAB that show a capacity to excrete EPS can be used to replace thickeners and stabilizers such as polysaccharides of animal (gelatin), plant (starch, pectin) or other microbial origin (xanthan). A further advantage is their GRAS (Generally Recognized As Safe) status, which means that LAB and their metabolites are considered safe and no declaration is needed when they are added to food.

The mechanism of EPS synthesis has been extensively studied for years (Joly et al., 2002; Wellman and Maddox, 2003). The amounts of EPS produced by LAB is strongly dependent on the growth medium composition (carbohydrate and nitrogen source, C/N ratio, vitamins, salts and other supplements) (De Vuyst and Degeest, 1999; De Vuyst et al., 2001; Seesuriyachan, 2012). Moreover, fermentation conditions such as temperature, environmental pH, and the presence of oxygen also have a significant impact on EPS synthesis (Boels et al., 2003; Svensson et al., 2005).

The aim of this study was to isolate and characterize some EPS produced by LAB isolated from traditional Romanian fermented vegetables and to study the influence of growth medium composition and the incubation temperature on the biosynthesis of these EPS.

MATERIALS AND METHODS

Bacterial strains and media

LAB used throughout this study were isolated from traditional Romanian fermented vegetables: green tomatoes (1 strain), cauliflower (2 strains), carrots (4 strains) or brine (14 strains). Strains were isolated by plating on MRS agar (de Man et al. 1960), purified, identified to species level by $(GTG)_5$-PCR fingerprinting and 16S rRNA sequencing (Wouters et al., 2013). Strains were stored at -75°C in MRS broth containing 25% (v/v) of glycerol as a cryoprotectant.

When screened for EPS production, LAB strains were grown in modified MRS medium containing 50 g/L sucrose instead of glucose. Glucomannans that could interfere with the EPS screening were removed through ultrafiltration according to the method described by Van der Meulen et al. (2007).

Screening for EPS production

A preliminary method to evaluate the capacity to produce EPS, was by observing the mucoidness/ropiness of the colonies developed on MRS agar media containing different carbon sources: glucose (20 g/L), sucrose (50 g/L), fructose (20 g/L), galactose (20 g/L), lactose (20 g/L), or xylose (20 g/L).

The 21 LAB strains were then screened for EPS production through gel permeation chromatography (GPC), using a Jasco HLPC system (Jasco Europe, Cremella, Italy), equipped with an Ultrahydrogel™ Linear column (Waters Corp., Milford, Mass., USA), kept at 35°C, and coupled to a RI-2031 refractive index detector (Jasco). Samples were prepared according to the method described by Van der Meulen et al. (2007). The EPS were eluted with 0.1 M $NaNO_3$ at a flow rate of 0.6 mL/L. Dextran standards ranging from 80 kDa to 1.4 Mda (Sigma-Aldrich, Switzerland) were used to estimate the molecular mass of the EPS.

EPS Isolation and Quantification

Isolation of EPS was carried out from 24 h cultures obtained in 25 mL of filtered MRS supplemented with 50 g/L of sucrose, without pH control or agitation. EPS was isolated according to De Vuyst et al. (1998). Total EPS yields were determined gravimetrically by measuring the polymer dry mass (PDM). Further purification of the EPS was done by dialysis against distilled water at 4°C for 7 days, with a water replacement twice a day.

Monomer Analysis

The purified EPS were hydrolysed for 6 h at 100°C with 8N HCl, evaporated in an Eppendorf AG centrifugal concentrator (Eppendorf, Hamburg, Germany) and resuspended in ultrapure water. Monosaccharide composition of EPS was determined by automated thin-layer chromatography (TLC) (CAMAG, Muttenz, Germany) using the ascending technique with silica gel 60 F254 precoated glass sheets (Merck, Damstadt, Germany). The sugars were eluted with a mixture of 1-butanol/acetic

acid/water, 6/1/2 (v/v) and the bands were visualized by spraying with p-aminobenzoic acid (Wall, 2005). Glucose, galactose, rhamnose, fructose, mannose, ribose (Fluka, Sigma-Aldrich), arabinose (Veb Berlin Chemie, Germany), glucosamine and galactosamine (Calbiochem, Inc. San Diego, Calif., SUA) were used as standards.

Effect of NaCl and incubation temperature on EPS production

Three high EPS-producing LAB strains belonging to *Leuconostoc* genus have been selected to determine the effect of incubation temperature and addition of NaCl on the biosynthesis of EPS.

Different medium variants were inoculated with 2% of fresh cultures obtained in filtered modified MRS (with 50 g/l of sucrose). Four incubation temperatures were tested: 20°C, 28°C, 37°C, 42°C, and three concentrations of salt: 1%, 3%, and 5%, respectivelly (both at 20°C and 28°C).

Growth parameters (final pH, optical density at 600nm and viable cell counts) were determined after 24 h, except when no visible growth was observed and cultures were left for 24 or 48 h more. EPS were isolated and quantified as previously mentioned.

RESULTS AND DISCUSSIONS

Screening for EPS production

Most of the strains used in this study grew in all media tested, but it was observed a preference for glucose, sucrose and fructose as energy source. In some cases, due to the small size of the colonies, the mucoid or ropy phenotype could not be detected. Eight strains were very mucoid on media containing sucrose (Fig. 1, Table 1). None of the tested strains showed a ropy phenotype.

The nomenclature used to describe the different EPS-producing phenotypes of LAB is confusing, and terms as “ropy”, “mucoid”, and “slime” have been indistinctly used. However, not all the mucoid or slime-producing strains are ropy. The mucoid colonies have a glistening and slimy appearance on agar plates, but are not able to produce strands when extended with a stik, whereas the ropy colonies form a long filament by this method (Knoshaug et al., 2000). Some LAB can express both ropy and mucoid phenotypes depending on the culturing conditions (Dierksen et al. 1997; Cerning et al., 1994).

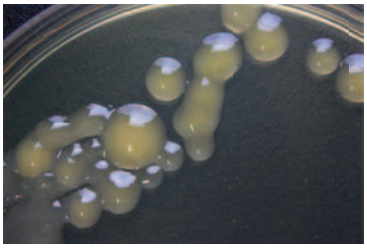


Figure 1. Mucoidness of the colonies developed on agar plates by *Lactobacillus plantarum* 235

EPS characterization

The GPC-based screening revealed five EPS-producing LAB strains. An individual peak, eluted at around 10 minutes, could be detected for all these strains, as shown in Fig. 2 for strain *Leuconostoc mesenteroides/ pseudomesenteroides* 406. For the EPS-negative strains, no peaks could be detected, thereby proving that contaminants were removed from the medium through ultrafiltration.

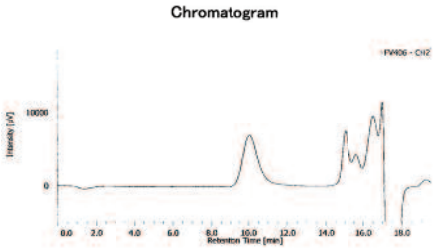


Figure 2. Gel Permeation Chromatography (GPC) of the EPS produced by the positive strain *Leuconostoc mesenteroides/ pseudomesenteroides* 406

All five EPS-producing strains belong to the genus *Leuconostoc*. A high incidence of EPS production among *Leuconostoc* strains was previously observed in a screening of LAB strains isolated from fermented dairy products (Grosu-Tudor et al., 2013) and in fermented vegetables (Grosu-Tudor and Zamfir, 2013). The high incidence of the EPS-producing strains compared with the total number of tested strains is in agreement with other studies (Adebayo-tayo and Onilude, 2008). However, the authors admit that mesophilic strains have

the highest potential for EPS production and this concurs with our findings. The EPS production of the five selected strains isolated from Romanian fermented vegetables could be correlated with the mucoidness of the colonies developed on MRS with 50 g/L of sucrose (Table 1). From all GPC-positive strains, EPS could be isolated in various amounts from cultures obtained in filtered modified MRS, by acetone precipitation. *Leuconostoc mesenteroides/pseudomesenteroides* 406 has been shown to produce large amounts of EPS, of about 15 g/L and two strains, *Leuconostoc citreum/lactis/garlicum* 167 and *Leuconostoc* sp. 208 were able to produce around 6 g/L of EPS. For two *Leuc. citreum* strains 96 and 247, EPS yielded 0.21 and 1.02 g/L, respectively.

Table 1. Growth and mucoidness of LAB strains on agar MRS with different carbon sources

Strain	**MRS-glc**	**MRS-suc**	**MRS-fruct**	**MRS-galact**	**MRS-xil**	**MRS-lact**
unidentified 56	+	m	+	+/-	+	+/-
Leuc. mesenteroieds 69	+	+	+	+	+	+
***Leuc. citreum* 96**	**+/-**	**vm**	**+**	**-**	**-**	**-**
Leuc. mesenteroides 97	+	vm	+	+	+	+
***Leuc. citreum/lactis/garlicum*167**	**+**	**vm**	**+**	**-**	**+**	**-**
Lb. parabrevis 196	+	+/-	+	+/-	+	+/-
Leuc. mesenteroides 197	+	+	+/-	+	+	+
Lb. plantarum 198	+	-	+	-	-	-
***Leuc*. sp. 208**	**+**	**vm**	**+**	**-**	**+/-**	**-**
unidentified 234	+	+	+	+	+	+
Lb. plantarum 235	+	vm	+	+	+	+
Leuc. mesenteroides/ pseudomesenteroides 246	+	vm	+	+	+/-	+
***Leuc. citreum* 247**	**+/-**	**vm**	**+/-**	**+/-**	**-**	**+/-**
Lb. pentosus 265	+	+	+	+	+	+
Lb. parabrevis 341	+	+	+	+	+	+/-
Leuc. mesenteroides 355	+	+	+	+	+	+
Lb. brevis 403	+	+	+	+	+	+
***Leuc. mesenteroides/ pseudomesenteroides* 406**	**+**	**vm**	**+**	**-**	**-**	**+/-**
Lb. brevis 530	+	+	+	+	+	+
Lb. plantarum 616	+	+	+	+	+	+
Lb. plantarum 619	+	+	+	+	+	+

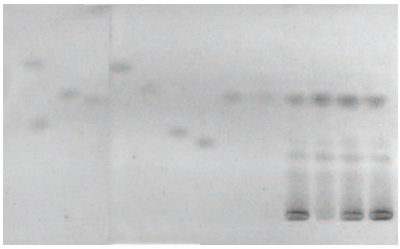
vm = very mucoid; m = mucoid;
+ = good growth; +/- = poor growth; - = no growth

The GPC chromatograms revealed that all EPS eluted before the elution of the largest dextran standard available (molecular mass of 1.4 MDa), indicating that the molecular mass of all EPS exceeded this value. High molecular mass EPS (both HoPS and HePS) produced by LAB strains isolated from fermented dairy products or fermented vegetables have been previously described (Vaningelgem et al., 2004c; Van der Meulen et al., 2007; Grosu-Tudor and Zamfir, 2013). An estimation of the molecular mass of a certain EPS can be important for its characterization, taking into account that the molecular mass is an important factor in determining the intrinsic viscozity and functional properties of EPS (Ruas-Madiedo et al., 2002). High molecular mass polymers can be used as viscosifiers, emulsifiers, gelling, or stabilizing agents to modify the rheological properties and texture of food product (Joly et al., 2002; Patel et al., 2010).

The monomer composition of the isolated EPS was determined by TLC of the hydrolyzed samples. For the strain *Leuc. citreum* 96, a light fading band was detected, corresponding to glucose, while for the other four strains tested, several bands could be observed (Fig. 3).

The major band corresponds to glucose, while the others might be other monosaccharides from the EPS composition, which could not be correlated with any of the sugars used as standards. However, the presence of these additional bands might be also explained by an incomplete hydrolysis of the EPS. Further use of additional standards or the use of complementary analysis, such as HPLC, would

bring more accurate information about the exact monomer composition of the isolated EPS.



1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 3. Monomer composition analysis by TLC: 10 = *Leuc. citreum* 96; 11 = *Leuc. citreum/lactis/garlicum*167; 12 = *Leuc*. sp. 208; 13 = *Leuc. citreum* 247; 14 = *Leuc. mesenteroides/ pseudomesenteroides* 406. Rhamnose (1), galactose (2), mannose (3), fructose (4), ribose (5), arabinose (6), glucosamine (7), galactosamine (8), and glucose (9) were used as standards.

Effect of NaCl and incubation temperature on EPS production

EPS production has been shown to be strictly correlated with bacterial growth, being in general higher when the producing strain is grown under optimal conditions. Therefore, the medium composition, the addition of extranutrients or nutrients for growth enhancement, the carbon to nitrogen ratio, but also the incubation temperature, the oxygen tension, the pH level and the incubation time are important factors affecting both the growth and the EPS production.

Three LAB strains producing high amounts of EPS – *Leuc.* sp. 208 and *Leuc. mesenteroides/ pseudomesenteroides* 406 from the present screening and *Leuc. citreum* 52 from a previous screening (Grosu-Tudor and Zamfir, 2013) - were selected to be used further to evaluate the EPS production in different growth conditions. Firstly, different incubation temperatures (20°C, 28°C, 37°C and 42°C) were tested.

The three strains grew well at all these temperatures (Table 2). The strains *Leuc. citreum* 52 and *Leuc*. sp. 208 showed the largest number of viable cells after 24 h of incubation at 20°C, whereas *Leuc. mesenteroides/ pseudomesenteroides* 406 after 24 h of incubation at 28°C. The poorest growth was shown for *Leuc. mesenteroides/pseudomesen-teroides* 406 after 48 h of incubation at 37°C and 42°C. This was expected, since leuconostocs are known to be mesophilic, with optimal growth temperature between 20°C and 30°C (Wood and Holzapfel, 1995). The pH dropped during the fermentation, reaching final values around 3.5 – 4.0 (Table 2).

The EPS yields varied significantly with the incubation temperature. EPS biosynthesis has been shown to be growth-related, the optimal growth conditions being favourable to the production (Torino et al., 2001, Zhang et al., 2011). However, there are also reports that optimal conditions for EPS production by some LAB strains might be different from those for their oprimal growth (Gamar et al, 2007) and EPS production has been considered by some authors as a mechanism of bacterial self-protection against unfavourable conditions (Ruas-Madiedo et al., 2002; Lin and Chang Chien, 2007).

In our studies, this was the case for two strains, *Leuc. citreum* 52 and *Leuc*. sp. 208, reaching the highest EPS yields of 14.52 g/L and 8.43 g/L, respectively, at incubating temperature of 20°C, although the best growth was obseved at 28°C. *Leuc*. sp 208 doubled the EPS yield at 20°C compared with the optimal growth temperature (Table 2). On the contrary, EPS biosynthesis of *Leuc. mesenteroides/ pseudomesenteroides* 406 reached the highest yield of 16.02 g/L at the optimal growth temperature, 28°C. Zamfir et Grosu-Tudor (2014) reported similar results in case of some EPS-producing LAB strains isolated from Romanian dairy products.

The EPS yield is generally affected by the composition of the medium used for microbial cultivation (De Vuyst et al. 2001), and an enhancement of EPS production by modifying the growth medium has been also investigated (Svensson et al., 2005; Boels et al., 2003; Seesuriyachan, 2012). Sometimes, EPS has been shown to be produced in higher amounts in order to protect microbial cells from stress conditions (Jolly et al., 2002). Therefore, the cultivation of LAB under several stress conditions in order to obtain a higher yield of EPS was investigated. As a stress factor, the presence of different concentrations of NaCl in the growth medium was used.

Table 2. Growth parameters and EPS yields of the tested strains at different temperatures

Strain	Temperature	O.D. 600nm	Final pH	Cell count (CFU/mL)	EPS yield (g/L)
Leuc. citreum 52	20°C	5.753	4.12	3.5×10^{13}	14.52
Leuc. citreum 52	28°C	7.807	3.80	5.5×10^{12}	11.73
Leuc. citreum 52	37°C	6.304	3.83	5.7×10^{11}	5.28
Leuc. citreum 52	42°C	3.108	4.27	3.05×10^{8}	0.31
Leuc. sp. 208	20°C	6.202	4.11	4.05×10^{13}	8.43
Leuc. sp. 208	28°C	8.333	3.72	4.2×10^{13}	4.56
Leuc. sp. 208	37°C	6.323	3.63	9.3×10^{12}	0.12
Leuc. sp. 208	42°C	3.939	3.95	2.2×10^{11}	0.28
Leuc. mesenteroides/pseudomesenteroides 406	20°C	5.215	4.38	2.6×10^{12}	13.47
Leuc. mesenteroides/pseudomesenteroides 406	28°C	6.126	4.33	1.8×10^{13}	16.02
Leuc. mesenteroide/pseudomesenteroidess 406	37°C	0.357	5.47	7.9×10^{8}	0.90
Leuc. mesenteroides/pseudomesenteroides 406	42°C	0.288	5.63	2.5×10^{7}	0.38

All tested strains showed a good growth in the presence of NaCl at 28°C (Table 3). Concerning the EPS production, *Leuc. mesenteroides/pseudomesenteroides* 406 reached the maximum yield in the presence of 5% NaCl, after 24 h of incubation at 28°C (25.83 g/L), higher than the amount produced under optimal conditions (16.02 g/L) and three times the value obtained in the presence of 1% NaCl (8.68 g/L). This is in accordance with the results obtained by Seesuriyachan et al. (2012), who report increased EPS production by *Lactobacillus confusus* under high salinity stress. Although *Leuc. mesenteroides/ pseudomesenteroides* 406 reached the highest EPS yields at 5% NaCl, the biomass production was the lowest, showing that high salinity stress can have a negative impact on microbial growth. On the contrary, *Leuc.* sp 208 and *Leuc. citreum* 52 showed a good growth in the presence of all concentrations of NaCl used, but they lost the ability to synthesise EPS under salinity stress, except for *Leuc. citreum* 52, which still produced 1.68 g/L EPS in the presence of 1% NaCl. Evidence of inhibition of EPS production by NaCl has been also reported in the cultivation of *Lactobacillus helveticus* ATCC 15807 (Torino el al., 2005) and *Pediococcus parvulus* 2.1 (Velasco et al., 2006).

Table 3. Growth parameters and EPS yields under salinity stress at 28°C

Strain	NaCl	O.D. 600nm	Final pH	Cell count (CFU/mL)	EPS yield (g/L)
Leuc. citreum 52	1%	7.461	3.69	5×10^{13}	1.68
Leuc. citreum 52	3%	5.863	3.71	2.7×10^{12}	0
Leuc. citreum 52	5%	5.042	3.52	1.2×10^{13}	0
Leuc. sp. 208	1%	7.759	3.66	1.5×10^{13}	0
Leuc. sp. 208	3%	6.080	3.67	7.6×10^{13}	0
Leuc. sp. 208	5%	5.751	3.40	7.3×10^{13}	0
Leuc. mesenteroides/pseudomesenteroides 406	1%	5.995	4.15	2.6×10^{13}	8.68
Leuc. mesenteroides/pseudomesenteroides 406	3%	5.789	4.09	1.6×10^{13}	16.53
Leuc. mesenteroides/pseudomesenteroides 406	5%	4.748	4.18	8.05×10^{11}	25.83

When incubated at 20°C, *Leuc. mesenteroides/pseudomesenteroides* 406 grew well in the presence of all concentrations of NaCl tested (Table 4). The other two strains showed a good growth in the presence of 1% NaCl (24 h) and a slower growth in the presence of 3% NaCl (48 h), while at higher concentration of NaCl (5%) they needed 72 h for growth. Under these growth conditions, *Leuc*. sp. 208 has lost again the ability to synthetize EPS, while *Leuc. citreum* 52 was still able to produce low amounts in the presence of 1% NaCl, although much lower compared with the yields obtained under

normal conditions. *Leuc. mesenteroides/ pseudomesenteroides* 406, was able to produce very high EPS amounts (over 11 g/L) at 20°C, in the presence of NaCl upto 5%. However, in the presence of 5% NaCl, the production was lower, probably due to the combined effect of two stress factors (lower incubation temperature and high salinity) (Table 4).

Table 4. Growth parameters and EPS yields under salinity stress at 20°C

Strain	NaCl	O.D. 600nm	Final pH	Cell count (CFU/mL)	EPS yield (g/L)
Leuc. citreum 52	1%	2.083	5.04	3.1×10^{10}	0.716
Leuc. citreum 52	3%	5.096	3.98	1.2×10^{13}	0
Leuc. citreum 52	5%	2.274	4.35	2.4×10^{13}	0
Leuc. sp. 208	1%	2.684	4.80	9×10^{10}	0
Leuc. sp. 208	3%	5.728	3.80	1.8×10^{13}	0
Leuc. sp. 208	5%	4.589	3.64	3.1×10^{12}	0
Leuc. mesenteroides/pseudomesenteroides 406	1%	6.405	3.95	1.7×10^{13}	17.42
Leuc. mesenteroides/pseudomesenteroides 406	3%	5.611	4.03	3.2×10^{12}	17.60
Leuc. mesenteroides/pseudomesenteroides 406	5%	2.883	4.66	1.5×10^{10}	11.91

CONCLUSIONS

In conclusion, this study provides information about new LAB strains isolated from fermented vegetables, able to produce large amounts of EPS, with potential application in food biotechnology (i.e. to improve the rheological properties of fermented products). The EPS isolated during this study are, most probably, HoPS, composed of glucose solely, and they have a high molecular mass. The incubation temperature and the presence of NaCl in the growth medium significantly affected the EPS production. The highest EPS yield, of over 25 g/L, was obtained for *Leuc. mesenteroides/pseudomesenteroides* 406 grown at 28°C, in the presence of 5% NaCl.

ACKNOWLEDGEMENTS

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OPTIMIZATION OF PROANTOCYANIDINE EXTRACTION PROCESS FROM *FRAGARIA VESCA* L. LEAVES

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Abstract

Wild strawberries Fragaria vesca L. have been traditionally used in herbal medicine in treating rashes, as well as internally for treating gastrointestinal catarrh (mucous), diarrhea, intestinal toning, liver health maintenance, catarrh of respiratory passages, rheumatism, nervousness, bladder health maintenance, gravel, fever, in support of vascular health and as a diuretic. The leaves of Fragaria vesca L. are natural source of biologically active substance, such as condensed tannins (epigallocatechins), ellagitannins (pedunculagin and agrimoniin), flavonoids (kampferol and quercetin glucosides) and proantocyanidins (catechin, procyanidin B1). The aim of the current investigation is connected with selection of the best conditions for proanthocyanidins extraction. The influence of the duration of the ultrasonic extraction and solvent system (acetone-water) in different concentration ratio over the extraction process was studied. The optimal conditions for the extraction of proantocyanidins from strawberries leaves were as follow 56% acetone-water solvent system, time of ultrasonic extraction 50 min in ultrasonic bath with frequency 35 kHz. Under these conditions the maximum amount of proantocyanidins 124.0 mg/100 g dry biomass were obtained.

Keywords: *Fragaria vesca L., proanthocyanidins, ultrasonic extraction*

INTRODUCTION

Wild strawberry (*Fragaria vesca* L.) is a widely growing plant with medicinal properties (Figure 1). In the traditional system of medicine, the plant has been reported to possess diuretic and liver tonic properties and antimicrobial activity (Vennat et al., 1988, Kishore et al., 2012).

Figure 1. Wild strawberry (*Fragaria vesca* L.)

It has certain therapeutic application such as astringent, arthritis, diuretic, disturbances and liver tonic etc. It contains flavonoids, phenolic acids, tannins, anthocyanins, as well as anti-oxidants (Wang et al., 2000, Filippone et al., 2001).

Procyanidin dimers B1, B2, and B5 (Figure 2, 3 and 4) have been identified in *Fragaria vesca* L. (Vennat et al., 1988, 1989, Buricova et al., 2011). The biological properties of proanthocyanidins have been extensively studied.

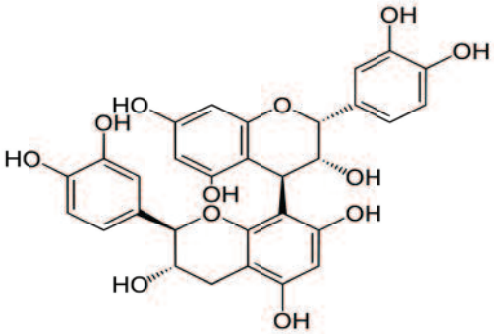

Figure 2. Procyanidine B_1 (epicatechin-(4β→8)-catechin)

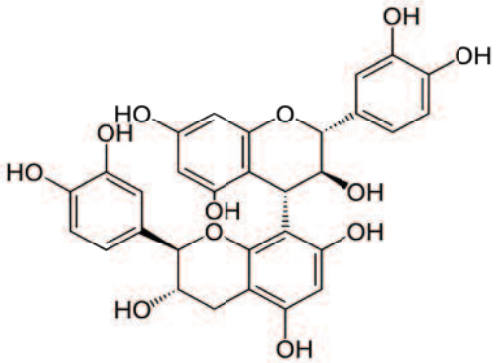

Figure 3. Procyanidine B_3 ((catechin-(4α→8)-catechin)

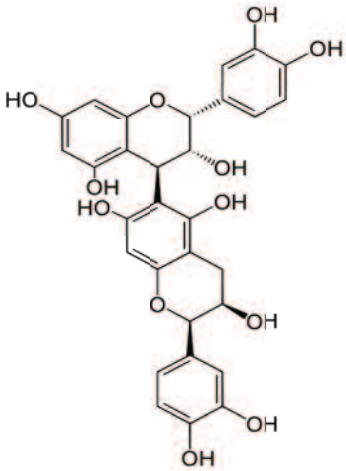

Figure 4. Procyanidine B_5 (epicatechin-(4β → 6)-epicatechin)

These substances have been possessed free radical scavenging and antioxidant activity (Dai and Mumper, 2010). Proanthocyanidins have been reported to have antibacterial, antiviral, anticarcinogenic, anti-inflammatory, anti-allergic, and vasodilators actions (Lin and White, 2012). They have also been shown to inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility, and to affect enzyme systems including phospholipase A_2, cyclooxygenase, and lipoxygenase (Dai and Mumper, 2010). Procyanidines have ability non-competitively to inhibit the activity of xanthine oxidase – a major generator of free radicals, elastase, collagenase, hyaluronidase, and beta-glucuronidase (Fine, 2000).

In nowadays application of ultrasonic irradiation techniques for extraction of biologically active substances constantly increased. It was known that ultrasonic waves accelerate the diffusion process and shortened the extraction time (Lingyun et al., 2007).

On this base, the aim of our investigation was connected with selection of the best ultrasonic conditions for proanthocyanidins extraction from *Fragaria vesca* L.

MATERIALS AND METHODS

Plant material

Aerial parts (leaves) by several random chosen plants of *F. vesca* L., were collected from their natural habitats near hut "Zdravec", Rhodopa mountain all in May 2013. The samples were dried in shade at ambient temperature for 7 days, and powdered by homogenizer. The powder was used for extraction of proanthocyanidins.

Extraction procedure

Half gram dry ground leaves were placed in a plastic vial and 50 mL solvent in different ratio mixture acetone and water was poured in it. Ultrasonic extraction procedure was performed in ultrasonic bath SIEL UST 5.7-150 (Gabrovo, Bulgaria) with frequency 35 kHz and power 240 W at temperature 50°C for different time. The ratio between acetone-water and extraction time were varied in order to obtain the highest yield of proantocyanidins.

Total proanthocyanidins assay

Acid butanol assay for proanthocyanidins, according to Porter et al. (1986), was used. Six milliliters of the acid butanol reagent (950 mL of *n*-butanol with 50 mL concentrated HCl), 0.5 mL aliquot of the fraction, and 0.1 mL of the iron reagent (2 % ferric ammonium sulfate in 2 mol/L HCl) were added to 10 mL screw cap tube and then vortexed. The tube was capped loosely and put in a boiling water bath for 50 min. The absorbance of formed colored complex was read at 550 nm. Condensed tannins were analyzed as leucocyanidin equivalent (Hagerman, 2011).

Response surface methodology (RSM) was used to optimize the variables to predict the best extraction conditions. In this investigation a series of statistically designed studies were performed to reveal the effect of the independent variables (solvent ratio and extraction time). To describe the nature of response surface in optimum region a

composite design with two coded levels (X_1 – solvent acetone-water in different ratio and X_2 – extraction time) was performed. The model for predicting the optimal conditions was expressed by the following equation (1).

$$Y = b_0 + \sum b_i \ x_i + \sum b_{ii} \ x_i^2 + \sum b_{ij} \ x_i x_j \ (1)$$

Where Y is response variable; *b* is regression coefficients and x is coded levels of the independent variable. The effects of acetone-water ratio and extraction time on proanthocyanidins were also analyzed by multiple regression techniques. The predicted equation for the proanthocyanidins extraction yield (Y) was given in the following equation as a function of the coded values.

Statistical analysis was performed with Statistical Software MINITAB 16. The adequacy of model was checked accounting for R^2. Numerical optimization techniques of designed experiment were used for simultaneous optimization response.

RESULTS AND DISCUSSIONS

Many researchers worldwide have been investigated the influence of various solvents on the extraction efficiency of proanthocyanidins from different plant sources. It has been established that the most suitable solvent was acetone-water mixture (Karamac et al., 2005, Liu and White, 2012). Kajdžanoska et al. (2010) analyzed polyphenolic compounds in cultivated strawberries (*Fragaria ananassa*) fruits by HPLC–DAD-ESI-MS. However until now, no information was available for extraction of proanthocyanidines from wild strawberry leaves under ultrasonic irradiation.

One of the most successive approach for the extraction process optimization of secondary metabolites from plant materials is the statistically optimization of extraction conditions (temperature, solvent and extraction time) (Lingyun et al., 2007).

On the base of this knowledge we carried out a detailed study for the influence of different acetone–water ratio and extraction time, accompanied with ultrasonic irradiation for acceleration of the extraction procedure.

The results described above clearly outlined that the investigated variables (solvent systems and time of extraction) significantly influenced proantocyanidins extraction by *Fragaria vesca* (Table 1). The statistical regression model was obtained, taking into account the influence of the solvent (X_1) and extraction time (X_2) on the amount of total proantocyanidins yield (Y) (2).

$$Y = -119.828 + 6.981X_1 + 1.999X_2 - 0.058X_1^2 - 0.014X_2^2 - 0.010\ X_1X_2 \qquad (R^2 = 86.8) \quad (2)$$

Table 1. Independent variable values of the process and their corresponding values

	X_1 (Solvent ratio Acetone – H_2O)	**X_2 (Extraction time, min)**	**Y (Total proanthocyanidins, mg/100 g DW)**
1	30	10	58,5
2	50	10	101,3
3	70	10	84,7
4	30	20	55,3
5	50	20	120,3
6	70	20	110,1
7	30	40	76,3
8	50	40	116,0
9	70	40	121,3
10	30	60	98,6
11	50	60	112,9
12	70	60	111,0

Table 2. Comparison between theoretically calculated and experimentally obtained yields of proanthocyanidins

	X_1[1]	**X_2[1]**	**$\hat{Y}^2$max**
Theoretically calculated	55.8 %	49.8 min	125.4 mg/100 g DW
Experimentally obtained	56 %	50 min	124.0 mg/100 g DW

[1] – independent variables;

[2] – maximum yield of proanthocyanidins.

The value of obtained coefficient of determination (R^2) was good enough. The optimization procedures carried out using "Response optimizer" of MINITAB 16 software gave the following values of variable X_1 and X_2 for maximum yield of proantocyanidins (Y) by *Fragaria vesca* L. (Table 2). The deviation between the theoretically studied maximal amounts of proantocyanidins and experimentally obtained (at 56 % acetone and 50 min time of extraction) was only 1.4 mg/100 g DW under ultrasonic influence (Table 2). On this basis we propose 56% acetone in water and 50 min time of extraction as optimal for yield of proantocyanidins by *Fragaria vesca* L leaves.

CONCLUSIONS

On the base of the obtained results from process optimization of proanthocyanidins extraction we proposed ultrasonic method for accelerated extraction procedure. These optimized methods will be applied in our future investigation for extraction of procyanidins and their further addition in drug, food products and cosmetics as a source of biological activity.

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STUDY OF THE EFFICIENCY OF THE METHOD USED FOR DETERMINATION OF THE STRAIN OF DISPLACEMENT OF RENNET GEL OBTAINED BY VARIOUS MILK CLOTTING ENZYMES

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Abstract

During the penetrometric study, the efficiency of the method for determining the strain of displacement in rennet gel obtained by chymosin of microbial and camel origin was studied.
Using the standard measuring time of 60 s, the monitoring of penetration in the gel for the time of 30 s was also investigated. This approach is applied in defining the strain of displacement from the 10 to 90 min during the formation of the gel.
The results obtained by the penetrometric study of the rennet gel are the basis for modification of the method of determination of the strain of displacement. The serum released from the gel during the penetrometric study affects measurement accuracy.
The results obtained for 30 s indicate better stability of the experimenal data, which shortening the time for its implementation.
The information obtained by the conducted study suggests new modification of the method related to the time of penetration from the 60 to the 30 s.

Keywords*: penetrometric method, rennet coagulum, milk-clotting enzymes, gel firmness.*

INTRODUCTION

Enzymatic milk coagulation for cheese manufacturing involves the cleavage of the scissile bond in k-casein by an acid protease. Bovine chymosin is strongly recommended enzyme, combining a strong clotting activity with a low general proteolytic activity (Valkova, 2005; Kappeler et al., 2006). Camel chymosin has different characteristics comparing with bovine chymosin. Camel chymosin demonstrate a 70% higher clotting activity than bovine milk and has only 20% of the unspecific protease activity of bovine chymosin. The camel chymosin, obtained by fermentation, is more thermostable than bovine chymosin (Kappeler et al., 2006).
Rates of increases in rigidity during the first half of gel assembly were increased by increases in rennet concentration but maximum gel rigidity was not strongly affected. Curd firming rates vary from enzyme to enzyme with the more specific enzymes producing a firm gel more quickly (Kowalchyk, 1978).

The amount of rennet added to the milk has a large effect on the rate of the overall process. An increased rennet concentration leads to a shorter flocculation time, gel firmness starts to increase earlier and the rate of increasing is higher (Zoon et al., 1988; Hyldig, 1993; Lomholt & Qvist, 1997). While the gels are at the same stage in gel formation with respect to gel firmness, and the enzymatic reaction is completed, other differences between the gels must be responsible for the differences in gel firming rate. In addition, while rennet concentration seems to affect the structure of initial aggregates, it seems reasonable to expect that structural differences between gels, made with different rennet concentrations, can explain this effect (Law and Tamime, 2010). Rate of curd firming is not important for determining properties of the curd (Green, 1982), but its control is important in cheese making (Yun, 1982).

Variations of curd firmness at time of cutting may result in greater losses of milk components and reduced cheese yield (Olson, 1982). Monitoring curd firmness during cheese making offers the potential for reducing such losses by cutting at consistent curd firmness to optimize cheese manufacturing.

Most fundamental studies on the gelation process involved use of instruments developed for the continuous monitoring of curd firmness. However, for the testing of a large number of milks, continuous monitoring is too time consuming and a single measurement of curd firmness at a given stage during coagulation is used as a basis for comparison. One of the most suitable equipment for this type of test is a penetrometer. Penetrometres generally measure the deformation of the coagulum under a compressive load. This deformation is then inversely related to the firmness of the coagulum (Burgess, 1978). The firmness of the gel increases for several hours after gel formation, depending on the conditions. Rennet gels show linear viscoelastic behaviour, i.e. deformation is proportional to applied stress, for relative deformations up to 0.026–0.05 Pa (van Dijk, 1982; Dejmek, 1987; Hyldig, 1993). At larger deformations the gel structure will be damaged.

Most detailed rheological investigations of renneting has been carried out on skimmed milk, but Storry et al. (1983) and Grandison et al. (1984) did not find any effect of fat content on the coagulation time or gel strength of unhomogenised milks.

The aim of this study is to identify the efficiency of the method for determination of the strain of displacement of rennet gel.

Penetrometric study was conducted with the use of coagulated cow's milk in order to identify the influence of the type of milk-clotting enzymes on the hardness of rennet-obtained gels.

MATERIALS AND METHODS

The milk used in this experiment had the following physicochemical parameters: Fat – 3,6 % (after degreasing – 2,76 %); Non-fat solids – 8,4 %; Density of milk – 1,027 kgxdm^{-3}; Protein – 3,11 %. These parameters were obtained using "Ekomilk" apparatus (Plovdiv, Bulgaria). Acidity was determined by titration – 16 °T; Active acidity (pH) – 6,8.

Partial skimmed milk was heated to temperatures of 65 ° C.

As coagulant was used camel chymosin (Cr. Hansen) with commercial name "CHY – MAX" ® M, with milk-clotting activity 1000 IMCU / ml (or strength of the enzyme 1:70000), stored refrigerated at 0 ÷ 8 °.

The milk-clotting enzyme of microbial origin (Cr. Hansen) was characterized by activity (1:50000) and other indicators in established certificate.

For the experiment, the enzyme activity of the aforementioned coagulants was reduced to 1:50000.

For determination of the density of the rennet-obtained gel was used a type of penetrometer, appliance developed by Todor Todorov Lyubenov, UFT, Plovdiv (Lyubenov, 1975). The device has a working body with cylindrical shape and a flat work surface area with F = $2x10^{-4}$ m^2, 0,0139 kg mass and constant k = 0,5 Nxkg^{-1}. The strain of displacement Θ was calculated by moving the operating body (h).

$\Theta = kxmxh^{-2} = 0{,}00695xh^{-2}$, Nxm^{-2}

All results were statistically processed to a level of significance of $\alpha = 0{,}05$. Statistical processing of the data obtained in the course of the experiment was carried out using specialized mathematical software - OriginPro 6.1.

RESULTS AND DISCUSSIONS

The aim of the conducted experiment was the examination of the efficiency of the method for determination of strain of displacement during the rennet gel formation with three different types of milk-clotting enzymes. The focus of the experiment was related to enzymes of camel and microbial origin, compared with calf chymosin. It was found that the strain of displacement of the rennet gel obtained was related with the time for the gel formation and the specific activity of the milk-clotting coagulants used.

The gel hardness and thickness were defined using penetrometric methods. The results for the penetration of the working body of the device applied in the rennet gel were obtained in mm. The amount of rennet for the specific experiment was taken as 30 $cm^3x10^{-2}xdm^{-3}$ for the three milk-clotting enzymes used for the preparation of the rennet gel.

The results obtained from the penetrometric study with the use of the three milk-clotting enzymes are presented in Table 1.

After mathematical and statistical processing was established that the values obtained for the penetration of the working body of the device after 60 min, for the interval of 30 s and 60 s related to the enzyme of microbial origin were statistically insignificant. For the milk-clotting enzyme of calf origin, this tendency was observed 50 min after the initial coagulation for both times of measurements; and finally, in the case of using the enzyme of camel origin – after 20 min for the same examinated times (30 s and 60 s).

This statistical insignificance of the values for the three enzymes applied can be explained by the uniform rate of increasing rigidity of the rennet gel.

Table 1. Pentration of coagulum obtained using milk-clotting enzymes with microbial, camel and calf origin

Penetration time, min	Penetration (mm) of coagulum, obtained by milk-clotting enzyme in an amount, 30 cm^3 x 10^{-2} x dm^{-3} from:					
	Microbial origin, s		Calf origin, s		Camel origin, s	
	30	60	30	60	30	60
10	45,1 ± 1,6	55,2 ± 5,1	30,7 ± 2,0	34,2 ± 2,8	37,4 ± 1,2	39,6 ± 2,1
20	38,2 ± 1,4	44,1 ± 3,2	26,8 ± 1,2	31,0 ± 2,3	33,2 ± 1,1	35,2 ± 2,5
30	33,4 ± 1,6	41,3 ± 3,1	26,2 ± 0,8	29,0 ± 1,8	29,8 ± 1,8	30,1 ± 2,8
40	28,2 ± 1,3	36,5 ± 2,4	25,4 ± 1,0	28,2 ± 2,0	24,6 ± 1,1	27,0 ± 2,6
50	27,3 ± 1,5	32,3 ± 2,6	25,0 ± 1,1	27,4 ± 2,4	23,4 ± 1,4	25,0 ± 1,8
60	26,0 ± 1,0	30,0 ± 2,8	24,1 ± 1,1	27,0 ± 2,0	22,2 ± 1,6	23,2 ± 2,2
70	25,0 ± 1,1	26,4 ± 1,9	23,2 ± 1,4	26,2 ± 2,3	22,1 ± 1,8	23,0 ± 2,4
80	24,2 ± 1,2	25,3 ± 2,4	21,2 ± 1,2	25,1 ± 2,0	19,0 ± 1,8	22,0 ± 2,1
90	22,2 ± 1,2	23,4 ± 1,5	20,0 ± 1,0	23,0 ± 2,4	17,1 ± 1,4	21,0 ± 1,8

Whereas, observing the results obtained for the both times of measurement, it was defined that the values of intrusion in depth in the rennet gel for a period of 60 s using all three types of enzymes were higher in comparison with the values for a time of 30 s.
The strain of displacement is directly related to the amount of the lactoserum separated of the rennet gel, i.e. the duration of the measurements define the amount of the serum released from the rennet-obtained gel.
The results for the strain of displacement of the rennet gel obtained with the use of microbial coagulant are presented in Figure 1.

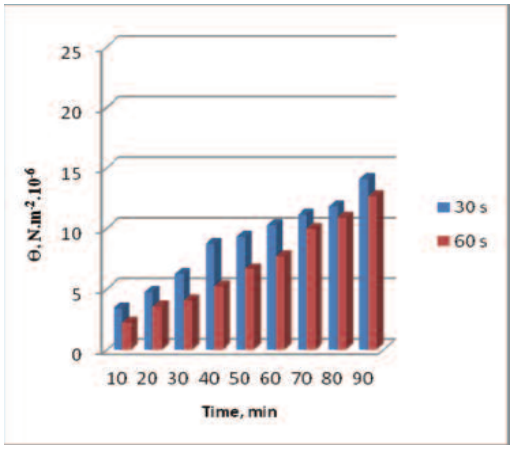

Figure 1. Strain of displacement ($Nxm^{-2}x10^{-6}$) of coagulum obtained using microbial chymosin.

The slow rate of compaction of the rennet gel with the use of the microbial enzyme resulted in low values of the strain of displacement – from 3,42 ± 0,94 Nxm^{-2} x 10^{-6} to 14,10 ± 1,04 Nxm^{-2} x 10^{-6} for a measuring time of 30 s and from 2,28 ± 0,81 to 12,69 ± 1,14 Nxm^{-2} x 10^{-6} - for 60 s. The use of this type of milk-clotting enzyme led to a formation of rennet gel having a structure very easy to destroy and releasing very high quantities of lactoserum. This milk coagulant showed significant variations of the values for the strain of displacement. In comparison with the other two clotting enzymes, the differences observed and related to the examinated factor were more noticeable and essential for the gel structure.

The results for the strain of displacement of rennet gel obtained with the use of bovine coagulant are presented in Figure 2.

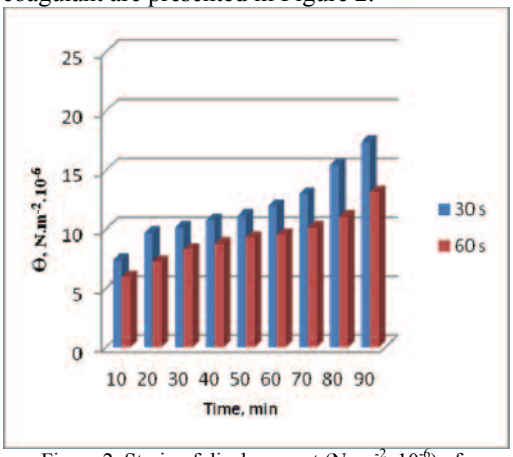

Figure 2. Strain of displacement ($Nxm^{-2}x10^{-6}$) of coagulum obtained using calf chymosin

Using the calf chymosin, it was observed that in comparison with the enzyme with microbial origin, the firming process of the gel was faster and the gel structure separated low quantities of milk serum. The strain of displacement and the variation of the obtained values were from 7,37 ± 0,60 to17,38 ± 1,55 Nxm^{-2} x 10^{-6} for 30 s and from 5,94 ± 0,64 to 13,14 ± 1,25 Nxm^{-2} x 10^{-6} for 60 s. The gel formed with the use of bovine chymosin showed significant differences of the values of the strain of displacement during the period of 50 min for both measured times (30 s and 60 s). The values for the calf chymosin were statistically insignificant 50 min after the initial coagulation and 70 min after it for the enzyme with microbial origin.
The results observed for the enzyme with camel origin described a process of formation of the rennet gel with intermediate characteristics - between these of a rennet gel formed with the use of bovine and with the use of microbial chymosin. The results for the strain of displacement of this enzyme are presented in Figure 3. The coagulum had a thick structure and a very low quantities of serum is separated. At the end of the experiment, the values of the strain of displacement were the highest compared to the values presented for the other two enzymes - 23,78 ± 1,84 Nxm^{-2} x 10^{-6} for 30 s and 15,76 ± 1,64 Nxm^{-2} x 10^{-6} for 60 s.

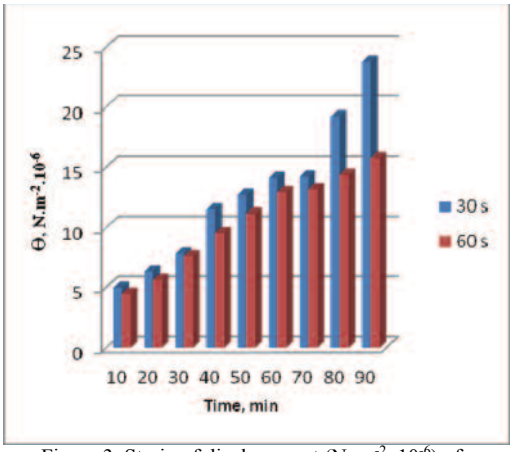

Figure 3. Strain of displacement ($Nxm^{-2}x10^{-6}$) of coagulum obtained using camel chymosin

The differences between the three studied enzymes were that the gel formed with the use of camel chymosin had no statistically significant differences between the values of the strain of displacement 20 min after the initial coagulation until the end of the experiment for both times of measurement - 30 s and 60 s.

CONCLUSIONS

The results of the penetrometric study with the use of three different genetic variants of milk-clotting enzymes indicate that during the experiment (90 min), the rennet gels obtained with the enzyme with camel origin have the most significant and uniform rate of compaction and the coagulum releases small amounts of serum.

The serum released from the gel during the penetrometry affects measurement accuracy.

The results obtained for a period of 30 s indicate better stability of the experimental data, and shortening the time for its implementation. These results are of great importance when the enzyme of microbial origin was used, because of the amount of serum released from the gel during the times of measurement - 30 s and 60 s.

The results obtained by the conducted experiment of the rennet gel are important for modification of the method of determination of the strain of displacement; also, their application suggests new modification of the method, related to changing the time of penetration from 60 s to 30 s.

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DETERMINATION OF BIOLOGICALLY ACTIVE SUBSTANCES IN TAPROOT OF COMMON CHICORY (*CICHORIUM INTYBUS L.*)

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Abstract

The object of our current study is to determinate the biologically active substance presented in the taproots of Bulgarian medicinal plant common chicory (Cichorium intybus L.). The carbohydrate composition, the amount of total phenols, the total flavanoides content and the antioxidant activity in the obtained sequential ethanol and water extracts has been evaluated. The amount of inulin-type fructans was defined by the resorcinol assay. The sugars, fructooligosacharides and inulin contents of the obtained extracts were analyzed by TLC and HPLC-RID methods. The total phenolic and flavonoid quantities were analysed by using Folin–Ciocalteu's and $Al(NO_3)_3$ reagents, respectively. The antioxidant activity was defined by four method (DPPH, ABTS, FRAP and CUPRAC). The total fructan content in taproots is in range 23 % dw. The presence of monosaccharide glucose, fructose, sucrose and trisaccharides 1-kestose, nystose in the ethanol extracts was observed in the ethanol extracts. The analysis of water extracts revealed the high level of inulin (14 %), total phenols and flavonoids (7 mg/g GAE dw and 2 mg QE/g dw, respectively). The 95 % (v/v) ethanol extracts of roots collected during autumn showed the most well-pronounced antioxidant activity as followed: DPPH - 31 mM TE/g dw, ABTS - 49 mM TE/g dw, FRAP - 28 mM TE/g dw and CUPRAC - 127 mM TE/g dw.

Key words*: fructooligosacharides, inulin, common chicory, antioxidant activity*

INTRODUCTION

Cichorium intybus L. known as common chicory or wild succory is a member of Compositae family and has been considered as an important medicinal herb (Kocsis et al., 2001). It is an erect, glandular, biennial plant with a taproot, rosette of 30-70 leaves and stem up to 90 cm height (Ilaiyaraja and Khanum, 2010) (Figure 1). This herb usually grows like a weed or flower near to the roadside and meadows (Ozuturk et al., 2006).

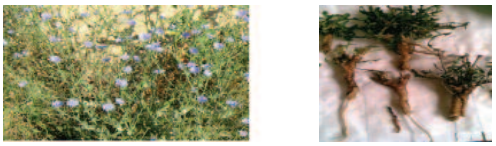

Figure 1. Common chicory (*Cichorium intybus* L.) - aerial parts and roots

The whole plant has numerous applications in food industry and medicine (Ilaiyaraja and Khanum, 2010). Its dried roots were used as a substitute or adulterant in coffee powder (Jung et al., 1996). The young leaves can be added to salads and vegetable dishes, while chicory extracts are used for the production of invigorating beverages. Except because of its nutritive value, wild chicory is used for winter forage for ruminant animal (Ozturk, 2006).

It has been reported that this plant is an important source of fructans and chicoric acid (Milala et al., 2009). It contains also saccharides, organic acids, polyphenols (Jurgonbski et al., 2011) such as chlorogenic acid, caffeic acid derivatives (Kumari et al., 2007). Because of its rich content of biologically active substances such as inulin, coumarins, vitamins, bitter compounds, flavonoids and sesquiterpene lactones, the whole plant extracts is used as anti-diabetic (Pushparaj et al., 2007), antioxidant (Gazzani et al., 2000; Papetti et al., 2002), antibacterial (Petrovic, 2004), immunotoxic (Kim et al., 2003), antihepatotoxic (Zafar and Ali, 1998; Ahmed et al., 2003), antiulcerogenic, anti-inflammatory, appetizer, digestive, stomachic, depurative (Rastogi et al., 1994), for curing

different diseases connected with gastrointestinal system. Chicory roots have been used in folk medicine for livers disorders, gallstone and inflammations of the urinary tract since 17 th century (Kocsis et al., 2001). It has been traditionally used to cure various ailments in Ayurvedic and Unani systems (Ilaiyaraja and Khanum, 2010). Leaves and roots of *Chicorium intybus* L. were also used for purification of blood, for curing arteriosclerosis and they are also considered to possess anti-arthritis, anti-spasmodic, hypotensive and laxative action (Tiwari, 2008).

During the past decade, there is a growing interest in natural plant extracts with potential antioxidant activity, because of their improved healthy effect (Aleksieva et al. 2013; Mihaylova et al. 2013). The expanded application is due to their protective properties against oxidative stress disorders, as well as oxidative damage in food products (Ivanov et al., 2014).

It is well known that polyphenols from plant extracts possessed strong antioxidant activities. Their presences in medicinal plants that are natural source of inulin-type fructans prebiotics additionally increase the biological activity of the obtained extracts (Petkova et al., 2012; Vrancheva et al., 2012). However, there are uncompleted information about presence of inulin, total phenols and flavonoids in root of medicinal plants common chicory (*Cichorium intybus* L.), growth in Bulgaria. Not detailed investigations have been reported regarding evaluation of antioxidant potency of common chicory roots.

Therefore, the aim of the current study was to determinate the content of biologically active substances and to evaluate antioxidant activities of extracts obtained from roots of common chicory (*Cichorium intybus* L.) gathered during the spring and autumn seasons from Bulgaria.

MATERIALS AND METHODS

The taproots of several random chosen plants of common chicory (*Cichorium intybus* L.) were collected from territory of Bulgaria - Kresna (Blagoevgrad region) and Chehlare village (Sredna Gora Mountain) during May and November 2012. The underground parts were air-dried, finely ground and passed through a 0.5 mm sieve. The prepared samples with approximately 8.7% moisture content were stored in dry containers for further use.

All used reagents and solvents were of analytical grade scale. Carbohydrate standards fructose, sucrose, 1-kestose and nystose have been purchased from Sigma-Aldrich (Steinheim, Germany). Fructooligosacchrides Frutafit®CLR, and inulin Frutafit®TEX were supplied by Sensus (Roosendaal, the Netherlands). Frutafit®CLR contains high level of oligofructoses with the average chain length of 7-9 monomers. Frutafit®TEX is characterized with mean degree of polymerization DP 22.

Ethanol and subsequent water extraction procedure was applied to obtained fructans, total phenols and flavonoids from the taproots of common chicory. The extraction process was carried out as follow: 0.7 g dry chicory roots were extracted three hours with 95 % (v/v) boiling ethanol. Then, the residue was treated three hours with boiling distilled water. The fructan content in the obtained extracts expressed as fructose equivalent were analyzed spectrophotometrically at wavelength 480 nm by resorcinol-thiourea reagent (Petkova and Denev, 2013).

TLC analysis were used to elucidate the presence of mono-, di-, fructooligosaccharides (FOS) and inulin in the ethanol and water extracts from common chicory roots. Five microliters of each sample were performed on silica gel 60 F_{254} plates (Merck, Germany) with mobile phase *n*-BuOH:*i*-Pro:H_2O:CH_3COOH (7:5:4:2) (v/v/v/v). The TLC plates were dipped in the detecting reagent diphenylamine-aniline-H_3PO_4–acetone (Lingyun et al., 2007), heated and scanned as previously described (Petkova and Denev, 2013).

The sugars, FOSs and inulin content in root extracts were analyzed by HPLC methods. Chromatographic separations were performed on HPLC Shimadzu, coupled with LC-20AD pump, refractive index detector Shimadzu RID-10A. The control of the system, data acquisition, and data analysis were under the control of the software program LC solution version 1.24 SP1 (Shimadzu Corporation, Kyoto, Japan)

The determination of inulin and sugars in water extracts were performed on a Shodex® Sugar SP0810 with Pb^{2+} a guard column (50 × 9.2 mm i.d.) and an analytical column (300 mm × 8.0 mm i.d.) at 85 °C. The mobile phase used for separation was distilled water with flow rate 1.0 ml/min. The injection volume of the samples was 20 µL (Petkova et al., 2013).

Folin–Ciocalteu's method was used for determination of total phenols. The results were expressed as mg gallic acid equivalent (GAE) on dry weight bases (Ivanov et al., 2014).

The total flavonoids content was analysed by $Al(NO_3)_3$ reagent. The absorbance was measured at 415 nm. The results were presented as mg equivalents quercetin (QE) per g dry weight (DW), (Kivrak et al., 2009) according to the calibration curve, linear in range of 10-100 µg/mL quercetin as a standard.

The total antioxidant activity of ethanol and water extracts from taproots of common chicory (*Cichorium intybus* L.) was estimated by forth methods as follows: DPPH (1,1-diphenyl-2-picrylhydrazyl radical), ABTS (2,2`azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)), FRAP (ferric reducing antioxidant power) and CUPRAC (cupric reducing antioxidant capacity).

DPPH assay: 0.15 ml of each extract was mixed with 2.85 ml freshly prepared DPPH solution (0.1 mM in methanol). After incubating for 15 minutes at 37 ºC in darkness, the absorbance at 517 nm was measured with spectrophotometer in comparison to the blank containing methanol and % inhibition were calculated.

For ABTS assay, 0.15 ml extract was mixed with 2.85 ml of the ABTS solution previously diluted with methanol (1:30; v/v). After 15 min at 37 ºC in darkness, the absorbance of formed complex was measured spectrophotometrically at 734 nm (Ivanov et al., 2014).

FRAP assay: 0.1 ml of investigated extracts were added to 3 ml FRAP reagent (0.3 M acetate buffer (pH 3.6): 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ): 20 mM $FeCl_3 \times 6H_2O$ (10:1:1; v/v/v) and allowed to react for 10 min at 37 °C in darkness. The absorbance of the formed coloured product was measured at 593 nm (Benzie and Strain, 1996).

CUPRAC assay: The reaction was started by mixing of 1 ml $CuCl_2 \times 2H_2O$, 1 ml Neocuproine (7.5 ml in methanol), 1ml 0.1 M ammonium acetate buffer; 0.1 ml of analyzed extracts and 1 ml d. H_2O. The reaction time was 20 min at 50 °C in darkness. After cooling the absorbance was measured at 450 nm (Marchev et al., 2012). All the results from the determination of antioxidant activity were performed in triplicates and expressed as mM Trolox equivalents (mM TE) by dry weight. All the data were expressed as mean ± standard deviation (SD).

Statistical analysis was performed using MS Excel 2010. The p values less than 0.05 were considered as significantly different.

RESULTS AND DISCUSSIONS

The results from the TLC analysis of the sequentially obtained ethanol and water of common chicory (*Cichorium intybus* L.) showed that a large number of carbohydrates were successively extracted (Figure 2). The presence of sugars fructose, sucrose, FOSs including 1-kestose, nystose and 7-9 oligomers, equivalent with used standards Frutafit CLR, was established in all investigated 95 % (v/v) ethanol extracts (Figure 2 A).

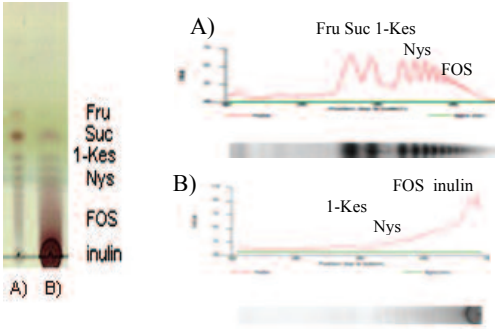

Figure 2. Thin-layer chromatograms of 5 µl A) ethanol and B) water extracts from root of *Cichorium intybus* L., where Fru - fructose, Suc - sucrose, 1-Kes - 1-kestose (GF2), Nys - nystose (GF3), fructooligosaccharides (respectively GF4, GF5, GF6, GF7, GF8) and inulin

In the water extracts, obtained after ethanol pretreatment, except 1-kestose, nystose, FOSs, the presence of high molecular fraction of inulin with DP 22 similar to the used standard was observed (Figure 2 B).

The spectrophotometric analysis showed the low and high molecular fructan fractions expresses as fructose equivalents (Table 1). The

established total carbohydrate content was 23 g/100 g dw. Bagaoutdinova et al. (2001) reported similar results. The detailed analysis of HPLC results of the studied extracts (Table 2) showed that the taproots of common chicory

Table 1. Fructan content expressed as fructose equivalents in the extracts from of common chicory (*Cichorium intybus* L.), g/100 g dw[1] (mean ± SD[2], n=4)

Location	Harvest time	Low-molecular fraction (Fru[3], Suc[4] and FOSs)	High-molecular fraction (inulin)	Total
Kresna	May 2012	9.3 ± 0.7	14.1 ± 1.2	23.4 ± 0.7
Chehlare	November 2012	6.8 ± 0.3	16.8 ± 0.3	23.1 ± 0.6

[1]dry weight, [2]SD – standard deviation, [3]Fru – fructose, 4 - sucrose

Table 2. Carbohydrates content in root extracts *Cichorium intybus* L.

Location	Harvest time	Fructose	Sucrose	1-Kestose	Nystose	Inulin
Kresna	May 2012	1.6	2.4	1.3	0.9	12.6
Chehlare	November 2012	1.2	1.9	1.5	1.2	16.2

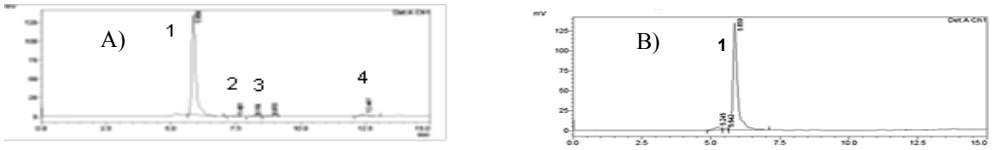

Figure 3. HPLC chromatograms of water extract obtained from taproots of chicory harvest from different locations and season: a) Kresna (May) and b) Chehlare (November), where 1. inulin; 2. nystose 3. sucrose, 4 fructose

are rich source of sugars and short chain fructooligosacharides. Sucrose was presented in both extracts. The content of 1-kestose and nystose, which possessed well pronounce probiotic effect (Van Loo, 2004), reached above 2 % dw. Moreover, the content of inulin in the investigated plant samples was relatively high - 16 % dw. HPLC chromatograms of carbohydrates in collected root are presented in Figure 3. The obtained results for inulin content in November taproots were with 2 % higher than reported by Milala et al. (2009). Therefore, the investigated plants contained constant level of inulin type fructans independently from harvest time and location. Chicory roots can be applied as a rich source of soluble dietary fibers in preparation of healthy food and nutrition formula.

The obtained extracts from taproots of common chicory harvest during the autumn showed the highest total phenolic contents: 7.9 ± 0.9 (in 95 % (v/v) ethanol) and 6.7 ± 0.9 (subsequent water extraction) mg GAE/g DW, respectively (Table 3). The similar results for 95 % ethanol were reported by Jurgonbski et al. (2011) and Özgen et al., (2004). Koleva et al. (2012) obtained 70 % EtOH extract from *Cichorium intybus L.* with flavonoid content less than 1 mg/ml. However, until now the detailed results for quantitative evaluation of total flavonoids in 95 % v/v EtOH and water extracts for roots of common chicory have not been reported. In our study the highest level of total flavonoids was observed in water extracts obtained after ethanol pre-treatment for both plants (2.8 ± 0.2, mg EQ/g dw) (Table 3).

Table 3. Total phenolic contents and total flavonoids contents of *Cichorium intybus* L. extracts.

Harvest time	Extracts	Total polyphenols, mg GAE[1]/g dw	Total flavonoids, mg EQ[2]/g dw
		Mean±SD[3]	
May	95% EtOH	4.3 ± 0.5	0.6 ± 0.1
	water	3.7 ± 0.4	2.7 ± 0.2
November	95% EtOH	7.9 ± 0.9	1.0 ± 0.1
	water	6.7 ± 0.9	2.8 ± 0.2

[1]Expressed as milligram of gallic acid per gram dry material, [2]Expressed as milligram of quiercetin per gram dry extract, [3]SD – standard deviation (n=6)

To evaluate the antioxidant activities of obtained ethanol and subsequent water extracts from common chicory roots, their abilities to scavenge the synthetic DPPH and ABTS radicals, as well as their power to reduce ferric (FRAP) and cupric (CUPRAC) ions were

investigated. The analysis were performed in triplicates and expressed as mM Trolox equivalents (mM TE) by dry weight (Table 4).

Table 4. Antioxidant activity of the extracts obtained from roots of common chicory (*Chicorium intubus* L.)

Harvest time	Extracts	DPPH	ABTS	FRAP	CUPRAC
May 2012	EtOH	29.2±0.5	8.6±0.6	1.2±0.2	64.6±2.0
	H_2O	16.1±2.8	1.8±0.3	1.5±1.5	58.5±3.3
November 2012	EtOH	31.3±0.1	9.9±0.7	8.7±0.2	123.6±0.6
	H_2O	29.1±0.8	6.1±0.5	5.7±0.5	114.2±4.3

Until now, not detailed information was available for antioxidant activity of root from common chicory grown on territory of Bulgaria. Only radical scavenging activities of methanol and water extracts from *Chicorium intubus L.* evaluated by DPPH method were reported (Özgen et al., 2004; Ilaiyaraja and Khanum, 2010; Nikolova et al., 2011).

For the first time we evaluated the antioxidant potential of 95 % (v/v) EtOH and subsequent water extracts by four methods DPPH, ABTS, FRAP and CUPRAC. Among the results obtained during our investigation, the 95 % (v/v) EtOH extract showed the highest antioxidant activities (31.3±0.1, 49.9±0.7, 28.7±0.2, 123.6±0.6 mM TE/g dw for methods DPPH, ABTS, FRAP and CUPRAC, respectively). In addition the ethanol extracts also had the highest total phenolic content (Table 3) 4.3 mg/g and 7.9 mg/g GAE, that may be the possible explanation about their enhanced antioxidant activity. The most common explanation of the observed tendency was the presence of phenolic acids and flavonoids in them (Ivanov et. al., 2013). According to Ilaiyaraja and Khanum (2010) the hydrogen donating ability of these compounds is responsible for their effective antioxidant property and used for protecting against cellular oxidative damage. It has been reported that not only phenolic compounds can be responsible for the *in vitro* antioxidant activity of chicory preparations. Jurgonbski et al. (2011) proposed that sugars themselves, especially sucrose and fructans, could also act as radical scavengers in plant cells. The presence of inulin and FOS in roots of common chicory additionally improve their biological activity.

CONCLUSIONS

The current report is the first comprehensive study that presented detailed information for inulin, FOSs, phenolic, flavonoids content and antioxidant activity of edible taproot from common chicory (*Cichorium intybus* L.) grown in Bulgaria. The antioxidant potential of extracts positively correlated with their phenolic contents and flavonoids contents, respectively. Except important source of inulin-type fructans the determined metabolites profile of its roots revealed their potential application as radical scavengers due to the presence of polyphenols. Therefore, this complex of biologically active substance in their roots offers many future applications in field of herbal medicine and nutrition for production of healthy food with well-pronounced healthy effect.

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NEW APPROACHES TO REMOVING ALKYL-METHOXYPYRAZINES FROM GRAPE JUICE AND WINE

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Abstract

Grape-based products, including wine, are amongst the world's most important value-added horticultural commodities, both economically and culturally. 3-isobutyl- (IBMP) and 3-isopropyl- (IPMP) 2-methoxypyrazine are important grape- and insect-derived flavour compounds in some grape juices and wine, and are responsible for undesirable green characters associated with under-ripe grapes or infestation from Coccinellidae. Here we present data on two novel approaches to removing these compounds from juice and wine. Firstly, we describe a protein-based technology that binds and removes IPMP and IBMP in juice. The lipocalin Mouse Major Urinary Protein 1 (mMUP) was expressed in Pichia pastoris, secreted, and purified using anion exchange chromatography. mMUP, combined with a 10 KD cut-off PES membrane filtration system, resulted in a reduction of IPMP and IBMP in juice of > 98%. However, removal of methoxypyrazines from wine using this technique may be limited by ethanol-induced changes in the mMUP structure. Therefore, a 2nd approach is being developed that takes advantage of the sorptive properties of various polymeric materials. A range of food-grade polyethylene-, polypropylene- and silicon- based polymers were evaluated for their capacity to remove IPMP and IBMP from red wine. Candidate polymers were standardized to a common surface area and added to red wine for 2 hrs. Quantification of IPMP and IBMP using HS-SPME-MD-GC-MS showed reductions of up to 40% for some polymers, and minimal changes to the sensory characteristics of the wine. We conclude there is capacity to significantly mediate the impact of methoxypyrazines on juice and wine quality using these biotechnology tools.

Keywords: *methoxypyrazines, wine taint, ladybug taint, Coccinellidae, remediation.*

INTRODUCTION

Flavour is a critical driver of consumer acceptance of and preference for agri-food products. Alkyl-methoxypyrazines (MPs) are aroma-active constituents of several species of vegetables, nuts (Boubee et al., 2000; Buchbauer et al., 2000; Sala et al., 2002), fruits (Schieberle et al., 2003) and spices (Jagella and Grosch, 1999). MPs have also been identified in several grape cultivars and their wines. In wine, they can have a positive impact on the aroma profile of certain varietals, particularly Sauvignon blanc (Allen and Lacey, 1998), but in general are considered detrimental to quality, contributing undesirable characters such as "green" and "unripe" (Allen et al., 1991). 3-isobutyl- (IBMP), 3-sec-butyl- (SBMP) and 3-isopropyl- (IPMP) 2-methoxypyrazine have all been confirmed in grape and wine, with IBMP

the most prevalent. IBMP concentrations in grape decrease with ripening; thus elevated levels in wine are indicative of the use of grapes of sub-optimum maturity or poor viticultural management (Lacey and others, 1991).

In addition to being found as intrinsic compounds in grapes, MPs in wine may also be derived from Coccinellidae (ladybeetles); specifically, *Harmonia axyridis* (Pallas) and *Coccinella septempunctata* (Botezatu et al., 2013). When grapes are harvested, beetles resident in grape clusters may also be inadvertently collected, and following crushing and pressing operations in the winery, impart the resulting juice and wine with an unpleasant aroma and flavour coined 'ladybug taint' (Figure 1).

Figure 1. *Harmonia axyridis* beetles can infest grapes and taint the resulting juice and wine with alkyl-methoxypyrazines (photo courtesy of Kevin Ker and Ryan Brewster).

Juice and wine affected by ladybug taint are characterised by undesirable peanut and bell-pepper aroma and increased bitterness (Pickering et al., 2004). IPMP – a component of Coccinellidae haemolymph – has been identified as the main compound responsible for the taint (Pickering et al., 2005), while other MPs may contribute to a lesser extent (Botezatu et al., 2013).

Importantly, the human threshold for both IPMP and IBMP in juice and wine is very low - down to 300 pg/L (Pickering et al., 2007) - meaning little is needed to compromise wine quality, and creating challenges for efforts aimed at remediating affected juice and wine. Indeed, traditional winery processes and fining agents have only proven partially effective in reducing MP levels (Pickering et al., 2006; Kotseridis et al., 2008; Kögel et al., 2014), in part due to a lack of specificity for MPs. Interestingly, some preliminary data suggests that polyethylene-based polymers may have capacity for removing MPs from wine through sorption (Blake et al., 2009; Pickering et al., 2010).

The objectives of the current study were to assess the efficacy of 2 technologies at reducing IPMP and IBMP in grape juice and wine. In the first study, an odorant binding protein (mMUP) with high specificity for MPs was trialled for use in grape juice. In the second study, a range of polyethylene and other polymers was evaluated for their potential to remove MPs from wine.

METHODS AND MATERIALS

In Study 1, we evaluated 2 lipocalins with purported high affinity for MPs (Kd values as low as 0.8 µm) - Mouse Major Urinary Protein 1 (mMUP) and Porcine Odorant Binding Protein (plOBP) - for their MP-fining potential in buffer and juice; we report on the results for mMUP here. mMUP was expressed in the methylotrophic yeast *Pichia pastoris*, and the secreted protein was purified using anion exchange chromatography. The protein was then added to MP-spiked Phosphate Citrate Buffers or a Chardonnay juice. In related assays, we also assessed the capacity of a polyethersulfone membrane (10kDa MW cutoff) and a bentonite fining agent to remove the mMUP-MP complexes from the buffer/juice. Full details can be found in Inglis et al. (2010).

In a second study, we investigated the capacity of a range of 14 synthetic and natural polymers to remove MPs from red wine. These included

low-, medium-, and high- density polyethylenes, polypropylene, and related copolymers. The 3 best-performing polymers were selected for further study, which involved standardizing their area to approx 68 cm^2 and soaking them in a 200mL aliquot of red wine (Cabernet sauvignon and Merlot blend) spiked with MPs, for 2 hrs.

IPMP and IBMP were quantified in both studies using the sensitive headspace solid-phase microextraction multidimensional gas chromatography-mass spectrometry method of Botezatu et al. (2013). Wine samples were treated with NaOH, diluted to volume and then volatiles extracted from the headspace using a Gerstel autosampler and a DVB/Carboxen/PDMS Stable Flex fiber. Analyses were performed using an Agilent 7890A Gas - Chromatographer coupled with an Agilent MS 5975 Mass Spectrometer with triple detector. The GC was equipped with a Dean Switch that permitted switching the effluent flow between the two columns (Column 1: HP5MS; Column 2: DBWAX). For IPMP, ions 137 and 152 were monitored as quantifying and qualifying ions, respectively. For [$^2H_3$] – IPMP, ions 140 and 127 were monitored as the quantifying and qualifying ion, respectively. The selected mass channels for IBMP were m/z 124 and 81, and for [$^2H_3$] – IBMP they were 127 and 154. Ions 124 and 127 were used as quantifying ions while 81 and 154 were qualifying ions. The MS was operated in total ion chromatogram mode (TIC), with a gain factor of 7.

RESULTS AND DISCUSSION

Study 1

As shown in Figure 2, IPMP and IBMP were reduced from 300ng/L to < 2ng/L and 5 ng/L, respectively, in the juice.

Importantly, the mMUP-methoxypyrazine complexes were successfully counter-fined out of the juice using bentonite; a commonly employed fining agent used in juice and wine production (data not shown). In a related trial, we demonstrated that bovine serum albumin, used as a non-specific protein control, had no impact on reducing MPs (Inglis et al., 2010).

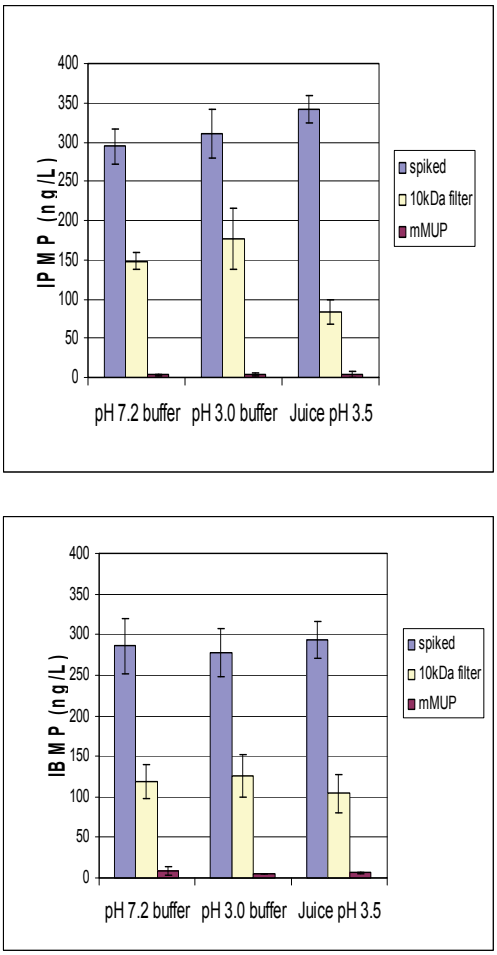

Figure 2. Reduction of 3-isopropyl- (top) and 3-isobutyl- (bottom) 2-methoxypyrazine in Phosphate Citrate Buffer and Chardonnay juice using mMUP and the 10 MWCO PES filtration system. Data represent the mean values of 9 replicates for each matrix. Figure adapted from Inglis et al., (2010).

Study 2

As shown in Figure 3, all 3 polymers significantly reduced both IPMP and IBMP concentrations in the wine. On-going research in our labs is focused on optimising Polymers B and C for use on a commercial scale.

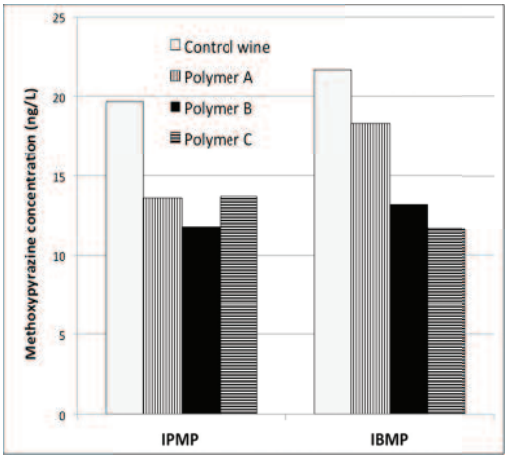

Figure 3. Reduction of 3-isopropyl- (IPMP) and 3-isobutyl- (IBMP) 2-methoxypyrazine in red wine after treatment with selected polymers.

Other considerations

Reduction of both IPMP and IBMP in juice to below their limit of quantitation with the mMUP system represents an important result, given the resistance of these compounds to more conventional additives and treatments (Pickering et al., 2006). We speculate this system will be similarly effective with other fruit and vegetable juices containing elevated MPs, given its efficacy across a wide pH range. The significant reduction in MPs due to the membrane alone (Figure 2) is unexpected, however likely to have limited commercial applicability; the 10 kDa pore size would be too small for industrial juice and wine processing. Further research from our labs is directed at immobilizing mMUP on solid supports for the extraction of MPs from wine and juice where membrane pore size can be varied. The immobilization of proteins on solid supports often results in increased protein stability, which would be advantageous in developing a system that may be regenerated for use multiple times. Preliminary results indicate reduced efficacy of the mMUP system in wine - possibly due to ethanol-induced changes in the protein's structure - and is also under active study in our labs.

While the reduction in IPMP and IBMP in wine using the various polymers is relatively modest, it may be sufficient to decrease their concentration to below the human detection threshold, particularly if combined with treatment of juice with mMUP prior to fermentation. Additionally, the sensory quality of polymer-treated MP-enhanced wines shows minimal differences compared to non-treated control wines (data not shown). On-going work in both the mMUP and polymer studies is assessing the specificity of these treatments for MPs by assessing their impact on other key volatile compounds in juice and wine.

CONCLUSIONS

Alkyl-methoxypyrazines are high impact, taint compounds found in some juices and wines, and are generally resistant to traditional remediation approaches. We developed and evaluated 2 treatments aimed at removing 3-isobutyl- and 3-isopropyl- 2-methoxypyrazine from grape juice and wine. The lipocalin mMUP was very effective, and selected polymers were moderately effective, at reducing these compounds in juice and wine, respectively. Current research is focused on optimising these tools for use on a commercial scale.

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THE SIGNIFICANCE OF SOME FLOUR QUALITY PARAMETERS AS QUALITY PREDICTORS OF BREAD

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Abstract

The purpose of the present research has been the highlighting of the correlation between the protein content, the wet gluten content and the gluten index of flours, and some characteristics of bread, such as volume and the ratio height / diameter (H / D). In this respect, were analysed 19 samples of flour obtained from Romanian wheat, determining the protein content, wet gluten content and gluten index. At the same time were carried out baking tests corresponding to the 19 loaves of bread and were determined the parameters volume and the height / diameter ratio (H /D).
*The results showed that the best predictor for the bread quality parameters: volume and H / D ratio, is the gluten fraction of the gluten index parameter which remains on the sieve (highly significant positive correlation r = 0.79***, respectively r = 0.73***). Gluten index parameter correlates insignificantly with bread volume (r = 0.18) and significantly with the height / diameter ratio (0.51*). In conclusion, the parameter Gluten index itself, is not relevant for the baking qualities of flour; these quality parameters could be better predicted by remaining fraction of gluten on the sieve.*

Keywords*: bread volume, gluten index, wheat flour.*

1. INTRODUCTION

The correlation of the analysis methods of flours quality with their technological performance has always represented a major interest for the experts in the field. The technological performance of flours depends on complex factors which are only partially revealed by the usual assessment tests for the flours quality. These factors are consisting of both physical and chemical parameters, such as protein content, ash content, wet gluten content, gluten index etc., as well as a range of parameters concerning the flours behavior in gel or dough stage, namely: falling number, amylografic viscosity, extensibility, resistance, strain energy, elasticity, development time, stability, softening, etc. [3, 5, 7, 10, 12, 14, 18, 19].
Besides this factors also the variability of the analysis methods by which these factors are determined, which can be very high, has to be considered.
The mixer, farinograph, extrudograph, mixograph, valorigraph, rheograph, give us indications about dough behavior during mixing.
The extensograph, alveograph, extensometer and glutograph give indications about dough behavior to stretch.
The fermentograph, the maturograph, the microclimate room, the zimotachigraph, the rheofermentometer, give indications about the behavior of dough during fermentation.
The amylograph, the viscograph, the rheotron, the consistometer, the penetrometer, the viscometer, give indications of penetration, viscosity and so on [13].
Several studies showed that the best predictor for bread volume is the protein content of wheat or flour [1, 4, 11].
R. Koppel and A. Ingver (2004) demonstrated interesting correlations for the flour processed from the Estonian wheat cultivated in the 1999 – 2003 period. Specifically, the researches focused in this case, on the linkages between physical and chemical parameters,

extensographic and farinographic parameters and the volume of the bread [8].

Gaines et al. (2006) tested 33 wheat samples of some varieties cultivated in the United States. Three of the parameters taken into consideration were proven to be superior with respect to predictability of technological characteristics: the alveographic mechanical work (W), the height of the mixographic peak and the capacity of retention of the solvents (Solvent Retention Capacity, AACC Method 56 – 11), against the gluten index ant the sedimentation indices (SDS)[6].

Similar investigations have been made by *Bettge et al.* (1989), *Kostyukovsky and Zohar* (2004), *Rashed et al.* (2007) [2, 9,15].

Różyło and Laskowski (2011) showed that the best predictors of bread quality on the volume and core are combinations of alveographic, physical and chemical parameters, namely: Zeleny sedimentation index , falling number, and alveographic work (W) or protein content, the falling number and alveographic extensibility [16] .

Sapirstein and Suchi (1999) obtained some results showing that the height of flour gel, obtained after centrifugation, in certain conditions of the flours dispersed in sodiumdodecilsulphate (SDS), correlates very strongly with bread volume (r^2 between 0.89 and 0.95) [17].

The purpose of the present research was to highlight the level of correlation between various parameters of flours (protein content, wet gluten content, gluten index) and some qualitative characteristics of bread, such as volume and the ratio height / diameter (H/D).

MATERIALS AND METHODS

We analyzed 19 samples of flour from the Romanian wheat harvest of the year 2012. We determined the following quality parameters: protein content (ICC 159-95 - NIR method, Perten Inframatic 8600), wet gluten content (ISO 21415-2:2007) and gluten index (SR ISO 21415-2:2007) [20,21].

Starting from the values of the parameter gluten index and wet gluten of the flour samples, we also calculated a different parameter. This refers to the fraction of wet gluten remained on the sieve (GRS), after centrifugation, shown in the standard for determining gluten index. Mathematically speaking, the amount of gluten fraction was calculated using the formula: GRS = (WG * GI) / 100.

For each of the 19 flour samples we carried out baking tests, in accordance with the prescription and technological parameters described in Table 1.

Table 1. Technological parameters and the recipe for baking samples

Specification	Value
Wheat flour	1 kg
Salt	13 g
Yeast	23 g
Water	Variable, depending on the technological requirements of flour: 550 – 607 ml
Slow kneading time	3 min
Intensive kneading time	Variable, approx. 5 – 8 min, depending on the technological requirements of flour
Bench proofing time (at room temperature)	Variable, approx. 20 – 25 min, depending on the technological requirements of flour
Proofing time (35^0C, 78 % humidity)	Variable, approx. 43 – 54 min, depending on the technological requirements of flour
Baking	220^0C, for 20 min

The dough was divided into portions of 350g each, in order to allow obtaining of a final product with the weight of 300g.

The equipment we used for making the baking samples included an intensive mixer with spiral and having the tank capacity of 30 kg, a dough moulder (for long format), a baking proofer with controlled temperature and humidity and an electric baking oven.

For each of the 19 samples we selected two loaves for which we measured, at 2 hours after baking, the following quality parameters:

- Volume (V, cm^3/100g) according to SR 91:2007, using a Fornet apparatus [22];
- Height/Diameter ratio (h/d). Bread height and diameter was measured by a calliper and the shape (height/diameter) was calculated.

The values used in the study represent the arithmetical average of the determinations

carried out for the two loaves of bread, selected at each baking sample.

RESULTS AND DISCUSSIONS

The results obtained by determining the quality parameters of flours and corresponding samples of bread are shown in Table 2.

Table 2. Estimates of the quality parameters variability of flours and bread

Specifications	X ± s_x	Range of variation		CV (%)
		Min.	Max.	
Protein content (P %)	13.42 ± 1.17	11.79	16.50	8.74
Wet gluten (WG, %)	33.37 ± 6.48	19.73	43.40	19.43
Gluten index (GI)	83.81 ± 13.34	56	99	15.92
Bread volume (V, cm^3/100 g)	441.48 ± 73.54	290	573	16.66
Height/Diameter ratio (h/d)	0.71 ± 0.06	0.65	0.88	8.41
Gluten remaned on the sieve (GRS, %)	27.62 ± 5.59	18.95	39.25	20.26

From Table 2 we can see that the analyzed flour samples were characterized by average values of quality parameters, excellent for the bread production process (more than 13.0% protein content, wet gluten content more than 30.0% and gluten index over 80).

Regarding the gluten quality, it may be described as being tough, with very good qualities for the baking process. Except for protein content, which showed relatively low variability (CV = 8.74%), all other quality parameters of flours had relatively high coefficients of variation.

Note that the analyzed flours have a very wide range of quality parameters, from flours with low wet gluten content (19.73%), to flours with high wet gluten content (43.40%), from flours with gluten of extremely poor quality (GI = 56), to flours with very strong gluten (GI = 99).

In terms of volume, the obtained bread showed high variability (CV = 16.656%), similar to the variability of quality parameters of the flours from which were derived (gluten index and wet gluten content).

Thus, we obtained improperly bread volume (290 cm^3/100 g), but also excellent bread volume ((573 cm^3/100 g).

The average volume of bread (441.484 cm^3/100 g), obtained from the 19 flour samples, do not reflect properly the average values of the quality parameters of these flours.

This is probably due to a big variability of these quality parameters.

Ratio h/d of bread was characterized by a rather small variability, similar to that observed for the protein content in the 19 analyzed flours.

The average value of the ratio h/d for the 19 obtained loaves of bread discloses a bread product with a curved profile, rather typical for strong gluten flour (see Figure 1).

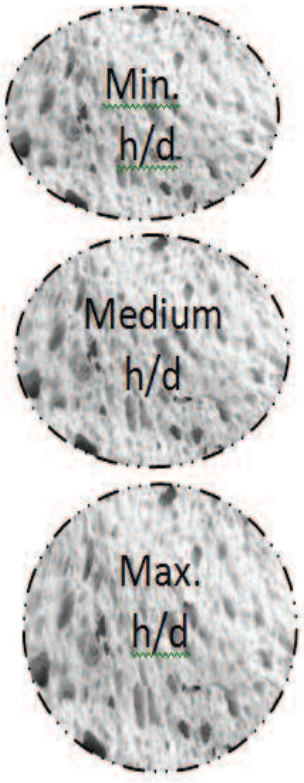

Figure 1. The bread loaves profile obtained from the 19 flours tested

Table 3 highlights the correlation between the technological parameters of flours and the quality parameters of obtained bread.

Table 3. Correlations between the technological parameters of flours and quality parameters of bread

Variable	P	WG	GI	V	h/d	G
P	1.00					
WG	0.51*	1.00				
GI	0.23	-0.43	1.00			
V	0.35	0.62**	0.18	1.00		
h/d	0.65**	0.27	0.51*	0.55**	1.00	
GRS	0.66**	0.66**	0.39	0.79***	0.73***	1

*p<0.05; **p<0.01; ***p<0.001

In table 3 we can notice that the volume of bread for the analyzed samples increased distinct significantly with increasing the wet gluten content of flour (r = 0.62**).

The protein content of the analyzed flours was not significantly correlated with bread volume, although this correlation is frequently described in the literature.

Apparently, the quality of gluten flours, as explained by the gluten index parameter, did not affect significantly the value of bread volume (r = 0.18 ns).

However, the fraction of gluten that remains on the sieve (GRS), which represents the percentage of the amount of strong gluten in flours, was the best predictor for the value of bread volume (r = 0.79***). Basically, this gluten fraction described in a proportion of 62.4% (r^2 = 0,624) the volume variability in analyzed bread samples.

The result suggests that the use of this fraction in assessing the quality of the flours, used to obtain bread, can be more useful than the value of the gluten index parameter itself.

The regression line and the corresponding regression equation are shown in Figure 2.

The amount of wet gluten remained on the sieve was also the best predictor for the height/diameter ratio of bread. This ratio has increased very significantly as the amount of gluten remained on the sieve was higher (r = 0.73***).

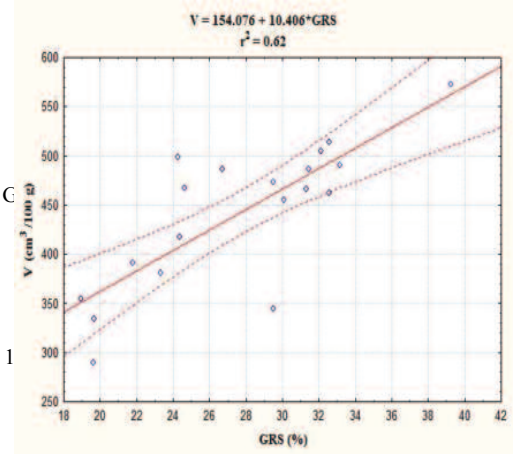

Figure 2. Regression of the bread volume and the amount of gluten remained on the sieve

Over 50% of the variation of the ratio h/d for the analyzed bread loaves is explained by the variation of the quantity of gluten remained on the sieve (r^2 = 0.53, Figure 3).

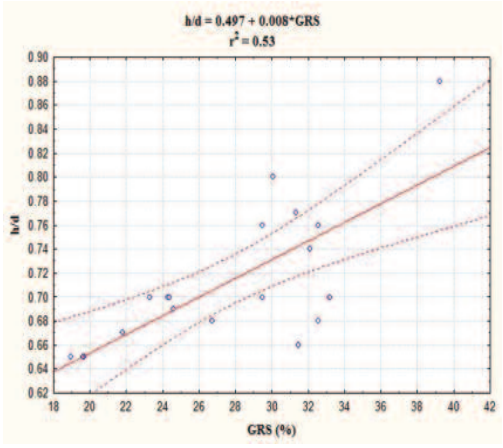

Figure 3. Regression of the h / d ratio and the amount of gluten remained on the sieve

The ratio between height and diameter at most of the 19 loaves increased distinct significantly as flour protein content increased (r = 0.66**). Also, the ratio h/d increased significantly as the value of the gluten index parameter was higher (r = 0.51*). These data suggest that the ratio h/d is strongly dependent on the tenacity of gluten, being higher as the more gluten is stronger.

Between the two quality parameters of bread, volume and h/d ratio, there have been noticed a distinct significant positive correlation, so that

bread loaves with a higher h/d ratio had a larger volume (0.55**).

We believe that our results can be a starting point to conduct more extensive researches that take into account the evaluation of the gluten index parameter of flours as predictor of bread quality.

CONCLUSIONS

1. Our results showed that the best predictor of bread quality is not the gluten index parameter as such, but the amount of wet gluten remaining on the sieve (GRS) during the determination of this parameter. GRS correlated very significantly with both the volume of bread loaf (r = 0.79***) and the h/d ratio (r = 0.73 ***);
2. The gluten index parameter correlated insignificantly with the bread volume (r = 0.18 ns), but wet gluten content flours distinct correlated significantly (r = 0.62**) with bread volume;
3. The protein content of flours was not significantly correlated with bread volume (r = 0.35 ns);
4. The h/d ratio increased distinct significantly as the protein content of flours was higher (r = 0.66**) and significantly as the value of the gluten index parameter was higher. These data suggest that the ratio h/d is strongly dependent on the tenacity of gluten, being higher as the gluten is stronger.

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OLFACTOMETRIC CHARACTERIZATION OF *TAMAIOASA ROMANEASCA* WINE COME FROM DIFFERENT WINE REGIONS

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Abstract

The work refers to the analysis of aroma compounds identified in Tamaioasa Romanian wines from 2 distinct Romanian wine-growing areas: vineyard Stefanesti-Arges and Pietroasa, watching, and in particular the variation of flavorings depending on the region of origin.
Gas chromatographic method coupled with mass Spectrometry were identified 6 esters, 3 higher alcohols, 1 aromatic alcohol, 2 terpenes, 1 lactone, 1 acid and 1 aldehyde. The high concentration of ethyl acetate, ethyl butanoate, isoamyl alcohol were identified; flavor specific Tamaioasa Romanian wine is given by 1-α-terpineol, terpenic alcohol has been identified in this wine in large quantities.
Research has shown that wine-growing region influence the organoleptic characteristics of wine and aromatic content of their wines, so the wines can be very quickly recognized when tasting.

Keywords**:** *gas chromatography, mass spectrometry, volatile compounds, Tamaioasa Romaneasca wine*

INTRODUCTION

The specific character of wines produced from a grape variety aromatic flavor is given by variety, on the one hand, the way the flavors evolve during fermentation and during aging wines but also, the one-off character printed wine-growing zone (Baek H et al., 1997). Several studies have referred to the volatile aroma compounds of musts and wines from aromatic varieties, especially those of *Muscat* (*Muscat Ottonel*, *Muscat of Alexandria, Muscat of Hamburg*), semiaromatic varieties (*Sauvignon*) (Chandary S. et al., 1964) or neutral varieties (*Riesling*) (Chisholm M. G. et al., 1994). Flavor of these wines is determined by terpene alcohols, aromatic terpenes are specific to each variety (ex. the linalool for *Muscat Ottonel*, geraniol for *Muscat of Alexandria*, nerol for *Muscat of Hamburg* etc) (Guth H., 1997).
Our studies concerns the characterization aromatic potential of *Tamaioasa Romaneasca* wine and wine-growing region influence the aromatic character of these wines. Grape and wine aroma of *Tamaioasa Romaneasca*, (aromatic variety as *Muscat* group) is on terpenes and especially the 1-α-terpineol, a compound that is found in large quantities in these wines and ranging, in rather large limits depending on wine region. Variation of chemical compounds of wine is even more striking if the areas are much different in terms of growing conditions and climate ecopedological.

MATERIALS AND METHODS

Tamaioasa Romaneasca wine was analyzed in terms of physico-chemical characteristics: alcoholic strength (vol% alcohol), sugar content (g/L), total acidity (g/L sulfuric acid), total dry extract (g/L), acidity volatile (g/L acetic acid) and glycerol (g/L). All analyses were performed by the standard methods in the field (*Recueil des methodes internationales d'analyse des vins et mouts*, 2011). Chemical tests were followed by organoleptic analysis (tasting laboratory) and GC/MS (gas chromatography coupled with mass spectrometry method) analyses for identification and determination of volatile

compounds in the wines (Campeanu G. et al., 2001). Determination of volatile aromatic compounds in wine was performed using a Hewlett Packard 5890 gas chromatograph series II coupled to a mass spectrometer Hewlett Packard 5972 series II.

Extraction methods

Volatile compounds were isolated by solvent extraction. 75 mL of wine (containing internal standard IS, 2-ethyl hexanol) were extracted with 15 mL solvent of pentane (Serot T. et al., 2001): dichloromethane 2:1 in a separation funnel. Internal standard solution in absolute ethanol (Merck, Germany) was previously prepared by adding 20 µL (16.2 mg) of ethyl hexanol to 4 mL of ethanol. From this solution 10 µL were introduced in the wine sample. The solvent extract was placed in a 25 mL round bottom flask, then concentrated to about 1 mL under a gently nitrogen flow, at 25°C. This 1 mL volume was further concentrated in a 2 mL vial under nitrogen flow to about 100 µL. From this solution 1 mL was injected in split less injection mode.

Chromatographic conditions

A Hewlett Packard gas chromatograph equipped with split/splitless injector was used. 1µl from each extract was injected into an HP 5-MS capillary column with dimensions: 30 m x 0.25 mm x 0.25 µm film thickness. Column temperature: 30° C for 10 min., followed by temperature gradient 10° C/ min up to 80° C, then gradient of 25° C/min up to 250° C where stationed 10 minutes. The temperature of the injection port was 280° C and the detector (FID) temperature was set at 250° C. MSD conditions are: temperature 180° C ion source, ionization energy 70 eV, mass limit of 20-400 amu, electronic multiplier voltage 1700V, scan rate 1.60 s^{-1}. Injection mode: split, opening after 60 sec, and the split flow: 20 ml/ min. Quantitative determination and identification of volatile compounds based on the comparison of retention indices (RI), mass spectra and the estate of odors. Identification is based on the standard MS library Wiley.

RESULTS AND DISCUSSIONS

Chemical and organoleptic analyses of wine

The two wines *Tămâioasă Românească,* from the two Romanian wine regions, Stefanesti-Arges (TR I) and Pietroasa (TR II) were analyzed in terms of key physical, chemical and organoleptic parameters (Tardea C., 1980) and results are presented in Table 1. Organoleptic analysis of wines showed: *Tamaioasa Romaneasca* wines are aromatic white wines, high quality, with a sugar content of 38 respectively 45 g/L and an alcoholic strength of 11.4 respectively 12.5 vol% alcohol (fig. 1). Wines are rich, complex, balanced taste, smoothness and softness sensation is printed, probably higher in glycerol content (16 and 18 g/L). Wine *Tamaioasa Romaneasca* of Pietroasa is a high class wine, with a strong flavor and total particular flavor, honey and lime and acacia flowers and amber color.

Table 1 - The Main Physico-Chemical Parameters Analyzed wines

Wine	total acidity (g/L sulfuric acid)	sugar content (g/L sugar)	alcoholic strength (vol% alcohol)	Glycerol (g/L)	total dry extract (g/L)	volatile acidity (g/L acetic acid)
TR I	4.9	38	11.4	16	27	0.3
TR II	4.1	45	12.5	18	29	0.5

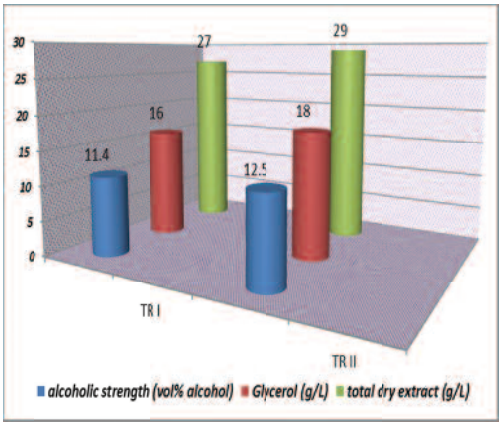

Figure 1. Physico-chemical analysis of the wines

GC/MS analyses of wines. Identification of volatile compounds

Results of the wine volatile compounds determined in *Tămâioasă Românească* wine are given in table 2. Given the large number of alcohols and acids contained in wine, the number of possible combinations (esters) is also very high, primarily ethyl esters, ethanol is mainly because the wine. Esters are formed in grape fermentation process in large quantities by enzymatic esterification and in the process of maturation and aging of wine by chemical esterification (Visan L. et al., 2010). Neutral esters (ethyl acetate, ethyl lactate etc.) are volatile and the wine bouquet, and affects acid esters are not volatile and have more influence on the taste of wine (fig. 2 and fig. 3). The *Tămâioasă Românească* wine stands for ethyl acetate, smelling the flowers and ethyl butanoate, smell of pineapple. Only *Tămâioasă Românească* wine of Stefanesti Arges region decanoate ester was identified as ethyl ester odor of chemicals.

Table 2- The Concentration of Wine Volatile Compounds Determined in *Tămâioasă Românească* Wines, (µg/L)

No.	Compound	Concentration (µg/L)	
		TR I	TR II
The concentration of esters			
1	Ethyl acetate	645	683
2	Butyl acetate	0.2	1
3	Ethyl butanoate	155	233
4	Ethyl octanoate	2	2
5	Methyl butanoate	90	56
6	Ethyl decanoate	2	-
The concentration of aliphatic alcohols			
7	2-methyl-1-propanol	19	22
8	3-methyl-1-butanol	200	280
9	1-hexanol	1	0.3
The concentration of aromatic alcohols			
10	Benzetanol	820	630
The concentration of terpenes			
11	Linalol	0.1	0.1
12	1- α -terpineol	2	3.7
The concentration of lactones			
13	γ-butyrolactone	56	78

Terpene alcohols are volatile class of compounds with the highest importance to wine aroma, terpenes, however, being found only wines from aromatic varieties: *Muscat Ottonel, Muscat of Alexandria, Muscat of Hamburg, Tămâioasă Românească* etc. Identification of terpenes in wine flavored variety shows the specific nature of each part, these compounds represent "key substances" (fig.4) that may contribute to the identity of a wine. Thus, terpene content varies by variety flavored: linalool is found in high concentration in wine *Muscat Ottonel,* geraniol in *Muscat of Alexandria* etc.

The *Tămâioasă Românească* wines analyzed are present 1-α-terpineol (an odorant monoterpenes with a very high potential, and linalool), α-terpineol printing wines sweet *incense* smell of lilac (Visan et al., 2012). Linalool is found in wines, but in very low concentrations. Although 1-α-terpineol was identified in both wines, noticed the wine TR II, by Pietroasa, with a about double content of 1-α-terpineol.

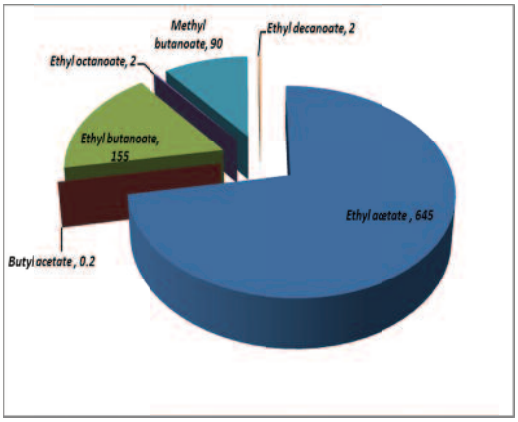

Figure 2. The main esters of a *Tamaioasa Romaneasca* wines, µg/L (TR I, Stefanesti)

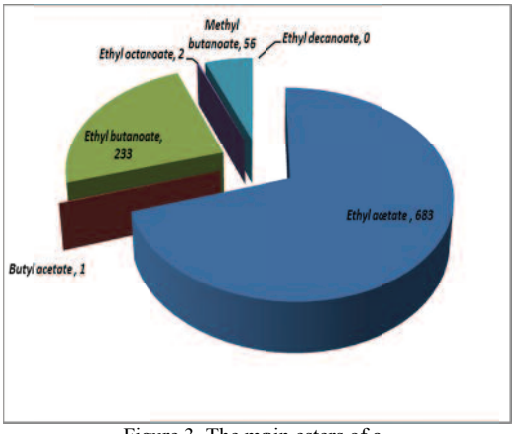

Figure 3. The main esters of a *Tamaioasa Romaneasca* wines, µg/L (TR II, Pietroasa)

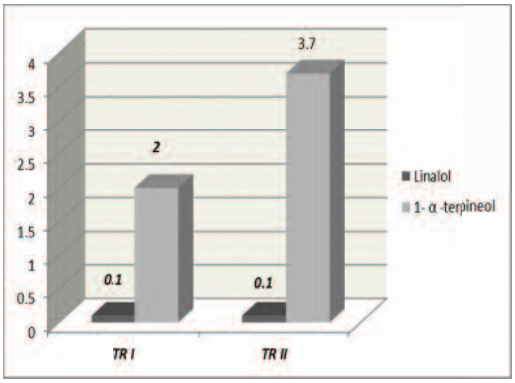

Figure 4. Concentration of terpenes in *Tamaioasa Romaneasca* wines (µg/L)

The higher alcohols were represented by 2-methyl-1-propanol, 3-methyl-1-butanol, 1-hexanol and benzetanol, in slightly different concentration (Fig. 5).

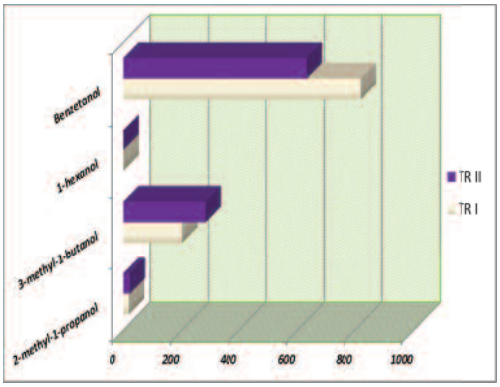

Figure 5. Concentration of higher alcohols in *Tamaioasa Romaneasca* wines (µg/L)

CONCLUSIONS

Aroma *Tămâioasă românească* wines is striking, but especially the standout wines from wine-growing center Pietroasa; wines have a strong and distinctive flavor, honey and lime and acacia blossom and amber color. Wine can be classified in the category high-class wines, is complex, robust, very well balanced.

Of esters were identified in both wines: ethyl acetate, butyl acetate, ethyl butanoate, ethyl octanoate, methyl butanoate and ethyl decanoate, the latter being found only in wine from Stefanesti-Arges wine-growing region. Concentrations in esters were slightly different, slightly higher Pietroasa wine-growing region.

Aromatic alcohols were represented by benzetanol, in slightly different concentration, higher in wine from Stefanesti-Arges wine-growing region.

Terpenic compound that print flavor of *Tamaioasa Romaneasca* variety is α-terpineol; the compound was identified in both wines, in wine from the Pietroasa wine-growing region, α-terpineol still being found in almost double the concentration Stefanesti-Arges wine.

Although flavored varieties presents a varietal aroma, though a great influence on the aromatic character of grapes and wines has wine-growing region, which can lead to large differences in the typical character of wines.

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ANALYSIS OF FERMENTED LACTIC ACID DAIRY PRODUCTS ENRICHED WITH INULIN-TYPE FRUCTANS

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Abstract

The production of functional lactic acid dairy products constantly increases nowadays. They are usually used to enrich with soluble dietary fibers that enhance their functional characteristics. The aim of the current research was to investigate the lactic acid dairy products enriched with inulin-type fructans with different degree of polymerization and to analyse the fructooligosacharides and inulin content in them after lactic acid fermentation. For the yoghurt preparation inulin and fructooligosacharides extracted from tubers of Helianthus tuberosus L. and commercially available inulin from chicory were used. The amount of fructans from inulin-type was determined by spectrophotometric, TLC and HPLC-RID methods. The results from the analysis showed that after lactic acid fermentation the content of inulin changed in a very small amount and the obtained product possessed improved healthy and potential prebiotic effect.

Keywords: *fructoligosaccharides, inulin, Helianthus tuberosus L., yoghurt, HPLC-RID analysis*

INTRODUCTION

Inulin is a fructan, that consist mainly of β-(2→1) fructofuranosyl units (Fm) and usually, but not always ended with a terminal α-glycopyranose unit (1→2) (GFn) (Van Laere and Van Den Ende, 2002) (Figure 1). Its degree of polymerization (DP) varies from 2 to 70 (De Leenheer and Hoebregs, 1994). Fructooligosaccharides (FOSs) are a subgroup of inulin, with DP 2-10 (Niness, 1999).

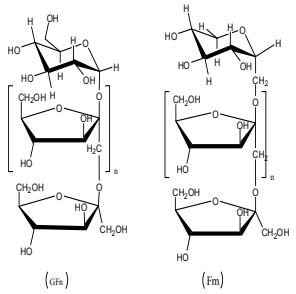

Figure1. Chemical structure of inulin

The primary plant sources of inulin are Jerusalem artichoke (*Helianthus tuberosus* L.) and chicory. Its content in Jerusalem artichoke varies in the range of 7 and 20 % on a fresh weight basis and >75% on a dry weight basis (De Leenheer, 1996).

During the last decade, the application of inulin and fructooligosacharides in food constantly increases due to their diverse range of potential health benefits (Niness, 1999; Roberfroid, 2005). Because of the β-configuration of the anomeric C_2 in the fructose monomers inulin-type fructans are resistant to hydrolysis by human small intestinal digestive enzymes, being therefore classified as dietary fibers (Roberfroid, 2005). They are selectively fermented by β-fructofuranosidase-producing bacteria. FOS and inulin were considered as potential prebiotic ingredients in foods (Gibson and Roberfroid, 1995). They offer a unique combination of nutritional properties and important technological benefits, which depends on the degree of polymerization DP. Therefore, fractions with variable DP can be used to formulate special food products (Yi et al., 2010).

Inulin is legally classified as a food ingredient and it is used in foods to improve organoleptic characteristics, to replace fats and carbohydrates and to enrich products with dietary

fibers. Inulin and FOS are often used to improve the functional properties of dairy products as replace fat and provide creamy mouthfeel in low-fat dairy products such as drinks, yogurts, dips, cream cheese, and process cheese (Franck, 2002). This makes inulin favorable as an ingredient for dietetic food (Roberfroid, 1999; Roberfroid, 2002; Roberfroid, 2005).

Many reports are dedicated to enrichment of yoghurt with inulin or/and fructooligosacharides extracted from chicory or Jerusalem artichoke as prebiotic food ingredients (Hempel et al, 2007, Paseephol T., Sherkat F., 2009). Yogurt is one of the most popular dairy products, obtained as the result of lactic acid fermentation of the milk. It is a traditional product in the everyday meal of Bulgarian people. The addition of inulin-type fructan influences the texture of fermented lactic acid product (Yi et al., 2010; Khalifa et al, 2011). They not only improve the textural properties but also enrich the product with soluble dietary fibers. The incorporation of inulin in dairy products is connected with the health benefits of this fructans, together with their immunomodulating and functional properties (Barclay et al., 2010).

The increasing application of inulin and FOS in the food industry needs the methods for their quantitative determination for food labeling and control of production process. Several methods are available in the literature for quantification of inulin-type fructans in milk or yogurt. Due to the high protein content, analysis of inulin in dairy products remains a challenge. Most of the spectrophotometric methods are based on enzyme hydrolysis with endo- and exo-inulinase (Borromei et al. 2010). Most of them are based on the quantification of fructose and glucose released after the enzymatic hydrolysis of inulin. Analysis with HPTLC was recommended by AOAC (Simonovska, 2000). The other analytical methods are based on HPLC separation with ELSD detection (Kristo, 2011) or using anion-exchange chromatography equipped with pulsed amperometric detection (HPAEC-PAD) (Yi et al., 2010; Borromei et al. 2010). Another developed method for FOS analysis in yoghurt is MALDI-TOF MS (Borromei et al. 2009).

The production of yoghurt prepared with traditional Bulgarian starter culture and dietary fibers extracted from Jerusalem artichoke tubers will be considered as a new functional dairy product with improved health benefits. Until now no report were available about enrichment of traditional Bulgarian yoghurt with FOS and inulin fractionated from *Helianthus tuberosus* L.

Therefore, the aim of the current study was to analyse inulin-type fructans with different degree of polymerization in yoghurt after lactic acid fermentation.

MATERIALS AND METHODS

Fructooligosacharides (DP 4-8) and inulin were extracted from tubers of previously investigated varieties *Helianthus tuberosus* L. (Petkova et al. 2013). The sequential extractions by ultrasonic irradiation, previously described by Peshlova et al. (2013) were applied. The obtained fractions were cleaned by ion-exchange resin and evaporated under reduce pressure. The resulting inulin and FOS from *Helianthus tuberosus L.* were dissolved in milk in final concentration 1 % and were used to prepare yoghurt enriched with inulin-type fructan. Inulin and fructooligosacharides from Jerusalem artichoke tubers were compared with commercial chicory root inulin Raftiline HP (DP 25) for preparation of yoghurts. Comparative study of yoghurt prepared with 1 % chicory inulin were carried out. Fructans were added to milk in the form of a solution prior to pasteurization. Standardized milk with 2.5 % fat was inoculated with 2 % *Lactobacillus delbrueckii* subsp *bulgaricus* and *Streptococcus thermophilus* as previously described by the authors (Vlaseva et al., 2013).

The moisture content in yoghurt was determinated by drying the samples at 102 °C for 4 hours (AOAC, 2007).

The changes of inulin content were followed on the first and twentieth after coagulation. Prior to analysis the samples were well homogenized.

The extraction of inulin and sugars from yoghurt was done as follows: 10 g of yoghurt were extracted with 30 ml boiling deionized water for 10 min. The proteins were precipitated by addition of 5 ml Carrez I reagent ($K_4Fe(CN)_6 \times 3H_2O$, 15 g/100 ml) and

5 ml Carrez II reagent ($Zn(CH_3COO)_2 \times 2H_2O$, 30 g/100 ml). The sample was filtered through 0.45 µm paper filter, transferred in 50 ml volumetric flask and made up to the mark with deionized water.

The carbohydrate content in yoghurt were analyzed by TLC analysis. For determination of inulin-type fructan in the obtained water extracts spectrophotometric and HPLC-RID method were performed (Petkova and Denev, 2013).

Chromatographic separation was performed on HPLC Shimadzu, coupled with LC-20AD pump, refractive index detector Shimadzu RID-10A. The control of the system, data acquisition, and data analysis were under the control of the software program LC solution version 1.24 SP1 (Shimadzu Corporation, Kyoto, Japan). The HPLC separation was carried out on a Shodex® Sugar SP0810 with Pb^{2+} a guard column (50 × 9.2 mm i.d.) and an analytical column (300 mm ×8.0 mm i.d.) at 85 °C. The column was placed in thermostat LCO 102 (ECOM, Czech Republic). The mobile phase was deionized water, vacuum-filtered through 0.2 µm membranes (Germany) before use**.** All samples were passed through the cellulose acetate filter with pore size 0.2 µm (Sartorius AG, Goettingen, Germany) before injection into the HPLC column.

Statistical analysis was performed by Excel 2010. Treatment means were considered significantly different ($P<0.05$).

RESULTS AND DISCUSSIONS

The changes in carbohydrate composition of fermented lactic acid dairy products enriched with inulin-type fructans were shown (Figure 3). In water extracts of yoghurts enriched with inulin the presence of fructose, galactose, and lactose, FOS and inulin similar to the used standards was observed. It was found that for the storage period of twenty days only the levels of lactose was significantly changed.

The HPLC-RID chromatograms of analyzed inulin in yogurts were presented (Figure 4 and Figure 5). The total fructan content of analyzed samples lactic acid fermented dairy products were expressed as fructose equivalent. Their content in FOS is reduced twice due to the lactic acid fermentation process, while the quantities of inulin isolated from Jerusalem artichoke and commercial one remained significantly constant (Table 1).

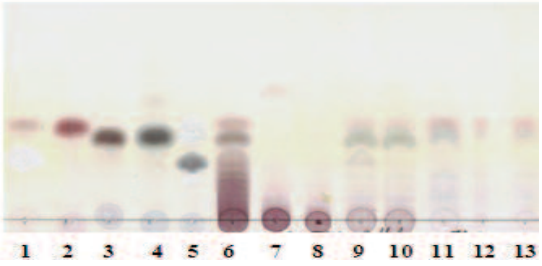

Figure 3. TLC chromatogram of water extracts from yogurt enriched with inulin, where 1. glucose, 2. fructose, 3. sucrose, 4. galactose, 5. lact artichoke, 8. inulin, 9. 10. water extract from yoghu m chicory, respectively

1 2 3 4 5 6 7 8 9 10 11 12 13

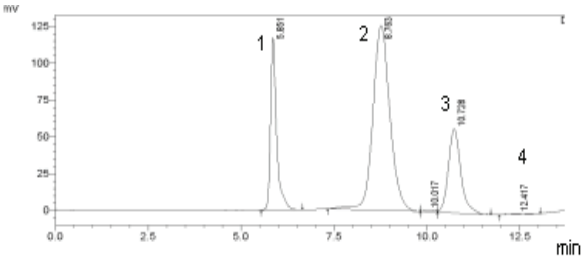

Figure 4. HPLC chromatogram of yogurt enriched with inulin extracted from tubers of Jerusalem artichoke (*Helinathus tubersus* L.), where 1. inulin, 2. lactose, 3. galactose, 4. fructose

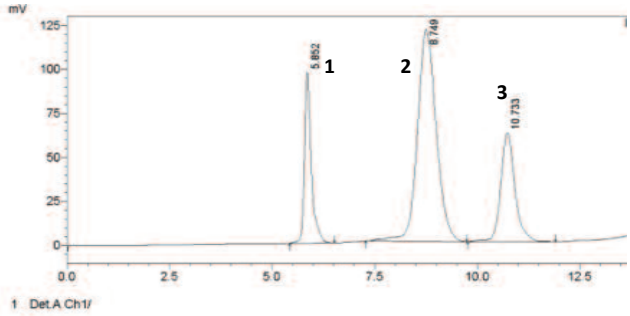

Figure 5. HPLC chromatogram of yogurt enriched with chicory inulin Raftiline HP (DP = 25), where 1. inulin, 2. lactose, 3. galactose

Table 1. Changes in inulin-type fructan content in yoghurt during storage at 4°C, g/100g dw[1] (Mean±SD[2]; n=3).

Sample	Inulin	Fructose	Total fructans	Inulin	Fructose	Total fructans[3]
	1 day after coagulation			20 day after coagulation		
Yoghurt wih 1 % FOS from *Helianthus tuberosus* L.	0.43	0.23	0.82	0.37	0.40	0.80
Yoghurt with 1% inulin from *Helianthus tuberosus* L.	0.90	-	0.92	0.83	0.09	0.90
Yoghurt with 1% chicory inulin Raftiline HP	1.00	-	1.00	0.86	0.08	0.98

[1]dry weight, [2]standard deviation, [3]express as fructose equivalent

On 20 days small amount of fructose 0.09g/100 g appeared in yogurt enriched with inulin with higher DP. FOS due to their shorther fructan chains was more preferable to be fermeted by the strains used for lactic acid fermentation. The small changes in FOS and inulin level could be explained with patrician hydrolysis due to the pH of the dairy products or to the ability of used microorganism to metabolized them. According to Kaplan and Hutkins *Lactobacillus bulgaricus* fermented inulin, whereas *Streptococcus thermophilus* is FOS non-fermentable (Borromei et al. 2010).

According to Kaplan and Hutkins (2000), Ivgantova et al., (2012) all studied *Lactobacillus* strains fermented FOS in different manner. These results show the differences in the ability to metabolize FOS by the isolated strains, which could be owed to different production levels of β-fructofuranosidase to break up the β (2→1) bonds presented in FOS. Makras et al. reported that lactobacilli have different capability to ferment fructooligosacharides and inulin and they conclude that not all probiotics degrade inulin in higher degree of polymerization (Borromei et al. 2009).

CONCLUSIONS

From the results obtained during the analysis of lactic-acid fermented dairy products enriched with fibers, we can conclude that the levels of inulin and fructooligosacharides in them remained constant. Therefore, the bacteria *Lactobacillus bulgaricus* and *Streptococcus thermophilus* used for lactic acid fermentation process were not capable to ferment inulin and FOS. Analysis of yoghurt enriched with inulin-type fructans extracted from tubers of Jerusalem artichoke (*Helianthus tuberosus* L.*)* presented a new functional food with improved nutritional values due to the presence of soluble fibers in it. On the basis of our investigation and considerably constant level of fructans added in lactic-acid fermented dairy products, we recommend application of inulin and FOS in preparation of functional dairy products. The final product will characterized with improved healthy effect because of fibers, presence of lactic acids and typical Bulgarian yoghurt starter culture.

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STUDY OF THE STRAIN OF DISPLACEMENT VARIATION DURING THE COMPACTION OF RENNET GEL OBTAINED BY VARIOUS MILK-CLOTTING ENZYMES

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Abstract

The variation in the rate of formation of rennet gel depending on the use of different types of milk-clotting enzymes for a set time was studied.
The intensity of development of the phases for the research process was determinate by the specificity and activity of coagulants. It was found that the strain of displacement was directly related with the time for the gel formation and also with the types of rennet enzymes used for its preparation.
The study was conducted with the amounts of rennet enzyme from 10 to 60 cm^3 $x10^{-2}dm^{-3}$ and the strain of displacement of the gel changes as values for a period of 90 min. The data obtained from the measuring instrument for the strain of displacement using milk-clotting enzyme with camel origin was in the range of $7,2x.10^{-6} \div 1,3x10^{-5}$. It was determined that under the similar conditions after 30 min there were no significant changes, these results demonstrate the ability of this enzyme to finalize the process more quickly. Using the enzyme of microbial origin, the values derived for the strain of displacement are $\Theta = 3,4x10^{-6}$ after 30 min of the experiment and $\Theta = 1,20x10^{-5}$ at the end of the conducted experiment. The use of this milk coagulant indicates significant changes in the strain of displacement at the initial stage of formation of the gel. It was recorded that differences were reduced as the experiment progresses.
The data obtained from the penetrometric study were processed statistically and the results can be apply in laboratory and manufacturing practices using the chymosin of microbial origin and camel chymosin obtained by fermentation.

Keywords: *rennet coagulation, penetrometry experiment, milk-clotting enzymes*

INTRODUCTION

The first stage of cheese manufacture is the conversion of liquid milk to cheese curd. This process is achieved by the addition of rennet to coagulate the milk and by the subsequent expulsion of the whey by syneresis (Home, 2004; Banks, 2004).
Rennet is the generic name for an enzyme preparation used to coagulate milk in the production of rennet coagulated cheese and rennet casein. The principal role of the enzymes in rennet is to coagulate milk, but they also contribute to proteolysis during the ripening of most cheeses (Bansal et al., 2009; Valkova-Jorgova, 2005; Danov, 2009).
The increase in cheese production, coupled to a diminishing supply of natural animal rennet, is responsible for increases in the demand for alternative milk-coagulating sources. Due to this and a variety of outher factors attention is being turned to the use of microbial coagulants (Garcia et al., 2012).
With developments in recombinant DNA technology, the gene for calf chymosin has been cloned into microorganisms, permitting the production of chymosin by fermentation. Such ‘‘fermentation produced chymosins’’ are now widely used for cheese manufacture in

many countries and give excellent results (Ernstrom and Wong, 1974; Fox and McSweeney, 1997; Green, 1977; Nelson, 1975; Phelan, 1985; Sardinas, 1972; Sternberg, 1976). For milk-clotting enzymes other than calf rennet, curd firmness and syneresis ideally should be similar to those when calf rennet is used, and there should be no significant losses of fat or protein. Actual rate of curd firming may not be important for determining properties of the curd (Green, 1977) but it's control is important in cheese making (Yun, 1982). Variations of curd firmness at time of cutting may result in greater losses of milk components and reduced cheese yield (Olson, 1982). Monitoring curd firmness during cheese making offers the potential for reducing such losses by cutting at consistent curd firmness to optimize cheese manufacturing (Olson, 1982). Rate of increase of curd firmness is reduced as the extent of proteolysis of casein increases (Yun, 1981). Excess proteolysis apparently inhibits curd firming by degrading the protein molecules involved in curd formation (Yun, 1982). Some methods of monitoring curd firmess include: vibrating reed viscometer (Marshall, 1982), pressure transmission systems (PTS) (Garnot, 1982; Marshall, 1982; Vanderheiden, 1976), Formagraph (McMahon and Brown, 1982; McMahon and Brown, 1983; Reuter et al., 1981), and resistance to penetration or cutting of the curd (Ritchardson et al., 1971; Storry and Ford, 1982).

MATERIALS AND METHODS

In order to clarify the influence of the type of milk-clotting enzymes on the firmness of rennet-obtained gels, penetrometric study was conducted with the use of coagulated cow's milk.

The physicochemical parameters of the cow's milk were: Fat – 3,6 % (after degreasing – 2,76 %); Non-fat solids – 8,4 %; Density of milk – 1,027 kgxdm^{-3}; Protein – 3,11 %; Acidity – 16; Active acidity (pH) – 6,8.

Before processing, the milk was subjected to a partial degreasing to reduce the effect of milk fat on the obtained results, and was heated to temperatures of 65 ° C.

For a period of 90 min, measurements were made every 10 min, and the duration of the measurements was 60 s.

As coagulant was used camel chymosin of „Cr. Hansen", under the name "CHY – MAX" ® M, with milk-clotting activity 1000 IMCU / ml (or enzymatic activity 1:70000), stored refrigerated at 0 ÷ 8 °C.

The milk-clotting enzyme of microbial origin, produced by "Cr. Hansen", was described as activity (1:50000) and other indicators in established certificate. For the experiment, the enzymatic activity of the aforementioned coagulants was reduced to 1:50000.

For determination of the density of the rennet-obtained gel was used a type of penetrometer, appliance developed by Todor Todorov Lyubenov, UFT, Plovdiv (Lyubenov, 1975). The device has a working body with cylindrical shape and a flat work surface area with F = 2x10^{-4} m^2, 0,0139 kg mass and constant k = 0,5 Nxkg^{-1}. The strain of displacement Θ was calculated by moving the operating body (h).

$\Theta = k.m.h^{-2} = 0{,}00695xh^{-2}$, Nxm^{-2}

All results were statistically interpretated to a level of significance of $\alpha = 0{,}5$. Data were statistically processed using specialized mathematical software - OriginPro 6.1.

RESULTS AND DISCUSSIONS

The aim of the conducted experiment was the examination of the process of rennet gel formation using different types and amounts of milk-clotting enzymes, also called chymosin or rennin. The rate of gel formation for a set time, with three different genetic variants of enzymes was studied. The experiment was focused on the enzymes of camel and microbial origin, compared with calf chymosin. It was found that the strain of displacement of the rennet obtained gel was related with the gel formation time and the specific activity of the coagulants used in the experiment.

Penetrometric methods were used for the gel hardness and firmness determination. The results of the penetration determination were obtained in mm. The data obtained from the analysis for the three different types of milk-clotting enzymes are presented in Table 1. The results demonstrate variations of the values for

a period of 10 to 90 min with the amounts of rennet enzyme from 10 to 60 cm^3x10^{-2} x dm^{-3}. The amount of rennet for the specific experiment was taken as a constant value (30 $cm^3x10^{-2}xdm^{-3}$) for the three milk-clotting enzymes used for the preparation of the rennet gel.

Table 1.Penetration of coagulum obtained using milk-clotting enzymes with microbial, camel and calf origin

Penetration time, min	Penetration (mm) in the rennet- gel, obtained using milk-clotting enzymes, (30 $cm^3x10^{-2}x$ dm^{-3}) for 60 s.		
	Microbial chymosin	Calf chymosin	Camel chymosin
10	57,2 ± 5,1	32,0 ± 2,8	38,0 ± 3,1
20	47,1 ± 3,8	29,2 ± 2,3	31,6 ± 2,5
30	42,3 ± 3,4	29,0 ± 1,9	29,2 ± 1,8
40	35,5 ± 2,2	28,2 ± 2,1	28,6 ± 2,4
50	31,3 ± 2,6	28,1 ± 2,2	26,1 ± 1,8
60	29,0 ± 2,8	27,6 ± 1,7	25,6 ± 2,2
70	27,6 ± 1,9	27,2 ± 2,3	25,2 ± 2,1
80	26,0 ± 2,6	26,4 ± 1,9	24,0 ± 2,0
90	24,1 ± 1,4	25,2 ± 2,1	23,0 ± 1,8

Table 2. Strain of displacement of coagulum, obtained using milk-clotting enzymes with microbial, camel and calf origin

Time for penetration, min	Strain of displacement ($Nx.m^{-x2}.10^{-6}$) in rennet-gel, produced using milk-clotting enzymes, in an amount of 30 $cm^3.10^{-2}.dm^{-3}$ for 60 s.		
	Microbial chymosin	Calf chymosin	Camel chymosin
10	2,12 ± 0,14	6,79 ± 0,11	4,81 ± 0,16
20	3,13 ± 0,11	8,15 ± 0,14	6,96 ± 0,16
30	3,88 ± 0,12	8,26 ± 0,18	8,15 ± 0,17
40	5,51 ± 0,18	8,74 ± 0,23	8,50 ± 0,24
50	7,09 ± 0,16	8,80 ± 0,41	10,20 ± 0,34
60	8,26 ± 0,24	9,12 ± 0,34	10,60 ± 0,43
70	9,12 ± 0,24	9,39 ± 0,42	10,94 ± 0,58
80	10,28 ± 0,66	9,97 ± 0,74	12,07 ± 0,62
90	12,87 ± 0,84	10,94 ± 0,64	13,13 ± 0,81

Using the data from Table 1 and after mathematical processing of the results presented, the average values of the strain of displacement are defined. The results for the strain of displacement for the tested enzymes are presented in Table 2.

The results demonstrate great variations between the values obtained for the enzyme with microbial origin compared with those obtained from the calf chymosin. The differences were explained through the mechanism of the gel forming: in the first 30 to 40 min the formation and the firming process of the gel produced with the microbial chymosin were very slow and the coagulum was too fragile and easy to be destroyed. The milk gel was enough tick and had the density needed for cutting at the end of the conducted experiment. The phases of coagulation process using this type of enzyme were delayed in time. The final values of the strain of displacement after 90 min of the experiment demonstrate the similarity in the results for these three different coagulants.

Typical for the milk-clotting enzyme with calf origin the strain of displacement had values almost similar during all the experiment, and the variations between the different periods of measurements were not significant.

The data between 30 and 60 min showed almost the same values for the strain of displacement i.e. using this type of milk-clotting enzyme the gel density reaches certain state and after that the hardness of the coagulum rest unchanged or varies slightly until the final phase of the rennet coagulation.

The results of the experiment using camel chymosin produced by fermentation demonstrate a faster rate of coagulation during the first two phases of the process and a relatively slower rate in the next phases of the process. The use of minimal amounts of the aforementioned coagulant delays the compaction of the rennet gel in comparison of the control chymosin. Using quantities of 30 cm^3 $x10^{-2}$ xdm^3, the strength of the rennet gel after 20 min was similar to that of the other two milk-clotting enzymes.

The values of the strain of displacement with the use of the coagulant of camel origin at the 90 min of the experiment are relatively equal and have no significant differences in comparison with the other two milk coagulants used to conduct the penetrometric study.

The mathematical and statistical processing leads to a nonlinear regression model, and the polynomial liner equation having the following form:

$$\mathbf{f = y_0 + a*x} \quad (1)$$

The mathematical models derived can be used in laboratory and manufacturing practice directly in the coagulation process consistent with the specific factors and the particular conditions.

Figures 1, 2 and 3 represent the mathematical modeling for the three types of the coagulants studied in the renneting process and the formation of the gels.

For the milk-clotting enzyme of camel origin the correlation coefficient R = 0,9930, the quadratic coefficient R_{sqr} = 0,9861 obtained, and coefficients of significance of the aforementioned value shows that the mathematical model describes correctly the relation studied and the research process of milk coagulation. The mathematical model that characterizes the formation of the gel using chymosin of camel origin has the following form (Figure 1):

$$f = 0,4444 + 0,1295*x \quad (2)$$

Verification of the adequacy of the obtained mathematical model was made according to the tables in the confidence level α (0,5).

It was determined that under similar conditions after the 30 min there were no significant changes in the values obtained for the strain of displacement, which shows the ability of this enzyme to finalize the process more quickly in comparison with the other two enzymes that were used.

The experiment with the use of calf chymosin as a control was also conducted. The statistical processing was made analogous to the previous experiment. For the bovine chymosin the results shows variations of the strain of displacement in the confidence and prediction bands (intervals). Verification of the adequacy of the obtained mathematical model was made according to the tables in the confidence level α (0,5), and the correlation coefficient R = 0,9815, the quadratic coefficient R_{sqr} = 0,9633 obtained, and coefficients of significance of the aforementioned values shows that the mathematical model describes correctly the examined process. The mathematical model that describes the investigated dependence with the use of bovine chymosin is in the form (Figure 2):

$$f = 4,7936 + 0,0938*x \quad (3)$$

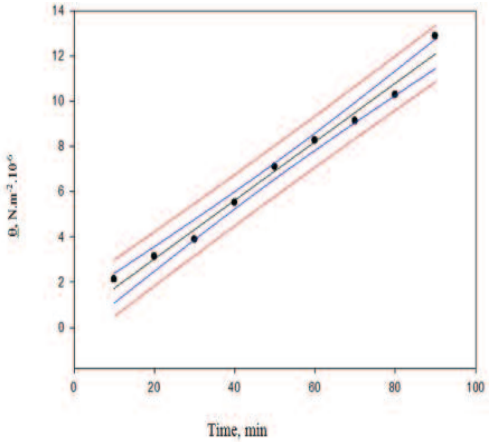

Figure 1. Variation of the strain of displacement in time using camel chymosin

Coefficient		Standard Error	t	P
y_0	**0,4444**	**0,3273**	**1,3580**	**0,2166**
a	**0,1295**	**0,0058**	**22,2607**	**<0,0001**

The mathematical model with the use of microbial chymosin was obtained following the same mathematical and statistical procedure as in the previous experiments and has the following form (Figure 3):

$$f = 6,8483 + 0,0412*x \quad (4)$$

According to the correlation coefficient R = 0,9591, R_{sqr} = 0,9198 obtained and coefficients of significance of the aforementioned values, the mathematical model describes properly the process of milk coagulation with the use of microbial chymosin.

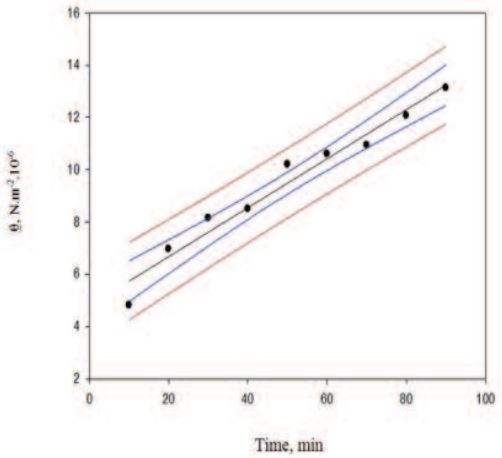

Figure 2. Variation of the strain of displacement in time using calf chymosin

Coefficient		Standard Error	t	P
y_0	**4,7936**	**0,3897**	**12,3014**	**<0,0001**
a	**0,0938**	**0,0069**	**13,5479**	**<0,0001**

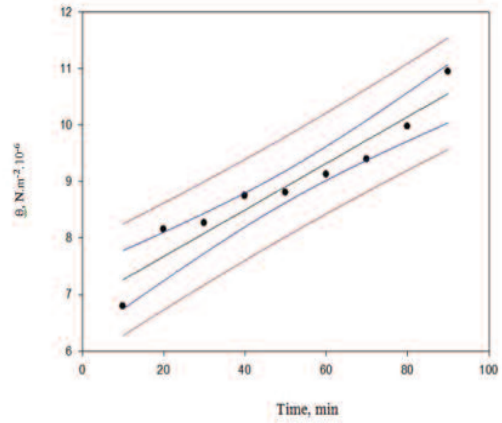

Figure 3. Variation of the strain of displacement in time using microbial chymosin

Coefficient		Standard Error	t	P
y_0	**6,8483**	**0,2585**	**26,4932**	**<0,0001**
a	**0,0412**	**0,0046**	**8,9618**	**<0,0001**

CONCLUSIONS

The results of the conducted penetrometric studies indicate that the modifications of the structure and the formation of the rennet gel obtained by using milk-clotting enzyme with camel origin for a period of 90 min were with uniform compaction unlike the fast rate of gel compaction with the use of bovine chymosin and the slow one when microbial chymosin was used.

Based on the statistical analysis, in case of coagulation induced by calf chymosin, it can be concluded that there is no significant differences between the values of the strain of displacement in the period 30 - 50 min of the gel formation. The coagulation process can be divided in three phases: the first one is the period up to 20 min, second one is the stage between 20 and 50 min, and the final phase of the formation of coagulum is between 60 and 90 min.

The gel formation with the use of microbial coagulant presents statistically significant differences between the values of the strain of displacement during the entire coagulation process, excluding the data from 80 to 90 min. These results suggest smooth and uniform changes during the formation of the gel.

Compared with the rate of coagulation of the before mentioned coagulants, the course of gelation with the use of chymosin with camel origin is intermediate.

The three types of gels are formed for a period of 90 min. Between 80 and 90 min there are no significant variations in the values of the strain of displacement, thus it can be concluded that the three different types of gels have similar structural and mechanical properties.

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PRELIMINARY STUDIES ON THE CAPACITY OF SOME MICROORGANISMS FOR THE SYNTHESIS OF PHYTASES

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Abstract

Phosphorus is one of the major constituents of which are involved in metabolic processes, nucleic acid and cell membranes biosynthesis as well as in the regulation of a large number of enzymes. Phytate (myo-inositol hexakisphosphate 3-phosphorilase) is the main storage form of phosphorus in various crops (cereals, legumes, and oilseed crops) and its accumulation in natural ecosystems could reduce the availability of various metal ions such as Fe, Zn, Mg or Ca, and could cause environmental pollution effects. Phytases are enzymes that catalyze the hydrolytic phosphate cleavage of phytic acid to lour inositol phosphate esters and inorganic phosphate. In the case of monogastric animals which do not have microbial phytases in their digestive system, the formation of insoluble metal cation-phytate complexes at physiological pH values is regarded as the major reason for poor mineral availability, because these complexes are essentially nonabsorbable from the gastrointestinal tract. However, phosphorus from soil is largely unavailable due to its rapid immobilisation of the organic and inorganic soil constituents. Several studies are focused on the exogenous and endogenous microbial phytases (produced by fungi - Aspergillus niger, Aspergillus ficuum, yeasts - Saccharomyces cerevisiae, or bacteria - Leuconostoc mesenteroides, Bacillus amyloliquefaciens) and their influence in the phytic acid dephosphorylation. The identification and the characterization of new microbial strains able to produce phytase and possible other important compound continue to be of large interest both for fundamental studies and for practical applications. For this reason, the aim of the present work was the identification of new phytase producing microbial strains from soil samples of different origins and by using collection microbial strains. The phytase activity was detected by cultivation on phytase specific medium (PSM) [1.5% glucose, 0.5% $(NH_4)_2SO_4$, 0.05% KCl, 0.01% $MgSO_4.7H_2O$, 0.01% NaCl, 0.01%, $CaCl_2.2H_2O$, 0.001% $FeSO_4$, 0.001% $MnSO_4$, pH 6.5 with 0.5% sodium phytate). Six bacteria strains (BPA, OS15, OS17, B4, B5 and B6) and one fungal strains (A.niger An) capable of hydrolyzing sodium phytate were recognized by their surrounding clear halo on PSM containing plates. Preliminary experiments on the characterization of the new isolates were also realized.

Keywords*: phytase, phytate*

INTRODUCTION

One of the macroelements existing in the living cells is phosphorus in the form of ortho or pyrophosphoric acids. Phosphorus is a part of the structure of nucleic acids, phospholipids, enzymes, hormones, etc. In the agricultural sector, the biggest part of the phosphorus-containing fertilizers (>90%) becomes unavailable to plants. This is due the interaction with the soil structure, adsorption and precipitation, which includes phosphorus into organic conglomerates or insoluble inorganic minerals (Mukhametzyanova et al., 2012). Important proportion of the supplied phosphorus is immobilized in the soil as phosphates of several divalent and trivalent metals like iron, aluminium or calcium (Kaur and Reddy, 2013). Though, the lack of phosphorus bioavailability leads to the need of frequent application of phosphate fertilizers, its presence causing environmental pollution, specifically eutrophication of rivers, lakes and water basins (Haefner et al., 2005; Naves et al, 2012; Szara and Sosulski, 2012).

Phytic acid is a chemical derivate of a six fold alcohol, inositol, with six molecules of phosphoric acid residues bound to its six

hydroxyl groups. Phytic acid is a source of phosphorus which can be used by human, animals, plants and microorganisms. Phytic acid or its salt form, phytate, [myo-inositol(1,2,3,4,5,6) hexakisphosphate], is able to bind the ions of divalent or trivalent metals like sodium, calcium, copper, with aminoacid residues, proteins or carbohydrates.

Phytate is the major storage form of phosphorus in plants. During plants maturation, phytic acid is accumulated in plant seeds, mostly as salts of mono and bivalent cations. It is known that phytic acid acts as phosphorus, cations and myoinositol source. Phytic acid also acts as a natural antioxidant in seeds during dormancy (Graf et al 1987). Phytic acid was also found in roots and tubers, vegetables, fruits, nuts, and pollen of various plant species. In feeds, its concentration varies considerably. In most grains and oilseeds the concentration of pytate is around 0.7-2%. It was stated that about 14.4 mil of tones is annually produced by seeds and fruits production at global level (Kumar et al., 2011).

Its presence in the monogastric intestinal tract is being the primary reason for poor mineral availability because of its ability to form insoluble metal cation-phytate complexes at physiological pH values, causing the lack of nutrients absorbance by the organism (Lopez et al, 2002).

The interest in phytate-degrading enzymes and their application in nutrition, agriculture and environmental protection have recently advanced significantly (Hussin et al., 2010). Phytase (myo-inositol hexakisphosphate 3-phosphorilase) catalyses the sequential release of phosphate by the hydrolysis of phytic acid in a stepwise manner, releasing lour inositol phosphates and inorganic phosphate (Naves et al., 2012). Phytases are divided in to categories, 3-phytase (myo-inositol hexaphosphate 3-phosphohydrolase) and 6-phytase (myo-inositol hexaphosphate 6-phosphohydrolase). Based on their catalytic characteristic, phytases are classified as HAP (histidine acid phosphatase), BPP (ß-propeller phytase), CP (cysteine phosphatase) and PAP (purple acid phosphatase (Mullaney and Ullah, 2003).

Phytase reduces the antinutritional properties of phytic acid and eutrophication (Khumar and Batt, 2011). Also, lately, phytase has been used in aqua feed industries in order to enhance the growth performance, nutrient utilization and bioavailability of macro and micro minerals in fish and also to reduce the P pollution into the aquatic environment (Kumar et al., 2011).

They can be synthesized both by micro organisms (bacteria, fungi and yeast) including the rumen microbes in ruminants, and soil microbes, and plants like wheat, barley, rice, corn or rye (Paik, 2003). According to Zhang et al. (2013), phytases can be isolated even from mushrooms. They isolated a phytate-degrading enzyme from *Lentinus edode*, a shitake mushroom, which has been shown to be relatively tolerant to high pH and is thermostable. The activity of endogenous phytase is comparatively higher in cereals and cereal by-products than in legume seeds (Kumar et al., 2011). The microorganisms able to produce phytases can also be isolated from various plants. Marlida et al. (2010) isolated species of *Rhizoctonia* and *Fusarium* as phytases producers from stem and root fragments from soybean and (Dave and Modi, 2013) from mangroves rhyzosphere.

The most phytases which are currently available for application as feed enzymes have shortcoming such as a narrow pH optimum and lack of resistance in high temperatures. Because of the fact that most feed manufactured for non-ruminants are pelleted at temperatures between 65 and 80^{o}C, most of the phytase activity will be lost under the processing conditions. Moreover, the phytases which are commonly used are rather susceptible to proteolysis (Elkhalily et al., 2007). The plant phytase activity in mature seeds is very low, contrary to its activity at the level of germination stage. The second reason for which the phosphorus from seeds used as feeds doesn't reach to animals is the fact that the phytases are highly thermolabile. Moreover, it appears that in feedstuffs, the phytases activity present great variations due its dependency on the genetic and environmental factors (Steiner et al., 2007).

The phytases which are secreted into soil by the microorganisms are participating to decomposition of plant debris and also to releasing of phosphorus from organic compounds.

Among the fungi which are able to produce phytases, we remind the *Aspergillus spp.*, *Fusarium spp.*, *Mucor spp.*, *Penicillium spp.* and *Rhizoctonia spp.* Several bacteria genus are synthesizing as well phytases, like *Bacillus*, *Enterobacter*, *Klebsiella*, *Escherichia*, *Leuconostoc*, etc. Also yeasts which are capable to produce these enzymes are *Saccharomyces* spp. and *Candida* spp. Also, during the fermentation process of bread dough, according to Nuobariene et al (2012) study, all yeasts involved are synthesizing pytases.

It is still a controverse in the scientific community whether the lactic acid bacteria are synthesizing or not phytases. During the sourdough fermentation, the lactic acid bacteria are either involved in phytate degradation or they decrease the pH, caused by lactic acid produced, and the plant's intracellular phytases are activated due this pH level. Although it was demonstrated that some strains of *Lactobacillus san franciscensis* was one of the best lactic acid bacteria which are able to produce phytases.

The optimum pH levels of phytases vary from 2.2 to 8. Generally, fungal phytases are activated in the pH range between 4.5 and 5.6. The optimal pH levels for those synthesized by bacteria and especially by *Bacillus spp*., are 6.5 – 7.5. Elhadi et al. (2011) observed that some strains of *E. coli* and *Klebsiella* spp. are able to produce phytases which have optimal enyzimatic activity to neutral, respectively acid pH.

The optimum temperature of phytases varies from 45 to 77°C (Caipang et al., 2011). Dechavez et al. (2011) stated that most phytases from *Bacillus* strains shows maximum activity to 37^{o}C, but are maintaining its activity even until 80^{o}C. However, Yu and Chan (2013) were able to isolate a strain of *Bacillus nealsonii* which shows a strong thermal stability, reaching the highest enzymatic activity at 55^{o}C and neutral pH.

The main objective of the present study was to detect the ability of some microbial strains from different species of *Bacillus sp*. (*Bacillus subtilis, B.amyloliquefaciens, B.pumilus*) to synthesize phytases in *in vitro* conditions. *Bacillus sp.* in one of the most studied and used bacteria genus for phytate-degrading enzyme synthesizing (Fu et al., 2008). Currently, about 60 % of the worldwide industrially produced enzymes are due to *Bacillus sp* (Fu et al. 2007).

According to Maksimov et al. (2011), *Bacillus subtilis* is able to produce extracellular phytases. In 1992 Shimizu was able to obtain a 36 kDa phytase from a strain of *B.subtilis* with the activation energy of 9.87 kcal/mol for sodium phytate, at 60^{o}C and pH 6,0 – 6,5. Kim et al. (1998) has isolated some *Bacillus* species producing a thermostable phytase from the soil of Korean cattle shed. They were also able to isolate a phytase which demonstrated that it has optimal activity at 70^{o}C and pH 7. Hong et al. (2010) has succeeded to isolate a strain from cattle feces which produced a phytase of 46 kDa molecular weight and having an optimal activity at 60^{o}C, pH 7.

Bacillus amyloliquefaciens is another species intensively used as phytase producer (Mukhametzyanova et al., 2011). Oh et al. (2011) observed that a thermostable phytase from *B.amyloliquefaciens DS11* is Ca^{2+}-dependent in order to catalyse the sequential release of phosphate from phytic acid. Also it was observed that the thermal stability of *B.amyloliquefaciens* phytases is strongly dependent on calcium ions (Ha et al. 1998). Although *Bacillus pumilus* did not receive the same consideration as other *Bacillus* species as phytase synthesizing bacteria, it was brought into attention its ability to be efficiently genetically manageable in order to produce qualitative phytases because its high capacity to secrete extracellular enzymes and to exhibit a pronounced resistance against oxidative stress (Wemhoff and Meinhardt, 2013). One of the currently uses is the control of several soil fungal pathogens (Maksimov et al., 2011). According to Vassilev et al. (2012), there were discovered phytase-producing strains of *Bacillus* sp. which are resistant to salinity, high pH and high temperature, thus helping mitigate the problems caused by the stress factors in agriculture.

The aim of our work was the screening of some phytate degrading microorganisms, isolated from natural sources.

MATERIALS AND METHODS

Microorganisms and culture preservation

The microorganisms used in the present study were presented in table 1.#

Table 1. Microbial strains used in experiments

Strain designation	Species	Source
Bl	*Bacillus licheniformis ATCC 14580*	Fac. Biotechnology collection
7.1.	*B.amyloliquefaciens*	Soil (Israel et al., 2012)
BN7	*B.amyloliquefaciens*	
Omf	*B.amyloliquefaciens*	
BW	*B.amyloliquefaciens*	Soil (Sicuia et al, 2012)
OS17	*B.amyloliquefaciens*	Onion rhizosphere (Sicuia et al, 2012)
OS15	*B.amyloliquefaciens*	
B2Vio	*B.subtilis*	Oil contaminated soil (Olteanu et al., 2011)
BPA	*Bacillus subtilis*	Soil
BIR	*B.pumilus*	Soil
B1	Unindentified	Compost
B2	Unindentified	
B3	Unindentified	
B4	Unindentified	
B5	Unindentified	
B6	Unindentified	
An	*Apergillus niger*	Airborne sprores

The bacteria were routinely maintained on nutrient agar and the filamentous fungi were cultivated on Potato Dextrose Agar (PDA).

The screening method

In order to select the phosphate solubilising microorganisms, three type of culture media were used: phytase specific medium (PSM): 1,5% glucose, 0,5% $(NH_4)_2SO_4$, 0,05% KCl, 0,01% $MgSO_4.7H_2O$, 0,01% NaCl, 0,01% $CaCl_2.2H_2O$, 0,001% $FeSO_4$, 0,001% $MnSO_4$, pH 6,5 and 0,5% sodium phytate (Bae et al., 1999); Pikovskaya's agar plates (PKA) (g L-1): glucose: 10.0; $(NH_4)_2SO_4$: 0.50; KCl: 0.20; $MgSO_4.7H_2O$,: 0.010; MnSO4·H_2O: 0.0001; FeSO4·7H2O: 0.0001, yeast extract: 0.50; 0.5% tri-calcium phosphate as the sole P source, pH 7.0 (Kaur and Reddy, 2013); wheat bran extract agar containing $(NH_4)_2SO_4$-0.04%, $MgSO_4.7H_2O$-0.02%, casein-0.1%, KH_2PO_4-0.05%, K_2HPO_4-0.04% and agar-2% (Sreedevi and Reddy, 2012). The inoculated plates were incubated for 3-7 days at 30°C. The experiment was realized with three repetitions in order to obtain accurate results. The detection of clear zone surrounding the bacterial colonies is the sign of phosphate solubilising activity or phytase biosynthesis. The efficiency of hydrolysis was determined by the formula Z-C/C, were Z = halo diameter and C = colony diameter (Joseph and Jisha, 2008).

RESULTS AND DISCUSSIONS

The great industrial significance of phytases explains the interest in isolation of new microbial strains producing such enzymes and in optimization their biosynthesis. Moreover, the ability of phytase producing bacteria for phosphate solubilization could be of interest for agronomic applications (increase soil fertility in an organic fields) (Kaur and Reddy, 2013).

Among the 16 bacterial strains used in experiments differences were observed on the three culture media tested. When wheat bran extract agar was used, the majority of the bacterial strains tested presented clear halo around their colonies (fig.1)

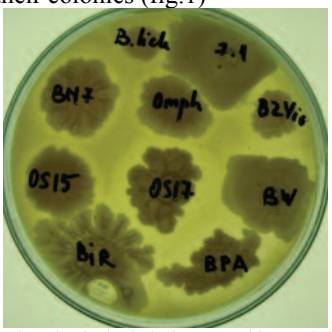

Figure 1. Clear hydrolysis halo around bacterial colonies on wheat bran agar

On PKA plates, small halo were detected only for B4, B6, BPA and OS15 bacterial strains.

Positive reaction on PSM medium was obtained for the strains 7.1, OS15, OS17, B4, B5, B6 BPA (fig.2) as well as for *A.niger* strain An.

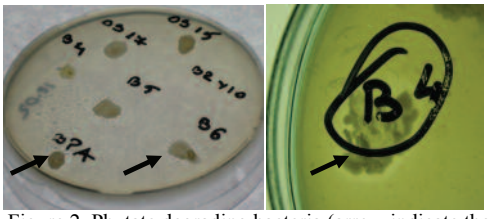

Figure 2. Phytate degrading bacteria (arrow indicate the hydrolysis area) on PSM medium

Best results, as hydrolysis efficiency were obtained with the strains BPA (over 81%), B6 (66%) and B5 (50%). Interesting aspect was observed for the *A.niger* strain: the hydrolysis area started after 48 h of incubation and its diameter continued to increase for the next 5 days (Fig. 4).

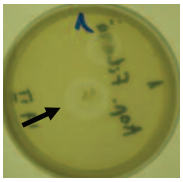

Figure 3.Hydrolysis area for *A.niger* strain on PSM mediu.

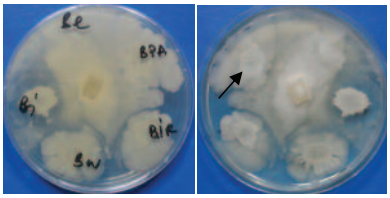

Figure 4. Growth inhibition of *Pythium debarianum* by bacteria. The arrow indicate the strain BPA

The results suggest that despite the positive reaction of bacteria on wheat bran agar, not all the strains are able to produce phytate. In this order, this medium could be used for a preliminary screening of possible hydrolysis of phytate from wheat bran but the confirmation of phytase biosynthesis need to be performed on PSM medium.

The results obtained in these experiments with soil derived bacteria are in accordance with the data of Gulati et al. (2007) and Singh et al. (2013) that isolated several strains of *B.subtilis* from rhizosphere, which were able to produce phytases.

Moreover, the strains designated B5 and B6 that presented large hydrolysis halo on PSM medium (fig.3) were isolated from compost, natural medium with increased phosphorus content, and they could be involved in the reduction of this compound from the environment (the presence of microbial phytases can reduce the environmental pollution of phosphorus in areas of intensive animal production)(Lei and Porres, 2003).

Regarding the strain designated as OS15, it was identified as *B.pumilus* (using Biolog GEN III system, data not shown); its ability for phytate hydrolysis is in accordance with the results of Watharkar et al. (2013).

Interesting results were obtained with *B.amyloliquefaciens* strains (described in literature for the thermostablitity of the phytases)(Haefner et al., 2005): clear phytate degrading area presented the strains OS17. It has to be noticed that the strains B6, BPA and OS15 presented also clear halo on PKA medium, suggesting their ability to hydrolyse both inorganic phosphorus (insoluble tri-calcium phosphate) and phytate.

Moreover, the strains BPA, BW, B5 and B6 presented antagonistic activities against several plant pathogenic fungi (like *Pythium debarianum*) (Fig. 4).

These results allow the conclusion that the selected bacterial strains could be of great interest not only for the phytate degrading activity but also for their biocontrol properties.

CONCLUSIONS

Several microbial strains were selected for phytate degrading abilities. The highest hydrolysis areas were detected for the strains BPA, OS15, OS17, B4, B5, B6 and *A.niger* An. The strains B4, B6, BPA and OS15 have inorganic phosphate solubilising properties.

It is obvious that the source of isolation (i.e. compost) is very important in the detection of the bacteria able of phytate degradation, but also the quality of nutritive media and parameters like temperature or pH.

The results of the present study suggest that the use for inoculation of such bacteria (with phytate degrading and antifungal abilities) is a good tool to improve the soil's available P level without the use of chemical phosphorous fertilizers and also to provide plant protection.

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ACTIVE PRINCIPLES WITH POSITIVE EFFECTS ON LIPID METABOLISM

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Abstract

Today attention is given increasingly higher to plants, considering that they represent an inexhaustible source of raw materials that regenerates every year. Plants can be used as raw materials in the preparation of drugs or in industrial extraction of active principles. The advantage of phytotherapy is that plant based drugs have a positive impact not only on a single organ or a specific system, but on the whole body. Experimental research focused on the following plant materials: sea buckthorn (Hippophae rhamnoides), hawthorn leaves and flowers (Crataegus oxyacantha), garlic bulbs (Allium sativum) and maple buds (Acer campestre). They were tested in order to characterize, identify and determine the content of active compounds. The analysis of the extracts revealed a high content of lipase in sea buckthorn, 6,3 U/g, as well as in garlic, 4,9 U/g, while the buds maple had a very low lipolytic activity, only 0,4 U/g. The highest content of flavones was determined in leaves and flowers of hawthorn and sea buckthorn. Reducing compounds were determined in large quantities in sea buckthorn, while the other materials tested showed a 50% lower content. The highest content of polyhydroxyphenols was determined in hawthorn and sea buckthorn. Sea buckthorn was also noted by a very high content of vitamin C, 483 mg/100 g. The results obtained in these experiments allowed the selection of plant products rich in many active principles involved in the regulation of lipid metabolism.

Keywords: *phytotherapy, active principle, lipases, lipid metabolism.*

INTRODUCTION

Currently phytotherapy has become an important science, practiced by competent doctors. The effects of medicinal plants on the human body was demonstrated by numerous studies of organic chemistry, phytochemistry, pharmacology, pharmacognosy, biochemistry and microbiology (Grigore, 2005).

Though phytotherapy is based on the curative effects of certain biological compounds from plants or other plant part, this ancient science of healing follows the classical rules, exploiting the plant material without selectively extract certain substances or principles from plant tissues, as do other sciences as pharmaceutical or aromatherapy (Grigore and Grigore, 2008).

For this reason we can say that phytotherapy remains the cleanest and most natural of sciences. (Parvu, 2002)

The active principles from medicinal plants are synthesized in the plant cells. In order to properly exploit them, these natural substances must be found in various phytotherapeutic forms (Grigore, 2005).

The main purpose of our research was to obtain active principles with positive effect on lipid metabolism.

The main objectives were: a) selection of plant materials with high content of active principles involved in the regulation of lipid metabolism; b) pharmacognostical characterization of the selected plant products; c) qualitative and quantitative analysis of the active compounds found in the plant extracts.

MATERIALS AND METHODS

Experiments focused on the following plant materials: sea buckthorn (*Hippophae rhamnoides*), hawthorn leaves and flowers

(*Crataegus oxyacantha*), garlic bulbs (*Allium sativum*) and maple buds (*Acer campestre*).
The selected materials were tested in order to characterize, identify and determine the content of active compounds.
The pharmacognostical analysis consisted of a set of qualitative and quantitative methods carried out to determine the identity, purity and quality of the plant product. Thus, several determinations have been performed: macroscopic examination, determination of purity, determination of humidity and imbibition factor (Gird et al., 2008).
Extraction of the biologically active substances from plant materials was performed by maceration using alcoholic and hydroalcoholic solutions (Dragota, 2005; Ionescu and Savapol, 1997).
Qualitative determinations of the active principles in the plant products aimed mainly to identify flavones, phytosterols and reducing compounds.
Quantitative determinations aimed: lipolytic activity, flavones, phytosterols, reducing sugars and ascorbic acid.
Lipolytic activity was evaluated by a titrimetric method, based on the determination of fatty acids released into the reaction medium after the action of the enzyme on the substrate, olive oil. One unit of lipase activity is defined as the amount of enzyme that releases 1μmol of fatty acid (Gropoşilă-Constantinescu, 2010).
Determination of flavones was performed by a spectrophotometric method, based on the determination of absorbance of the chelate complexes resulted from the reaction of flavones with aluminum chloride (Hooper et al., 2008). The amount of reducing sugars was determined by a widely used titrimetric method, known as Schoorl method (Jurcoane et al., 2010). The amount of ascorbic acid was determined by a titrimetric method, based on the property of vitamin C to reduce 2-6-dichlorophenol-indophenol to a leucoderivate (Dumitru and Iordachescu, 1981).

RESULTS AND DISCUSSIONS

1. Pharmacognostical determinations
From the point of view of macroscopic characterization of plant materials, they have met all the quality requirements of their use for subsequent qualitative and quantitative analysis, as described in the Romanian Pharmacopoeia, Xth Edition (FR X, 1998).
Determination of humidity and imbibition factor led to the following results:

Table 1. Humidity and imbibition factor of the tested plant materials

Plant material	Humidity (%)	Imbibition factor (%)
Pulverized sea buckthorn	1	10,3-10,9
Pulverized hawthorn leaves and flowers	0,8	10,7-11,2
Garlic bulbs	93	2,8-2,9
Maple buds	82	2,1-2,3

Relatively high values obtained in case of pulverized products can be correlated with the presence of relatively large amounts of various mucilaginous substances.

2. Identification of active principles
Identification reactions of active principles revealed the targeted compounds in most of the materials tested.
Evaluation of the results obtained in this analysis is presented in the following tables:

Table 2. Identification of flavones

Plant material	Type of reaction: Shibata reaction/ NaOH reaction	Intensity of the reaction
Pulverized sea buckthorn	orange/yellow	++ / +++
Pulverized hawthorn leaves and flowers	orange/yellow	+++ / +++
Garlic bulbs	- / -	- / -
Maple buds	orange/yellow	+ / +

Table 3. Identification of reducing compounds

Plant material	Intensity of the reaction
Pulverized sea buckthorn	+++
Pulverized hawthorn leaves and flowers	++
Garlic bulbs	++
Maple buds	-

Table 4. Identification of phytosterols

Plant material	Intensity of the reaction
Pulverized sea buckthorn	+++
Pulverized hawthorn leaves and flowers	+++
Garlic bulbs	++
Maple buds	+

3. Quantitative determination of the active principles

Analyses conducted to determine the lipolytic activity of plant extracts showed a high lipolytic activity in sea buckthorn fruits, 6.3 U/g and garlic bulb extract, 4.9 U/g. Also, a high lipolytic activity was found in leaves and flowers of hawthorn, 3.2 U/g, while the maple buds extract was very low, only 0.4 U/g.

The results obtained for the determination of lipolytic activity in plant extracts are shown in the following figure:

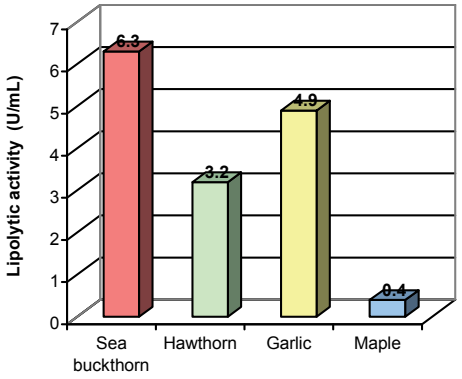

Figure 1.Lipolytic activity of plant materials

Thus, the results obtained for flavones content are shown in the figure below.

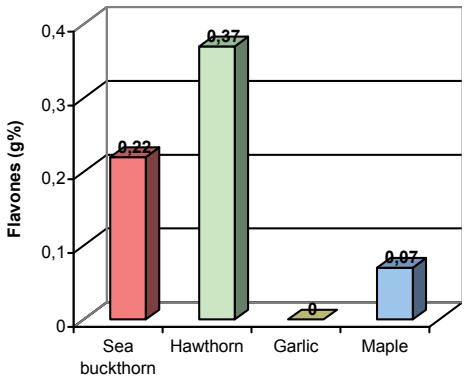

Figure 2. Flavones content of plant materials

The highest values of flavones content was obtained in the leaves and flowers of hawthorn. A fairly large content was also obtained in case of sea buckthorn fruit. A lower content was observed in maple buds. In case of garlic bulbs analysis confirmed the preliminary identification of these compounds in which the results of flavones content was zero.

For the quantitative determination of polyhydroxyphenols, a spectrophotometric method was used, in which was determined the izonitrozoderivates formed from the phenols in the alkaline media, in the presence of nitrous acid.

The content of polyhydroxyphenols can be seen in the following figure.

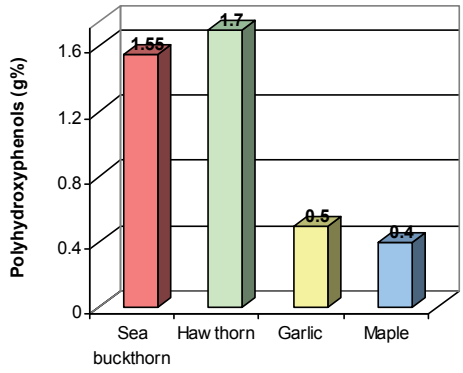

Figure 3. Polyhydroxyphenols content of plant materials

From the results shown in Figure 4, it can be observed that the highest values of reducing sugar content was obtained in case of sea buckthorn fruit. Also, a relatively high content of reducing sugar was obtained in garlic and hawthorn. A very low content was observed in maple buds.

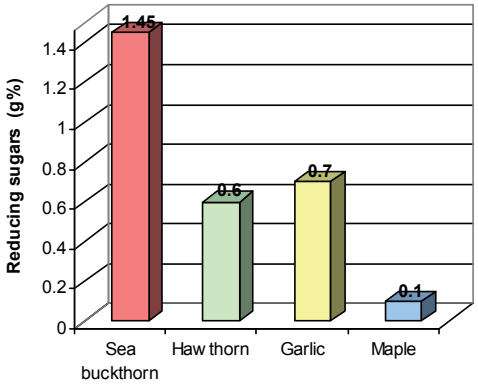

Figure 4. Reducing sugars content of plant materials

The results showed that, as mentioned in the literature, the highest content of vitamin C was

obtained for sea buckthorn fruit, reaching a maximum of 483 mg/100 g dry product.
In case of the leaves and flowers of hawthorn, the amount of vitamin C was 23.4 mg/100 g dry product and for garlic bulbs, 17.3 mg/100 g fresh product.
Maple buds did not presented in their composition viatmina C.

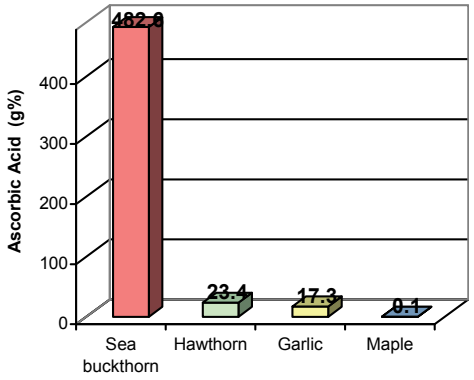

Figure 5. Ascorbic acid content of plant materials

CONCLUSIONS

Of all the plant materials tested, sea buckthorn fruit have the highest content of flavones, lipases, ascorbic acid and reducing sugars.
Corroborating the results obtained in the qualitative and quantitative analyzes of the plant materials tested, we selected, in order to obtain a complex bioproduct, the sea buckthorn fruit powder, the powder of hawthorn leaves and flowers and the extract of garlic bulbs, rich in many active principles involved in regulation of lipid metabolism.

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BACTERIAL PROTEOLYTIC ENZYMES TESTED ON KERATIN AND COLLAGEN BASED MATERIAL

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Abstract

Biodegradation of fibrous protein is a challenge due to the resistance of the raw material. Enzymes based processes are an alternative to conventional chemical ones because they are environmental friendly, but their efficiency is still limited. They are used mainly in the process of leather obtaining. In the last decade they gain a lot of credit for waste degradation purposes. The main enzymes involved in leather and fur degradation are collagenases and keratinases. The aim of the reported researches is to test a proteolytic complex for its capacity of destroying leather and fur wastes. Four bacterial strains were tested regarding their capacity of hydrolyzing collagen and keratin from bovine leather and sheep fur. For comparison synthetic fur was considered too. Best results were obtained when using sheep leather. The influence of leather dyes was also investigated. Three types of sheep fur were tested: no painted, white painted and black painted. For the painted materials the degradation was less than half, especially when assessing collagenases activity.

Ke words*: collagenase, fur, keratinase, leather, wastes.*

INTRODUCTION

Leather and leather-based industries represent one of the most important economic sectors, but each year large amounts of solid wastes is produced, mainly collagen and keratin proteins. These wastes may be a source of valuable products, with many applications, so a degradation of the rigid structure of collagen and keratin is necessary.

Destroying of these proteins is usually made by thermal hydrolysis with acid or alkaline solutions, or by enzymatic digestion with specific proteases (collagenases and keratinases). Enzyme based processes are an alternative to conventional chemical ones because they are environmental friendly.

The protein products that result from these treatments have potential as a fertilizer and as an animal feed additive (Aftab et al., 2006). Also, the collagen degradation produced peptides, which were shown to have some biological activities with industrial and medical uses (Braikova et al., 2007), such as immunotherapeutic agent, moisturizer in cosmetic or food additives with preservative and seasoning role (Ku, 1993; Honda, 1998; Ravanti, 2000).

Microorganisms are an attractive source of proteases owing to the limited space required for their cultivation and their ready susceptibility to genetic manipulation (Rao et al., 1998). Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Godfrey, 1996). Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications.

Collagenase was first discovered in the broth of the anaerobic bacterium *Clostridium hystolyticum* as a component of toxic products. Later, it was found to be produced by the aerobic bacterium *Achromobacter iophagus* and other microorganisms including fungi (Godfrey, 1996).

Different authors reported as collagenase-producing strains *Bacillus* and *Aspergillus*: *B. subtilis* (Okamoto, 2001; Tran and Nagano, 2002; Rui et al., 2009), *B. licheniformis* (Baehaki et al., 2012), *A. fumigatus* (Reichard et al., 1990).
Also, keratinases have been isolated from various microorganisms and introduced into a wide range of biotechnological applications, including those in the feed, fertilizer, detergent, leather and pharmaceutical industries (Gupta et al., 2006). They have been purified from diverse microorganisms, including fungi, such as *Purpureocillium lilacinum* (Cavello et al., 2013) and *Chryseobacterium gelum* (Chaudhari et al., 2013), and bacteria, such as *Streptomyces* (Jaouadi et al., 2010) and *Bacillus subtilis* (Pillai et al., 2008).
The aim of the reported researches was to test a proteolytic complex for its capacity of destroying leather and fur wastes. Four bacterial strains were tested regarding their capacity of hydrolyzing collagen and keratine from bovine leather and sheep fur. For comparison synthetic fur was considered too. The influence of leather dye was also investigated. Three types of sheep fur were tested: not dyed, white dyed and black dyed. Enzymatic activity and protein content were determined using spectrophotometrical methods.

MATERIALS AND METHODS

Microorganisms and growth conditions

Four bacterial strains were used: three *Bacillus amyloliquefaciens* strains (7.2, BN7 and OMF) were recently isolated and belong to the collection of Faculty of Biotechnology, University of Agronomical Sciences and Veterinary Medicine Bucharest, while Bl strain is a collection strain (*Bacillus licheniformis* ATCC 14580).
The inoculation was done in a basal salt medium supplemented with 0.3% sucrose and 0.6% bovine leather or sheep fur meal. Synthetic fur was also tested for comparison. Proteolytic enzymes were produced in 500 ml flasks, kept at 30 - 32^{o}C, agitated at 140 rpm, for 10 days. The culture was centrifuged at 5000 rpm, at 4^{o}C, for 30 minutes.
White sheep fur, black sheep fur and no dyed fur were used in order to study the effect of fur dyes on the enzymatic activities of proteolytic complex.

Enzymatic assay of collagenase, based on some classical refferences (Moore and Stein, 1948; Mandl et al., 1953), was made as following: after collagenase catalytically promote hydrolysis of collagen, the degree of proteolysis is measured by color development with ninhydrin (absorbance was measured at 570 nm).
The enzymatic activity of collagenase was measured in U/ml (*unit definition*: one unit liberates peptides from collagen equivalent in ninhydrin colour to 1 μmol of leucine in 2 hours at pH 7.4 and at 37 ^{O}C).

Enzymatic assay of keratinase was performed using 4 mg keratin azure as substrate. One unit of keratinase activity was defined as the amount that caused an increase in absorbance of 0.01 at 595 nm within 60 min reaction at 60°C.

RESULTS AND DISCUSSIONS

Enzymatic activity of proteolytic complex on synthetic and natural leather and fur

The four bacterial strains: 7.2, BN7, OMF, and Bl were tested regarding the collagenolytic and keratinolytic activity on different materials. Bl strain cultivated on medium containing sheep fur registered the highest collagenase activity (23.7 U/ml) (fig. 1). Also the BN7 strain manifested a remarkable collagenase activity both in the samples with bovine leather (18.2 U/ml) and in the ones with sheep fur (18.07 U/ml).
As we expected, the keratinolytic activity was higher at the strains cultivated in medium with sheep fur, which contained both leather and hair, last one being the specific substrate for keratinases. The best results were again obtained with *Bacillus licheniformis* strain Bl which showed the highest activity (4.44 U/ml) (fig. 2). When using the bovine leather the keratinases synthesized by 7.2 and BN7 strains registered a better activity (1.25 U/ml, respectively 1.15 U/ml).

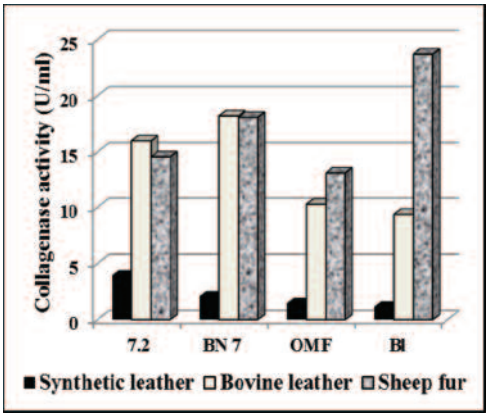

Figure 1. The collagenase activity of enzymatic complex on different materials

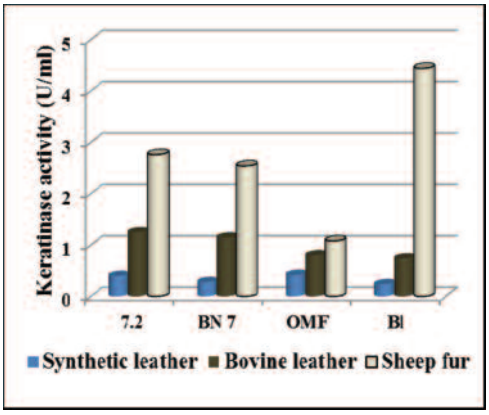

Figure 2. The keratinase activity (of enzymatic complex) on different materials

The obtained results indicated a slightly enzymatic activity also in the samples containing synthetic leather, which may be a residual activity derived from inoculum (bacterial suspension cultivated on natural fur).

Effect of fur dyes on the activity of proteolytic complex

In order to emphasize the effect of fur dyes on the enzymatic activity of the proteolytic complex, an experiment was performed: white and black painted samples of sheep fur were added in the cultivation medium used for the bacterial strains (Figure 3) and then the enzymatic activities were measured. Unpainted white fur was used in the same conditions and considered as control.

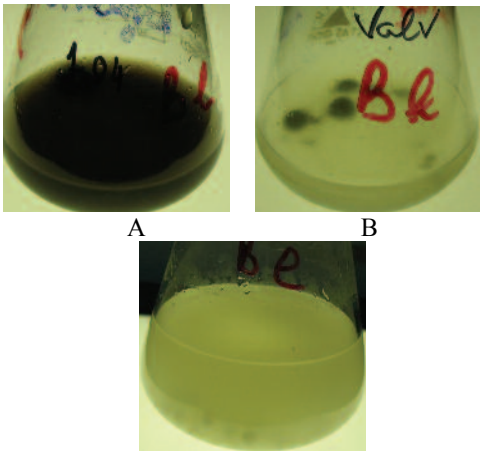

Figure 3. Degradation degree of some samples of natural sheep fur under action of *Bacillus licheniformis* ATCC 14580 strain (Bl). A = black painted fur; B = white painted fur; C= no painted fur

Table 1. Collagenase activity of enzymatic complex on different types of fur

Fur type	Bacterial strain	Collagenase activity (U/ml)
no painted fur	7.2	5.81
	BN7	18,55
	Bl	21.59
white painted fur	7.2	1,7
	BN7	6.13
	Bl	10.44
black painted fur	7.2	0
	BN7	5.81
	Bl	9.81

The obtained results indicated that the enzymatic activity of proteolytic complex decreased in the presence of specific dyes used for painting the natural furs. Bl strain manifested the highest collagenase activity (21.59 U/ml) followed by BN7 strain. During this test 7.2 strain showed very low collagenase activity (Table 1).

This trial showed lower results for keratinase, probably due to the fur biochemical compositon. Anyhow, the highest values were obtained for Bl strain (Table 2). Poor keratinase activity was registered for 7.2 bacterial strain when using undyed sample, while the enzyme activity was absent for dyed samples.

Table 2. Keratinase activity of enzymatic complex on different types of fur

Fur type	Bacterial strain	Keratinase activity (U/ml)
no painted fur	7.2	0.38
	BN7	1.33
	Bl	3.29
white painted fur	7.2	0
	BN7	1.12
	Bl	1.61
black painted fur	7.2	0
	BN7	1.05
	Bl	1.22

The activity of the proteolytic complex was highly affected by the dying process. Regarding collagenase activity, it is obviously that the values registered in the samples with painted fur are at least 50% lower than the ones measured in the control samples (with no painted fur) (Figure 3). When using black painted fur, 7.2 strain showed no collagenase activity.

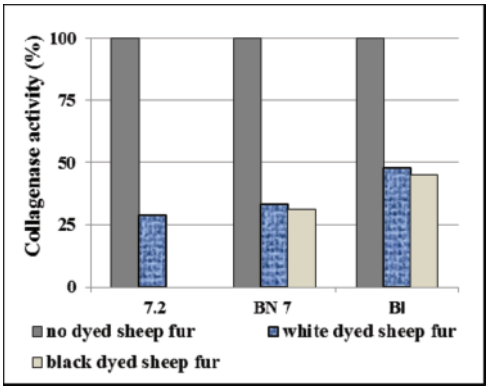

Figure 3. The percentage decreasing of collagenolytic activity under dyes effect

The keratinase activity of BN7 and Bl cultivated with dyed fur samples was not so deeply affected, especially for the first one. For these strains the enzymatic activity was 15-60% lower for the painted samples comparing with no painted fur, while for 7.2 bacterial strain the activity was absent for both dyed samples (Figure 4).

Considering collagenase activity, Bl strain was the most resistant to dyes effect, while BN7 strain showed higher resistance regarding keratinase activity. Unlike, the 7.2 strain was significant affected by the presence of dyes, considering that the collagenase activity was 75% lower only for white dyed fur and the keratinase activity was totally inhibited in the sample containing painted fur.

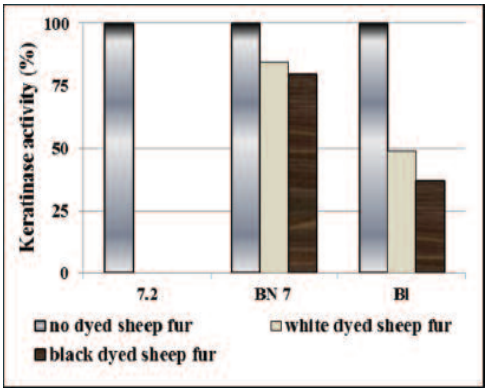

Figure 4. The percentage decreasing of keratinolytic activity under dyes effect

Moreover, the colour of the used dyes influenced differently the enzymatic activity of proteolytic complex: lower values were measured in the samples containing white painted fur than in the ones with black painted ones. It is not sure if the dye itself is the single cause of the enzyme inhibition or it can be also the result of the chromium oxide content in the tested samples. White painted fur contained 1.6 up to 2.06% chromium oxide, comparing with black painted fur with 2.32 up to 3.09%. This significant difference in chromium oxide concentration (used in the tanning process) may influence in a different measure the enzymatic activity of the proteolitic complex.

CONCLUSIONS

Enzymatic activity of proteolytic complex was higher in the samples containing sheep fur added in cultivation medium than in the ones with bovine leather, both for collagenase and for keratinase.

Bl and BN7 strains registered the best results regarding the enzymatic activity of the proteolytic complex.

The use of fur dyes determined a 50-70% decreasing of the collagenase activity and 15-60% decreasing of the keratinase activity. Both enzymatic activities were less affected when using white fur than black painted ones.

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CONCENTRATION AND IMMOBILIZATION OF A PROTEOLYTIC COMPLEX

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Abstract

Proteases are very important industrial enzymes, accounting for more than a half of the total enzymes used in the world. For leather industry they have an important technological role as well as for waste degradation.This paper reports the researches done for obtaining immobilized proteolytic complexes for using in biodegradation purposes. Three bacterial strains were cultivated in minimal medium with 0.6% sheep fur as carbon source. The concentration of the culture medium was performed by rotaevaporator, at 35 C, followed by lyophilisation at 0.04 mbar, for 18 hours. The concentrated complex was immobilized on clay and adabline, which are inert and cheap supports that are technically convenient because we used the adsorption process for immobilization. Collagenase and keratinase activity was assayed for estimation of the immobilization efficiency. The best results were obtained when the immobilization support was clay, using a 2:1(ml enzyme/g immobilization support) ratio.

Keywords*: collagenase, concentration, keratinase, immobilization, lyophilization.*

INTRODUCTION

Leather industry is among the major pollution causing industries and has a high environmental impact. A lot of chemicals mainly responsible for pollution are used in tanning processes, therefore using proteases for processing tanneries materials and wastes has been recognized as a reliable alternative (Sivasubramanian et al., 2008).

Proteases are industrially important due to their wide applications in leather processing, food industries, textile industry etc (Deng et al., 2010). Proteases are obtained from plants, animal organs and microorganisms, but currently a large proportion of commercially available proteases are derived from bacteria and fungi (Raj, 2012).

The keratinase productions by various microorganisms were reported by a number of authors. It was found that keratinase produced by fungi, *Streptomyces* and bacteria were produced at alkaline pH and almost thermophilic temperatures (Allpress, 2002).

Although microbial collagenases have been found in a wide variety of mesophilic bacterial strains, the industrial-scale application of known bacterial collagenase for collagen peptide production has been disturbed because of their insufficient stability (Tsuruoka, 2003). Maintenance of their structural stability during any biochemical reaction is highly challenging. Enzyme immobilization provides an excellent base for increasing their stability and repetitive use. Attaching enzymes to a solid support results also in an improved availability to the substrate, so that several natural and synthetic supports have been assessed for their efficiency (Datta, 2013).

This paper reports the researches done for obtaining immobilized proteolytic complexes for using in biodegradation purposes. Three bacterial strains were cultivated in minimal culture medium with 0.6% sheep fur as carbon source. The concentrated complex was immobilized on some inert and cheap supports as clay and adabline, which are technically convenient because the adsorption process may be used. Collagenase and keratinase activity was assayed for estimation of the immobilization efficiency. Enzymes activity

and protein content were determined using spectrophotometrical methods.

MATERIALS AND METHODS

Microorganisms and growth conditions Three bacterial strains were used from the collection of Faculty of Biotechnology, University of Agronomical Sciences and Veterinary Medicine Bucharest: 7.2 and BN7 strains were recently isolated, while Bl strain is a collection strain (*Bacillus licheniformis* ATCC 14580).

The inoculation was done in a minimal salt medium supplemented with 0.6% sheep fur as carbon source. Proteolitic enzymes were produced in 500 ml flasks, kept at 27^{o}C, agitated at 150 rpm, for 15 days. The culture medium was centrifuged at 6000 rpm, at 4^{o}C, for 20 minutes.

Concentration of the culture medium. The concentration of the culture medium was performed by rotaevaporator, at 35^{o}C, followed by lyophilisation at 0.04 mbar, for 18 hours.

Testing of some immobilization supports. The concentrated complex was immobilized on clay and adabline. They are inert and cheap supports that are technically convenient because the adsorption process for immobilization was used. Different immobilization ratio was used (1:2; 1:1; 2:1 ml enzyme/g immobilization support).

Obtaining of the immobilized enzymatic complex. For this purpose two methods were tested: drying at 40^{o}C in air stream for 24 hours and vacuum drying.

Enzymatic assay of collagenase, based on some classical refferences (Moore and Stein, 1948; Mandl et al., 1953), was made as following: after collagenase promotes hydrolysis of collagen, the degree of proteolysis is measured by color development with ninhydrin (absorbance was measured at 570 nm). The enzymatic activity of collagenase was measured in U/ml (*unit definition*: one unit liberates peptides from collagen equivalent in ninhydrin colour to 1 μmol of leucine in 2 hours at pH 7.4 and at 37 ^{O}C).

Enzymatic assay of keratinase was performed using 4 mg keratin azure as substrate. One unit of keratinase activity was defined as the amount that caused an increase in absorbance of 0.01 at 595 nm within 60 min reaction at 60°C.

The protein concentration was determined by Lowry method, which is based on the reactivity of the peptide nitrogen with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteu phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids (Lowry et al., 1951).

RESULTS AND DISCUSSIONS

Selection of the most efficient method for concentration of culture medium

The bacterial collagenases registered the highest values of the enzymatic activity in the lyophilized preparates compared to the ones concentrated by rotary evaporation for all the tested strains (Figure 1).

Remarcable among these was Bl strain, which reached initial the highest collagenase specific activity (18.21 U/mg protein); after lyophilization the activity of collagenase produced by this strain increased 1.3 times. The collagenase activity of Bl strain decreased 1.56 times after concentration by rotary evaporation. Also the BN7 strain manifested initial a good specific activity of collagenase (11.27 U/mg protein), which increased 1.87 times by lyophilization. The rotary evaporation leads to a decreasing of collagenasic activity also for this strain.

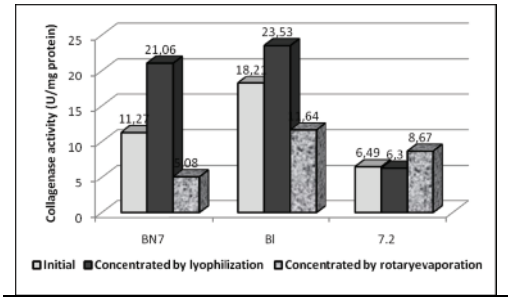

Figure 1. Activity of colagenase in concentrated enzymatic complex

Regarding the specific activity of keratinase, the obtained results indicated also the lyophilization as the most efficient method of

concentration (Figure 2). Initial, the highest keratinase activity was registered by the Bl strain (5.67 U/mg protein); a slight increasing of keratinase activity was observed after lyophilization (6.19 U/mg protein).

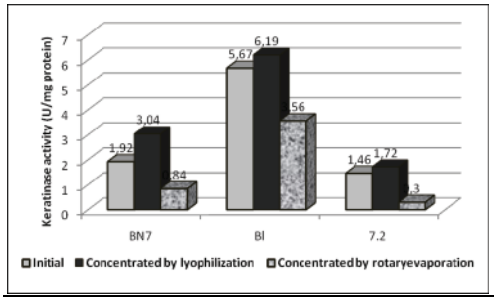

Figure 2. Activity of keratinase in concentrated enzymatic complex

Generally, the values of collagenase and keratinase activities were significant lower after rotary evaporation than after lyophilization for all the tested strains. Considering the fact that rotary evaporation was performed at 40° C, probable the enzymatic activities of collagenase and keratinase were partly inhibated by this temperature value.

In conclusion, among the tested methods of concentration, the best results were obtained by lyophilization for all the used strains, such that the further experiments were performed on lyophilized enzymatic complex.

Selection of the most efficient immobilization support

The concentrated complex was immobilized on clay and adabline by drying at 40°C in air stream for 24 hours. The obtained results indicated a good conservation of the enzymatic activities in the immobilized preparates with differences depending on the support type. One can observe that the proteolytic complex manifested a higher enzymatic activity on clay as immobilization support than on adabline for all the tested bacterial strains (Figure 3 and 4).

For example, the BN7 strain, which generally proved a good colagenase activity, registered a value of 6.96 U/ml on clay support and only 3.67 U/ml on adabline (Figure 3).

Also regarding the keratinase activity the same tendency can be noticed (fig. 4): higher values were determined on clay support (1.92 U/ml for Bl strain and 2.12 U/ml for BN7 strain) compare to the ones measured on adabline support (1.02 U/ml for Bl strain and 1.01 U/ml for BN7 strain).

The rate of residual enzymatic activity after immobilization was calculated for Bl strain, which manifested the highest proteolytic activity. So, if the activity of collagenase synthetized by Bl strain measured initial 18.02 U/ml, the value found after immobilization on clay support was 7.38 U/ml, which indicated 40.62% preservation of collagenase activity. On adabline support only 24% of collagenase activity was maintained.

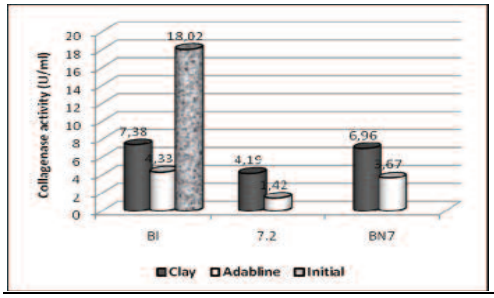

Figure 3. Activity of colagenase in immobilized enzymatic complex

The keratinase activity of Bl strain initial registered a value of 4.11 U/ml. Subsequent, a preservation of 46.71% of keratinase activity was noticed on clay support and only 24.81% when adabline was used as immobilization support.

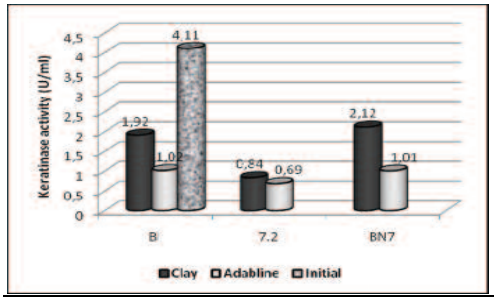

Figure 4. Activity of keratinase in immobilized enzymatic complex

Although clay was established as the most efficient immobilization support, the loss of enzymatic activity after immobilization was high (more than 50%), probably because of a partial inhibating of proteolytic activity at 40°C, the temperature value used by this immobilization method.

Selection of the most efficient method for enzyme immobilization

Further researches were performed in order to find an efficient immobilization method, which can preserve most of enzymatic activity of the proteolytic complex. For this purpose drying under vacuum at room temperature was used as method of immobilization on clay support.

Under these circumstances, the measured collagenase activity for Bl strain was initial 17.81 U/ml and 16.09 U/ml after immobilization, therefore 90.34% of enzymatic activity was preserved (Figure 5).

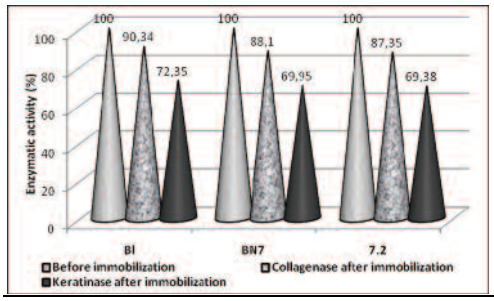

Figure 5. Preservation capacity of proteolytic activity after immobilization

Regarding the keratinase activity, also the Bl strain initial registered the highest value (4.16 U/ml), from which 72.35 % was preserved in the immobilized complex.

Analyzing these results, one can observe a great efficiency of this immobilization method, considering the high preservation capacity of colagenase activity (87–90%). Instead, keratinase proved less resistance to immobilization, taking account of lower remain activity measured after immobilization (70–73%).

CONCLUSIONS

Among the tested methods of concentration, the best results were obtained by lyophilization of cultivation medium.

The proteolytic complex manifested a higher enzymatic activity on clay as immobilization support than on adabline for all the tested bacterial strains.

Drying under vacuum at room temperature was proved as the most efficient method of immobilization.

Immobilization on clay by drying under vacuum leaded to the highest preservation of the enzymatic activity of the immobilized proteolytic complex: 87–90% of collagenase activity and 70–73% of keratinase activity.

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EFFECT OF NON VOLATILE COMPOUNDS OF *TRICHODERMA SPP.* AGAINST *FUSARIUM GRAMINEARUM, RHIZOCTONIA SOLANI* AND *PYTHIUM ULTIMUM*

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Abstract

Biological control represents an important approach of agricultural biotechnology for controlling many fungal plant pathogens. Trichoderma spp. are the most promising and effective bioagents against many plant pathogenic fungi. In present paper, two strains of Trichoderma aspellerum isolated from soil were screened for their efficacy against some common soil borne plant pathogens by dual culture technique. Trichoderma strains were grown in potato dextrose broth and collected metabolites were amended to PDA medium to obtain 5, 10, 25 and 50% concentration in Petri plates. The solidified agar plates were inoculated with pathogen and incubated at 25 ± 2ºC for 7 days. The colony diameter was measured and percentage inhibition of radial growth was calculated. Both antagonists' strains produced non-volatile metabolites and inhibit the mycelial growth of Fusarium graminearum, Rhizoctonia solani and Pythium umtimum.

Key words : *biocontrol, non volatile, phytopathogens, Trichoderma*

INTRODUCTION

Biological control represents a viable alternative to the use of chemical fungicides and it is considered to be a safe, effective and eco friendly method for plant disease management [1].

Trichoderma is known to be one of the best candidates of biocontrol agents. Modes of action of this fungus include mycoparasitism, antibiosis, competition for nutrients and space, tolerance to stress through enhanced root and plant development [1; 2]. Many *Trichoderma* strains, mainly *T. harzianum*, *T. asperellum, T. viride* and *T. virens* have been identified as having potential applications in biological control. The list of genera of plant pathogenic fungi affected by *Trichoderma* includes: *Armillaria, Botrytis, Colletotrichum, Dematophora, Endothia, Fulvia, Fusarium, Fusicladium*, *Monilia, Nectria,Phytophthora, Plasmopara, Pythium, Rhizoctonia, Rhizopus, Sclerotinia, Venturia Sclerotium, Verticillium,* and wood rot fungi [3-4].

In present study, two strains as *Trichoderma aspellerum* T75 and *T. aspellerum* T83 were evaluated against pathogens in dual culture techniques and through production of non-volatile compounds. The targeted pathogens were some common soil borne plant pathogens, such as *Rhizoctonia solani, Fusarium graminearum* and *Pythium ultimum*

MATERIALS AND METHODS

Microorganisms

Potential biocontrol agents *Trichoderma asperellum* T57 and *T. aspellerum* T83 were provided from Microbial Collection of ICECHIM. The strains isolated from forest soil

were maintained on potato dextrose agar (PDA) slants at 4°C. Plant pathogens were obtained from Microbial Collection of DSMZ (Germany).

Evaluation of antagonistic activity through production of antifungal non-volatile metabolites

The effect of non-volatile substances on pathogen was studied following the method elaborated by Dennis and Webster [5], developed by Kamala and Indira [6], and Mishra [7]. *Trichoderma* isolates were inoculated in 100 ml sterilized potato dextrose broth (PDB) in 250 ml conical flasks and incubated at 25 ± 1° C on a rotatory shaker Heidolph Unimax 1010 at 150 rpm for 14 days. The mycelium was collected after 14 days and filtered through Whatman filter paper. The culture filtrate was sterilized by passing through a microbiological membrane filter. Different volumes of filtrates were added to the molten PDA medium (at 40 ± 3 °C) to obtain final concentrations of 10, 25 and 10% (v/v). The medium placed into Petri plate was inoculated with mycelial plug of pathogen at the centre. The plates were incubated at 25±1°C for 7 days. Control plates were maintained without culture filtrate. All experiments were done in triplicate. The colony diameter was measured and percentage inhibition of radial growth was calculated by following formula:

Inhibition (%) = D1 – D2/D1 x 100,

where: D1 is the diameter of radial growth of plant pathogens in control; D2 is the diameter of radial growth of plant pathogens in treatment

RESULTS AND DISCUSSIONS

The visual observations of antagonist microorganisms and pathogen cultures carried out on solid medium in Petri plates are presented.

The growth inhibition of *F. graminearum* in presence of different concentrations of *T. asperellum* culture extracts as non-volatile compounds is presented in Fig. 1 and Fig. 2.

These results showed that culture filtrates from *Trichoderma* T83 was more active against pathogens than the other strain. The lowest inhibition was produced by *T. asperellum* T57 at a concentration of 10% (69.44%). The highest inhibition of 88.88% was produced by *T. asperellum* T83 at 50% filtrate concentration.

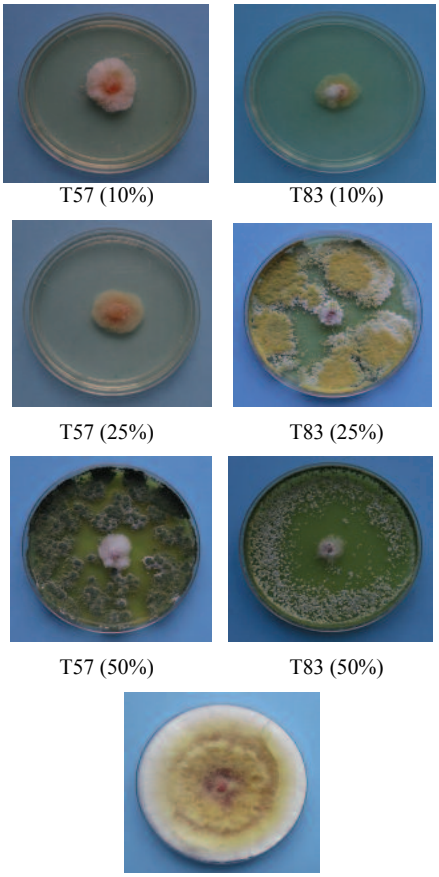


Figure 1 Results of *in vitro* antagonism testing for *T. aspellerum* T57 and T83 against *F. graminearum*

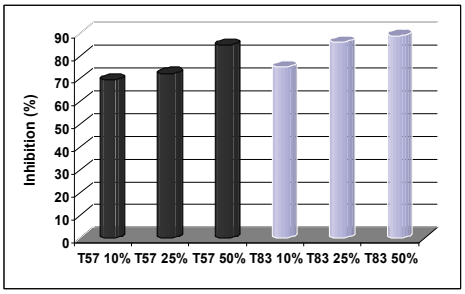
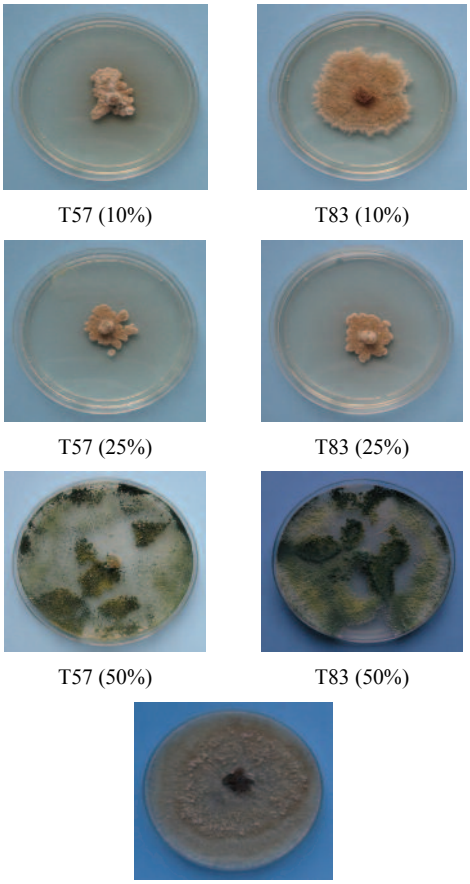


Figure 2. *In vitro* inhibition of *F. graminearum* by non-

volatile compounds from *T. asperellum* T57 and T83

The performance of antagonistic strains against *R. solani* is presented in Fig. 3. In the case of this pathogen, the inhibition induced by non-volatile metabolites varied from 48.71% for 10% filtrate concentration at *T. aspellerum* T83, to total inhibition at 50% filtrate concentration for both *Trichoderma* strains (Fig. 4).



T57 (10%) T83 (10%)

T57 (25%) T83 (25%)

T57 (50%) T83 (50%)

Control – *R. solani* culture

Figure 3. Results of *in vitro* antagonism testing for *T. aspellerum* T57 and T83 against *R. solani*

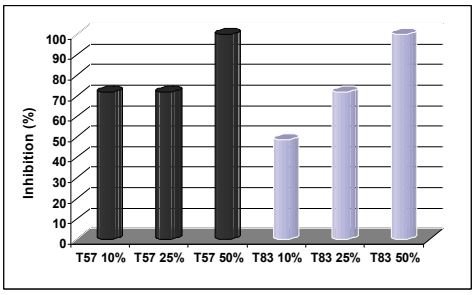
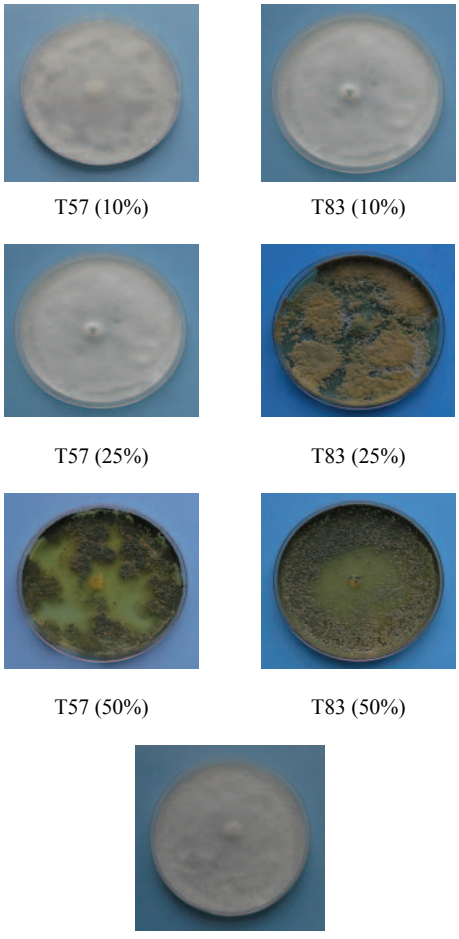


Figure 4. *In vitro* growth inhibition of *R. solani* by non-volatile compounds from *T. asperellum* T57 and T83



T57 (10%) T83 (10%)

T57 (25%) T83 (25%)

T57 (50%) T83 (50%)

Control – *P. ultimum* culture

Figure 5. Results of *in vitro* antagonism testing for *T. aspellerum* T57 and T83 against *P. ultimum*

The non-volatile metabolite activity against *P. ultimum* is presented in Fig. 5 and Fig. 6.
In test with *T. aspellerum* T57 strain, the growth inhibition of pathogen was zero for 10% and 25% filtrate concentrations, while the increase of filtrate concentration to 50% produced 65% inhibition.
T. aspellerum T83 was more active against *P. ultimum* producing 75% growth inhibition even at 25% filtrate concentration. For 50% filtrate concentration, the pathogen inhibition was 75%.

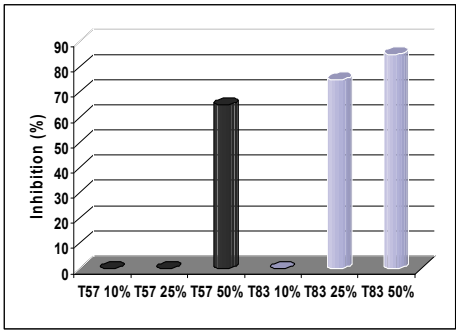


Figure 6. Effect of non volatile compounds produced by *Trichoderma* strains on the growth of *P. ultimum*

CONCLUSIONS

This study demonstrated the efficacy of isolates of *Trichoderma asperellum* T57 and T83 in controlling *Fusarium graminearum*, *Rhizoctonia solani* and *Pythium ultimum.* The antagonist strains produced non-volatile metabolites that inhibit the mycelial growth of plant phytopathogen. The antifungal activity of the culture filtrate was dose-dependent. At higher concentrations, the culture filtrate of the antagonist restricted the growth of all pathogens with better efficacy. The tests showed that *T. aspellerum* T83 was more active in controlling all pathogens tested. Out of tested pathogens, *R. solani* was more sensitive to the inhibitory effect of tested biological agents.

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ORGANIC SOLVENT RESISTANCE MECHANISMS IN *RHODOCOCCUS* sp. Po_4

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Abstract

Crude oil extraction, transportation and storage facilities are frequently the source of soil and water pollution in Romania. Rhodococcus sp. Po_4 showed good tolerance to both 1 % (v/v) alkanes (cyclohexane, n-hexane, n-decane) and aromatics (toluene, styrene, ethylbenzene) with log P_{OW} (logarithm of the partition coefficient of the solvent in n-octanol-water mixture) values between 2.64 and 5.98. However, 1 % (v/v) alkanes were less toxic for Rhodococcus sp. Po_4 cells, compared with 1 % (v/v) aromatics. The high organic solvent tolerance of Rhodococcus sp. Po_4 could be due to the presence in their large genome of some catabolic and trehalose-6-phosphate synthase genes. In addition, Rhodococcus sp. Po_4 exhibits potential to synthesize carotenoid pigments which can improve the cell membrane impermeability to toxic organic solvents.

Keywords: *mechanisms, resistance, Rhodococcus, organic solvents.*

INTRODUCTION

The genus *Rhodococcus* belongs to mycolic-acid containing actinomycetes. Many bacteria from the order *Actinomycetes* are known to be antibiotic producers (e.g., *Streptomyces*), while rhodococci are known to exhibit a broad range of catalytic activity that has applications in industrial, pharmaceutical, and environmental biotechnology (Kagawa et al., 2013). Strains of *Rhodococcus* have been reported to be particularly solvent tolerant, while presenting a broad array of enzymes with potential for the production of commercially interesting compounds and/or for the metabolism of recalcitrant organic solvents. The adaptability and versatility of *Rhodococcus* cells can further broaden their application scope (de Carvalho, 2010).

Several reviews have been published on the tolerance of bacterial cells to organic solvents in which the mechanisms of cell adaptation were discussed (Sikkema et al., 1995; de Carvalho, 2010; Torres et al., 2011; Segura et al., 2012). Most of the papers published on this subject dealt with Gram-negative bacteria. Although the interest in using Gram-positive bacteria in biocatalysis and bioremediation processes is increasing, studies on the adaptation of *Rhodococcus* strains to toxic organic solvents are still scarce (de Carvalho, 2010; Torres et al., 2011). *Rhodococcus* cells can adapt the cell wall and membrane compositions, as well as the physicochemical properties of the cell surface, can degrade or bioconvert toxic compounds (e.g., benzene, toluene), and can aggregate and produce exopolymeric substances to protect the cell population from stressful environments (de Carvalho, 2010).

The possible cell adaptation mechanisms pursued by *Rhodococcus* sp. Po_4 after 1 % (v/v) alkanes and aromatics exposure was studied by following the changes in the cell viability, carotenoid pigments production and in their genomic fingerprinting.

MATERIALS AND METHODS

Organic solvent resistance mechanisms in *Rhodococcus* sp. Po_4. This bacterial strain was cultivated on liquid LB-Mg medium (Stancu and Grifoll, 2011) and incubated at 28 °C on a rotary shaker (200 rpm) until they reached a turbidity of 0.600 (OD_{660nm}). Then, 1 % (v/v) organic solvents (alkanes: cyclohexane, *n*-hexane, *n*-decane; aromatics: toluene, styrene, ethylbenzene) were supplied to the culture broths. Flasks were sealed and incubated for 1 and 24 hours at 28 °C on a rotary shaker (200 rpm).

Cell viability. Serial dilutions of the culture broths were spotted on LB-Mg agar and the number of viable cells (CFU ml^{-1}) was determined after 24 hours incubation of the Petri plates at 28 °C.

Pigments production. Culture broths were spotted on LB-Mg agar and the pigments production was observed after 24 hours incubation of the Petri plates at 28 °C. The Petri plates were visualized under a 254 nm ultraviolet light, and the fluorescence of the cultures is due to carotenoid pigments. Carotenoids were extracted from the culture broths with acetone. UV/visible scanning spectra of the samples were recorded between 220 and 700 nm using a NanoDrop1000 UV-visible spectrophotometer. For HPTLC analysis, the samples were spotted with a Linomat 5 sample applicator (CAMAG), on a 10×20 cm precoated silica gel 60 TLC aluminium sheets (Merck). The separation was performed using acetone-*n*-hexane (7.5:92.5 v/v) mixture (Tao et al., 2004) as mobile phase. The TLC plates were visualized under a 254 nm ultraviolet light.

Genomic fingerprinting. DNA from culture broths was isolated using the method of Whyte et al. (1996). For rep-PCR (repetitive sequence-based PCR) amplification, 1 µl of DNA extract was added to a final volume of 25 µl reaction mixture, containing: 5×GoTaq flexi buffer, $MgCl_2$, dNTP mix, specific primers (REP 1R-Dt and REP 2-Dt, BOXA 1R, Versalovic, et al. 1994), and GoTaq DNA polymerase (Promega). PCR was performed with a mastercycler proS (Eppendorf). The PCR program consisted in initial denaturation for 6 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 40 to 50 °C for 1 min, extension at 65 °C for 8 min, and a final extension at 65 °C for 16 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 rep-PCR products were analyzed.

Reagents used during this study were procured from Merck, Sigma-Aldrich, Promega, Invitrogen, Zymo Research, Applied Biosystems, Biolog or Bio-Rad Laboratories. The PCR primers were purchased from Biosearch Technologies, Integrated DNA Technologies and Invitrogen.

RESULTS AND DISCUSSIONS

Rhodococci are often isolated from soils contaminated with crude oil and/or xenobiotic compounds, and these organisms can utilize such compounds as carbon sources (Kagawa et al., 2013). The strain used in the present study was *Rhodococcus* sp. Po_4. This nonpathogenic Gram-positive bacterium was isolated previously from Poeni oily sludge by enrichment cultures method (Stancu and Grifoll, 2011). Their taxonomic affiliation was determined on the basis of phenotypic characteristics, G+C content of the bacterial chromosome, and the 16S rRNA gene sequence (Stancu and Grifoll, 2011). In the 16S rRNA gene phylogenetic tree obtained using the neighbour-joining method in MEGA5.1 program (Tamura et al., 2011), *Rhodococcus* sp. Po_4 formed a cluster with four strains of *Rhodococcus qingshengii* (Ba49, PT3-14, PT2-14B, BLH-Y4) and two strains of *Rhodococcus* sp. (Y2-2-10, G1-2-10) (Figure 1).

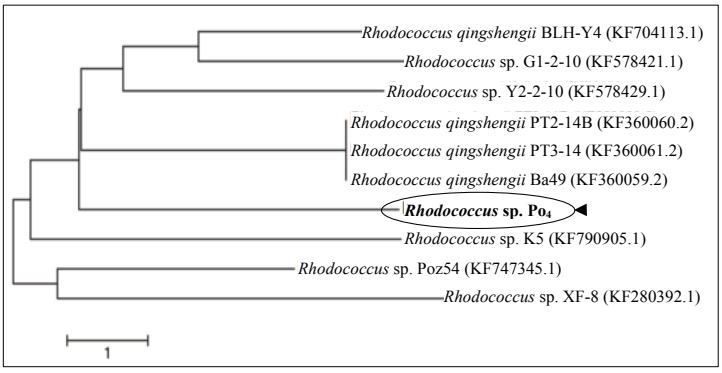

Figure 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the position of *Rhodococcus* sp. Po_4 with respect to other *Rhodococcus* strains from GenBank public database
The scale bar indicates substitutions per nucleotide position.

Organic solvent resistance mechanisms in *Rhodococcus* sp. Po$_4$.

Cell viability. The exposure for 1 and 24 hours of *Rhodococcus* sp. Po$_4$ cells to 1 % (v/v) alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene) had different effects on their survival rate (Table 1).

Table 1. Viability of *Rhodococcus* sp. Po$_4$ cells after 1 % (v/v) organic solvents exposure

Cell viability[a]	Organic solvents (log P_{OW}[b])						
	Control	Cyclohexane (3.35)	*n*-Hexane (3.86)	*n*-Decane (5.98)	Toluene (2.64)	Styrene (2.86)	Ethylbenzene (3.17)
1 hour	2.7×10^{9}	3.3×10^{7}	4.7×10^{8}	2.0×10^{8}	4.0×10^{5}	8.3×10^{6}	1.2×10^{7}
24 hours	3.1×10^{12}	2.2×10^{10}	1.7×10^{11}	2.3×10^{11}	4.3×10^{7}	1.4×10^{8}	2.1×10^{9}

Legend: [a] = serial dilutions of cultures were spread on LB-Mg agar and the number of viable cells (CFU ml^{-1}) was determined; [b] = logarithm of the partition coefficient of the solvent in *n*-octanol-water mixture.

The viability of *Rhodococcus* sp. Po$_4$ cells 1 and 24 hours after alkanes and aromatics exposure was lower (10^5-10^{11} CFU ml^{-1}), compared with the controls (10^9, 10^{12} CFU ml^{-1}). One hour after alkanes and aromatics exposure the survival rates were 10^5-10^8 CFU ml^{-1}, and after 24 hours the survival rates were 10^7-10^{11} CFU ml^{-1}. Tolerance of bacteria to organic solvents has been estimated by the solvent parameter log P_{OW}, which is an index of biological toxicity (Sikkema et al., 1995). It is generally accepted that solvents with log P_{OW} values below 5 are considered extremely toxic because of their high degree of partitioning into the aqueous layer surrounding the cells, and from there into the lipid membrane bilayer (Torres et al., 2011). In our study the results showed higher survival rates (10^7-10^{11} CFU ml^{-1}) when *Rhodococcus* sp. Po$_4$ cells were exposed to alkanes (cyclohexane, *n*-hexane, *n*-decane) with log P_{OW} between 3.35 and 5.98, as compared with the survival rates of cells (10^5-10^9 CFU ml^{-1}) exposed to aromatics (toluene, styrene, ethylbenzene) with log P_{OW} between 2.64 and 3.17. This is in agreement with a previous study which found that organic solvents with lower log P_{OW} value bound more abundantly to bacterial cells thus being more toxic for them (Sikkema et al., 1995; Torres et al., 2011).

The high organic solvent tolerance of *Rhodococcus* sp. Po$_4$ could be due to the presence in their large genome of some catabolic (i.e., *alkB/alkB1*) (Stancu and Grifoll, 2011) and trehalose-6-phosphate synthase (*otsA1*) genes. The DNA extracted from *Rhodococcus* sp. Po$_4$ was screened by PCR for the presence of *otsA1* gene using otsA-f and otsA-r degenerate primers (Tischler et al., 2013). PCR amplification was performed as described by Tischler et al. (2013). As expected, *Rhodococcus* sp. Po$_4$ possesses the *otsA1* gene (760 bp). According to literature (Tischler et al., 2013), this gene seems to be involved in the overproduction of trehalose lipids by *Rhodococcus opacus* 1CP during growth on different *n*-alkanes.

Pigments production. It is well known that the carotenoid pigments are present in some bacteria, and they have important functions in photosynthesis, nutrition, and protection against oxidative damage (Tao et al., 2004). Some natural carotenoids are asymmetrical molecules that are difficult to produce chemically, and the biological production of carotenoids using specific enzymes is a potential alternative to extraction from natural sources (Tao et al., 2004). As observed in Figures 2a-2c, *Rhodococcus* sp. Po$_4$ exhibits potential to synthesize carotenoid pigments. Therefore, we further investigated the effect of 1 % (v/v) alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene) to the carotenoids in *Rhodococcus* sp. Po$_4$ cells.

On the Petri plate, no significant modifications in pigments production was observed in *Rhodococcus* sp. Po$_4$ cells 1 and 24 hours after alkanes and aromatics (except toluene) exposure, as compared with the controls (Figure 2a). The fluorescence of the cultures was similar in control cells and those of the cells exposed 1 and 24 hours to alkanes and aromatics. Only in the case of the cells exposed to toluene the fluorescence of the cultures was lower compared with the controls.

The carotenoids synthesized by different *Rhodococcus* strains were previously (Tao et al., 2004) characterized to be 4-keto-γ-carotene (KγC), γ-carotene (γC), chlorobactene (CB) and β-carotene (βC). The carotenoids (Figure 2b) found, based on their R_f values, in *Rhodococcus* sp. Po_4 cells were 4-keto-γ-carotene (with R_f 0.03-0.06), chlorobactene (with R_f 0.09-0.11), γ-carotene (with R_f 0.19-0.21), and β-carotene (with R_f 0.37-0.42).

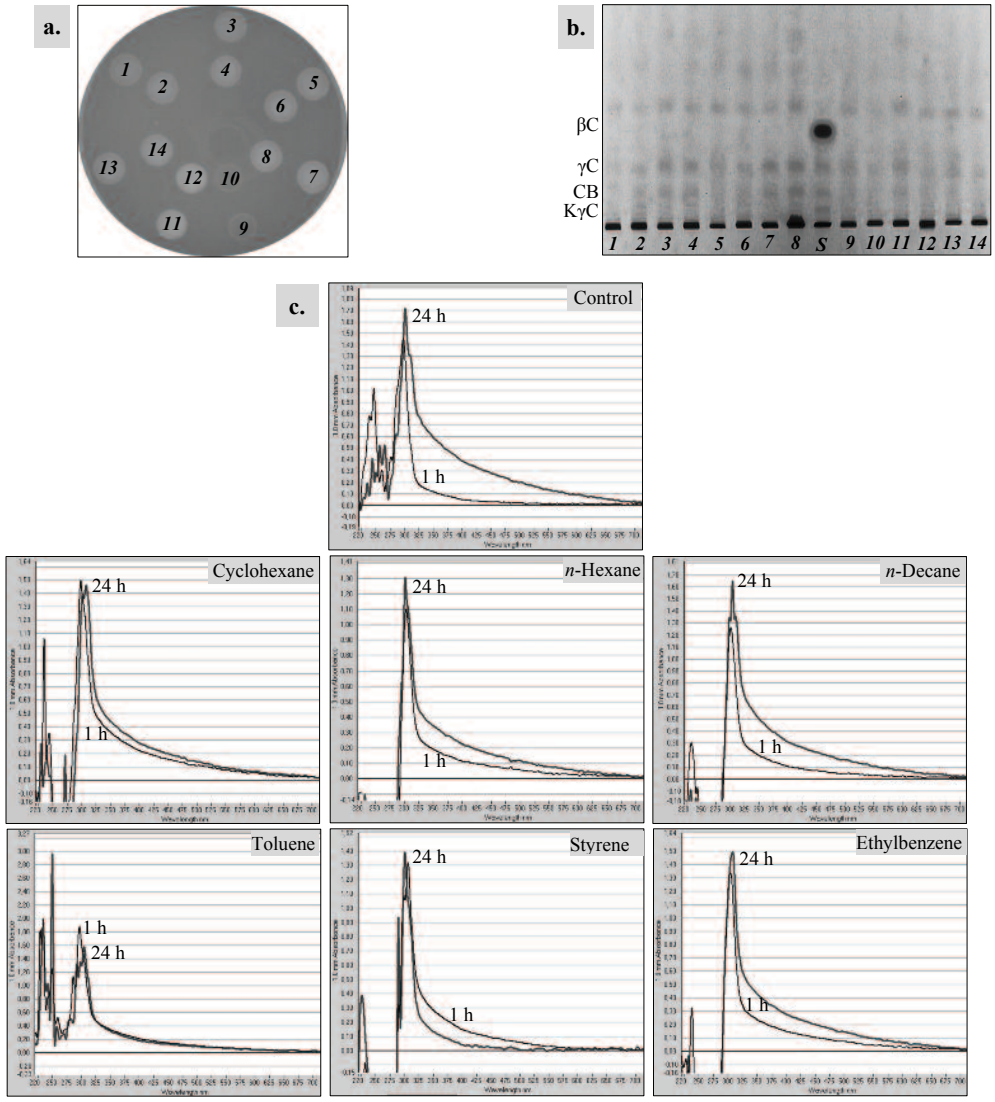

Figure 2. Pigments production by *Rhodococcus* sp. Po_4 after 1 % (v/v) organic solvents exposure
Bacterial cells cultivated 1 hour (spots or lanes ***1***, ***3***, ***5***, ***7***, ***9***, ***11***, ***13***) and 24 hours (spots or lanes ***2***, ***4***, ***6***, ***8***, ***10***, ***12***, ***14***) in LB-Mg medium, control (***1***, ***2***), cyclohexane (***3***, ***4***), *n*-hexane (***5***, ***6***), *n*-decane (***7***, ***8***), toluene (***9***, ***10***), styrene (***11***, ***12***), ethylbenzene (***13***, ***14***).
Panel a. The Petri plate was visualized under a 254 nm ultraviolet light.
Panel b. The TLC plate was visualized under a 254 nm ultraviolet light, carotenoids standard (S), 4-keto-γ-carotene (KγC), chlorobactene (CB), γ-carotene (γC), β-carotene (βC).
Panel c. UV/visible scanning spectra of the extracts were recorded between 220 and 700 nm.

On the TLC plate, no significant modifications in pigments profiles was observed in *Rhodococcus* sp. Po_4 cells 1 and 24 hours after alkanes and aromatics exposure, as compared with the controls. However, the spectral studies (Figure 2c) revealed the existence of some differences between carotenoid pigments extracted from *Rhodococcus* sp. Po_4 control cells and those extracted from cells exposed 1 and 24 hours to 1 % (v/v) alkanes and aromatics (especially toluene). According to literature (Godinho and Bhosle, 2008), the UV/visible absorption spectra of carotenoid pigments are of immense importance, since they aid a great deal in determining the structure of carotenoids. The UV/visible absorption scanning spectra of the pigment extract of *Rhodococcus* sp. Po_4 cells cultivated 1 and 24 hours on LB-Mg medium (control) showed peaks with absorption maxima at 290 nm and 300 nm, respectively. Different peaks with absorption maxima between 280 and 310 nm were detected in the pigment extract of *Rhodococcus* sp. Po_4 cells exposed 1 and 24 hours to 1 % (v/v) alkanes and aromatics.

Genomic fingerprinting. We showed previously (Stancu, 2012) that simultaneous exposure of *Vibrio alginolyticus* IBB_{Ct2} cells to salt stress and toxic organic solvents induced considerable modifications in their genomic fingerprinting 1 hour after solvent exposure, and these variations imply complex genomic rearrangements. Therefore, genomic DNA extracted from *Rhodococcus* sp. Po_4 control cells and those extracted from cells exposed 1 and 24 hours to 1 % (v/v) alkanes and aromatics were analyzed for their rep-PCR fingerprinting. Amplification using a gradient of annealing temperatures indicated optimal annealing temperatures of 40 °C for REP primers, 50 °C for BOXA primer (data not shown). BOXA primer produced the most complex amplified banding patterns for *Rhodococcus* sp. Po_4, with sizes ranging from 200 to 2,000 bp (Figure 3).

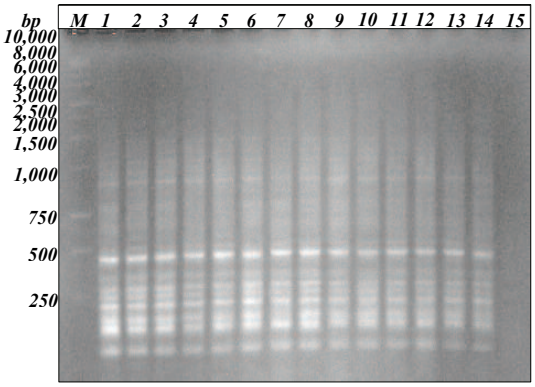

Figure 3. Genomic fingerprinting of *Rhodococcus* sp. Po_4 after 1 % (v/v) organic solvents exposure
Bacterial cells cultivated 1 hour (lanes ***1***, ***3***, ***5***, ***7***, ***9***, ***11***, ***13***) and 24 hours (lanes ***2***, ***4***, ***6***, ***8***, ***10***, ***12***, ***14***) in LB medium, control (***1***, ***2***), cyclohexane (***3***, ***4***), *n*-hexane (***5***, ***6***), *n*-decane (***7***, ***8***), toluene (***9***, ***10***), styrene (***11***, ***12***), ethylbenzene (***13***, ***14***), negative control DNA (***15***), 1 kb DNA ladder, Promega (***M***).

Exposure for 1 and 24 hours of *Rhodococcus* sp. Po_4 cells to alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene) had no effect on their genomic fingerprinting, compared with control. Whole genome analysis of many *Rhodococcus* strains which have very large genomes indicates that they have acquired many gene copies, including the genes important for DNA-damage repair, in contrast to other bacteria with much smaller genomes but which are lacking DNA repairing functions (Larkin et al., 2006).

CONCLUSIONS

Rhodococcus sp. Po_4 showed good tolerance to both 1 % (v/v) alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene). Nevertheless, 1 % (v/v) alkanes were less toxic for *Rhodococcus* sp. Po_4 cells, compared with 1 % (v/v) aromatics.

The high organic solvent tolerance of *Rhodococcus* sp. Po_4 could be due to the presence in their large genome of some catabolic and trehalose-6-phosphate synthase genes. Additionally, *Rhodococcus* sp. Po_4 exhibits potential to synthesize carotenoid pigments which can improve the cell membrane impermeability to toxic organic solvents. By TLC analysis the same pigments profiles were revealed in *Rhodococcus* sp. Po_4 cells exposed or not to 1 % (v/v) alkanes and aromatics. However, the spectral studies revealed the existence of some differences between carotenoid pigments extracted from *Rhodococcus* sp. Po_4 control cells and those extracted from cells exposed to 1 % (v/v) alkanes and aromatics.

Exposure of *Rhodococcus* sp. Po_4 cells to 1 % (v/v) alkanes and aromatics had no effect on their genomic fingerprinting.

Due to its environmental origin and its metabolic potential, *Rhodococcus* sp. Po_4 could be used in the bioremediation of soil and water contaminated with different oily sludge.

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BIOPOLYMERS BASED ON RENEWABLE RESOURCES - A REVIEW

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Abstract

It is well known that plastic waste has become a great problem for the environment all over the world. Conventional polymeric materials are not easily degraded because they are resistant against microbial attack; they accumulate in the environment and represent a significant source of environmental pollution. These problems caused by synthetic waste have led to the need for developing new polymeric materials that can be biodegradable and biocompatible with the environment, to replace the conventional ones. Over the past years a lot of attention has been paid to biodegradable polymers based on renewable resources because of their wide range of applications in packaging, agriculture or biomedical fields. This paper aims to present a review regarding the development of biopolymers and biocomposites based on renewable resources, their properties and the area of their application.

Key words: *biodegradable, biocomposites, biopolymers, renewable resources.*

INTRODUCTION

Materials made from synthetic polymers are not biodegradable and are often improperly discarded (Suchada C., 2010). The huge development of conventional plastics made from petroleum-based synthetic polymers unable to degrade in landfill or compost-like environment had led to serious environmental issues. In response to this increasing awareness, the use of polymers stemming from renewable and sustainable resources to develop biopolymers constitutes an innovative and promising alternative to reduce greenhouse gas and toxic emissions, reduce energy demand and the use of non-renewable resources (Godoia F.C. et al., 2011; Hassan M.A. et al., 2013; Payam M. et al., 2010; Chevillard A. et al., 2011).

Natural polymers derived from agricultural products (such as starch, proteins, cellulose and plant oils) are the major resource for developing renewable and biodegradable polymer materials to replace petrochemicals in many industrial applications due to increased environmental concern and diminishing petrochemical resources (Raquez J.M. et al., 2013; Xiaoqing Z. et al., 2010). Particularly, renewable agricultural and biomass feedstock have shown much promise for use in eco-efficient packaging to replace petroleum feedstock without competing with food crops (Abdelwahab M.A. et al., 2012). However, as compared to thermoplastic synthetic polymers, biopolymers present problems when processed with traditional technologies and show inferior performances in terms of functional and structural properties (Mensitieri G. et al., 2011).

In recent years, there has been an important increase in interest in the use of biodegradable materials for packaging, agriculture, medicine and other areas. A number of blends using biopolymers can be the alternative of currently used synthetic polymeric materials. The most common and potential biopolymers are starch,

chitosan, alginate, gelatin, PLA, PHAs, etc. (Akter N. et al., 2012).

The term *biopolymer* is generally understood as an organic polymer that is produced naturally by living organisms (Armentano I. et al., 2013). One major advantage of biopolymers is that they are also fully capable of biodegradation at accelerated rates, breaking down cleanly into simple molecules found in the environment, such as carbon dioxide, water or methane, under the enzymatic action of microorganisms, in a defined period of time. Polymeric materials derived from renewable resources can be biodegradable or compostable under specific environmental conditions. They are classified according to the method of production or their source:

- Polymers directly extracted or removed from biomass such as polysaccharides and proteins.
- Polymers produced by classical chemical synthesis starting from renewable bio-based monomers such as polylactic acid (PLA).
- Polymers produced by microorganisms or genetically modified bacteria such as polyhydroxyalkanoates, bacterial cellulose, etc. (Mensitieri G. et al., 2011).

POLYSACCHARIDES AND PROTEINS

Starch is a potentially interesting biodegradable material due to its availability, low cost and renewability. Moreover, the use of starch in the plastics industry can reduce dependence on synthetic polymers. Although its structure has not been fully elucidated, it was established that starch is a heterogeneous material consisting primarily of two types of polymers: amylose (Figure 1) and amylopectin (Figure 2).

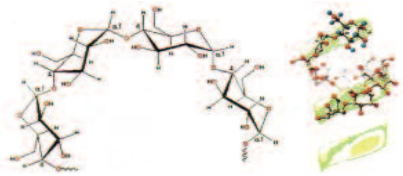


Figure 1. Chemical structure for amylase

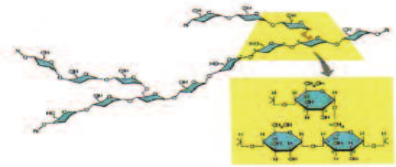


Figure 2. Chemical structure for amylopectin

Starch is a widely used material for making biodegradable plastics, but pure starch based films possesses low mechanical properties (Akter N. et al., 2012). Starch is the most important polysaccharide; it is the most abundant in nature and relatively inexpensive. Natural starch exists in granular form and, as such, it has been used as filler in polymers, but it can also be processed with classical plastic processing technologies such as extrusion, foaming and film blowing after thermoplasticization. The main limitation for starch is its hydrophilic nature, which limits its use in high moisture environments (Mensitieri G. et al., 2011). Starch can be successfully used in PLA composites (Qingfeng S. et al., 2011), and it can also be mixed with polyvinyl alcohol, poly-hydroxybutyric acid, polycaprolactone, chitosan, derivatives, and other degradable polymers to prepare fully degradable biomaterials (Wang Z. et al, 2008). Starch can be used as drug delivery carriers in tissue engineering applications (Thombre N. A. et al., 2009), membranes in direct contact with living tissues (Baran E.T. et al., 2004), microcellular foams (Manoi K. and Rizvi S.S.H., 2010) and food industry (Majzoobi M. et al., 2009; Omojola M. O. et al., 2012; Li B.Z. at al., 2009; Chung H.J. et al., 2008; Dang H.V. et al., 2008; Anand U. and Ambarish J., 2011).

Chitosan, as a unique positively charged polysaccharide, has been one of the most popular biopolymers for development of drug delivery systems for various applications, due to its promising properties, including high biocompatibility, excellent biodegradability, low toxicity, as well as abundant availability and low production cost (Bomou M. et al., 2014; Yangchao L. and Qin W., 2014). Chitosan is a biopolymer derived by deacetylation of chitin, which is the second

most abundant biopolymer in nature after cellulose. Chemical structure of chitin and chitosan is shown in Figure 3.

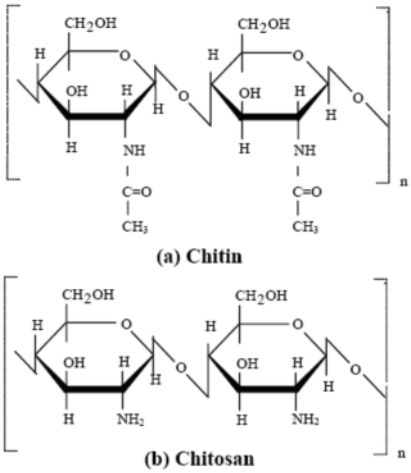


Figure 3. Chitin and chitosan structures

Chitin is present in the exoskeleton of arthropods such as insects, crabs, shrimps, lobsters and certain fungal cell walls. The production of chitosan from crustacean shells, wastes of the seafood industry, is economically feasible. Chitosan has been reported as a potential material of food packaging, especially as edible films and coatings due to its film forming properties. However, one of the main drawbacks of chitosan based materials relates to its relatively weak mechanical strength. Because of reactive amino and hydroxyl functional groups, chitosan is frequently blended with other polymers or crosslinked to improve their functional properties by inducing inter- or intra-molecular crosslinking in the polymer matrix (Yangchao L. and Qin W., 2014; Akter N. et al., 2012; Liang S. et al., 2009; Belalia et al., 2008; Vroman and Tighzert, 2009; Khwaldia et al., 2010; Agostino et al., 2012; Zuang et al., 2012).

The antibacterial activity of chitosan is affected by molecular weight and degree of deacetylation. Low molecular weight chitosan has strong antibacterial properties and it is also harmless to human body. For the food packaging industry, food quality and safety to human health are the two major concerns as consumers prefer fresh and minimally processed products. Chitosan has proven a useful antimicrobial agent in food processing, particularly for improving the shelf life of food materials (Bano I. et al., 2014; Jooyeoun J. and Yanyun Z., 2014; Sanches-Silva et al., 2010). Chitosan and its derivatives have been receiving significant scientific interests and became one of the hottest topics in recent decades, especially for its food, medical and pharmaceutical applications, including drug delivery and tissue engineering (Huang J. et al., 2012; Jooyeoun J. and Yanyun Z., 2014; Yangchao L. and Qin W., 2014; Lavorgna et al., 2014; Sweetie R., et al., 2012, Dash M. et al., 2011). Chitosan membrane, an important form of chitosan, presents potential application in tissue engineering, food preservation, wastewater purification, environmental protection, fuel cell and separation technology (Bomou M. et al., 2014).

Cellulose is a very important and fascinating biopolymer and an almost inexhaustible and sustainable natural polymeric raw material, which is of special importance both in industries and in daily lives (Weili H. et al., 2014). It is found in the cell walls of superior plants in the form of microfibrils with a helical organization on several levels containing crystalline domains (domains with ordering high cellulose chains) and amorphous (segment fields distorted, twisted and deformed) (Figure 4).

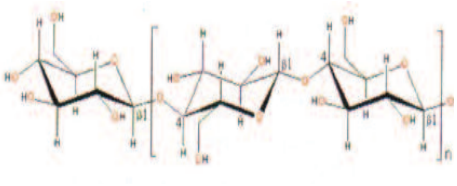


Figure 4. Chemical structure of cellulose

Cellulose is the most abundant biomass material in nature, and possesses some promising properties, such as mechanical robustness, hydrophilicity, biocompatibility, biodegradability, relative thermostability, high sorption capacity and alterable optical appearance (Weili H. et al., 2014; Xiaoyun Q. and Shuwen H., 2013). Cellulose has been widely applied in many fields. Biocomposites

based on cellulose have great advantages (especially their intelligent behaviors in reaction to environmental stimuli) and they can be applied to many circumstances. Approximately 5×1011 metric tons of cellulose is being generated yearly. Unfortunately, only 2% is recovered industrially. The great properties of cellulose enable it to be applied to a vast array of fields (Xiaoyun Q. and Shuwen H., 2013).

Collagen has been extensively used as a biomaterial in many biomedical applications. Collagens are the most abundant proteins found in extracellular matrices of vertebrate animals (Vroman I. and Tighzert L., 2009). In animal hides and skins, the dominant collagen is type I and it is also the major structural component of tendon, bone and connective tissue. Collagen exists in the form of fibrils and they provide the main mechanical support and structural organization of connective tissues. Because collagens provide natural structure, biodegradability, and biocompatibility, they have extensive applications as a biomaterial in tissue engineering, wound healing, as drug carriers, and cosmetics. For example, fibril-forming collagens provide a scaffold for cell attachment and migration, as well as providing specific mechanical properties (Ganesh S. et al., 2014, Hoyer B. et al., 2014). Because of the presence of collagen, the animal derived tissues are used for the replacement of human tissues that results in the improvement of the wound-healing process. Furthermore, it has been used as the main component in the design of biomaterials such as artificial dermis, wound dressings, tissue engineering devices, tendon substitutes, and injectable material in plastic surgery. Collagen from animals, particularly from bovine species, is more advantageous due to the possibility of extraction of a large quantity of pure type I collagen. The structure and stability of collagen are thus an important factor as it is widely used as biomaterials (Ganesh S. et al., 2014).

The major challenge in the material research is to develop suitable modification methodologies to improve the properties of natural polymers. One example is the development of wheat proteins or wheat *gluten* - based natural polymer materials. As one of the cheapest plant proteins derived from the second largest cereal crop wheat (after maize), wheat proteins or wheat gluten have excellent properties in viscoelastic performance, tensile strength and gas barrier performance (Xiaoqing Z. et al., 2010). Wheat gluten, a by-product of the starch industry with a high protein content (>75 wt %), could be considered suitable for lots of applications because of its good thermoplastic properties, good processability and its remarkable biodegradability. Moreover, the use of protein as raw material offers a wide spectrum of chemical functionalities due to the large variety of amino acids, and also represents a significant source of nitrogen for the crops nutrition, a huge advantage for agricultural applications (Chevillard A. et al., 2011, 2012). Wheat gluten is mainly constituted of two main storage proteins that are gliadins (monomeric proteins with molecular weight ranging from 15 to 85 kDa) and glutenins (macro polymer with molecular weight ranging from 150 to more than 103 kDa). Gluten proteins can undergo disulphide interchange upon heating, which leads to the formation of a three-dimensional macromolecular network (Chevillard A. et al., 2011).

CHEMICAL SYNTHESIS PRODUCED POLYMER (PLA)

In the framework of environmentally friendly processes and products, polylactide (PLA) represents the best polymeric substitutes for various petropolymers because of its renewability, biodegradability, biocompatibility, good thermomechanical properties and relatively low cost (Armentano I. et al., 2013; Raquez J.M. et al., 2013). Chemical structure of PLA is shown in Figure 5.

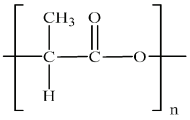


Figure 5. Chemical structure of PLA

Initially, most of its applications concerned biomedical sector and short time uses such as packaging, particularly for the biodegradable

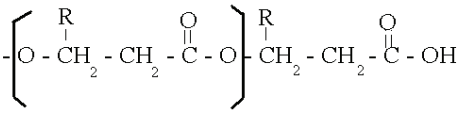
properties of PLA. Interestingly, due to the depletion of petroleum resources, PLA is now seen more and more as a valuable biosourced polymer alternative in long term applications such as automotive and electronics (Abdelwahab M.A. et al., 2012; Lasprilla A.J.R. et al., 2012; Raquez J.M. et al., 2013). PLA is an eco-friendly product with better features for use in the human body (nontoxicity). Lactic acid polymers can be synthesized by different processes so as to obtain products with an ample variety of chemical and mechanical properties. Due to their excellent biocompatibility and mechanical properties, PLA and its copolymers are becoming widely used in tissue engineering for function restoration of impaired tissues. It is a highly versatile biodegradable polymer, which can be tailored into different resin grades for processing into a wide spectrum of products. Polymers based on lactic acid (PLA) are a most promising category of polymers made from renewable resources (Lasprilla A.J.R. et al., 2012). PLA exhibits several advantages in relation to the petroleum-based polymers usually used for packaging (Armentano I. et al., 2013): (i) Good transparency, usually defined as the transmission of visible light in the range of 540–560 nm, slightly higher than that of poly(ethylene terephthalate) (PET) and poly(styrene) (PS); (ii) Degradation in biological environment such as soil or compost; (iii) Biocompatibility: PLA has been demonstrated to be biocompatible and to degrade into non-toxic components and it has been approved by the Food and Drug Administration (FDA) for implantation in the human body; (iv) Process ability: The main conversion approaches of PLA are based on melt processing.

Unfortunately, PLA is rigid and brittle at room temperature due to its glass transition temperature (Tg) close to about 55°C. PLA is a glassy polymer with poor elongation at break (typically less than 10%) (Xiao L. et al., 2012). To improve the ductility of PLA-based materials, a large number of investigations have been made to modify its properties via plasticization and blending with other polymers. However, a great number of variables, i.e. nature of PLA matrix, type, and optimal percentage of plasticizer, thermal stability at the processing temperature, etc. must be considered (Hassouna F. et al., 2011, Abdelwahab M.A. et al., 2012; Halasz K. and Csóka L., 2013). PLA is a linear aliphatic thermoplastic polyester derived from 100% renewable resources such as sugar, corn, potatoes, cane, beet, etc. (Armentano I. et al., 2013).

POLYMERS PRODUCED BY MICROORGANISMS

Polyesters are now universally used as fibers and films in various areas, while plastic waste management recently became a critical problem of global environment. *Polyhydroxyalkanoate* (PHA), which is produced from renewable carbon resources by many microorganisms, is an environmentally compatible polymeric material and can be processed into films and fibers. Also, findings suggest that PHA is a suitable material for fabrication of resorbable medical devices, such as sutures, meshes, implants, and tissue engineering scaffolds (Brigham C.J. and Sinskey A.J., 2012; Chen G.Q and Wu Q., 2005). A wide variety of bacteria can accumulate these polymers as a carbon and energy storage material under nutrient imbalanced condition such as nitrogen, phosphorous or oxygen limitation together with excess carbon. They can be composed of over 100 different monomers and they act as a carbon/energy store for more than 300 species of Gram-positive and Gram-negative bacteria as well as a wide range of archaea (Davis R. et al., 2013; Keiji N. et al., 2009; Laycock B. et al., 2014; Suchada C. et al., 2010). PHAs can be produced from renewable raw materials and are degraded naturally by microorganisms that enable carbon dioxide and organic compound recycling in the ecosystem, providing a buffer to climate change (Suchada C., 2010). Biodegradation of PHA material occurs due to the action of extracellular PHA depolymerase secreted from microorganisms in various natural environments (Davis R. et al., 2013; Keiji N. et al., 2009; Laycock B. et al., 2014). Currently, new research efforts are proceeding

towards developing PHAs in transgenic plants (Reddi M.M.et al., 2013). Polyhydroxybutyrate (PHB) and poly(hydroxybutyrate-cohydroxyvalerate) (PHBV) are the most well-known polymers of the polyhydroxyalkanoates family. Chemical structure of the most representative PHA is shown in Figure 6.



Poly (3-hydroxyalkanoate) (PHA)

R = CH3, Poly(3 hydroxybutyrate)
R = CH2-CH3, Poly(3-hydroxyvalerate)

Figure 6. PHA structure

Polyhydroxybutyrate (PHB) is polyester from the PHA family and is accumulated by a wide variety of micro-organisms as an intracellular storage source of organic carbon and chemical energy (Bertini F. et al., 2012).
The properties of the PHBV co-polymer can be easily tailored by varying the valerate content.
PHB is biodegradable thermoplastic polyester that can be considered analogous to many conventional petroleum-derived plastics currently in use. Furthermore, it has some additional advantages such as being biocompatible and can be produced from a renewable raw material in a sustainable technology from economical to ecological point of view (Godoia F.C. et. al., 2011).

Bacterial cellulose (BC) is a fascinating and renewable natural nanomaterial characterized by favorable properties such as remarkable mechanical properties, porosity, water absorbency, mold ability, biodegradability and excellent biological affinity.
Intensive research and exploration in the past few decades on BC nanomaterials mainly focused on their biosynthetic process to achieve the low-cost preparation and application in medical, food, advanced acoustic diaphragms, and other fields (Weili H. et al., 2014, Zhang S. and Luo J., 2011). Bacterial cellulose has the same molecular formula as plant cellulose, but with unique and sophisticated three-dimensional porous network structures. The cellulose obtained from bacteria is known to have unique properties over plant cellulose such as: (i) Absence of lignin and hemicellulose, making it a highly pure source of cellulose, (ii) High degree of polymerization combined with crystallinity (60–70%), leading to high Young's modulus at room and elevated temperatures, (iii) Extremely high water-holding capacity, up to 100 times its self-weight; (iv) Excellent biodegradability and biological affinity (Vitta S. and Thiruvengadam V., 2012).
Various modification methods have been explored to open up possibilities for endowing BC with new functionalities. In the last few years, growing worldwide activity can be observed regarding extensive scientific investigation and increasing efforts for the practical use of the BC materials. There is an increasing annual publication activity on BC (also known as microbial cellulose or bacterial nanocellulose). In recent years, the investigation and utilization of BC in functional materials have been the focus of research, and a growing number of works have been included in this field. Functional BC-based nanomaterial's are especially an attractive topic because they enable the creation of materials with improved or new properties by mixing multiple constituents and exploiting synergistic effects, such as electronic, optical, magnetic, catalytic properties and bioactivity (Weili H. et al., 2014, Castro C. et al., 2012).

CONCLUSIONS

Biodegradable natural polymers are mainly based on renewable resources (like starch, collagen, cellulose, etc.) and can be produced naturally or synthesized from renewable resources. Starch, collagen, chitosan, wheat gluten have all applications in agriculture, food industry, medicine, cosmetics, etc. PLA also has applications in many areas such as packaging industry and biomedical sector. Biobased and biodegradable polyesters like PHAs have been in demand in order to reduce carbon dioxide emissions from plastic waste and to build a sustainable society.
In view of expanding the scope of BC applications, it is important to take full advantage of the unique structure and

properties of BC nanomaterials to develop novel BC-based nanomaterials with ground-breaking new features.

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BIO-FILTRATION STEP USE IN DRINKING WATER TREATMENT

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Abstract

The paper presents a case study, for the drinking water plants which involve a bio-filtration step used to reduce simultaneously, certain pollutants (iron, manganese, ammonia) in a single stage filtration. The bio-filtration step, with microorganisms multimedia doped layers was compared with a classic filtration step. The new approach shows the advantage of: higher yields of water pollutants reduction at low specific energy consumption. The bio-filtration process is preceded by an electrochemical pre-treatment whose role is: oxidation of dissolved substances from water, turn non biodegradable organic substances in biodegradable, favour coagulation and flocculation processes, bio-filtration process improvement. In order to optimize the process, was developed and validated a mathematical model of bio-filtration process.

***Keywords*:** bio-filtration, electrochemical treatment, multiple oxidants, ammonia, drinking water

INTRODUCTION

Given the implementation of the water supply programme in the current phase for many rural human settlements from Romania whose consumption does not exceed 100...300 m^3/day, the only source of water available in the neighbourhood is the under-ground water extracted from medium depth wells (150...200 m). These sources of water have constant parameters in time for many regions (Crişana, Banat, Oltenia, Bărăgan, Moldavia, Dobrogea) and they are impurified with iron ions, manganese, ammonia, nitrates and arsenic, in a higher concentration than the maximum value allowed by the legal norms. Water drinking stations have to run several treatment stages, which use quite difficultly purchasable chemical reacting agents that call for experienced water operators and all this effort is not justified for these relatively area where the water consumptions is relatively low. In order to meet these requirements and to mitigate the aforementioned pollutants, the biofiltration stage within the water drinking station is suitable for relatively because of is relatively easy to automate energetic and chemical consumptions. The process of biofiltrating the pollutants from the water meant to make drinkable water was highlighted by chance in certain water drinking stations when no reacting agents were added during the chemical treatment stage and the filters were cleaned more rarely, and an improvement of the processed water parameters has been noticed. When the processed water parameters (in-out) were correlated to the population of bacterial cultures developed in the filtrating environments, the processes began to be understood. They were subsequently applied in order to enhance the performances of certain existing processes and especially for being used as a basis for a new generation of biological treatment processes, providing a sudden improvement of the biofiltrating performances. Technically speaking, drinkable water obtaining by biofiltration consists in 'preparing the water for biofiltration', which most of the times means airing the water, with holding the unwanted substances (the water pollutants) in the filtrating layers by developing specific bacterial cultures that feed on these pollutants, followed by a stage of enriching the water in oxygen in order to enhance the stability of the water parameters in its distribution system and the disinfection stage, for hindering the water from transmitting diseases.

WATER POTABILISATION FLOW PRESENTATION

The water potabilisation process generally used by authors for the three treatment stages was implemented in the township station. The stations supply water to the human settlements where the sources of water are the wells from the second source of ground water.

The ***'Water Preparation for Biofiltration'*** by ozonisation or electrochemical treatment supplemented with water aeration under pressure were used. The process of ozonisation or electrochemical treatment followed by aeration aims at oxidising the ions dissolved in the water at a superior valence condition, in order to retain them in the filtrating materials. The aim is to diminish the natural organic matter (NOM) by total or partial oxidation until inorganic oxides or a biodegradable organic matter and to enrich the water with oxygen for favouring the nitrification-denitrification reactions in the biolfilter. In the water treatment electro-chemical process, the electrodes (at least one cathode and an anode) are inserted either directly in the amount of water that needs treating or in a by-pass pipe. A direct or pulsating current is applied between the electrodes, thus giving birth to water electrolysis and causing the reaction to take place in situ.

At the anode, the main product is oxygen and water acidification takes place near the anode :

$$2H_2O \rightarrow O_2 + 4H^+ + 4e^- \quad (1)$$

At the cathode, hydrogen gets formed, the water near the cathode thus becoming alkaline :

$$2H_2O + 2e^- \rightarrow H_2 + 2OH^- \quad (2)$$

The utilisation of current pulses and the turbulent flow on small radius edges lead to local areas with high density dissipated energy, which make possible certain chemical reactions that do not take place under normal conditions, followed by a nanometrical dispersion of the reaction products. Electro-chemical oxidation is an efficient electro-chemical technique, which is used to oxidise various water compounds. This technique is also environmentally friendly, as no chemical reacting agents are used. Electrochemical oxidation depends on the anode selection. This can be initiated by the direct transfer of one or several electrons from the sublayer to the anode or the sublayer can be indirectly oxidised by the intermediaries formed as a result of water oxidation. The electrochemical treatment at the cathode leads to hydrogen release in small amounts (approx. 0.4 l of hydrogen under normal conditions are generated by an amper*hour), which in most cases does not cause problems.

The following effects arise after the electrochemical treatment: water pre-disinfection, organic substances, ammonia and nitrates reduction.

One-stage ***'Biofiltration'*** was applied in a filter doped with specific target pollutant-mitigating bacteria, at an adjusted concentration of the oxygen from the water. The granular active carbon (GAC) filters with quartz sand were noticed to be able of eliminating NOM beyond the adsorption capacity, which has led to the development of certain biofiltration processes for treating drinkable water. Seeing the low maintenance costs and the pollutant mitigation efficiency, biofiltration has become an attractive treatment process. When new biofilters are commissioned, they undergo the colonisation phase for a full biodegradation activity. Biofilm increase in the new filters is a slow process, which takes months. In order to reduce the biofilter generation time, the filtrating materials upon mounting are doped with specific pollutant mitigation bacteria.

'Water Disinfection and Oxygen Enrichment' take place in a stage with ozone obtained from oxygen. In case that the drinkable water supply systems do not seem safe, namely they are old, full of deposits and run a high risk of infesting the water in the mains, the water disinfection flow is supplemented by dosing the sodium or calcium hypochlorite.

PRESENTATION OF A BIOFILTER WITHIN THE POTABILISATION STATION

In nature, bacteria do not exist as isolated cells, but they grow and survive in organised communities. In suspensions they can generate flakes, liquid films and the germ communities that get developed at such stage interfaces as solid-liquid or air-liquid are called biofilms. Generally speaking, biofilms can be used to treat waters, to perform biological purifications – to remove unwanted substances from the water, in food – for food digestion, but they can also be harmful – for instance the biofilms that get formed on the dental plate and the medical prosthetics or in the drinkable

water tanks and conveying pipes. The aggregates of cells and other particles accumulated on the surfaces are called biofilms. They can inevitably get formed at any interface (liquid or solid) in contact with water. In the drinkable water, the biofilms are composed of functionally organised complex microbial communities incorporated in a jelly matrix of extracell polymers excreted by microorganisms. Any inorganic or organic substances that pass close to biofilms (corrosion products, clay, sand) may also be incorporated into biofilms. Biofilm development can take days or even months, subject to the organic concentration of the influent. The critical point for a successful biofilter operation consists in controlling and maintaining the biomass in a good condition on the surface of the supporting medium. Since the biofilter perfor-mance depends on the microbial activity for a long-lasting operation, a constant quantity of sublayer (organic substances and nutrients) is needed in the influent. According to the manner of using energy, the biochemical processes from the living cell, called metabolism, can be divided into: a) disassi-milation or catabolism processes, which consume organic matters and produce energy as a result of substance degradation (exothermal); b) assimilation or anabolism processes, which encompass the reactions that lead to the synthetisation of a new cell material according to the energy released in the catabolism phase (endothermal). In both phases, the extremely complex biochemical reactions take place in successive stages, each stage being catalysed by specific enzymes. The biofilm development on the surface of the solid matters in contact with water undergoes four evolution phases

- the formation of the conditioning film;
- the initial microorganisms adhesion onto the surface;
- biofilm increase;
- biofilm maturation – a balance between accumulation and detachment.

The biofilms development, morphology and physical stability are influenced by several factors: temperature and pH, the chemical and hydrodynamic conditions, the sublayer nature and nutrient supply. Any changes in the water chemistry can unstabilise the biofilm, causing its fracture. Any high flowing speeds (turbulent flow rates) can lead to biofilm shearing. Both processes may lead to the mobilisation of the microbial material through the pore system, thus determining germ enhancement in the effluent, with negative results for biofiltration. The bacteria cultures seen in the drinkable water transport and processing are linked to the substances dissolved in the water, which alongside carbon are used by them as nutrients. They are :

- bacteria from the nitrogen cycle, especially the nitrification and denitrification ones;
- bacteria from the iron and manganese cycle;
- bacteria from the sulphur cycle.

The nitrification filter (ammonia reduction)

These cultures adhere to the surface of the filtering material and consume the ammonia, which they turn into nitrites (the specific culture of *Nitrosomonas spp.*).

$$NH_4^+ + O_2 \rightarrow 2H^+ + NO_2^- + H_2O \quad (3)$$

In the latter stage, the nitrite-oxidising bacteria (*Nitrobacter)* oxidise nitrites at nitrates. The reaction speed of the nitrite oxidation in nitrates is lesser than the nitrification reaction, so that the speed of the entire reaction chain is determined by the speed of this reaction :

$$NO_2^- + 0{,}5O_2 \rightarrow NO_3^- \quad (4)$$

For these reactions to be able to take place, the concentration of the oxygen dissolved in the influent water must be sufficient, taking into account that each mg of ammonia needs 4.5 mg of oxygen. If the ammonia concentration in the influent is higher than 3 mg/l, the necessary oxygen is greater than the concentration dissolved in the influent and the difference is added to the basis of the filter, like in Figure 1. In order to keep the biofilm active, the filter must be filled with water all the time, even if it is not functioning (the exit piece in the figure). Moreover, the biofilm needs a source of organic carbon or carbon dioxide and phosphorus for breathing and growing. If these components do not exist in the incom-ing water, the indicated substances are dosed in the influent.

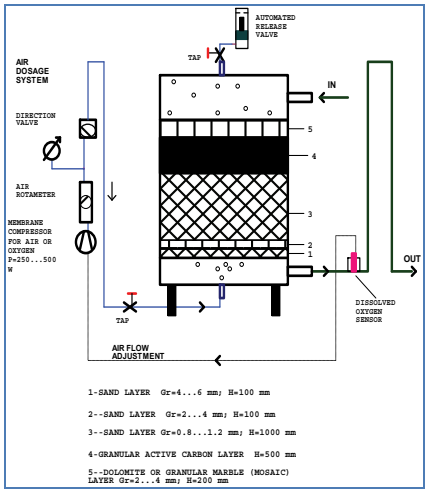

Figure 1. Theoretical scheme of the nitrification biofilter (ammonia)

The filtering layers within a nitrification filter considered from upstream to downstream are :

1. the 0.2 m high granular marble layer (granulation: 2...4 mm), meant to correct the pH and to fix the lower filtration layer – in case of acid waters
2. the 0.8 m high granulated active carbon (charcoal) layer, meant to support the biofilm and to be an additional source of carbon for it;
3. the 1 m high quartz sand layer (granulation: 0.8...1.2 mm), meant to adsorb unwanted compounds and to support the biofilm;
4. the 0.1 m high quartz sand layer (granulation: 2...4 mm), meant to support the upper filtration layers and to even the water and air flow rates;
5. the 0.1 high quartz sand layer (granulation: 4...6 mm), meant to support the upper filtration layers and to even the water and air flow rates. Total height of the filtrating layers: 2.2 m.

In case that the amount of oxygen inserted at the basis of the filter is reduced, the afore described nitrification reaction undergoes changes: the ammonia ion reacts with the nitrite ion (resulted from the nitrification reaction, as well), the result being gazeous nitrogen and approximately ¼ of the nitrate ion, as compared to the nitrification that takes place under a sufficient concen-tration of oxygen. This partial nitrification reaction, called ANAMOX, is very useful in the biofiltration step, as the concentration of the nitrate ions dissolved in the effluent is low. The Anamox reaction is given below:

$$NH_4^+ + 1{,}2NO_2^- + 0{,}066HCO_3^- + 0{,}13H^+ \rightarrow 1{,}02N_2 + 0{,}066CH_2O_{0{,}5}N_{0{,}15} + 0{,}26NO_3 + 2{,}03H_2O \quad (5)$$

In order to provide a good filter control and stability, in the sense of maintaining the partial nitrification reactions (ANAMOX), the oxygen at its basis must only be dosed as much as the process requires. This can be achieved by measuring the oxygen dissolved in the water that comes out of the biofilter and by inserting this size in a loop of adjusting the flow rate of the oxygen injected at its basis. In case that the filter is used for denitrification purposes, no air or oxygen is inserted at the basis of the filter. If there is iron and manganese in the water, they are mainly retained in the upper layers of the biofilter, in the layer of marble and active carbon. The filter is usually washed up every 2...3 weeks only with water, from the bottom upwards, under moderate conditions, that is at a flow rate approximately double the nominal one. Since the concentration of the oxygen dissolved in the effluent is lower than the minimal concentration imposed for the drinkable water, the water after biofiltration has to be enriched in oxygen, which takes place in the ozone disinfection stage, the goal being water taste correction, too.

BIOFILTRATION STAGE MODELLING

A mathematical template is a systematic attempt of conceptually translating the understanding of the real world phenomena into mathematical terms in order to assess the future statuses of the template, departing from a certain current status. That is why a template is a valuable system for testing our understanding about how a system works. By simulating various scenarios (the incoming parameters course in time), the templates allow the users to adjust such operational parameters as the processed water flow rates, the air or ozone doses, the operation times etc. at optimal levels. From the viewpoint of the course in time, the templates are static (they are not time-dependent or they are templates that reach

equilibrium) and dynamic (templates in transition).

For modelling a process produced in a a bioreactor (biofilter), like in any other case, one departs from the weight and energy conservation relation for every chemical species and for the entire system. In this case we treat all microorganisms as a system to which an average growth rate is assigned. By applying the law of conservation :

$$\frac{Accumulated\ rate}{in\ the\ volume\ V} = \frac{incoming\ rate}{in\ the\ volume\ V} - \frac{outcoming\ rate}{in\ the\ volume\ V} + \frac{Reaction-derived\ rate}{in\ the\ volume\ V} \quad (6)$$

Total weight result :

$$\frac{d(V \cdot \rho)}{dt} = \rho(Q_0 - Q_1) \quad (7)$$

where :

- V: the reactor (biofilter) volume – measured in m^3 ;
- Q_0: the incoming flow rate – measured in m^3/s ;
- Q_1: the outcoming flow rate – measured in m^3/s ;
- ρ: the liquid (water) density – measured in kg/m^3.

For a certain sublayer S (sublayer meaning a chemical substance undergoing a reaction – for instance ammonia, nitrate):

$$\frac{d(V \cdot S_1)}{dt} = Q_0 \cdot S_0 - Q_1 \cdot S_1 + r_S \cdot V \quad (8)$$

where :

- S_0: the incoming concentration – measured in kg/m^3 ;
- S_1: the outcoming concentration;
- r_s: the rate of reducing the pollutant S by the biofilm X – measured in $kg/(m^3 \cdot sec.)$

Reaction rates (speeds):

$$r_X = \mu \cdot X_1 = \frac{\mu_{MAX} \cdot S_1}{K_s + S_1} \cdot X_1$$

and

$$r_S = \frac{-r_X}{Y_{X/S}} = -\frac{\frac{\mu_{MAX} \cdot S_1}{K_s + S_1}}{Y_{X/S}} X_1 \quad (9)$$

where:

- μ: is the specific growth rate of the microorganisms
- μ_{max}: is the maximum specific growth rate of the microorganisms
- K_s :is the "half-velocity constant"

The reaction speeds r_i are reduced from the stationary status (the equilibrium one, namely dS/dt = 0 and dX/dt = 0). The reaction rates are of the Monod-type. The system of differential equations that gets formed departing from relation 8, where the reaction rates rendered by relation 9 are inserted, turns into a system of integral equations (10), which represents the sublayer and biomass course subject to time. This is the simplest biofilter template for reducing ammonia and it expresses the template transition from the initial status to the dynamic equilibrium one.

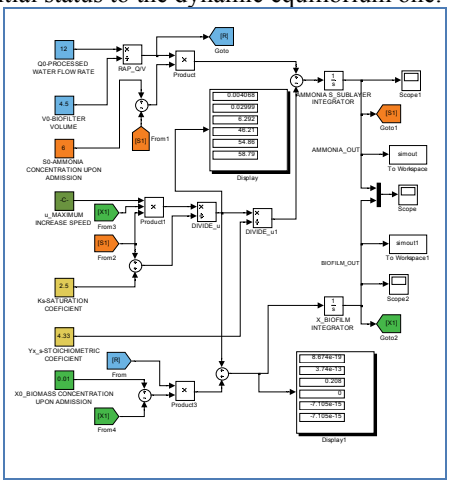

Figure 2. The Simulink template of the equation system (10)

$$dS_1 = \int \left(\frac{Q_0}{V} \cdot S_0 - \frac{Q_1}{V} \cdot S_1 - \frac{\mu_{MAX} \cdot S_1 X_1}{K_s + S_1} \cdot \frac{1}{Y_{X/S}} \right) \cdot dt$$

$$dX_1 = \int \left(\frac{Q_0}{V} \cdot S_0 - \frac{Q_1}{V} \cdot X_1 - \frac{\mu_{MAX} \cdot S_1 X_1}{K_s + S_1} \right) \cdot dt \quad (10)$$

The system of integral equations from relation 10 can be simulated by means of the SIMULINK template within the Matlab software – set out in Figure 2.

The nitrification reaction in the volume of the biofilm fixed onto the filtering material surface takes place in two steps: ammonia oxidation at the nitrites, followed by the nitrites oxidation at the nitrates. This means that the biofilter is deemed to be composed of two subsystems of micro-organisms, to which average growth rates are assigned. The sublayer penetrates inside the biofilm by diffusion. The biomass growth rates inside the

biofilm are neglected, so the biofilm is considered to be stationary. The biochemical reactions that are taken into account are rendered by the relations 3 and 4 from the paper. The oxygen requirements give rise to the stoichiometric coefficients of the reactions; thus : Y_1 = 3.5 mg O_2/mg NH_4^+ and Y_2=1.1 mg O_2/mg NO_2^-. The rates of the biochemical reactions mentioned above are rendered by relation 11 and have the form of the twofold Monod relations :

$$r_{NH_4^+} = \mu_{Max\,1} \cdot \left(\frac{NH_4^+}{K_{NH_4^+} + NH_4^+}\right) \cdot \left(\frac{O_2}{K_{O1} + O_2}\right)$$

And nitrites oxidaction

$$r_{NO_2} = \mu_{Max2} \cdot \left(\frac{NO_2^-}{K_{NO_2} + NO_2^-}\right) \cdot \left(\frac{O_2}{K_{O2} + O_2}\right) \quad (11)$$

The mass result for the system constituted from the biofilm can be written as follows:

$$\frac{Accumulated\,rate}{in\,the\,biofilm} = \frac{diffused\,rate}{incoming} - \frac{diffused\,rate}{outcoming} + \frac{Reaction-derived\,rate}{inthe\ \ biofilm}$$

For the water filters, so for biofilters too, the system of axes can be reduced to two sizes: a height axis, usually put down as x on the water flowing direction and a radius axis, usually put down as z, on the biofilm development direction. Deeming that the biofilm got developed evenly on the x water flowing direction, the result relation for the sublayer can be written in the following way :

$$\frac{\delta S}{\delta t} = D_S \cdot \frac{\delta^2 S}{\delta Z^2} + r_S \quad (12)$$

As a particularisation for each sub-layer chemical species :

$$\frac{\delta NH_4^+}{\delta t} = D_{NH_4^+} \cdot \frac{\delta^2 NH_4^+}{\delta Z^2} - r_{NH_4^+}$$

$$\frac{\delta NO_2^-}{\delta t} = D_{NO_2^-} \cdot \frac{\delta^2 NO_2^-}{\delta Z^2} + r_{NH_4^+} - r_{NO_2^-}$$

$$\frac{\delta NO_3^-}{\delta t} = D_{NO_3^-} \cdot \frac{\delta^2 NO_3^-}{\delta Z^2} + r_{NO_3^-}$$

$$\frac{\delta O_2}{\delta t} = D_{O_2} \cdot \frac{\delta^2 O_2}{\delta Z^2} - s_1 \cdot r_{NH_4^+} - s_2 \cdot r_{NO_2^-} \quad (13)$$

Like in case of the equation system 10, by means of the system 13 of partial derivative equations, a SIMULINK template is elaborated in the Matlab software. The time-dependent sublayer course in the biofilter complies with relation 14. The flow of processed pollutant (sublayer) depends on the moment from which the biofilter was commissioned. For each pollutant (ammo-nium, iron, manganese), the coefficient that represents the oxygen demand has another value, namely:

$$I_{pollutant}(t) = \frac{J_{O_2\,max}}{C_{needO_2}} \cdot \left(\frac{C_{pollutant}}{K_{pollutant} + C_{pollutant}}\right) \cdot E_0 \cdot \exp(-k_N(t) \cdot H) \quad (14)$$

where :

- $J_{POLUANT}$(t): the flow of (POLLUTANT) that enters the biofilm (g/m^2 x day) ;
- $J_{OXIGEN-MAX}$: the oxygen flow, which depends on the filtrating material characte-ristics (g/m^2 x day) ;
- $C_{OXYGEN-NEED}$: the stoichiometric coefficient of the biochemical reaction ;
- $K_{POLUANT}$: the half value of the pollutant concentration saturation value (mg/l) ;
- $C_{POLUANT}$: the pollutant concentration upon admission (mg/l) ;
- E_0 the (no size) biofilter efficiency ;
- k_N(t): the empirical constant for reducing the pollutant to time t since the biofiltration begins (m^{-1}) ;
- H: the biofiltrating layer height (m).

The previous relation lead to relation 15, which is a dynamic equilibrium one, used for designing purposes. In this relation, the parameter E_0 has to be known, as it represents the efficiency of reducing the pollutant by the biofilter, which is generally achieved after several days of biofilter operation and which depends on the hydraulic load of the filter (the water flow rate as compared to the filter surface). The values of this size come out after experiments.

$$C_{NH_4^+\,out}(t) = C_{NH_4^+\,in} \times \exp\left[-(k_{coal}(t) \cdot H_{coal} + k_{sand}(t) \cdot H_{sand}) \cdot Q^m\right] \quad (15)$$

where

Q is the specific flow rate and the value of m is – 0.68.

$C_{NH_4^+\,out}$ - ammonium concentration at the outlet

$$C_{NH_4^+\,out}(t) = C_{NH_4^+\,in} \cdot \exp\left[-(k_{biofilter}(t) \cdot H_{biofilter}) \cdot Q^m\right] \quad (16)$$

RESULTS AND DISCUSSIONS

The biofilter was tested in two stages, at a 15 m^3/h flow rate. In the first stage, the height of the active carbon layer was 0.3 m and the height of the sand layer was 1.2 m. As the results regarding the concentration of the ammonia in the effluent were not satisfactory, the height of the active carbon layer was increased to 0.65 m, the height of the sand layer being the same. The concentration of the ammonia in the influent remained constant all along the water station commissioning tests and the water from the wells preserved its parameters. Figure 3 graphically renders the ammonia concentrations calculated by means of the relation 16 upon the biofilter exit, subject to time for the aforesaid water flow rate and the measured average values.

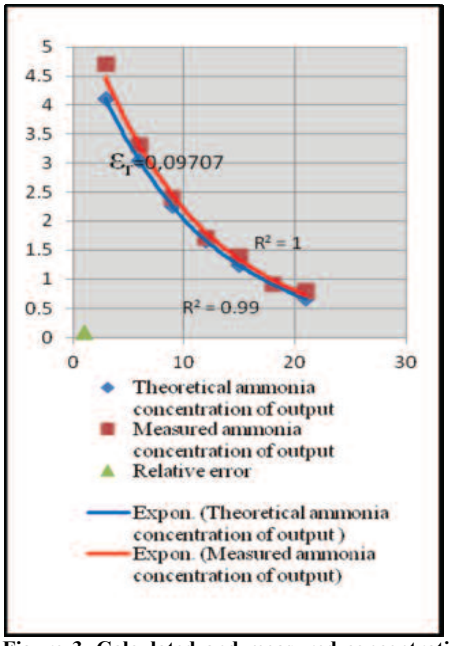

Figure 3. Calculated and measured concentration of ammonia upon exiting the biofilter : case 1

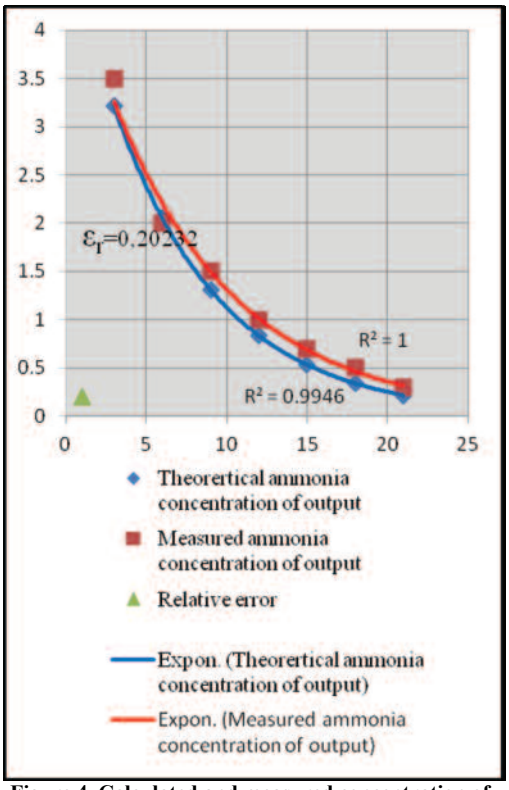

Figure 4. Calculated and measured concentration of ammonia upon exiting the biofilter : case 2

Relative error calculation formula :

$$\varepsilon_r = \sqrt{\frac{1}{n}\left(\frac{\Sigma(C_{outm}i - C_{out}i)^2}{C_{outm}i}\right)}$$

ε_r = relative error

n = number of determinations

$C_{outm}i$ = measured ammonia concentration of output

$C_{out}i$ = theoretical ammonia concentration of output

CONCLUSIONS

The result of monitoring the potabilisation station with the ammonia reduction biofilter showed that the ammonia was better reduced in the biofilter and the amount of carbon was greater.

The concentration of ammonia obtained upon the exit from the biofilter falls into the limits imposed by Act 458/2002, as supplemented by Act 311/2004, republished in 2012.

The biofiltering stage has got the following advantages:

- No chemical reacting agents are used in the treating process, as the bacteria consume the ammonia and the nitrates present in the water; in addition, no reaction toxic by-products come out.
- It is efficient in the potabilisation process by the fact that it greatly contributes to the pollutant reduction.
- The energetic consumption versus efficiency is low.

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FOOD SAFETY

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INHIBITORY EFFECT AND PRIMARY MECHANISM OF PROANTHOCYANIDINS FROM GRAPE SEEDS AGAINST ACRYLAMIDE FORMATION IN A MAILLARD REACTION MODEL SYSTEM

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Abstract

Acrylamide, a potential health hazard with carcinogenic risk, is formed during frying or baking carbohydrate-rich foods such as potatoes and cereal products. Addition of phytochemicals during food processing may be a feasible way to reduction of acrylamide formation in food processing. Proanthocyanidins from grape seeds (PGS) consist of monomeric flavan-3-ol units (the degree of polymerization DP = 2-10, sometimes > 10-25) which are mainly linked through C4 → C8 or C4 → C6 bonds. We demonstrated that a suitable concentration of PGS could cause a significant reduction of acrylamide formation in an asparagine-glucose Maillard reaction model system at pH 6.8. The presence of PGS substantially mitigated the extent of Maillard browning. Possible inhibitory pathways and primary mechanism of PGS against acrylamide formation is discussed. PGS, a kind of condensed tannins, could precipitate amino acids through complexation, thus possibly making asparagine less available for the corresponding Maillard reaction so as to reduce acrylamide formation.

Key words*: acrylamide, grape, proanthocyanidin, Maillard reaction, asparagine-glucose*

INTRODUCTION

Acrylamide is formed during frying or baking carbohydrate-rich foods such as potatoes and cereal products (Mottram and Wedzicha, 2002). It is a potential health hazard with carcinogenic risk. This issue has raised global concerns and led to extensive studies in this field (Friedman and Levin, 2008). Many studies have shown that addition of phytochemicals during food processing may be a new feasible way for reduction of acrylamide in fried and baked carbohydrate-rich foods (Zhang et al., 2009; Zhu et al., 2009). Proanthocyanidins from grape seeds (PGS) consist of monomeric flavan-3-ol units (the degree of polymerization DP = 2-10, sometimes > 10-25) which are mainly linked through C4 → C8 or C4 → C6 bonds (Fig. 1) and possess a wide range of biological activities (Xia et al., 2010). The aims of this study were to investigate the reducing effect of PGS against acrylamide formation in a Maillard reaction model system, and to elucidate its primary mechanism of action.

MATERIALS AND METHODS

The effect of different concentrations (0.001, 0.01, 0.05, 0.1, 0.5 and 1.0%; 0% as control) of proanthocyanidins isolated and purified from grape seeds (PGS > 90%) against acrylamide formation was evaluated in an equimolar asparagine-glucose model system at pH 6.8 (phosphate buffer) (Zhu et al., 2009). The relevant Maillard reactions were conducted in an oven at 120-220°C for 10-25 min.
HPLC-DAD (HP 1100 series, Germany) and LC-PDA-APCI-MS (LC-MS-2010A system, Shimadzu, Japan) were used for quantifying acrylamide and monitoring important intermediates produced via Maillard reaction in the tested samples according to the previous methods (Zhu et al., 2009; Zhu et al., 2010; Channell et al., 2008). The color changes (L^*, a^*, and b^*) after Maillard reactions of the asparagine-glucose samples added with PGS were monitored using a colorimeter (Minolta Chroma Meter CR-300, Japan).

RESULTS AND DISCUSSIONS

Figure 2 shows that PGS could significantly reduce acrylamide formation in a dose-dependent manner, with a decrease of 23.2-58.4% acrylamide compared with control, although not in a linear manner. Surprisingly and interestingly, a significantly positive PGS-concentration-dependent relationship of acrylamide inhibitory rate occurred with the PGS treatment range of 0.001-0.1%, but a slightly negative relationship with the PGS treatment range of 0.1-1.0%. In comparison with control (0% of PGS), 0.1% PGS treatment had the highest inhibitory rate (58.4%) against acrylamide formation.

It was also found that the addition of PGS in the model system considerably mitigated the extent of Maillard browning (Table 1). The *a** and *L** values are the most commonly used parameters to monitor browning, with a lower *a** value and a higher *L** value indicating a lower degree of browning.

Data obtained by LC-PDA-APCI-MS in the present study could be helpful for primary analysis of inhibitory mechanism of PGS against acrylamide formation in the model system. The proposed main pathways of acrylamide formation in asparagine-glucose model system via Maillard reaction are summarized in Figure 3, based on the review reports[8,9]). PGS, a kind of condensed tannins, could precipitate amino acids through complexation, thus possibly making asparagine less available for the corresponding Maillard reaction so as to reduce acrylamide formation. Other possible pathways for inhibitory effect of PGS against acrylamide formation (Figure 3): (1) PGS likely reacted directly with acrylamide precursors to generate new Maillard intermediates for changing the pathways of acrylamide formation; (2) PGS might influence and inhibit some key procedures of the Maillard reaction in Figure 2, such as the formation of Schiff base, Strecker type degradation, N-glucoside pathway and β-elimination reaction of the decarboxylated Amadori compounds.

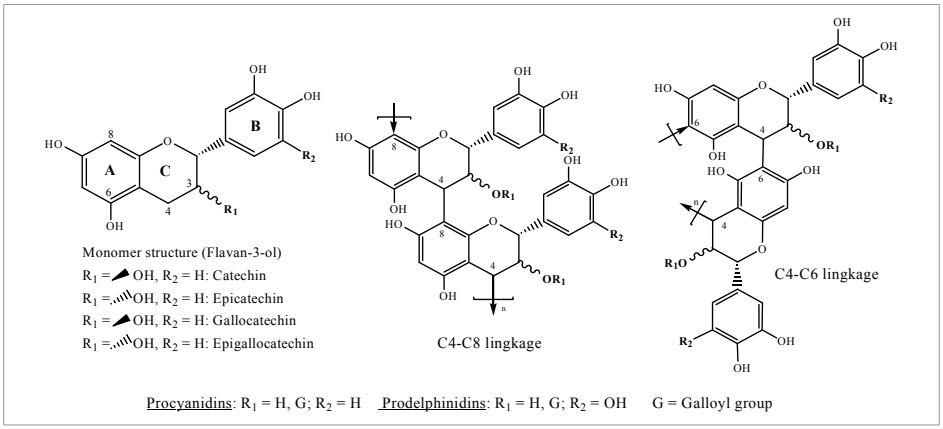

Figure 1. Basic chemical structures of proanthocyanidins (flavan-3-ol monomers and polymers) from grape seeds)

Table 1. Changes of color parameters of the asparagine-glucose samples added with different concentrations of proanthocyanidins from grape seeds (PGS) after Maillard reaction under the selected conditions

Concentration of PGS (%)	*L**	*a**	*b**
0 (control)	15.4 ±	19.3 ± 0.11	13.7 ± 0.28
0.001	17.3 ±	14.6 ± 0.26	15.6 ± 0.22
0.01	22.7 ±	11.4 ± 0.24	21.5 ± 0.18
0.05	27.5 ±	9.6 ± 0.31	24.9 ± 0.15
0.1	33.9 ±	8.1 ± 0.29	27.3 ± 0.30
0.5	30.8 ±	6.8 ± 0.38	28.1 ± 0.27
1.0	31.2 ±	6.0 ± 0.25	27.4 ± 0.32

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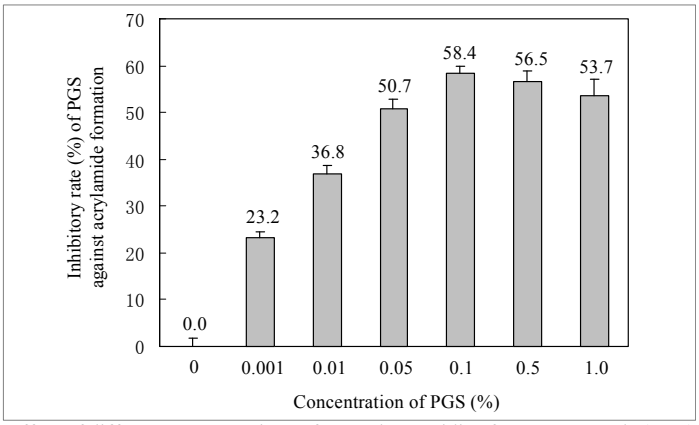

Figure 2. Inhibitory effect of different concentrations of proanthocyanidins from grape seeds (PGS) against acrylamide formation in an asparagine-glucose model system for Maillard reaction at 180°C for 20 minutes

CONCLUSIONS

Suitable concentration of PGS could cause a significant reduction of acrylamide formation in the Maillard reaction model system. The presence of PGS in the tested model system considerably mitigated the extent of Maillard browning. Possible inhibitory pathways and primary mechanism of PGS against acrylamide formation was also discussed in this study.

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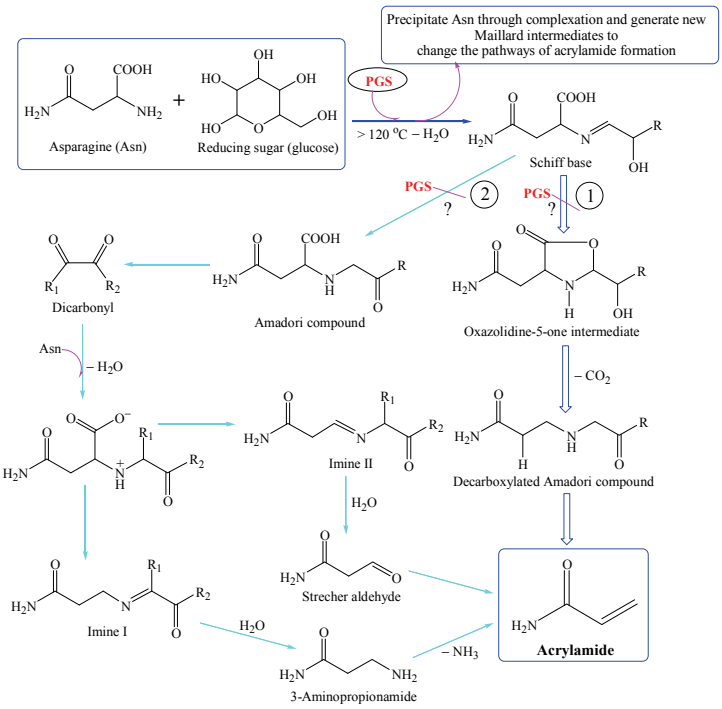

Fig. 3. The proposed main pathways of acrylamide formation in asparagine-glucose model system through Maillard reaction and the possible inhibitory mechanism of PGS against acrylamide formation

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MODERN TECHNIQUES OF ANALYSIS FOR THE PESTICIDE RESIDUES ASSESSMENT IN PLANT MATERIALS

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Abstract
Organochlorine pesticides were tested in order to establish optimal conditions for rapid detection in dried leafy matrices, using DRS software. In tests we have used dried Trifolium pratense herba fortified with OC pesticide mix, processed with modified QuEChERS method. Instrumental analysis was carried out complying Agilent "key" condition, column of 15 m, in which case the retention time for chlorpyrifos-methyl was 8,296 min. DRS allows linear calibration in the concentrations range 0,05-1 mg/kg for most of pesticides; for concentrations ≤ of 0.05 mg / kg is needed most advanced purification method or acquisition in SIM mode.

Keywords: *DRS, organochlorine, pesticides residues, Quechers.*

INTRODUCTION

The pesticide residues presence in plant products is a current problem and implementation of quick and accurate determination methods is of real importance.
Although organochlorine pesticide (OC) treatments was forbidden for the most part, however, such residues are found quite frequently in products. Monitoring OC has been justified by their persistence and the possibility of bioconcentration in organisms or extracts, through certain manufacturing stages. Nowadays, the pesticide monitoring is expanding beyond food, for example, to botanical dietary supplements (Meng et al., 2007). Food extracts for pesticide residue analysis contain a lot of chemical compounds. Consequently, one risks removing pesticides along with endogenous compounds if elaborate cleanup steps are used. So, the challenge is to detect traces of pesticides in samples that contain a lot of interferences (Wylie, 2008). Thus, monitoring of OCs residues in food becomes a routine analysis of pesticides monitoring laboratories. Official pesticides datasets showed that persistent organochlorine esidues were surprisingly common in certain foods despite being off the market for over 30 years. Residues of dieldrin, in particular, posed substantial risks in certain root crops. About one quarter of samples of organically labelled fresh produce contained pesticides residues, compared with about three quarters of conventional samples (Panseri, 2013). Although QuEChERS started out as a method for the extraction of multiclass, multiresidue pesticides from fruits and vegetables, it now is being used for a wide variety of analytes (for example, vet drugs, PAHs and antibiotics) in a wide variety of matrices (for example, plasma, meat and soil (Lehotay et al., 2010). Could be mentioned diverse applications for determination of pesticide residues, i.e. flax samples, nuts and pastry (Urairat et al., 2010), green tea, red tea and chamomile (Lozano et al., 2012) fresh spices (Sadowska-Rociek et al., 2012) honey and beeswax (Mullin et al., 2010), gingko-biloba leaf etc (Zhou, 2009). **D**econvolution **R**eporting **S**oftware (DRS) allows the identification of target compounds eliminating interferences and involves four consecutive steps: (I) noise analysis, (II) separation of pure components, (III) deconvolution of spectra, (IV) identification of

pure compounds. Determination is based on comparison with a library of spectra acquired in standard conditions. DRS is a mathematical technique that separates overlapping mass spectra into deconvoluted spectra of the individual components (Meng et al., 2007). The technique began to be used in laboratories, and the underlying problem is the limit of detection (LOD) of the subjected residues, considering that the technique is recommended for "dirty" samples analyzis. However, setting the limit at which residues can be detected is the first step to the method develop. Kirchner et al. estimated the lowest concentration of pesticide residues (mix formed by 18 residues) in non-fatty food matrix at which the residues can be successfully identified by automatic spectral deconvolution software and they found that the identification was successful at concentration levels ranged between 4 and 0.4 mg kg^{-1} in real matrix samples (apples). Also, with decreasing concentration, the number of identified pesticides and the quality of deconvoluted spectra decreased. The calculated limits of full-scan detection ranged from 0.20 ng for chlorpyrifos to 1.10 ng for captan (Kirchner et al., 2007).

MATERIALS AND METHODS

Testing technique, in order to establish the lower limit of determination was performed using red clover (*Trifolium pratense*) matrix and OC pesticide standards. Extraction was performed with modified QuEChERS protocol. Extraction step (1 g sample) was performed with 10 mL acetonitrile working variant "without citrates". Clean-up step was performed using 1 ml extract with a mixture of sorbents (50 mg Primary Secondary Amine (PSA) 12 mg active C and 150 mg MgSO4 anh) by SPD (solid phase dispersion) technique. The analysis was performed using an Agilent GC-MS equipment (7890A-5975C) - SCAN mode. The acquisition parameters were enforced by the software algorithm (HP-5MS column, 15 m x 0.25 mm, 0.25 µm, MMI Inlet - 60^{O} C (0.35 min) -900 ^{O}C/ min to 280 ^{O}C (15 min) to 900 ^{O}C to 300 ^{O}C (0 min); 2 µL injection volumes; Oven profile: 70 ^{O}C (1 min) -50 ^{O}C/ min to 150 ^{O}C, 6 ^{O}C/ min to 200 ^{O}C, 16 ^{O}C/ min to 280 ^{O}C (5 min), run time = 22 min; He, constant pressure.

Identification and quantification through DRS using the specific file associated to separation by 15m column (2Xdata1X.cal); retention time of chlorpyrifos -methyl was 8,296 min.

Steps to lock and adjust the retention time, for DRS applications are:

1) running a sample representing the analyzed mixture, which contains chlorpyrifos methyl
2) required chromatograms acquisition for the **R**etention **T**ime **L**ocking (RTL), using Chemstation software specific option
3) RT checking for chlorpyrifos methyl and its adjustment, if necessary at RT = 8.29 min
4) lock method towards chlorpyrifos methyl
5) running the standard mixture for verification

RESULTS

- Analytes can be separated and properly identified by DRS (standard chromatogram and DRS report for 1µg/ml mixture concentration is represented in figure 1 and 2 respectively).
- DRS allows linear calibration for the pesticide residues concentrations in the range 0.05-1 mg/ kg for the majority of the studied analytes .
- analyzed pesticides for which the DRS allowed quantitation at the concentration values less than maximum residue limit (MRL) **()** were: α-HCH, β-HCH, γ-HCH, δ-HCH, dieldrin, p,p-DDE, p,p-DDD, p,p-DDT, β-endosulfan, endosulfan sulphate, methoxychlor.
- QuEChERS extraction protocol and DRS quantitation enable the determination at certain concentrations for: *α*-HCH, *β*-HCH, *γ*-HCH, *δ*-HCH, heptachlor, adrin, heptachlor epoxide, chlordane-*trans*, chlordane-*cis*, dieldrin, p,p-DDE, p,p-DDD, p,p-DDT, endrin, β-endosulfan, endosulfan sulphate, endrin-ketone, methoxychlor.
- a DRS quantitation report for 1 ppm concentration in sample is represented in figure 3.

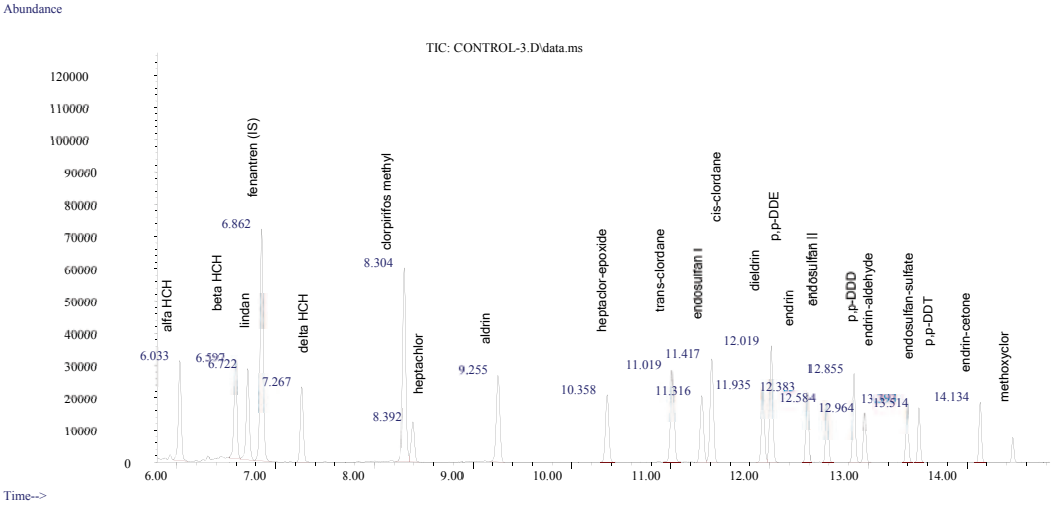

Figure 1. Standard chromatogram for 1µg/ml mixture concentration

Document

MSD Deconvolution Report
Sample Name: MIX-OC
Data File: C:\msdchem\1\DATA\2014\22.01.2014\MIX-OC.D
Date/Time: 2:45:27 PM Monday, January 27, 2014

Adjacent Peak Subtraction = 2
Resolution = Medium
Sensitivity = High
Shape Requirements = Medium

The NIST library was searched for the components that were found in the AMDIS target library.

			Amount (µg/ml)		AMDIS		NIST	
R.T.	Cas #	Compound Name	Chem station	AMDIS	Match	R.T. Diff sec.	Reverse Match	Hit Num.
4.9870	84662	Diethyl phthalate			99	1.0	94	1
6.0298	319846	BHC alpha isomer	1	0.95	99	-1.5	88	1
6.5898	319857	BHC beta isomer	1	0.87	97	-1.2	89	1
6.7155	58899	Lindane	1	0.98	84	-1.8	84	3
6.8571	1517222	Phenanthrene-d10			100	-0.7	84	2
7.2621	319868	BHC delta somer	1	0.89	98	-1.2	90	1
7.9729	84695	Diisobutyl phthalate			86	3.1	80	30
8.3002	5598130	Chlorpyrifos Methyl	1.9	1.74	98	0.4	90	1
8.3882	76448	Heptachlor	1	0.83	91	-1.2	83	1
9.254	309002	aldrin	1	0.59	95	-0.9	85	1
10.3569	1024573	Heptachlor exo-epoxide isomer B	1	0.57	95	-0.2	86	1
11.0128	5103742	trans-Chlordane	1	0.88	97	-1.0	88	1
11.3122	959988	Endosulfan (alpha isomer)	1	0.97	88	-0.8	81	2
11.4137	5103719	cis-Chlordane	1	0.92	96	-0.3	86	2
11.933	60571	dieldrin	1	0.81	86	0.0	90	1
12.0165	72559	p,p'-DDE	1	0.91	99	0.7	89	1
12.1646	53190	o,p'-DDD			82	-1.4	73	6
12.3764	72208	Endrin	1	0.74	90	0.5	81	1
12.5812	33213659	Endosulfan (beta isomer)	1	0.82	88	0.3	81	2
12.8531	72548	p,p'-DDD	1	0.91	99	1.2	87	1
12.9602	7421934	Endrin aldehyde	1	0.8	86	0.6	83	1
13.391	1031078	endosulfan sulfat	1	0.55	92	1.4	79	1
13.5099	50293	p,p'-DDT	1	0.89	96	2.3	85	2
14.1286	53494705	Endrin ketone	1	0.85	85	1.8	78	1
14.4592	72435	Methoxychlor	1	0.81	90	3.4	78	1
6.859		fenantren	1					

Figure 2. DRS report for 1µg/ml mixture concentration

MSD Deconvolution Report
Sample Name: proba TRF-OC-1 mg/kg
Data File: C:\msdchem\1\DATA\2014\22.01.2014\TRF-1.D
Date/Time: 10:04:53 AM Tuesday, January 28, 2014

Adjacent Peak Subtraction = 1
Resolution = Medium
Sensitivity = Medium
Shape Requirements = Medium

The NIST library was searched for the components that were found in the AMDIS target library.

			Amount (mg/kg)		AMDIS		NIST	
R.T.	Cas #	Compound Name	Chem station	AMDIS	Match	R.T. Diff sec.	Reverse Match	Hit Num.
2.6818	87627	2,6-Dimethylaniline			57	9.9	62	25
2.7069	91203	Naphthalene			64	4.1	79	2
3.1240	89838	Thymol			96	7.4	88	3
3.4080	97530	Eugenol			95	1.2	88	1
3.9490	131113	Dimethylphthalate			61	-0.4		
3.9490	33933798	4-Octanol, 2,4-dimethyl-					78	1
4.2953	33704619	Cashmeran			68	-1.8	66	49
4.9964	84662	Diethyl phthalate			100	2.1	96	1
5.3409	119619	Benzophenone			97	0.9	91	1
6.0317	319846	BHC alpha isomer	1	0.96	93	-1.2	88	1
6.5908	319857	BHC beta isomer	1	0.77	85	-1.1	77	3
6.7184	58899	Lindane	1	0.56	89	-1.5	82	3
6.9046	85018	Phenanthrene			55	-0.8		
6.9046	0000	Benzo[f]isoindol-1,3(1H,3H)-dione, 3a,4,9,9a-tetrahydro-4,9-O-benzeno-2-hexyl-					57	1
7.2668	319868	BHC delta isomer	1	0.95	89	-0.6	83	2
7.6639	106025	Exaltolide [15-Pentadecanolide]			45	13.1		
7.6639	502692	2-Pentadecanone, 6,10,14-trimethyl-					94	1
7.9721	84695	Diisobutyl phthalate			100	2.9	88	1
8.2937	5598130	Chlorpyrifcs Methyl	1.88	1.79	83	-0.3	77	1
8.3831	76448	Heptachlor	1	0.74	72	-1.8	66	1
9.2085	84742	Di-n-butylphthalate			63	0.1	72	53
9.2518	309002	Aldrin	1	0.84	76	-1.5	65	1
10.355	1024573	heptaclor epoxid	1	0.41	76	-0.4	64	1
11.0124	5103742	trans-Chlordane	1	0.77	84	-1.1	72	2
11.3062	959988	Endosulfan (alpha isomer)	1	0.31	59	-1.5	53	2
11.4105	5103719	cis-Chlordane	0.99	0.88	84	-0.7	72	3
11.9323	60571	Dieldrin	1	0.91	56	-0.3	66	2
12.0157	72559	p,p'-DDE	1	0.94	96	0.6	89	1
12.1939	53190	o,p'-DDD			60	2.2	42	24
12.3816	72208	Endrin	1	0.95	63	1.1	57	1
12.5799	33213659	Endosulfan (beta isomer)	1	0.64	64	0.1	61	1
12.8499	72548	p,p'-DDD	1	0.9	92	0.8	85	1
13.3876	1031078	Endosulfan sulfate	1	0.67	73	0.9	64	1
13.5095	50293	p,p'-DDT	1	0.83	86	2.2	75	4
14.1273	53494705	Endrin ketone	1	0.88	46	1.6	54	2
14.4597	72435	Methoxychlor	1	0.88	82	3.4	70	2
14.8605	117817	Bis(2-ethylhexyl)phthalate			93	4.4	86	3

Figure 3. DRS quantitation report for 1 ppm concentration in sample

CONCLUSIONS

- DRS is a time saving method which allows the determination of the OC residues in complex matrices.
- In case of exceeding the MRL, DRS can be used to confirm the results for most pesticides residues.
- Using the DRS at lower detection limits is conditioned by the optimization of the clean-up method and/ or the use of SIM (**S**elected **I**on **M**onitoring) mode acquisition.

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ROMANIAN CONSUMERS' BEHAVIOR ON PACKAGING MATERIALS RISKS PERCEPTION

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Abstract

Romanian consumers' concern for food safety and health has increased in recent years due to media news about cases of illness due to the use of improper packaging of various food stuffs.
Also, environmental protection actions have contributed to the development of research in the direction of obtaining biodegradable packaging materials but also to educate consumers to use and to promote "eco"packaging concept
Our study indicates that the consumer is interested to purchase packaged foods, without incurring increased costs due to the use of a package, to improve the quality of life,and to maintain a good health status.
The European Commission indicates to mobilize municipalities towards the achievement of 2020 target 50% recycling to improve waste management and to reduction the amount of disposas.

Keywords: consumers' behavior, biodegradable materials.

INTRODUCTION

In recent years, Romanian consumer faced with the situation of the economic recession, and changed his behavior, trying to save money, but also sometimes consume more than it can afford. These approaches creates a personal duality which is reflected in price increases and shortages of consumer goods.
According to studies carried out in 2010, GfK consumer cuts in expenses that have with clothing and footwear in a percentage of 32%, to eat in the city (23%), while 25% have saved and have made investments. It can be concluded, regarding the changes of behavior visible in 2010 so far, that the Romanians buying increasingly more cautious, no more deviations from their priorities list for shopping and postponing major shops.
Recent studies conducted by the Unilever highlight that 56% of Romanian consumers do not take into account the environment-friendly packaging when buying a product. The major objective of our study is to analize how Romanian consumers react about packaging materials used on food stuffs.

MATERIALS AND METHODS

It was created a research theme aiming high educated consumers from urban environment, to achieve a objective observation of consumers toward Romanian awareness materials used for food packaging. For this, we choose a set of questions about social criteria's and technical criteria's; we conducted a questionnaire investigation on 50 persons involved in this study and interpretation by quantitative methods of the obtained results. Among the questions it was elaborated a measurement scale about level of importance in food packaging technical characteristics and use the semantic scale technique.

RESULTS AND DISCUSSIONS

Subjects questioned have answered to a number of 9 questions regarding their personal data for their engagement in social demographic range, but also questions relating to consumer risks perception-

correspondence materials used for packaging of food products.

Partial or total transparency of the package was marked as very important by 60% of the subjects, which leads to the conclusion that the visibility of the product is very important to most consumers.

How important is protection of the environment and how we protect the environment fails to constitute a priority in the selection of food products due to this issue, only 40% of the subjects were scored as being very important, with the maximum score, for this issue.

Packaging manufacturers are permanently studying newest packaging materials and make considerable efforts to provide consumers with better quality products. What opinion have Romanian consumers about "environment-friendly" materials?

In the Figure 1 was represented the distribution of opinions from unimportant (0) to very important (8) related to the materials considered to be environmentally friendly.

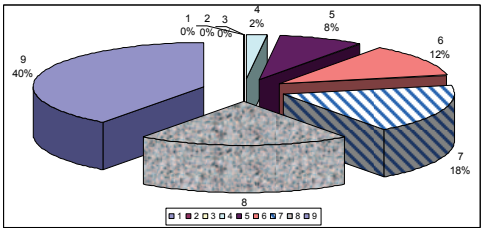

Figure 1. The importance of environmental issues in consumer opinion

The importance of environmental protection in the opinion of questioned consumers are reflected in the following ways: in favor of re-use (40%), in favor of recycling (60%) and in favor of biodegradable materials (58%).

As it can be seen in the Figure 2, 64% of the subjects mark as very important increasing the product shelf life by means of packaging.

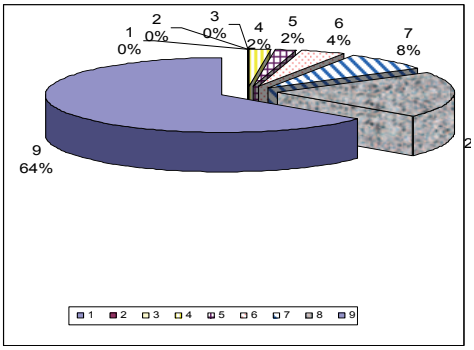

Figure 2. The importance of increasing food product shelf life in the consumers ' opinion

Most consumers (54%) want to buy food that is packed in Ziplock bags.

Table 1

The importance of using Ziplock packages

The subjects ' opinion	Points	% achieved
No opinion	0	2
Unimportant	1	6
Diferent levels of importance	2	0
	3	4
	4	4
	5	6
	6	4
	7	20
Very important	8	54

The packaging system Ziplock can be used several times. The thickness of the Ziplock packages protecting objects stored in them.

According to the study conducted for the Ministry of Agriculture and Rural Development in 2009 the Romanian consumer behavior Analysis of meat products, 61,6% of consumers paid their attention to packaging and labelling, the clarity of information and especially the contents of the respective product information is particularly important.

From this question how interested is the consumer to know the technical characteristics of the packaging when they are buying food stuffs, has been assessed. Subjects questioned responded with a tremendous majority (92%) they are familiar with this, 67% stating the information obtained in this respect helps in choosing the product they have purchased. The data collected are described in the Figure 3.

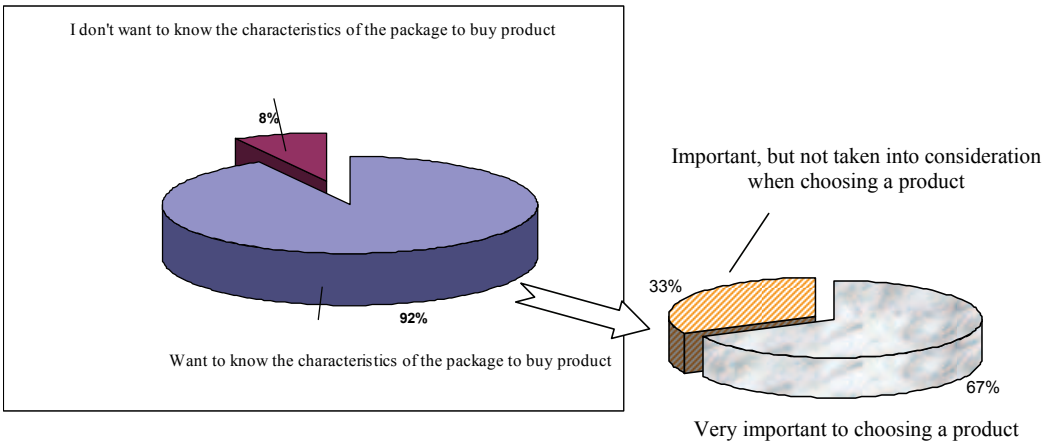

Figure 3. Importance of knowing the technical characteristics of the package in the decision to purchase the product

Generally speaking, any material in contact with food should not release the chemicals in foods at levels that can cause harm to human health (EU 1935/2004, article 3 and the U.S. 21CFR174). To be able to assess the risk associated with food packaging should be better understand the chemical composition of packing material and the levels at which these compounds can migrate into the food; however these issues are solved by the packaging material producers just meeting the legislation requirements for the food contact materials.

How Romanian consumers are preocuppied about this information and what opinions their have can be found in the answers to the following question:

- Do you consider that, at present, the materials used for packaging affect your health?
- The scores scale was from 1 (very unfavorably) to 5 (very favorable) for consumer health relationship with food packaging materials.

For the first question, the answers were Yes for 38% of subjects, no for 20% of subjects and I don't know for 42% of the subjects, as it can be seen in the Figure 4.

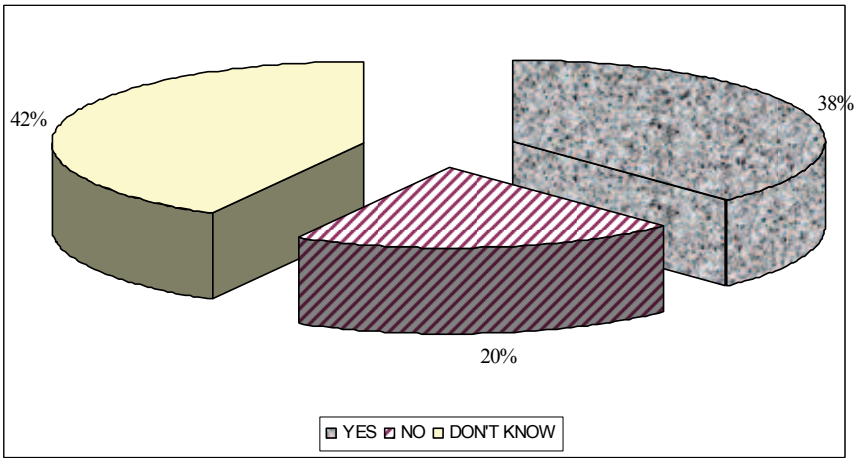

Figure 4. Consumers ' opinion about the health risk of the food packaging materials

About the consumer health relationship with food packaging materials, each 2% of the respondents scored as very unimportant and inconsequential the relationship quality of packaging materials -consumer health, 36% marked as important this relationship and very important for 28% of subjects (Figure 5).

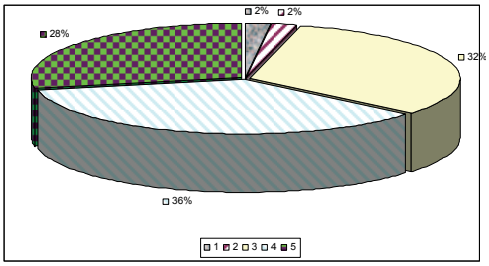

Figure 5. Consumer health relationship to-quality of food packaging materials

Tremendous majority is represented by the subjects who are buying individually packed food (92%) and only 8% of subjects are buying foods packaged in large packages or large packages with individual servings (Figure 6).

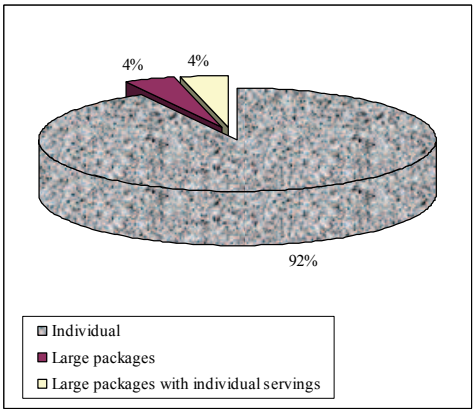

Figure 6. Consumer opinion on type of packaging of food

Consumers are not willing to pay more for food packaging that contributes to improve the quality of life compared with conventional ones in proportion of 42%.
Compared to these, 30% agree with a higher price to 5%, 22% agree with a price increasing between 5-10%, and 6% are in agreement with the higher price between 10-20%.

CONCLUSIONS

The consumer is interested to purchase packaged foods, more safety for the health but without any additional costs due to the use of more sustainable materials which should improve the quality of life. Nearly half of respondents don`t know whether packages used today affect their health, but most of them would like to read the proper information on the label of the purchased food product.
An increasing number of consumers appreciate Ziplock packages, so to consume foods in smaller portions, maintaining at the same time the product quality. Protection of the environment, in our opinion is not a priority in the opinion of consumers when they are choosing packaged foods.

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INHIBITORY EFFECTS OF ESSENTIAL OILS WITH POTENTIAL TO BE USED IN FOOD INDUSTRY

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Abstract

Lately, essential oils are trying to be used in food industry because of their biological activity. Essential oils (EOs) are natural compounds with a complex composition which have the potential to be used as antimicrobial agents in food system due to the fact that contain active principles present in plants, as many research have been already demonstrated.
The objective of this study is to show that EOs have the potential to be used in food system like antifungal agents by inhibiting the growth of pathogenic moulds.
The method used to evaluate the in vitro inhibition effect of essential oils is Agar diffusion disk method. A known amount of limonene, nonane, pinene and nerol was applied on 6 mm diameter disks and placed onto the agar surface where an inoculum with filamentous fungi from the genus Aspergillus was spread. After this, the plates were incubated for 9 days at 25^{0}C under aerobic conditions. By evaluating and measuring the colony diameter of the fungus colonised daily and comparing it with the diameter of the control it could be told that nerol and pinene have antifungal activity.
Essential oils can be used in consumer goods as a natural alternative to conventional food preservatives because it can prevent the spoilage of the products and can increase the shelf life, this way ensuring the microbiological safety of food products.

Keywords*: antimicrobial, essential oils, food processing, microorganism inactivation*

INTRODUCTION

The contamination of spoilage microorganisms during storage is a major problem for the food industry and consumers. Fungi, especially some *Aspergillus* species, are responsable for spoilage and poisoning of food, having the ability to grow in various climates (Garcia et al., 2011). It is established that the microorganisms from the *Aspergillus* genus represent very serious risks for the health of consumer because they produce dangerous mycotoxines, which are a chemical risk in food products. *Aspegillus niger* and *Aspergillus ochraceus* are known to produce ochratoxine (OTA). The mycotoxin OTA derives its name from *Aspergillus ochraceus*, the first mould from which it was isolated. It is the main toxic component in cultures of this mould, but it is also produced by other ubiquitous moulds such as various strains of *Aspergillus* and *Penicillium* (Meca and Ritieni, 2009). *Aspergillus flavus* is known to produce aflatoxins which are a group of common, extremely hazardous, and carcinogenic metabolites (Passone et al., 2012).

Curently, the demand of consumers to reduce or eliminate these food-related microorganisms during the shelf life of food products is growing. This fact has led to the research and development of alternative treatments. There is a strong debate about the safety aspects of chemical preservatives since they are considered responsible for many carcinogenic and teratogenic attributes, as well residual toxicity. For these reasons, there is an increasing interest in the use of natural compounds, antimicrobial agents from herbs and plants. Antimicrobial properties of herbs and spices have been recognized and used since ancient times for food preservation and in medicine (Omidbeygi et al., 2007). EOs may be an alternative to common chemical control agents because they constitute a rich source of bioactive compounds (Burt, 2004) which can reduce the environmental risk, increase the shelf life and safety of food products and satisfy the consumer's request.
According to the 8th Edition of the French Pharmacopeia (1965), EOs are products of complex general composition that contain volatile principles present in plants, more or less modified during their preparation.

The composition of EOs includes a complex mixture of several compounds. In general, most chemical components of essential oils are terpenoids, including monterpenes, sesquiterpenes, and their oxygenated derivates, all characterized by low molecular weight. Terpenes are among the most active antimicrobial compounds of essential oils (Bakkali et al., 2008, Tian et al., 2012).
Volatile oils contain two or three major components at high concentrations (20-70%) compared to the other components which are found in trace amounts. Mono- and sesquiterpenoids are the major components of essential oils, which are known as phenolic compounds in nature. Aromatic compounds occur with less frequency than the terpenes, but the antimicrobial effect of essential oils depends on the content of phenolic components (Cakir et al., 2004).
Several authors have demonstrated the antifungal activity of plant extracts and their ability to inhibit mycotoxin production (Rasooli et al., 2006, Tzortzakis et al., 2007, Tian et al., 2011, Phillips et al., 2012, Tian et al., 2012, Ferreira et al., 2013). In addition, they have attempted to elucidate the effect of bioactive chemicals on growth and morphological features and on primary and secondary fungal metabolism (Ferreira et al., 2013).
The present study aimed to determine the antifungal efficacy of 4 essential oils (nonane, pinene, nerol and limonene) against three common food spoilage-related fungi from the genus *Aspergillus* (*A. ochraceus*, *A. niger*, *A. flavus*) with emphasis for the future use of the essential oils as alternative antimould compounds.

MATERIALS AND METHODS

1. Essential oils
The commercial EOs used in this study were purchased from MERCK and Sigma-Aldrich. S-(-)-Limonene for synthesis, n-Nonane, 1 S-(-) -α-Pinene is from Merck. Nerol 97% and Malt Extract Broth is from Sigma Aldrich Fluka.

2. Microbial strains and growth conditions
Three food spoilage-related microorganisms were used to assess the antifungal properties of essential oils used in this study. All strains of *Aspergillus* (aflatoxin producer *Aspergillus ochraceus* mi 152, *Aspergillus flavus* 3-88 and *Aspergillus niger* 3-200) were provided from the collection of Faculty of Biotechnology, University of Agronomic Sciences and Veterinary Medicine Bucharest and maintained on Malt Extract Broth at 25^0C for 15 days. Spore suspensions were prepared and diluted in sterile water to a concentration of approximately 10^6 spores/ml. Spore population was counted using a haemocytometer.

3. Agar disk diffusion assay
The EOs were screened for antimicrobial activity using the agar disk diffusion method and 3 target microorganims. Malt Extract Broth was sterilized in an autoclave and cooled to 45-50^0C before being poured into 90 mm Petri dishes. After solidifying, under aseptic conditions, in the middle of each Petri dish was seeded 2 µl inoculum suspension of each microorganism. Sterile filter diskes Whatman (6 mm diameters) containing 0.5/1/2/5/7,5/10µl of pinene, nonane and limonene, respectively 0.25/0.5/1/2 µl of nerol were applied to the surface of agar plates. Control plates (without essential oils) were inoculated using the same procedure. All inoculated plates were incubated at 25^0C for 9 days. Fungal growth was visually appreciated by measuring the colony diameter of the fungus daily based. The diameter of the growing fungal colonies was measured with a rule, in two directions at right angles to each other to obtain the mean diameter for each colony. The values were expressed in mm diameter/day. The growth of fungal cultures containing different concentrations of all EOs was compared with that of the control culture that was grown with no EOs. Samples were examine in duplicate.

4. Statistical analysis
Data were analyzed statistically using analysis of variance (ANOVA) and differences among the means were determined for significance at $P<0.05$ by SPSS software. All experiments were repeated twice.

RESULTS AND DISCUSSIONS

The activity of the four essential oils used in this study was evaluated against the strains *A.*

ochraceus, *A. niger* and *A. flavus*. The best result were obtained for nerol and pinene.

Mycelia growth of the three fungi species treated with nerol during the nine days incubation at 25^0C are shown in figure 1. The results indicated that mycelia growth was significantly ($P<0.05$) influenced by incubation time and essential oil concentration. Mycelia growth was considerably reduced with increasing concentration of essential oil while their growth increased with incubation time. It is evident that nerol exhibited both fungistatic and fungicidal activities on the test molds, depending on the concentration used. From figure 1 it can be noticed that nerol completely inhibited the growth of all the food spoilage fungi tested when it was used in a higher concentration (1 and 2 µl). In the case of *A. flavus*, nerol exhibited fungicidal activity starting with the concentration of 0.5 µl. The mycelia growth was retarded by 4 days for *A. flavus* when it was used the smallest concentration of nerol (0.25 µl nerol). The mycelia growth inhibition percentage was determine at day 9. When nerol oil was used in a concentration of 0.25 µl it significantly reduced mycelium growth of *A. ochraceus, A. niger* and *A. flavus* (61.82%, 19.43%, and 63.14% reduction respectively). When used in a higher concentration at 0.5 µl, the percentage reduction was 73.96% for *A. ochraceus*, 44.53% for *A. niger*, respectively 100% for *A. flavus*. The fungal inhibition observed when nerol was used may be caused by the hydroxyl group present in this compound that can form hydrogen bonds with active enzymes resulting the deactivation (Tian et al., 2011).

In the case of nonane and limonene, these essential oils didn't inhibit the mycelial growth of the tested microorganisms. Limonene is a terpene that can be found in citrus oil. It has been reported that his antifungal activity is weakly inhibitory against *A. niger* (Moleyar et al., 1986). Caccioni (1998) showed in his study that the antimicrobial effect of citrus oil was produced by the synergy between monoterpenes other than limonene and sesquiterpene content. In a study made by Matan N. and Matan N. (2008) it was shown that lime oil and tangerine oil, which contain limonene as their main constituents can inhibit the growth of *A. niger* but the concentration needed is higher than that used to inhibit *Penicillium sp.*

In a study realized by Phillips et al. (2012) it was tested the effect of the citrus EO vapour on mycelial growth and spore germination of the *A. niger* in culture and also determined the growth of this mould on grain. The citrus EO vapour under test (Citri-V™®) contains limonene which is being present in the highest quantities and linalool, citral and β-pinene also present and the later three components having been previously identified as the active antimicrobial compounds. A 15 min treatment with the citrus EO vapour used in this study reduced growth of *A. niger* in culture by 66.9%, although the citrus EO vapour was an effective treatment to reduce the growth of *A. niger* by 50% on grain over 10 days, suggesting its possible use in reducing spoilage in grain by this specie, especially as this treatment has previously been shown not to affect the organoleptic properties of raw vegetables. Similar results were obtained by M. Viuda-Martos et al. (2008) who showed that essential oils of lemon, mandarin, grapefruit and orange inhibited completely the growth of *A. niger* when a concentration of 0.94% of any of these EOs was used. Orange EO is the most effective against *A. niger*, while mandarin is the best inhibitor of *A. flavus*.

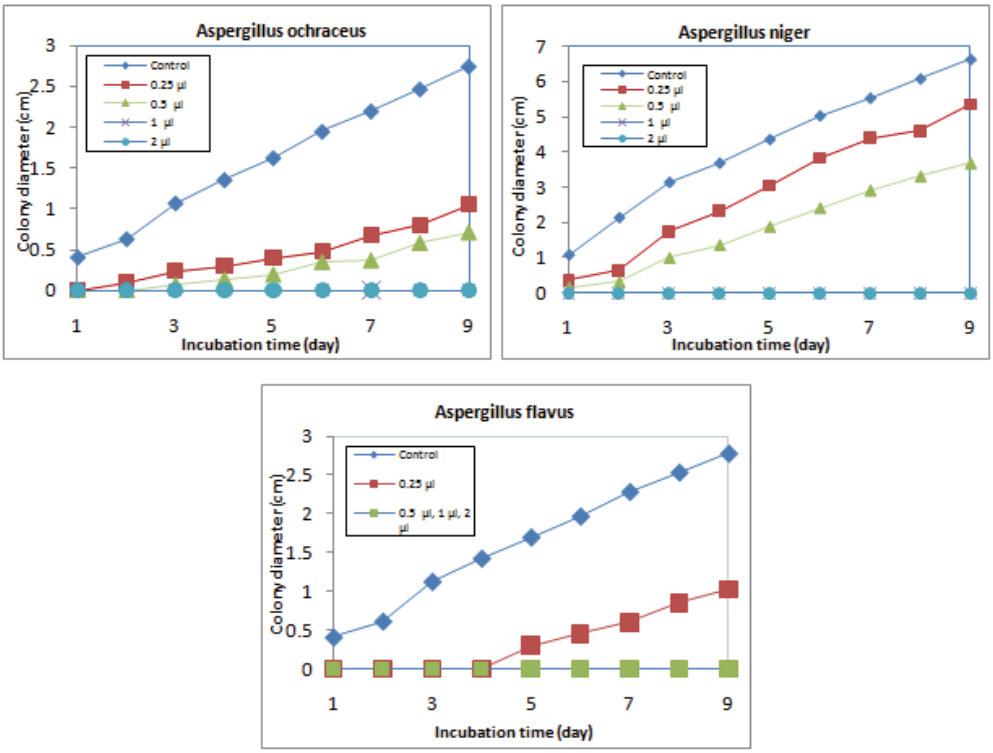

Figure 1. Effect of different concentrations of nerol on colony growth of *A. ochraceus*, *A. niger* and *A. flavus* raised in MEA. Plates were incubated at a temperature of 25^{0}C for 9 days.

When pinene was used against the tested moulds, it was noticed that this monoterpene has antifungal activity only against *A. flavus*. From figure 2 it can be seen that when the concentration of pinene was higher (7.5 and 10 µl), this monoterpene had fungicidal activity. The antifungal activity of pinene was in direct ratio to the amount of pinene added to the tested samples. The mycelia growth of *A. flavus* was reduced with a percentage reduction of 17.30% for 2 µl pinene, respectively 26.28% for 5 µl. Similar results were obtained by Lopez-Malo et al. (2007) who showed that cinnamon extracts have fungistatic effect when the concentration was smaller and fungicidal effect at higher concentrations. Colony diameter decreases with the increasing antimicrobial concentration.

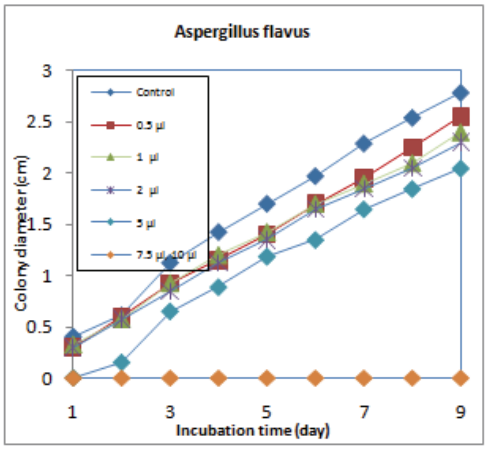

Figure 2. Effect of different concentration of pinene on colony growth of *A. flavus* raised in MEA. Plates were incubated at a temperature of 25^{0}C for 9 days.

Several authors study the mechanism of inhibition of *Aspegillus* growth at ultra-structural level. Rassoli et al. (2006) showed with a transmission electron microscopy (TEM) that *A. niger* exposed to MIC levels of *Thymus eriocalyx* and *Thymus x-porlock* presented irreversible damage to cell wall, cell membrane and cellular organelles. The mycelium exposed to the thyme oils showed morphological changes in the hyphae, plasma membrane

disruption and mitochondrial destruction. In accordance with this study, Tolouee (2010) reported the same result when it was used *Matricaria Chamomilla* L. flower essential oil against *A. niger.* When it was used a scanning electron microscopy, the major changing observed were swelling and deformation of hyphal tips, formation of short branches, and collapse of entire hyphae. A change in cell permeability might result in an imbalance in intracellular osmotic pressure, subsequent disruption of intracellular organelles, leakage of cytoplasmic contents and finally cell death.

CONCLUSIONS

In the present study, nerol and pinene were reported as potential inhibitors of the in vitro *Aspergillus* tested strains.

The results of this study showed that only nerol and pinene have antifungal activity, the highest effect on the growth of the three tested species of *Aspergillus* being demonstrated by nerol. Nerol exhibited 100% inhibition of the growth of *A. ochraceus*, *A. niger* and *A. flavus* when it was used in a higher concentration (1, 2 µl). It can be concluded that nerol can inhibit ochratoxigenic and aflatoxigenic moulds. Due to the antifungal activity of nerol, it can be exploited as a suitable alternative to chemical additives for use in food industry, attending to the needs for safety and satisfying the demand of consumers for natural components.

Pinene also can be an alternative antifungal agent, our study showing that the highest action of this essential oil was against *A. flavus.*

These oils could be used as food preservatives in some food products in which *A. flavus*, *A. niger* and *A. ochraceus* growth and potential production of mycotoxines are considered healthy hazards. For the practical use of these oils as novel fungal-control agents, further research is needed on safety issues for human health. It is also necessary to establish the level of essential oil needed to inhibit the fungal growth in food matrix, knowing that in food products the amount of essential oil is higher than *in vitro*, most probably due to interactions between phenolic compounds and the food matrix.

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WINE TRACEABILITY AND AUTHENTICITY – A LITERATURE REVIEW

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Abstract

In the actual economic context, the agro-food economy is focused on the consumer demands regarding quality, safety and security of food and foodstuffs. Traceability of wine can be defined as a method through which anybody in the wine supply chain can be able to verify the origin and composition of each batch of wines, its conditions of storage, and all the products that were in contact with the wine after the production. Traceability in the wine industry has an important role in a quality assurance management system. It ensures the registration on specific documents of all manipulations of raw materials, ingredients and final products. They are created especially to allow a rapid identification of the product history. The wine supply chain requires traceability from grape production to processing and wine distribution. Authenticity of wine has been extensively investigated because wine is an easily adulterated product due to its chemical composition and its availability throughout the world. Responsible and continuous controls are required to maintain the quality of wine. Usually volatile compounds are used to characterize varieties, whereas minerals are used for geographical differentiation. Amino acids as well as phenolic compounds evaluation are used for both. The development of advanced techniques for wines authentication is a challenge, which currently is given a special attention. In this literature review, latest scientific papers on this subject will be assessed in order to establish the state of the art in the proposed field and to establish further research needed.

Keywords: *wine, traceability, authenticity, food safety.*

INTRODUCTION

Wine is a beverage which value is influenced by many factors amongst which the origin, vintage, grape variety and the growing condition play the major role. Wine is characterized by a wide variety of sensory characteristics. The information indicated on the wine label is connected to special consumer's expectations concerning the sensory and quality criteria. The label may indicate, therefore, significant and price-determining characteristics such as the brand, type, vintage, variety and origin of the product, which represent the wine identity. Wine control is traditionally strongly associated with proof of authenticity.

Generally, chemical falsifications to deal with are the addition of water, glycerol, alcohol, dyes, sweeteners, flavor substances, the non-authorized addition of sugars, preservatives and acidity adjustments. However, wine authentication aims to confirm all declarations of label descriptions or to detect fraudulent statements by various analytical methods (Schlesier et al., 2009).

The analysis of wine is of great importance since wine components strongly determine its stability, organoleptic or nutrition characteristics. In addition, wine analysis is also important to prevent fraud and to assess toxicological issues (Grindlay et al., 2011).

The great number of parameters affecting wine quality has initiated the development of different protocols for analysis. Wine constituents are strictly regulated by international organizations (OIV, 2014) or government agencies to avoid fraud and health risks. Luque de Castro et al. (Luque de Castro et al., 2005) have recently reviewed methods of analysis for the most commonly determined parameters in wine such as ethanol, sulphur dioxide, reducing sugars, polyphenols, organic acids, total and volatile acidity, Fe, soluble solids, pH and color (Grindlay et al., 2011).

For the wine industry and market sector, it is particularly essential that the intended value traits created via genetics (variety), origin of production (typicity), and unique inputs or

processing method (vinification technology) are preserved. In other words, it must be ensured that a product's label is accurate and not misleading, since consumers distinguish peculiar commodities from a mass of other similar ones, on the belief that they bear a superior quality. However, wine is a product that can be easily adulterated, and for this reason wine authenticity is guaranteed by strict guidelines laid down by responsible national authorities, and includes sensory evaluation, chemical analyses, and examination of the records kept by wine producers (Makris et al., 2006).

The EC regulations dealing with geographical indications (GI) all include traceability provisions. These are built into the provisions dealing with certification schemes and the various requirements concerning the overall control of the producers' operations.

For example, Article 118p of EU Regulation 1234/20073 incorporated EU Regulation 479/2008 (dealing with wine) provides for annual verification of compliance with GI product specifications by relevant bodies (e.g. product certification bodies, public authorities, etc.). In all cases, producers will need to be able to show inspectors how and where products were produced using reliable traceability systems.

In the certification and control processes provided for GIs under these EU regulations, traceability is a core element. Only by recording the origin of the various components and tracking the production chain it is possible for the certification and control bodies to verify, certify, and monitor whether the final product actually has the claimed link with its geographical origin and conforms to the relevant specification and quality requirements (Sciarra and Gellman, 2012).

Traceability is defined as the ability to follow a product batch and its history through the whole, or part, of a production chain from raw materials through transport, storage, processing, distribution and sales (called chain traceability) or internally in one of the steps of the chain, for example the production step (called internal traceability). Traceability of products has been introduced since the 1990s (Cimino and Marcelloni, 2012) and is still under investigation by scientific and industrial bodies (Bevilacqua et al., 2009) (Gandino et al., 2009). A number of traceability systems, technologies and standards have been developed to carry out supply chain traceability and internal traceability, with different business objectives (Bechini et al., 2008) (Bertolini et al., 2006). Nevertheless, only large enterprises, which are characterized by a tightly aligned supply chain and supported by a considerable use of information and communication technology, employ very efficient and fully automated traceability systems. On the contrary, small enterprises only rarely implement traceability and, when they do, they add the traceability management to their normal operation, decreasing the efficiency and increasing the costs. Thus, today, a considerable challenge is to develop agile and automated traceability platforms for communities of small-scale enterprises (Cimino and Marcelloni, 2012).

Figure 1 highlights the main actors of the supply chain. Each actor is responsible for specific activities which have to be traced so as to enable supply chain traceability. For each actor, the activities and the corresponding data which have to be collected are described as follows:

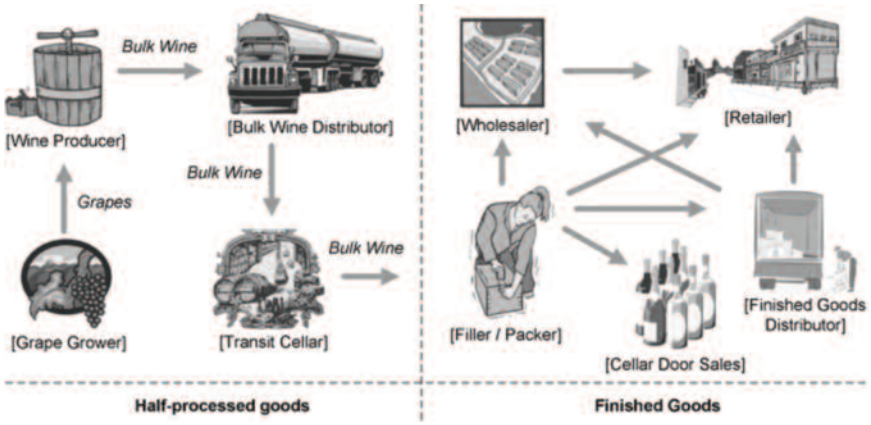

Figure 1. A representative scenario of wine supply chain. Source: (Cimino and Marcelloni, 2012)

Grape Growers are responsible for the production, harvest and delivery of grapes. Growers should record, details about the location, type of the vines, annual production record, origin and chemical content of water used for cleaning and irrigation, and the annual treatment. For each treatment product, growers should record the supplier's details, a description of the product received, as well as applicable batch numbers.

The growers supply, with each delivery, the location number of the plot from which it comes and the date of picking, so that the receiving wine producers can link the related details to the wine made from these grapes.

Wine Producers are responsible for the production, manufacture and/or blending of wine products. Wine producers should record where, in the winery, grapes or juice was stored. They must keep accurate records for the procedures and operations performed. The wine producer is responsible for identifying each production run with a batch number. For the receipt of additives, the producers should record the supplier's details, receiving date, a description of the product received, as well as applicable batch numbers.

The *Bulk Distributor* is responsible for receipt, storage, dispatch, processing, sampling and analysis of bulk wine. The bulk distributor checks the receiving documents, records all the information including the amount of received wine and takes samples for tasting and analysis. If the wine is rejected, the wine returns to the source, otherwise, two distinct processes are performed:

(i) storage and dispatch of bulk wine without any blending or any other processing;

(ii) storage, blending of different wines and dispatch of the new bulk blend. Identification is handled for the bulk distributor and the bulk wine container. To ensure forward tracking, it is essential to record references of the delivery items and to link these to the recipient.

The *Transit Cellar* is responsible for the receipt, storage, dispatch, processing, sampling and analysis of bulk wine. The transit cellar receives bulk wine from bulk distributors in different kinds of containers. Each of these containers is identified with a proper code. The transit cellar sends batches of bulk wine to the filler/packer. Each container sent is identified with a unique number, and with the associated quantity of wine (liters). In order to maintain accurate traceability throughout the chain, it is necessary that the transit cellar records the item and batch numbers, as well as the identifier of each dispatched item. To ensure forward tracking, it is necessary to record the global identifiers of the shipped items and link these to the location of the recipient.

The *filler*/*packer* is responsible for the receipt, storage, processing, sampling, analysis, filling, packing and dispatch of finished goods. The filler/packer receives containers of bulk wine from the transit cellar, and also "dry goods" in contact with wine (bottles, caps, corks, etc). Each of the containers of bulk wine and logistic units of dry goods are identified with a proper batch number. During this stage, the wine is poured into different kinds of containers, such as bottles, bags, kegs or barrels, and a lot

number is allocated to them. A link between these components (bulk wine, finished product) should be maintained. The next step is the packaging into cartons and pallets and the dispatch of these cartons and pallets (identified with a lot number) to the finished goods distributor. The lot number must be linked to the batch (es) of bulk wine used to fill the bottles. To ensure forward tracking, it is necessary to record the global lot number of the shipped items and link these to the location number of the recipient.

The *finished goods distributor* is responsible for the receipt, storage, inventory management and dispatch of finished goods. The finished goods distributor receives pallets and cartons from the filler/packer and dispatches them to the retailer. These trade items are identified with lot numbers. To ensure forward tracking, it is necessary to record the global lot number of the shipped items and link these to the location number of the recipient.

The *retailer* receives pallets and cartons from the finished goods distributor and picks and dispatches goods to the retail stores. The container number of an incoming pallet is recorded and linked to the location number of the supplier. The retailers keep a record of the container number and the lot numbers of the components of the pallets and cartons they receive. The retailers sell consumer items (bottles, cartons) to the final consumer. These items are identified with a number allocated by the brand owner.

This brief description of the wine supply chain has highlighted that all the processes from the grape grower to the consumer can be traced by associating appropriate identifiers with the traceability entities managed by the single supply chain actors and, for each identifier, creating a record with all the information required about the entity. Each actor of the supply chain is therefore responsible for recording traceability data corresponding to specific entities. Further, each actor has to create the links between identifiers which identify correlated entities (Cimino and Marcelloni, 2012).

MATERIALS AND METHODS

The authenticity of wine is guaranteed by strict guidelines laid down by the responsible national authorities that include official sensory evaluation, chemical analyses and examination of the register kept by the wine producer (Rapeanu et al., 2009).

1. Authentication of geographical origin (production area)

Climatic, edaphic and orographic factors influence the process of vine growth, with direct influence on the compositional and sensory parameters of wines (Ballabio et al., 2006). Since the area of production raises the visible mark on originality and quality characteristics of products, the determination of geographical origin is a main requirement for wine authenticity. The concept of "*terroir*" has been introduced within Europe, considering the specific characteristics of a wine that are induced mainly by geographical location and characteristics of production in the concerned areas. The “denomination of origin” (DOC) regions are areas within designated traditional wines, which have great quality features.

Sensory evaluation done by specialists (tasters) was the only way to determine the geographical origin of wines. The method has a high degree of uncertainty; therefore, instrumental analysis is used to identify the compounds which are in very small quantities (Rapeanu et al., 2009).

The principal methods used for authentication of geographic origin are:

1.1. Analysis of volatile compounds profile

Based on the content in 1-hexanol and cyclohexanone, it was able to differentiate between Pinot noir wines of French and American origin (Kwan and Kowalski, 1978). Further, it was able to identify the geographic area of French red wines and Spanish white wines by use of more volatile compounds (ethyl esters, isoamyl esters, aldehydes, acetals etc.) (Rapeanu et al., 2009).

1.2. Analysis of amino acidic profile

By investigating the amino acids arginine, alanine, tyrosine, valine and leucine, which are responsible for the amino acidic profile of wines, it is possible to identify the geographical origin of wines (Flamini and De Rosso, 2006) (Chambery et al., 2009).

1.3. Mineral profile analysis

The "fingerprinting" of mineral profile of wines is the most valuable method of assessing the geographical origin. Investigation of mineral elements of wine is the main procedure to authenticate the geographical origin of wines. Since some of the macro and micronutrients have great changes during technological process, such as Na, K, Ca, Fe, Cu, Zn and others, attention has been focused on the elements which show very small changes during technological process, although they are found in small quantities or as traces (Cr, Co, Sb, Cs, Sc, Eu, Hf, Ta etc.). Lithium and rubidium are the most relevant to geographical origin authentication. They are in very small quantities, 1-200 ppm lithium and rubidium 0.5-5.0 ppm, but may be quantified in a relatively simple manner by modern methods (flame atomic emission spectrometry). Investigation of rare earth elements (lanthanides) also provides valuable information in order to detect the growing areas of the vine, by using mass spectrometry with inductively coupled plasma (ICP-MS) (Dutra et al., 2011).

1.4. Analysis of stable isotopes and organic compounds of lead

Determination of stable isotopes and isotopic reports and subsequent interpretation of experimental data using chemometric methods: principal component analysis (PCA), linear discriminate analysis (LDA), is another way to authenticate the geographical origin of wines.

Since the contents of stable isotopes of water and alcohol of wine, and their isotopic ratios (D/H, 18O/16O, 13C/12C) had variations caused mainly by climatic factors they may be used to locate areas of growing, especially depending on their climate (cold and dry, cold and wet, hot and humid, hot and dry).

There was a decrease in content of oxygen isotopes (18O) and of deuterium (2H) of wine water, when they move from warmer areas to areas with a temperate climate and from West to East. The isotopic ratio value of 13C/12C is depending of the weather conditions, particularly the temperatures at which are grapes ripening.

Recorded values of the ratio generate reliable information for elucidation of the area production of a wine (country, continent). Moreover, because the values of isotopic ratios D/H and H2 18O/H2 16O are dependent on soil water isotopic composition (and climatic conditions, especially precipitation), investigation of these ratios provide relevant information's to authenticate the geographic origin of wines (Rapeanu et al., 2009).

2. Authentication of grape variety

Biotic factor has a major contribution to the formation of qualitative characteristics of wine, so the compositional and sensory parameters are largely dependent on a variety of vines.

2.1. Sensory Analysis

Sensory is limited by the influence of several factors (ecological, agro and technological) that modify significantly the primary characteristics of the variety.

Although sensory analysis is still influenced by human subjectivity, statistical interpretation of results and using equipment such as "electronic nose" and "electronic tongue" improved the performance of sensory evaluation (Biernacka and Wardencki, 2012).

2.2 Mineral profile analysis

As the vine varieties selectively accumulate various metals, their identification can be achieved by the content of certain elements of the wine like: lithium, nickel, calcium, rubidium etc (Rapeanu et al., 2009) (Dutra et al., 2011).

2.3. Amino acid and protein profile analysis

While soil, agro-technical and technological factors causes the large variations, the content and nature of amino acids may contribute to recognition of the vine variety. Recent research has shown that molecular weights of proteins recorded small variations in wines by variety. Proteins are stable in must and can be found in wine and even those subject to stabilization. Protein quantification is relatively simple and can be done by conventional methods (electrophoresis) or by modern methods (mass spectroscopy etc.) (Rapeanu et al., 2009).

2.4. Analysis of polyphenolic profile

Their evaluation is useful to authenticate individual wines by botanical origin. Investigation of phenolic compounds by multidimensional nuclear magnetic resonance allows the differentiation of wines by variety, and even the clones of the same variety. The most representative phenolic compounds of red

wines are the anthocyanins. They are in the free form, anthocyanins /anthocyanidins (malvidol, delfinidol, cyanidol, peonidol and petunidol), as well as acyl and coumaryl compounds. Cabernet Sauvignon is characterized by a higher content in malvidol and coumaryl anthocyanins while Merlot is highlighted by a higher content in peonidol and coumaryl anthocyanins (González-Neves et al., 2004).
Because during the grapes processing and wine preservation acyl and coumaryl anthocyanins are more stable, it was proposed that anthocyanins fingerprinting of red wines to express the amount of acyl anthocyanins + coumaryl anthocyanins and ratio of acyl anthocyanins/coumaryl anthocyanins (von Baer et al., 2008).
Since the authentication variety by using the fingerprint anthocyanins is operational only in red wines, white wines, roses and even the red is investigating by the content of shikimic acid (Mardones et al., 2005). Shikimic acid is found in small amounts in various fruits, including grapes. In wine is found in concentration of 10-150 mg/L. The content of shikimic acid is especially recommended for white wines authentication and as the additional indicator for red wines authentication (Makris et al., 2006).

2.5. Analysis of volatile compounds profile

Quantifying the odorant substances of wines, compounds that usually are found in extremely small quantities, is achieved by their extraction with various solvents and determination with modern methods such as gas chromatography coupled with spectrometry mass (GC-MS, LC/ESI-MS, MALDI-TOF-MS) (Nasi et al., 2008).

2.6. Residual DNA analysis of grapes

Since it was found that wines have small amounts of DNA from grapes, their analysis is done in order to identify the variety of the vine (Baleiras-Couto and Eiras-Dias, 2006).
The difficulties like - low content of DNA in wine, contamination (DNA from yeast, bacteria), changes during processing and inhibitors for the PCR reaction - were solved by using appropriate methods of DNA extraction and improving performance analysis using microsatellite PCR amplification.

3. Authentication of wine age

During time, the wines suffer a number of beneficial changes such as physical-chemical and even biochemical changes. They cause the development of refined sensorial qualities, thus, old wines are more valuable.
Moreover, the crop year, which benefited from favorable weather conditions, also give great features to the wines, therefore, being appreciated by consumers.
The analytical method that is frequently used is to determine the radioactivity of the 14C isotope of the ethanol molecule in wine. The method is relatively simple and consists in determination of the 14C isotope radioactivity of the concentrated solution (minimum 95%) of alcohol obtained by wine distillation or other alcoholic beverages. For red wines, to increase certainty, it is recommended to combine this results with those obtained from analysis of polyphenolic compounds and color indices. Determination of radioactivity by measuring the activity of wine sediment isotopes such as 210Pb, 210Po, 239Pu, 240Pu, 13 Cs, etc. is another way of knowing the wine age. The validity of the method was verified by appropriate dating of wine during 1850-1968, for every 6 years.
Age rating wines and other alcoholic beverages by determining radioactivity of isotopes have a high degree of reliability only for products with a considerable age (Dutra et al., 2011).

4. Authentication technology for production

There are a variety of techniques and processes of winemaking, authorized or used fraudulently altering appreciably the compositional and sensorial wine parameters.
This aspect of authenticity has a particular relevance to special wines like the sparkling wine, the oxidative wines etc.

4.1. Authentication of sparkling wines

Sparkling wines, which included class sparkling wines (sparkling), semi-sparkling, sparkling, pearl etc., are elite alcoholic beverages, much appreciated by consumers. There are a wide range, determined both by the diversity and quality of raw materials (wine based) but also technology development. Besides the general aspects of wines authentication, sparkling wines should represent be a clear distinction between

products which contain endogenous origin CO_2 (sparkling wine) and those with totally or partially exogenous CO_2 (Martinelli et al., 2003). The most information is provided by the component amino acid analysis certainty being higher when is associated with the study of other compounds like: odorant substances, polyphenols content, carbohydrates etc.

Isotopic analysis is generating the most rigorous investigation results by determining the amount of isotope ratio 13C/12C. In this way, is precisely the origin of carbon dioxide in the product, if whether it comes from plants with C3 metabolism (the vine) or C4 (sugar beet etc.) or from other sources (industrial, synthesis etc.) (Calderone et al., 2007).

4.2. Authentication of oxidative type wines

Oxidative type wines, with the main representatives of Port wine, Madeira, Jerez etc. are characterized by sensorial properties, generated mainly by biochemical or physical-chemical oxidative processes.

Authentication aims to detect the original products of imitation. In addition to sensory evaluation, content analysis of metals (Rb, Li, Mn, Fe and Al), phenolic compounds, the carbohydrates (including the ratio of glucose and fructose), presence and content of amino acids etc. generate information that is useful to identify the original products (Câmara et al., 2006).

4.3. Authentication of rose wines

Rose wines are at the border between white and red wines, having specific chemical compounds, so it is difficult to license their origin. Authentication of rose wines has two major objectives. The first step is to identify the source of raw material in the sense that comes from red grapes or a blend of white grapes and red or white and red wines, and secondly to assess the authenticity of color, to detect possible fraud color of white wines by oenocyanin addition. Analysis of compounds and parameters such as polyphenols, sugars, volatile acidity, extract and especially the color indices etc. and comparing the results with reference values provide a high degree of certainty.

The recommended method for defining the color is the spectrophotometric method, the reference method, which monitors the three color indices: bright, chromaticity and purity (Rapeanu et al., 2009).

CONCLUSIONS

The ability to trace and authenticate a food product is of major concern to the food industry.

Wine authenticity is very important, especially in the case of quality control and consumer information.

Since wine quality is dependent on the consumer demands, compliance with traceability provisions satisfies the associated economic needs.

It is necessary that a traceability information system exists on the wine supply chain, in order to provide a better management of all representative events which may arise on this chain, from grape production to wine selling.

Traceability documents allow the detection of certain deviation from the usual process, that may render the final product unsafe or of a lower quality than the one expected.

Therefore, identifying the safety and quality issues during the process, by means of authentication and traceability, assures the compliance, within the framework of international regulations, with consumers' request for the certification of quality attributes.

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ANTIFUNGAL ACTION OF LACTIC ACID BACTERIA ISOLATED FROM PLANT MATERIALS AGAINST MYCOTOXIGENIC FUNGI

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Abstract

The contamination of food and feed by mycotoxigenic fungi that belong to genera Aspergillus, Alternaria, Fusarium and Penicillium poses significant risks for animal and human health. The mycotoxins (aflatoxins, trichothecenes, ochratoxins, fumonisin etc) produced by such spoilage fungi can be carcinogenic, hepatotoxic, teratogenic or immunosuppressing (Logrieco et al., 2003; Atanda et al., 2013). The limitation of the mycotoxin contamination of food and feed could be achieved by specific methods that inhibit the fungal growth. Several approaches are described by now to avoid the accumulation of mycotoxins, directed towards: prevention of contamination; decontamination of mycotoxin-containing food and feed; and inhibition or absorption of mycotoxin content of consumed food into the digestive tract (Juodeikiene et al., 2012). The use of biological control microorganisms (bacteria, yeasts or fungi) to inhibit the growth of mycotoxin producing fungi and/or degrade the mycotoxins is an important strategy for prevention/decontamination of products (Tsitsigiannis et al., 2012).
The aim of this work was the screening of new lactic acid bacteria (LAB) strains isolated from plant materials or some Romanian traditional foods for antifungal action againstplant pathogenic fungi and mycotoxin producing Aspergillus strains. 123 strains of LAB were examined for anti-Aspergillus action and 24 out of them (more than 21%) exhibit strong inhibitory activity against the growth of A.flavus, A.ochraceus and A.niger strains after 3 days of incubation. For most of the LAB strains the inhibitory action was maintained even after 14 days of incubation. The principal antifungal compound is represented by lactic acid, quantified by HPLC. Important damages of the fungal hyphae were observed at microscopic level (fragmentation, loss of the cellular content, twisting of hyphae or apical swelling). 17 new isolate of Aspergillus spp. were obtained and their ability to produce mycotoxin was evaluated by qualitative and quantitative methods. Four of them were used for interactions with LAB: strong and stable inhibition was obtained with the LAB strains designated as 35, 58 and 26. The results suggest that selected LAB could be used for fungal growth inhibition and possible for mycotoxin content reduction.

Key words: *antifungal LAB, mycotoxin producing Aspergillus*

INTRODUCTION

Biological means for yield protection and food preservation are becoming increasingly interesting for both food industry and consumers. Spoilage fungi not only that determine quantitative looses but could induce qualitative depreciations such as mycotoxin contamination. The contamination of food and feed by mycotoxigenic fungi that belong to *Aspergillus, Alternaria, Fusarium* and *Penicillium* poses significant risks for animal

and human health. The mycotoxins (aflatoxins, trichothecenes, ochratoxins, fumonisin, etc) produced by such spoilage fungi can be carcinogenic, hepatotoxic, teratogenic or immuno-suppressing (Logrieco et al., 2003; Atanda et al., 2013). Aflatoxins (B1, B2, G1 and G2) are a group of secondary metabolites predominantly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, considered as the most potent hepato-carcinogens affecting animal species, including humans (Mishra and Das, 2003). The limitation of the mycotoxin contamination of food and feed could be achieved by specific methods that inhibit the fungal growth. To avoid fungal contamination and mycotoxins accumulation in foods and feeds, several approaches are described by now and directed towards: prevention of food and feedstuff contamination, decontamination of mycotoxins-containing products, and inhibiting the absorption of mycotoxin content into the digestive tract when consuming contaminated aliments (Griessler et al., 2010; Juodeikiene et al., 2012). The use of biological control microorganisms to inhibit the growth of mycotoxin producing fungi and/or degrade their mycotoxins is an important strategy to prevent infections or aliment decontamination (Tsitsigiannis et al., 2012).

Several microorganisms, especially bacteria, have been examined for their potential to inhibit fungal growth and production of aflatoxins, the best results being obtained, under laboratory conditions, with spcies of *Bacillus*, *Lactobacillus*, *Pseudomonas*, *Ralstonia, Burkholderia, Enterococcus or Rhodococcus* (Alberts et al., 2006; Reddy et al., 2010; Topcu et al., 2010). It was shown that microorganisms (both living or dead cells) could bind aflatoxins to their cell walls components or could produce extra- or intracellular enzymes involved in degradation/modification of aflatoxins. The results obtained by various authors proved that the methods of biodegradation can be o promising choice to reduce or eliminate the contaminations of aflatoxins in food and feed (Wu et al., 2009).

The inhibition of spoilage fungi using beneficial microorganisms such as lactic acid bacteria (LAB) has been the subject of various investigations due to the practical importance of LAB as active ingredients in food and feed preservation. LAB not only that are widely used in food and feed processing but they have probiotic activity and, in some cases, they can produce antimicrobial metabolites with antifungal effects, inhibiting also mycotoxigenic fungi (Nyoman Pugeg Aryantha and Lunggani, 2007). Several studies reported LAB strains that could bind aflatoxin (Peltonen et al., 2001; Kankaanpää et al., 2000, Gratz et al., 2004; Shah and Wu, 1999, El-Nezami et al., 2002), inhibit aflatoxin accumulation (Coallier-Ascah and Idziak, 1985; Onilude et al., 2005), or even remove AFB1 (Shah and Wu, 1999; El-Nezami et al., 2002). Fuchs et al. (2008) reported LAB strains that could remove ochratoxin A mycotoxin from solutions.

The aim of this work was the screening of new lactic acid bacteria (LAB) strains isolated from plant materials or some Romanian traditional foods for antifungal action against plant pathogenic fungi and mycotoxins producing *Aspergillus* strains.

MATERIALS AND METHODS

Microbial strains

One hundred and twenty three lactic acid bacteria were used in this study. The LAB were isolated from plant materials or some Romanian traditional foods such as borş (fermented wheat bran), sauerkraut or pickles. Three collection strains of LAB were used as reference strains: *Lactobacillus plantarum* IC12353, *Lb.paracasei* CCM 1837 and *Lb.acidophilus* IC11692. Routinely, LAB strains were grown on MRS broth at 36°C.

Fungal isolation and identification

Two groups of fungi were used in experiments: plant pathogenic fungi and aspergilli. The plant pathogens, like *Botrytis cinerea, Alternaria alternata, Penicillium spp., Monilinia fructigena, Fusarium spp.* were isolated from fruits, seeds or vegetables (Cornea et al., 2013). *Aspergillus spp.* strains were isolated from cereal seeds (wheat, Triticale, barley, oats) on Potato Dextrose Agar (PDA), and their identification was based on macroscopically and microscopically features. 17 strains of aspergilli were isolated and identified as *A.flavus/A.parasiticus* (13 strains), and *A.niger* (4 strains). Other eight *Aspergillus spp* strains,

previously isolated and identified as *A.niger* (An4 and An5), *A.ochraceus* (2 strains), *A.oryzae* MI156, *Aspergillus spp.* (Asp1 and Asp 4.3)(Cornea et al., 2011) and *A.flavus* (Judet et al., 2006) were also used in our experiments.

Screening for mycotoxin-producing aspergilli

The mycotoxin producing capability of the fungal strains was evidenced on coconut agar medium (Davis et al., 1987; Heenan et al., 1998; Lin and Dianese, 1976), under UV light, after 7 days of incubation in the dark, at 26°C, and by cultivation of fungi on PDA supplemented with 0.3% methyl-β-cyclodextrin (MβCD) and 0.6% sodium deoxycholate (a beige opaque ring surrounding the fungal colonies is associated to aflatoxin production)(Jaimez Ordaz et al., 2003; Yazdani et al., 2010).

Mycotoxin production and quantification

For mycotoxin production, fungi were grown in APA broth (NH_4 H_2PO_4 10g, K_2HPO_4 1g, $MgSO_4 \cdot 7H_2O$ 0.5g, KCl 0.5g, $FeSO_4 \cdot 7H_2O$ 0.01g, sucrose 30g, $HgCl_2$ 5×10^{-4} M, corn steep liqueur 0.5g, distilled water 1L) (Hara *et al*., 1974) for 4 days at 26°C. Quantitative analysis of aflatoxin production was performed with RIDA® QUICK Aflatoxin RQS kit from R-Biofarm AG, according to kit protocol.

Antagonistic interactions

The antifungal activity of LAB strains against *Aspergillus* was first examined using the double layer technique. MRS plates were first spotted with fresh LAB suspensions (5 µl) and after 48 h of incubation at 36°C there were covered with 5ml of 0.6% PDA containing fungal spores. Plates were than incubated at 27°C for 3 days, and up to 14 days at room temperature. Antifungal activity of the LAB strains was appreciated based on the fungal growth inhibition all around the bacterial spot. Microbial interactions were microscopically examined.

In a second method, the fungi were streaked diagonal in plates containing MRS or PDA. The LAB were subsequently placed at the both sides o the fungi. The incubation was performed at 37°C or 30°C for at least 5 days, and inhibitory areas were examined.

HPLC analysis of LAB antifungal compound

Using HPLC method two antifungal compounds were determined, lactic and acetic acids. Therefore, LAB broth cultures were centrifuged, and clear supernatant was diluted 10 times. Samples were filtered through 0.22µm PVDF filter discs before HPLC analysis. Chromatographic separation and quantification were done using Alliance HPLC System (Waters). Data acquisition and management were completed with the Empower 2 software.

RESULTS AND DISCUSSIONS

1. Screening of antifungal action of new LAB isolates

123 isolates of LAB strains were selected from various plant materials. Among them, about 45% presented inhibitory action against at least one plant pathogenic fungi (fig.1).

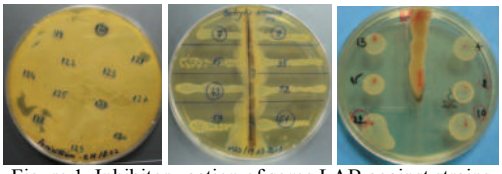
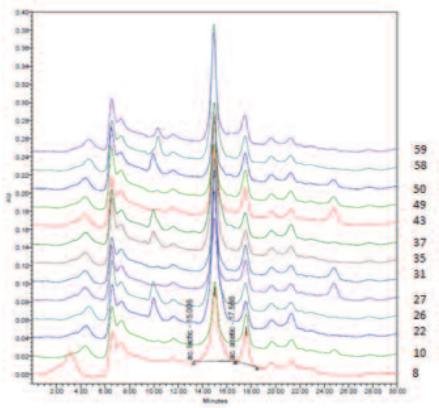


Figure 1. Inhibitory action of some LAB against strains of *Penicillium spp., Botrytis cinerea* and *Alternaria alternata* (Alt)

The best results were obtained with 12 LAB strains isolated from fermented wheat bran and from sauerkraut: they presented a broad spectrum of activity against selected fungi. The production of organic acids is clearly involved in antifungal action, at least for the most active LAB: strains 22, 26, 35 and 58 (fig.2).

These results suggest that inhibitory activity of the selected LAB is due mainly to the production of fermentation end products such as lactic acid, acetic acid (as it was proved by HPLC analysis) or diacetyl, acetaldehyde and hydrogen peroxide. Although, some of the selected LAB strains used in these experiments produce bacteriocins, no antifungal activity was associated with this (data not shown).



Sample	Lactic Acid (µg/ml)	Acetic Acid (µg/ml)
8	18,325	6,228
10	12,002	5,723
22	**32,781**	**3,831**
26	**28,754**	**4,105**
27	12,095	3,991
31	15,303	4,814
35	26,82	3,513
37	25,662	2,764
40	17,479	4,821
43	11,687	4,459
49	9,278	5,042
50	10,118	4,342
58	**25,689**	**3,805**
59	18,969	3,648

Figure 2. HPLC quantification of lactic and acetic acids produced by selected LAB strains

2. Inhibition of mycotoxigenic fungi by selected LAB

Aflatoxin and ochratoxin contamination of food and feed cause negative effects on human and animal health and the prevention of mycotoxins production/accumulation could be achieved by LAB, as presented various authors (Reddy et al., 2010). For these reason, the ability of selected LAB to inhibit the growth of several *Aspergillus flavus*, *A.oryzae* and *A.ochraceus* strains was examined. Results shown that, among the 123 LAB isolates tested, at least 24 were able to inhibit almost all the aspergilli strains tested (table 1).

Table 1. Growth inhibition of different aspergilli by LAB

LAB	*Afl*	Asp 4.3	Asp1	MI156	Ochra5	*Ochra* Am
7	+	±	+	+	+	+
8	+	+	+	+	+	+
9	+	±	+	+	+	+
10	+	+	+	+	+	+
13	+	+	+	+	+	+
15	+	±	+	+	+	+
22	+	+	+	+	+	+
26	+	±	+	+	+	+
27	±	+	+	+	+	+
31	±	+	+	+	+	+
35	+	+	+	+	+	+
37	+	±	+	+	+	+
40	±	+	±	+	+	+
43	+	+	+	+	+	+
49	+	+	+	+	+	+
50	±	±	+	+	+	+
53	+	±	+	+	+	+
58	+	+	+	+	+	+
61	+	+	+	+	+	+
94	–	–	–	+	–	+
115	+	±	+	+	+/±	+
120	+/±	+	+	+	+	+
126	+	+/±	+/±	+	±	+
135	+/±	+	+	+	+	+
Lpl	+	+	+	+	+	+

Where: + = inhibition of the fungal growth; ± = slight inhibition of the fungal growth; – = no inhibition

Moreover, important damages of the fungal hyphae (fragmentation, loss of the cellular content, twisting of hyphae or apical swelling) due to interactions with the strains LAB58 and LAB35 were observed for *A.oryzae* and *A.ochraceus* (fig.3).

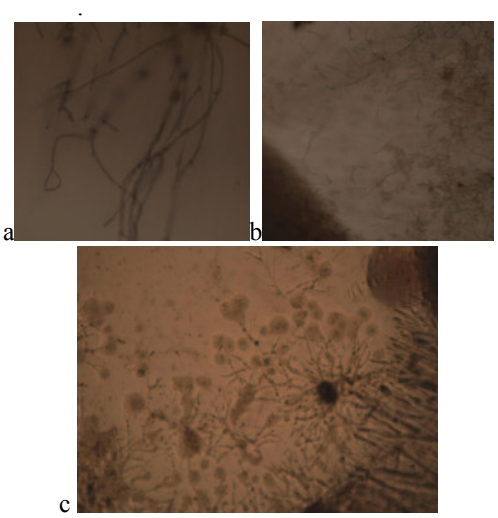


Figure 3. Microscopically aspects of LAB action against *A.oryzae* mycelium (a, b) and *A.ochraceus* mycelium (c)

Similar effects were also observed against *A.niger* strains, growth inhibition (fig.4, left) and mycelium alterations were induced by LAB (Fig.4, right).

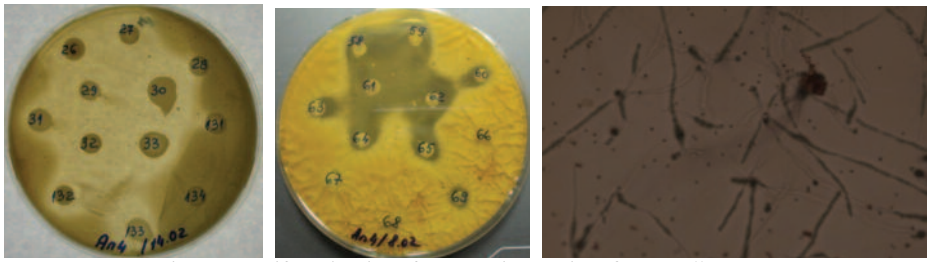


Figure 4. Antifungal action of LAB against strains of *Aspergillus niger*

The ability of natural *Aspergillus spp.* to synthesize mycotoxins is not a general feature, but higly mycotoxigenic strains could be isolate from infected materials and products. The most rapid and simple screening methods of aflatoxin producing fungi involve cultivation on selective media and/or examination of culture characteristics. In this respects, 17 natural isolates of *Aspergillus spp.* were selected from cereal seeds and preliminary identified as *A.flavus/A.parasiticus* (13 strains), and *A.niger* (4 strains). Their ability to produce mycotoxins was examined on selective media. On coconut medium, blue fluorescence (associated with aflatoxin production) on reverse side of some fungal colonies, as well as green-blue fluorescence (associated with ochratoxin production) was observed in UV light (Fig. 5).

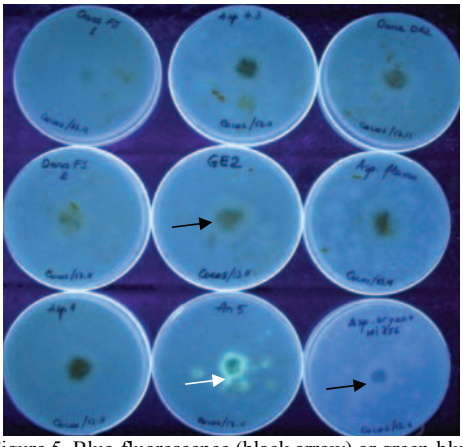


Figure 5. Blue-fluorescence (black arrow) or green-blue fluorescence (white arrow) on reverse side of some fungal colonies grown on coconut medium

Better results were obtained when fungi were cultivated on PDA supplemented with MβCD and sodium deoxycholate, were the presence of an opaque (white) halo around the colonies suggest aflatoxin production. Significant differences were noticed between the tested fungal strains, when this medium was used (Fig. 6).

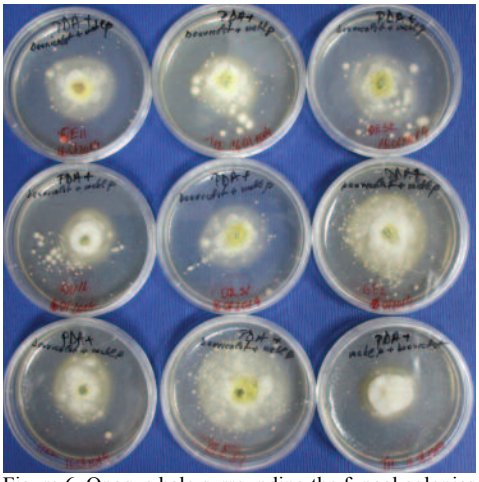


Figure 6. Opaque halo surrounding the fungal colonies associated with aflatoxin production.

The high mycotoxin producing strains selected in these experiments were GE21, T11, GE2 and G32. Significant amounts of aflatoxin were detected using RIDA® QUICK Aflatoxin RQS kit, when these strains were grown in APA broth for 4 days. The aflatoxin level was between 6.1-6.5 ppb, comparing with 5.2. ppb detected for *A.flavus* used as reference.

The effect of LAB against the four aflatoxigenic strains was examined (fig.7) and at least three LAB strains (35, 58 and 26) confirm their high and stable inhibitory action (the areas of inhibition were maintained even after 14 days of incubation). These results suggest that the antifungal action could be due not only to organic acids, but to other possible mechanisms.

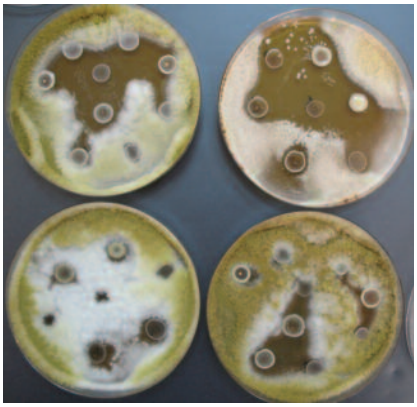


Figure 7. Inhibitory action of some LAB against selected aflatoxigenic strains

CONCLUSIONS

Among the 123 LAB isolates, 45% presented inhibitory action against at least one plant pathogenic fungi and 21% of the strains were active against *Aspergillus spp*.

Important damages at cellular level (fragmentation, loss of the cellular content, twisting of hyphae or apical swelling) due to interactions with the strains LAB58 and LAB35 were observed.

The production of organic acids is clearly involved in antifungal action, at least for the most active LAB strains: 22, 26, 35 and 58.

17 natural isolates of *Aspergillus spp.* were selected from cereal seeds and preliminary identified as *A.flavus/A.parasiticus* (13 strains), and *A.niger* (4 strains).

Their ability to produce mycotoxins was determined on coconut medium and on PDA medium supplemented with 0.3% MβCD and 0.6% sodium deoxycholate, and quantified using RIDA® QUICK Aflatoxin RQS kit.

Four isolates, designated as GE21, T11, GE2 and G32 were selected for their higher level of aflatoxin and used for interactions with LAB.

At least three LAB presented a broad spectrum of activity against almost all fungi used in experiments, including the natural aflatoxigenic isolates (GE21, T11, GE2 and G32), the inhibitory action being strong and stable even after 14 days of cultivation

The selected LAB strains could represent good candidates as biocontrol agents for fruits/ vegetables protection and prevention of contamination with aflatoxigenic fungi.

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THE INFLUENCE OF CHILLING STORAGE ON COLOR, pH AND ACIDITY OF FRUIT SMOOTHIE BEVERAGES

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Abstract

Recently, for increasing smoothies shelf life, food industries used thermal processing, which has been shown in some studies to affect the sensorial and physic-chemical properties of these products.
Color, flavor, texture and physico-chemical properties of food products have an important role in correlation with taste, sensory perception and consumer acceptance. These are critical quality attributes affecting the acceptability of fruits, fresh or processed, thus being of major concern in product design.
The aim of this study is to observe the influence of chilling storage on color, pH and acidity of some products based on mixtures of pressed and squeezed fruits without adding preservatives or stabilizers.
For this purpose, some mixtures of several pressed fruit like apples-pears, peaches-mango and sour cherry –bananas were purchased from a local market. These mixtures were stored in climate chamber at 5^0C, the color, pH and acidity being analyzed throughout the 27 days chilling storage period. During chilling storage the lightness index, L, yellowness index, b and the redness index, a, were measured and it was observed color changes for all of fruit smoothie samples. pH and acidity values*, showed that the fruit smoothie samples registered insignificant changes in this comparative study between the same samples after different chilling storage period.*

Keywords*: color, fruit smoothie, chilling storage, shelf life*

INTRODUCTION

Nowadays, there is a strong tendency toward consumption of fresh foods using minimal processing or reducing chemical preservatives. Juices directly obtained from fruits (not from concentrate), distributed through the refrigerated chain with a relatively short shelf-life are good examples of this (Esteve, Frigola, Rodrigo, & Rodrigo, 2005).
Smoothies are an example of this trend to increase the consumption of vegetables and fruits, as an alternative and/or a complement to fresh products. Smoothies were first introduced in 1960 in United States. Further, they re-emerged worldwide in 2000 (Titus, 2008). The manufacture of smoothies is based on the use of a mixture of fruits and vegetables, after removing seeds and peel, which are processed into pulp or puree (Qian, 2006). In most of the cases, the selection of the mixtures is based on the sensorial characteristics like colour, flavour, texture and, trying at the same time to ensure high concentration of nutrients with low energy content (Watzl, 2008).
The Food Standards Agency (FSA) has established its message that a minimum of five portions of fruit and vegetables a day contributes towards a healthy diet (FSA, 2010).
Also, scientific evidences encouraged the consumption of vegetables and fruits to prevent chronic pathologies such as hypertension (Dauchet et al., 2007), coronary heart diseases and the risk of stroke (He et al., 2007).
Unfortunately, the daily intake of vegetables and fruits is estimated to be lower than the doses recommended by the World Health Organization (WHO), and Food and Agriculture Organization (FAO) (www.who.int/; www.fao.org/).
The demand for high quality fruits, which provide healthful substances, high nutritional values and first-rate taste, highlights the importance of methodologies able to monitor quality along the postharvest chain from the field to the consumer.

The aim of this study is to observe the influence of chilling storage on color, pH and acidity of some products based on mixtures of pressed and squeezed fruit without preservatives or stabilizers, throughout the shelf life period.

MATERIALS AND METHODS

Samples

The fruit smoothie beverages like apples-pears, peaches-mango and sour cherry –bananas without preservatives or stabilizers, were purchased from a local market. These products were stored in climate chamber at 5^0C and monitoring. After 6, 13, 20 and 27 chilling storage days since production date, the samples were analysed.
Every time, two bottles from each type of smoothie were opened for duplicate measurements.

Physico-chemical analysis

pH determination

pH was determined with a pH meter WTW INOLAB 720 series type with automatic temperature compensator, whose pH domain is between 0,00-14.00, with a precision of ± 0,01.

Titratable acidity (TA)

Titratable acidity was determined by titrating 10 g of homogenized smoothie sample with 0.1 N NaOH to an end point of pH 7.3 using Schott automatic titrator type Titronic basic. TA was analyzed in triplicate and expressed as citric acid/100 g product.

Color

Color assessment of the samples was conducted at room temperature using a HunterLab colorimeter, Miniscan XE Plus. This instrument was calibrated using the black and white tiles provided. Instrumental color was measured using Illuminant D65 and 10^0 observer angle. Smoothies were filled into a low reflectance sample container and placed over the colorimeter chamber. For each sample of smoothie, measurements were made in ten different points and results were averaged. Therefore the total color change (ΔE) was calculated with the following equation (Hunter Lab, (1996)):

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}.$$

RESULTS AND DISCUSSIONS

pH and acidity evolution

The obtained results showed that the pH and acidity values of smoothie samples like apples-pears, peaches-mango and sour cherry – bananas without preservatives or stabilizers, were recorded insignificant changes throughout the chilling storage period, as can be seen in Figures 1 and 2.

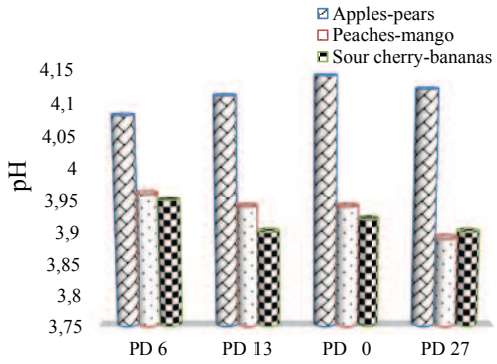

Figure 1. Variation of the pH values of the smoothie samples after the different chilling storage period, PD 6- after 6 chilling storage days PD 13- after 13 chilling storage days, PD 20- after 20 chilling storage days, PD 27- after 27 chilling storage days

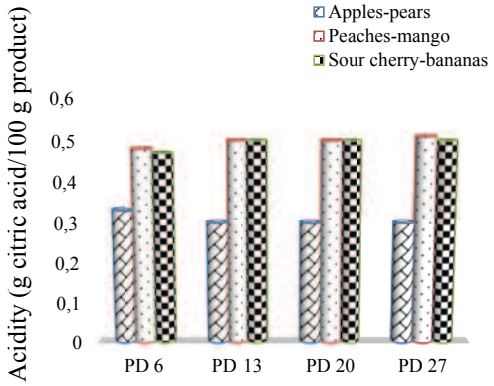

Figure 2. Variation of the tritratable acidity values of the smoothie samples after different chilling storage period, PD 6-after 6 chilling storage days, PD 13- after 13 chilling storage days, PD 20- after 20 chilling storage days, PD 27- after 27 chilling storage days

Color changes during chilling storage period

During the chilling storage period were not observed visually detectable color changes for any of the analysed smoothie samples. Changes were observed when the color characteristics were analysed with colorimeter Hunter Lab according to Universal Software V4.01 MiniScan^{TM} XE Plus program.

During chilling storage period, at 5^0C in climate chamber, the L (lightness), a (redness) and b (yellowness) values of apple-pear and peaches-mango smoothie samples tended to increase, indicating lower color changes, as can be observed in figures 3 and 4. For sour cherry-bananas smoothie samples, the L and b values tended to increase but values of a (redness) decrease during storage period, indicating a low discoloration of the samples (figure 5).

The ΔE values, which are an indicator of total color difference (table 1), showed that chilling storage at 5^0C affected insignificant color attributes of apples-pears, peaches-mango and sour cherry-bananas smoothie.

Table 1. Instrumental color variables of fruit smoothie samples throughout the chilling storage period at 5^0C in climate chamber

Samples	Analysis day	L	a	b	ΔE
Apples - pears	PD6	49.57	2.51	16.56	-
	PD13	50.11	2.78	16.99	0.74
	PD20	50.87	2.99	16.82	1.40
	PD27	49.28	2.8	16.7	0.43
Peaches - mango	PD 6	45.59	9.04	24.18	-
	PD13	45.71	9.49	24.32	0.48
	PD20	46.17	10.06	24.59	1.24
	PD27	45.99	9.92	24.52	1.02
Sour cherry - bananas	PD 6	36.85	18.76	8.33	-
	PD13	36.42	17.75	8.19	1.10
	PD20	37.79	17.83	8.79	1.40
	PD27	37.56	16.47	9.09	2.51

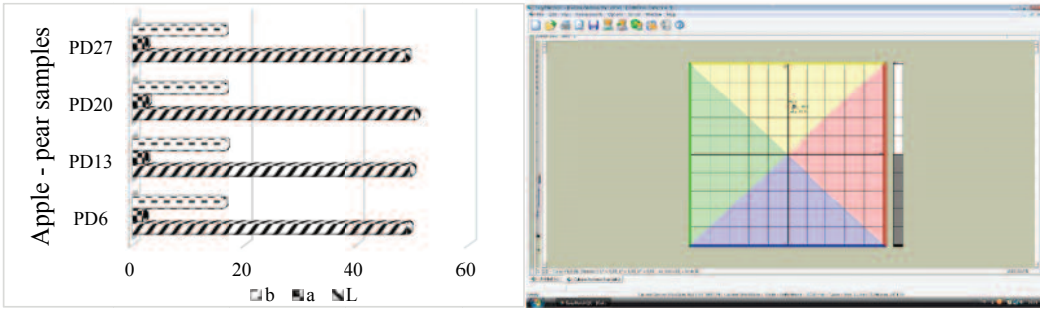

Figure 3. Graphical representation of the values of L, a, b, according to Universal Software V4.01 MiniScan^{TM} XE Plus program for apple - pear smoothie samples, PD 6-after 6 chilling storage days, PD 13- after 13 chilling storage days , PD 20- after 20 chilling storage days, PD 27- after 27 chilling storage days

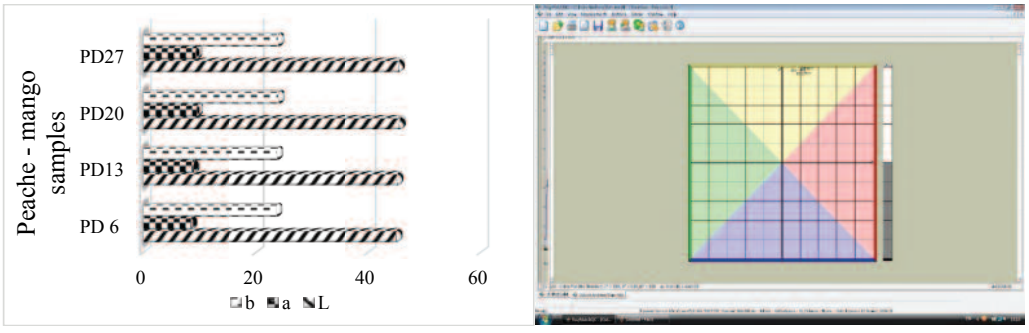

Figure 4. Graphical representation of the values of L, a, b, according to Universal Software V4.01 MiniScan^{TM} XE Plus program for peaches - mango smoothie samples, PD 6-after 6 chilling storage days, PD 13- after 13 chilling storage days, PD 20- after 20 chilling storage days, PD 27- after 27 chilling storage days

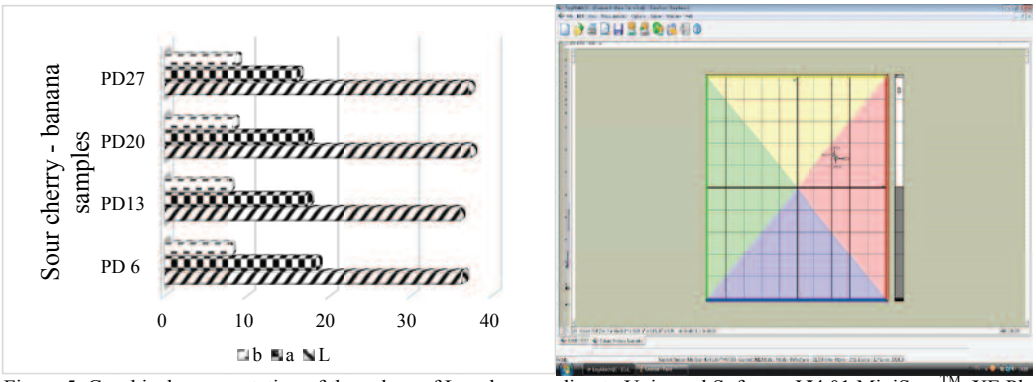

Figure 5. Graphical representation of the values of L, a, b, according to Universal Software V4.01 MiniScan™ XE Plus program for sour cherry - banana smoothie samples, PD 6-after 6 chilling storage days, PD 13- after 13 chilling storage days, PD 20- after 20 chilling storage days, PD 27- after 27 chilling storage days

CONCLUSIONS

The physico-chemical properties of food products have an important role in the creation of taste and sensory perception.

These are critical quality attributes affecting the acceptability of fruits, fresh or processed, thus being of major concern in new product design.

Selection of the smoothies is based on the colour, flavour, texture and, especially, ability to ensure high concentration of nutrients with low energy content.

During the chilling storage period were not visually detected any color changes for the smoothie samples analysed.

During the storage period the pH and acidity values of apples-pears, peaches-mango and sour cherry-bananas smoothies without preservatives or stabilizers, were recorded insignificant changes.

Also, insignificant changes were observed when color characteristics of apples-pears, peaches-mango and sour cherry-bananas smoothie were analysed with colorimeter Hunter Lab according to Universal Software V4.01 MiniScan™ XE Plus program.

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MISCELLANEOUS

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HAZARD ANALYSIS AND CRITICAL CONTROL POINTS SYSTEM OPTIMIZATION IN A STARCH FACTORY

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Abstract

The starch is a product obtained through the wet-milling of corn after the germs, hulls and protein separation. Because it is used as raw material in the food industry, safety assurance during its production is important. The hazard analysis and critical control points is a management system which aims to assure the safety of the food products by the identification, controlling and prevention of microbiological, chemical and physical hazards. Even if the production process of the corn starch is an aggressive one and unfavorable to the microorganism's multiplication, some food safety hazards still exist. This paper aims to review the international literature and the general guidelines of food safety assurance in order to optimize the HACCP system already implemented in a starch factory. Several control and critical control points were identified and for each one a specific monitoring procedure was elaborated. Also, several preliminary programs were identified and centralized in order to prevent the hazards occurrence.

Keywords*: food-safety, HACCP, starch production*

INTRODUCTION

Hazard analysis and critical control points system is an "essential element" in the production process of any food product and it should be guided by specific scientific research in order to be efficient for the intended use of the respective product.
The cornstarch is a product used as raw material in the food industry (e.g.: dough, sweets, sauces, preserves, emulsified products, etc).
It is obtained through a wet-milling process of corn, after the separation of the germs, fiber and gluten. In the food industry the cornstarch has the role to stabilize the composition and to interact with other components in order to maintain the food's nutritional value and flavor (Liu, 2005).

MATERIALS AND METHODS

In the international literature there are several research papers which discuss topics such as difficulties and barriers for implementing HACCP system (Baş *et al.*, 2007), factors which affect the food safety management system (Sampers *et al.*, 2012), different tools able to ease the evaluating risk level of hazards (Ryu *et al.*, 2013) and even models of the HACCP implementation in several food industries.
The HACCP study followed the tasks included in the seven principles of the HACCP system described in the second edition of the joint FAO/WHO Food Standards Programme Codex Alimentarius Commission, taking into account the most recent research from literature. The steps, the specific activities and the possible improvements for each of them are presented in Table 1.
For the validation of the HACCP plan presented in Table 6 we used two methods: determination of the foreign bodies in corn and tests for surfaces hygiene (bioluminescence method).

Table 1. Application of the HACCP program in the glucose syrup production process

Task according to FAO	Activity description	Improvements
Assemble HACCP Team	It is primordial to first establish a multidisciplinary team which can be able to develop an effective HACCP plan.	
Describe Product and Identify Intended Use	The cornstarch is a product obtained through the wet-milling process of corn. It is used as raw material in the food industry.	The product was described in detail in internal product data sheet, taking into account its applications in the food industry.
Construct Flow Diagram; On-site Confirmation	Every operation from the flow diagram was analyzed considering also the preceding and following steps and verified in the factory.	For an easier understanding and tracking, the Flow Diagram was split in 2 parts (starch slurry and starch), and after that each of them was completed with the modified or new-introduced steps. It is presented in figure 1.
List all Potential Hazards Conduct a Hazard Analysis Consider Control Measures	The food safety team conducted the hazard analysis by centralizing all the steps mentioned in the diagram flow, the hazards that may be reasonably expected to occur at each step (physical, chemical, microbiological), the gravity, the frequency, the hazard class and the control measurements for each hazard (Chira, 2010).	The **specific** microbiological hazards presented in Table 3 were taken into consideration, while the chemical and physical ones remained as previously determined.
Determine CCPs	The critical control points were identified using the decision tree presented by FAO/WHO, considering only the steps which were identified to have the risk class 3 or 4 (Chira, 2010).	The previous critical control points, the sulphur dioxide concentration from the steeping solution and the pH of starch slurry were re-evaluated and transformed in operational prerequisite programmes (oPRPs). Two new critical control points were identified and marked on the flow diagram.
Establish a Monitoring System and corrective actions for each CCP	The monitoring system was developed for each CCP by setting the critical limits to be observable and measurable (Chira, 2005). The corrective actions were established.	In Table 6 are presented, for the new critical control points, the monitoring system, the corrective actions and the responsible persons for each action.
Establish Verification Procedures, Documentation and Record Keeping	The verification procedures were established using the literature, sampling plans, analysis results, corrective actions in order to demonstrate that the HACCP plan is correctly functioning.	The validation of the HACCP plan was made by centralizing and interpreting the analysis results for each CCP during one year. The analysis results are presented in Tables 4 and 5. A plan for the checking the preliminary programs, PRPs and HACCP plans was developed. It should be able to assure that the HACCP system is periodically implemented, updated and improved.

For the determination of the foreign bodies in corn we used the method described in STAS 1069/1977 – "Seeds for consumption. Determination of foreign bodies and seeds with defects". For the validation process we considered the values of foreign bodies as being the same with the values for wheat seed content, but for the analysis made for the CCP's control we calculate only the wheat seed content (WS) from the sample using the formula: $WS\,(\%) = \frac{Nws}{m}$, where *Nws* is the number of wheat seed from the sample and *m* is the sample weight.

The tests for surfaces hygiene were performed using the bioluminescence method based on the chemical reaction which produced light when ATP come in contact with the enzyme called luciferase (figure 1), with the SystemSURE II ATP Detection from Hygiena. This system includes three components:

- SystemSURE II Luminometer – device for reading and displaying the results.
- Ultrasnap Sample Testing Device – sampling device and analysis kit in which the bioluminescence reaction takes place.
- DataSURE II Data Analysis Software –software application which allows transferring data to a computer.

The results were interpreted according to Table 2. The values from this table were taken from Hygiena - "A Guide to Rapid ATP Monitoring" and they are the producer recommended limits for any high-risk food factory.

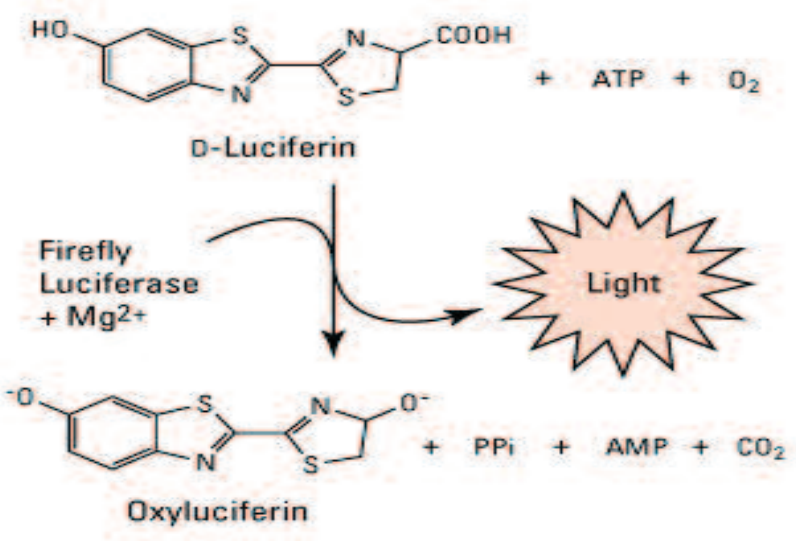

Figure 1. Bioluminescence reaction

Table 2. Setting the thresholds of surfaces hygiene for containers and materials in contact with the finished product

Thresholds	Corresponding limits	Interpretation
Pass	≤ 10 RLU	The surface has been adequately cleaned
Caution	11 – 29 RLU	The control point surface may not have been adequately cleaned.
Failed	≥ 30 RLU	The surface is dirty or contaminated, and must be cleaned again and re-tested until a Pass or Caution level is achieved.

RESULTS AND DISCUSSIONS

The first and most important step in the development of a HACCP study is the establishing of the HACCP team. The starch factory from Tandarei has established a multidisciplinary team called "food safety team" which includes only factory employees from seven departments, as follows: production, maintenance, quality control, sales, purchasing, human resources and quality management. The team members have knowledge and experience regarding the glucose syrups as well as the technology used and they are trained regarding the food safety.

The factory took as reference has a HACCP plan which proved to be efficient until now. However, external auditors and authorities recommended a revision of this plan, in order to take into consideration the effects of changes made in the last year into the factory.

After the new hazard analysis we found that the changes made in the factory did not affect the product from the viewpoint of food safety. Instead, we found that the existent critical control points were insufficient to reduce the

danger of microbiological contamination, the real hazard being the cross-contamination.

The old CCPs, concentration of sulphur dioxid solution having the critical limit 0.16% and the pH of starch slurry having the critical limit 6.9, were consider redundant due to the fact that according to the production procedures these values are impossible to be achieved. Taking into account that the concentration of sulphur dioxid solution used for corn steeping must be included in the range 0.18 – 0.22% and the pH-value for the food starch must be included in the range 4.5 – 5.5, the old CCPs were kept only as oPRPs.

From a hygienic point of view, the hazards from cross-contamination, briefly presented in Table 3, could come from equipment, tanks, storage tanks, packaging and personnel manipulation (Samuels, 1993). On the other hand, the chemical and physical hazards could appear from the facilities, equipment and also from the personnel. Some of these possible hazards can be eliminated through preliminary programs, but the others only with specific preventing measures.

A special topic took into account in the HACCP study was the gluten free starch status. To assure it, the HACCP team fixed as new CCP the wheat seed content in corn to be max. 0.5%.

The updated flow diagram is presented in figure 2 and includes the CCPs newly identified. For them we establish a monitoring plan presented in Table 3. In order to obtain a fast result, these new CCPs will be monitored using a daily tests based on wheat seed identification and ATP detection.

In table 4 we centralized the results obtained during a whole year (October 2012 – September 2013), after the evaluation of hygiene status of surfaces which come in contact with finished products. The results show that the working equipment and operators hands have a higher contamination than the packaging, but without any value higher or equal to 10 RLU, meaning that the prerequisite programs are implemented, complied and effective. For the starch packaging the results were around the values 0 and 1, very rarely achieving the values 4, 5 or 6 RLU.

Table 3. Microbiological criteria for corn starch process (Samuels, 1993)

Contaminated place	**Identified microorganisms**
Starch slurry storage tanks	*Fusarium sp., Absidia sp., Penicillium glaucum, Aspergillus niger, Lactic bacteria (Lactobacillus)*
Dehydrating starch slurry centrifuges	*Faecal streptococci, Staphylococcus aureus, Faecal coliforms, Fusarium spp., Aspergillus spp., Penicillium spp., Geotrichum spp.*
Process water	*Faecal streptococci, Staphylococcus aureus, Faecal coliforms, Fusarium spp., Aspergillus spp., Penicillium spp.*
Mixing dehydrated starch bunker	*Faecal streptococci, Staphylococcus aureus, Faecal coliforms, Fusarium spp., Aspergillus spp., Penicillium spp., Mucor spp., Rhizopus spp.*
Walls	*Aspergillus niger*
Finished product (cornstarch)	*Escherichia coli, Coliforms, Staphylococcus aureus, Faecal Streptococci*

Table 4. Centralization of the analyzes results of hygiene status surfaces for the period Oct. 2012 – Sept. 2013

	Hygiene status of surfaces: MAX. 10 RLU																							
Surface	Oct.12				Nov.12				Dec.12				Jan. 2013				Feb. 2013				Mar. 2013			
Equipment	8	7	6	2	5	4	3	5	2	4	5	1	4	3	6	4	5	7	7	6	4	3	5	7
Hands	8	5	8	1	2	5	3	4	3	5	5	2	4	6	2	3	4	5	3	5	2	3	4	5
Starch packaging	2	0	1	0	3	0	1	1	0	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0
Surface	Apr. 2013				May. 2013				Jun.13				Jul.13				Aug.13				Sep.13			
Equipment	3	3	3	3	3	4	2	4	5	4	7	4	5	3	4	5	2	6	1	3	6	6	4	3
Hands	3	2	1	4	2	1	2	1	3	2	5	3	2	3	4	3	3	4	1	1	2	4	1	0
Starch packaging	0	1	1	0	0	1	0	1	0	0	0	0	3	4	0	0	0	0	0	0	0	0	0	0

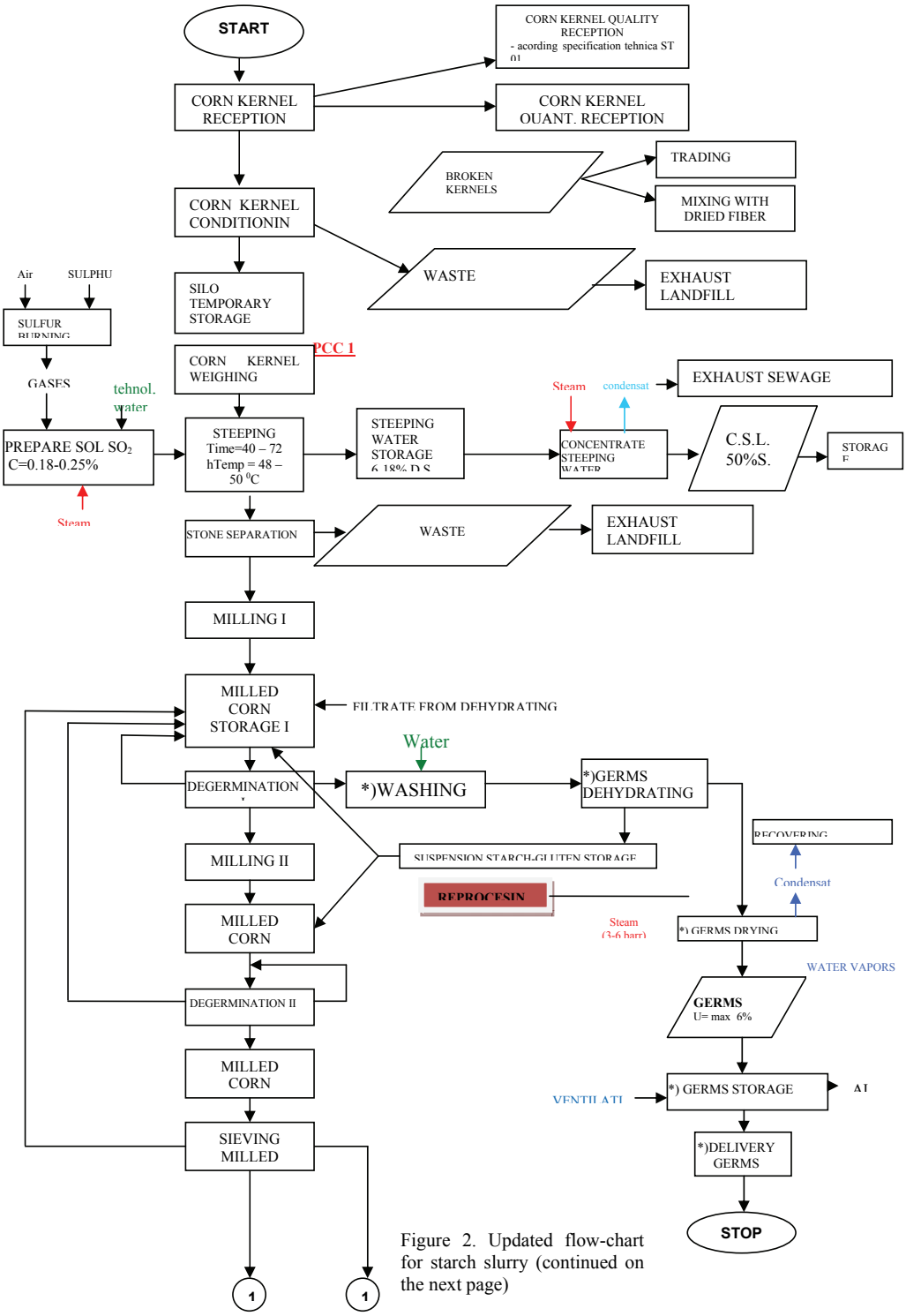

Figure 2. Updated flow-chart for starch slurry (continued on the next page)

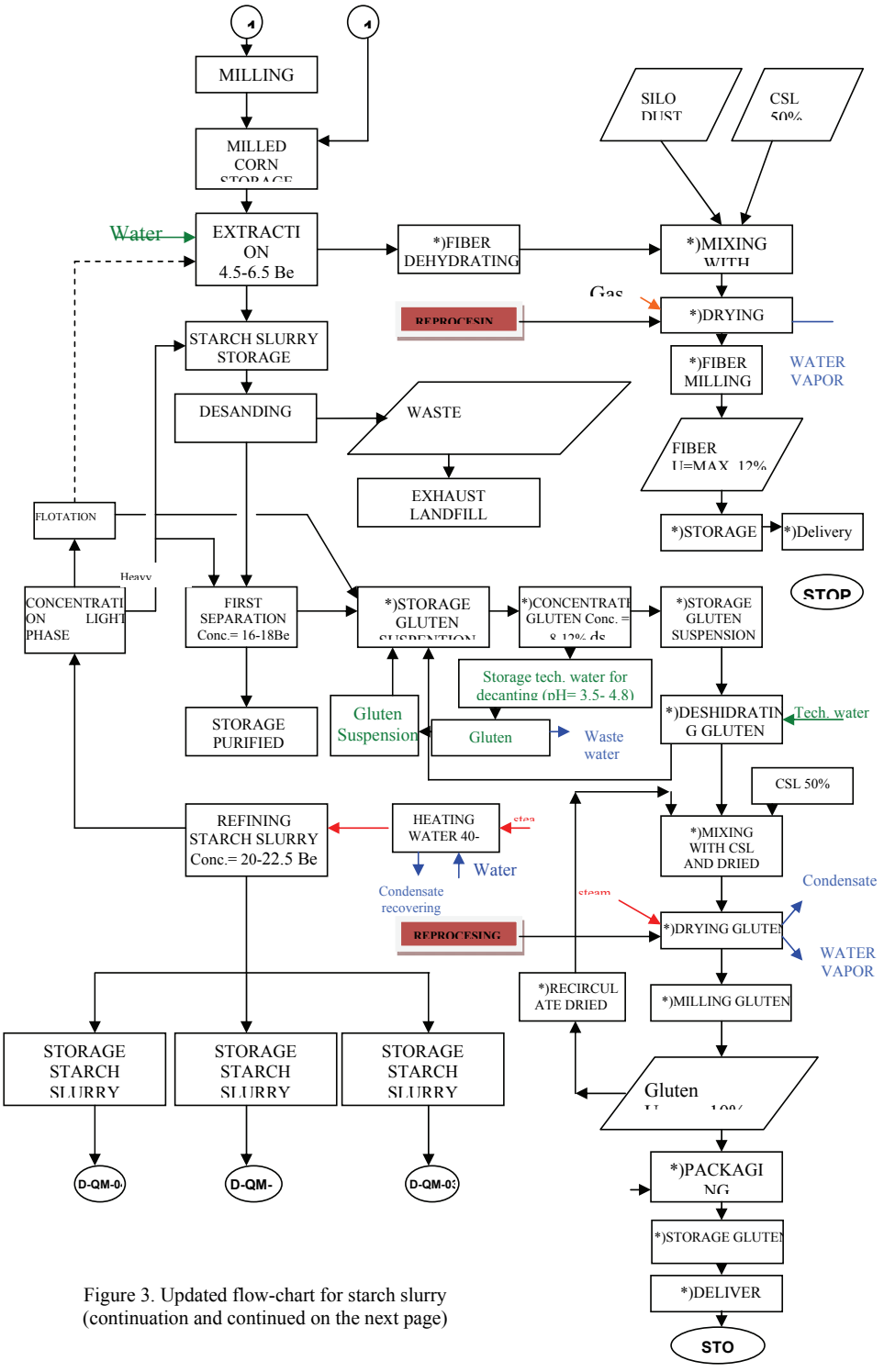

Figure 3. Updated flow-chart for starch slurry
(continuation and continued on the next page)

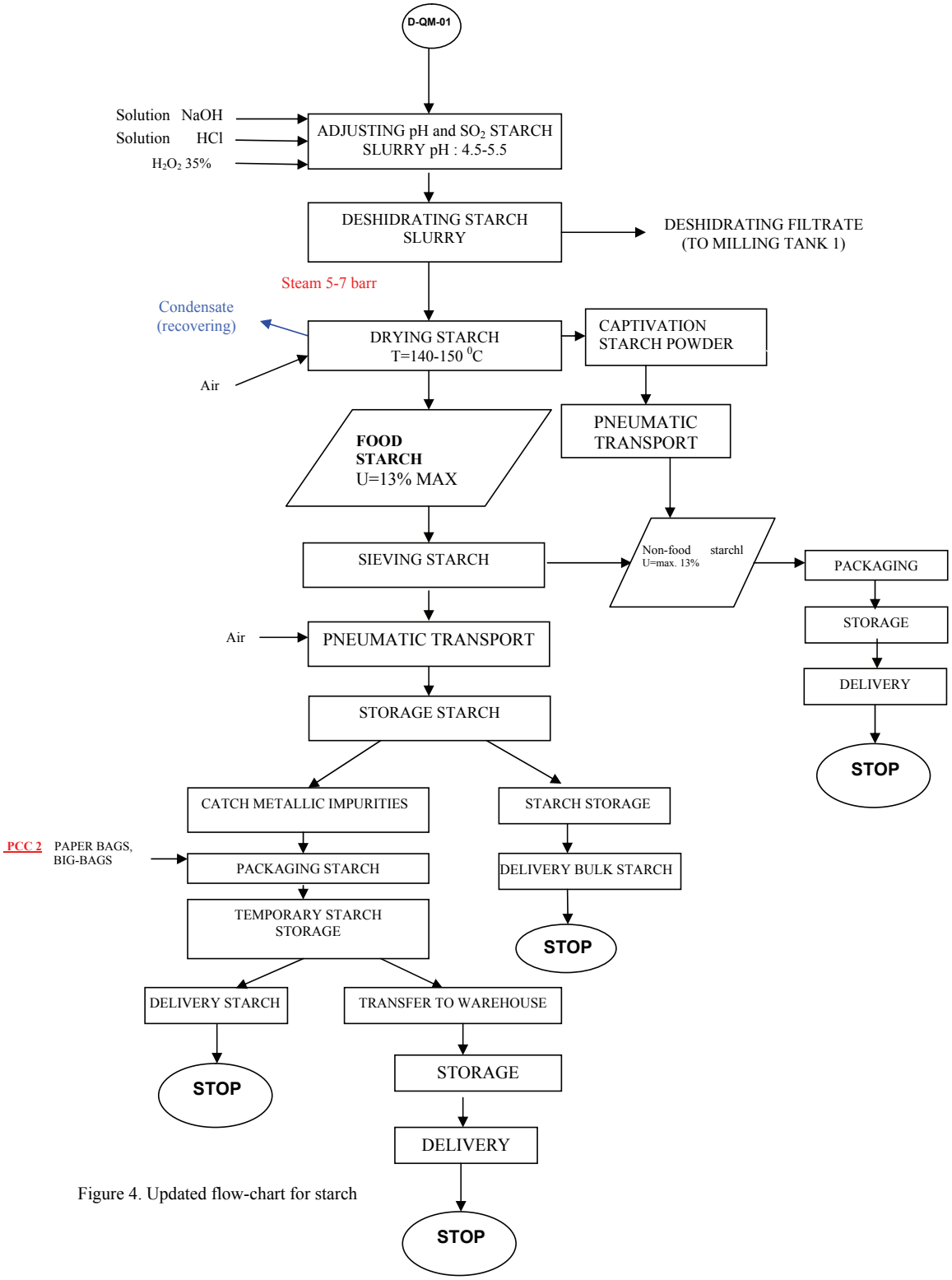

Figure 4. Updated flow-chart for starch

Table 5. Centralization of the foreign bodies analyzes results in corn for the period Oct. 2012 – Sept. 2013

	Foreign bodies in corn: MAX. 0,50 %											
	Oct.12	**Nov.12**	**Dec.12**	**Jan.13**	**Feb.13**	**Mar.13**	**Apr.13**	**May.13**	**Jun.13**	**Jul.13**	**Aug.13**	**Sep.13**
Foreign bodies (%)	0.32	0.33	0.31	0.32	0.27	0.27	0.27	0.28	0.25	0.23	0.36	0.36

Table 6. Monitoring plan for the proposed CCPs in the corn starch production process

Important hazard	**Control measures**	**Critical limits**	**Monitoring**			**Corrective action**	**Responsible**
			Responsible	**Method**	**Frequency**		
Allergens (wheat gliadin)	Identification of wheat seed content in the corn entered in the steeping tanks	0.5 %	Raw material technician	Instruction for determination of foreign bodies in corn	After each steeping tank filling	Produce non-food starch	Production Manager
Bacteria and moulds - packaging	Test for hygiene of surfaces at the batch reception	10 RLU	Hygiene Responsible	Test for hygiene of surfaces	Once per day randomly (min. 1% from packaging)	Change packaging batch, contact packaging supplier	Hygiene Responsible

Data centralized in Table 5 represents the foreign bodies' analysis results averages for corn made in the period October 2012 – September 2013. These values include the wheat seed content in corn and we considered that they couldn't be higher than the foreign bodies' content. Even if we supposed that the values from the table represent itself the wheat seed content they are below the maximum limit, 0.5%.

In conclusion, the values presented in Tables 4 and 5 lead to the validation of the HACCP plan presented in Table 6.

CONCLUSIONS

The HACCP system already implemented by the factory, although proven as efficient until this moment, was re-evaluated in this study. By reviewing the newly technical and scientific proofs and possibilities it turned out that the current HACCP plan needed some improvements in order to be more effective and to take into account the real hazards, CCP and critical limits.

Although the cornstarch is used in the food industry only as raw material and it is undergo supplementary treatments before becoming a "ready to eat" product, the microbiological hazards still exist and have to be carefully monitored. Even if the production process is not favorable for the growth of microorganisms, the microbiological hazard can appear from cross-contamination and this fact was taken into consideration in this study.

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WET-MILLING HIGH-AMYLOSE CORN IN THE INDUSTRIAL SCALE

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Abstract
Due to its properties, high-amylose corn containing about 70% amylose can be better wet milled by including some modifications in the process conditions. The most important changes can be made in the steps of steeping and protein separation. In this paper we compared the results obtained during three procedures of high-amylose corn processing performed in a starch factory located in the south-eastern part of Romania. The aim is to establish the optimal processing procedure for high-amylose corn in an industrial scale. To obtain a reliable process evaluation, the amylose content of the starch produced was analysed by using two different conventional colorimetric methods, a potentiometric method and a spectrofotometric one. As compared to the laboratory and pilot scale the industrial scale wet-milling has some advantages materialized in higher starch yields and protein content of starch.

Key words*: high-amylose corn, industrial scale, wet-milling*

INTRODUCTION

High-amylose corn is grown to be used exclusively to wet-milling processes, the starch obtained having applications in the textiles, candies and adhesives industries (Thomison, 2013). High-amylose starch is composed by round and filamentous granules with length of approx. 15 µm (Liu, 2005). It can be consumed without any problems also by diabetics and others who try to control insulin levels in blood, because it has a glycemic index lower than conventional corn. Another advantage is that the high-amylose starch is an excellent resistant starch source, considered to be functioning as prebiotic and reducing the colorectal cancer incidence. In addition, this starch type can be used to manufacture the biodegradable packaging materials and adhesive materials (Polaske *et al.*, 2005). High-amylose corn is processed through the wet-milling process, too being necessary some small changes due to its properties. The most important changes were observed in steeping and protein separation steps. A comparative study of three starch types with different amylose content (63%, 23% and 2%), performed in 2010 by Kibar *et al.,* showed several specific characteristics for the high amylose corn, such as higher water absorption, lower degree of swelling due to its internal structure and the presence of endogenous lipid granules.

The steeping of high-amylose corn kernels is made more rapidly than for conventional corn, while it is observed that supplementary steeping water of 6 to 9% is consumed. For example, Anderson *et al.*, in 1959 observed that for corn with amylose content of approx. 49%, the steeping water consumed was 6% higher and in 1960 for corn with amylose content of 68% was 9% higher than for the conventional corn. The steeping time and temperature seem to be the same, but the steeped corn total volume achieves 128%, while for conventional corn is only 68% (Anderson *et al*., 1959). In the protein separation step appear some difficulties due to the small dimensions of granules, which are of 11 µm in the high-amylose starch instead 14 µm in the conventional starch

(Wittenberger, 2003). Also, this fact is easily observed in the gluten protein content, 35-44% (Anderson *et al*., 1959, Anderson *et al*., 1960) and in the starch protein content 0.5-0.7% (Anderson *et al*., 1959, Anderson *et al*., 1960). As the amylose content is high, the starch gelatinization is more difficult and increases susceptibility to retrogradation, the main mechanism of resistant starch formation in processed food (Hódsági and Salgó, 2011).
The most high-amylose studies found in the literature were performed in laboratory scale and only some in pilot scale; therefore in this study we present the wet-milling high-amylose corn process in the industrial scale, considering this approach to be more useful for the starch producers.

MATERIALS AND METHODS

In the literature there can be found some studies made in the period 1959-1961 by several researchers in order to observe the processing behaviour of high-amylose starch containing 57, 66, 68 and 75% amylose. The tests were performed in the laboratory and confirmed in pilot stations. In the starch factory from Tandarei, where our study took place, there were performed until now three high-amylose corn processing cycles, the first one in the beginning of 2011 (batch 2011) and the others two, in the beginning and the end of 2012, batch 2012-1 and 2012-2, respectively. For the first high-amylose processing we took as reference the test made in 2003 by the pilot station of innovation centre Zuckerforschung Tulln from Austria and for the others, in addition we used the experience acquired. The high-amylose corn used was cultivated in Hungary for the first processing cycle and in Romania for the other two processing cycles.
The analysis methods performed for the corn and for the final products and by-products were the same as those used for the conventional corn, as following:

- The starch content in corn, germs, fiber and gluten was analyzed using the polarimetric method of Ewers.
- The protein content in corn, starch, corn gluten meal and corn gluten feed was analyzed using Kjeldahl method.
- The fat content in germs was analyzed according to Soxhlet method.
- The moisture of steeped corn was monitored at each 5 h until achieving 35 h of steeping, then every hour, using the rapid drying method halogen lamp moisture analyzer.
- The sulphur dioxide content from steeping water was performed through iodometric method.
- The soluble substances from steeping water were analyzed using a refractometer capable to measure 0 – 95 brix degrees.

The analysis of starch amylose content and starch viscosity were specific, as follows:

- The amylose content for the samples obtained in the first two processing cycles was analyzed using only the potentiometric method, but for the samples obtained in the third processing cycle we used both potentiometric and spectrofotometric method.
- Beginning with the third processing high-amylose corn, in addition we analyzed also the starch viscosity using a Brookfield viscometer, type RV DV-E equipped with small volume sample adapter (measure chamber SC4-13R and spindle SC4-27.

RESULTS AND DISCUSSIONS

Studies made in the factory from Tandarei upon the high-amylose corn wet-milling process and high amylose starch characteristics had as the main goal to establish the optimal high-amylose wet-milling procedure. This study shows some advantages as compared to other studies found in literature, because the results were obtained in a production plant which processed between 75 and 350 to of high-amylose corn for these three processing cycles.
As we mentioned above, the production steps in which appear differences over the conventional corn wet-milling process are corn steeping and protein separation from starch suspension. In the following two tables (1 and 2) we centralized the average results obtained during the three wet-milling processes for corn characterisation, steeping and protein separation processes. The values presented in

Table 1 show a decreasing of starch content in high-amylose corn from the first to the third processing, apparently unexplainable. After a literature review we can conclude that it is possible that these results are not reliable due to the fact that amylose hydrolysis with hydrochloric acid is quite difficult and the method SR EN ISO 10520:2002 (polarimetric method of Ewers) excludes the high-amylose starch from its field of application. The steeping time varies from 46 to 61 hours, but this fact did not have any impact on the corn moisture content after steeping, this being maintained at around 50%. The soluble substances and SO_2 content in steeping water increased with steeping time, most probably because in the industrial scale production the recirculation in counter-flow process is used. This steeping industrial way brings in the steeping vessel (which is filled with dry high-amylose corn) the steeping water circulated through all steeping vessels in different steeping phases, leading to a higher content of soluble substances and the lowest SO_2 content. In the same time the SO_2 solution, with the lowest content of soluble substances and the higher SO_2 content is introduced in the steeping vessel filled with a corn having the highest moisture content.

Table 1: Corn and steeping process characteristics

	Batch 2003 Austria	**Batch 2011**	**Batch 2012-1**	**Batch 2012-2**
Starch content (%/DS)	59	53.1	41.5	35.8
Corn moisture after steeping step (%)	53	52.2	51.1	49.3
Steeping time (h)	48	46.0	61.0	57.0
Soluble substances in steeping water (%)	-	4.2	8.7	6.7
SO_2 in steeping water (%)	-	0.047	0.069	0.048

Table 2: Protein separation process and starch characteristics

	Batch 2003 Austria	**Batch 2011**	**Batch 2012-1**	**Batch 2012-2**
Starch slurry concentration (^{0}Be)	15	22.1	18.5	20.0
Ratio washing water / starch slurry	-	1.2	1.5	1.3
Protein in starch (%)	0.72	0.48	0.43	0.45
Starch content in gluten (%/DS)	-	10.8	19.8	17.5
Protein in gluten (%/DS)	-	41,3	47,2	47,6
Amylose (%/DS)	55.3	65.6	71.0	75.3

We have observed (Table 2) that the concentration of the starch slurry does not negatively influences the protein content in the starch, for the highest concentration of the starch slurry obtained during the three processing, 22.1 ^{0}Be in the batch 2011, the highest protein content in the starch being obtained (0.48 %/DS). Conversely, if we take into consideration the values recorded for the ratio washing water / starch slurry and the protein content in the starch, we can easily observe that the value of the ratio is influencing in a favourable way the protein separation from the starch slurry. The lowest protein content from starch, 0.43 %/DS, was obtained in the batch 2012-1 for which the ratio was the biggest, 1.5. If we compare the protein content of high-amylose starch reported in the literature, either obtained in laboratory (average 0.59 %/DS) or in pilot scale (average 0.72 %/DS) we can say that the industrial scale processing is more advantageous for the protein separation step, the average value being 0.46 %/DS.

The yields of high-amylose starch and by-products obtained for these three high-amylose corn processing are summarized in Table 3. We can observe that for the batch 2012-1 the yield was the lowest both for high-amylose starch and by-products, which is related to the lowest quantity of the raw material processed. If we try to correlate the high-amylose corn processed quantity with the total yield, we observe that the yield increases with quantity, but a reasonable correlation coefficient could not be obtained. The same remark is also valid for the starch and germs yields, but not for fiber and gluten yields, the latter two being influenced by the CSL dosed quantity used in order to adjust the protein content and increase the yield.

The diagram presented in figure 1 shows that during the three high-amylose corn processing cycles the starch content (SC) had a decreasing evolution, while the amylose content increased. This comparison between starch and amylose content confirms the above mentioned statements regarding the difficulty of quantifying the starch content in high-amylose corn and moreover, indicates a possible explanation of starch content decreasing from the first to the last high-amylose corn processing cycle. Figure 2 shows a comparison of the amylose content determined in the high-amylose starch obtained in the batch 2012-2 using either the potentiometric or the spectrofotometric method. As it is also known from the literature, the iodometric methods seem to overestimate the amylose content (Zhu *et al.* 2008; Vilaplana *et al.*, 2012), but remain the most reliable of all conventional methods (Duan *et al.*, 2012). With the exception of a single sample (no. 6) all the analysis results showed that the potentiometric method gives higher values than the spectrofotometric method.

In order to validate more correct analysis methods for high amylose starch this study should be continued.

Table 3: Yields after high amylose corn processing

Yield (%)	**Batch 2011**	**Batch 2012-1**	**Batch 2012-2**
High-amylose corn quantity (to)	**195**	**79**	**347**
High-amylose starch	53.0	51.8	57.4
Germs	8.5	7.1	9.0
Fiber *	22.1	19.0	19.8
Gluten *	7.8	6.5	6.9
TOTAL	**91.4**	**84.4**	**93.1**

* to which was added CSL (steeping water concentrated till DM of approx. 50%) to adjust the protein content.

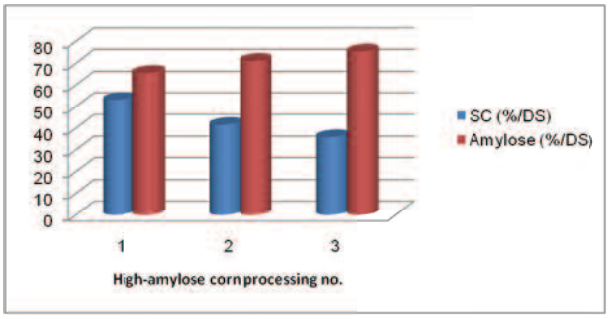

Fig. 1: Starch content vs. amylose content for the batch 2012-2

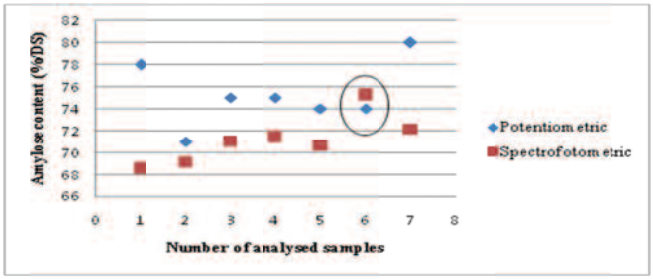

Fig. 2: Comparison of amylose content results obtained by two colorimetric methods for the third processing

CONCLUSIONS

The high-amylose corn can be processed following the classic wet-milling process for the normal corn, but making some small changes at corn steeping and protein separation steps, in order to accommodate the higher capacity of this type of corn to absorb water and also to cope with the smaller dimensions of its starch granules.

The stepping process in counter-flow used only in industrial scale is more advantageous than any stationary process used in laboratories or pilot stations, allowing a more rapid disaggregation of corn starch bonds and a much better separation of protein from starch slurry.

Even if the analysis methods for conventional corn were useful to a certain extent in the high-amylose corn processing control too, it is recommendable for future batches that the polarimetric method of Ewers for starch content to be replaced with another method, more specific for this type of corn. More studies are needed in order to find a more appropriate method for amylose determination in high-amylose corn products.

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