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Faculty of Biotechnologies

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# SUMMARY

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# AGRICULTURAL BIOTECHNOLOGY

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# *IN VITRO* CULTIVATION OF *LAETIPORUS SULPHUREUS* AND EVALUATION OF ITS ANTIMICROBIAL PROPERTIES

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***Abstract***

*Laetiporus sulphureus (Bull. Fr.) Murill., is a wood-rotting basidiomycete mushroom well known for it nutritional value. In this study, alcoholic and aqueous extracts obtained from a Romanian isolate of L. sulphureus cultivated on various culture media were investigated for the antimicrobial properties. PDA, malt extract as solid media and PD (I), malt extract (II), YPG (III) and Hwang (2008)(IV) -as liquid media were used for in vitro cultivation of L. sulphureus, in order to evaluate the optimal medium for an efficient biomass production of L. sulphureus. Between all media tested, best results regarding the growth of mycelia, were obtained when I, II, IV media were used. Only on IV (Hwang) culture medium was observed the typically orange pigment elaborated by fungus. Alcoholic and aqueous extracts of fruit bodies and submerged mycelium developed in liquid media were analyzed against strains of Candida albicans ATCC10321, Candida parapsilopsis CBS604, Escherichia coli, Bacillus cereus, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas fluorescens and Pseudomonas aeruginosa. The results shown that the types of culture media for biomass production of L. sulphureus and their aqueous and alcoholic extracts tested against these pathogens have different effects on inhibitory activity. Between the two types of extracts tested aqueous extracts were inferior to alcohol extract in their inhibitory activity on all organisms except Candida sp. in interaction with L. sulphureus aqueous extract from biomass developed on IV medium.*

***Keywords:*** *antimicrobial properties, in vitro cultivation, Laetiporus sulphureus*

## INTRODUCTION

*Laetiporus sulphureus* (Bull. Fr) Murril (Aphyllophorales, Polyporaceae) is a wood – rotting basidiomycete mushroom which grow on mature and old-growth trees in forests or in urban parks. It is know as a destructive pathogen of the trees that cause butt and trunk rots (Holsten et al., 2001; Sinclair and Lyon, 2005). *L. sulphureus* is characterized by an intense orange colour, fleshy basidiocarps and tubular hymenopores (Banik et al., 1998) and can be harvested as an edible fungus with reliable nutritional value. Because of its medical properties *L. sulphureus* has been used in some therapies as antitumor, antiviral, antimicrobial treatments (Wasser and Weis, 1999). Among several other mushrooms like *Trametes versicolor, Ganoderma applanatum* and *G.lucidum*, *Laetiporus sulphureus* has been found to be an excellent source of natural products with therapeutic properties. Those mushrooms provide a rich variety of secondary metabolites and polysaccharides that have been proved to posses significant antimicrobial activities (Siljegovic et al., 2011). The fruiting bodies of *L. sulphureus* contain N-methylated tyramine derivatives (Rapior et al., 2000), polysaccharides (Alquini and Carbonero, 2004), terpenoids, laetiporic acids and other compounds (Weber et al., 2004; Davoli et al., 2005). From *L. sulphureus* submerged mycelia cultures have been isolated various polysaccharides (Hwang et al., 2008; Hwang and Yun, 2010) with therapeutic evidences. For these reasons, the goals of our studies were to

find the optimal medium for an efficient biomass production of *L. sulphureus* and to investigate the antimicrobial activities of alcoholic and aqueous extracts from fruit bodies and submerged mycelium developed in liquid media, against some pathogenic agents.

## MATERIALS AND METHODS

***In vitro* culture establishment.** Samples of the fruit bodies of *Laetiporus sulphureus,* collected from Sinaia woods, were surface sterilizing and cutting out a piece of trama using a sterile scalpel. The pieces were placed in Petri dishes on 2% malt extract agar and PDA (potato-dextrose-agar) media and incubated at $25^0$C for a week. After the mycelium growing on the medium surface, mycelia agar discs (5 mm diameter) obtained from the active growth areas were placed in 100 ml Erlenmeyer flasks, each with 50 ml PD (potato-dextrose) (I), 2% malt extract (II) and YPG (yeast peptone glucose) (III) liquid media. We also used a medium (IV) prepared according Hwang et al (2008) (20g/l glucose, 2g/l peptone, 2g/l yeast extract, 0,46g/l $KH_2PO_4$, 1g/l $K_2HPO_4$, 0,5 g/l $MgSO_4$), in order to determine the optimal growing medium for *in vitro* culture of *L. sulphureus*. After inoculation the samples were incubated at $25^0$C in a rotary shaker at 148 rpm for 10 days. The biomass obtained from each liquid medium was filtrated and weighed.

**Extracts preparation.** For extracts preparation the biomass developed on each liquid medium tested was used. A mixture of mycelium and medium from the *in vitro* culture was separated by filtration. The filtrated mycelia mass was grounded and used for the extracts preparation. For aqueous extract, 1 ml of distilled water per 1 g of mushroom material was added. In the case of alcohol extract, 1 ml 70% ethyl alcohol was added to sample (1 g wet weight). Also, ethyl alcohol (70%) was used as negative control. The aqueous and alcoholic solutions were then centrifuged at 5000 rpm for 10 minutes. The supernatant was kept at 4 $^0$C and used to determine the antimicrobial activity of *L. sulphureus* extracts.

**Antimicrobial activity.** Alcoholic and aqueous extracts of submerged mycelium developed in liquid media were analyzed against strains of *Candida albicans* ATCC10321*, Candida parapsilopsis* CBS604 (from the collection of MICROGEN, Bucharest)*, Escherichia coli, Bacillus cereus, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas fluorescens* and *P. aeruginosa* (from the collection of Faculty of Biotechnologies Bucharest). To determine the antimicrobial activities of tested extracts, 1 ml from each bacterial and yeasts suspensions was inoculated on Luria Broth or YPG media, respectively, in Petri plates. After removing the excess suspension using a micropipette, sterile filter paper discs soaked in fungal extracts were placed on the surface of the inoculated medium. 24 hours after incubation at $37^0$C and $27^0$C respectively, occurrence of inhibition halos was observed.

## RESULTS AND DISCUSSIONS

The submerged cultivation of mushrooms is a promising method for obtaining pharmaceutical compounds and was applied for various edible and medicinal species (Petre et al., 2012). For *L. sulphureus* there are only few reports regarding the submerged cultivation and fungal pellet obtaining in order to obtain various metabolites (Sivulski et al., 2009; Hwang et al., 2008).

For this reason, one of the aims of our experiments was to evaluate the effects of different media compositions on the submerged mycelium growth of *L. sulphureus* and to achieve maximum biomass production. For this purpose *L. sulphureus* fresh mushroom was firstly cultivated on two agar media (malt extract and PDA). The mycelium grown on the surface of these media was used for inoculation of four different liquid media: I-PD (potato-dextrose), II- malt extract, III-YPG (yeast peptone glucose) and IV- Hwang (2008). After ten days of culture on these tested media the results shown that the best growth of the mycelia biomass (as pellets) was observed on Hwang medium followed by PD and malt extract media. The poorest growth was encountered on YPG medium. The wet weight of filtrated mycelia biomass harvested from Hwang medium (8.26 g/100ml) was significantly superior to that obtained on PDA

(2.4 g/100 ml) and malt extract (1.30 g/100ml) media, respectively. The mycelia growth as pellets is of great interest from practical point of view, facilitating the extraction procedures of biological active compounds and which can be used to prepare functional food (Petre et al., 2012).


I-PD II-Malt extract III- YPG IV- Hwang

Figure 1. Aspect of submerged mycelia of *Laetiporus sulphureus* in different liquid media

An interesting aspect of our results was the accumulation of orange pigment in mycelium in submerged conditions (in Hwang medium) (figure 2); in the other media the mycelium was uncoloured.

Figure 2. *Laetiporus sulphureus* orange pigment elaborated in Hwang medium

Davoli et al (2005), investigating the orange pigment produced by fruit-bodies of *L. sulphureus* using modern spectroscopic techniques, found that the pigment is a polyene of non-isoprenoid biosynthetic origin named laetiporic acid. The practical significance of this compound is not very clear but it could be used as food colorant.

In the second investigation, alcoholic and aqueous extracts obtained from biomass developed on each liquid medium tested (I, II, III, IV) were analyzed for their ability to inhibit six strains of Gram positive and Gram negative bacteria: *E. coli, B. cereus, S.aureus, E. faecalis, P. fluorescens* and *P. aeruginosa* and two pathogenic yeasts: *Candida albicans* ATCC 10231 and *Candida parapsilopsis* CBS 604. The results obtained shown that the culture media used for submerged cultivation of *L. sulphureus* mycelium influenced not only the growth rate but also the biological activities of aqueous and alcoholic extracts against pathogens (Table 1).

Table 1. Pathogen interactions with *L. sulphureus* extracts obtained from biomass developed on different liquid culture media (I, II, III, IV)

| Variant | Liquid media | Extracts | Pathogens | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *E. coli* | *B. cereus* | *S. aureus* | *E. faecalis* | *P. fluorescens* | *P. aeruginosa* | *C. albicans* | *C. parapsilopsis* |
| I | PD | aqueous | - | - | - | - | - | - | - | - |
| | | alcoholic | + | - | ++ | + | + | - | ++ | ++ |
| II | Malt extract | aqueous | - | - | - | - | - | - | - | - |
| | | alcoholic | - | - | - | - | - | - | - | - |
| III | YPG | aqueous | - | - | - | - | - | - | - | - |
| | | alcoholic | ++ | - | - | - | - | - | + | - |
| IV | Hwang | aqueous | - | - | - | - | - | - | + | + |
| | | alcoholic | - | - | ++ | - | - | - | + | - |

It was shown that the inhibitory action of **aqueous extracts** were inferior to alcohol extracts against all the pathogenic organisms tested. However, the aqueous extract from biomass developed on IV (Hwang) medium presented clear inhibition area against both *Candida* species (Figure 3). **Alcoholic extracts** of biomass provided from IV (Hwang) medium were optimal for inhibition of *S. aureus* and *C. albicans.* Clearly inhibition halo was observed on *S. aureus, E. coli E. faecalis, P. fluorescens* and against both *Candida* species when the alcoholic extract from biomass provided to I (PD) medium was used (Figure 3).

Alcoholic extract from biomass growth on III (YPG) medium had the best inhibitory activity against *C. albicans* and *E. coli*, but the alcoholic extracts from mycelium growth in malt extract medium inhibited only the growth of *E. coli* strain.

a b

Figure 3. Inhibitory activity of *L. sulphureus* alcoholic extracts from biomass developed on I (PD) medium against *Candida* sp. (a, b -1). M=control (70% alcohol)

## CONCLUSIONS

The goals of our studies were to find the optimal medium for an efficient biomass production of *L. sulphureus* and to investigate the antimicrobial activities of alcoholic and aqueous extracts from submerged mycelium developed in liquid media, against some pathogenic agents. The data from *in vitro* growth tests provides significantly evidences on biological differences between mycelia biomass developed on various media used. Between all media tested for maximum biomass production, the best growth of the fungal biomass, including as pellets, was observed on Hwang medium follow by PD and malt extract media. Only in Hwang culture medium was observed the typically orange pigment elaborated by fungus. Alcoholic and aqueous extracts from submerged mycelium cultivated in different liquid media tested against several microbial strains demonstrate the influence of culture conditions on inhibitory activity. It was shown that aqueous extract from submerged mycelia developed on Hwang media was inferior to alcohol extract in their inhibitory activity on all pathogenic organisms tested except *Candida* sp. Alcoholic extracts of biomass provided from IV (Hwang) medium had the best inhibitory action for inhibition of *S. aureus* and *C. albicans*. Moreover, alcoholic extract from *L. sulphureus* biomass developed in PD medium inhibited *Candida* sp., *S. aureus, E. coli E. faecalis* and *P.fluorescens*.

The results obtained are promising but optimisations of the growth conditions as well as the extraction procedures are necessary in order to recover larger quantities of biological active compounds.

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# INDUCTION OF INDIRECT ORGANOGENESIS *IN VITRO* IN *RHODIOLA ROSEA*– AN IMPORTANT MEDICINAL PLANT

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***Abstract***

*Rhodiola rosea L. is an endangered medicinal plant due to over harvesting in Bulgaria and in other European countries. The root and rhizomes are rich in pharmacologically and therapeutically active substances like flavonoids, phenolic acids that make the plant of commercial importance. The seed propagation in nature is very poor as far as only of 2 to 30 % of the seeds germinate depending on the ecologically geographic conditions. Thus, in vitro techniques are suitable for propagation of this species. In vitro propagation is possible by direct and indirect organogenesis. The latter is much more difficult in many species, including golden root. The present study aimed to understand the conditions, which provoke undifferentiated tissue initiation and development of shoots.*

*The effects of different plant growth regulators in various concentrations oncallus induction and indirect plant regeneration were investigated. Regenerative callus was received on Murashige and Skoog medium containing 2,4-Dichlorophenoxyacetic acid (0.1 mg $l^{-1}$)within 28 days. The adventitious buds were differentiated when the calli were subcultured on Murashige and Skoog (1962) medium supplemented with 6-benzylaminopurine (1.0 mg $l^{-1}$) and indole-3-acetic acid (0.1 mg $l^{-1}$) within 4 - 5 weeks. Half-strength solid MS with indole-3-butyric acid (2.0 mg $l^{-1}$)and IAA (0.2 mg $l^{-1}$) exhibited the best in vitro rooting.*

*These results contribute to the understanding of processes of growth and development in vitro of Rhodiola rosea and possibilities for selected of valuable clones and establishment of propagation schemes.*

***Keywords****: calli, golden root, indirect organogenesis, regeneration.*

## INTRODUCTION

More than 2000 plant species in Europe are used for production of medical and nutraceutical herb preparations. The wild species represent 70 % from this production and only 30 % from the species are cultivated in the field. However, a significant number of the wild species are rare, disappeared and/or under protection (Varbanova, 2002; Evstatieva et al., 2007). One of the reasons is the increasing interest of pharmaceutical industry towards these plants and respectively more intensive use of their natural resources which are not unlimited. This raises the question of searching for and development of alternative methods friendly to the environment. (Tasheva and Kosturkova, 2013). Such ones are the biotechnological methods which are important for breeding, propagation and conservation of valuable medicinal plants.

Biotechnological techniques using cells, tissues, organs or whole organisms, growing and developing in *in vitro* conditions, are suitable for genetic manipulations (Khan et al., 2009), and to obtain valuable compounds (Rao andRavishankar, 2002).

During the last years, propagation of medicinal plants in *in vitro* conditions has gained increasing interest to the industry and has been more widely used for the necessities of the pharmaceutical industry. The way leading to production of great number of identical plants, what is a subject of clonal/micro-propagation, is one of the most preferable approaches from a commercial viewpoint. This approach could be used to create *ex situ* and *in vitro* collections, as well as for commercial propagation to obtain raw material for pharmacetical and cosmetic industries (Julsing et al., 2006). *In vitro*propagation allows obtaining of a large plant mass for a short period of time (Tripathi and Tripathi, 2003). Developed

micropropagation methods have been reported for many medicinal plants but this list is increasing (Tasheva and Kosturkova, 2013).

Experimental approaches used for *in vitro* propagation of medicinal plants could be broadly divided/classified in three categories: (1) Isolation of meristems and stem tips and stimulation of their growth which depends on different factors. The effect of plant regulators and their combinations to micropropagation of medicinal plants is reported by Makunga et al., 2003; Debnath, 2009; Keng et al., 2009; Yusuf et al., 2011.

(2) The second way includes induction of adventitious buds from leaves, stems and root segments or obtaining callus from these organs. The plant regeneration by organogenesis is well developed in some medicinal and aromatic plants, but it varies widely for different plant species, which requires an individual approach to study the conditions for regeneration, as well as determining the factors controlling growth and differentiation for each type of species (Patra et al. 1998).

(3) The third way is somatic embryogenesis, which is the theoretically most effective to obtain plants (Martin, 2004; Paramageetham et al., 2004; Fiuk and Rybczynski, 2008, Robinson et al., 2009). To determinate, the relationship between different groups of phytoregulators is essential for these because success of tissue culture work depends on phytoregulators′ type and concentration. The process of differentiation of unorganized callus tissue, initiation of the buds and roots depends on the appropriate combination of auxins and cytokinins in the nutrient medium (Sagare et al., 2000). The influence of the cytokinins and auxins on *in vitro* cultures depends on the *in vitro* systems, plant species and in many cases even to the variety or/and ecotype. The natural and synthetic auxins and cytokinins induce typically physiological responses in plants. The specificity of the investigated object from one side and endogenous phytohormones from other side determine the effects of the exogenously applied phytoregulators.

*Rhodiola rosea* is a medicinal plant under protection in Bulgaria and other European countries. The extract from root and rhizomes has a number of applications. It has adaptogenic, antitoxic and antihypoxic action, and increases resistance to infection diseases. Due to the low toxicity and the absence of side effects of its extract, golden root is widely used in food and cosmetic industry. In Bulgaria, there are no systematic and comprehensive studies of this endangered species and data on the application of modern biotechnological and phytochemical approaches. Creating integrated technologies for breeding, preservation and cultivation of this valuable species is a major premise for its cultivation in different mountain regions in Bulgaria.

The few biotechnologically investigations concerning species from *Rhodiola* genus refer particularly to the process of indirect organogenesis. Callus induction followed by plant regeneration of *Rh. coccinea, Rh. sachalinesis, Rh. rosea* were studied by some authors (Kirichenko et al., 1994; Furmanova et al., 1995; Ishmuratova, 1998; Yin et al., 2004; Sha Hongetal., 2008; Liu Jianfeng et el., 2007; Liu Jian-feng et al., 2009).

The present study aimed to understand the conditions which provoke undifferentiated tissue initiation and development of buds and shoots investigating the effects of different plant growth regulators in various concentrations oncallus induction and indirect plant regeneration.

## MATERIALS AND METHODS

**Plant material.** Explants isolated from *in vitro* propagated plants, obtained in our previous work were used in these experiments (Tasheva and Kosturkova, 2010a, b).

**Culture media.**

***Nutrient media composition for callus induction*** was Murashige and Skoog (1962) medium containing zeatin 2.0 mg/l (Trans form), IAA 0.2 mg/l, casein hydrolisate 1000 mg/l, sucrose 3%, and agar-agar 0.6% (Tasheva and Kosturkova. 2010a, b).

***Nutrient medium composition for induction of organogenic callus*** designated as mediumDwas basic Murashige and Skoog (1962) nutrient medium enriched with 0.1 mg/l 2,4-D, 3% sucrose and 0.6 % agar-agar.

***Nutrient medium composition for bud formation*** was Murashige and Skoog (1962) nutrient medium containing BAP 1.0 mg/l, IAA

0.1 mg/l, sucrose 3% and agar-agar 0.6% (designated as BA medium).

***Nutrient medium composition for shoot formation and plant*** was Murashige and Skoog (1962) medium containing zeatin 2.0 mg/l (mixed isomers), IAA 0.2 mg/l, casein hydrolisate 400 mg/l, sucrose 3%, and agar-agar 0.6% (Tasheva and Kosturkova. 2010a, b). The pH of all culture media was adjusted to 5.7 – 5.8 prior and autoclaved at 1.1 kg.cm$^{-2}$, 121°C for 20 min.

**Culture conditions.** The calli explants were cultivated in test tubes or Petri dishes.

The frequency of regenerants induction was evaluatedperiodically with an interval of 28 days. Necrotic tissue was removed during each sub-cultivation. The average number and size of the buds, shoots and regenerants from one explant were scored. Obtained regenerants were separated from the callus and cultivated on media for multiplication followed by rooting.

The cultures were cultivated (induced and maintained) in a growth room with artificial illumination (fluorescent lamps) under a 16 h photoperiod at temperature of 21 - 23°C and light intensity 20 μM m$^{-2}$ s$^{-1}$ for callus initiation and maintenance and 40 μM m$^{-2}$ s$^{-1}$ for organogenesis induction and bud/shoot formation. The regenerants which had formed roots *in vitro* were transferred to small pots containing soil, peat and perlite.

## RESULTS AND DISCUSSIONS

**Callus induction.** Callus like structures or callus in the shoot basis was formed using the developed scheme for micropropagation (Tasheva and Kosturkova. 2010a, b). About 0.6 – 1.0 % from the plants formed non differentiated tissue. In most of the cases (about 85 – 90%) callus was solid, grainy, with green-brown color (Figure 1 and Figure 2) and was considered appropriate for the present experiments.

Auxins, cytokinin and auxin/cytokinin interactions are usually considered the most important factors for regulating growth and organized development in plant tissue and organ culture. Plants generally require these two classes of growth regulators. The formation of calli may be due to accumulation of the auxins in the main cutting edges which stimulates cell proliferation especially in the presence of cytokinins (Martin 2000). The application of plant growth regulators tested concentrations significantly affects the growth and the development of callus and its differentiation.

Figure 1 Regenerated plants and calli formation on MS medium containing 2.0 mg/l zeatin, 0.2 mg/l IAA and 1000 mg/l casein hydrolisate

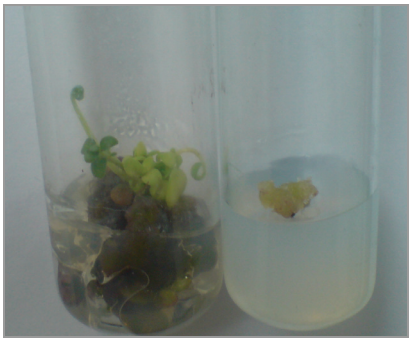

Figure 2. Formed and isolated callus tissue

**Induction of organogenic callus and proliferation.** Obtained callus when transferred to fresh nutrient medium D **(**containing MS salts and vitamins, 0.1 mg/l 2,4-D, 3% sucrose and 0.6% agar-agar) formed buds for 2 passages with 28 days duration of every passage (Figure 2 and Figure 3). Cultivation of the calli on this media for a one passage did not bring to revealing of organogenic capacity of calli.

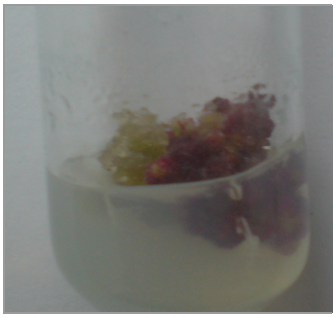

Figure 3. Isolated calli and cultivated on MS medium containing 0.1 mg/l 2,4-D

**Bud and shoot induction.** Bud formation was stimulated when the calli were cultivated on BA medium (MS nutrient medium containing BAP 1.0 mg/l, IAA 0.1 mg/l, sucrose 3% and agar-agar 0.6% (Figure 4).Thus, in the present study it was found that the BAP and BA concentration with auxin (IAA and BA) induced the organogenesis.

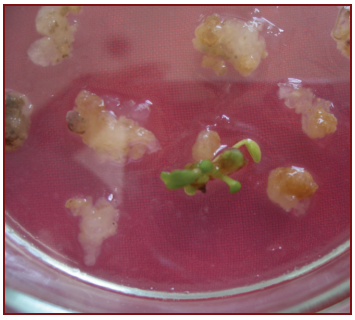

Figure 4. Indirect organogenesis and bud formation on MS medium containing BAP 1.0 mg/l, IAA 0.1 mg/l

**Multiplication of the obtained regenerants.** The frequency of regenerated plants from callus cultures was very low not exceeding 5 – 6 %. However, it was possible obtained mini plants to be multiplied on the same medium (Figure 5) or on a medium containing zeatin (cis/trans = 82/18% and IAA (for a short period of time) (Figure 6).

Studied concentrations of growth regulators and their combination, stimulated cell division in the tissues, buds growth and appear of the first leaf. Received healthy plants are ready for *in vitro* cultivation. These preliminary results show that BAP supported indirect organogenesis process by stimulating the cells to reach the competent phase for cell division.

Figure 5. Isolated buds and shoot formation on MS medium containing BAP 1 mg/l and IAA 0.1 mg/l

A high concentration of cytokinin inhibits callus formation and increases buds induction. The combinationof cytokinin and auxin has a positive effect on cell division and differentiation.

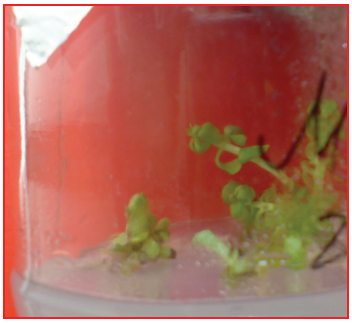

Figure 6. Plant multiplication on MS medium containing BAP 1 mg/l and IAA 0.1 mg/l

Callus is dedifferentiated and unorganized mass of parenchyma cells formed by the proliferation of parent tissue. Callus tissue is a good source of genetic variability and adventitious shoot formation. Callus induction is a prerequisite for

adventitious shoot formation and also for the other *in vitro* genetic improvement including induction of somaclonal variations and embryoids.

Difficulties in working with callus cultures of different *Rhodiola* species were reported by other authors, too. Kirichenko et al. (1994) and Yin et al. (2004) studied the possibilities for callusogenesis, organogenesis and regeneration form leave explants of *Rh. rosea*. Furmanova et al. (1995) examined the effects of the different nutrient media at the regeneration and callus formation capability of *Rh. rosea* and study biologically active substances in the roots in wild species. Ishmuratova (1998) demonstrated that the ecotype is also a factor influencing the processes of effective callusogenesis and organogenesis. The latest capability is studies in seeds and rhizomes from three ecotypes Rhodiola growing in high Altai using MS nutrient medium containing different phytoregulators – BA, IAA, NAA, IBA and 2,4-D. The author specified that the choice of the optimal nutrient medium (MS containing BAP (0.2 mg/l) and IAA (0.1 mg/l) for explant development is depended on ecotype.

Callus induction followed by plant regeneration of *Rhodiola coccinea* was studied byof ShaHongetal. (2008). The authors obtained three-type calli and noted that only one type of them was embryogenic. Similarly to our observations the role of BA and IAA was crucial. The embryogenic callus was obtained on MS nutrient medium containing BA (0.5 mg/l) and IAA (2.0 mg/l) and is suitable for regeneration. Callus induction (88.33 %) in different colors (yellow, green and red) was obtained of *Rhodiola sachalinsis* using leaf explants and cultivated on MS medium additionally with BA (2.0 mg/l) and NAA (0.5 mg/l) (Liu Jianfeng et el., 2007). They concluded that only green callus is able to regeneration on MS media containing BA (1.0 mg/l) and NAA (0.1 mg/l) with comparatively low percent (21.33 %) after long period of cultivation 50 days. The necessity for a long cultivation period was noted in our experiments, too. Regenerants were successfully rooted on½ MS. In the same species *Rhodiola* LiuJian-feng et al. (2009) obtained protoplast cultures from leaves of *in vitro* propagated plants, followed by callus induction and bud formation. The protoplasts formed callus on MS medium containing 2,4-D (1.0 mg/l), zeatin (0.5 mg/l), 0.5 M/l manitol and500 mg/l casein hydrolysate for 40-days period of cultivation. The callus formed adventitious buds on MS nutrient medium added with BA and NAA (1.0 mg/l and 0.1 mg/l, respectively). The buds grew and induced roots on ½MS for 30 days.

***In vitro* rooting.** The process of mini plants rooting is an important for their next step to adaptation in *ex vitro* conditions (Hazarika, 2003). The processes of rooting have specific requirements and rhyzogenesis was not obtained in all of the cited references. The rooting process on MS media without auxins has been studied in many plant species, such as *Echinaceapurpurea* (Korachetal., 2002), *Carlinaacaulis* (Trejgelletal., 2009). The frequency of the induced roots on medium without auxins may be due to the presence of endogenous auxins in regenerated buds/shoots (Minocha, 1987). Root induction of the growing *in vitro* shoots was achieved by adding auxins in nutrient media or on media without regulators which depends on species genotype (Rout et al., 1989). Different species had different potential to form roots and the optimal conditions are determined empirically. Moderate to high concentration of all auxins in the media inhibited root growth.

Figure 7. Root induction on MS medium containing BAP 1.0 mg/l and IAA 0.1 mg/l after 36 days cultivation.

The presence of auxin for the rhizogenesis process is necessary for many medicinal species. The concentration of IBA, for example, plays key role for stimulation of root formation for a number of plants as *Centaurea rupestris* (Perica, 2003), *Wedelia chinensis*

(Kameri et al., 2005), *Emilia zeylanica* (Robinson et al., 2006). Reducing the amount of salts in nutrient media for rhizogenesis had different effects to root formation and depend on the species. In many medicinal plants, rooting is more successful when the macro/micro salts (sometimes vitamins) are reduced twice or more *Saussurea obvallata* (Joshi and Dhar, 2003); *Ensete ventricosum* (Birmeta and Welander, 2004); *Ecliptaalba* (Baskaran and Jayabalan, 2005; *Carlina acaulis* (Trejgell et al., 2009), and sucrose is reduced to 2.0, 1.0 or 0.5%.

Figure 8. Roots formed on MS medium containing 2.0 mg/lIBA, 0.2 mg/lIAAand 0.4 mg/l$GA_3$

Figure 9. Propagation on MS medium containing zeatin (cis-trans form).

When regenerated shoots attained a height of 1.0-1.3 cm they were excised and transferred to MS medium for root induction. In our experiments rooting was observed on the same medium used for multiplication (Figure 7). Root formation needed about 28-36 days period of time, which is longer period in comparison with the use of half strength MS nutrient medium containing 2.0 mg/lIBA, 0.2 mg/lIAAand 0.4 mg/l$GA_3$, reported in our previous papers (Tasheva and Kosturkova, 2010 a,b) (Figure 8 and 9).

**Adaptation *ex vitro*.** Determination of the optimal conditions for adaptation of the invitro obtained plants in *exvitro* condition after the stage of rooting is a significant step for completed propagated scheme report. Adaptation of the regenerants in external conditions is the one critical stage of micripropagation. Adaptation of the plants obtained from this process of indirect regeneration was performed by the use of the scheme mentioned in our previous studies (Tasheva and Kosturkova, 2010a,b)

A model protocol for indirect organogenesis:

1. Callus induction on nutrient media containing zeatin 2.0 mg/l, IAA 0.2 mg/l and casein hydrolisate 1000 mg/l (MSZ1 medium). The callus was isolated and cutting on the explants with 0.5/1.0 cm in size.
2. Cultivation of the calli on MS media containing 0.1 mg/l 2,4-D (D medium) for 28 days, 22$^{o}$C and 16/ 8photoperiod - 2 passages
3. Buds regeneration on MS media containing BAP 1.0 mg/l and IAA 0.1 mg/l for 28 days, 22$^{o}$C and 16/ 8photoperiod
4. Propagation of plants regenerants on MS nutrient medium containing1.0 mg/l BAP and 0.1 mg/l IAA (BA medium) or on MS containing 1.0 mg/l zeatin and 0.1 mg/l IAA for 28 days, 22$^{o}$C and 16/ 8photoperiod
5. Rooted of the regenerants on ½MS media containing 2.0 mg/lIBA, 0.2 mg/lIAAand 0.4 mg/l$GA_3$(½ MS rooting medium**)** for 28 days, 22$^{o}$C and 16/ 8photoperiod.
6. Adaptation in *exvitro* conditions (Tashevaand Kosturkova, 2010 a,b)

## CONCLUSIONS

As a result of our experiments the following scheme of consecutive culture media for indirect organogenesis and regeneration could be proposed: **MSZ2 →D→D→BA→½ MS.**

Plants produced in this study appeared normal without observed morphological or phenotypical abnormalities and successfully developed in pots. The present system could be used as an alternative one for multiplication of selected valuable clones of this important

medicinal plant. This gives possibility to enrich the spectrum in a population including by the methods of gene transfer.

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# CAMELINA CULTIVATION FOR BIOFUELS PRODUCTION

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***Abstract***

*Recently studies have been carried out regarding Camelina sativa benefits. This oilseed plant that belongs to Brassicaceae family presents a major interest due to the fact that it can be a renewable resource for sustainable biofuels production. The oil obtained by crushing the seeds is rich in polyunsaturated fatty acids and can be processed in order to obtain biokerosene for aviation. Camelina oil has the property to resist at minus 47-48°C, the requirement for aviation fuel, due to negative temperature in the area the aircraft fly. In our study, Camelina sativa was cultivated in several locations from Romania: in the North (Iasi and Satu-Mare county), in the West (Arad county) and in the South (Calarasi county). Two camelina varieties were tested: GP 202 and GP 204. The aim of this study is to assess the camelina yield potential and identify the optimum cultivation technology. The climatic conditions and the soils were monitored during the tested period. The production was influenced by the sowing date. The best results were obtained at Iasi (2.9 t/ha), Calarasi (1.2 t/ha) and Satu-Mare (1.2 t/ha).*

***Keywords:*** *aviation, biofuel, Camelina yield, Camelina sativa, cultivation technology.*

## INTRODUCTION

The issue of plant-based biofuel sustainability is very complex. The whole chain involved in biofuel production depends on multitude of factors, such as: GHG (greenhouse gases) emissions reduction, land use change, indirect land use change, land availability (food vs. fuel dilemma), agricultural practices, water consumption reduction, limiting negative effects on biodiversity, biofuels efficient use in the economic and energy sectors, logistics and social impact (Lee et.al, 2008; Moser, 2010; Shonnard et al., 2010; Amigun et al., 2011). According to EPA (Environmental Protection Agency). *Camelina sativa* can be successfully used for advanced biofuel production, meeting all the above mentioned criteria.

*Camelina sativa* is an annual plant belonging to *Brassicaceae* family. It adapts to cold climate and can be cultivated in northern regions (Gugel and Falk, 2006; Moser, 2010). Its recent research has focused on introducing it in rotational crops with cereals (Moser, 2010), as well as in double cropping (Schillinger et al., 2012; Gesch and Archer, 2013). Its vegetation period is short (85-100 days) and, unlike rapeseed, it is more resistant to drought, pests and diseases (Putnam *et al*. 1993; Gugel and Falk, 2006; Martinelli and Galasso, 2010; Moser, 2010) It requires minimal or no tillage and no special equipment (Dobre *et al,* 2011).

The oil obtained from *Camelina sativa* seeds is rich in polyunsaturated fatty acids (Abramovic and Abram, 2005) and can be used for various purposes: biodiesel (Fröhlich and Rice, 2005; Kruczyński, 2013), biojet fuel (Moser, 2010; Shonnard *et al*., 2010, Jurcoane *et al*. 2011), heating oil, naphtha, liquefied petroleum gas (EPA, 2013), paint, varnish and cosmetics industry (Zubr,1997). Moreover, the by-product that remains after camelina seed crushing can be used in animal feeding in small amounts (Putnam *et al*. 1993, Jurcoane *et al*. 2011).

Our study is aimed at identifying the best camelina cultivation technology using low-inputs and testing the potential of *Camelina sativa* varieties in several locations from Romania.

## MATERIALS AND METHODS

### *Camelina sativa* cultivation and harvesting

In Romania, during 2011-2012, *Camelina sativa* was cultivated in the North (Iasi and Satu-Mare county), in the South (Calarasi

county) and in the West (Arad county) in non-irrigated conditions. Two *Camelina sativa* varieties were tested: *GP 202* and *GP 204,* German varieties. The camelina crop was sown in different periods at the depth of 1-2cm. The climatic conditions and the soil were monitored during testing period. Soil samples were taking during camelina vegetation at the depth of 20cm.

In Iasi county (Tiganasi village location), 2 ha were sown with *Camelina sativa GP 202* and 1 ha with *GP 204* variety, using an Amazone sowing machine. The distance between rows was 25 cm, according with Imbrea *et al.* recommendations, 2011. The previous crop was represented by mustard. Before sowing, the following soil works were carried out: stubble ploughing after previous crop harvesting, rolling and leveling. Inputs applied for camelina crop were the highest from all locations. On the middle of August 2012, 200 kg/ha N: P: K 15:15:15 and 200 kg/ha N: P: K-5:15:30 were applied. During flowering period and when the plants had almost all the capsules formed, 3 kg/ha of foliage fertilizer were applied. The seeding rate was 5 kg/ha for GP 202 and 5.5 kg/ha for GP 204. Only in this location phytosanitary treatments were applied (1 l/ha *Folicur solo* and 0.1 l/ha *Calypso*) and also a desiccant (3l/ha *Reglone*) was used a week before harvesting time. The harvest took place on 27 June 2012 using a Class Lexion combine, seeding conditioning being done in the same time with harvesting, using a Selector EF 3802 equipment that had very low diameter sieves.

In Satu-Mare County, the trial was located in Acas village. 2 ha were sown with camelina using a SUP 21 seeding machine. Only *Camelina sativa GP 202* was tested. The previous crop was maize. The soil tillage works made were: ploughing, disc harrowing, milling and rolling (before and after sowing). The seeding rate was 10 kg/ha and 200 kg/ha NPK 15-15-15 were applied in autumn. The harvesting was done on 10 July 2012 using a Claas Lexion combine.

In Calarasi County the trial was located near Fundulea city (about1 km). 1 ha for each variety was tested. The seeding rate was 6 kg/ha for both varieties. The previous crop was represented by maize. The distance between rows was 25 cm. The following soil works were carried out: ploughing, disc harrowing, combinatory and rolling (before and after sowing). In March 2012, 150 kg/ha ammonium nitrate was applied. Harvesting was done on 27 June 2012 with a Fendt combine.

In Arad County, the tests were carried out in Buteni village. In this location, 2 ha were sown with *Camelina sativa GP 202* using a SUP 21 seeding machine. The seeding rate was 10 kg/ha. The distance between rows was 12.5 cm. Soil tillage was represented by ploughing, disc harrowing and rolling (before and after sowing). The harvesting took place on 11 July 2012 using a Claas Lexion combine.

**Camelina seeds conditioning**

After the harvest, the seeds were stored by farmers and were dried naturally (not by using special equipment). They were aired by farmers until they reached a humidity level of 8-9%. The entire yield obtained from all the demo trials was sent to Iasi for conditioning because this location was best equipped i.e. a suitable sieve with round holes of 1,5 mm. The separation process was made for each variety and impurities were removed. A second conditioning was necessary to be performed. The quantity left this process was also stored in Iasi.

**Analysis methods**

After harvesting and conditioning, IBNA-Balotesti (National Research Development Institute for Animal Biology and Nutrition) analysed the seed oil content. Regarding the soil samples the following analyses were conducted: soil pH using a pH meter, the total carbon and nitrogen content using LECO methodology and the available soil phosphorus content after Olsen's method.

## RESULTS AND DISCUSSIONS

In 2011-2012, the climatic conditions recorded in Romania were not favourable for agriculture. In November in the tested locations a low amount of precipitation was recorded (< 2mm). For this reason, the camelina crop planted in autumn did not emerge in December. For the most areas from Romania, in May was recorded a prolonged period with high amount of precipitation followed by a period of drought and high temperatures in June. For instance, In Iasi county from 15 May 2012 to 25 May 2012

daily rainfall was recorded, the amount of precipitations reaching 78.4 mm (from the amount recorded during the whole month).
The land from Iasi and Calarasi locations and Fundulea are preferred for agricultural activities.
Regarding the soils from Satu Mare, Iasi and Calarasi (see Table 1), these had a slightly acid pH, and the soils from Arad a neutral pH. The C/N ratio showed that soil fertility had normal values in Calarasi (C/N: between 10 and 15) and higher values for Iasi trial. The phosphorus concentration determined by Olsen method varied in the tested soils. None of these samples had a very low P concentration (< 4 ppm). The samples taken from Sate Mare, Arad, Calarasi had a low P concentration, which proves that for future tests P fertilization is mandatory.

Table 1. Soil tests results from different locations from Romania during camelina flowering period

| **Location (county)** | pH | Total Nitrogen (%) | Total Carbon (%) | C/N | P (ppm) |
|---|---|---|---|---|---|
| Iasi | 6.25 | 0.14 | 2.52 | 18 | 20.31 |
| Calarasi | 6.62 | 0.15 | 1.84 | 12.27 | 11.83 |
| Satu-Mare | 6.67 | 0.14 | 1.03 | 7.36 | 15.84 |
| Arad | 7.15 | 0.14 | 1.35 | 9.64 | 15.13 |

The camelina crop cultivated in autumn had a good resistance over the winter period and started to emerge in April (Table 2). In April 2012 the plants had 4-6 leaves. During April and May the plants grew rapidly. At the end of the May in Satu Mare, Iasi and Calarasi locations, and all the plants had their capsules formed (Figure 1). The weed infestation was low and no effects on camelina production. In Calarasi county the presence of few plants infested with *Peronospora camelinae* was noticed but no phytosanitary treatments were required. When the distance between rows was 25 cm, the plants were more robust and had numerous branches. Only in Iasi an insecticide and a fungicide with systemic action were applied to prevent *Meligethes aeneus* and *Peronospora camelinae* from damaging the plant.

Table 2. Sowing, emergence and harvesting period.

| **Location (county)** | **Sowing date** | **Emergence date** | **Harvesting date** |
|---|---|---|---|
| Iasi | 21 November 2011 | 05 April 2012 | 27 June 2012 |
| Calarasi | 9 November 2011 | 20 March 2012 | 27 June 2012 |
| Satu-Mare | 10 November 2011 | 27 March 2012 | 10 July 2012 |
| Arad | 4 March 2012 | 11 April 2012 | 11 July 2012 |
| | | | |

For the spring trial carried out in Arad county, the flowering occurred in the middle of May and continued at the beginning of June (see Figure 2). No fertilizers no phytosanitary treatments and no desiccant were used.
Harvest reaping started in mid June and finished in July. The harvesting period was the same for both *Camelina sativaGP 202* and *GP 204* varieties.
In Arad County the yield obtained was 0.76 t/ha but the seed oil percentage was higher (32.38%). We consider that the optimal period for harvesting was exceeded.

Figure 1. *Camelina sativa* GP 202 variety, Calarasi County, 31 May 2012

Figure 2. *Camelina sativa* GP 202 variety, Arad County, 3 June 2012

In Satu-Mare, only a part of trial was infested with *Peronospora camelinae* (Figure 3, 4). No phytosanitary treatments were applied. In Satu-Mare with low inputs application, 1.2 t/ha were obtained. The seed oil percentage was the best from all locations (32.95%). Therefore, *Camelina sativa* is not a demanding plant but its cultivation technology requires further study.

Figure 3. Satu Mare County-Plants infested with *Peronospora camelineae*, 2 June 2012

Figure 4. Satu Mare County – General aspect of *Camelina sativa* crop, 2 June 2012

In Iasi County, the harvesting was done at the right time and the losses were minimal. The most predominant weed was *Chenopodium album* but after treatment it was eliminated (see Figure 5). The seed oil percentage was higher for GP 202 variety (28.91%) and lower for the GP 204 variety (23.88%). The best obtained yield, 2.9 t/ha was obtained for *GP 202* variety. For *GP 204* variety, 2.2 t/ha were obtained.

Figure 5. *Chenopodium album* in camelina crop, Iasi County, 07 June 2012

In Calarasi, the harvesting was done using a combine with minimum air flow. Also, the harvesting was done too late. Especially in Calarasi county plants are harvested earlier than in other regions. No dessicant was applied. The first capsules containing high amount of seeds matured earlier and were the first to shake. It is necessary to identify the optimum harvesting period. The gross production for each variety was 1.8 t/ha for *GP 202* and 1.2 t/ha for *GP 204*. The seeds had 25.23% oil content for *GP 202* variety and 22.23% for the *GP 204* variety.

## CONCLUSIONS

The technology of camelina cultivation is a complex one which needs attention by the farmers, although the plant itself is not a demanding one.

The best obtained yield, 2.9 t/ha, was obtained in Iasi for *Camelina sativa* GP 202 variety.

We consider that the harvest occurred too late in the South of Romania when the yields are harvested earlier than the rest of the country. It is necessary to identify the optimal harvesting date.

The seed oil percentage was higher for GP 202 variety and lower for the GP 204 variety.

The crops that are sowing later in spring mature non-uniformly, leading to yield losses.

We consider that both *Camelina sativa* varieties (*GP 202* and *GP 204*) meet the growth and development conditions for Romania.

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# *IN VITRO* STUDY ON THE INTERACTION BETWEEN *BACILLUS THURINGIENSIS* AND CHEMICAL PESTICIDES USED FOR CORN CROP PROTECTION

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***Abstract***

*Interactions between the entomopathogenic bacteria Bacillus thuringiensis and chemical pesticides used for corn crop protection is one of the most important factors that influence the effectiveness of the entomopathogenic microorganism. Bacterial biopreparates based on B. thuringiensis could be used along with chemical pesticides. The effect of chemical ingredients on bacteria viability is mandatory and it should be conducted first. Interactions between the entomopathogenic bacteria B. thuringiensis and chemical pesticides occurs when chemicals and bacterial biopreparates are applied simultaneously or mixed together. Selectivity on some biological parameters of B. thuringiensis was tested by making a mixture of B. thuringiensis with chemical pesticides. Different concentrations of chemical pesticides were mixed with sporulated and vegetative bacterial cultures. The effects of chemical plant protection products on sporulation and vegetative growth of B. thuringiensis were monitored. The paper presents the results of experiments aimed at determining the influence of some chemical pesticides used for corn crop protection on the B. thuringiensis multiplication and sporulation.*

***Keywords:*** *Bacillus thuringiensis, chemical pesticides, corn crop.*

## INTRODUCTION

*Bacillus thuringiensis* is an endospore-forming Gram-positive bacterium of economic importance due to its entomopathogenic capability and has been used as a safe microbial insecticide for over 50 years for caterpillars' pest control. The insecticidal action of *B. thuringiensis* is attributed to protein crystals produced by the bacterium. *B. thuringiensis* based insecticides are popular with organic farmers because they are considered 'natural insecticides'. They differ from most conventional insecticides because they are toxic to only a small range of related insects (Hellmich, 2012). This is because specific pH levels, enzymes, and midgut receptors are required to activate and bind a given Cry toxin to midgut cells, which leads to pore formation in the insect's intestine and death (Federici 2002). Modern technology involves *B. thuringiensis* gene responsible for the production of the insecticidal protein incorporation into the maize genome for the corn borer control. Although *B. thuringiensis* based insecticides are an important tool for maize growers, they cannot completely replace the chemical control methods. That is why the bacterial entomopathogenic insecticides should be especially compatible with traditional pest management practices. Chemical plant protect-tion products is one of the most important factors that influence the effectiveness of entomopathogic bacteria *B. thuringiensis* used in corn pest control.

Mixtures of bacterial biopreparates based on *B. thuringiensis* with different chemicals are possible and can be used in practice. The effect of chemicals on active substance of bacterial bioproducts must be checked.

The interaction between entomopathogenic bacteria and pesticides can occur in the following ways:

(1) Corn pests and diseases form a rich complex of species that cause damages in our country. Simultaneous control of diseases and pests could be made using plant protection chemicals. It is also very important to maintain a natural biological balance, to protect the environment and the useful insects. The systems of integrated protection in agriculture use chemical pesticides to control (a) Diseases: seedling damping-off (*Pythium* sp.), foot rot (*Fusarium* spp.), head smut (*Sorosporium*

*holci-sorghi*), corn smut (*Ustilago maydis*), (b) Pests: maize leaf weevil (*Tanymecus dilatecollis*), European corn borer (*Ostrinia nubilalis*), wireworms (*Agriotes* sp.), locusts and (c) Annual and perennial monocotyledonous and dicotyledonous weeds (*Amaranthus* sp., *Chenopodium* sp., *Sinapis* sp., *Capsella* sp., *Thlaspi* sp., *Cirsium* sp., *Hibiscus* sp., *Xanthium* sp., *Abutilon* sp., *Raphanus* sp., *Solanum* sp., *Polygonum convolvulus*, *Setaria* sp., *Echinochloa* sp., *Digitaria* sp., *Convolvulus arvense*, *Calystegia sepium*).

(2) Application of chemical pesticides in the mixture or simultaneously with bacterial bioproducts, depending on the evolution of insect pest, in order to make treatments more profitable.

(3) Application of some pesticides containing bacterial preparation in order to obtain adequate efficacy.

## MATERIALS AND METHODS

T4 strain of *B.thuringiensis* var. *thuringiensis,* from Research-Development Institute for Plant Protection collection of micro-organisms, was used for this experiment.

The following working method was used in order to identify the selectivity of plant protection products against entomopathogenic bacteria. Bacterial culture was grown in corn extract agar media which was mixed with each of the chemicals in the following three concentrations: the recommended concentration for use (c.u.), ½ (1/2c.u.) and ¼ (1/4c.u.) of recommended concentration for use.

Test mixtures were sown in Petri dishes, which were incubated at $28^0$C for 72 hours.

Different chemicals from fungicides, insecticides and herbicides groups were tested (Table 1).

Table 1. Plant protection chemicals tested in combination with bacterial culture

| **FUNGICIDES** | | | |
|---|---|---|---|
| **Chemical group** | **Product (s.a.)** | **Target organism** | **Dose (conc.)** |
| Dithiocarbamates and thiuram derivatives | ROYAL FLO 42 S (thiram 480g/l) | *Pythium* spp. *Fusarium* spp. | 3,0 l / t seeds |
| Triazoles and imidazoles | VITAVAX 200 FF (carboxina 200g/l+thiram 200 g/l | *Pythium* spp. *Fusarium* spp. | 2,5 l / t seeds |
| **INSECTICIDES** | | | |
| Synthetic pyrethroids | SIGNAL (cypermethrin 300 g/l) | *Agriotes* spp. | 2,0 l/t seeds |
| Various | ACTARA 25 WG (thiamethoxam 25%) | *Tanymecus dilaticolis* | 0,100 kg/ha |
| | GAUCHO 600 FS (imidacloprid 600 g/l) | *Tanymecus dilaticolis* *Agriotes* spp. | 6,0-8,0 l pc/t seeds |
| | COSMOS 250 FS (fipronil 250 g/l) | *Agriotes* spp. | 5,0 l/t seeds |
| | CRUISER 350 FS (thiamethoxam 350 g/l) | *Tanymecus dilaticolis* *Agriotes* spp. | 1,2 µl/one seed |
| **HERBICIDES** | | | |
| Aminofosfats | DOMINATOR (glyphosate 360 g/l) | Annual and perennial weeds | 4,0 l/ha |
| | ROUNDUP (Glyphosate isopropylamine salt 360 g/l) | Monocotyledonous and dicotyledonous weeds, annual and perennial (+*Sorgum halepense* from rhizomes) | 4 l/ha (mixed with 100-150 l water/ha) |
| Picoline derivatives | CERLIT (fluroxypyr 250 g/l) | *Convolvulus arvensis* *Calystegia sepium* | 1,0 – 2,0 l/ha |
| Sulfonylureas | MISTRAL 4 SC (nicosulfuron 40g/l) | *Sorghum halepense* | 1,5 l/ha |
| | TITUS 25 DF (rimsulfuron) | Monocotyledonous weeds, annual and perennial – including *S.halepense* from seeds and rhizomes- and some annual dicotyledonous weeds | 60 g/ha |
| Isoxazoles | CALLISTO 480 SC (mesotrine 480 g/l) | Annual weeds | 0,350 l/ha |
| Mixtures | CALLAM (tritosulfuron 12,5% + dicamba 60%) | Annual and perennial dicotyledonous weeds | 0,4 + 1,0 l/ha |

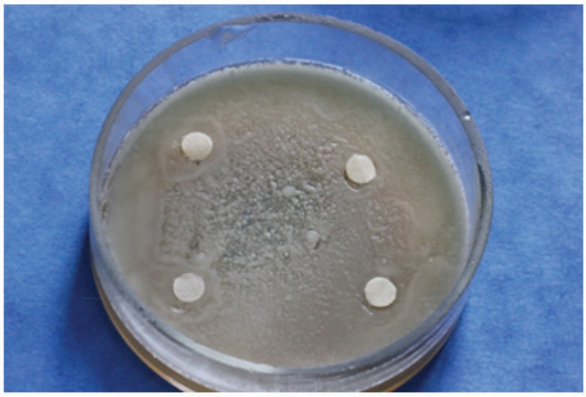

Figure 1. Filter paper discs with sterilized distilled water on *B. thuringiensis* bacterial lawn (corn extract agar media)

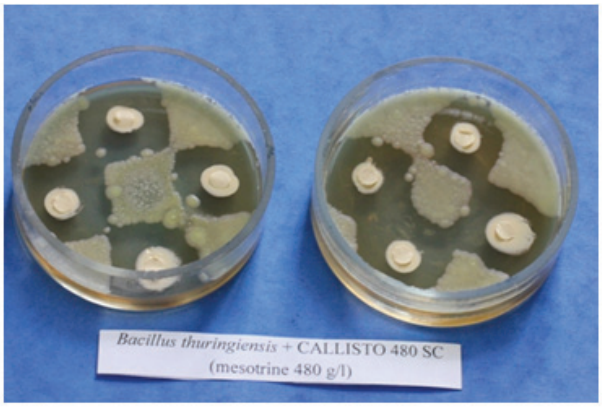


Figure 2. Effect of mesotrine on halo formation and inhibition of the growth of *Bacillus thuringiensis* on corn extract agar media

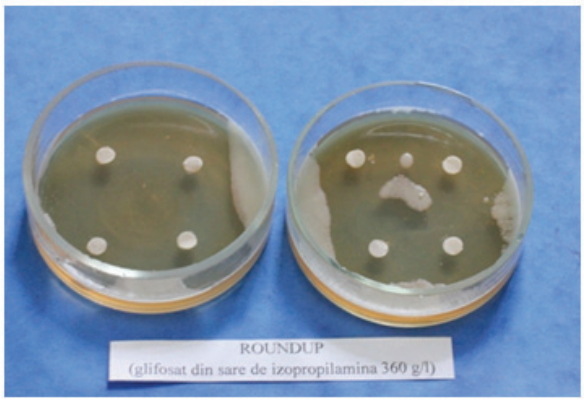


Figure 3. Effect of glyphosate isopropylamine salt on halo formation and inhibition of the growth of *Bacillus thuringiensis* on corn extract agar media

In order to make a better observation on selectivity of plant protection products against *B. thuringiensis* strain, a common working method was used for some of the variants.

Bacteria was inoculated in Petri dishes on corn extract agar media, followed by the placement of 4 paper discs with the evaluated insecticide in 4 points of the dish. In the control treatment, four filter paper discs with sterilized distilled water replaced the insecticide (Figure 1). The data was analyzed on the 4th and 7th day after the treatment application. Each Petri dish was analyzed for the absence or presence of the bacterial growth inhibition halo (Figures 2, 3)

The tested chemicals are commonly used in maize crop protection. Observations followed *B. thuringiensis* colony diameter treated with pesticides variants, compared with untreated control variants.

## RESULTS AND DISCUSSIONS

Results on the effect of chemicals on spore germination and vegetative multiplication of *B. thuringiensis* are presented in Tables 2, 3 and 4. Bacterial growth on agar media was noted as follows: + + + = very good growth, + + = good growth, + = weak growth.

An overall analysis of the data presented reveals that the tested products fall into one of these 3 categories: (a) products with high selectivity towards *B. thuringiensis* (b) products with average (good) selectivity and (c) products with low selectivity (bacteriostatic). This analysis refers to how bacterial growth and spore germination of *B. thuringiensis* have been influenced by contact with plant protection chemicals

Bacteriostatic properties of some chemicals influenced spore germination and vegetative multiplication in varying proportions. This effect was revealed through the partial inhibition of vegetative and sporal multiplication at high concentrations of the chemical. Bacterial cultures mixed with recommended concentration for use of chemicals belonging to this category and inoculated on agar media, developed colonies with a diameter from two to eight times lower than the bacterial colonies of untreated control variant.

By decreasing the concentration of the chemical in the mixture experiments, development of bacterial cultures was registered normal parameters. The size of bacterial colonies was close or equal to those of the control variant.

Microscopic analysis showed no changes in vegetative or sporulated bacterial cells.

The following issues were observed for the groups of chemicals: (a) fungicides have generally manifested stronger inhibitory effect on bacterial spores; (b) insecticides manifested, in general, high degree of selectivity against *B. thuringiensis* and (c) herbicides manifested the lowest degree of selectivity.

There was an almost complete inhibition of bacterial growth, both in vegetative and sporulated culture when mixed concentration of DOMINATOR and ROUNDUP herbicides dose corresponded to recommended concentration for use.

Partial inhibitory effect was maintained when the concentration was reduced by two or four times.

Recent studies aimed at rice crops protection, revealed the compatibility between insecticides (thiamethoxam, labdacyhalothrin, malathion and fipronil) and *B. thuringiensis* strains (*B. thuringiensis* subsp. *dendrolimu*s, *B. thuringiensis* var. *kurstaki*, *B. thuringiensis* var. thuringiensis and *B. thuringiensis* subsp. entomocidus) interaction. However, at a $10^1$ concentration, ten times higher than the recommended concentration, some insecticides presented inhibitory effect in the bacterial development. The insecticide malathion inhibited the development of six out of seven evaluated *B. thuringiensis* strains at the higher concentration (Pinto et al., 2012).

Batista-Filho et al. (2001) and Almeida et al. (2003) reported compatibility between *B. thuringiensis* bacterial growth and thiamethoxam insecticide.

Silva et al. (2008) reported resistant *B. thuringiensis* var. *kurstaki* colonies expressing inhibition in the presence of some herbicides.

Field toxicity studies have shown that when chemical insecticides manifest in vitro toxicity against *B. thuringiensis*, this does not suggest necessarily high field toxicity (Alves et al., 1998).

It is recommended, though, chemical insecticides be used in the advised doses when using *B. thuringiensis*-based products.

Table 2. Influence of some fungicides on *Bacillus thuringiensis* Experimental mixture tested Bacterial lawn /Fungicide concentration

| Variants | Chemical substance + | *Bacillus thuringiensis* | c.u. | ½ c.u. | ¼ c.u. |
|---|---|---|---|---|---|
| I (a) | ROYAL FLO 42 S | vegetative | +++ | +++ | +++ |
| I (b) | | sporulated | +++ | +++ | +++ |
| II (a) | VITAVAX 200 FF | vegetative | +++ | +++ | +++ |
| II (b) | | sporulated | +++ | +++ | +++ |

Table 3. Influence of some insecticides on *Bacillus thuringiensis* Experimental mixture tested Bacterial lawn /Insecticide concentration

| Variants | Chemical substance + | *Bacillus thuringiensis* | c.u. | ½ c.u. | ¼ c.u. |
|---|---|---|---|---|---|
| I (a) | SIGNAL | vegetative | +++ | +++ | +++ |
| I (b) | | sporulated | +++ | +++ | +++ |
| II (a) | ACTARA 25 WG | vegetative | ++ | +++ | +++ |
| II (b) | | sporulated | ++ | ++ | +++ |
| III (a) | GAUCHO 600 FS | vegetative | +++ | +++ | +++ |
| III (b) | | sporulated | +++ | +++ | +++ |

Table 4. Influence of some herbicides on *Bacillus thuringiensis* Experimental mixture tested Bacterial lawn / Herbicide concentration

| Variants | Chemical substance + | *Bacillus thuringiensis* | c.u. | ½ c.u. | ¼ c.u. |
|---|---|---|---|---|---|
| I (a) | DOMINATOR | vegetative | - | + | ++ |
| I (b) | | sporulated | - | + | ++ |
| II (a) | ROUNDUP | vegetative | - | + | ++ |
| II (b) | | sporulated | - | + | ++ |
| III (a) | TITUS 25 DF | vegetative | +++ | +++ | +++ |
| III (b) | | sporulated | +++ | +++ | +++ |
| IV (a) | CALLISTO 480 SC | vegetative | +++ | +++ | +++ |
| IV (b) | | sporulated | +++ | +++ | +++ |
| V (a) | CALLAM | vegetative | +++ | +++ | +++ |
| V (b) | | sporulated | +++ | +++ | +++ |

## CONCLUSIONS

The overall effect of the chemicals on *B. thuringiensis* efficiency is difficult to assess in field conditions.

On one hand, we consider the average concentration of pesticides with which bacteria come into contact is in a lesser amount than the one tested in the laboratory. Occasionally, those which can be applied directly to the soil could exceed the normal dose. On the other hand, growth of *B. thuringiensis* on agar media, with optimal conditions, makes it more tolerant for chemicals compared to bacteria released into nature where it has to face less favorable conditions, competition with antagonists etc. Therefore, experimental variants which showed good selectivity of chemicals against bacteria *B. thuringiensis* in controlled laboratory conditions should be tested under field conditions too.

Based on the data presented, the tested chemicals fit within these degrees of selectivity in relation to *B. thuringiensis.*

Table 5. Selectivity of chemicals against *B. thuringiensis*

| | High selectivity | Good selectivity | Low selectivity |
|---|---|---|---|
| Fungicides | ROYAL FLO 42 S<br>VITAVAX 200 FF | - | - |
| Insecticides | SIGNAL<br>GAUCHO 600 FS<br>CRUISER | ACTARA 25 WG | - |
| Herbicides | MISTRAL 4 SC<br>TITUS 25 DF<br>CALLISTO 480 SC<br>CALLAM. | ESTERON 60<br>ATRANEX 80 WP<br>ROMANEX 500 SC<br>ALANEX 48 EC | DOMINATOR<br>ROUNDUP |

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# DIVERSITY ANALYSIS OF TUBERED-BEARING *Ipomoea trifida* (H.B.K.) G. DON. ORIGINATED FROM CITATAH WEST JAVA INDONESIA BASED ONCHROMOSOME TRAITS

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***Abstract***

*Ipomoea trifida is considered as a wild crop relative to sweet potato Ipomoea batatas. A set of 10 accessions was selected for tubered-bearing I. trifida originated from Citatah West Java was evaluated on their diversity based on chromose traits. A field trial and laboratory analysis were conducted at Universitas Padjadjaran Bandung Indonesia. Relationship between species identified by cluster analysis and Principal Component Analysis (PCA). The results showed that the observation on the 10 accession of tubered-bearing I. trifida using nine chromosome traits produced dissimilarities distance (Euclidean coefficient) ranging from 1.75 to 6.22. Dendogram generated at a dissimilarity distance of 5.23 showed the formation of three main clusters. Principal Component Analysis (PCA) produced first two principal component ($PC_1$ and $PC_2$), which has been able to explain 89.64% of the total variation. It is concluded that thare are highy diveristy between 10 accession of tubered-bearing I .trifida based on chromosomes traits.*

***Keywords:*** *Chromosome, Ipomoea trifida, Principal Component Analysis.*

## INTRODUCTION

Wild relative of sweet potato *Ipomoea trifida* is considered as having a potential source of genes to support plant breeding programs of domesticated sweet potato (*Ipomoea batatas* (L.) Lam.). Hambali (1988) reported that the highest genetic and phenotypic diversity of wild relatives of sweet potato in Indonesia is in the Citatah-West Java. The results of field observations in Citatah by a Padjadjaran University Team in 2010 have collected 168 accessions of wild relatives of sweet potato that have not been identified. Based on morphological variation in flowers and leaves, supposedly 168 accessions comprising *I. trifida* and *I. triloba* (Agung Karuniawan, personal communication, 2010). Wild relatives of sweet potato were found in Citatah known by local residents as "huhuian" and "boled areuy". Naturally wild relatives of sweet potato were found in Citatah grow as weeds in agricultural land of sweet potatoes, and other calcareous slope areas. Wild relatives of sweet potato *I. trifida* has been used as a source of genes in sweet potato breeding to improve the character of the yields, levels of dry matter, starch, increased levels of protein (Kobayashi & Miyazaki, 1976) and resistance to certain pests and diseases as black rot root diseases (Shiotani and Kawase, 1989; Komaki, 2001) and scab disease (Hartana, 1994). Wild relatives *I. trifida* originated from Citatah West Java has morphotype variations, so to determine the level of genetic diversity and relationship among accessions need to be done the clustering by morphological or cytogenetic studies (chromosome traits). Morphological characterization done through observation of the phenotypic appearance, while cytogenetic studies done through observation of chromosome number or ploidy level and form of chromosomes. These characters can be used as a chromosome differentiating factor for identifying genetic variation in plants that will be useful for breeders in developing and improving the quality of crops. Most wild relatives of sweet potato are found of tetraploid or diploid (Renwarin *etal*., 1994) and have not been characterized, so many potential sources of genetic diversity is unknown. So far it is not known the level of morphotype variation,

genetic diversity and relationship among accessions of wild relatives of sweet potato originated from Citatah West Java. This information is necessary to support the management and utilization of germplasm. Information of the level of genetic diversity on germplasm material is needed by breeders to identify potential progenitor and will be useful also to prevent the use of closely related progenitor closely in crossing.

## MATERIALS AND METHODS

### Materials

Research materials consisted of ten accessions of tubered-bearing *I. trifida* collection of the Faculty of Agriculture, Universitas Padjadjaran. Root tips used as materials for preparations for observation of chromosomes. The chemicals required include a solution of 0.002 M 0.8-hydroksiquinolin, fixative solution (ethanol: glacial acetic acid = 3:1), a solution of 4N HCl, solution of 45% acetic acid, and 2% orcein.

### Methods

*Chromosome preparations*

Root tips that meristimatis obtained from stem cuttings grown on medium (soil: compost: manure = 1:1:1). Chromosome preparations made using the squash method of Darnaedi (1990). The root was cut 1 cm from the root tip and soaked in a solution of 0.002 M 8-hydroksiquinolin for 3-5 hours at 18-20$^0$C. Subsequently the roots were fixed in a mixture solution of ethanol: glacial acetic acid (3:1) for 48 hours and transferred to a solution of 4N HCl for 10 minutes. Subsequently the root was immersed in 45% acetic acid solution for 10 minutes. Staining of preparations carried out using 2% orcein for 10 minutes on top of a glass object, then closed, heated and pressed.

*Observations and data analysis*

Observation of chromosome using light microscopy. Chromosomes on prometafase or early metaphase stage photographed and made the micrography. Chromosome captured images magnified and printed with a computer program, then print out of the chromosome picture was used for observation of chromosome number, chromosome size (the length of the long arm (q), the length of the short arm (p) and total length (q + p)), centromere index (CI), shape of chromosomes, haploid complement chromosome lenght (HCL), the value of intrachromosomal index ($A_1$) and interchromosomal index ($A_2$). Analysis of genetic diversity performed using cluster analysis and Principal Component Analysis (PCA) with the software of XLstat 2009.

## RESULTS AND DISCUSSIONS

### Cluster analysis on ten accession of tubered-bearing *I. trifida* based on chromosome traits.

Dissimilarities distance (Euclidean coeffisien) between ten accession of tubered-bearing *I. trifida* based on nine chromosome traits ranged from 1.755 to 6.224 (Figure 1).

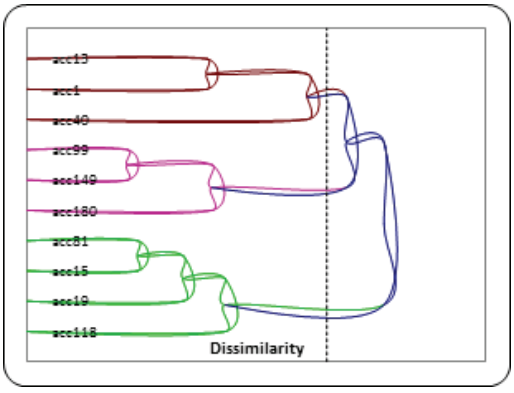


Figure 1. Dendogram of clustering on ten accession of tubered-bearing I. trifida

Dendogram generated at a dissimilarities distance of 5.228 showed the formation of three main clusters, namely cluster I consists of 4 accessions (accession 118, 19, 15, and 81); Cluster II consists of 3 accessions (accession 180, 149, and 99); and cluster III consists of 3 accessions (accession 40, 1 and 13). Highest dissimilarities distance of 6.224 possessed by the accession of 118, 19, 15, and 81 are joined in cluster I. Thus it can be assumed the four accession have the most distant relationship to other accession based on the chromosome traits were observed. The lowest dissimilarities distance of 1.755 is owned by the accession of 99 and 149 showed that the two accessions have a high similarity of chromosome traits so that the realtionship between the two accessions are very close. The high degree of similarity between the two accessions probably

that the two accessions is the same material. Afuape *et al.* (2011) state that the genotypes that showed high similarity can be expected as duplicate genotypes so besides morphological characterization, molecular characterization is needed to confirm whether these genotypes are the same material with a different name or whether these genotypes came from the same parent.

**Principal component analysis on ten accessions of tubered-bearing *I. trifida* based on chromosome traits.**

Principal component analysis performed on ten accessions of tubered-bearing *I. trifida* to see relationship and chromosome traits affecting variation appeared between accessions. Based on Eigen value > 1, then there are two pricipal components that have been able to explain 89.64% of the variation total of accessions tested (Table 1.).

Table 1. Principal component analysis (PCA) on ten accession of tubered-bearing I. trifida based on nine chromosome traits

| No | Traits | Principal Component (PC) | |
|---|---|---|---|
| | | 1 | 2 |
| 1 | Lenght of the long arm (q) | **-0.892** | 0.414 |
| 2 | Lenght of the short arm (p) | -0.014 | **0.988** |
| 3 | Total lenght of chromosome (TL) | **-0.660** | **0.735** |
| 4 | Centomere Index (CI) | **0.863** | 0.484 |
| 5 | Number of metacentric chromosome (m) | **0.903** | 0.332 |
| 6 | Number of submetacentric chromosome (sm) | **-0.903** | -0.332 |
| 7 | haploid complement chromosome lenght (HCL) | **-0.660** | **0.733** |
| 8 | Intrachromosomal index ($A_1$) | **-0.851** | -0.500 |
| 9 | Interchromosomal index ($A_2$) | -0.155 | **0.589** |
| Eigen | | 4.792 | 3.275 |
| Proportion (%) | | 53.247 | 36.393 |
| Cumulative (%) | | 53.247 | 89.640 |
| | | | |

Note: The bold is the value of an influential traits because diskriminant> 0.5 (Zubair, 2004). The first Principal Componen ($PC_{1)}$ contributes the proportion of variation by 53.247% in ten accessions of tubered-bearing *I. trifida* provided by almost all the tested chromosome traits except the traits of the short arm (p) and interchromosomal asymmetry index ($A_2$). The traits of the short arm (p), centromere index (IS), the number of metacentric chromosomes (m), the number of submetacentric chromosome (sm) and intrakchromosomal asymmetry index ($A_1$) on $PC_2$ contributes for 36.393% of the variation that arises among the tested accessions. Therefore, until the second principal component ($PC_2$) was able to explain 89.640% of the total variation (Table 1.). To see the pattern of distribution of ten accessions tubered-bearing *I. trifida* can be seen in biplot graphic (Figure 2.). Accessions that are in the same quadrant indicates that the accessions have a very close relationship, however if they are in a different quadrant with an angle > $90^0$ then the accessions have a distant relationship.

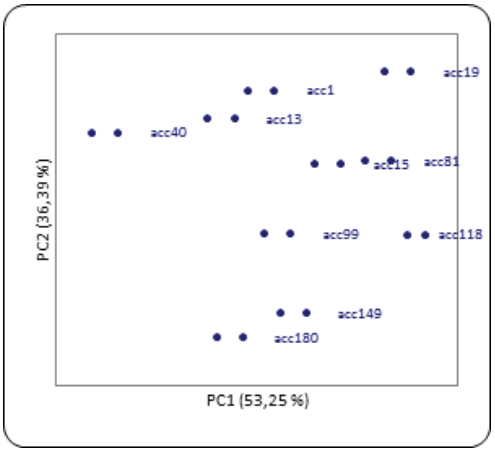


Figure 2. Biplot of PC1 and PC2 of ten accession tubered-bearing I. trifida base on chromosome traits

Ten accessions spread in four quadrants of biplot. Accession 15, 19, and 81 are in the same quadrant, indicating that the three accessionshave a close relationship. The three accessions also have close relationship with accession 118 that was in different quadrants as forming an acute angle (<$90^0$).

## CONCLUSIONS

Ten accessions of tubered-bearing I. trifida originated from Citatah West Java has a broad genetic diversity.Tests on the ten accession of tubered-bearing I. trifida using nine chromosome traits produced dissimilarities distance (Euclidean coeffisien) ranged from 1.755 to 6.224. Dendogram generated at a dissimilarities distance of 5.228 showed the

formation of three main clusters.Principal Component Analysis (PCA) produced two first principal component (PC1 and PC2), which has been able to explain 89.64% of the total variation.

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# INFLUENCE OF FERTILIZATION OF SOILWITH WORM COMPOST ON THE QUALITY OF PEAS

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***Abstract***

*Worm compost is one of the final products obtained as a result bioconversion technology of organic waste by worm cultivation. The product obtained is a natural organic fertilizer ecological, composed of grains of various sizes, color dark brown, odorless, hygroscopic with long acting.*
*In this article are presented the results of research obtained in the field experiment during the period of three years. The experiment was organized in field conditions of Technological-Experimental Station 'Maximovca'.As research material was used worm compost-organic fertilizer and peas variety 'Renata'. In experiment were included 3 groups, with a surface such (two-experimental, one-control). Experimental on the batches before sowing, was incorporated organic fertilizer dose of 4t/ha and 3t/ha from dejections of cattle, as a result of use of technology bioconversion of organic waste by worm culture. The control group was grown peas with natural background. The research was conducted in order to assess the influence worm compost on the quality of peas.*
*In the outcome research found that worm compost influences the beneficial the development at the phases phenology of the agricultural crops. Early development of agricultural crops at different phases phenology conditional increasing the quality of production the harvest, resistance in the adverse climatic conditions and at different maladies, allowing obtaining output ecological agricultural.*
*Analysis of research results obtained revealed that worm compost has a positive impact on the quality of output of pea by increasing total nitrogen, crude protein and the decrease content of nitro compounds. The nitrogen content as total and crude protein in the samples of pea grown with fund worm compost, increased by properly with 42,38%-126,50% si 42,37%-127,50% and nitro compounds diminished by 5,76%-65,11%.*
*Thus, into results of the investigations it was found improve the quality of peas intended grown with fund worm compost compared with the cultivated fund natural.*

***Keywords:*** *fertilizer organic, peas, quality, nitro compounds, protein gross.*

## INTRODUCTION

To obtain ecological production both as animal feed and for human alimentation, it is necessary to use certain technologies to improve the environment. Branches of the national economy are important producers of organic waste that pollutes components of nature (soil, water, air).

In science and practice of world were performed research directed at reducing negative influence of organic waste on the environment, paying special attention to their performance methods of bioconversion. An effective method is considered bioconversion of organic waste by worm cultivation that as the new direction of agrobiological science and practice worth fundamental research. The purpose of this biotechnology is to obtain worm compost-ecological organic fertilizer, the use of which help increase of soil fertility, harvest and improving the quality of agricultural production.

In worm compost considerable are concentrated qualities of enzymes, vitamins, stimulators of growth, non-pathogenic microflora. Worm compost rests role in the development of ecological agriculture (Cremeneac, 2003).

As a result of the investigations it was found that in the worm compost is well balanced content of macro-and microelements, which allows dose reduction of incorporation into the soil, which is 8-12 times lower than dose of ordinary compost. It was found that a tonne of worm compost contain 270-300 kg of humus. It allows to significantly decreasing period for completing the amount of humus in the soil, thus restored soil fertility and resistance to wind and alluvial erosion.

Worm compost can be used in cultivation of all agricultural crops (greenhouse and open field). Influence on their development at different phenological stages, harvest and quality of agricultural production. According to research found that using worm compost is welcome in the greenhouse practice of vegetable growing, where of rule uses considerable amounts of mineral fertilizers, chemicals for pest and disease destroying crops. This hampers to obtain ecological agricultural production, thus reducing demand for agricultural products on the market (Cosolapova et al., 1996). As a result of previous studies found that worm compost had a positive effect on the quality of forage crops (maize alfalfa and fodder beet) grown with worm compost fund. Quantity of nitro-compounds in maize alfalfa and fodder beet grown with worm compost fund reduced corresponding from 2,10 to 2,66, 3,47 to 3,76, 1,10 to 1,14 times compared with plants grown with mineral fertilizer (Boclaci et al., 2012).
So, incorporation of worm compost in the soil improves soil fertility, influence early development of agricultural crops in different phenological stages, increasing yields and improved quality of obtained production.

## MATERIALS AND METHODS

To appreciate the influence of worm compost on quality of peas experiment was organized under the field of Technological-Experimental Station 'Maximovca'. As research material was used organic fertilizer and peas variety 'Renata'. In experiment (Table 1) were included 3 groups, with a surface such (two-experimental and one-control).

Table 1. The scheme of the experiment

| No | Type of culture | Lots | Condition of experiment |
|---|---|---|---|
| 1 | Peas | I-experimental | Worm compost, 4t/ha |
| 2 | Peas | II-experimental | Worm compost, 3t/ha |
| 3 | Peas | III-control | Natural fund |

On experimental lots before sowing, was incorporated organic fertilizer dose of 4t/ha (experiment I) and 3t/ha (experiment II) from cattle manure as a result of use bioconversion technology of organic waste by worm growing. On control lot was grown peas with natural fund. The research was conducted over three years, including the first, second and third year of action of worm compost.
During the experiment was determined by using the usual methods, total nitrogen content and crude protein (Petuhova et al., 1989), and nitro-compounds content-electro-colorimetric method (Razumova et al., 1986). All analyzes were conducted using samples of peas in their natural state. In order to assess the influence of soil fertilization with worm compost on quality of peas in different phenological stages, research has been carried out over three years. Each year was conducted investigations over phases: early flowering, total flowering, pod formation and total ripening. During the experiment has been observed that in all phenological phases of the experimental plants I and II growing by worm compost fund, total nitrogen content, crude protein and nitro-compounds was higher than in the control group cultivated naturally fund. As a result of studies found that incorporation of worm compost in the soil, at a dose of 4t/ha-3t/ha, the quality of peas has improved in all phenological stages.
During the experiment the dependence of phenological stages of development samples of peas were taken in order to assess its quality.

## RESULTS AND DISCUSSIONS

Analyzing the results of research conducted in the first year of action of organic fertilizer (Table 2) revealed a significant increase of total nitrogen and crude protein content in plants of experimental groups.
This increase constituted duly 126,50% 127,50% in plants of the experimental group I and 65,66%, 65,58% in plants of the experimental group II. Regarding the content of nitro compounds in the first year of action of worm compost in peas samples collected from experimental group I, the nitrate content decreased by 53,69%, and nitrite 53,70% compared with control group. In peas samples collected from the experimental group II nitrate and nitrite content decreased, corresponding to 53,74% and 52,67%.

Table 2. Assessing the influence of worm compost on quality of peas from the experiment

| No | Indices | Variant of experiment | | |
|---|---|---|---|---|
| | | Control | Experiment I-4t/ha | Experiment II -3t/ha |
| 1 | Total nitrogen,% | 1,66 | 3,76 | 2,75 |
| 2 | Crude protein,% | 10,40 | 23,66 | 17,22 |
| 3 | Nitrites, mg/kg | 289,80 | 134,20 | 138,20 |
| 4 | Nitrate, mg/kg | 4,86 | 2,25 | 2,30 |

This increase constituted duly 126,50% 127,50% in plants of the experimental group I and 65,66%, 65,58% in plants of the experimental group II. Regarding the content of nitro compounds in the first year of action of worm compost in peas samples collected from experimental group I, the nitrate content decreased by 53,69%, and nitrite 53,70% compared with control group. In peas samples collected from the experimental group II nitrate and nitrite content decreased, corresponding to 53,74% and 52,67%.

So the results obtained show beneficial influence of worm compost on quality of peas in the first year of action of fertilizer, increasing total nitrogen and protein contentand diminished content of nitrates and nitrites.

The same regularity was followed in the second year of action of fertilizer on peas cultivation with fund of worm compost (Table 3).

Table 3. Assessing the influence of worm compost on quality of peas from the experiment

| No | Indices | Variant of experiment | | |
|---|---|---|---|---|
| | | Control | Experiment I-4t/ha | Experiment II -3t/ha |
| 1 | Total nitrogen,% | 3,15 | 4,76 | 4,88 |
| 2 | Crude protein,% | 19,69 | 29,75 | 28,63 |
| 3 | Nitrites, mg/kg | 265,00 | 155,00 | 130,24 |
| 4 | Nitrate, mg/kg | 3,12 | - | 5,00 |

Total nitrogen and crude protein values?? both in samples of plant collected from the control group and in the-in the experimental groups increased corresponding to 89,80%, 89,33% (control group), with 26,60%, 25 70% (experimental lot I) and 66,55%, 66,26% (experimental lot II). The content of nitro-compounds decreased the exception of the nitrate content in peas samples collected from experimental group I and nitrite content in plants of the experimental group II. Compared with control lot in the second year of action content of crude protein and of total nitrogen increased corresponding by 51,11,% and 51,09% (experimental lot I) and 45,40% (experimental lot II). Comparing the amount of nitro-compounds in plants of experimental groups with those of their control group was found reduction with 41,51,%-50,85,% exception being nitrite content of peas samples collected from the experimental lot II.

So the results obtained and in the second year of action worm compost quality of peas was directly related to the influence of worm compost.

Table 4. Assessing the influence of worm compost on quality of peas from the experiment

| No | Indices | Variant of experiment | | |
|---|---|---|---|---|
| | | Control | Experiment I -4t/ha | Experiment II -3t/ha |
| 1 | Total nitrogen,% | 3,02 | 4,34 | 4,30 |
| 2 | Crude protein,% | 18,88 | 27,13 | 26,88 |
| 3 | Nitrites, mg/kg | 190,00 | 66,30 | 72,30 |
| 4 | Nitrate, mg/kg | - | - | - |

The results presented in Table 4 show that the values of total nitrogen and crude protein in peas, in the third year of action of worm compost, compared to their plants collected in second year both the control lot and the experimental decreased as corresponding with 4,13%, 4,11% (control group), 8,82%, 8,81% (experimental lot I) and 6,11% (experimental lot II). Comparing the results obtained in the third year with those obtained in the first year of action of worm compost was found that the value of these indices has increased accordingly but it has exceeded that of the first year of action of worm composting with 81,93%; 81,54% (control group), 15,43%, 14,67% (experimental lot I) and 56,36%, 56,10% (experimental lot II).

In peas collected from the control lot, experimental I and II experimental, in the third year of action of worm compost nitrate value decreased, corresponding to 34,44%, 50,60%

and 47,61% in comparison with the results in the first year and corresponding to 28,30%, 57,23% and 44,48% compared with the results obtained in the second year of action of worm compost.

In comparison to the control group in samples of peas, the experimental lot I, total nitrogen content and crude protein increased, corresponding to 43,71% and 43,70%, and the ones collected from experimental lot II-with 42,38% and 42,37%. The amount of nitrates in samples of peas from the experimental group decreased by 65,11% I and experimental group II-with 61,95% compared to that in samples collected from the control lot. In samples of peas from all groups, in the third year of action of worm compost nitrite were not detected.

So, researches that were made in the third year of action of worm compost found the influence of fertilizer on quality of peas.

## CONCLUSIONS

Fertilization of soil with worm compost in doses of 3t/ha and 4t/ha improved quality of peas over three years:

total nitrogen and crude protein content in peas plants in experimental groups increased by 42,38% 126,50% and 42,37%-127,50% compared to that in the control lot plants; the value of nitro-compounds diminished on experimental plants with 5,76%-65,11% compared to the control group of plants.

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# GENERAL ASPECTS REGARDING WASTE MANAGEMENT IN SUSTAINABLE DEVELOPMENT OF AGRICULTURE

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**A*bstract.***

*The paper summarizes the world and own experience using worm culture (earthworms) for utilization of various organic wastes, use of products worm cultivation (worm compost, biomass, worms and alarm pheromone) in a number of industries. Recognized features of physiological development and maintenance of worm culture, the stages and the basic conditions of industrial cultivation technology, the product features and their use. The bioconversion process of organic wastes is implemented in the Experimental Section of the Scientific and Practical Institute of Biotechnologies in Animal Husbandry and Veterinary Medicine. The studies conducted by several states, including Moldova, concluded that worm compost has a positive influence on crop productivity, reducing growth period, their resistance to unfavourable climatic conditions and common plant diseases.*

*Worm cultivation opens up new perspectives and opportunities for introducing technology of obtaining the protein not only for feed purposes, but also in the food industry for the production ecological products. The results of research showed that the products obtained from biomass worms and alarm pheromone, can serve as raw material for the preparation of medicinal products used in medicine and in veterinary medicine for the control and prevention of various diseases.*

*Technology of bioconversion of organic waste using worm culture is designed for landowners, farmers, ranchers, and agricultural enthusiasts, and others as an alternative method for the sustainable development of agriculture.*

*The research conducted and analysis of results about the development of worm cultivation found that: worm cultivation is a branch that has the ability to solve stringent ecological problems existing in some sectors of the national economy, contributing to the development of sustainable agriculture and otained ecological agricultural productions, products obtained as a result of worm cultivation can be widely used in crop, livestock, human and veterinary medicine.*

***Keywords:*** *bioconversion, organic wastes, organic fertilizer, worm compost, worm culture.*

## INTRODUCTION

Organic waste management, the diverse origin of ancient times had spreading range of practical people. Livestock waste, plant growing and food can be used as raw material in organic waste bioconversion technology by worm cultivation to obtain valuable organic fertilizer and biomass with a high content of protein (Condîreva et al., 1994; Cremeneac, 2003).

Biotechnology used, along with organic waste management and production aspect, manifest an environmental aspect, which allows solving the problems of environment protection and sustainable development of agriculture.

Special attention in the last decades of the twentieth century was directed to use worm culture biotransformed as organic waste. Was found that worm culture (earthworms), as biotransformed, is able to process organic waste of different origin, transforming them into valuable organic fertilizer - worm compost (biohumus, biocompost).

An important support in the elaboration bioconversion technology of organic waste has served long observations made on the lives of earthworms in nature. It was found that search of food earthworms swallow soil particles with organic waste, which enter the digestive tract of worm culture is enriched with micro- and macro turning into worm compost, characterized by major agrochemical properties. "Domestication" of

earthworm served as the basis for the management of organic waste and use of worm culture in the process of bioconversion of organic waste in the branches of the economy in general and especially in agriculture,of food industry, of household waste and of sludge from wastewater as a result of treatment.

Based on the research conducted and results obtained (Gorodniy, 1990; Melnik, 1994; Cremeneac, 2003) was elaborated a complex program of management and bioconversion of organic waste by worm cultivationthat includes following directions: bioconversion of organic waste by worm cultivation to obtain valuable organic fertilizer, biological transformation of organic waste and getting worm compost, obtaining biologically active preparations of tissues worm culture, prospects and effectiveness in agriculture of worm compost (as fertilizer) and biomass earthworm as the protein supplement in animal feed rations) to obtain otained ecological agricultural production (Cremeneac et al., 2012).

So in the article are described aspects of organic waste management for use in environmental quality of life and nutrition of worm culture and possibilities of using products from sustainable development of agriculture in addressing issues of environmental protection in society.

## MATERIAL AND METOD

Research in the domain management of organic waste and appreciation of worm compost have been carried out in the Technological-Experimental Station "Maximovca", where he was held the household for worm cultivation and the experiment in field conditions in order to evaluate the influence of worm compost on quality and yield of agricultural crops.

In organizing of household for worm cultivation was taken into account the objectives and tasks that must be solved. It was found that the dominant household for worm cultivation will be: processing of organic waste, increasing the population of earthworm, production of worm compost for resort needs.

The field chosen for worm cultivation was located in an area accessible for use the organic waste, less inclined, with drainage into the ditch, for the water from sectors because worm culture not support high humidity. Sectors for worms cultivation were oriented in the direction of predominantly wind in order not be situated in the wind and to receive the sufficient amount of warmth, with south exposition. Formed sectors for worm cultivation in household were oriented from north to south.

The next step was to prepare the sectors and departments. for worm cultivation According to the technology of bioconversion the organic waste for worm cultivation in households is practiced by pairs sectors 1m wide and 50m long. The distance between the two sectors can be 1m, and of the pair - 4,0m -4,5m Taking into account these requirements STE "Maximovca" were used 5 sectors for worm cultivation, in which initially were placed about 125 tons of organic waste (manure of cattle) that were previously undergone fermentation during 6 months. During six months in sectors were added as supplementary food for still about 25 tonnes.

So, in total have undergone processing about 150 tons of organic waste. Each sector was divided into 25 sections, with dimensions 1,0m × 2,0m. Bioconversion technology of the organic waste by worm cultivation in this household was held at the open air.

In the first year of work the main objective was the accumulation of increased population the earthworm Red Hybrids of California therefore the process has been used only two sectors that have been placed organic waste and worm culture. In each sector for worm cultivation were placed about 25 tons of organic waste subjected to bioconversion to increase population of earthworm, which can then be used to manage organic waste and obtain worm compost. Each sector for worm cultivation were divided into sections, where was placed each a ton of organic waste. Nutritive substrate thickness in the sectors for worm cultivation was 25-35cm - summer and 35-45cm - winter. Under one month nutritive substrate from sectors was sprayed for a week - every day, then once a week. After the period of

spraying, the substrate prepared for worm cultivation has undergone biochemical analysis to determine quality indices.

Initially, and then permanently in order to determine the quality of the organic waste placed in areas for worm cultivation was make analyzes: biochemical, determination of active acidity (pH), ammonia nitrogen content, total nitrogen, organic matter, magnesium, phosphorus, potassium and calcium and microbiological taking into consideration pathogenic and non-pathogenic microflora present, using the usual methods (Razumova, 1986). Also to determine the quality of nutritional substrate for worm culture is used "50-the earthworm test" that provide control of the organic waste by using 50 earthworms. In a container were placed 2-3kg of organic waste that is located 50 earthworms. If after 24 hours the earthworms does not leaving the substrate and were active when it is considered that organic waste can be used as a nutritional substrate for worm culture.

Complete cycle of processing of organic waste vary from 5 to 8 months, depending on climatic conditions earthworms and population density located to substrate .

As a result of use of bioconversion technology of organic waste by worm cultivation final products were obtained: worm compost - valuable organic fertilizer, organic mass of earthworms and ecologycal production.

For assessment purposes the influence of viermicompostului on quality and yield of agricultural crops in conditions of field was organized experiment in which research materials have served viermicompostul and agricultural crops: peas, fodder beet and corn. For each agricultural crop were used a control group (with natural background) and two experimental groups (with background of worm compost). Worm compost was incorporated into the soil at a dose of 4t/ha (experimental group I) and 3t/ha (experimental group II).

To determine the quality of nutrient substrate, organic fertilizer produced (of worm compost) and cultivated agricultural crops with fund of worm compost were used following methods: the determination of active acidity - using pH meter, total nitrogen - using the Kjeldahl method; nitro-compounds – using the electro colorimetric method, magnesium, calcium, phosphorus and potassium - using the usual methods (Petuhova et al.,1989). Determining the harvest productivity was made by weighing. Control counting of earthworm population was carried out by special method, which consists in using a form with size 10cm × 10cm, with a length of 20 cm (corresponding nutritive substrate thickness of sectors). With this form are taken three samples of nutritive substrate from each section. By counting is determined the number of earthworms of all ages in all three samples, and then the necessary calculations were made taking into account the surface section and then sectors.

Subsequently, in the second and subsequent years, the population of earthworm obtained was used as organic waste biotransformed in the 5 sectors.

So, the objectives and the proposed duties for organic waste management in sustainable agriculture have been met. Statistical processing of the results was performed according to the method developed by E.Mercurieva

## RESULTS AND DISCUSSIONS

As a result of biochemical analyzes of nutritive substrate placed for worm cultivation sectors, it was found that it coincided with necessary parameters for bioconversion of organic waste by worm cultivation, being used as a nutritive and life medium for worm culture (Table 1).

Through the quality control of nutritive substrate according to test "50 of earthworm" also was confirmed quality of nutritive substrate because after 24 hours of placement of earthworms in substrate, all 50 earthworms used in testing, remained alive and active. In April in each section were placed each 2,000 mature earthworms. Placement of earthworms was made early morning, since under the influence of sunlight they quickly hide in the substrate.

Table 1. Chemical composition of the nutrient substrate

| No | Indices | Index value, M ±m |
|---|---|---|
| 1 | Active acidity (pH), units | 7,57 ± 0,08 |
| 2 | Ammoniacal nitrogen, mg / kg | 5,56 ± 0,57 |
| 3 | Total nitrogen, % | 0,83 ± 0,63 |
| 4 | Organic substanc, % | 30,35 ± 0,60 |
| 5 | Magnesium, % | 1,17 ± 0,52 |
| 6 | Phosphorus($P_2O_5$), % | 0,65 ± 0,32 |
| 7 | Potassium (K2O), % | 0,68 ± 0,01 |
| 8 | Calcium, % | 1,55 ± 0,35 |

During the first week, daily, investigations were performed to establish the presence of abnormalities in behavior and development of worm culture. As a result of investigations it was found that at the end of the first week, mature individuals began to use in nutrition the substrate in which they were placed. This showed that the substrate is benific for use as living environment for worm culture. During of investigations, over 6 months, were made observations on the process of reproduction of worms culture by conducting monthly control count of individuals of all ages present in sections. Results regarding the developer population earthworm are presented in Table 2.

Table 2. Dynamics of population development of earthworm of California Red Hybrid

| No. | Population of earthworm | During of investigations, month | | |
|---|---|---|---|---|
| | | One month | Three month | Six month |
| 1 | Mature earthworms | 2000 | 2000 | 10077 |
| 2 | Juvenile earthworms | 5125 | 17490 | 70113 |
| 3 | Cocoons | 4320 | 5200 | 20740 |
| 4 | Total: (mature earthworms + juvenile earthworms) | 7125 | 19490 | 80190 |

Analysis of the results has found that over six months, the number of mature earthworms in a section increased 5 times compared to their number in the initial stage. During the investigation were found significant changes in correlation: Cocoons: mature earthworms, earthworms juvenile: cocoons and juvenile earthworm:mature earthworms. It was found that in the first month these indices were 2,2 cocoons to one mature earthworm, 1,2 juvenile earthworms to a one cocoon and 2,6 juvenile earthworms to one mature earthworm. After three months, this correlation has changed constitute: 2,6 cocoons to one mature earthworm, 3,4 a juvenile earthworms to one cocoon and 8,7 juvenile earthworms to one mature earthworm. To the end of the sixth correlations cocoons: mature earthworms and juvenile earthworms: earthworms mature decreased and correlation juvenile earthworms: cocoons remained at level of the first month. These changes at the end of the sixth were influenced by increased earthworm population in sectors, which resulted decline in reproduction. According to the results obtained after six months of earthworms population in a sector was about 2004750 individuals of all ages. Such in both sectors was obtained for worm cultivation a population of about 4 million earthworms, which was later used for biotransformed as the organic waste 5 sectors ready for bioconversion of waste by worm cultivation. After being determined quality of nutritional substrate for worm cultivation in the sectors in these worms were placed culture reasons in a section 32000 earthworms or earthworm 800000 in a sector. Complete duration of processing of nutritional substrate is 6 months. During the experimental period nutritive substrate was sprayed with water (according to necessity). For worm cultivation sectors were covered with straw in order to reduce evaporation - summer and protection from the cold - winter.

During the experimental period requirements have been bioconversion technology of organic waste by worm cultivation (humidity - 70-80 %, content of ammoniacal nitrogen 1,0 to 20mg / kg, active acidity (pH) - 6,8 to 7,6 units and content of cellulose - 30 %). After 30 days from the beginning of the experiment, it was found that worm culture completely processed the nutritive substrate, so started adding new amounts of nutritive substrate, as additional food. This process was conducted (according to necessity) every 10-14 days. At the end of the experimental period worm culture was separated from the substrate and placed in other sectors, prepared

in advance. As a result of bioconversion was obtained about 90 tons of worm compost, valuable organic fertilizer.

In Table 3 are exposed quality indices of worm compost obtained as a result of bioconversion of organic waste by worm cultivation.

Table 3. Quality indices of worm compost obtained from cattle manure

| No | Indices | Index value, M ±m |
|---|---|---|
| 1 | Active acidity (pH), units | 7,81 ± 0,03 - 8,08 ± 0,02 |
| 2 | Organic substance, % | 32,92 ± 0,02 - 39,96 ± 0,03 |
| 3 | Total nitrogen, % | 1,09 ± 0,01 - 3,00 ± 0,04 |
| 4 | Potassium ($K_2O$), % | 1,92 ± 0,02 - 2,80 ± 0,05 |
| 5 | Magnesium, % | 1,18 ± 0,03 - 2,50 ± 0,04 |
| 6 | Phosphorus ($P_2O_5$), % | 1,37 ± 0,08 - 2,50 ± 0,06 |
| 7 | Calcium, % | 1,62 ± 0,02 - 3,80 ± 0,05 |
| 8 | Humus, % | 29,66 ± 1,40 - 35,91 ± 1,90 |
| 9 | Nonpathogenic bacterial flora, colonies | $2x10^{12}$-$3x10^{12}$ |

Comparing the values of worm compost obtained with the the nutritive substrate was found that the amount of active acidity, total nitrogen, potassium, magnesium, phosphorus and calcium have exceeded that of a the nutritive substrate, ie 3,17 % - 6,74 % , 31,32 % - 261,14 % 182,40 % - 311,8 %, 110,77 % - 284,62 % and 4,52 % - 145,20 %. Organic matter content decreased by 8,46 % - 31,66 % respectively. As a result of the investigations it was found that worm compost contain 100 times more non-pathogenic microflora (2x1012 colonies) than traditional compost. In worm compost are concentrated considerable quantities of enzymes, vitamins and stimulators of growth. It was also found that in the worm compost is well balanced content of macro-and microelements, which allows decreasing the dose of incorporation in soil of 8-12 times compared to ordinary compost. When using worm compost as organic fertilizer savings are considerable taking into account that at one hectare using 3-6 tons of worm compost compared to 40-70t/ha of traditional compost. Efficacy action of worm compost is kept over 3-4 years.

So, according to the results obtained it was found that after the biochemical composition and usage, worm compost is upper traditional compost.

According to the research found that one tonne of compost worm contained an amount of 270-300kg of humus. This allows to significantly decreased period for completing the amount of humus in the soil, thus restored soil fertility and soil resistance to wind and alluvial erosion.

In order to determine the particularities of the development process of plants and agricultural productivity cultivated with fund of worm compost in field conditions of Technological-Experimental Station "Maximovca" was organized experiment.

At the initial stage of the experiment it was found that all cultures of the experiment cultivated with fund of worm compost sprang with 5-7 days earlier than those in control groups. This demonstrates that the beneficial influence viermicompostul germination and springing of agricultural crops.

Comparing the development of plants in all variants was found that in lots of worm compost fund of agricultural crops have grown more intense, early flowering of peas and formation earlier of maize cobs took place 5-6 days earlier than in control groups.

So, as a result of studies found that incorporation of worm compost in the soil, at a dose of 4t/ha, resulting in faster development of crops, reduce the period of flowering and ripening of crops. At the end of the experiment there was an essential difference in the productivity of crops, depending on the fund that was grown and crop type (Table 4).

Analyzing obtained found that harvested pea obtained varied from lot to lot. The experimental groups I and II harvested grains peas exceeded that of the control group, respectively 47,50 % and 35,30 %.

Table 4. Indices of agricultural crop productivity

| No. | Agricultural crop | Lots and quantity of yield, kg | | |
|---|---|---|---|---|
| | | Control | Experi-mental I | Experi-mental II |
| 1 | Peas | 10,640 | 15,700 | 15,400 |
| 2 | Maize | 57,000 | 77,000 | 69,000 |
| 3 | Fodder beet | 630,000 | 1115,000 | 907,000 |

Harvest of maize in the experimental group was 35,08 % and 21,05 % more increased than the control group of plants.

On the experimental groups where fodder beet was cultivated with worm compost fund

has collected 76,98 % and 43,96 % more productive than the control group.

So, the results of investigations involving different crops and incorporation of worm compost in the soil reduce phenological stages of plant development increases yield of agricultural crops.

Thus, as a result of organic waste management and their use as living environment and nutrition for the worm culture was possible solve some problems of the environment protection and obtaining increased quantities of agricultural production.

## CONCLUSIONS

Bioconversion of organic waste by worms cultivation solves some important issues in the sustainable development of agriculture: the complete processing of organic waste, protect the environment, obtaining organic ecological fertilizer, long-acting, increasing crop yield and improving the quality of agricultural production.

Qualitative indexes values of worm compost have exceeded those essential the nutritive substrate.

Worm compost used for growing crops, beneficial influence their developers in different phenological phases contributing to increased harvest obtained per unit of area.

Incorporation of compost worm in the soil, at a dose of 4t/ha and 3t/ha, expected decrease springing and ripening period and increases of agricultural crops harvest by 21,05 %-76,98 %.

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# THE SELECTION OF SOME TISSUE LINES PRODUCERS OFANTHOCYANINS IN BILBERRY (*Vaccinium mytillus* L.) CALLUSCULTURE

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***Abstract***

*Our research aimed the obtaining a proliferative tissue at 7 bilberry (Vaccinium mytillus L.) local population, to select some anthocyanins producing tissue lines by in vitro culture. The tissue lines selected from Arieşeni, Semenic, Retezat, Valea Sebeşului, Buces-Vulcan, Vadul Dobrii and Cornereva local population, with good results of callus growth capacity in subculture, produced anthocyanins (646 mgC3GE/100g). These results demonstrated the possibility to elaborate a production system of these metabolites in controlled conditions.*

***Keywords:*** *bilberry (Vaccinium mytillus L.) local population, callus, anthocyanins.*

## INTRODUCTION

Among the plants that provide raw material for obtaining a wide range of natural medications (*phytotherapeutical* products), food supplements, natural colorants and preservatives, there is also included the species *Vaccinium myrtillus* L. In our country, but also worldwide, a sustaining preference for the consumption of *phytotherapeutical* preparations can be recognized, to the disadvantage of medicines prepared by chemical synthesis. This is due to the fact that herbal therapeutic products are better tolerated by the human body (on the background of a better compatibility at a metabolic level). The curative medicinal properties of bilberry are known since antiquity and they were exploited under various forms, from fruit and leaf teas, poultices, tinctures, powders, to complex drugs obtained nowadays (Maillefert, 2010). The bilberry plants have therapeutic importance both in human medicine and in veterinary medicine. Among the many effects of the active principles of bilberries there we can find: hypoglycemic, hypotensive, antiseptic, cholagogue, vasoprotectives of capillaries (eye, brain, peripheral), antidiarrheal, antioxidant (Martz et. al, 2010).

## MATERIALS AND METHODS

We used 7 local populations of bilberry collected from the central-west part of Transilvania: *Arieşeni, Semenic, Retezat, Valea Sebeşului, Buces-Vulcan, Vadul Dobrii and Cornereva*. For callus inducing three types of explants were used: meristem, leaf and stem, grown *in vitro* on three culture media (Lloyd and McCown Woody plant medium-WPM, Anderson′s Rhododendron medium-AND and Mourashige-Skoog medium-MS), under the influence of ANA (naphtalen-acetic acid) two hormonal variants ($V_1$, $V_2$, table 1). Undifferentiated tissues obtained were sub-cultured on media supplemented with the same hormonal balances. Callus was sub-cultured in three culture cycle, on WPM medium supplemented with 1,5 mg/l ANA,

Table 1. Hormonal balances tested for callus induction on culture media (WPM, AND, MS)

| Variant | Hormons mg/ml | | |
|---|---|---|---|
| | ANA | BAP | AS |
| 1 | 1,5 | 1 | - |
| 2 | 1,5 | 1,5 | 40 |

The total antioxidant capacity of the selected tissue lines from the Arieşeni, Retezat and

Valea Sebesului populations has been studied, achieving good results concerning the ability of callus growth in subculture and obtaining plant biomass, with rich anthocyanin pigments (those calluses were visually selected that showed red cells, signaling the presence of anthocyanins). To determine the total antioxidant capacity we use extracts obtained from callus and foliar tissue of bilberry mother plant, using a rapid method, elaborated by Benzie and Strain (1996), modified by Varga et al. (2000); Szollosi and Varga (2002). The total anthocyanin content was expressed in cianidin 3-glucoside equivalents (C3GE) /100g fresh vegetal material-FW.

## RESULTS AND DISCUSSIONS

### 1. Experimental results regarding the callus induction at bilberry local population

Abundant and proliferative callus was obtained from stem and foliar explants, grown on WPM media, supplemented with 1.5 mg/l ANA + 1.5 mg/l BAP + 40 mg/l AS, demonstrating that the culture media, explant type and association of citokinine with auxine, in equal proportions and in the presence of adenine sulphate-AS (40 mg/l) are key factors in inducing callus at spontaneous bilberry.

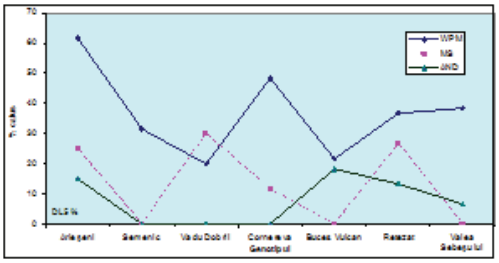


Figure 1. Graphical representation of bilberry callus percentage for different genotypes and culture medium on bilberry meristem explants.

AND medium determine the lowest variability between populations in terms of callus production. The best values registered WPM medium when it used meristem explants (fig.1).

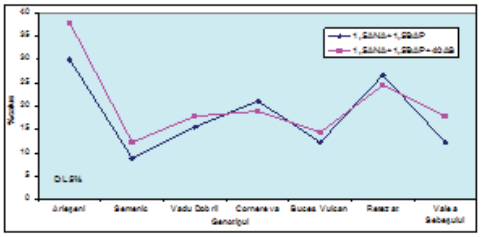


Figure 2. Graphical representation of bilberry callus percentage for different genotypes and hormonal balances on bilberry meristem explants.

However, meristem explants produced less callus (fig. 2).

The genotype is another factor which influences callus production at bilberry. Only at the populations from Arieseni, Cornereva, Retezat and Valea Sebesului, callus was obtained in greater quantities comparing to the other studied genotypes, over 90% callus from leaf and stem explants (fig.1, 2, 3, 4, 5 and 6).

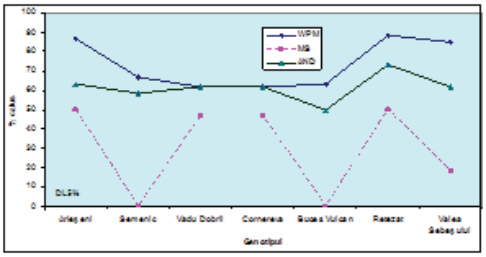


Figure 3. Graphical representation of bilberry callus percentage for different genotypes and culture mediums on bilberry leaf explants.

Under WPM medium influence the leaf explant produced callus at all bilberry local population, with values between 61,67% and 88,33% (fig.3).

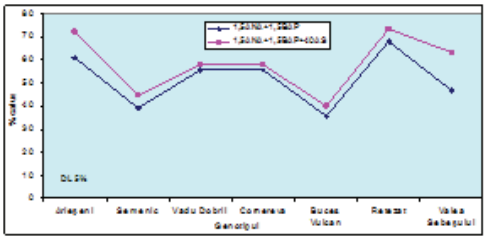


Figure 4. Graphical representation of bilberry callus percentage for different genoyupes and hormonal balances on bilberry leaf explants.

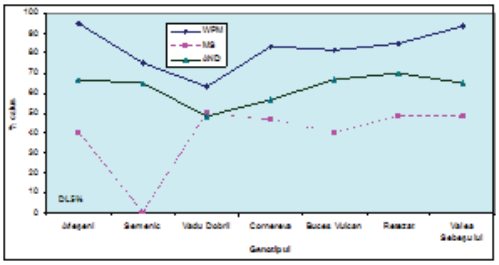


Figure 5. Graphical representation of bilberry callus percentage for different genotypes and culture media on bilberry stem explants.

The stem explant produced callus in the largest amount on WPM medium, with values between 63,33% at Vadul Dobrii population and 95% at Arieseni population.

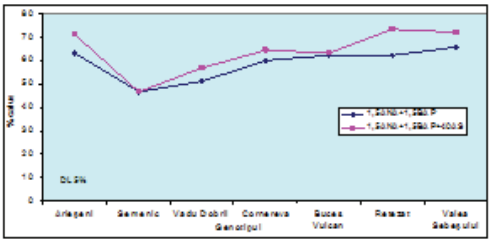


Figure 6. Graphical representation of bilberry callus percentage for different genotypes and hormonal balances on bilberry stem explants.

The two populations *Buces-Vulcan* and *Vadul Dobrii* present lower results in callus growth. The best medium which provided a good callus growth was WPM and it was used in callus subculture. The callus growth in subculture is significantly influenced by the genotype and the presence of AS. Our results are in concordance with those obtained by Litwinczuk and Wadas (2008).

The growth bilberry callus in subculture on WPM medium was influenced by genotype, callus type and AS concentration (table 2).

It can observe that a significantly callus growth in subculture was registered at Retezat, Valea Sebesului and Arieseni populations (2,70-2,83) in presence of 60 mg/l AS.

The origin of callus is a factor influencing in a certain extent the callus growth in subculture. The stem callus has a good growth compared with foliar callus at bilberry local population.

Table 2. The effect of the genotype, callus type and AS concentration on the growth of the bilberry callus in subculture on the solid medium WPM (1,5 mg/l ANA+1,5 mg/l BAP)

| Genotype | **Arieseni** | |
|---|---|---|
| Concentration | Callus type | |
| (AS) | **Foliar** | **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 | **Vadu Dobrii** | |
| Concentration | Callus type | |
| (AS) | **Foliar** | **Stem** |
| **40 AS** | x2,13 a | x2,25 b |
| **60 AS** | y2,10 a | x2,68 a |
| **80 AS** | y1,99 a | x2,65 a |
| Genotype | **Cornereva** | |
| Concentration | Callus type | |
| (AS) | **Foliar** | **Stem** |
| **40 AS** | y1,71 a | x2,07 b |
| **60 AS** | y1,60 a | x2,35 a |
| **80 AS** | y1,69 a | x2,22 ab |
| Genotype | **Buceş Vulcan** | |
| Concentration | Callus type | |
| (AS) | **Foliar** | **Stem** |
| **40 AS** | x1,74 a | x1,62 b |
| **60 AS** | y1,61 ab | x1,90 a |
| **80 AS** | y1,50 b | x2,04 a |
| Genotype | **Retezat** | |
| Concentration | Callus type | |
| (AS) | **Foliar** | **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 | Callus type | |
| (AS) | **Foliar** | **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 |

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

**2. Results regarding the evaluation of the total anthocyanin content (on solid WPM media)**

The callus produced by *Retezat, Valea Sebesului* and *Arieseni* populations in subculture synthesised anthocyanins that was analysed (fig.7).

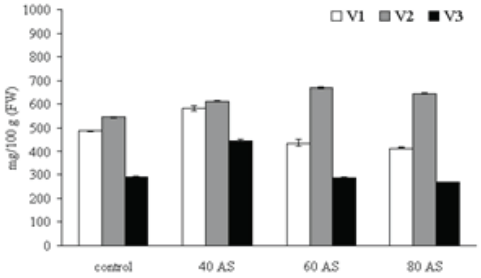


Figure 7. The variance analysis for the total anthocyanin content (mg C3GE /100 g fresh weight) of the bilberry tissue lines obtained on solid WPM medium for the Arieseni (V1), Retezat (V2) si Valea Sebesului (V3) populations

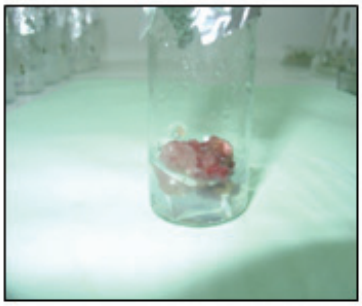

Figure 8. Callus with anthocyanins at the bilberry tissue lines from Retezat population

We selected tissue lines with higher anthocyanin content than control samples, for all three populations, due to the contribution of AS we suppose. The best antioxidant capacity was registered at *Retezat* bilberry population in presence of 60 mg/l AS (V2, 646 mg/ 100 g fresh weight).

## CONCLUSIONS

Our results demonstrated the strong influence of genotype, culture medium and hormonal balance on callus induction and anthocyanin biosynthesis capacity of bilberry tissue cultivated *in vitro*. An abundant callus can be induced from leaf end stem explants at wild bilberry, on WPM medium, in presence of ANA and BAP in equal amount (1,5 mg/l) and 40 mg/l AS. In callus subculture, the genotype and amount of AS are important factors that allow selection of tissue lines with anthocyanin biosynthesis capacity.

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# ANTIFUNGAL ACTIVITY OF *ASTRAGALUS ONOBRYCHIS* L. EXTRACTS

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***Abstract***

*Ethanol and water extracts from aerial parts of Astragalus onobrychis were tested against 12 species of Candida, using the broth microdilution method, in order to determine the MIC values. The study showed a more potent activity on the ethanol extract with a MIC value of 2,57 mg/mL and a weaker activity on the water extract of 3,32 mg/mL on most of the Candida species studied. The results indicate that Astragalus onobrychis is a promising candidate for developing antifungal products.*

***Keywords****: Astragalus onobrychis, antifungal activity, broth micodilution, Candida.*

## INTRODUCTION

*Candida albicans*, the most commonly isolated opportunistic human fungal pathogen, can cause skin and mucosal infections as well as life-threatening systemic infections (Wang et al., 2010). *Candida* infections, or candidiasis, are difficult to treat and create very serious challenge in medicine. Therefore, screening and testing various plants for potential antifungal activities is very important in order to develop antibiotics.

Although, *C. albicans* is the most commonly isolated yeast, other species are found with increasing frequency, including *C. parapsilosis* (Safdar, 2004). *C. parapsilosis* particularly affects critically ill neonates and surgical intensive care unit (ICU) patients, likely because of its association with parenteral nutrition and central lines (Kuhn et al., 2004).

In recent decades, many studies have been carried out on different plant species to discover compounds of possible interest for antifungal applications. Among these studies, several have focused on the biological and phytochemical properties of different species of *Astragalus*, the largest genus of the family Fabaceae and, with over 2500 species, probably the largest genus of flowering plants (Teyeb, 2012). Fabaceae is the third largest family of angiosperm plant with approximately 730 genera and over 19400 species worldwide, which includes the plants commonly known as legumes (Wojciechowski, 2004).

Astragalus is mainly distributed in cool to warm, arid and semiarid regions of the northern hemisphere, South America, and tropical East Africa; it is especially diverse in the south-western and Sino-Himalayan regions of Asia (ca. 1500–2000 spp.), in western North America (ca. 400–450 spp.), and the Andes of South America (ca. 100 spp.). Both the geographic center of diversity and the presumed origin of Astragalus in Eurasia – specifically in the steppes and mountains of south-western to south-central Asia and the Himalayan plateau. According to Ekuci and Ekim (2004) there are 142 species in Europe of which 50 are endemic. Several species of this genus are used in folk medicine due to their hepatoprotective, antioxidative biological activities and their antiviral properties. In Turkish folk medicine, the roots of *Astragalus* species are used for the treatment of leukemia and for the healing of wounds (Yesilada, 2005). Furthermore, *A. mongholicus* Bunge and *A. membranaceus* Bunge are among the most popular Chinese medicines and are used for a variety of diseases, including as an adjunctive in cancer chemotherapy. Some *Astragalus* products, such as gum tragacanth, are widely in use in the preparation of pharmaceuticals and as thickening agents in certain foods (Paul,

2007). Antibacterial activity has also been reported for some Astragalus species, such as *A. gymnolobus*Fisch and *A. brachystachys* DC. In addition, bioactive saponins have been extracted from *A. suberi* L. (Jassbi, 2002). Gođevac et al. (2008) investigated the antioxidant activity of methanol extract from the aerial part of A. *glycyphyllos* L. Another study showed that an ethanol extract of A. membranaceus roots may inhibit the growth of *Trypanosomacruzi* (Schinella, 2002).

*Astragalus onobrychis* has a wide distribution. It can be found in Europe, Asia and northern Africa. It is the first *Astragalus* species to be described by Linnaeus in his famous book, *Species plantarum* from 1753 (Ranjbar, 2007).

To the author's knowledge, there are no previous studies of antifungal properties of *A. onobrychis* on *Candida* species.

## MATERIALS AND METHODS

**Plant material**

Fresh, young plants were collected in the spring, from the Botanic Garden of the University of Szeged.

**Preparation of extracts**

The plants were cleaned, washed and air dried under shade with occasional shifting and then powdered with a mechanical grinder and stored in an airtight container.

Aqueous solutions were prepared with 10 g of plant powder and 100 ml distilled water. The alcoholic solution was made with 100 ml of 95% ethanol. The solution was put in an orbital shaker for 24 hours and kept in a dark room at all times. The aqueous extract was filtered with a vacuum pump and concentrated using the lyophilization process. The alcoholic extract was concentrated using a rotavap. The dried substance was measured on a digital scale. The solution for testing was made by diluting the dried substance and making solutions to a known concentration.

The final concentration of the solutions, are as follows:

*Astragalus onobrychis* ethanol extract: 41,2, 20,6, 10,3, 5,15 and 2,57 mg/mL.

*Astragalus onobrychis* water extract: 26,6, 13,3, 6,65, 3,32 and 1,66 mg/mL.

**Microorganisms**

The *Candida* strains used are: *C. albicans, C. glabrata, C guilliermondii, C. inconspicua, C. krusei, C. lusitaniae, C. norvegica, C. parapsilosis, C. pulcherrima, C. zeylanoides, C. orthopsilosis, C. metapsilosis.*

**Media**

YEPD agar and broth (1% (w/v) yeast extract, 2% peptone, 2% glucose/dextrose, 2% agar in distilled water),

RPMI 1640 medium (Sigma –Aldrich , St. Louis, MO, USA; with L-glutamine , without sodium bicarbonate powered, buffered whit 0,165 mol 1-1 4-morpholinepropanesulfonic acid at pH=7,0).

**Inoculum preparation**

It was prepared a number of test tubes with YEPD agar medium in which the yeast strains were inoculated. The tubes were then incubated for 3 days at 37$^{o}$C.

**Fungal Enumeration**

Fungal populations were determined by plate counting. A modified version of the method described by Montville (2008) was used. In this procedure, five samples of different dilutions were individually surface plated onto one plate, in the form of "lanes" and then incubated at 37$^{0}$C for 24 h. Plates with colonies ranging between 30 and 100 were considered for colony counting to determine the fungal populations.The densities for each strain is shown in (Table 1).

Table 1. The densities measured for each strain

| Fungal strains | CFU/ml |
|---|---|
| *C. inconspicua* | 7,5 x $10^{8}$ |
| *C. krusei* | 8,2 x $10^{8}$ |
| *C. glabrata* | 7,6 x $10^{9}$ |
| *C. pucherrima* | 9,6 x $10^{7}$ |
| *C. methapsilosis* | 9,8 x $10^{9}$ |
| *C. guilliermondi* | 9,2 x $10^{8}$ |
| *C. albicans* | 8,2 x $10^{8}$ |
| *C. norvegica* | 8,8 x $10^{8}$ |
| *C. parapsilosis* | 7,8 x $10^{9}$ |
| *C. kefyr* | 9,4 x $10^{7}$ |
| *C. lusitaniae* | 9,8 x $10^{8}$ |
| *C. zeylanoides* | 6,4 x $10^{8}$ |

**Antifungal assay:**

In each well was put 1 ml of RPMI and inoculated with yeast. 100 µl of the stock solution of plant extract was prepared at the concentration of 41,2 mg/mL for the ethanol

extract and 26,6 mg/mL for the aqueous extract and was added into the first wells. Then, 100 µl of their serial dilutions was transferred into four consecutive wells. The last well containing 100 µl of nutrient broth without the compound and 100 µl of the inoculum from each strip was used as negative control. The final volume in each well was 200 µl. The plates were covered with a sterile plate sealer. Contents of each well were mixed on plate shaker at 300 rpm for 20 s and then incubated at $37^0$C for 48 h. Microbial growth in each medium was determined at 24 hours and at 48 hours by reading the respective absorbance (A) at 620 nm using an ASYS Jupiter plate reader (Biochrom Ltd., Cambridge UK) and confirmed by plating 5 µl samples from clear wells on nutrient agar medium. The MIC was defined as the lowest concentration of the compounds to show no growth of microorganisms.

The tests were performed in triplicate. From the samples which showed activity, it was taken out 50 µl of solution and put in a Petri plate with YEPD agar medium and incubated at $37^0$C.

**Statistical analysis**

Data were averages of three results ± Standard Deviations (SD) by using Microsoft Excel.

## RESULTS AND DISCUSSIONS

The results obtained, show that *A. onobrychis* presents antifungal activity and inhibited the growth of most tested strains. As it can be observed from the tables below, a more intense inhibitory effect was noted in case of the ethanol extract and a weaker activity in the case of aqueous extract. As it can be seen from (Table 2), which shows the ethanol extract values, most of the 12 *Candida* species tested, had a MIC value of 2,57 mg/mL and a few were more resistant.

(Table 3) presents the aqueous extract values, which are higher, that means the aqueous extract presents a weaker activity. In the case of *C. lusitaniae, C. glabrata* and *C. metapsilosis*, the MIC values could not be determined. No significant change was found at the 48 hours.

Table 2: Ethanol extract

| Species | MIC value mg/ml | Control |
|---|---|---|
| *C. norvegica* | 2,57 | 62,5 |
| *C. inconspicua* | 2,57 | 70,1 |
| *C. zeylanoides* | 41,2 | 54,3 |
| *C. pulcherrima* | 2,57 | 55,6 |
| *C. guillermondi* | 41,2 | 64,3 |
| *C. albicans* | 2,57 | 41,5 |
| *C. krusei* | 41,2 | 45,3 |
| *C. lusitaniae* | 41,2 | 52,8 |
| *C. glabrata* | 2,57 | 67,3 |
| *C. parapsilosis* | 2,57 | 62,4 |
| *C. metapsilosis* | 2,57 | 54,3 |
| *C. orthopsilosis* | 2,57 | 54,1 |

The results are in accordance with the one obtained by Sulaiman (2012) on *A. atropilosulus* subsp. *abyssinicus* from Saudi Arabia on *Candida* sp. He tested leaf extracts and obtained a MIC value of 13 mg/mL with an ethanol extract and 30 mg/mL with an aqueous extract. Also, Mikaeili (2012) obtained a MIC value of 160 mg/mL on *Candida albicans*, with an aqueous extract from *Astragalus verus*.

Table 3: Aqueous extract

| Species | MIC value mg/ml | Control |
|---|---|---|
| *C. norvegica* | 3,32 | 54,2 |
| *C. inconspicua* | 26,6 | 65,2 |
| *C. zeylanoides* | 26,6 | 43,2 |
| *C. pulcherrima* | 26,6 | 44,7 |
| *C. guillermondi* | 3,32 | 46,2 |
| *C. albicans* | 26,6 | 57,3 |
| *C. krusei* | 26,6 | 53,2 |
| *C. lusitaniae* | ND | 63,2 |
| *C. glabrata* | ND | 61,6 |
| *C. parapsilosis* | 26,6 | 54,2 |
| *C. metapsilosis* | ND | 67,1 |
| *C. orthopsilosis* | 26,6 | 61,3 |

In the case of isolated constituents, tested separately, the MIC values are much lower, for example 31,25 µg/mL for soyasaponin I, isolated from *A. trimestris*, and tested on *C. albicans*, which shows that the isolated compounds have a much more intense antimicrobial properties (El-Hawiet, 2010).

Most *Astragalus* species exhibit a wide range of antimicrobial properties on very diverse microorganisms, like the ones tested by

Balachandar (2012). However, not all species do present antimicrobial properties, and one interesting example are the 13 *Astragalus* species from eastern Anatolia in Turkey, 4 of which are endemic, tested by Adigüzel (2009) on 40 different species of microorganisms. He found that at any concentration, "none of the extracts tested has inhibitory activity against any of the microorganisms tested" (Adigüzel, 2009).

## CONCLUSIONS

The extracts prepared from aerial parts of *A. onobrychis*, with different solvents, exhibit a different antifungal effect. The ethanol extract presents a stronger antifungal activity than the aqueous extract. *A. onobrychis* extracts, inhibited almost all of the *Candida* species.

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# ANTIBACTERIAL ACTIVITY OF *MOMORDICA CHARANTIA* L. GEMMOTHERAPIC EXTRACT

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***Abstract***

*The antibacterial activity of a gemmotherapic extract of Momordica charantia L., was investigated against 5 species of bacteria, Bacillus subtilis, Streptococcus faecalis, Serratiamarcescens, Pseudomonas aeruginosa and B. cereus v. mycoides, using the well diffusion assay. The extract was prepared from young buds of M. charantia, in according to the gemmotherapic principles. The result revealed that the extract had a significant inhibitory activity against Bacillus subtilis and Pseudomonas aeruginosa but moderate on the other species at a concentration of 50mg/mL. The results are indicating that the gemmotherapic extracts can be a viable alternative to the modern extraction techniques.*

***Keywords****: Momordica charantia, antibacterial activity, gemmotherapic extracts, well diffusion assay.*

## INTRODUCTION

Gemmotherapyor plant stem cell therapy uses a wide variety of embryonic plant parts, collected in the spring at a critical stage in the plants growth when much of the plants energy is directed to the growing areas. Gemmotherapy is an important subsection of phytotherapy.
Gemmotherapic extracts are known for their higher content in active compounds (Rozencwajg, 2008). They are prepared according to gemmotherapic principles from the French Pharmacopoeia, (as cited in Nicoletti, 2012), which consists in maceration of plant buds with equal thirds of water, alcohol and glycerin.
In recent years, there have been a revival of natural, plant based antimicrobial agents. This trend is the consequence of the limited effectiveness of synthetic products to fight against newer, drug resistant bacteria. For this purpose, the antimicrobial properties of many plant compounds from a wide variety of plant species have been assessed (Karuppusamy, 2009). Further, about 80% of the drugs used in modern medicine are the products of plant origin (Patwardhan et al., 2004). Also, food preservatives derived from plants and herbs are of growing interest, since plant compounds often possess antimicrobial properties thatprotect them from infection (Lou et al., 2010).
In this study, we tested a gemmotherapic extract from young buds of *M. charantia* based on the principle that gemmotherapic extracts have a more intense inhibitory activity compared with the traditional extracts (Braca, 2008).
*M. charantia* is a climber belonging to family Cucurbitaceae, is commonly known as bitter gourd or bitter melon. The plant is native to Asia and it was recently introduced in culture in Romania. It is cultivated in the western part of Romania since the year 2006 (Crisan, 2007).
The antimicrobial activity of *M. charantia* is well documented, the plant possessing high inhibitory effects on many species of bacteria and fungi (Abalaka et al., 2011), anti-inflammatory activities (Koboriet al., 2008), (Lii et al., 2009) and antioxidant (Wu and Ng, 2008).

## MATERIALS AND METHODS

### Plant material collection

Plants of *M. charantia* were cultivated in a garden at the University of Agricultural Sciences in Timisoara. Early buds were

collected from very young plants and put immediately in alcohol of 96% concentration.

**Preparation of gemmotherapic extracts**

The solutions were made with equal thirds of alcohol, glycerol and distilled water. The fresh buds were collected, cleaned, washed with distilled water and then put in the solution for extraction. The process of extraction took place for a week in a dark place at $10^0$C, using an orbital shaker. The extract was then filtered, concentrated by using a rotavap and weighted. The dry material was diluted for the tests and filtered through a sterile membrane filter. Two concentrations were tested: one of 50 mg/mL and the second, of 1:10 dilution factor solution.

**Microorganisms**

The bacteria used in this experiment are two Gram-negative bacteria species: *Pseudomonas aeruginosa* and *Serratiamarcescens* and three Gram-positive bacteria: *Bacillus subtilis, Bacillus cereus* var. *mycoides* and *Streptococcus faecalis*. The bacterial cultures were grown in Mueller Hinton Agar (Merck) and Mueller Hinton Broth (Merck).

**Bacteria counting**

The bacteria were counted using a Burker chamber. The values are shown in (Table 1).

Table 1: The Burker chamber count data

| Bacteria species | Number of cells/ml |
|---|---|
| *Pseudomonas aeruginosa* | $1.6x10^8$ |
| *Serratia marcescens* | $1.7x10^8$ |
| *Bacillus subtilis* | $8x10^7$ |
| *Bacillus cereus* var. *mycoides* | $15x10^5$ |
| *Streptococcus faecalis* | $2.2x10^8$ |

*Well diffusion assay and antibacterial activity*

The antibacterial activity was determined using the hole in plate assay procedure (Perez et al., 1990). All bacterial cultures were maintained on nutrient agar slants at temperature of $4^0$C and sub cultured onto nutrient agar broth for 24 hours prior to testing. The pure cultures of the microorganisms were inoculated onto Muller-Hilton nutrient broth incubated at temperature of $37^0$C for 24 hours. 25 ml of nutrient agar was poured into the 100 mm plate, with an even depth of 4 mm on a level surface shaken and allowed to cool.

The nutrient agar plates were seeded with 0.1 ml of standardized inoculums of each of the five test organisms. The inoculum was spread evenly over plate with a sterile glass spreader. Using a sterile cork-borer of 5 mm diameter, three holes were made into the Petri dishes seeded with bacterial culture. The bottoms of the holes were sealed with agar to avoid seepage. 50µl of extracts were introduced in the wells, using a micro liter syringe. Concentrations of 5 and 50 mg/ml extracts were reconstituted in distilled water and transferred into the wells. The plates were kept for 30 min at room temperature to allow diffusion of the extract, and then were incubated at temperature of $37^0$C for 24 hours. After the incubation period, the zones of inhibition were measured using a digital caliper. In this study, the measurement is taken including the 5 mm diameter of the hole.

Studies were performed in triplicates and the mean value was calculated. A solution of only alcohol, glycerol and water in equal ratios wasused as a negative reference.

**Statistical analysis**

Data were averages of three results ± Standard Deviations (SD) by using Microsoft Excel.

## RESULTS AND DISCUSSIONS

In the (Table 2) are presented the mean zone of inhibition measured after 24 hours and in the (Figure 1) is represented the graph with the measurements. The 1:10 dilution factor solution presents little or no visible inhibitory effect, most likely because the concentration here is too low as shown in the (Figure 2). All values were expressed as means ± standard error means.

From the measurements we obtained, it can be observed that *B.subtilis* and *P.aeruginosa* presented the highest sensitivity, the lowest being the *S.marcescens*. All bacteria species tested are susceptible to the extract, at 50 mg/mL concentration and intermediate at 5 mg/mL. The diameter of the zones of inhibition approximately doubles at a tenfold concentration.

These results are similar with those reported by Supraja (2013), in a test with a 50 mg/mL concentration of methanol extract of *M.*

*charantia* on *B.subtilis*, with a ZOI (zone of inhibition) of 16 mm diameter. In the present study, we obtained a 20 mm diameter at the same concentration.

Table 2: Antimicrobial activity of *Momordica charantia* by well diffusion method after 24 hours. Measurement taken including the 5 mm diameter of the hole

| Bacteria/Zone of inhibition in mm* | 50mg/ml | 5mg/ml | Control |
|---|---|---|---|
| *Pseudomonas aeruginosa* | 17.4±0.2 | 11.6±0.3 | 8.2±0.4 |
| *Serratia marcescens* | 11.4±0.5 | 10.2±0.4 | 9.0±0.2 |
| *Bacillus subtilis* | 20.8±0.2 | 12.4±0.4 | 7.8±0.3 |
| *Bacillus cereus var. mycoides* | 15.4±0.4 | 7.6±0.2 | 5.8±0.4 |
| *Streptococcus faecalis* | 13.6±0.2 | 11.4±0.3 | 6.2±0.2 |

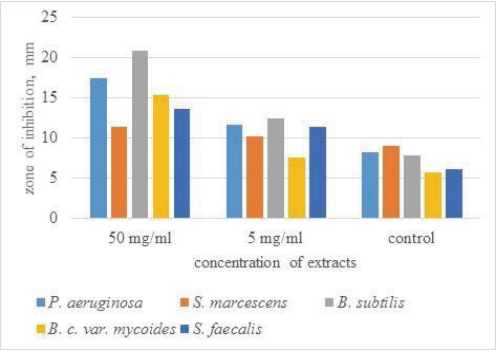


Figure 1. Antimicrobial activity of *Momordica charantia* solutions and the reference, zone of inhibition in mm

In another study made by Leelaprakash (2011), on a methanol leaf extract, he obtained a ZOI of 22 mm at *B.subtilis* but at 100 mg/mL concentration and an 18 mm at *P.aeruginosa* at the same 100 mg/mL concentration.

Although similar tests were performed with slightly different types of cork borers with diameter ranging from 5 to 8 mm, different extract volumes and different concentrations, the results from the present study, confirms the findings of Roopashree et al. (2008) and Costa et al. (2011) on crude alcoholic and water extracts of *M. charantia.*

The data in this study indicate that Gram-positive bacteria were more susceptible to inhibition as compared to Gram-negative bacteria. This finding confirms numerous previous similar reports regarding this aspect (Somchit, 2010; Rahman, 2011).

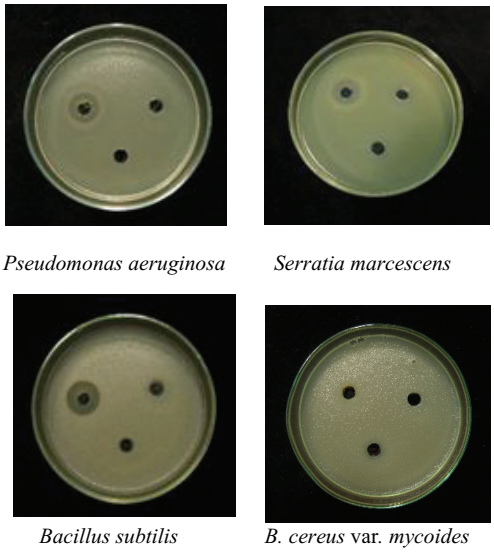


Figure 2. The zones of inhibition for the extracts of *M. charantia* at 50 mg/ml (left), 1:10 dilution (right) and reference (bottom), for *Bacillus cereus* var. *mycoides, Bacillus subtilis, Pseudomonas aeruginosa* and *Serratia marcescens.*

The use of antibiotics has reduced the incidence of infectious diseases but their extensive uses in therapy, has led to the appearance of drug-resistant bacteria (Normanno et al., 2007), which is a major public health issue worldwide. For this purpose, numerous plant extracts were screened for antimicrobial properties that could protect people from microbial infections (Serra et al., 2008; Lou et al., 2010).

The plant extracts can also be used in combination with traditional antibiotics.In the literature, there are reports regarding the use of plant crude extracts (Aqil et al., 2005, 2006)in combination with fewer amounts of antibiotics for anti-bacterial activities, especially for antibiotic-resistant bacteria, compared to antibiotics alone (Schmidt et al., 2008).

**CONCLUSIONS**

Based on the present study, it can be concluded that there is a great potential in using the gemmotherapic principles for plant extracts in the development of more potent and efficient antimicrobial agents.

Also, the gemmotherapic extracts obtained from *M. charantia*young shoots, using the classic gemmotherapic principles, exhibit an intensive antimicrobial activity.
Further investigation is needed in order to study the synergy of fractions from *Momordica charantia.*

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# ESTIMATING FREE RADICALS SCAVENGING ACTIVITY OF SOME BERRIES SPECIES

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***Abstract***
*Berries fruits contain phytochemical components with antioxidant activity, such as polyphenols, ascorbic acid, that have possible protective effects on human health. Free radicals can induce changes in different cell biomolecules such as lipids, proteins, and nucleic acids. This oxidative stress is involved in pathogenesis of many human diseases: cancer, cardiovascular diseases, osteoporosis, neurodegenerative processes. The objective of these researches was to estimate and compare the free radicals scavenging activity of total extracts of certain berries fruits species: raspberry (Rubus idaeus), strawberry (Fragaria ananassa), sea-buckthorn (Hippophaea rhamnoides). The evaluation involved determination of total phenols using spectrophotometrical method and of ascorbic acid content by HPLC method. Total antioxidant capacity was determined using the stable free radical diphenylpicrylhydrazyl (DPPH) method and calculating the parameter $IC_{50}$ (the concentration of sample which is required to scavenge 50% of DPPH free radicals). As expected, sea-buckthorn fruits manifested the highest radical scavenging activity expressed as $EC_{50}$ value (512.76 µg/ml). A linear correlation was obtained for total phenols content (Pearson's correlation coefficient -0.945) and for ascorbic acid (-0.8607).*

***Keywords:*** *ascorbic acid, berries, scavenging activity, total phenols*

## INTRODUCTION

Berries fruits are an important source of active natural biocompounds known to be responsible for free radicals scavenging activity. These fruits contain phytochemical components with antioxidant activity, such as polyphenols, ascorbic acid, that could be involved in preventing the occurrence of oxidative-stress related diseases, caused by the attack of free radicals on key biocomponents like lipids, proteins or nucleic acids (Mayne S.T., 2003; Pisoschi A., 2011). This oxidative stress is involved in pathogenesis of many human diseases: cancer, cardiovascular diseases, osteoporosis, neurodegenerative processes. Vitamin C is an electron donor, and this property accounts for all its known functions. As an electron donor, vitamin C is a potent water-soluble antioxidant in humans. Antioxidant effects of vitamin C have been demonstrated in many experiments *in vitro* (Kelly F.J., 1998; Padayatty S., *et a*l, 2002).
The food industries used some synthetic antioxidants for the protection against the oxidizing agents, but recent researches emphasized their possible toxicity for human health, therefore a preference for antioxidants from natural rather than from synthetic sources have imposed. Considering that, an increasing interest for investigating the antioxidants provided by berries fruits was observed in recent years.
The objective of these researches was to estimate and compare the free radicals scavenging activity (antioxidant activity) of total extracts of certain berries fruits species: raspberry (*Rubus idaeus*), strawberry (*Fragaria ananassa*), sea-buckthorn (*Hippophaea rhamnoides*). The evaluation involved determination of total phenols using spectrophotometrical method and of ascorbic acid content by HPLC method. The total antioxidant capacity was determined using the stable free radical diphenylpicrylhydrazyl (DPPH) method and calculating the parameter $EC_{50}$ (the concentration of sample which is required to scavenge 50% of DPPH free radicals).

## MATERIALS AND METHODS

**Biological materials.** The analyzis were performed on three wild berries fruits species: three samples of raspberry (*Rubus idaeus*), five

samples of strawberry (*Fragaria ananassa*), and two samples of sea-buckthorn (*Hippophaea rhamnoides*) provided by the local market. The determinations were made in triplicate, using fresh fruits. The extractions were conducted according to the protocol used for each determination.

**Total phenolic content** was performed according to the modified Folin-Ciocalteu assay (Singleton *et al*., 1999). The method consists in chemical reduction of Folin-Ciocalteu reagent (which is a mixture of tungsten and molybdenum oxides) and measuring the intensity of the obtained blue colour at 750 nm. The measurements were achieved with a UV/Visible ThermoSpectronic Helios spectrophotometer. Total phenols values were expressed in terms of gallic acid equivalent (GAE), which is a common reference compound.

**The ascorbic acid content**was determined by HPLC-RP with UV detection. The mobile phase consisted in 0.1% phosphoric acid. Samples were centrifuged at 4,000 rpm for 10 minutes; 1 ml supernatant was ten times diluted with elution solvent. Before injection, samples were filtered using 0.22 µm PTFE filters. Data acquisition and processing were realized with EMPOWER software. Ascorbic acid detection was performed at 210 nm. Calibration curve was achieved using a 0.1 µg/ml standard solution of L-ascorbic acid, in five concentration levels with three injections for each level.

**The free radical scavenging activity (total antioxidant capacity)** was determined using the stable free radical diphenylpicrylhydrazyl (DPPH) method according to Blois, M.S. (1958) procedure adaptated by Brand-Williams W. (1995) for complexes matrices. Briefly, a 100 µM solution of DPPH in methanol was prepared and 2 ml of this solution was mixed with 1 ml of different concentrations of berries fruits extract in 80% aqueous methanol. After 30 min incubation in dark at room temperature, absorbance was measured at 515 nm. The percentage of the radical scavenging activity (RSA) was calculated as follows:

$$\% \text{ RSA} = (1-[A_{sample}/A_{control\ t=0}])/100$$

DPPH solution in 80% methanol was used as a control. Gluthation at various concentrations (25 to 200 µg/ml) was used as a standard.

The $EC_{50}$ parameter for each sample, defined as the concentration of sample which is required to scavenge 50% of DPPH free radicals, was calculated from the non linear regression curve of Log concentration of the sample extracts (µg/ml) against the percentage of the radical scavenging activity.

**The statistical analysis** was performed using the one-way Analysis of Variance (ANOVA). Pearson's correlation coefficient (*r*) was used to calculate the relationship between the DPPH and total polyphenol contents and ascorbic acid content of the three berries fruits species.

## RESULTS AND DISCUSSIONS

The phenolics constitute a very divers and widespread group of biochemical compounds occurred in natural vegetal sources. Biological effects of polyphenols are attributed to their antioxidant effects, so that their determination is of considerable interest.

The biochemical analysis performed in this study indicated high values of the total phenols content in the tested berries (table 1), well known for their rich content in bioactive compounds (Battino M. *et al*, 2009; Ribera A.E. *et al*., 2010). The results emphasized sea-buckthorn as the richest phenolics source among the tested fruits. The total phenols content in sea-buckthorn fruits was 669.63 mg GAE/100g, which is 1.93 times higher than in strawberry and 1.63 times higher compared to raspberry. Other authors found that the total content of phenols in sea-buckthorn depends on the cultivar and varies from 828.7 to 1099.6 mg/100g (Novruzov E., 2005; Seglina D. *et al*., 2008).

Table 1. The total phenols content in the tested berries

| **Samples** | **Total phenols** (mg GAE/100g fresh weight) |
|---|---|
| Strawberry | 345.34 ± 3.93 |
| Raspberry | 410.78 ± 6.39 |
| Sea-buckthorn | 669.63 ± 8.18 |

Regarding the level of the ascorbic acid content (table 2), also sea-buckthorn fruits reached the highest amount (1154.24 mg/100g), which was according with the expectations. The results of ascorbic acid content in sea-buckthorn agree with those reported by Gutzeit D. *et al*. (2008) and Christaki E. (2012).

Table 2. The ascorbic acid content in the tested berries

| Samples | Ascorbic acid (mg/100g fresh weight) |
|---|---|
| Strawberry | 64.21 ± 2.18 |
| Raspberry | 52.02 ± 1.50 |
| Sea-buckthorn | 1154.24 ± 4.15 |

For estimating the antioxidant capacity of tested berries fruits was used the DPPH method because is adequate for screening antioxidant compounds formed by small molecules (such as phenols or ascorbic acid), considering that the reaction intensity can be measured using a spectrophotometrical method (Nickavar B. *et al*, 2009). The $EC_{50}$ values for every tested fruits were calculated for further comparison.

For this purpose the extracts of selected berries were screened for their possible radical scavenging activity (RSA). Extracts in different concentrations of selected berries exhibited a high antioxidant activity, expressed as percentage of DPPH reduction (figure 1). The measurements indicated the highest antioxidant activity for sea-buckthorn fruits, confirming the expectations due to their rich content in total phenols and ascorbic acid.

The $EC_{50}$ values were calculated for all the tested berries fruits (table 3), showing that sea-buckthorn fruits manifested the highest scavenging activity (512.76 µg/ml).

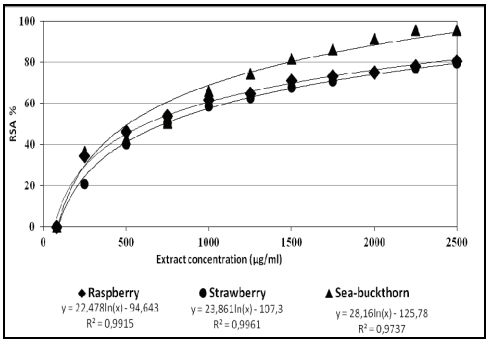


Figure 1. Radical scavenging activity (%) of the tested berries fruits

The extract with the lowest scavenging capacity was strawberry, which required a higher concentration (727.63 µg/ml) to scavenge 50% of DPPH free radicals.

However, this results are significant low compare to the standard (gluthatione) scavenging power (79.66 µg/ml), but we must take into account that, in contrast with the standard, the berries extracts are complex mixtures of numerous compounds with different properties.

Table 3. $EC_{50}$ values of DPPH scavenging activities of the studied berries

| Samples | $EC_{50}$ (µg/ml) |
|---|---|
| Strawberry | 727.63 |
| Raspberry | 620.05 |
| Sea-buckthorn | 512.76 |
| Standard (gluthatione) | 79.66 |

The rich content in phenols and ascorbic acid of the berries may cause the antioxidant properties of these fruits.

The correlation between total phenols content, as well as ascorbic acid content, and the antioxidant activity of certain fruits and vegetables extracts was studied by different authors (Pellegrini N. *et al*, 2003; Franco D. *et al*, 2008; Dvorakova M. *et al*, 2008), which reported an increasing antioxidant activity correlated with the concentration of these active biocompounds.

In the present paper a correlation study was performed between the radical scavenging activity (expressed as $EC_{50}$) and the content in total phenols and ascorbic acid in order to reveal the contribution of these biochemical compounds to the total antioxidant capacity of the berries fruits (figure 2).

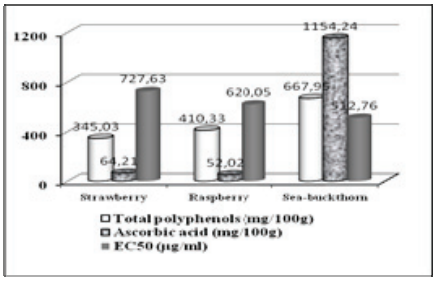


Figure 2. Antioxidants content and radical scavenging activity in berries

A strong linear correlation with Pearson's correlation coefficient - 0.945 was obtained for total phenols content. For ascorbic acid the linear relationship had a lower value, respective - 0.8607.

The obtained correlation coefficients were in accordance with those reported by Rufino M.S.

et al. (2010) and Arancibia-Avila P. et al. (2012)

## CONCLUSIONS

Biochemical analysis performed indicated sea-buckthorn fruits as the richest phenolics sources among the tested fruits (669.63 mg GAE/100g).

Sea-buckthorn fruits reached also the highest amount of ascorbic acid content (1154.24 mg/100g).

As expected, sea-buckthorn fruits manifested the highest radical scavenging activity expressed as $EC_{50}$ value (512.76 µg/ml).

The radical scavenging activity reached high values in the extracts rich in total phenols and ascorbic acid, so that a linear correlation was obtained for total phenols content (Pearson's correlation coefficient -0.945) and for ascorbic acid (-0.8607).

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# THE INFLUENCE OF ENVIRONMENTAL CONDITIONS AND PLANTING DATE ON SUNFLOWER OIL CONTENT AND FATTY ACIDS COMPOSITION

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***Abstract***

*The sunflower oil is very important in human food because it contains a high percentage of unsaturated fatty acids. This oil ideally combines high nutritional value due to its content in linoleic acid with the high stability resistance during cooking because of oleic acid. This paper aims to show the variation of the main fatty acids in sunflower oil depending on environmental conditions and sowing date. In the studied area, adjusting the planting date and considering climatic factors, we can obtain the desired fatty acid percentage in our inbreed lines, used further for obtaining valuable hybrids. The same set of sunflower inbred lines was planted in two stages: April 10 and the May 27. In this period the environmental factors varied from low temperatures and rainfalls up to very high temperatures and severe drought, year 2012 being marked by extreme phenomen.*

***Keywords:*** *fatty acids, linoleic acid, oleic acid, planting date, sunflower.*

## INTRODUCTION

Sunflower is a typical oleic plant due to its high content of oil from seeds, which often exceeds 50% of the dry matter. Whole seed oil content used to be determined as the relationship between the percentage of hulls and the percentage of kernel oil. Now, the oil content is expressed as percentage of whole seed weight. In cultivars with low percentage of hulls (20-24%) the oil content exceeds 50% of dry matter. Sunflower oil is considered first-class edible oil due to its content of linoleic acid, followed by oleic acid, together representing approximately 90% of the fatty acids composition.The fats found in food represent a combination in different proportions of saturated, monounsaturated and polyunsaturated fatty acids.

Sunflower oil contains saturated and monounsaturated fats but in much smaller amounts than polyunsaturated fats (Vrânceanu, 2000). The new mutant variety called oleic sunflower has a high content of monounsaturated fats, namely oleic acid and very low proportion of linoleic acid (Soldatov, 1976).

Burr and Burr's demonstrated in their experiences the importance of this specific group of polyunsaturated fatty acids called essential fatty acids, human and animal organisms being unable to synthesize them (Vles and Gottenboss, 1989).

Unlike other vegetable oils, the sunflower oil has a great nutritional value due to its high linoleic acid content and it is very stable due to lack of linolenic acid, so it can be preserved for a long period of time. The linoleic/oleic ratio is not constant and it can be changed by many factors, the most important being temperature during oil accumulation and genotype. (Vrânceanu, 2000).

This paper aims to show how the oil content and the main fatty acids varies depending on sowing date and climatic conditions of the year 2012 in Fundulea area.

## MATERIALS AND METHODS

The biological material used for this experience consisted in a set of 5 sunflower lines which was sown on two different dates: on April 10 and May 27. The climatologically data were collected from a meteorological institute nearby

the studied area. The seeds were sown in 2 rows for each sunflower line. The distance between rows was 0.75 m and the length of a row was 4.8 m.

Sunflower seeds were ground with laboratory mill Knifetech Foss, the oil content being determined with Foss 1241 equipment which uses NIR technology. For fatty acids the oil is extracted with diethyl-eter, which is an organic solvent, in Soxleth extractor. The obtained oil is subjected to a transesterification reaction and the fatty acid methyl esters will be determined by gas chromatography.

The materials used for this method are: n-heptane, internal standard methyl heptadecanoat 10 mg/ml, gas chromatograph (Trace GC Ultra) with FID detector and split injector, DBWAX column (30 m-0.25 mm-0.25 µm), analytical balance, vials (with capacity of 10 ml), flask, pipette (5 ml). The chromatographic conditions are: oven temperature 210 ºC, injector temperature 250 ººC, gas pressure 80 kPa, gas flow 1-2 ml/min, injector flow 50 ml/min, analysis time 25 min.

The work procedure consists in weighing of 250 mg of sample in a 10 ml vial then adding 5 ml of methyl heptadecanoate. Homogenized mixture is injected and the chromatogram is obtained. The peaks from C 14 to C 24:1 are integrated.

## RESULTS AND DISCUSSIONS

The period Between January and August was marked by extreme phenomena from low to very high temperature and from heavy rain falls in May to severe draught and high temperatures in July.

The ratio of the major fatty acids in sunflower oil is not constant, changing during the oil accumulation. Thus, the concentration of linoleic acid increases until the end of seed maturity, while oleic acid concentration decreases. There exists a negative correlation because oleic acid is a precursor of fatty acids with higher degree of unsaturation. This explains the variation of linoleic and oleic acid between the seeds of the same head (Canvin, 1965; Robertson *et alii*, 1978; Goyne *et alii*, 1979; Unger and Thompson, 1982; Downes and Tonnet, 1982; Simpson and *alii*. 1989; Connor and Sadras, 1992).

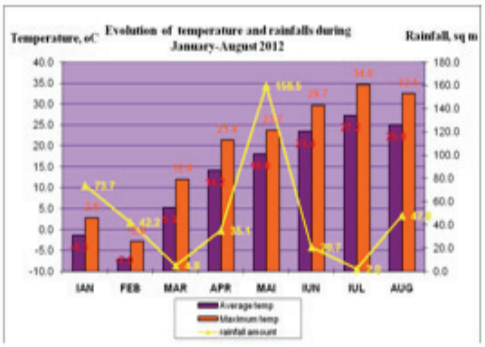


Figure 1. Evolution of temperatures and rainfalls during January-August 2012

The linoleic and palmitic acid contents increase, while the oleic acid content decreases in seed from the perimeter towards the centre of the head (Fick and Zimmerman, 1973; Zimmerman and Fick, 1973).

Regarding the two main fatty acid of sunflower oil, it is well known that they closely depend on the environmental conditions. Temperature, especially day/night temperature differences are the most important environmental factors driving seed oil percentage and oil chemical composition (Izquierdo et al., 2002; Rondanini et al., 2003, 2006; Qadir et al., 2006; Echarte et al., 2010).

Data obtained after processing the samples are presented in table 1 and table 2.

Table 1. Oil and fatty acid composition for sunflower lines sown on April 10, 2012

| Line | Oil | Humidity | Palmitic | Stearic | Oleic | Linoleic |
|---|---|---|---|---|---|---|
| **V1** | 42.0 | 8.5 | 6.1 | 2.5 | 36.4 | 53.1 |
| **V2** | 46.8 | 7.9 | 5.8 | 4.4 | 33.0 | 55.3 |
| **V3** | 47.6 | 8.0 | 5.8 | 2.6 | 28.7 | 61.7 |
| **V4** | 48.0 | 8.4 | 5.6 | 3.6 | 32.0 | 57.4 |
| **V5** | 47.7 | 8.2 | 5.8 | 4.1 | 30.5 | 58.0 |

Table 2. Oil and fatty acid composition for sunflower lines sown on May 27, 2012

| Line | Oil | Humidity | Palmitic | Stearic | Oleic | Linoleic |
|---|---|---|---|---|---|---|
| **V1** | 38.2 | 7.9 | 6.1 | 3.2 | 31.7 | 57.1 |
| **V2** | 46.5 | 8.5 | 5.9 | 4.2 | 33.7 | 54.4 |
| **V3** | 41.8 | 8.6 | 5.4 | 2.5 | 33.6 | 57.2 |
| **V4** | 48.4 | 7.5 | 6.3 | 4.5 | 29.1 | 57.6 |
| **V5** | 42.5 | 8.6 | 6.1 | 4.4 | 28.7 | 58.1 |

Different planting dates and water regimes cause different environmental conditions during seed-filling and oil synthesis of

sunflower seed and therefore a possible alteration in oil content and fatty acid composition of the seed (Flagella *et al*., 2002). Thus, in our results we can observe that the concentration of oleic acid decreased for 4 of 5 lines until the end of seed maturity (figure 2), while for 4 of 5 lines the linoleic acid concentration increased for plants sown later (figure 3).

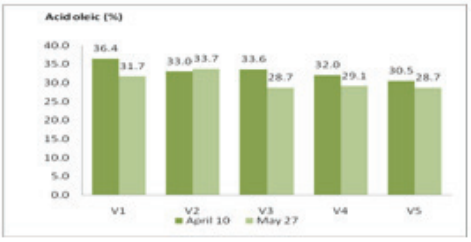


Figure 2. Variation of oleic acid depending on sowing date

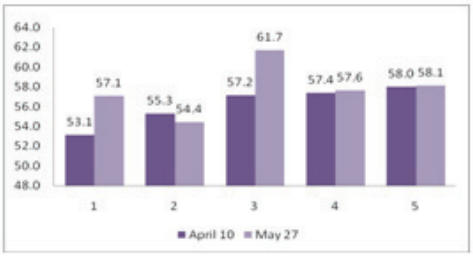


Figure 3. Variation of linoleic acid depending on sowing date

It is known that the oil content varies very much, depending on genotype and environmental factors. The main factor in oil accumulation is water, thus in southern regions, that are more draughty, the oil content is lower than in humid regions.

The rain fallen during seed formation have favorable effects on oil accumulation not only by improving the water supply but also because decrease temperature and increase atmospheric humidity. Even if it is a year with heavy rainfalls, but with high temperatures, the oil content decrease. In case of heavy rainfalls during the second half of summer they will lead to stimulate the secondary growth, which will also have negative effects on oil content (Vrânceanu et al., 1969).

As can be seen in figure 2, the oil content varied very much, thus sunflower lines sown on May 27 showed a significant decrease in oil content, even *with 5% less than in the first set.*

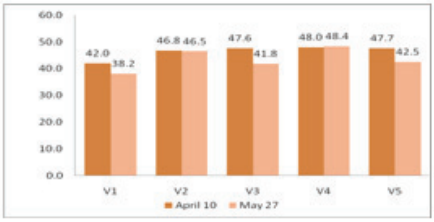


Figure 4. Oil content depending on sowing dat

## CONCLUSIONS

It was noted from results obtained for the five sunflower lines that the oil concentration varied up to 5% from one set to another, this trait being very strong affected by draught. Regarding fatty acids, there are also differences between the two set of sunflower lines; the linoleic acid increased in the second set sown later, while the oleic acid concentration was affected by late planting.

In order to obtain optimal results, it is recommended to adjust sowing date according to the objectives pursued.

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# EXTRACTION, PURIFICATION AND CHARACTERIZATION OF LECTIN FROM *PHASEOLUS VULGARIS* L. CV. WHITE SEEDS (WHITE KIDNEY BEAN)

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***Abstract***

*The purpose of the research was to study the purification and characterization of lectin from Phaseolus vulgaris L. cv.white seeds. The lectin was purified by sequence of steps , namly , first with ammonium sulfate precipitation followed by ion exchange (DEAE cellulose) and gel filtration (sephadex G-200) chromatographies , and finally by polyacrylamide electrophoresis (PAGE). Single band was observed in native PAGE . The lectin was shown to have molecular weight of 33 kDa in SDS PAGE and about 35 kDa in gel filtration and purified about 9.01 fold to final specific activity of 64 titer/mg of protein. The hemagglutination activity of the lectin was stable within the pH range from 4-11 and temperature range from 0°-50°C. Chemical modification results indicate that lysine and tryptophan were crucial for the hemagglutination activity of lectin. The results of carbohydrates specificity showed that the lectin was had complex sugar specifities, but not specific to xylose and mannose.*

***Keywords:*** *lactin, purification, characterization, Phaseolus vulgaris L.*

## INTRODUCTION

Lectins are defined as proteins/glycoproteins possessing at least one non-catalytic domain which binds reversibly to a specific mono-or oligosaccharide (Van damme et al., 2003). Over the last few decades, lectins have become a topic of interest to a large number of researchers owing to their potentially exploitable biological properties including antitumor (Abdullaev et al., 1997; Ye et al., 2001), immunomodulatory and anti-insect (Rubinstein et al., 2004), antifungal (Barrientos et al., 2005), antibacterial (Pusztai et al., 1993), anti-HIV (Tsang et al., 2001; Barrientos et al., 2005; Pollicita et al., 2008), and mitogenic (Wimer, 1990) activities. Because of their sugar binding properties, lectins have been extensively studied and used as molecular tools for the study of carbohydrate architecture and dynamics on the cell surface, and have been exploited for such practical applications as distinguishing between normal and malignant cells (Sharon, 1993; Padma et al., 1998), purification of glycoconjugates (Yamamoto et al., 1984), and coating of drugs to enhance their gastrointestinal tract absorption (Naisbett & Woodley, 1990; Leher, 2000). Further, specific amino acid residues are essential for maintaining the carbohydrate binding and hemagglutinating activities of lectins (Bao et al., 1996; Ba˘i˘miev et al., 2007). Identification of these amino acid residues is a prerequisite for investigating the structure-function relationships of lectins. Chemical modification with group-specific modifying agents provides a general approach for identification of the amino acid residues present in the functional or active site of proteins, including lectins (Bao et al., 1996; Nadimpalli, 1999). Hence, elucidation of biological activities of lectins and amino acid residues essential to these activities is a meaningful undertaking. Although lectins are found ubiquitously in plant species, they have variable structures and specific activities according to the plants they originate from cells (Sharon, 1993; Padma et al., 1998). Thus, purification and characterization of lectins from a variety of plant species interests researchers in the field of glycobiology. The more is known about the lectins, the wider the applications of this type of proteins that can be achieved. This study reports the purification and some properties of a new lectin isolated from seeds of the *Phaseolus vulgaris* L. cv.white cultivar (common name, white kidney bean). To date, the isolation of a lectin from the *Phaseolus*

*vulgaris* L. bean and examining it for various potentially exploitable biological activities such as mitogenic, antitumor, immunomodulatory, and HIV-1 reverse transcriptase inhibitory activities have not been attempted. In this study, a lectin was isolated from Phaseolus vulgaris L. beans. It was assayed for the various aforementioned activities. In order to further characterize the lectin, a chemical modification study was undertaken to determine the involvement of different amino acid residues in its hemagglutinating activity.

## MATERIALS AND METHODS

### 2.1. Materials

*Phaseolus vulgaris* L. cv.white (White kidney bean) from local source, Hilla, Babylon, Iraq, human red blood cells (healthy persons), ammonium sulfate, DEAE cellulose, sephadex G-200, PAGE, electrophoretic reagents were purchased from Sigma (USA), 5mMphenylmethylsulfonyl fluoride (PMSF), 5, 5-dithiobis- (2 nitrobenzoic acid (DTNB), NaBH4, Nbromosuccinimide (NBS) were obtained from Himedia (India), BSA, alkaline phophatase, RNase and Trypsin obtained from Biobasic .

### 2.2. Methods

*2.2.1. Isolation of Phaseolus vulgaris lectin*

By using, the soaking Method white kidney beans were ground to a powder in filtered through 80-mesh grit. The powder (5 g) was mixed with 0.15M NaCl (1:8, w/v) for 48 h at $4^0$C, and filtered through 80-mesh grid. Subsequently, the filtrate was centrifuged at 9168×g for 30 minutes, and the supernatant was fractionally precipitated with ammonium sulfate at 10%-100% saturation, respectively. The four pellets were combined, dissolved in a minimal volume of water, and dialyzed against distilled water at 4°C (Yufang et al., 2010).

*2.2.2. Determination of protein concentration.*

Determination of Protein Concentration. Bradford′s method (Bradford, 1976) was used for protein quantification, using bovine serum albumin (BSA) as the standard.

*2.2.3. Hemagglutinating activity assay.*

Serial two-fold dilutions of the lectin solution in microtiter v-plates (25 µL) was mixed with 25 µL 2% human red blood cell suspension in saline (pH 7.2). Readings were recorded after about 30 minutes at room temperature, when the blank had fully sedimented. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was treated as one hemagglutination unit. Specific activity was expressed as the number of hemagglutination units per mg protein (Yufang et al., 2010).

*2.2.4. Purification of Phaseolus vulgaris L. cv.white lectin (White Kidney Bean).*

Purification of white kidney beans lectin firstly at precipitation by ammonium sulfate at 10%-100% saturation rate to Crude extract from the soaking and then dialyzed against distilled water,then loaded on a DEAE cellulose column (2.6 cm × 60 cm) that had been equilibrated with the same buffer, and subjected to ion exchange chromatography. The column was washed initially with 0.1M NaCl in 0.02M Tris-HCl (pH 8.0) to remove proteins that had not specifically absorbed to the column, then washed with linear salt gradient elution. Fractions showing hemagglutinating activity were further purified by sieve chromatography on a Sephadex G-200 column in 0.15M NaCl in 0.02 M Tris-HCl (pH 8.0).

*2.2.5. SDS-PAGE.*

SDS-PAGE. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in accordance with the method of Laemmli (Laemmli, et al., 1973) using a 15% separating and a 10% stacking gel.

*2.2.6. Molecular mass determination.*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in accordance with the procedure of Laemmli and Favre (Laemmli, et al., 1973). The molecular mass of the lectin was estimated from the standard curve plotting electrophoretic mobility against molecular mass. Gel filtration was carried out using a Sephadex G-200 column that had been calibrated with molecular-mass standards proteins (BSA, Alkaline phophatase, RNase and Trypsin) to estimate the molecular mass of the purified lectin.

*2.2.7. Sugar specificity.*

The Sugar specificity investigate inhibition of lectin-induced hemagglutination by various carbohydrates were performed in a manner analogous to the hemagglutination test. Prepare 1-100 mM of each sugar samples was prepared

in phosphate-buffered saline. All of the sugar samples were mixed with an equal volume (25 µL) of a solution of the lectin. The mixture was allowed to stand for 30 minutes at room temperature and then mixed with 50 µL of a 2% human erythrocyte suspension. The known sugar which gives agglutination with blood suspension and no precipitate of red blood cell that means the tested lectin specific to that sugar type (Wang et al., 2000).

*2.2.8. Effect of temperature on purified lectin-induced hemagglutination.*

The effect of temperature on hemagglutinating activity of the purified lectin was examined as previously described (Wang et al.., 2003). A solution of the lectin was incubated at various temperatures for 30 minutes: 0°C, 10°C, 20°C, 30°C, 40°C, 50°C, 60°C and 70°C. The tubes were then put on ice, and assay of hemagglutinating activity was then carried out.

*2.2.9. Effect of pH on purified lectin-induced hemagglutination.*

The pH stability of the lectin was determined by incubation of the lectin (1 mg/mL) in buffers of different pH values ranging from pH 4.0–12.0 for 60 minutes. The pH of the lectin solution was adjusted to 7.0 by the addition of 0.1N HCl or 0.1N NaOH before hemagglutination activity was determined (Yufang et al., 2010).

*2.2.10. Effect of chemical modification of amino acid residues on hemagglutinating activity.*

For serine modification, the lectin (100 µg) in 0.1mL of 50mM Tris-HCl buffer (pH 7.4) was incubated with 5mMphenylmethylsulfonyl fluoride (PMSF) at 27°C for 1 hour (Habeeb, 1966). Aliquots were removed at 15 minutes intervals.followed by determination of residual hemagglutinating activity. Lectin incubated without PMSF served as a control.

Reduction of the thiol groups of white kidney beans lectin was carried out by incubating the lectin (100 µg) in 0.1mL of 50mM phosphate buffer (pH 8.0) with 0.1mM 5, 5-dithiobis- (2-nitrobenzoic acid (DTNB) at 27°C for 1 hour. Aliquots were removed at different time intervals, followed by determination of residual hemagglutinating activity. Lectin incubated in the absence of DTNB served as a control (Fraenkel, 1957).

For lysine modification, 0.5 mg of NaBH4 was added to the lectin (5 mg) in 2mL of 0.2M sodium borate buffer (pH 9.0) at 4°C, followed by six aliquots (5 µL each) of 3.5% formaldehyde at 10 minutes intervals. Excess reagent was removed by ultrafiltration. Lectin incubated in the absence of sodium borohydride (NaBH4) served as a control (Mean & Feeney, 1968).

Modification of tryptophan residues was carried out according to the method of Spande and Witkop (Spande & Witkop, 2006). The lectin was dissolved in NaOAc buffer (0.1 M, pH 5.0) to 1mg/mL. The modification was carried out at 20°C. Nbromosuccinimide (NBS) (10 µL, 10mM) was added every 5 minutes and then assay of hemagglutinating activity was carried out. Lectin incubated in the absence of NBS served as a control.

## RESULTS AND DISCUSSIONS

Soaking Method showed spicfic activity about 7.1 titer/mg of white kidney beans. The protein concentration was calculated based on the regression equation (Figure 1), and results showed protein concentration about 2.74 mg/ml by precipitation by ammonium sulfate at 70% saturation rate. Purification of white kidney beans lectin show two fractions were obtained from ion exchange chromatography with DEAE cellulose (Figure 2) just the first peak showed hemagglutination activity about 45.5 titer/mg,so just the first peak concentrated and loaded on sephadex G-200 to gel filtration step which show only one peak with hemagglutination activity about 57.07 titer/mg (Figure 3), the volume obtained from peak of sephadex G-200 was concentrated and reloaded on sephadex G-200 to improved the purification step of lectin and the result of second loading of white kidney beans lectin on sephadex G-200 is also one peak show specific activity as 64 titer/mg (table 2). The peak of second loading of gel filtration was dialyzed and concentrated, then stored at-20°C. The purified lectin (fraction I in Figure 3) formed a single band with a molecular mass of about 33 kDa in SDS-PAGE electrophoresis and three bands was showed in results of ione exchange electrophoresis (Figure 4), which confirmed the effectiveness of the purification method used and about 35.5 kDa in gel filtration method to determination molecular mass.

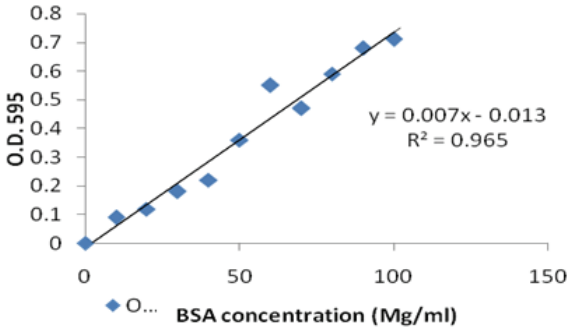



Figure 1. Standard curve of bovine serum albumin

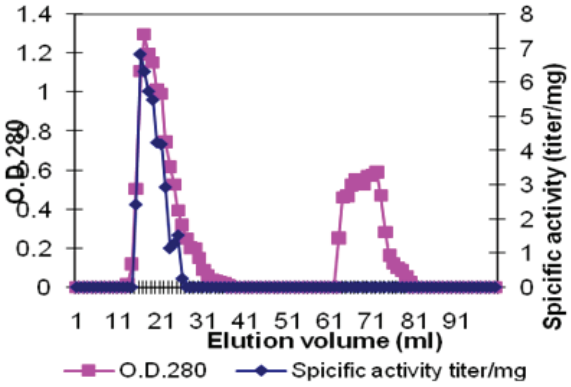



Figure 2. Fractionation of the crude extract of Phaseolus vulgaris L. cv.white bean lectin by DEAE cellulose ion-exchange chromatography equilibrated with 0.1M NaCl in 0.02M Tris-HCl (pH 8.0). The column was washed initially with the same buffer to remove proteins that were not specifically absorbed to the column (data not shown on the figure) then washed with buffer in which the NaCl concentration increased linearly from 0.1M to 1.0M. Tow peaks were obtained, among which peak I exhibited hemagglutinating activity. Column: 2.5 cm × 50 cm; flow rate: 0.3 mL/min.

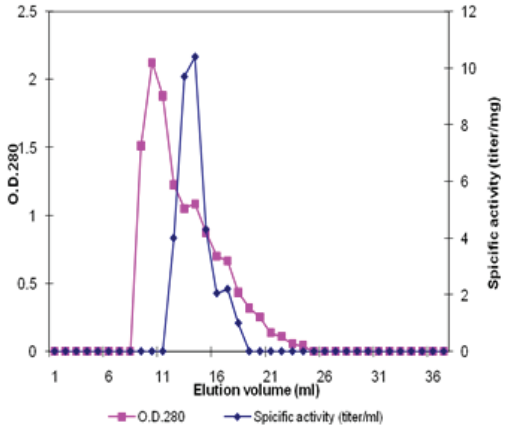



Figure 3. Fractionation of peak I on a Sephadex G-200 column with 0.15M NaCl in 0.02M Tris-HCl (pH 8.0). Column: 2 cm × 50 cm; flow rate: 0.5 mL/min. Only the major peakv exhibited hemagglutinating activity

The hemagglutinating activity of purified white kidney beans lectin could not be inhibited by many of the simple sugars tested at 1−100 mM of D (+) glucose, D (+) galactose, D (+) fructose, D (+) lactose, (+) ribose, D (+) arabinose, L (+) maltose, D (+) sucrose and N-acetylglucoseamine, but the hemagglutinating activity inhibited with D (+) xylose and D (+) mannose as show in figure (Figure 5).

The hemagglutinating activity was completely stable between 0°C and 50°C. Considerable loss in activity occurred at 60°C. Some activity was discernible at 60°C (Figure 6a). The lectin exhibited remarkable stability over the range of pH 4−10 and loss the activity at pH 11 (Figure 6b).

Residues, respectively, did not play any important role in its hemagglutinating activity. However, 85% loss of hemagglutinating activity after NBS treatment was noted, whereas no change in the control was detected. These results strongly suggest a considerable involvement of tryptophan residues in hemagglutinating activity, and stability of the lectin. NaBH4 treatment resulted in also 86% loss in hemagglutination activity suggesting partial involvement of lysine in the lectin activity. A concomitant drop in lectin activity was clearly seen upon modification of tryptophan residues.

The effects of various types of chemical modifications on hemagglutinating activity of the purified lectin are summarized in Table1, DTNB, reductive methylation and PMSF treatments did not produce any alterations in the hemagglutinating activity of white kidney beans lectin, suggesting that cysteine, cystine and serine

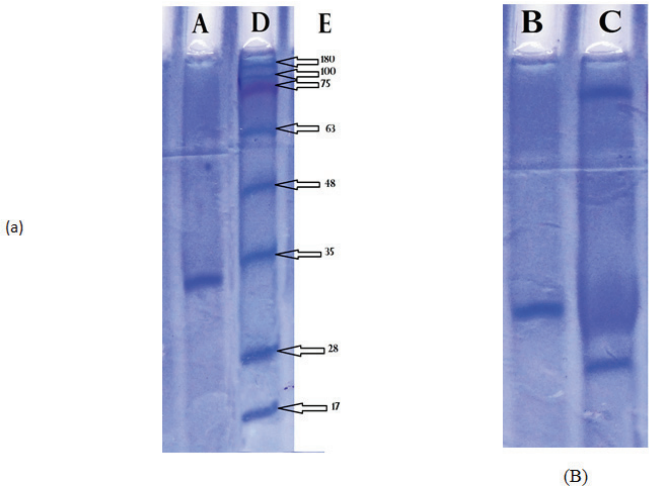



Figure 4. (a) Molecular weight determination of purified Phaseolus vulgaris L. cv.white bean lectin in SDS-PAGE electrophoresis. Line A: purified lectin. Line D: protein ladder line E: molecular weights (b) purification of lectin. Line C: crude lectin. Line B: purified lectine by gel filtration.

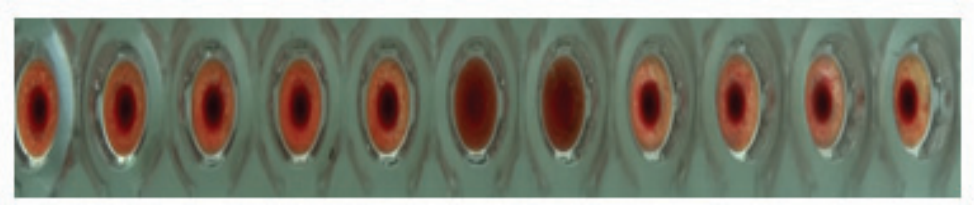
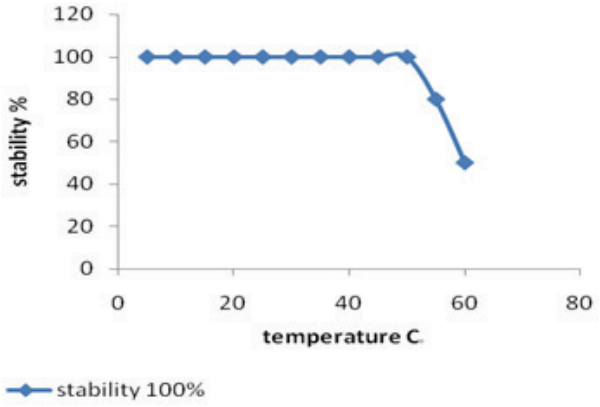


Figure 5. Sugar specificity of Phaseolus vulgaris L. cv.white bean lectin image showed the agglutination of red blood cell with (Glucose galactose lactose ribose arabinose xylose mannose fructose maltose sucrose Nacetylglucoseamine, respectively) xylose and mannose mean the tested lectine not specific for that simple sugar.



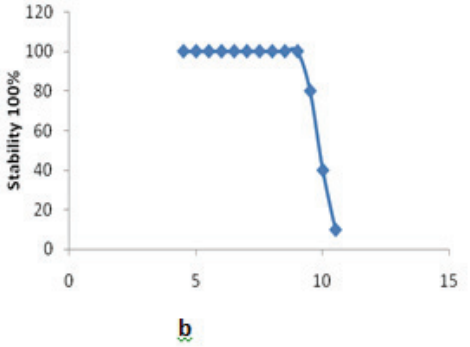




Figure 6. (a) Effect of temperature on hemagglutinating activity of Phaseolus vulgaris L. cv.white bean lectin. (b) Effect of pH on hemagglutinating activity of Phaseolus vulgaris L. cv.white bean lectin.

| Treatment | Modified group/amino acid | % Hemagglutinating activity remaining |
|---|---|---|
| Phenylmethylsulfonyl fluoride (PMSF) | Serine | 100 |
| 5, 5-Dithiobis-(2-nitrobenzoic acid(DTNB) | Thiol group | 100 |
| Reductive methylation | Lysine | 14.2 |
| N-Bromosuccinimide (NBS) | Tryptophan | 14.9 |

Figure 7. Effect of chemical modification on hemagglutinating activity of *Phaseolus vulgaris L.* cv.white beans lectin.

| Sample | H. U. | Total protein mg | Total activity titer | Specific activity Titer/mg | Fold | Yield % |
|---|---|---|---|---|---|---|
| Crude extract | 6 | 27.4 | 192 | 7.1 | 1 | 100 |
| Precipitation by ammonium sulfate at 70% saturation rate | 12 | 65.3 | 24567 | 37.61 | 5.29 | 128 |
| Ion exchange purification step | 8 | 22.5 | 1024 | 45.5 | 6.41 | 5.3 |
| Gel filtration purification step1 | 7 | 7.85 | 448 | 57.07 | 8.03 | 2.3 |
| Gel filtration purification step2 | 7 | 7 | 448 | 64 | 9.01 | 2.3 |

Figure 8. Purification table

Lectins possess many bioactivities that have important practical applications, but the high price resulting from the low extraction rate restricts practical application of lectins. In this study, a plant lectin has been purified by three-step chromatography from seeds of the white kidney beans. The homogeneity of the white kidney beans lectin preparation was evidenced by the presence of a single band in SDS-PAGE. The results of SDS-PAGE and gel filtration chromatography together revealed that the lectin exists as a monomer of one subunit. The molecularmass and monomeric nature of Phaseolus vulgaris L. cv.white lectin are similar to those of Anasazi bean lectin and most of the other Phaseolus lectins (Tsang et al., 2001; Reynoso et al., 2003). On the other hand, it differs from a tetrameric 115–120 kDa lectin from tepary bean (Phaseolus acutifolius) (Reynoso et al., 2003). Phaseolus acutifolius var. latifolius lectin from which consists of four subunits of 21 kDa molecular mass (Vargas et al., 1987), and a tetrameric 94 kDa immunosuppressive lectin isolated from seeds of Phaseolus vulgaris L. cv. Cacahuate. (Vargas et al., 1993).

Lectins from some cultivars of Phaseolus vulgaris are oligomeric (Felsted et al., 1977), whereas Phaseolus vulgaris bean lectin is dimeric. Isolectins are absent in Phaseolus vulgaris L. cv.white beans but present in some cultivars of P. vulgaris such as red kidney bean (Felsted et al., 1977; Leavitt et al., 1977). The isolectins differ from one another by the number of erythrocytereactive (E) subunits and lymphotcyte-reactive (L) subunits that they possess. There are five such isolectins: L4, L3E1, L2E2, L1E3, and E4 (Yufang et al., 2010).

Although there is striking homology between white kidney beans lectin and other Phaseolus lectins in N-terminal sequence, white kidney beans lectin exhibit of some simple sugar specificity. Simple sugars as D (+) glucose, D (+) galactose, D (+) fructose, D (+) lactose, (+) ribose, D (+) arabinose, L (+) maltose, D (+) sucrose and N-acetylglucoseamine are not able to inhibit the hemagglutinating activity of white kidney beans.

White kidney beans lectin is fairly thermostable because its hemagglutinating activity is stable at temperatures up to 40°C, and is reduced only at 50°C. Interestingly, some activity remained even after heating at 60°C for 30 minutes. However, have been shown to lose activity beyond 50°C in a temperature-dependent manner (Laemmli, et al., 1973; Wang et al., 2003). The lectin shows remarkable pH stability, its activity being unaffected throughout the entire range of pH from 4 to 10. This is in contrast to lectin from Parkia javanica beans which is stable in pH 7–10 (Utarabhand & Akkayanont, 1995).

Chemical modification studies were carried out to investigate the role of specific amino acids in the hemagglutinating activity of white kidney beans lectin. The results disclose that tryptophan and lysine are important to the hemagglutinating activity, the contribution of tryptophan being more important. Previous studies have reported that lysine, tyrosine, and tryptophan (e.g., in Dolichos lab-lab bean) (Nadimpalli, 1999), or tryptophan alone are indispensable for the hemagglutinating activity of some legume lectins (Das, 1995). Specific amino acids may be involved in either direct interaction with the sugar or may have a role in maintaining conformation of the sugar binding pocket, and hence contribute to the hemagglutinating activity of lectins (Ba? i? imiev et al., 2007).

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# EXPERIMENTAL RESULTS ABOUT POTATO CALLUS INDUCTION

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***Abstract***

*The callus is an unorganized mass of parenchymal proliferate cells that through cultivation, forming groups of meristematic cells, elements of leading system, pigmented cells, etc.. Using other explants than meristem, for regeneration neoplantlets require mandatory completion of a stage of callus culture. To obtain callus is need an agarose to support the cellular mass in growth. In 2012, at Brasov was fitted trifactorial experience, in which two clones of Christian variety were studied, 6 media for callus induction and 2 explants sources consisting of leaf disc and petiole segment. The following results were obtained: medium, explant source (foliar disc, petiole segment) and variety have different influences on callus proliferation. The callus explants responded better to the foliar disk (72.5%) than petiole segments (40%). Media containing 3 mg / l 2,4-D and 3 mg / l BAP x 3 mg / l 2,4-D favored callus induction rate of 90%. Differences obtained by using BAP citokinine are statistically assured, very significant, negative compared to 2,4-D auxine (2mg/l- concentration regarded as control), by -3.5 explants / that induced callus. The callus which was obtained will be used for plantlets regeneration and to identify any somaclonal variation.*

***Keywords:*** *leaf disc, petiole segment, callus.*

## INTRODUCTION

Callus is an unorganized mass by proliferate parenchymal cells that through cultivation, form centre of meristematic cells, elements of system leading, pigmented cells (Rodica Pop, 2008).

Callus is a particular formation consisting by an undetermined mass of cell holding a uniform histological structure. Usually, when it is young, undifferentiated cells possess, actively dividing.

Friable as possible callus induction its principal purpose when aiming is initiation of cell culture and from this a culture of protoplast.

Callus growth is considered to be indefinite, because it can be multiplied and grown indefinitely when periodically is done subculturing, respectively fragmentation of it.

Callus is often associated with somaclonal variability, variations derived from callus being called caliclones (Skirvin et al., 1976, quoted by Skirvin, 1994).

At the beginning cultivars obtained „in vitro", regardless of species, were called caliclones. Variation is associated, in caliclones case with direct regeneration from callus or cell suspension and not with micropropagation or meristem culture (Karp, 1989).

Somaclonal variation, a common phenomenon in tissue culture includes all variations and derived from all tissue culture (Skirvin et al., 1993). Somaclonal variation is also called tissue variation or culture-induced (Kaeppler, et al., 2000).

Somaclonal variation control represents a challenge (Amirato, 1991). Variations had as result: changes in structure and / or number of chromosomes, a gene changes as a result of structural change in chromosome (Rao *et al*., 1992; Kaeppler, *et al*., 2000). Evans & Sharp (1988) reported four critical variables for somaclonal variation: genotype, explant origin, period of cultivation and culture conditions.

Plant genotype can have significant effects on somaclones regeneration. These effects are very obvious for potatoes: differences are observed in the number of plants regenerated from cultivars grown under identical conditions (Gunn & Shepard, 1981). Explant source is considered most essential variable in somaclonal variation.

Because explants may present different stages of regeneration, selection procedures may differs between different types of explants (De Jong & Custers, 1986).

## MATERIALS AND METHODS

In laboratory Plant Tissue Cultures of NIRDPSB Brasov was assembled an experience that followed the influence of growth regulators and explants of Christian variety (two clones).

The experience was by type 2x2x6, made by combining three experimental factors, placed by Latin rectangle method, the number of studied variants was 24, set in three repetitions, and on each experimental variant were inoculated 5 explants.

- Experimental factor A-clone with two graduations:
- $a_1$- Christian Cl2
- $a_2$- Christian CHR 01 ROU
- Experimental factor B - explants, with two graduations:
- $b_1$- leaf discs;
- $b_2$- petiole segments.
- Experimental factor C - nutrition environment, with six graduations:
- $c_1$ - MS medium and citokinine BAP (2 mg/l);
- $c_2$ - MS medium and citokinine BAP (3 mg/l);
- $c_3$ - MS medium and auxine 2,4-D (2 mg/l);
- $c_4$ - MS medium and auxine 2,4-D (3 mg/l);
- $c_5$ - MS medium and BAP x 2,4-D (2 mg/l x 2 mg/l);
- $c_6$ - MS medium and BAP x 2,4-D (3 mg/l x 3 mg/l).

The proposed objective of this research is to determine the influence of hormonal composition of the culture medium on callus induction of different types of potato explants grown *in vitro*.

Experience in which six medium were used to induce callus was organized in the laboratory on culture vessels, aiming on callus process from leaf discs and petiole segments.

Experimental conditions:

The experience was mounted in the laboratory using conditions required by „in vitro" technology; experimental conditions were those specific to growth chamber of plantlets, provided in the working protocol, sterilization of culture vessels was performed in a drying chamber at $180^0$C and culture media was sterilized by autoclaving at $121^0$C for 20 minutes at pressure of 1.25 atmospheres.

Cultures were transferred to growth chamber under conditions of darkness; after crossing this period light regime is 4000 lux, with a period of 16 hours light and 8 hours dark at a temperature of $20^0$C.

Observations concerning callus growth were made weekly. Also, all sampling operations, inoculation subculturing were performed under sterile conditions in a laminar flow hood.

The biological material used in the production experiences of clonal variability in laboratory conditions, consisted of two clones of the same variety (Christian) from NIRDPSB Brasov. All analyzes were performed in the laboratory plant tissue cultures from NIRDPSB Brasov.

Other materials used in the experiments:

-to initiate callus culture, the biological material used was represented by two types of explants (leaf discs of 1 $cm^2$ and petiole segments of 1.5-2 cm).

Explant source:

When intended to make somaclonal variability for a new species or cultivar is better to use several types of explants and compare descendants from each. Not to all types of explants are supposed the same capacity for manifestation of variation. In general the variation is more difficult to observe in preformed shoots (derived from axillary buds, shoot tips and meristems), unless meristem explants were not preformed, such as leaves, roots or protoplasts (Rodica Pop, 2008).

Regenerates obtained from organized explants with preformed meristems are genetically stable. For many plant species, cell differentiation is accompanied by qualitative and quantitative changes of genomic DNA. Therefore, when used as explants for callus obtained segments from leaf or root, cells begin to divide (Rodica Pop, 2008).

The culture media used:

To obtain callus is need by a medium with agarose to support cell mass in growth. Periodically, callus must be fragmented and subcultivated in order not enter into senescence. Callus formation can be induced and it proliferates, especially on culture media with 2,4-D. Also, other growth regulators: auxine or citokinine who are in high concentrations in the culture medium can generate callus.

Vegetal explants can be kept alive by detaching their mother's body by growing and raising on aseptic media with a complex chemical composition. The success *in vitro* cultivation of

vegetal explants depends very much on achievement of nutritional composition that best suits with vital requirements of cultured tissues. One of the most common culture media used Murashige-Skoog is (MS) (Rodica Pop, 2008).
In our experience, basic medium used was Murashige-Skoog (1962) supplemented with different growth regulators. As a source of carbohydrates sucrose was used at a concentration of 2% and that phytoagar gelling agent was used in concentration of 0.9%.
Growth regulators used:
Growth regulators are organic compounds other than nutritive substances that in small amounts stimulate, inhibit or modify physiological processes in plants (Rodica Pop, 2008). Growth regulators, particularly 2,4-D (dichlorophenoxyacetic acid 2.4) and benzylaminopurine (BAP), are involved in inducing variability but their direct relation with this phenomenon is still discussed.
Auxins
Auxins are natural compounds that, in small doses, directly or indirectly, can stimulate the growth and development of plants, or forming of vegetative organs and regenerative.
Between auxines, 2,4-dichlorophenoxyacetic acid (2,4-D), in concentrations ranging from 0.5 to 2.0 mg / l, has proven very efficient in callus induction and maintenance of various types of somatic tissues (Tang şi Mullins, 1990 ; Castillo şi colab., 1998, quotation by Rodica Pop, 2008).
Citokinins
Citochinins are substituted with a purine nucleus. They stimulate cell division and have important role in stimulating vegetal cells mature unmeristematic (Cachiţă, 2000).
Caulogenesis is stimulated by the presence in culture substrate of a particular ratio between auxine and citokinine.
Specific technology applied in experiments
From existing material from greenhouse during plant vegetation, aerial plant samples (leaves and stems) were harvested manually which constituted the biological material under experimentation. It was followed the influence of genotype on callus induction and influence of different medium variables used
It was respected the following stages: vegetative material sterilization it requires a few minutes washing, sterilization with sodium hiploclorit - 10 minutes, after which explants are rinsed with doubly distilled water; culture media preparing; sterilization of vessels and growing medium; preparing of plant material for explants sizing, inoculation, incubation either at dark or light, usually at $24^0$C, regular observation and passing callus fragments (about 4-6 weeks) in fresh medium.
Explants were represented by foliar disc (1 $cm^2$) and petiole segments of 1.5 - 2 cm in length (Fig. 1), taken from 4 potato genotypes. After performing disinfection (Figure2), biological material was transferred to Erlenmeyer flask (Fig. 3) on a basic medium: Murashige-Skoog (1962) enriched with vitamins, 20 g / l sucrose, 9 g / l agar and different growth regulators, different concentrations.

Figure 1. Fragment of leaf and petiole segment

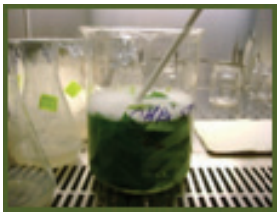

Figure 2. Sterilization of explants

Figure 3. Inoculated explants for callus induction

After incubation of leaf and petiole segment in the dark for two weeks, were registered some features. These include:- Type of callus and callus color:
After callus was initiated (Fig. 4), at about 4-6 weeks, from each recipient was fragmented into three segments and subcultivat on the same type of medium (Fig. 5). Callus was then exposed to UV radiation (Fig. 6), operation repeated three times at an interval of one week.

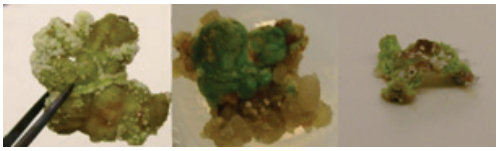

Figure 4. Initiated callus

Figure 5. Recipents with callus fragmented

Figure 6. The exposure callus on UV radiation

## RESULTS AND DISCUSSIONS

Analyzing Table 1, we see that when using citokinine BAP, callus induction did not take place. In the case of auxine 2,4-D callus was brittle, with a brownish color, and if the used medium contained both growth regulators, callus was hard, its color being green.

Clones explants of Christian variety were cultured on MS medium containing different concentrations of 2,4-D, BAP or in combination. Explants which produced callus were analyzed at 6 weeks, and the results show that there is a wide variation in the percentage of explants which initiated callus, callus texture, color callus, it depends on the culture.

It was found a positive response of leaf fragments and auxine 2,4-D and its combination with citokinine BAP in callus proliferation. Reaction manifested callus after 8 weeks after inoculation.

Medium did not contain 2,4-D (which contained the BAP 2mg, 3mg respectively - media c1 and c2) did not produce callus induction (0%).

Leaf segments and internode segments explants were cultured on MS medium containing different concentrations of 2,4-D, BAP and combinations thereof.

Table 1. Effects of substances 2,4-D and BAP on callus induction

| Growth regulator (mg/l) | | Callus texture | Color callus |
|---|---|---|---|
| 2,4-D | BAP | | |
| 0 | 2 | - | - |
| 0 | 3 | - | - |
| 2 | 0 | friable | brownish |
| 3 | 0 | friable | brownish |
| 2 | 2 | strong | green |
| 3 | 3 | strong | green |

Table 2. Influence of variety and medium in the callus culture

| Explant source, b | Culture medium, c | Callus induction/variety (clones) (%) | | Average % variety Christian |
|---|---|---|---|---|
| | | CHRISTIAN Cl 2 | CHRISTIAN CHR 01 ROU | |
| Fragment of leaf | $c_3$ | 100 | 40 | 70 |
| | $c_4$ | 80 | 100 | 90 |
| | $c_5$ | 60 | 20 | 40 |
| | $c_6$ | 100 | 80 | 90 |
| Average | | 85 | 60 | **72,5** |
| Segment of petiole | $c_4$ | 40 | 40 | 40 |

Note:
c3= Medium MS – 2 mg/l 2,4-D
c4= Medium MS – 3 mg/l 2,4-D
c5= Medium MS - 2 mg/l BAPx2 mg/l 2,4-D
c6= Medium MS - 3 mg/l BAPx3 mg/l 2,4-D

From Table 2, it can be noted the superiority of the Christian variety clone 2 of the callus, in case of disc explant foliar (85%).

About the influence of culture medium on callus, it appears that for clone 2, using media containing: 2.0 mg / l 2,4-D, and 3 mg / l BAP x 3 mg / l 2.4 -D is achieved highest percentage of callus (100%), followed by media containing 3 mg / l 2,4-D and 2 mg / l BAP x 2 mg / l 2,4-D (80% and 60%).

For CHR 01 ROU clone, of Christian variety, the best results were registered in callus induction on c4 medium using, leading to callus proliferation at 100%.

On the variety, the best results were registered using c4 and c6 the media, achieving a rate of 90%.

In case of using petiole segment, the callus induction had low intensity of only 40% (average of two clones), only at variants that experienced the quantity of 3 mg 2,4-D/l; the remaining samples were affected by infections.

Statistical analysis of the influence of growth regulators on callus induction shows that all

data derived from experimentation showed statistically differences. Considering auxine 2,4-D (the quantity of 2mg / l) control, the results showed significant increases in the number of explants (1 explant) for citokinine BAP (in the case of both concentrations) decreases of explants numbers are significant of -3.5 explants; the combination of 2,4-D * BAP (2mg / l) are distinct differences significant negative (-1.5 explants) and for 2,4-D * BAP (3 mg / l) increases of explants number are significantly positive (1explant), resulting that induction of callus was directly influenced by growth regulator and quantity of this (Table 3).

Table 3. Influence of growth regulators used in the callus induction

| Growth regulator used | Number of explants that induced callus | Procent (%) | Differences (explants number) | Signification |
|---|---|---|---|---|
| 2,4-D (2 mg) (ct) | 3,5 | 100.00 | - | - |
| 2,4-D (3 mg) | 4,5 | 128.57 | 1 | * |
| BAP (2 mg) | 0 | 0 | -3.5 | ooo |
| BAP (3 mg) | 0 | 0 | -3.5 | ooo |
| 2,4-D*BAP (2 mg) | 2 | 57.14 | -1.5 | oo |
| 2,4-D*BAP (3 mg) | 4,5 | 128.57 | 1 | * |

DL 5% =0.80 (explants)
DL 1% =1.13 (explants)
DL 0,1% =1.64 (explants)

## CONCLUSIONS

Medium, explant surse (foliar disc, petiol segmente) and variety have different influences in callus proliferation.

The callus induction responded better to explants from foliar disk (72.5%) than petiole segments (40%).

C4 (3 mg / l 2,4-D) and c6 (3 mg / l BAP x 3 mg / l 2,4-D) media favored callus induction in proportion of 90

Differences obtained using BAP citokinine are statistically very significant negative towards auxine, 2,4-D (concentration of 2mg/l-considered control), -3.5 explants / that induced callus.

Medium that contained 3 mg / l 2,4-D * BAP determined obtaining an increase in the number of expants that induced callus (1 explant).

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# A STOCHASTIC EPIDEMIC MODELFOR DYNAMIC OF INFECTIOUS DISEASES

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***Abstract***

*After a short introduction of the deterministic SIS and SIR models, we present three types of stochastic epidemic models: discrete times Markov chain (DTMC) model, continuous times Markov chain (CTMC) model and stochastic differential equation (SDE) model. We discuss a stochastic epidemic model for dynamic of infectious diseases with variable population size, one which varies according to some population growth laws. Finally, we compare the stochastic differential equation of a SIS epidemic model having a constant population size with a stochastic differential equation having a variable population size.*

***Keywords*****:** *model basic reproduction number, discrete times Markov chain (DTMC) model, continuous times Markov chain (CTMC) model and stochastic differential equation (SDE) model*

## INTRODUCTION

The beginnings of mathematical applications in epidemiology relate to the "smallpox" model, by Daniel Bernoulli (1760) and a primary theory was developed between 1900-1935. The research continued and recent progresses recorded in our days, a good example being "The SARS" (Severe Acute Respiratory Syndrome) epidemic (2002-2003). Establishing forecasts on the evolution of infectious disease and the comparison of different control methods maintain at a high level the interest of researchers in mathematical modeling of epidemiology.

## MATERIAL AND METHOD

### 1. The deterministic SIS and SIR Epidemic Models

Most epidemic models are based on the dividing of target population into a small number of compartments, each of them containing members that are identical in terms of their relationship towards a certain disease.

In a SIR model, the population is divided into three groups:

a) **S - susceptible**: individuals who don't have immunity to infectious agents and who have been exposed to the disease contact;
b) **I - infected**: individuals who have been recently infected and who transmit the infection to susceptible individuals who are in contact with them;
**c) R- removed**: individuals who are immune to the infection and who are not contaminated even if they come in contact with those categories a), b).

An example of SIR Epidemic Model is represented by childhood diseases.

If we denote $S(t), I(t), R(t)$, the number of individuals in each category in relation to time, the total population will be:

$$N(t) = S(t) + I(t) + R(t) \tag{1}$$

We observe that $S(t), I(t), R(t) \in N$ and for a sufficiently large volume of the total population, they are continuous random variables, their variation is characterized by the following system of differential equations:

$$\begin{cases} \dfrac{dS}{dt} = -\dfrac{\beta}{N}\cdot S\cdot I + b\left(I+R\right) \\ \dfrac{dI}{dt} = \dfrac{\beta}{N}\cdot S\cdot I - \left(b+\gamma\right)\cdot I \\ \dfrac{dR}{dt} = \gamma\cdot I - b\cdot R \end{cases} \quad (2)$$

where $\beta > 0$ is the transmission rate, $\gamma > 0$ is the recover rate and $b \geq 0$ is the birth rate.

The initial solution of the system (1) is:

$$S(0) > 0, I(0) > 0, R(0) \geq 0, \quad S(0) + I(0) + R(0) = N \quad (3)$$

where $N(0) = N$ is the population volume at the initial moment, when it downgraded the epidemic.

**Observation:**

If we note $c$, the death rate, we assume that in a SIR model, $b = c$ i.e. the birth rate is equal with the death rate, then the population volume is constant with respect to time, and $\dfrac{dN}{dt} = 0$.

Using the **basic reproduction number**, $R_0 = \dfrac{\beta}{b+\gamma}$, which represents the number of secondary infections caused by some infected individuals in the entire susceptible population (the fraction $\dfrac{1}{b+\gamma}$ is the interval of infection relative to deaths and recovery rate ) the authorsJ. Mena-Lorca, H.W. Hethcote [6] characterize the system solution (2), in the following theorem:

**Theorem 1**

If $S(t), I(t), R(t)$ is the system solution (2), then:

a) for $R_0 \leq 1$, $\lim\limits_{t\to\infty} I(t) = 0$ (disease-free equilibrium)

$R_0 > 1$, $\lim\limits_{t\to\infty}(S(t), I(t), R(t)) =$

b) for $\left(\dfrac{N}{R_0}, \dfrac{b\cdot N}{b+\gamma}\left(1-\dfrac{1}{R_0}\right), \dfrac{\gamma\cdot N}{b+\gamma}\left(1-\dfrac{1}{R_0}\right)\right)$ (endemic equilibrium)

c) for $b = 0$ and $R_0 \cdot \dfrac{S(0)}{N} > 1$, there is an initial increase of the number of infected cases $I(t)$ and if $R_0 \cdot \dfrac{S(0)}{N} \leq 1$, then $I(t)$ monotone decreasing.

In a SIS Epidemic Model, a susceptible individual, after contact with an infected person in his turn becomes infected or infectious, but he doesn't develop immunity, i.e., after recovery, he returns in the susceptible category, so in such a model, the population is divided only into two categories: S-susceptible and I-infectious.

An example of a SIS Epidemic Model is represented sexually transmitted diseases (STDs).

With the same notations as in the SIR epidemic model and if we assume that all individuals are born susceptible and we don't find the deaths caused by that epidemic, the dynamics of the SIS epidemic model is described by the following system of differential equations:

$$\begin{cases} \dfrac{dS}{dt} = -\dfrac{\beta}{N}\cdot S\cdot I + \left(b+\gamma\right)\cdot I \\ \dfrac{dI}{dt} = \dfrac{\beta}{N}\cdot S\cdot I - \left(b+\gamma\right)\cdot I \end{cases} \quad (4)$$

In this case, the population size is:

$$N(t) = S(t) + I(t) \quad (5)$$

**Observation**:

If we assume as in the case of the SIR model $b = c$, then in a SIS epidemic model the population size is constant with respect to time, i.e. $\dfrac{dN}{dt} = 0$. The following theorem [6] characterizes the system dynamics model of differential equations (4) in relation to the variation of the basic reproduction number:

**Theorem 2**

If $S(t), I(t)$ is the system solution (4), then:

a) for $R_0 \leq 1$, $\lim\limits_{t\to\infty}\left(S(t), I(t)\right) = (N, 0)$ (disease-free equilibrium)

b) for $R_0 > 1$, $\lim\limits_{t\to\infty}(S(t), I(t)) = \left(\dfrac{N}{R_0}, N\cdot\left(1-\dfrac{1}{R_0}\right)\right)$ (endemic equilibrium)

**Observation:**

The interpretation of the first statements of the theorem is that if the number of secondary infections generated by the infected individuals is less than 1, then, $I(t) \to 0$ si $S(t) \to N$.

**2. The Stochastic Epidemic Models**

In this section we present three types of stochastic modeling processes: (1) a discrete time Markov chain (DTMC) model, (2) a continuous time

Markov chain (CTMC) model, and (3) a stochastic differential equation (SDE) model. The differences between these processes refer to time and to the set of states. In DTMC model, the time and the state are random discrete variables. In a CTMC model, time is continuous, but the state variable is discrete, finally, the SDE model is based on a diffusion process, where both the time and the state variables are continuous. One of the most important differences between the deterministic and stochastic epidemic models is their asymptotic dynamics. Eventually stochastic solutions (sample paths) converge to the disease-free state even though the corresponding deterministic solution converges to an endemic equilibrium.

### 2.1. Discrete Time Markov Chain Epidemic Models (DTMC)

Let on consider $S(t), I(t), R(t)$ random variables representing the number of individuals susceptible, infected, respectively immune to the time *t,* in relation to a specific infectious agent.

In a DTMC epidemic model, $t \in T = \{0, \Delta t, 2\Delta t, ...\}$ and the discrete variables $S(t), I(t), R(t) \in \{0,1,...N\}$.

Further, we refer to a SIS Epidemic Model. We consider that $N =$ the population size is constant and $I(t)$ is independent random variable. Then $S(t) = N - I(t)$ and the stochastic process $\{I(t)\}_{t \in T}$ has the probability function

$$p_i(t) = P(I(t) = i), \quad i = \overline{0,N} \; t \in T,$$

$$\sum_{i=1}^{N} p_i(t) = 1 \tag{6}$$

If $p(t) = (p_0(t), p_1(t), ..., p_n(t))^T$ is the probability vector associated of stochastic process $\{I(t)\}_{t \in T}$, then the process has the Markov property:

$$P\left(I(t+\Delta t) \middle| I(0), I(\Delta t), ...I(t)\right) = P\left(I(t+\Delta t) \middle| I(t)\right) \tag{7}$$

i.e. the process state (the number of infected individuals) at the moment $t + \Delta t$, depends only on the process state at the moment *t*.

The probability of transition from the state $I(t) = i$, to the state $I(t+\Delta t) = j$ is given by relationship:

$$p_{ji}(t+\Delta t, t) = P\left(I(t+\Delta t) = j \middle| I(t) = i\right) \tag{8}$$

**Observation:**

If $\Delta t$ is a sufficiently small interval, the process $I(t)$ can move from the state $i \to i+1, i \to i-1$ or $i \to i$, i.e. the number of infected people can grow with one, or can be a birth, a death or a cure. In this case, the transition probabilities verify the relationships:

$$p_{ij}(\Delta t) = \begin{cases} \dfrac{\beta i(N-i)}{N} \cdot \Delta t, & j = i+1 \\ (b+\gamma) i \cdot \Delta t, & j = i-1 \\ 1 - \left[ \dfrac{\beta i(N-i)}{N} + (b+\gamma) i \right] \cdot \Delta t, & j = i \\ 0, & j \neq i+1, i, i-1 \end{cases} \tag{9}$$

The relations which are above can be interpreted as follows:

- probability of the occurrence of another infected person in an interval $\Delta t$, $(i \to i+1)$ is $\dfrac{\beta i(N-i)}{N} \cdot \Delta t$,
- probability of the occurrence of death or recover $(i \to i-1)$ in an interval $\Delta t$ is $(b+\gamma) i \cdot \Delta t$,
- probability that not occur any change in state $(i \to i)$in an interval $\Delta t$ is $1 - \left[ \dfrac{\beta i(N-i)}{N} + (b+\gamma) i \right] \cdot \Delta t$.

So that the population size remains constant as with the deterministic case, $b = c$ meaning the birth rate must be equal to the death rate.

**Observation**

If a SIS epidemic model (DTMC) is seen as a process of birth and death, then the system (9) can be written more simple:

$$p_{ij}(\Delta t) = \begin{cases} b(i) \cdot \Delta t, & j = i+1 \\ d(i) \cdot \Delta t, & j = i-1 \\ 1 - [b(i) \cdot \Delta t + d(i) \cdot \Delta t], & j = i \\ 0, & j \neq i+1, i, i-1 \end{cases} \tag{10}$$

with $b(i) \cdot \Delta t$ it noted the probability of new infections and the probability of death or recover $d(i) \cdot \Delta t$. If we apply the previous transition probabilities the Markov property, then $p_i(t+\Delta t)$ can be expressed in terms of probabilities at the time *t* :

$$p_i(t+\Delta t) = p_{i-1}(t)\cdot b(i-1)\cdot\Delta t + p_{i+1}(t)\cdot d(i+1)\cdot\Delta t + p_i(t)\left(1-\left[b(i)\cdot\Delta t + d(i)\cdot\Delta t\right]\right) \quad (11)$$

where

$$i=\overline{1,N},\quad b(i)=\frac{\beta i(N-i)}{N},\quad d(i)=(b+\gamma)i.$$

**2.2 Continuous Time Markov Chain Epidemic Models (CTMC)**

In a CTMC process, time is a continuous random variable, $t\in[0,\infty)$ and $S(t),I(t),R(t)\in\{0,1,...,N\}$ are discrete random variables.

Further we characterize a CTMC SIS epidemic model. The vector of probability functions associated to stochastic process $\{I(t)\}_{t\in[0,\infty)}$ is

$$p(t)=\left(p_0(t),p_1(t),...,p_n(t)\right)^T \quad (12)$$

with $p_i(t)=P(I(t)=i),\quad i=\overline{0,N}$

The process has the Markov property:

$$P\left(I(t_{n+1})\middle|I(t_0),...I(t_n)\right)=P\left(I(t_{n+1})\middle|I(t_n)\right), \quad (13)$$

$$(\forall)0\le t_0<...<t_n<t_{n+1}$$

The previous relationship indicates that the transition probability at the time $t_{n+1}$ depends only on the state of the process at the time $t_n$. If in a DTMC, the transition probability refers to a short period of time $\Delta t$, in the transition probabilities related CTMC process is included the term $o(\Delta t)$, with the property :

$$\lim_{t\to\infty}\frac{o(\Delta t)}{\Delta t}=0 \quad (14)$$

The infinitesimal transition probabilities are defined as:

$$p_{ji}(\Delta t)=\begin{cases}\dfrac{\beta i(N-i)}{N}\cdot\Delta t+o(\Delta t), & j=i+1\\ (b+\gamma)i\cdot\Delta t+o(\Delta t), & j=i-1\\ 1-\left[\dfrac{\beta i(N-i)}{N}+(b+\gamma)i\right]\Delta t+o(\Delta t), & j=i\\ o(\Delta t), & j\ne i+1,i,i-1\end{cases} \quad (15)$$

As $\Delta t$ is sufficiently small, there are three possibilities for mood swings: $i\to i+1$, $i\to i-1$ or $i\to i$. With the same notations as in the case of a DTMC process, the formulas (15) become:

$$p_{ji}(\Delta t)=\begin{cases}b(i)\cdot\Delta t+o(\Delta t), & j=i+1\\ d(i)\cdot\Delta t+o(\Delta t), & j=i-1\\ 1-\left[b(i)\cdot\Delta t+d(i)\cdot\Delta t\right]+o(\Delta t), & j=i\\ o(\Delta t), & j\ne i+1,i,i-1\end{cases} \quad (16)$$

If we apply the Markov property to the previous transition probability and given that $P(I(0)=i_0)=1$, then $p_i(t+\Delta t)$ can be expressed in terms of probabilities at the time $t$ :

$$p_i(t+\Delta t)=p_{i-1}(t)\cdot b(i-1)\cdot\Delta t+p_{i+1}(t)\cdot d(i+1)\cdot\Delta t+ +p_i(t)\left(1-\left[b(i)\cdot\Delta t+d(i)\cdot\Delta t\right]\right)+o(\Delta t)$$

**2.3. The Stochastic Differential Equations Epidemic Models (SDE)**

In a stochastic SDE epidemic model, time is a continuous random variable with $T=[0,\infty)$ and $S(t),I(t),R(t)$ are also continuous random variables, with the state-space interval $[0,N]$. Further we will stop to a SIS stochastic epidemic model. The stochastic process $\{I(t)\}_{t\in[0,\infty)}$ represents the number of individuals affected with respect to time. The random variable $I(t)$ has the probability density $p(x,t)$ and

$$P(a\le I(t)\le b)=\int_a^b p(x,t)\,dx.$$

The process $\{I(t)\}_{t\in[0,\infty)}$ has the Markov property:

$$P\left(I(t_n)\le y\middle|I(t_0),I(t_1),...I(t_{n-1})\right)= P\left(I(t_n)\le y\middle|I(t_{n-1})\right) \quad (17)$$

$(\forall)0\le t_0<...<t_{n-1}<t_n\in T$, and the transition probability density is

$$p(y,t+\Delta t;x,t)\ I(t)=x,\quad I(t+\Delta t)=y \quad (18)$$

In the paper [1], it is shown that a construction of SDE SIS model epidemic, starting from the CTMC SIS epidemic model.

## RESULTS AND DISCUSSIONS

We assume that $N$ - population size is not constant, but it varies in relation to the law of population growth. Formulation of an epidemic model requires

that the birth and death rates, which depend on population size.

We suppose that the birth rate is:

$$\lambda(N) = b \cdot N \tag{19}$$

and the death rate:

$$\mu(N) = b \cdot \frac{N^2}{k} \tag{20}$$

where $k > 0$ is the carrying capacity. Then,the number $N$ checks differential equation:

$$\frac{dN}{dt} = \lambda(N) - \mu(N) = bN\left(1 - \frac{N}{k}\right) \tag{21}$$

According to [2] there are many forms of birth rates and death choice, depending on population dynamics that will be modeled. If we assume that the population size checks the differential equation (21), then a deterministic SIS epidemic model can be characterized by the system of differential equations:

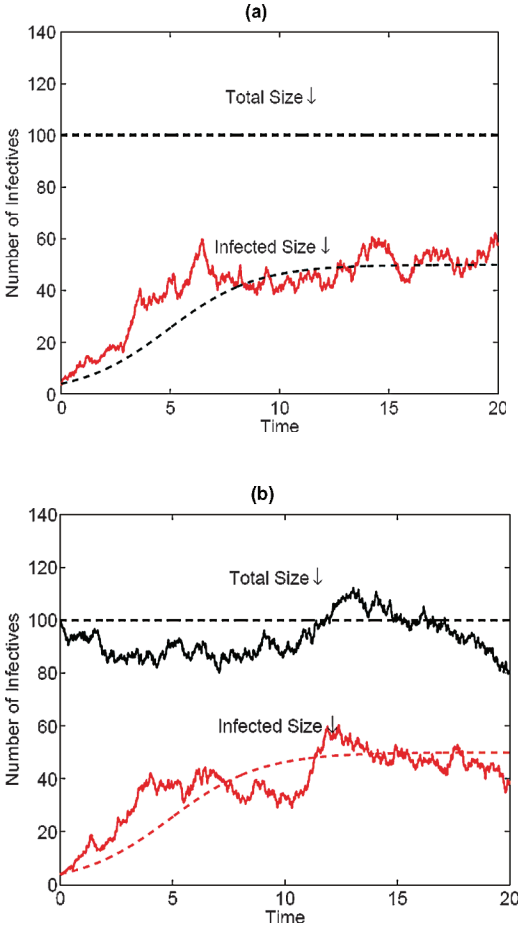
$$\begin{cases} \dfrac{dS}{dt} = \dfrac{S}{N}\left(\lambda(N) - \mu(N)\right) - \dfrac{\beta}{N} S \cdot I + (b+\gamma) I \\ \dfrac{dI}{dt} = -\dfrac{I}{N}\mu(N) + \dfrac{\beta}{N} S \cdot I - \gamma \cdot I \end{cases} \tag{22}$$

with $S(0) > 0$ and $I(0) > 0$.

The previous system solution depends on the basic reproduction number $R_0 = \dfrac{\beta}{b+\gamma}$.

Next, we formulate a stochastic model of type EDS SIS solution and we compare it with the deterministic model [1], [2]. Let us consider $S(t), I(t)$ variables representing the number of individuals susceptible, infected respectively at time $t$. Obviously $S(t) + I(t) = N(t)$ and $S(t), I(t) \in [0, \infty)$. If we apply the same method as for the model SDE SIS epidemic model, we obtain the system of differential equations

In [4] Brauer, F., Driessche P present a graph of a SDE SIS epidemic model (**a**) with constant population size, $N$ = 100 and (**b**) with variable population size, $N(t)$. The parameter values are $\beta$ = 1, $\gamma = 0.25 = b$, $K$ = 100, and $R_0 = 2$




## CONCLUSIONS

In many cases these three stochastic formulations generate similar results, if the time step $\Delta t$ is small [2]. There are numerical advantages in applying the discrete time approximations (DTMC model) in that the discrete simulations generally have a shorter computational time than the CTMC model. Mode and Sleeman [7] discuss some computational methods in stochastic processes in epidemiology. The most important consideration in modeling, however, is to choose a model that best represents the demographics and epidemiology of the population being modeled.

In the future we plan to continue studying to application of stochastic modeling in epidemiology to determine the final number of individuals of a population affected by an infectious agent but also for estimating the duration of an epidemic

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# *CHLAMYDIA PSITTACI* IN THE PARROTS, PIGEONS AND CANARIES IN THE CITY OF TIRANA

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***Abstract***

*The growth of decorative birds in Albania in the recent years has brought an increased frequency of chlamydial infection not only in the flocks of birds but, also to humans. According to the data from the Public Health Institute (PHI) in Tirana, is it has been observed an increase in the vulnerability of people, mainly in to those who are in the young age who have been in contact with decorative birds. This study, the first of its kind conducted in Albania, was based on a serological control of 573 blood samples which were taken from pigeons, parrots and canaries in Tirana, Albania. Sampling was carried out in all four seasons of the year 2011 and their control was carried out by the Institute of Veterinary Food Safety (FSV) Tirana. In order to check them it was used an indirect immunofluorescence test (IFT), combined with chlamydia's isolation in chicken egg embryo cells, as a comparative method, in which there were identified at least 51 cases with Ch. psittaci. Relative specificity of fluorescent antibody's test in the serum was approximately 95.3% and the relative sensitivity was about 60.3%. Results of this study, which as mentioned above were conducted for the first time in Albania, showed that immunofluorescence tests performed using diagnostic kits of Medical servicce-2000, combined with primary isolation of chlamydia in embryo of chicken eggs,were very specific and very useful for the identification of useful option for veterinary service.*

***Keywords:*** *fluorescent antibody, immunodepression, immunofluorescence overhead, relative sensitivity seropositivity.*

## INTRODUCTION

Cage birds parrots, canaries, and pigeons showed different sensitivity to *C.p*sittaci, causing their injuries, but also a risk for the spread of this disease with zoonotic nature. (Barnes, RC: 1989 and Pospisil, L., et al: 1996). Even in Albania from year to year, this category of birds has been increasing, serving as a potential risk in to the spread of the chlamydial infection not only in flocks of birds, but also in humans. (Pospisil, L., et al: 1996). Evidence shows that during the period 2001-2011 there have been reported about 635 cases of individuals infected with chlamydia (PHI: 2011) who mostly were pople in their young age or individuals involved decorative bird breeding. Expressed clinical signs were depending on chlamydia's pathogenicity, type, race, and physiological condition, age of the bird, route and time frame of exposure, stressful factors, immunodepresive and situation of the birds (Lublin, A., et al: 1993) and the presence of other infections in an interaction. Psittacosis' soft "explosion's" can often go unnoticed because there are no clear symptoms. The most obvious are those of airways and diarrhea (Avian Disease Manual, 1983). In adult wild birds,C. psittaci is often seen without clinical signs and they can serve as asymptomatic carriers**.** The infection can develop in the acute form, sub-acute, or it can be chronic. In the acute form the disease can cause severe damages, which can be fatal, especially for some species, while at younger birds, it appears to be highly sensitive. The most typical sings are seen on young birds, which often appear weak, showing anorexia, they faint, lie in a special position, from their eyes and nose there is a purulent flow, they usually are contracted and stay with disheveled feathers (Gerlach 1986).

Diagnostic methods used for detection of chlamydic infection in the birds are numerous. For specificity, sensitivity, speed and relativity

and its simplicity, the identification of chlamydial antibodies, especially in sub-clinical cases, is made by using the indirect immunofluorescence test which is considered to be the most effective test, whereas the primary isolation of chlamydia of chicken embryos **(**Andersen, A.A.1991) can also be used as comperative method (Kennedy G, et *al: 1985*). In different species such as, pigeons, parrots and canaries, it has been seen that their immunological responses towards their organism can be different (Geens, T. et al. 2005). The study showed that organs taken from dead birds which were infected with *C. psittaci* were important in identifying the infection by using diagnostic kits. The aim of the study was to develop and evaluate a rapid antemortem diagnostic technique for detection of *C. psittaci* in the serum of birds. Also as a quick and accurate method it can allow a veterinary doctor to immediately begin treating the sickening birds thus, preventing the infection to spread from domestic birds to humans, Greub,G.2010**)** which is the basic objective of the veterinary medicine.

## MATERIALS AND METHODS

For conducting the study, parrots and canaries, were selected inclusive, and for the control of pigeons were included and those races where as indicated had cases of people affected by this infection.The perennial scope had as an objective the study of the dynamics of the disease, as well as climatic factors correlating with other environmental issues. The process of taking the blood samples from decorative birds was carried out in batches, at 0.5 ml / head, which were marked and have been monitored throughout the year, 573 samples which prevailed according to their species were: 118 heads of parrots, 348 pigeons and 107 canaries, of which 30% were selected from young birds, varying from 2-4 months old. As a laboratory method it was selected the *indirect immunofluorescence test* (IFT), which because of its high specificity helps in all sub-clinical cases, when a seropositive birds lack a complete clinical framework. It is fast and simple to implement in the field and laboratory procedures, and also combined with primary isolation method of chlamydia in the chicken embryo, it can obtain rapid and accurated data (Andersen, AA, 2008). The IFT control procedure was based on the principle of reaction of 573 blood samples, using IgG Antibody Kit, imported by Medical Servicce-2000. The separation of serum was made using the usual metthod, by following rigorously all steps to preserve the kit, dilution, incubation, testing and control of the material prepared. At the end the reading of the small droplet shaped spots was made using a 400x magnification for each tile, and then they were compared with the visual intensity of basic troops, shown in the positive control well and negative.The tiles were stored in a dark room in a temp 2-8 degree celsius for a period of 24 hours. In the positive responses appeared a fluorescent glow, sharp, regular elementary troops and stained, which was rated at (1 +, 2 +). The isolation of chlamydia cells in chicken embryos was carried out according to standard procedures, injected into the viteline sac, up 0.5 ml.i emulsion prepared with positive material from positive birds and suspected, from the lungs, liver, spleen, trachea and air sacs injured birds and sacrificed, in chicken embryos aged 6-7 days. Then they were placed for incubation at 39 degree celsius. After that it was carefully observed the replication of chlamydia, and evaluation of results was done from the fetus state which, in case it results positive, usually dies within 5-12 days after inoculation. After the histopathological control of infected embryos with the typical chlamydie infection, the material was collected and homogenised with a 20% suspension sacus vitelinus. The Identification of the agent was carried out by preparation of an infected sakus vitelinus antigen.

## RESULTS AND DISCUSSIONS

Study shows that in areas chlamydic infection monitoring circulating in the following levels: in parrots: 12.7%, pigeons: 8.4%, and canaries: 7.47%. Chlamydia focus in birds' bodies but, more sensitive and higher concentrations they are found in the lungs. IFT method, combined with the isolation of chlamydia′s in embryos of chicken eggs, as well as histopathological control of the heads of damage, proved to be effective for disease control, the practical implementation of field and laboratory procedures. Chlamydia chicken embryo grew

and multiplied in the first pass, where the largest concentration of them is in the core of an egg. Death of embryos, under the action of chlamydia is high in percentage; the most pathogenic were chlamydia which was separate from the parrots and less pigeons and canaries. Identification of *C.psittaci* is done by the clinically healthy birds, and those coupled other pathologies, where the intensity of touching the bodies, in the first case has been much lower than in cases with pathology combined with other pathology, aspergilosis, parasites etc. The morbidity rate of birds only by chlamydia are observed to be as 10, (7 parrots and 3 pigeons), but always accompanied with other causes, which have enabled C.psittaci where after histopathology 'Basic ' are found localized in the brains of damaged birds. The clinical disease has been evident and obvious mainly in young birds, which make possible a veterinary service, whereas adults show a sub-clinical form, serving as an asymptomatic carrier, helping to permanent recycling of infection. The survey data show that chlamydia are ordinarily resident in the bodies of birds, which are activated when lowered their sustainability as a result of stress and other resources that accompany infection, findings that are compatible with those of many foreign authors. Recognition of favorable factors and the development cycle chlamydia′s can serve as options for preventive measures by the veterinary service to control the disease in poultry in general and particularly decorative, and as a zoonosis, prevention and protection of human health.

Table 1. Information about the positivity of chlamydiosis in parrots by the breeds (in%)

| Race | Spring | | | Summer | | | Autumn | | | Winter | | | Annual | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Birds Tested | Positive + | % infection | Birds Tested | Positive + | % infection | Birds Tested | Positive + | % infection | Birds Tested | Positive + | % infection | Birds Tested | Positive + | % infection |
| Total | **28** | **4** | **14.2** | **26** | **6** | **23** | **34** | **3** | **8.8** | **30** | **2** | **6.6** | **118** | **15** | **12.7** |
| Amazon | **8** | 2 | 25 | **7** | 3 | 42.8 | **10** | 1 | 10 | **10** | 1 | 10 | **35** | 7 | 20 |
| Ondule | **8** | 1 | 12.5 | **7** | 2 | 28.5 | **10** | 1 | 10 | **10** | 1 | 10 | **35** | 5 | 14.3 |
| Calopsitte | **6** | 1 | 16.6 | **6** | 1 | 16.6 | **7** | 1 | 14.3 | **5** | 0 | 0 | **24** | 3 | 12.5 |
| Cacatoe | **6** | 0 | 0 | **6** | 0 | 0 | **7** | 0 | 0 | **5** | 0 | 0 | **24** | 0 | 0 |

Table 2. Information about the positivity of chlamidiosis in pigeons by breeds (in %)

| **Race** | Spring | | | Summer | | | Autumn | | | Winter | | | Annual | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Birds Tested | Positive + | % infec-tion | Birds Tested | Positive + | % infection | Birds Tested | Positive + | % infection | Birds Tested | Positive + | % infection | Birds Tested | Positive + | % infection |
| **Total** | **84** | **8** | **8.5** | **89** | **11** | **12.3** | **95** | **6** | **6.3** | **80** | **3** | **3.3** | **348** | **28** | **8.04** |
| **Rancing** | **44** | 5 | 11.3 | **44** | 7 | 15.9 | **41** | 3 | 7.3 | **40** | 2 | 5 | **169** | 17 | 10.05 |
| **Other** | **40** | 3 | 7.5 | **45** | 4 | 8.8 | **54** | 3 | 5.5 | **40** | 1 | 2.5 | **179** | 11 | 6.14 |

Table 3. Information about the positivity of chlamydiosis in canaries by breeds (in%)

| **Race** | Spring | | | Summer | | | Autumn | | | Winter | | | Annual | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Birds Tested | Positive + | % infection | Birds Tested | Positive + | % infection | Birds Tested | Positive + | % infection | Birds Tested | Positive + | % infection | Birds Tested | Positive + | % infection |
| **Total** | **29** | **3** | **10.3** | **26** | **3** | **11.5** | **29** | **2** | **6.9** | **23** | **0** | **0** | **107** | **8** | **7.5** |
| **Serina** | **19** | 2 | 10.5 | **16** | 2 | 12.5 | **19** | 1 | 5.3 | **13** | 0 | 0 | **67** | 5 | 7.5 |
| **Belge** | **10** | 1 | 10 | **10** | 1 | 10 | **10** | 1 | 10 | **10** | 0 | 0 | **40** | 3 | 7.5 |

The presentation of data, in tables No. 1, 2, 3, in overall it has been identified a total of 51 heads of seropositive birds, of which 15 heads of parrots, 28 heads of pigeons, 8 heads of canaries, and by using the primary isolation method of chlamydia in chickens embryos, there were prepared 15 samples, of which 5 were parrots, 7 were pigeons and 3 canaries. Selecting the method of indirect immunofluorescence in determining the positive samples resulted to be very useful especially in sub-clinical cases, when complete clinical signs were missing, mostly in adult birds, and combined with the method of isolation chlamydia in chicken embryos, because the death of embryos occurred when they were at the age of 12-18 days, making

completely compatible with IFT score. (Andersen, A.A.2008). From the histologic control of typical chlamydia infection in chicken embryos, in general for all species it was observed vascular congestion of membranes sacus -vitelinus, (Andersen, AA 2008). An important element was observed and the dynamics of infection, which according to the species stands as follows : parrot′s, average annual level of infection has been 12.7%, in spring 14.2%, in summer 23%, in fall 8.8%,and in winter 6.6%. (Table.1). Pigeon′s, average annual level of infection was about 8.04%, 8.5% in spring, in summer 12.3%, in fall 6.3%, while in winter, 3.3%, where is almost is hidden completely. (Table.2) Canarie′s average annual level of infection was 7.47%, in spring 10.3%, in summer 11.5%, in autumn 6.89%, while in the winter it was 0, which means that is completely extinct (Table.3). Although in different species such as parrots, pigeons, canaries the frequency was different, it was strictly related to seasonal conditions of the weather. In spring the infection has shown a tendency to increase, in summer where the weather has been so hot it has shown it maximum value whereas in autumn, with the weather cooling the value has tended to decrease, while in the winter when the weather has been cold the infection is reduced, hidden or wiped out, facts which coincide with that of the foreign authors (Lublin, A., et al, 1995), etc. It is important to be considered as support for increasing the frequency of infection during the months with warm and hot weather there were also many other factors, individually or in the correlations between them have contributed to the situation. Those can be from the: increase of contact between birds with warm weather, activation of arthropods and hematophag insects which help spread the movement vectors of infection in decorative birds, which have also been observed on a case by case basis on the infected birds. While monitoring the incidence of pigeons (not decorative ones) and seropositive races, which make up about 30% of the samples, as a source of infection we should also evaluate the contact with water weeds, which have been polluted with eksements of porter birds. On the other hand unfamiliar areas affected by infection, and the transit during the races(Salinas, J., et al.1993), but also the use of uncontrolled food products with unsafe origin, low hygiene standards of breeding, stresses and strains circulation with high virulence, etc.. Should also be taken more into consideration (Geens, T., et al., 2005). During monitoring, it was noted that infection to young people is organized in acute form causing damage to former company because of their high sensitivity. Young birds have shown signs of weakness typical, anorexia, purulent leak from eyes and nose. Inactivity, stay in position to collect, disheveled feathers (Gerlach, 1986). In cases with mild to developments birds lacked clear symptoms, the most obvious would be those of the respiratory tract and diarrhea (Avian Disease Manual, 2007) making them serve as asymptomatic carriers, recycling permanently the infection. The isolation of chlamydia from chicken egg embryos aged 6-7 days, were made from 15 samples, of which 5 parrots, 7 pigeons and 3 canaries, which resulted seropositive IFT method. To avoid horizontal transmission of infection through eggs, before infecting, 6 embryos were checked aged 6-7 days, two for each species, with IFT method, which resulted in negative from chlamydia. Control embryos was carried out for 14-15 days in a row and the death rate of embryos infected with suspension by the parrots was 100% Infected embryos suspension pigeons was 4, or 57.7%, whereas embryos infected with suspension canary bodies 1, or 33%. Mortality dynamics was observed during the period 3-14 days after infection, and mortality to parrots, 70-80% of them occurred 3-8 days after infection, 10-12 pigeons after infection, while the canary in the day of 13 after infection. Histopathological control of dead embryos dominated by the presence of hemorrhage in the body, in the head region of the feet, thickening of the lining of an egg and the slowdown in their growth and development. Surviving embryos, especially those with suspension by pigeons and canaries, microscopic researches have been identified the 'Basic corpus'. By monitoring the people affected with C. psittaci, in the past three years, there have been a total of 132 cases, and after controlling almost 45 of them, it was found that the disease has been correlated directly from their contact with seropositive birds, parrots,

canaries and doves, and its frequency was variable, with age, the level of exposure and patogenicity were the determining factors for the occurrence and form infection clinic. (POSPISIL, L., et al., 1996)

## ACKNOWLEDGEMENTS

The circulating levels of decorative birds with chlamydia infections are considered to be relatively low.*C.psittaci* are mainly focused on birds' bodies but, more sensitive and higher concentrations are to be found in the lungs. The IFT method, combined with the isolation of chlamydia in embryos of chicken eggs, as well as histopathological control of the damaged heads, proved to be effective for controlling the disease, the practical implementation of field and laboratory procedures. Identification of 'elementary bodies' can be done with materials which are taken from bodies stained with May-method Grynvald-Giemsa and by controlling them under a microscope, shows that they are located mainly within the cytoplasm of the cell in the form of meal pomegranate and being a little pink in color. It was observed that the death of the embryos was higher in chlamydia isolated from parrots and less from those in doves and canaries. Identification was done by the clinically healthy birds, where the intensity was much lower however, in cases with combined pathology with mycotic causes, aspergilosis, parasites etc intensity was higher. Morbidity cases of birds just by chlamydia alone are 10 in total, (7 parrots and pigeons 3) but, cases coupled with other causes have dominated. The clinical disease has been evident and obvious mainly in young birds which make possible orientation in veterinary service, whereas adults show a sub-clinical form, serving as asymptomatic carriers, helping to permanent recycling of infection. The study shows that chlamydia are ordinarily resident in the birds' bodies which, are activated when their sustainability is lowered as a result of stress and other resources that accompany infections. The findings are compatible with those of many foreign authors. Recognition of favorable factors and the development cycle in chlamydia can serve as options for preventive measures by the veterinary service to control the disease in poultry in general and particularly decorative birds, and as a zoonosis, in prevention and protection of human health.

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# FOOD BIOTECHNOLOGY

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# *IN VITRO* EVALUATION OF ANTIOXIDATIVE PROPERTIES IN *CAPSICUM ANNUUM*, *VACCINIUM VITIS-IDAEA* AND *MELISSA OFFICINALIS* TINCTURES

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***Abstract***

*Several commercial varieties of tinctures were analyzed for determining their antioxidant potential, since most knowledge relating to therapeutic properties of medicines are obtained from folk phytomedicine. Antioxidant potential was determined by the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radicals, reducing power, and chelating activity. The best results against scavenging of free radicals were obtained with tincture from Vaccinium vitis-idaea,followed by Capsicum annuum and Melissa officinalis. There were obvious significant differences between the free radicals' scavenging activities. Results were correlated with values obtained for reducing power and chelating ability. This finding was also confirmed by the low values of the $EC_{50}$. Also, the results were positively high when correlated with total phenolic and flavonoidic contents from the tinctures. The results given herein are the scientific proof that confirms the empirical medical knowledge on how to use the three tinctures in oxidative dysfunctions.*

***Keywords:*** *scavenging activity, phenolic content, tincture.*

## INTRODUCTION

In recent decades, interest in the use of medicinal plants has significantly increased due to the isolation of biologically active substances that have a wide range of pharmacological effects. The phytotherapeutical action of herbal products is generated by the rich content of such substances, including phenolic compounds, vitamins, alkaloids, saponins, essential oils, and various minerals (Ivan, 2007). Their main and most evident effect is antioxidative, that acts upon the characteristics of reactive oxygen and nitrogen species. The antioxidative molecules existing in herbal products can react with free radicals and they are able to neutralize the latter by donating their own electrons. This process helps prevent the damage of cell tissues. Each cell protects itself from the influence of free radicals by its own prevention mechanisms, but in the event of excessive formation, oxidative stress occurs (Sen et al., 2010). Oxidative stress has been defined as the imbalance between the production of various reactive species and the ability of a living body's natural defense mechanisms (Oguntibeju et al., 2009). Under such circumstances, it is necessary to take antioxidative products which can restore the balance of a body's antioxidant status.

Polyphenols are the main secondary metabolites to be found in medicinal plants. Besides their antioxidant activity, such compounds are responsible for their antimicrobial actions (due to their content of flavonoids) and cytotoxic effects exerted against tumor cells Gharras, 2009). Their antimicrobial actions have been proven against potentially pathogenic strains, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida* strains, *Listeria* strains, or *Bacillus cereus* (Vamanu et al. 2011). On the other hand, their cytotoxic effects have also been associated with the content of alkaloids and triterpenes, which have been demonstrated in previous surveys as being directly related to the antioxidative response (Agarwal et al., 2012). This is the reason why the present research has undertaken to demonstrate the antioxidative properties of certain tinctures sold in Romania. Their properties have been linked to polyphenol and flavonoid contents. Highlighting the antioxidative effects has taken place by means

of the DPPH and ABTS radicals′ scavenging activity, reducing power, and chelating properties.

## MATERIALS AND METHODS

### Materials

The tinctures that have been used are: hot pepper tincture (*Capsicum annuum*), cranberry tincture (*Vaccinium vitis-idaea*), and lemon balm tincture (*Melissa officinalis*) manufactured by SC Dacia Plant SRL.


Figure 1. Marketing image of *Capsicum annuum, Vaccinium vitis-idaea* and *Melissa officinalis* tinctures

### DPPH radical scavenging activity assay

The sample (100 µl) was mixed with 3 ml of ethanol solution of 0.004% DPPH and the absorbance was read at 517 nm 30 min later. Standard antioxidant, tert-Butylhydroquinone (TBHQ), was used for comparison as positive control. EC50 value (milligram extract/mL) is the effective concentration at which DPPH radicals were scavenged by 50%. The DPPH radical-scavenging activity of the samples was expressed as% = ((Acontrol − Asample) /Acontrol) × 100, where Acontrol is the absorbance of the blank control (DPPH solution without test sample) and Asample is the absorbance of the test sample (Vamanu et al. 2011).

### ABTS Radical Scavenging Assay

ABTS radical was obtained by adding 7mM of the ABTS stock solution to 2.45mM potassium persulfate. The mixture was left to stand in the dark, at room temperature, for 12−16 h before use. The ABTS radical solution was then diluted with 5mM phosphate-buffered saline (pH 7.4) to an absorbance at 730nm of 0.70 ±0.02. After adding 10 µL of the sample to 4mL of the diluted ABTS radical solution, the absorbance was measured at 30 min. The ABTS radical-scavenging activity of the samples was expressed as% = ( (Acontrol − Asample) /Acontrol) × 100, where Acontrol is the absorbance of the blank control (ABTS solution without test sample) and Asample is the absorbance of the test sample (Vamanu, 2012a). Standard antioxidant (TBHQ) was used for comparison as positive control. EC50 value (milligram extract/mL) is the effective concentration at which ABTS radicals were scavenged by 50%

### Reducing Power

Each sample (2.5 mL) was mixed with 200 mM sodium phosphate buffer (2.5 mL, pH 6.6) and 1% potassium ferricyanide (2.5 mL), and the mixture was incubated at 50 °C for 20 min. Next, 10% trichloroacetic acid (2.5 mL) was added, and the mixture was centrifuged at 3,000 g for 10 min. The upper layer (2.5 mL) was mixed with distilled water (2.5 mL) and 0.1% ferric chloride (0.5 mL). Finally, the absorbance was measured at 700 nm and compared to a blank. The extract concentration providing 0.5 of absorbance ($EC_{50}$) was calculated from the graph of absorbance at 700 nm plotted against the extract concentration. TBHQ was used as positive control (Vamanu, 2012b).

### Ferrous Ion Chelating Assay

1mL of the sample was mixed with 3.7mL of ultrapure water, following which the mixture was reacted with ferrous chloride (2 mmol/L, 0.1mL) and ferrozine (5 mmol/L, 0.2mL) for 20 min. The absorbance at 562nm was determined spectrophotometrically. TBHQ was used as a positive control. The chelating activity on the ferrous ion was calculated using the equation following: chelating activity (%) = [(*Ab* −*As*) */Ab*] ×100, where *Ab* is the absorbance of the blank without the extract or TBHQ and As is the absorbance in the presence of the extract or TBHQ (Vamanu and Nita, 2013).

### Determination of total phenolic and flavonoid content

The total phenolic and flavonoid content of ethanolic extract, and several organic fractions, were determined using Folin-Ciocalteu reagent and aluminium chloride colorimetric method,

respectively (Vamanu, 2012c; Premanath et al., 2011).

**Statistical analysis**

All the essays were assessed in triplicate, and the results were expressed as mean ±SD values of three observations. The mean values and standard deviation were calculated with the EXCEL program from Microsoft Office 2010 package.

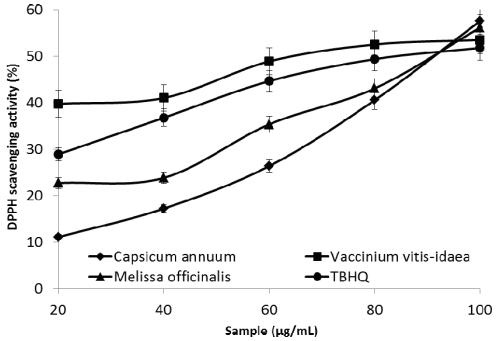


Figure 2. DPPH scavenging activity of *Capsicum annuum, Vaccinium vitis-idaea* and *Melissa officinalis* tinctures

## RESULTS AND DISCUSSIONS

Evaluating antioxidative potential by the DPPH and ABTS determination of radicals' scavenging activity requires a modern method to be applied due to the stability of these free radicals, and to the precise results that ensue thereof. The discoloration level of the reaction mixture (turning from mauve into beige for the DPPH scavenging assay; or turning from turquoise into colorless for the ABTS scavenging assay) is allocated in direct proportion to the ability of donating a hydrogen atom derived from the antioxidative compounds in the examined assay. The process leads to the reduction of existing free radicals, and also generating the cessation of the radical chain reaction which has determined the propagation of the oxidation mechanism (Kumaraswamy and Satish 2008). DPPH and ABTS free radicals accept hydrogen radical and become stable molecules (Nikhat et al., 2009). In this research, the standard (TBHQ) has proven a moderate scavenging activity against DPPH radicals, and a high scavenging activity against ABTS radicals. At a concentration of 100 µg/mL, the descending order of the scavenging activity against DPPH radicals has been: *C. annuum >M. officinalis >V. vitis-idaea* (Figure 2). Thus, in terms of the hot pepper tincture, the scavenging activity's difference from the standard has been on average 10% higher. With respect to ABTS radicals, the descending order has been: *V. vitis-idaea >M. officinalis >C. annuum* (Figure 3). In this context, the set value of the TBHQ reaching 100 µg/mL has been on average 2% higher. The differences noticed in relation with the three tinctures for scavenging properties against the two free radicals have been generated by the existing molecules that possess antioxidative effects. The type and presence of certain molecules have caused specificity differences as compared with the DPPH or ABTS radicals which have been best monitored in the *C. annuum* tincture.

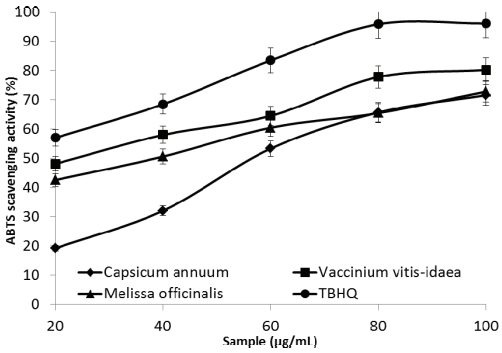


Figure 3. ABTS scavenging activity of *Capsicum annuum, Vaccinium vitis-idaea* and *Melissa officinalis* tinctures

For reducing power assays, the antioxidative molecules to be found in the three tinctures will determin the reduction of $Fe^{3+}$ into $Fe^{2+}$ (Moein et al., 2008). The molecules' reducing power in the three plant tinctures serves as a direct indicator of the extent of the antioxidative potential. According to the research in the field of natural supplements, reducing power is related to the existence of reductones (Li and Lin, 2010). Ascorbic acid is the best known reductone, and high reduction ability is generally associated with a significant amount of this compound which is frequently found in hydroalcoholic extracts. The absorbance increase has been set at 700 nm, as compared with the standard value (Figure 4). The value

set at the concentration of 100 µg/mL has ranged between 0.404 and 1.643. Compared with the standard, the reducing power value in the *V. vitis-idaea* tincture is 3.8 times higher which has indirectly confirmed the presence of molecules with obvious reduction properties. The same behavior has been noticed in the *M. officinalis* tincture as well. The difference between the two tinctures has been 28.4%, in favor of the cranberry tincture.

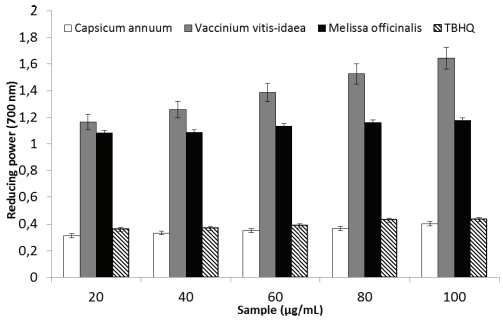


Figure 4. Reducing power of *Capsicum annuum, Vaccinium vitis-idaea* and *Melissa officinalis* tinctures

As far as the determination of ferrous chelating capacity is concerned, ferrozine forms $Fe^{2+}$ compounds, whereas in the presence of chelating agents from the three tinctures, compound formation is disturbed. According to the sample concentration, the reaction mixture is colored in several shades of red and pink in an inverse proportion. The decrease in absorbance has been measured spectrophotometrically (Grace-Lynn et al., 2012). Ferrum is essential as it is very important for oxygen conduction and acts as an activating agent for various enzymes. The ferrous ion (or copper ion) intervenes in a range of mechanisms that may contribute in the emergence of oxidative processes. Its role is very well known in the lipid peroxidation of the cell membrane, and its action upon the protein content is also recognized (Karthika et al., 2012). In the present research, the capacity of the examined tinctures to link to $Fe^{2+}$ in the presence of ferrozine as compared with the (TBHQ) standard has been shown in Figure 5. The maximum value of the ferrous ion chelating activity is 80.17%, at 100 µg/mL, with respect to *C. annuum* tincture. It has been 1.07% higher than the *V. vitis-idaea* tincture, and 5.61% higher than the tincture obtained from the *M. officinalis*. The standard has shown a low ferrous chelating activity at a significantly lower value than the three assays, up to a maximum of 46.61% in the highest concentration of the assay.

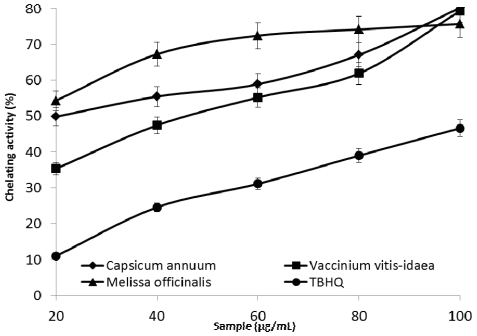


Figure 5. Ferrous ion chelating activity of *Capsicum annuum, Vaccinium vitis-idaea* and *Melissa officinalis* tinctures

The results of the above tests have been relatively different based on each individual biochemical test performed. This finding proves that certain molecules can exert their antioxidative properties in their own individual ways. This confirmation has ensued from the inversely proportional values of the $EC_{50}$ regarding its chelating capacity. From the perspective of this parameter, the maximum value has been reached for *M. officinalis* tincture (< 10 µg/mL). The $EC_{50}$ values of the other two tinctures have been ≈30 µg/mL in the *C. annuum*, and ≈65 µg/mL in *V. vitis-idaea*. The latter results confirm the high biological values of these tinctures, especially that of the cranberry one, because Ferrum immobilization within compounds stops its accumulation which has toxic effects at cellular level (Grace-Lynn et al., 2012). The same behavior has been assessed in the DPPH scavenging activity where the lemon balm tincture has shown its $EC_{50}$ value as being inferior to 90 µg/mL. On the contrary, the $EC_{50}$ values of the other two tinctures have been superior to the former, namely ≈95 µg/mL.

The scavenging activity against free radicals is due to the amount of phenol compounds. Subsequent to all the measurements performed, the *V. vitis-idaea* tincture has contained an amount of 106 ± 8.15 mg gallic acid/g extract,

*M. officinalis* tincture 96.4 ± 4.74 mg gallic acid/g extract, and 76.8 ± 3.98 mg gallic acid/g extract in the *C. annuum* tincture. The flavonoid content has proven a similar trend, with a maximum level of 4.43 ± 0.5 mg quercetin/g extract in the *V. vitis-idaea* tincture. This value has been 35.44% higher than the lemon balm tincture, and 64.78% higher than the hot pepper tincture. The calculated values of the main antioxidative compounds have corresponded with the increase in the $EC_{50}$ index value in terms of the scavenging activity, reducing power and ferrous ion chelating activity (Zhu et al., 2006).

Interpreting the results of the antioxidative properties′ analysis relating to the three tinctures has also taken place by calculating the index value of the correlation among various examination methods. In the hot pepper tincture, the $R^2$ value has been 0.8811, while the same value among the remaining methods has ranged between 0.9711 and 0.9891 ($p<$ 0.0005). The correlation coefficient has ranged from 0.828 to 0.9305, with a minimum value calculated for the ABTS ratio between radicals′ scavenging activity and ferrous ion chelating activity. The value ensuing thereof has confirmed the behavior of this tincture when expressing its antioxidative feedback. As to the lemon balm, there has been a low $R^2$ value in the relationship between the ferrous ion chelating activity and the reducing power, a value of 0.6311. In the relation with the ABTS assay, a 0.7129 value of the correlation index has been measured, whereas, the correlation level has been high in the other outcomes, ranging between 0.8621 and 0.9446.

Owing to the generally neutral pH contained in tinctures, the DPPH assay has been a less appropriate method where the sample′s correlation degree influences the final result. The differences when calculating the $R^2$ value related to the ABTS assay more precisely indicate the distinctions among the three tinctures, as the method is valid irrespective of the pH value, and the results do not directly depend on the coloring level of the sample (Bhoyar et al., 2011; Zhu et al., 2006).

## CONCLUSIONS

To conclude, the total phenolic content has been determined as significantly higher in the lemon balm tincture as compared with the other two. This ratio has been correlated in the case of flavonoid contents as well. According to the $EC_{50}$ values, the *V. vitis-idaea* tincture has had a more obvious antioxidative activity in compliance with its correlation to the scavenging activity against free radicals and the chelating activity, than in the hot pepper (*C. annuum*) tincture. These measurements comply with similar research, thus confirming that a tincture′s expression of its antioxidative value is not automatically and directly connected with its phenolic content. The antioxidative feedback is very complex due also to the existence of additional compounds that exert specific effects.

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# A COMPARATIVE STUDY ON MOUNTAIN AREA INFLUENCE OF MILK SAMPLES FROM COW AND SHEEP

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***Abstract***

*With the current rapid growth of world population and prolongation of human life, the raise of the living level and the targeting of food to a growing extent of agro-food products, with high nutritional and biological value, the need for food, especially of animal origin have increased ever more. This research was carried out to investigate and compare the physicochemical and microbiological parameters of milk samples of two different species like cow and sheep, including: pH, fat, protein, total solids, density, and somatic cells. Results showed that maximum fat and protein content were observed at sample 3 and sample 4, indicate that in mountain area sheep's are the most favorable animal breeding. The milk samples were collected nine month in the year 2011 from different farmers. The statistical analysis showed that the physicochemical and microbiological parameters of these milk samples were significantly different ($p<0.05$).*

***Keywords:*** *physicochemical parameters, microbiologic indices, cow milk, sheep milk.*

## INTRODUCTION

The animals have an important role for small farmers. The growth of these animals is a sector of livestock production, effective from the biological and economic point of view, constituting an important goal for researchers.
Due the biological recovery of nutritional substances from feedstuffs in valuable products for human health, in the future, major investments in raising farms will be needed, but also in milk processing centers, to support and develop the supply of milk and milk products. [1, 2].
A market place for milk and milk products constitutes a fundamental strategic objective of farmers whose purpose is the systematic adjustment of the food sector, its fulfillment estimating the agriproduct potential conferred by natural resources. [4,11].
The significant increase in the competitiveness of Romanian agricultural producers is restricted by limited financial possibilities of maintenance through public resources of the government actions in the field of agro-food sector, as well as qualitative losses in what regards human resources, especially the attractiveness of careers in research.
The raw material for the production of drinking milk and milk products is, in our study, the milk of cows and sheep. It is considered the most complete product and easily assimilated by the body, constituting one of the basic foods in human nutrition. [3, 7].

## MATERIALS AND METHODS

In the frame of selected farms for the determination of physic-chemical composition of Spotted breed's milk (called in Romania "Baltata") is predominate, but there are also a few specimens from the Holstein,the Romanian Spotted breed.
Sheep breeding is, through their social implications, a zoo-traditional representing for agricultural landowners source of food and raw material for their own needs, but also for their involvement in trade activities.
The prevalent breed in the farms taken into study, in Sibiu district is the breed Ţurcana. Milk samples were collected for 9 months in 2011 from different farmers.

A total of 210 samples of sheep's milk, 130 of cow's milk samples were analyzed from the physics-chemistry point of view, using the milk analyzer Ekomilk found in the equipment of the company ASI NATURE SRL Sibiu.
The microbiological analyses were made with MT-04. Sampling was done in sterile 50 ml vials, labeled and placed in a freezer at the device temperature 4°C up to the laboratory to be analyzed.

## RESULTS AND DISCUSSIONS

The variability presents significant differences regarding the physicochemical parameters, but also microbiological. The chemical composition of the milk is subject breed, individuality, area, age, level and nature of nutrition, lactation, season calendar, during milking and health status [6.9].

**The variability of the results of physicochemical and microbiological of cow's milk**

The study shows that the majority of the samples of milk fall within normal values by 3.5% to 4.00% of the fat content. Samples that have experienced the greatest amount of fat are during the months of October and February, with a mean value of 3,99% 3,90% respectively.

High fat content recorded in winter is due to complete the different ration of warm season forage. Hay's introduction of good quality alfalfa and oat sharps increases the fat percentage with 1.08% as compared to the value recorded in the month of May (3.68%) fat.

Considering that the total proteins are relatively comparable in the two types of milk (3.43 g/100 ml for cow's milk and 2 g/100 ml goat's milk), it follows that of cows ' milk has a lower protein content and non-nitrogen. [8, 14, 15]

Determination of solids (SNF) for farms taken into study reveals that SNF is directly related to our values, values of protein, fat, lactose and water.

The integrity of the raw milk is the farmers'concern, but at the same time processors'one.

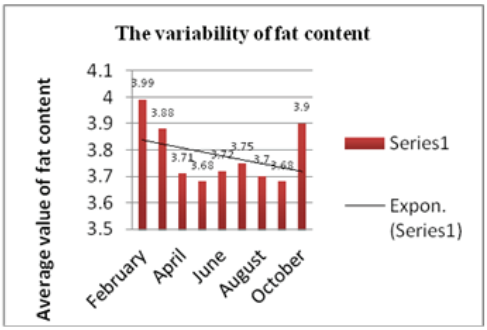


Figure 1. The variability of fat content

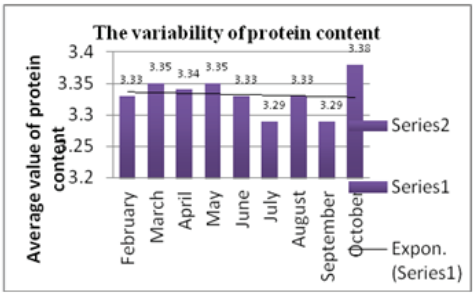


Figure 2. The variability of the protein content

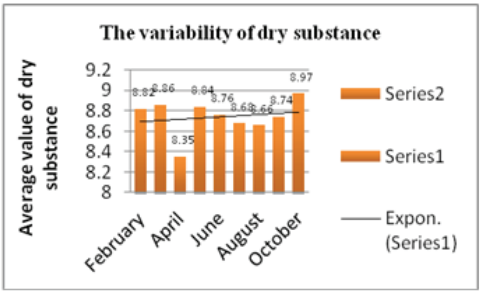


Figure 3. The variability of SNF content

The average value of the dry portion of the cows' light milk substance in 9 months is: 8,82% in February, 8,86% in March, April, 8,35% 8,84% in May, in June, the 8,68 8,76% in July, in August, 8,66% 8,74%, in September, 8,97% in October.

Cow's milk has a Newtonian behavior that depends on pH, in the sense that an increase or decrease in the pH of the milk causes an increase in the volume of casein micelles. [11].

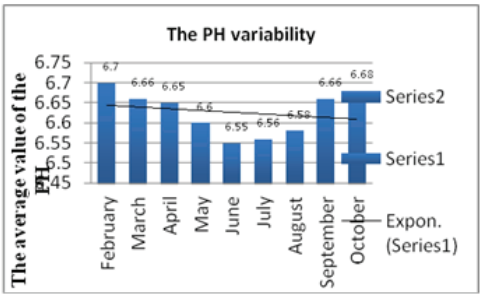


Figure 4. The variability of PH

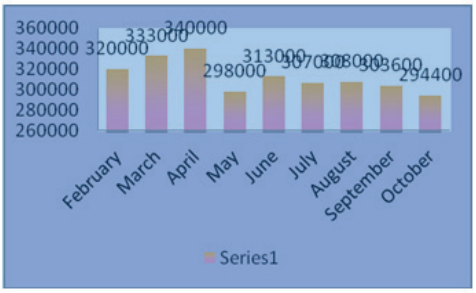


Figure 6. The variability of somatic cells number

The average value of pH of cow's milk caused by GARANIYA and associations in 2012, states that the pH value varies according to lactation, but remains around 6,62, the same values that it has been obtained in the present study.

The variability of the density of cow's milk according to lactation remains at around 21,904 g/cm³. The density (mass/volume) is dependent on the temperature at the time of determination, material composition, particularly in fat content, air inclusions.

The density varies between limits of l431-1,033 g/cm3.

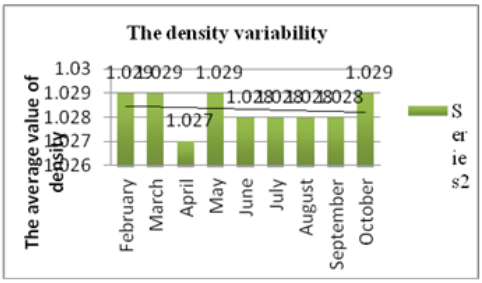


Figure 5. The density variability

As a result of the study on the quality of raw milk in the researched holdings regarding the evaluation of somatic cell counts (NCS), it is found that the samples analyzed milk fall in the standard of quality set by European Regulations 853/2004, 854/2004 and 2076/2005 and 479/2007.

The highest value registered was 340,000 M/m in April, and the lowest value was 294.400/ml NORTON in October.

On this basis, the correlation between the number of somatic cells equal to or greater than 400,000 cells/ml of milk and the presence of infection in cow's milk, has been determined using the test, Fisher associations (p = 0,2), under different production systems.

On this basis, the correlation between the numbers of somatic cells equal to or greater than 400,000 cells/ml of milk and somatic cell count was accepted as quantitative index in the case of mastitis in cows or to indicate the level of irritation of the mammary glands. [12, 13]

Among the diseases that can be transmitted through human milk consumption, include enterocolitis caused by salmonella, brucellosis, Q fever, listeriosis, toxoplasmosis, streptococe and stafilococe infections caused by Campylobacter. [8, 9]

Most dispersed agents, which causes mastitis, are: *Streptococcus agalactiae, Streptococcus pyogenes, Streptococcus dysgalactiae, Streptococcus zooepidemicus, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus pyogenes, Yersinia pseudo-tuberculosis, Enterobacter cloacae, Pseudomonas aeruginosa, Clostridium perfringens,* tipul C*, Corynebacterium ovis, Bacillus cereus, Klebsiella pneumoniae* şi *Mycopasma putrifaciens*. [16, 17]

**The variability of the results of physicchemical and microbiological milk sheep indices**

The type of diet can influence both the quantity of milk and milk quality indices. The milk fat may be influenced in terms of quantity and consistency. Reducing the fat content of the milk may also be caused by the lack of an adequate level in the diet, composed of cellulose by the presence of large amounts of food rich in unsaturated fatty acids and ruddy acidosis.

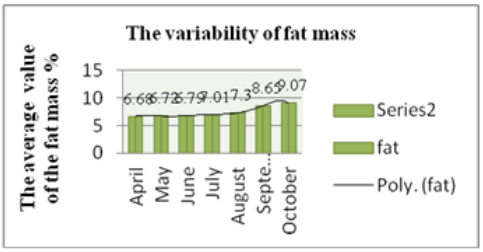


Figure 7. The variability of fat mass

Nutritional status, endocrine and physiological affects milk production and composition for shorter or longer periods.

Total protein from sheep's milk is higher in autumn than in the summer, in addition, the total content of protein depends on breed, researches conducted by the specialists' reveale that the highest concentration of the milk proteins has the milk of Turcana's breed.

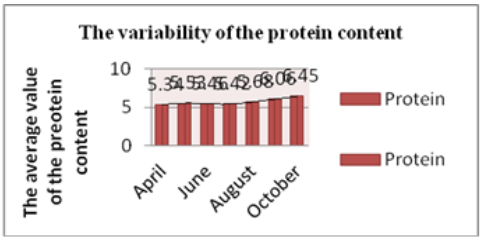


Figure 8. The protein content variability

Due to the introduction of good quality hay and fodder beet forage ration in dementrial of green mass, the non-fat solids record a maximum in winter (12%), the difference compared to the value recorded in summer being 10,81%.

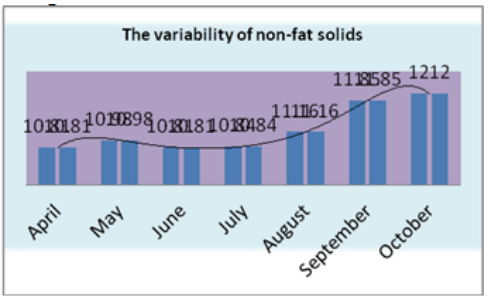


Figure 9. The variability of SNF

Density of milk is used for the conversion of mass to volume and vice versa, for estimating dry matter content, for calculating other properties, such as the kinematic viscosity.

The minimum density of sheep milk is determined by 1,032 + 0, 009 mg in the months of June and July.

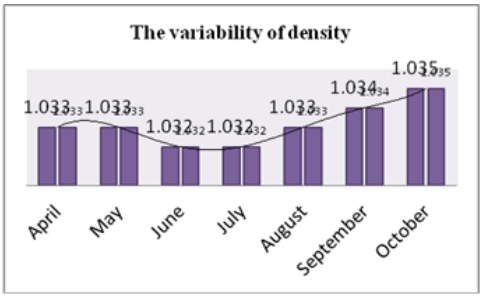


Figure 10. The density variability

As a result of researches carried out on the herd of sheep a minimum value of pH was registred for 6,58 milk in May, and in October of 6,70.

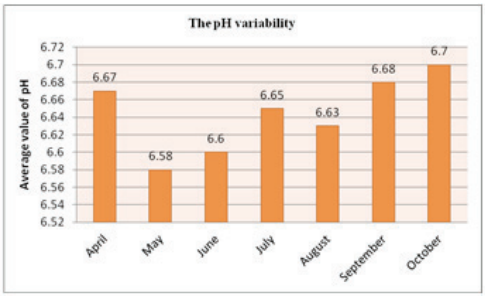


Figure 11. The PH variability

Some of them consider that the appeareance of intramamare infections with nonhemolitic staphylococci is random; others argue that these infections may become chronic and lead to irritation of the udder, increasing the number of somatic cells and decreased milk production. [5, 6].

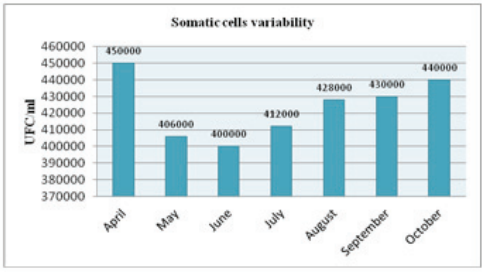


Figure 12. The variability of somatic cells number

## CONCLUSIONS

The results obtained in this study show us that the breed most suitable to be developed and exploited for milk production livestock in the mountain area is sheep breed.

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# SOME IMPORTANT QUALITY PARAMETERS OF PORK MEAT-BIODEGRADABLE PACK SYSTEM MONITORING AT REFRIGERATION STORAGE

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***Abstract***

*The quality of fresh meat is an extremely important characteristic influencing the consumer's purchase decision. The behavior of biodegradable tray in contact with meat is important also for food chain logistic management. The stability of this system meat-biodegradable tray is depending by a variety of factors and can be monitored through physic-chemical and microbiological assessment of meat on the one hand and tray material behavior on the other hand. The effect of chilling temperature on both meat and tray material have been experimented in this work. For this purpose, Longissimus dorsi from pork carcass was packed in biobased trays and stored at 4°C ±1 for 7 days. During the chilled storage physic-chemical analysis as pH, color, dry matter content, $a_w$, titratable acidity, presence of hydrogen sulfide, ammonia in the free state and microbiological analysis as total plate count, number of yeasts and moulds, coliform bacteria, the enterobacteriaceae, Salmonella sp., also thermo gravimetric analysis (TGA-DTG), differential scanning calorimetry (DSC), dynamic mechanical analysis (DMA) and Fourier transform infrared spectrometry (FTIR) of biobased trays were performed. Thermal analyzes showed no changes in the properties of polymeric materials used in the manufacture of food trays. FTIR and TGA analysis revealed the occurrence of some deposits consisting especially in moisture and organic material on the inside of the food trays.*

***Keywords:*** *meat quality, biodegradable trays, meat shelf life, refrigeration storage.*

## INTRODUCTION

Consumers find pork meat a very important food product because of its high nutritional value. *Longissimus dorsi* it is specifically important, from a scientific point of view, because of its uniformity, which helps getting very accurate results from different analyzes. Another important issue regarding pork meat is the preservation by freezing or chilling and the effects of low temperatures on meat. For a conclusive set of results, the meat was stored in biodegradable trays, as if it was on the commercial shelf. The behavior of biodegradable tray in contact with meat is important also for the food chain logistic management. The stability of this system meat-biodegradable tray is depending on a variety of factors and can be monitored through physical - chemical and microbiological assessment of meat on the one hand and tray material behavior on the other hand. In this paper, some important parameters have been assessed in order to establish both meat quality and biodegradable material in contact with meat behavior in certain storage conditions.

## MATERIALS AND METHODS

In this study, the system *Longissimus dorsi* pork muscle – biodegradable tray was analyzed in different conditions of low temperature preservation.

The meat was analyzed fresh as control, after 3 and 7 days of refrigeration (chilling) storage at 4°C, and after the freezing storage at - 18°C for 8 months, during which the meat was analyzed at the 1st, 2nd, 3rd, and the 8th month.

**Physical – chemical analysis**

In order to establish the degree of freshness of meat samples during the storage period, a certain number of physical-chemical analysis were performed.

- *Preparation and characteristics of watery extract.* For the preparation of the watery extract, 10 g of meat were put in an Erlenmeyer, along with 100 ml of distilled water for 15 minutes. They were given 2-3 shakes then filtered. Filtration time is shorter if the pork meat is fresh. If the product is fresh, filtering is done in a continuous jet and takes about 5 minutes. The filtration efficiency (the ratio between the amount of filtered and distilled water initially added) is 90 - 95%. For a fresh product, the watery extract has the following characteristics: it is clear, has clear pink color and specific odor.

- *Determination of the free ammonia with the Nessler reactive.* The free ammonia inside the watery extract forms with the Nessler reactive a yellow precipitate that shows the degree of alteration.

**-***Determination of dry matter.* Determination of the dry matter with the Precisa XM 60 thermal balance represents a fast and reliable determination of moisture content using thermo gravimetric principle. Thermogravimetry represents weighing the sample before and after heating to determine the moisture content by difference.

Sample preparation: the sample is prepared at the time of measurement. This prevents moisture exchange with the environment. Weigh 5 g of the sample, which are distributed in a thin and uniform layer over the entire surface of the dish.

- *Determination of $a_w$. $A_w$* index is a measurement of the energy status of water in a system. In this study, $a_w$ was determined with a NOVASINA system, which is very easy to use and provides both the $a_w$ index value and the temperature of the sample.

Sample preparation: using a spatula, pork meat samples were placed in boxes fitted with thermostatic apparatus for determining $a_w$, so the box is covered completely with the sample, and filled only halfway to avoid contact between the sample and the filter covering the reading sensor.

- *pH determination.* The samples′ pH was determined after each storage time with an INOLAB 720 WTW series pH-meter with an automated temperature compensator equipped with an insertion electrode. The electrode was inserted into the sample vertically, so that the glass electrode′s membrane is entirely in contact with the sample and was maintained until stabilization of pH on the screen. When the reading is over, the pH value is displayed. Three readings were taken at different positions in each sample.

- *Determination of titratable acidity.* Acidity of different products can be determined by titration of their watery, or other solvent solutions, with sodium or potassium hydroxide.

**Microbiological analysis**

*The total plate count* was determined using the SR ISO 4833-94 standard. The analysis method includes the following steps: 10 g of each sample were placed in Erlenmayer with 90 ml of distilled water. From the sample thus obtained 1 ml was placed in 9 ml of distilled water, producing the 1st decimal dilution. More decimal dilutions were produced, different for each sample. 1 ml of each dilution was inoculated, in duplicate, on RIDA COUNT type petrifilms. Petrifilms were incubated aerobically for 72 hours at a temperature of 30 ° C and then grown colonies were counted on each plate. The aerobic count of each sample was determined by applying the formula:

$$N = \frac{\sum C}{(n_1 + 0{,}1n_2)d}$$

where:

N = the microorganisms load in 1 ml of sample;

$$\sum C$$

=total number of colonies on the held plates;

$(n_1 + 0{,}1n_2)$ = total number of held plates, with the same dilution;

d = the first applied dilution;

The same method was used for the determination of coliform bacteria, *Enterobacteriaceae*, *E. coli* and *Salmonella sp.*

**Color determination**

The color of the food is a very important attribute in the food industry because food

products are frequently purchased by their color. Many of the consumers believe that if a food product looks good and has an appealing color, this means it′s safe to consume. Color measurements are made to determine the food quality.

After each storage period sample color evaluation was performed after opening the package using a MINISCAN$^{TM}$ XE PLUS colorimeter connected to a computer provided with the Universal Software V.4.01. The following settings were used for color index calculations: D65 illuminant, a 10º observer angle, LAV vision range and the CIELAB′76 color system. The *L**, *a** and *b** color indexes were obtained from the average values of ten readings on the surface of *longissimus dorsi* samples placed in the glass recipient provided by the colorimeter. The samples were then placed in the colorimeter′s reflectance port and color measurements were taken from ten different positions (Viana et al., 2005).

**Packaging Trays Behavioral analysis methods**

During this experiment thermal analysis were performed on the biodegradable trays which have been used for the packaging of the pork meat samples. The trays material was made as "sandwich" from 2 different components as follow: first layer – 10% PLA, second layer – 80% [(PLA-Ecoflex)- wood fiber - (50:50):15 wt%] and the third layer – 10% PLA. The brand name Ecoflex produced by BASF company is consisting of biodegradable aliphatic-aromatic copolyesters.

The TA Q2000 apparatus was used for differential scanning calorimetry (DSC). Differential scanning calorimetry (DSC) it′s a technique in which the heat needed to raise the temperature of the reference zone is measured in terms of time. Both the initial sample and the reference are kept at the same temperature through the whole experiment. The reference zone must have a heat capacity well-defined in scanner′s temperature range. DSC is used to confirm the formation of a complex in the solid state (Mourtzinos I. et al., 2007).

The thermo-gravimetric analysis (TGA) is a method that determines mass changes in a controlled atmosphere, under the effect of temperature. This analysis uses the TA Q5000 IR machine and is based on 3 accurate measurements: mass, temperature and temperature changes. The thermo-gravimetric analysis is a process that uses heat and stoichiometric ratios to determine the solution′s mass percentage. The mass loss is proportional to the increase of temperature, which in some cases goes above 1000 °C.

The dynamical mechanical analysis (DMA) uses the DMA Q800 machine and is used to study and characterize the mechanical properties of different materials, especially polymers. This is the best way to determine the visco-elasticity in the polymer matrix.

Fourier transform infrared spectroscopy (FTIR) is a technique which is used to obtain an infrared spectrum of absorption, emission, photoconductivity or Raman scattering of a solid, liquid or gas. An FTIR spectrometer simultaneously collects spectral data in a wide spectral range. This confers a significant advantage over a dispersive spectrometer which measures intensity over a narrow range of wavelengths at a time (Krishna G.M. et al, 2013).

## RESULTS AND DISCUSSIONS

**Physical – chemical analysis**

The results are shown in Table 1 and 2.

Over the refrigeration period the pH decreased from 5.97 initially to 5.77 after 7 days. During the freezing period the pH increased to 5.92 after 1 month, 6.03 after 2 months, 6.19 after 3 months. After the whole 8 months period, the pH decreased to 5.73.

The determination of the free acidity of pork meat samples during the refrigeration and the freezing periods showed a slight decrease.

Table 1. Values of physical-chemical analysis

| Period of cold storage | pH | $H_2S$ | Acidity (g oleic acid/100g ) | $NH_3$ (Nessler react) |
|---|---|---|---|---|
| Chilled samples | | | | |
| Control | 5.97 | Negative | 0,18612 | Negative |
| 4 days | 5.78 | Negative | 0.25116 | Negative |
| 7 days | 5.77 | Negative | 0.16926 | Positive |
| Frozen samples | | | | |
| 1 month | 5.92 | Negative | 0.37224 | Negative |
| 2 months | 6.03 | Negative | 0.24816 | Negative |
| 3 months | 6.19 | Negative | 0.1974 | Negative |
| 8 months | 5.73 | Negative | 0.24024 | Negative |

The fresh sample recorded a value of 0.18 g of oleic acid/100 g of product, while after 7 days of refrigeration the value decreased to 0.16 g of oleic acid/100 g of product.

During the freezing period the overall acidity was higher than the fresh sample, ranging from 0.37 g of oleic acid/100 g of product after 1 month, to 0.24 g of oleic acid/100 g of product after 8 months.

The results indicated that the meat samples kept their freshness during the experiment, except on the 7th day of refrigeration, in which case the meat showed signs of alteration ( through the Nessler reactive index which was positive).

The determination of aw index of pork meat samples has shown that during the refrigeration the $a_w$ index slightly increased, while during the freezing period the aw index decreased. Thus the fresh sample recorded a value of 0.973, while after 7 days of refrigeration the sample had a value of 0.976. During the freezing period the $a_w$ value decreased from 0.970 after one month to 0.950 after 8 months (see Table2).

The determination of dry matter in pork meat samples showed that during the refrigeration the dry matter percentage increased, while during the freezing period it decreased. Thus the fresh sample had a value of 71.21% dry matter, while after 7 days of refrigeration it increased to 82%. During the freezing period the meat samples recorded a value of 77.54% after 1 month, decreasing to 71.41% after 8 months (Table 2).

Table 2. Values of physical-chemical analysis

| Period of cold storage | Watery extract | Super natant | $a_w$ | Dry matter |
|---|---|---|---|---|
| Chilled samples | | | | |
| Control | Clear, normal, pink | Clear, yellow | 0.973 | 71.21% |
| 4 days | Clear, normal, pink | Clear, yellow | 0.973 | 83% |
| 7 days | Turbid, dark pink | Turbid, yellow | 0.976 | 82% |
| Frozen samples | | | | |
| 1 month | Clear, normal, pink | Clear, yellow | 0.970 | 77.54% |
| 2 months | Clear, normal, pink | Clear, yellow | 0.966 | 72.26% |
| 3 months | Clear, normal, pink | Clear, yellow | 0.960 | 76.07% |
| 8 months | Clear, normal, pink | Clear, yellow | 0.950 | 71.41% |

**Microbiological analysis**

The results of the microbiological analysis for the total plate count are shown in Figure 1.

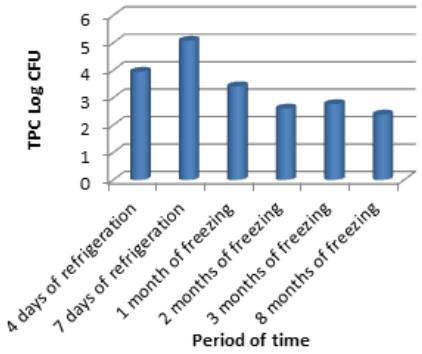


Figure 1. TPC evolution

It can be observed a slight decrease of CFU in all the samples except the fresh one in which case there were no CFUs at all.

The results of the microbiological analysis to determine the yeast CFUs are shown in Figure 2. It can be observed a slight increase of CFU during the refrigeration period, while during the freezing period the CFU value decreased.

Yeast was also present in all of the samples except the fresh one.

Molds were present in all of the samples but under the countable values in the first dillution.

Coliforms were only present in the meat samples that were analyzed after 4 and 7 days of refrigeration, and after 1 and 3 months of freezing.

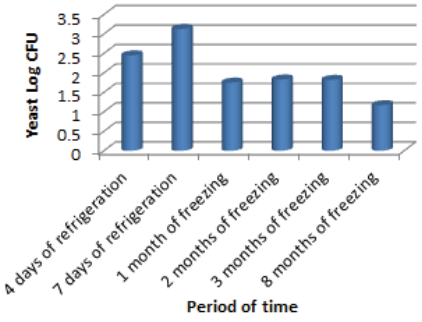


Figure 2. Yeast count evolution

The enterobacteriaceae analysis showed CFUs only after 7 days of refrigeration and after 1 month of freezing.
*E. coli* and *Salmonella* were not present in any pork meat sample during the monitoring period.

**Color measurements**

The values determined with the Hunter Lab colorimeter for L*, a* and b* are showedin Table 3 and they are graphically represented in Figure 3.Color measurements showed very little difference between fresh meat and stored meat. Some differences were noticed among samples related to yellow and red intensity, most probably due to heterogeneity of the sirloin fat content.

Table 3. The values of L*, a* and b

| Period of cold storage | L* | a* | b* |
|---|---|---|---|
| Control | 36,57 | 10,77 | 12,03 |
| 4 days | 37,28 | 11,42 | 11,88 |
| 7 days | 34,51 | 10,21 | 11,5 |
| 1 month | 39,15 | 12,1 | 15,54 |
| 2 months | 36,06 | 10,09 | 12,42 |
| 3 months | 28,8 | 10,46 | 9,06 |
| 8 months | 37,47 | 9,26 | 10,88 |

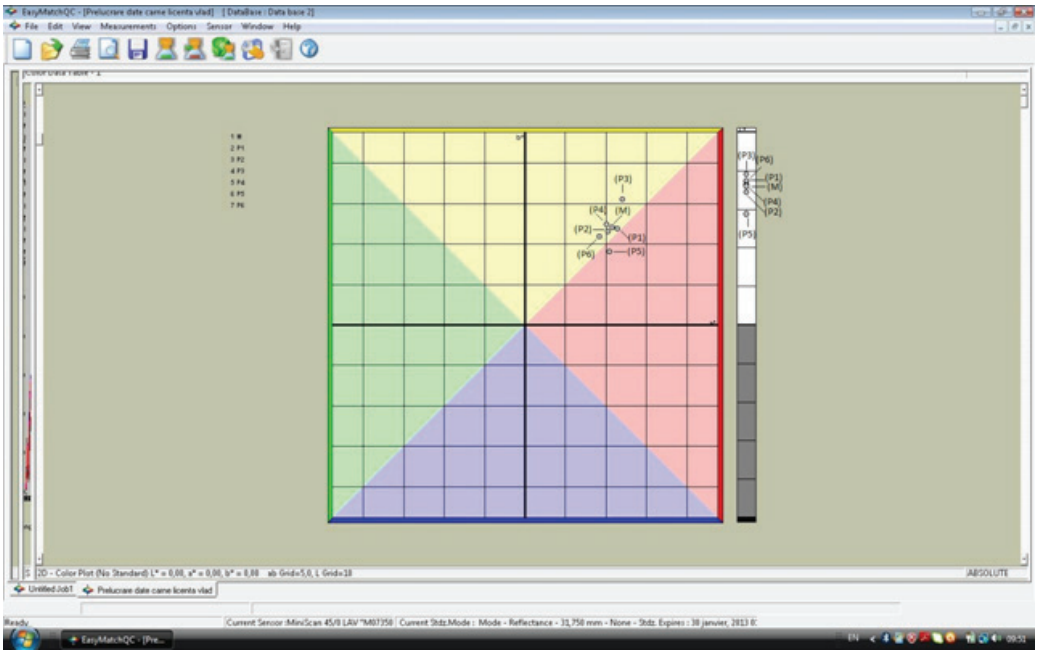


Figure 3. Graphic representation of L*, a*, b* values, according to Universal Software V4.01 MiniScanTM XE Plus program, for longissimus dorsi meat samples during the assessment period. M – fresh meat sample (control); P1 – 4 days of refrigeration sample; P2 – 7 days of refrigeration sample; P3 – 1 month of freezing sample; P4 – 2 months of freezing sample; P5 – 3 months of freezing sample; P6 – 8 months of freezing sample

However, in the graph presented in Figure 3, it can be observed that the values for a* and b* are grouped around the control for the chilled meat samples and slightly different positioned from control for the frozen meat samples.

**Biodegradabile food tray analysis**

The results for thermo – gravimetric analysis (TGA) of the biodegradable food trays are graphically represented in Figure 4 for the refrigerated samples, respectively in Figure 5 for the frozen samples.

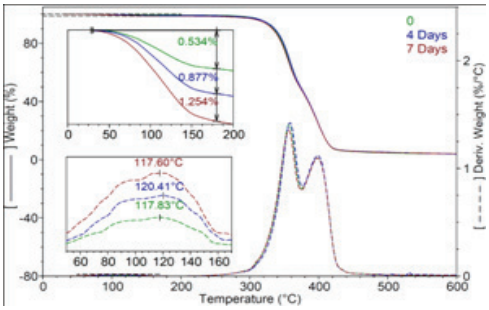


Figure 4. Standard TGA results of FT-Sandwich: 0 (reference), 4 D and 7D, refrigerated for 4 and 7 days.

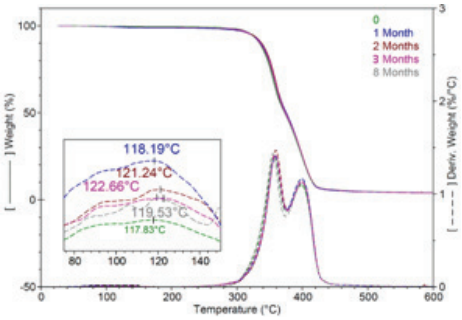


Figure 5. Standard TGA results of FT-Sandwich: 0 - reference, 1, 2, 3, 8 months.

In Figure 6 are represented the values obtained by analyzing the refrigerated biodegradable food trays with Differential Scanning Calorimetry (DSC). Also, the values obtained for the food trays that were kept at - 18ºC are graphically represented in Figure 7.

The differences between samples in terms of specific heat (ΔCp), enthalpy of cold crystallization (ΔHc) and total melting enthalpy (ΔHm) measured by DSC are probably due to absorption of water during your time spent in contact with meat.

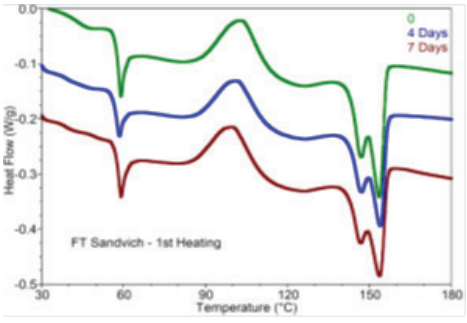


Figure 6. DSC curves of FT-Sandwich: 0 (reference), 4 D and 7D, refrigerated for 4 and 7 days

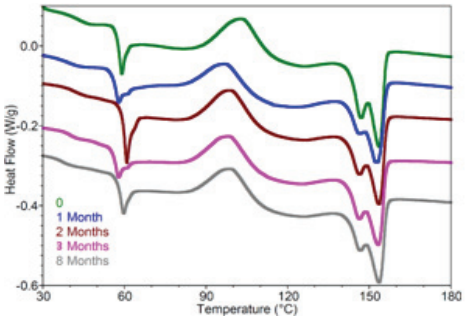


Figure 7. DSC curves of FT-Sandwich – 0 - reference, 1, 2, 3, 8 months

In the figures below (Figure 8 for the refrigerating period and Figure 9 for the freezing period) are graphically represented the values obtained after analyzing the biodegradable food trays from dynamic mechanical point of view.

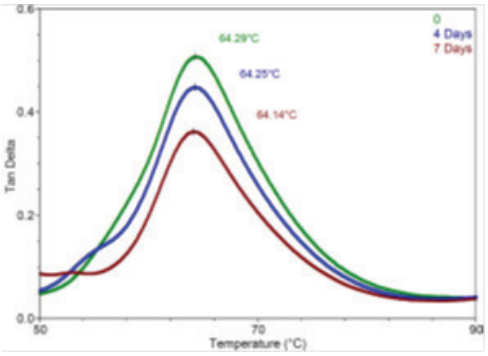


Figure 8. Graphic representation of Dynamic Mechanical Analyses (DMA) (Loss Factor – tan δ)

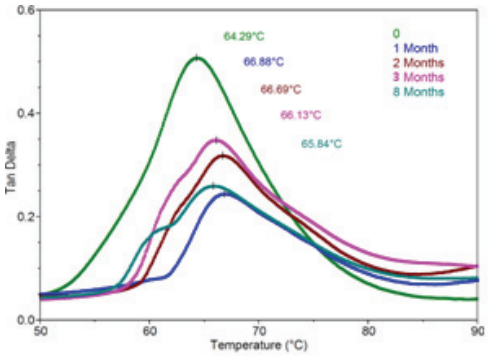


Figure 9. Figure DMA curves (Tan Delta) of FT-Sandwich: 0 - reference, 1, 2, 3, 8 months

The results for FT-IR analysis performed on the biodegradable trays are presented in the figures below. In Figure 10 are presented the results of

the analysis performed during the refrigeration storage time, respectively in Figure 11 are presented the results of the analysis performed on the food trays during the freezing storage time. FTIR analysis carried out on trays with frozen meat showed the occurrence of a layer that contains water and organic materials probably due to microbial cultures formed on the surface (peaks 3288cm-1, 1653 and 1544 cm−1), fact confirmed by thermal analysis which showed an increase in weight loss of around 120ºC.

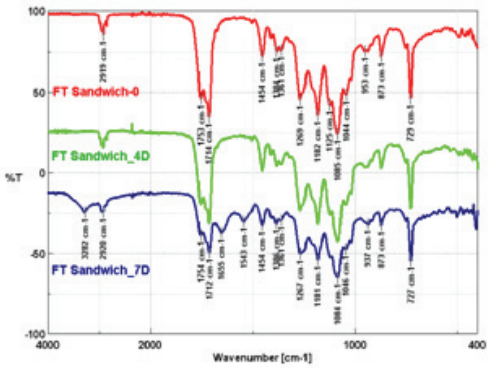


Figure 10. FT-IR Patterns of FT-Sandwich (0 - reference, 4D and 7D - with refrigerated meat for 4 and 7 days)

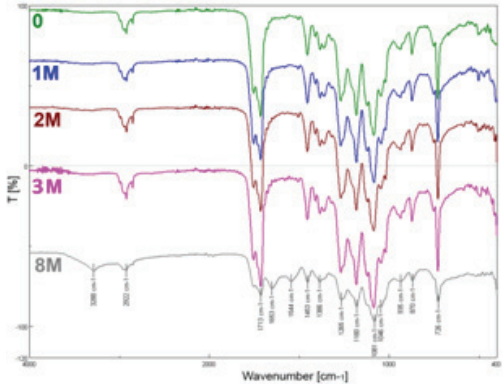


Figure 11. FT-IR Patterns of FT-Sandwich: 0 - reference, 1, 2, 3, 8 months

## CONCLUSIONS

Temperature is one of the major factors affecting physical chemical and microbiological quality of meat.

Storage at chilling temperature (4$^{o}$C) of the meat packed in trays for minimum 4 days according to present study, showed no significant quality degradation.

Storage at freezing temperature (-18$^{o}$C) for 8 months showed no significant quality changes from physical chemical point of view and an improvement for the microbiological parameters.

Thermal analysis (TGA, DSC and DMA) carried out on "Sandwich" Food Tray Prototype have shown that meat storage conditions did not lead to significant changes in terms of thermal degradation, glass transition, cold crystallization temperature or melting temperature. Any small differences between the analyzed samples are most probably due only to material heterogeneity.

## ACKNOWLEDGMENTS

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# EVALUATION OF FRUCTAN CONTENTS IN THE TAPROOTS OF PLANTS *LACTUCA SERRIOLA* L. AND *SONCHUS OLERACEUS* L.

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***Abstract***

*The current research aimed to present the evaluation of the underground parts of two widespread plants in Bulgaria- prickly lettuce (Lactuca serriola L.) and annual sow thistle (Sonchus oleraceus L.) as a source of inulin-type fructans. The sequential ethanol and water extractions from their dry taproots were carried out. The amount of extracted fructans was defined by the resorcinol assay. The fructooligosacharides and inulin contents of the obtained extracts were analyzed by TLC and HPLC-RID methods. The total fructan content in the weed plant Sonchus oleraceus L. (19.6% dw) is higher than the fructan level in the roots of Lactuca serriola L (9.56% dw). In the ethanol extracts were observed the presence of monosaccharide glucose and fructose, high level of sucrose and trisaccharides 1-kestose. In the result of the carried analysis, we can conclude that the roots are rich source of fructans as the fructoligosacharides fraction dominates in ethanolic extracts. These plants could not only be consider as weeds, but it have to pay attention to their future possibility to be used as a potential source of fructooligosacharides with prebiotic effect in nutrition formula for animals and human.*

***Keywords:*** *fructoligosacharide, inulin, Lactuca serriola, Sonchus oleraceus.*

## INTRODUCTION

Inulin is a polydisperse plant polysaccharide, member of fructan family, consisting mainly of β-(2→1) fructofuranosyl units ($F_m$), and a terminal α-glycopyranose unit (1→2) ($GF_n$) (Van Laere et al., 2002). The degree of polymerization (DP) of inulin varies from 2 to 70 (De Leenheer et al., 1994). Molecules with DP<10 are called oligofructoses or fructooligosaccharides (FOSs) (Figure 1) and they are a subgroup of inulin (Niness, 1999).

Inulin and FOSs are classified as soluble dietary fiber. They act as prebiotics, because stimulate growth of *Bifidobacteria.* Inulin is only hydrolyzed in small amounts in the stomach. In large intestine it is fermented by intestinal microflora into short-chain fatty acid (SCFA), lactic acid and gases (Gibson, 1995, Knudsen, 1995). Inulin-type prebiotics reduce blood levels of triglycerides (Roberfroid, 2005); prevent cardiovascular disease and osteoporosis (Delzenne, 2002). Inulin is helpful in the management of diabetes and blood sugar-related illness (Rumessen, 1998). In recent issues, inulin is presented as immunomodulator and anticancer agent (Barclay et al., 2010).

Depending on the conditions of extraction and the type of used raw material, a short-chain bioactive molecules (FOSs) or long-chain ones (inulin) could be achieved. Both they have different bioactivity as no digestible oligosaccharides of long chain length are typically less biodegradable than compounds of shorter chain length. Van Loo (2007) proposed that a combination of short-chain and long-chain fructans is physiologically more active that the individual fractions.

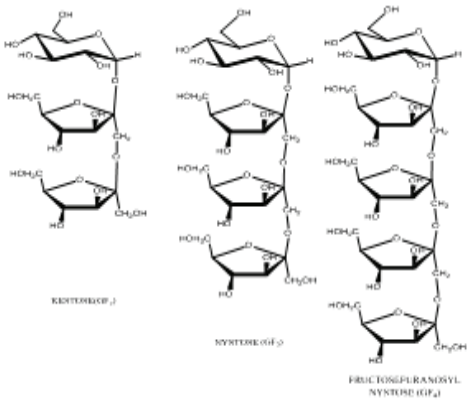

Figure 1. Chemical structure of fructooligosaccharides

Inulin serves as a reserve carbohydrate in underground part of the *Compositae* (*Asteraceae*) plants such as *Cichorium intybus, Inula helenium and Helianthus tuberosus* (Van Laere et al., 2002). Prickly lettuce (*Lactuca serriola*) and annual sow thistle *Sonchus oleraceus* L. also belong to this plant family.

Prickly lettuce (Lactuca serriola L.) is an annual or biennial plant, slightly foetid, that is commonly considered as a weed of orchards, roadsides and field crops. Many species in Lactuca are medical herbs, as well as wild vegetable. Scientists focused their research interest on searching for some promising compound with effectiveness and low toxicity for the benefit human`s health (Ren et al., 2004). The plant can be eaten as a salad, although it has a bitter taste. The young leaves can be eaten raw or cooked (Kleonikos, 2006)

*Sonchus oleraceus* L. is growing in cultivated fields and disturbed sites, ditch banks, bottomlands, city lots and alleys (Reaume, 2010). *Sonchus* wild food plants might be applicable in natural medicine and healthy food. *Sonchus oleraceus* L. and *Sonchus* sp.pl. eaten in several Italian regions, are cholagogue and laxative agents, due to their sesquiterpene lactones but also the high content of vitamin C, carotenoids and fatty acids of type ω-3 (Aliotta, 1981, Guil-Guerrero, 1998). In China, Sonchus wild vegetables are used mostly in infusion or decoction and are administered to treat acute icterohepatitis, cancer, inflammation, rheumatism, diarrhoea and snake venom poisoning (Dao et al., 2011). The underground roots of sow thistle store reserve carbohydrates, and inulin is the major storage carbohydrate in them (Lemna et al., 1990).

Figure 2. Photos of prickly lettuce (*Lactuca serriola* L.) and annual sow thistle (*Sonchus oleraceus* L.)

The variety *S. arvensis* can be used as a livestock feed and is considered to be highly nutritious for rabbits (Szcza-wenski et al., 1978). Boulos (1973) stated that *S. arvensis* roots can be used as a coffee substitute when is roasted.

According to Jana et al. (2010) the prebiotic effect of the *Taraxacum officinale, Sonchus oleraceus* and *Asparagus sprengeri* extracts on *L.lactis* and *L. reuteri* was higher than or equivalent to inulin - a commercial prebiotic, as *Sonchus oleraceus* exhibited the best prebiotic effect. It was the only plant to stimulate all the probiotics including *B. longum*.

In this context, the paper present an analysis of the fructooligosaccharides and inulin content in the roots of *Lactuca serriola* L. and Sonchus oleraceus L. from Plovdiv region of Bulgaria in order to study their inulin-type fructan content. This investigation aimed to present that these weeds can be potential and unstudied source of prebiotics.

## MATERIALS AND METHODS

The roots of *Lactuca serriola* L. and Sonchus oleraceus L. were collected from Thracian valley near to Plovdiv (Bulgaria) during the months September and November in 2012 year. The underground parts were dried and ground into a fine powder.

All used reagents and solvents were of analytical grade scale. Carbohydrate glucose, fructose, sucrose, together with high purity 1-kestose and nystose, used as standards for the identification of low molecular weight oligomers have been purchased from Sigma-Aldrich (Steinheim, Germany). Fructooligosacchrides Frutafit®CLR, HD 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®HD - with the average chain length of 8-13 monomers. Frutafit®TEX was characterized with mean degree of polymerization DP 22. Inulin Raftiline®HP (DP~25) was purchased from Orafti (Belgium).

Moisture content of the dried ground roots was determined according to AOAC 945.32.

Dried roots of weed plants were extracted in a Soxhlet apparatus successively with hexane,

$CHCl_3$, and ethyl acetate to remove phenolic and lipophilic compounds (Olennikov et al., 2009). Then the residue of roots was dried and the extraction process was carried as follows: 0.45 g dry sample (roots) was put into a round bottom flask and was extracted three times with 95% (v/v) boiling ethanol. For the first and the second extraction, 40 ml 95% (v/v) ethanol were used and 20 ml for the third one. The duration of each extraction procedure was 60 minutes. The extracts were collected in 100 ml volumetric flask. The low-molecular carbohydrate fraction composed of fructose and FOSs was obtained in the ethanol extracts. For extraction of high-molecular fraction (inulin), the residue in the flask after ethanol extraction was extracted by three following extractions (40, 40, 20 ml) with boiling water as it was described above. The content of mono-, di-, oligosaccharides and inulin in the obtained extracts was analyzed by TLC in order to observe the extraction rate of fructans.

Thin-layer chromatography (TLC) of the obtained ethanol and water extracts from roots of prickly lettuce and annual sow thistle were performed on silica gel 60 $F_{254}$ plates (Merck, Germany) with *n*-BuOH:*i*-Pro:$H_2O$:$CH_3COOH$ (7:5:4:2) (v/v/v/v) used as a mobile phase. The spots were detected by dipping the plates into the solution with detecting reagent diphenyl-amine-aniline-$H_3PO_4$–acetone (1:1:5:50) (Lingyun et al., 2007) and heating at 120 °C for 5 min. As carbohydrate standards were used glucose, fructose, sucrose, 1-kestose, nystose, fructooligosaccharides (Frutafit CLR and HD) and inulin (Frutafit TEX and Raftiline HP) all of them in concentration 2 mg/ml. Thin-layer chromatograms were generated by densitometry measurement of obtained spots with QuantiScan Version 3.0 software (Biosoft).

The fructan contents in ethanol and water extracts were analysed spectrophotometrically at wavelength 480 nm by resorcinol-thiourea reagent (Pencheva et al., 2012). The experiments were carried out on a Camspec M107 Vis spectrophotometer (UK).

The sugars and FOSs content in ethanol extracts was analyzed by HPLC. Chromatographic separations were performed on HPLC Shimadzu, coupled with LC-20AD pump, refractive index detector Shimadzu RID-10A, a column Supelcosil LC-NH2 (Supelco®, Sigma-Aldrich, Bellefonte, PA, USA) with pore size 5 μm and degasser Waters In-Line −IF (Milfrd, MA, USA ). The separations were performed on an analytical aminopropyl silica column SUPELCOSIL LC-NH2 (250 x 4.6 mm i.d.) equipped with a guard column (2.5 x 4.6 mm i.d.) of the same filling. The mobile phase used for separation of glucose, fructose, sucrose and FOSs was acetonitrile/water (83/17 v/v). The column was placed into a temperature-controlled unit LCO 102 (ECOM spol. s.r.o., Czech Republic) maintained at 40 °C. All samples were filtered through a 0.45 μm filter. Injection volume of the sample was 20 μL and the flow rate of the eluent was 1.5 $ml.min^{-1}$with an isocratic mobile phase. Detection and identification of sugars and fructooligosaccharides were performed using RID detector that operated at 40 °C. 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).

## RESULTS AND DISCUSSIONS

The moisture content in the taproots of plants prickly lettuce was 8.46% and 10.41 % in the roots of annual sow thistle, respectively.

The results from determination of fructan content in the extracts from the underground parts of prickly lettuce and annual sow thistle were obtained by our developed ketose-specific spectrophotometric method with resorcinol reagent (Pencheva et al., 2012). On the base of our previous investigations of the extracts from dandelion, elecampane and topinambour, our observation during analysis have been shown high levels of low moleculecular fraction in ethanol extracts. Therefore, after ethanol pre-treatment of the samples in water extracts have been remained FOSs with longer chain length and inulin. The ethanol and water extracts obtained from *Sonchus oleraceus* L. (8.26 ±0.22 g/100 g dw and 11.30±0.09 g/100 g dw) contained big quantity of low molecular fraction than the same extracts obtained from *Lactuca serriola* L. The ratio between fructans in the ethanol and water extracts from roots of prickly lettuce is almost equal. Therefore, the low and high molecular fractions have been extracted at the same extent. In the result of our

study we can conclude that from both plants *Sonchus oleraceus* is richer source of FOSs and inulin than prickly lettuce (Table 1 and Figure 3).

Table 1. Fructan content in the extracts obtained from the taproots of prickly lettuce and sow thistle (g/100 g dw1)

| Plant type | Low molecular fraction (fructose, sucrose & FOS[1]) | High molecular fraction (inulin) | Total fructants |
|---|---|---|---|
| | | mean ± SD[3] | |
| prickly lettuce (*Lactuca serriola* L.) | 5.39±0.22 | 4.17±0.50 | 9.6±0.86 |
| *annual* sow thistle (*Sonchus oleraceus* L.) | 8.26±0.22 | 11.30±0.09 | 19.56±0.14 |

[1]dw – dry weight; [2]FOS – fructooligosaccharides; [3]SD – standard deviation

The obtained results from TLC analysis of the ethanol and water extracts from the roots of prickly lettuce and annual sow thistle showed that extraction process in triplicate was efficient. Almost all carbohydrates presented in the samples have been successively extracted during these sequential extractions with ethanol and water used as solvents. All ethanol extracts (from 8 to 11 and from 16 to 19) contained fructose ($R_f$ = 0.55), sucrose ($R_f$ = 0.48) and FOSs which are equivalent to standards Frutafit CLR (7-9 oligomers) and HD (8-13 oligomers). The TLC analysis of the water extracts from the roots (12, 13, 14, 15, 20, 21, 22, 23) showed the presence not only of mentioned above FOSs, but also these extracts contained high molecular fraction of inulin with DP, similar to these of used as standards Frutafit TEX and Raftiline HP (DP 22-25). The water extracts obtained from the roots of annual sow thistle contained also and sucrose ($R_f$ = 0.48) (Figure 3).


Figure 3. Thin-layer chromatography of fructans in 5 µl ethanol and 5 µl water extracts obtained from plants a) prickly lettuce (*Lactuca serriola* L.) and b) annual sow thistle (*Sonchus oleraceus* L.), standards 1-glucose, 2-fructose, 3-sucrose, 4 and 5-FOSs Frutafit CLR and HD, 6 and 7 - inulin Frutafit, TEX and Raftiline HP; 8, 9, 10, 11 – first, second, third and common ethanol extract from prickly lettuce; 12, 13, 14, 15 - first, second, third and common water extracts; 16,17,18 and 19 - first, second, third and common ethanol extracts; 20, 21, 22, 23 first, second, third and common water extracts from annual sow thistle.

The results obtained from densitometry analysis of the thin-layer chromatograms showed presence of high level of trisaccharides 1-kestose ($R_f$ = 0.37) and tetrasaccharide nystose ($R_f$ = 0.34) in ethanol and water extracts from the roots of prickly lettuce (*Lactuca serriola* L.) and annual sow thistle (*Sonchus oleraceus* L.). Except sugars fructose and sucrose, the extracts contained FOSs like commercial FOSs or inulin, used as standards. Solvent ethanol have been extracted FOSs until 9 monomer units (from GF3 to GF8). In the water extracts except FOSs with GF9 also dominate and high molecular inulin (Figure 4).

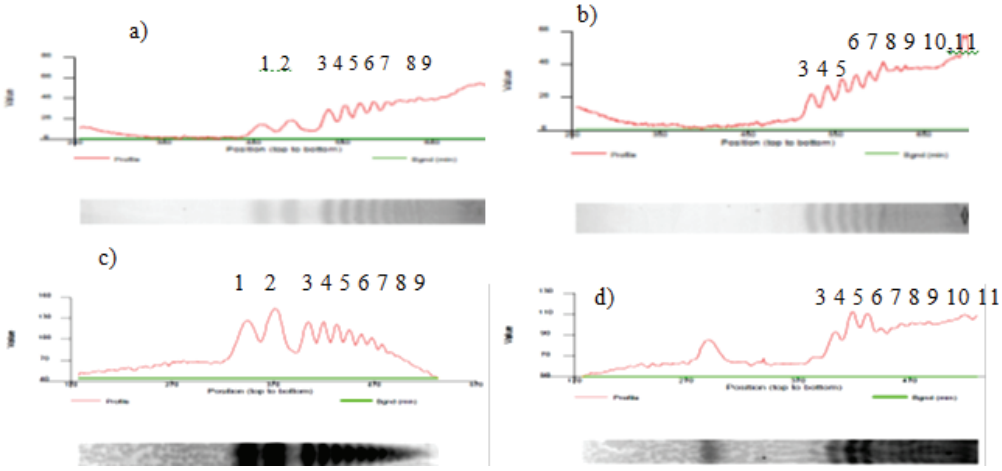


Figure 4. Thin-layer chromatograms of extracts from 5 µl a) ethanol and b) water extracts from roots of Sonchus oleraceus L. and 10 µl c) ethanol and d) water extracts from roots of Lactuca serriola L., where 1. fructose, 2. sucrose, 3.1-kestose (GF2), 4.nystose (GF3), 5.pentafructooligosaccharide (GF4), 6,7,8,9,10. fructooligosaccharides (respectively GF5, GF6, GF7, GF8, GF9) and 11. inulin

High-performance liquid chromatography with refractive index detection (HPLC-RID) has been widely used for determination of sugars and small oligosaccharides. After the ethanol extracts have been obtained from roots *Lactuca serriola* L. and *Sonchus oleraceus* L. these extracts have been analysed by the HPLC coupled with refractive index detector. These analyses help us to determinate the quantity of sugars and FOSs in their roots. The HPLC analysis proved the results obtained from the TLC analysis. The obtained chromatograms showed the presence of fructose ($t_R$=3,9 min), sucrose($t_R$=6,1 min), 1-kestose ($t_R$=14,1 min) and nystose ($t_R$=20,9 min) in the ethanol extracts and also showed the presence of glucose ($t_R$=4,7 min) in them (The HPLC chromatogram of *Lactuca serriola* was not shown) (Figure 5).

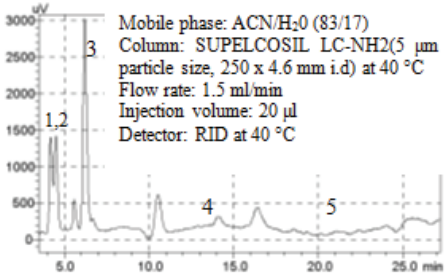


Figure 5. HPLC chromatograms of 95% (v/v) ethanol extracts of a) prickly lettuce (*Lactuca serriola* L.) and b) annual sow thistle (*Sonchus oleraceus* L.): 1.fructose, 2.glucose, 3.sucrose, 4.kestose and 5.nystose

The obtained results from HPLC analysis showed that the ethanol extract from roots of *Sonchus oleraceus* L. contained more 1-kestose and nystose (1.25 and 1.28 % dw, respectively) than prickly lettuce (Table 2).

Table 2. Mono- and oligosaccharides content (% d.w) in the ethanol extracts obtained from the roots of *Lactuca serriola* L. and *Sonchus oleraceus* L.

| Plant | fructose | glucose | sucrose | 1-kestose | nystose |
|---|---|---|---|---|---|
| prickly lettuce (*Lactuca serriola* L.) | 1.78 | 0.91 | 2.23 | 0.80 | 0.65 |
| sow thistle (*Sonchus oleraceus* L.) | 2.03 | 1.31 | 3.92 | 1.25 | 1.28 |

*Lactuca serriola* L. and *Sonchus oleraceus* L. contains in their roots high amount of frucooligosaccharides. The results of our research showed that the underground parts of annual sow thistle is rich source of trisaccharide kestose, tetrasaccharide nystose, FOSs and inulin. All these inulin-type fructans possess well-pronounced prebiotic effect. These taproots could be used in feed and foods to increase the dietary fiber content in them.

Our research explained and proved the statement of Jana et al. (2010) that *Sonthus oleraceus* L. possess the best prebiotic effect and stimulate growth of *B. longum*.

## CONCLUSIONS

The results from our analysis of the ethanol and water extracts obtained from the roots of *Lactuca serriola* L. and Sonchus oleraceus L. showed that these plants contain inulin-type fructan. Because of the absence of information in literature about the fructooligosaccharides and inulin contents in their underground parts for us it was a challenge to investigate these weed plants eaten as a salad in some countries in the world. The roots of annual sow thistle (Sonchus oleraceus L.) contains much more total inulin-type fructans (19.6 g/100g dw) than the roots of Lactuca serriola L. (9.56% dw) The levels of 1-kestose and nystose are higher in the ethanol extract of the annual sow thistle. The both plants are rich source of fructooligosaccharides that are in much more content in the ethanol extracts. The water extracts contain high molecular fructooligosaccharides and inulin. The findings of the current study showed that these two widespread weed plants are potential source of fructooligosaccharides (DP 3-5) and can be used as a new source of prebiotics that can find application in human or animal nutrition.

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# STUDY ON THE INTERACTION BETWEEN THE FOOD MATRIX AND THE METAL FOOD CANS

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***Abstract***

*The metal cans are used to preserve food and beverages for long periods of time, proving to be one of the most versatile packaging materials nowadays. Some of their vital functionalities are represented by ensuring that foods are not contaminated, providing physical protection and extending the shelf life of it.*
*Metal cans are protected on the inside by a thin coating layer, which can be affected by stroke. Thus, the canned food or beverage comes in direct contact with packaging metal. The coating is an epoxy lacquer that separates the liquid from the packaging material. Normally, the metal cans are affected by corrosion (primary, secondary or stress corrosion). However there are several factors which can accelerate the corrosion processes.*
*This study is conducted to determine the degree in which the damages caused by hitting causes the existence of substance traces in canned food or beverages (product contamination). While the final product is stored, the coating is damaged and the intensity and the type of damages are depending on the products characteristics, e.g. acidity, resulting higher pore-gaps in coating surface, which allow direct contact between the food and the metal from which the can is actually made (e.g. aluminium alloys, iron etc.) and also between the food and the coating epoxy resin.*
*In this paper were summarized the literature scientific approaches which revealed until now the chemical contamination occurring in canned food considering the packaging materials. This information have been completed by various tests carried out in specialized laboratories in order to determine aspects like: which type of mechanical actions generate damages to the cans coating integrity, the remanence of metals or other chemical substances within the product, the optimal variances of the internal coating, considering the various types of can strokes and other research evidence which may be needed. The measurement results are compared considering the food matrix remanence revealed through other researches (local, international). All in all, the study aims to offer a better approach regarding handling canned food and beverages without or with minimum damaging of their coating, in order to maximize the product's safety and guarantee its long shelf life.*

***Keywords:*** *canned food, corrosion, packaging materials, food matrix, chemical contamination.*

## INTRODUCTION

The damages made through the operations carried out on the production line or during the final storage phase, like transport, handling, bottling and can filling may alter the internal coating quality of the packaging cans. While the final product is stored, the coating is damaged due to the products characteristics, e.g. acidity, resulting higher pore-gaps in coating surface, which allow direct contact between the food and the metal from which the can is actually made (e.g. aluminum alloys, iron etc.) and also between the food and the coating epoxy resin.
These residual BPA and BADGE can be a potential contaminant to the food that is packed. When the cans are heated at high temperature as in case of commercial canning, BPA may leach out of can coating. Bisphenol A (BPA) and its condensation product with epichlorohydrin, bisphenol A-diglycidyl ether (BADGE) may remain unreacted if the curing process of lacquer coated can is insufficient (Mungia Lopez and Sato-Valdez, 2001).
Recent studies discovered many aspects regarding the connections between the materials from which the metal cans are made of and the food matrix. Thus were revealed a series of chemical changes that occur in canned food considering the packaging materials, such as the influence of aluminum particles which are migrating into the packed product or the Bisphenol A (BPA) toxic traces in food (a product found in the epoxy resins most commonly used as a protective lining in canned food and beverages). Some studies revealed that Bisphenol A diglycidyl ether traces were

discovered in fish tins. The Community legislation takes account of the new toxicological results (Regulation 1895/2005/EC) and sets a new, higher migration limit for BADGE and its hydrolysis products at 9 mg/kg/food, but for BADGE chlorohydrins it maintains a limit of 1 mg/kg/food (Barnes, K.A., et al., 2011). According to FERA′s research, the limit of dietary exposure of Melamine is 0.013 mg/kg body weight per day, and their highest recorded level of melamine was 0.33 mg/kg found in the coatings for can and jar lids for food packaging (Stollenwerk, J.D., 2012).

Metal can be considered to be a specific type of packaging-derived contaminant. Although there is no evidence that excess tin intake has any long-term health effects, some studies have shown that intake of high concentrations (more than 150 mg/kg in canned beverages; more than 250 mg/kg in canned fruit and vegetables) may cause short-term gastrointestinal problems. For most foods, this is of no significance, but for foods packed in cans with some unlacquered tinplate, high levels can sometimes occur. Tin dissolution in unlacquered tinplate cans is essential in that it confers electrochemical protection to the iron, which makes up the structural component of the can and so maintains the can's integrity. Without it, the can would quickly become corroded by its contents; this could cause serious discoloration, off-flavors in the product and swelling of the can.

Given all the factors mentioned above, were conducted three types of analyses aiming to establish the integrity level of lacquer.

## MATERIALS AND METHODS

In order to reveal the interaction between the food matrix and the metal cans were used special analyses tools and methods, including:

- Cans made of aluminum alloy or galvanized tray;
- Can lids;
- Chemical solutions, juices, beer, canned food, honey etc.;
- Liding machine (Seamer);
- Coating measuring device (Sencon);
- Induction meter (Sencon);
- Microscope with camera;
- Axial force measuring machine;

-ISM − Automatic spraying can machine (varnished); -gloves, protective glasses, scissors.

Batches of 2x10 boxes each have been analyzed. Using a special equipment, called Secon, they were classified in the proper coating category (1,2,3,4). The boxes have been filled with water or viscous solutions (food or chemicals) with different pH/acidity levels (according to the coating categories). Also, they were filled with corrosive chemical solutions (various concentrations of NaOH, CuSo4). The witness water-based solution should have a pH close to 7. Previously, the cans were marked and sealed with a lid (seaming), using the manual electrical-device from the laboratory.

After the storage period, the cans have been drained. In the laboratory have been measured, using the camera microscope, the internal size of the damage, the pores, in order to observe how the different solutions altered the coating and to conduct other relevant aspects (measurements of the coating integrity after storage).

Another Sencon device will quantify the electricity inductance through the damaged can and will generate reports that will help in determining what type of mechanical actions generate damages to the cans coating integrity.

Following the cans opening, samples have been analyzed by the chemical laboratory in order to measure the remanence within the product, for aluminum or other metals/chemical substances.

Also have been conducted tests in order to determine the optimal variances of the internal coating, considering the various types of can strokes.

These were made using the Sencon device, which determines the distribution of the interior lacquer over the can′s inner surface, returning the results in g/m2.

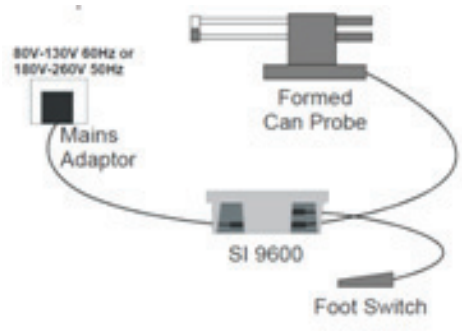


Figure 1. Lacquer measuring Sencon system

Considering A the capacitance between the probe tip and the metal of the can or sheet, which are separated by the lacquer, acting as the dielectric. The capacitance is given by the formula C = (ε x A) / d where ε is the dielectric constant of the lacquer, A is the probe surface area, and d is the lacquer thickness. By using the gauge as a comparator to a known calibration standard sample, both ε and A become constant. So variation in capacitance is due to the difference in d, which can thus be used to determine the lacquer thickness.

1) Determining the weight of the interior lacquer, using the analytical balance, by weighting the can before and after the lacquer operation.


Figure 2. Can measurements on the analytical balance

2) Measuring the integrity of the cans interior coating by measuring the conductivity using a saline solution (5%NaCl) and a measuring device (Auto EnamelRater).

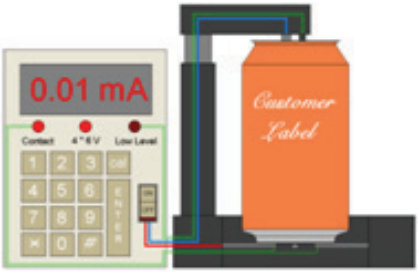


Figure 3 Auto EnamelRater – measuring the cans conductivity

Simultaneously have been analyzed the empty cans, without any lid or product inside, in order to see how the hits influence the axial endurance – respectively the cans resistance at these force actions. This parameter will also be monitored also by microscopic observation of the cans in order to see any damage over the cans (the aim is to obtain information regarding the behavior of the pallet stored can).

The measurement results have been compared considering the food matrix remanence revealed through other previous researches (local, international).

All measuring machines (except the manual ones) can generate reports, graphs, statistical data, regarding the measured product type, category, etc (having more options from which to select which are taken into the analysis).

3) Measuring the interior lacquer in 4 areas of the can (up, middle, base, bottom).The data obtain after the measurement are automatically stored within a server database. Thus, it can be obtained customized reports (e.g. daily, weekly, by type, by measurement area etc.).

## RESULTS AND DISCUSSIONS

The importance of measuring the integrity of the cans interior coating analysis is given by the fact that a can with high integrity of the interior coating represents a safe packaging material (the result should be close to zero).

```
Batch 3          13:04   02-Apr-01          Line 1
SODA POP         Lacquer 1 Epoxy 2603ST
=============================================================
Machine 1              Gun 1                Can 1

          Top of can       | Average
       8.6*   8.3    8.3   |   8.4
       8.4    8.2    8.2   |   8.3
       8.4    8.3    8.1*  |   8.3
  ------------------------------------
Average 8.5    8.3    8.2   |   8.3
Dome    8.1
```

Figure 4. Measurement results and mean values

Regarding the experiments the minimum values (near to zero at Auto Enamelrater) are the normal ones and the exponential increasing of values is directly linked to the deficiencies of the internal varnish (being the first part of experiment). The second method of testing shows values like eight (at Auto Enamelrater) are represented by small interior dots that could be provided by dust particle, undried water drops from process washer etc. Almost 90% of this process can imperfections are sorted by necking phase camera (Optic sorting). The

most considerable defects that could bring such values as eight are physical damages like those that are made from handling and presentation racks in stores. These are the precursors of corosivity and most of the damaged cans measured (shown in the third part of the research) are with values above two and the normal one between zero point one and two value.

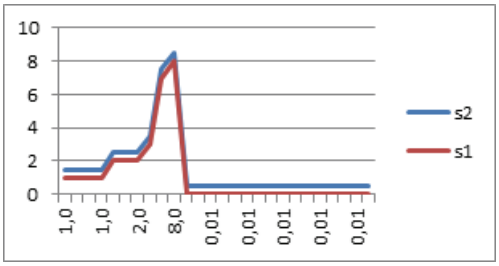


Figure 5. Conductivity results for 2 batches of tested cans, s1 and s2, including maximum and minimum values as per results (where 2 is the maximum value admitted)

## CONCLUSIONS

Most food products rely on their particular packaging to achieve their expected shelf life.

In some circumstances the desired shelf life can be a major factor in the selection of a packaging material.

Nowadays, in the EU there is a general requirement that food packaging components must not migrate into food during its normal shelf-life to the detriment of the food (i.e. to pose a health risk, or to adversely affect the quality of the food, its flavor, texture or appearance).

Most canned foods are now processed in lacquered cans which are one of the best solutions, but also but all these cans are susceptible to residual chemicals migration in food.

Test results of the present study indicate that in 90% of damaged cans, scratched or hitted, have lost their laquer integrity in a certain degree.

According to the test, weak results of the axial force determine changes within the cans structure (affecting the pressure rezistance) and is correlated with the measurings indicating that the laquer integrity is also damaged.

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** Coating Thickness Gauge User Manual – Sencon User Guide

*** Survey of Chemical Migration from Food Contact Packaging Materials in Australian Food, 2011

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# STUDIES ON THE AROMA OF *SAUVIGNON* WINE

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***Abstract***

*The Sauvignon variety is part of the grape varieties semi-aromatic; the flavor is not given by the terpenes, as the aromatic varieties (muscat) but is given by other compounds, found in grapes under the form of precursors flavor, odorless thiols and after the alcoholic fermentation are converted in corresponding aromatic thiols. The volatile compounds that participate at the Sauvignon flavor, according to some authors are mercaptans and especially the 4-mercapto-4-metylpentan-2-one and after others is methoxypyrazine, compound with a decisive role in the variety flavor. There were analyzed three Sauvignon wines from the wine region Dealu Mare, vintage from 2007, 2008 and 2011. The wines were analyzed physico-chemical and analytical technique of GC/MS. In the Sauvignon case of the 2011 year there was identified and determined quantitatively with the GC/MS method, three compounds from the mercaptans group with role in the Sauvignon aroma: 4-mercapto-4-metylpentan-2-one, 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate. The results led to the identification and dosing of seventeen volatile compounds. In the highest concentration was found phenyl-2-ethanol, made with rose flower scent. From the esters prevail the 2-hydroxy-ethyl propanoate and ethyl acetate. Regarding the mercaptans the results show that they have an important role in the flavor of Sauvignon, the determined concentrations are grater than their threshold of perception.*

***Keywords:*** *4-mercapto-4-metylpentan-2-one, GC/MS method,Sauvignon flavor*

## INTRODUCTION

The *Sauvignon* is part of the *Vitis vinifera* varieties and makes part of obtaining a higher semiaromatic wine, this variety is one of the most demanded in the international trade with wine.

The *Sauvignon* is originally from France (Sauternes) and was introduced in our country, like many other wines, after the phylloxera invasion (twentiethcentury).

The *Sauvignon* wines are semiaromatic of a outstanding quality, balanced, smooth, with plenty personality. Their flavor is appreciated by tasters as suave, delicate, with a aroma of citrus, flowers of vine etc. When the ecopedoclimatice conditions are met the *Sauvignon* wine can be an exceptionally wine and rarely equaled.

About the *Sauvignon* flavor there are many research and opinions. According to some authors the most important flavor compounds that participate at the *Sauvignon* flavor are methoxypyrazine (Allen M. et al, 1991) and volatile thiols (mercaptans) that are part of different fruit flavor (Chandary S. et al., 1984; Guth H, 1997) like: grapefruit, passion fruit, guava, pomelo, black currants.

Thiols are found in grapes as precursor form, odorless, bound of amino acids. They became flavored only after alcoholic fermentation.

The most studied mercaptans are: *4-mercapto-4-methylpentan-2-one* (broom flavor, tree bark), (Chandary S. et al., 1984, Guth H, 1997), *3-mercapto-3-methylbutan-1-ol* (flavor of leeks cooked), *3-mercaptohexan-1-ol* (pomelo, passion fruit, lemon), *3-mercaptohexyl acetate* (passion fruit) and *4-mercapto-4-methylpentan-2-ol*(flavored with lemon, pomelo, passion fruit) (Guth H, 1997, Tranchant J. et al., 1995).

Mecaptans are found in nature in different concentrations,their sensory perception is related to the concentration. Between thiols,*4-mercapto-4-metil-pentan-2-one* has a very low perception threshold and is essential in *Sauvignon* wine flavour.

Compoundsas *4-mercapto-4-methylpentan-2-one*, *3-mercaptohexan-1-ol* are also part of other wines like: *Gewurztraminer (Traminer roz)*, *Colombard*, *Chenin Blanc* etc.

However, it seems that to the distinct flavor of *Sauvignon* participate and other volatile

compounds: esters, aliphatic alcohols, aromatic alcohols, terpenes etc.

## MATERIALS AND METHODS

**Wines Analyzed**. Analyses were carried out on Romanian *Sauvignon* wines from the wine region *Dealu Mare*, vintage from 2007, 2008 and 2011. *Sauvignon* wines was analyzed in terms of physico-chemical characteristics: alcoholic strength (vol% alcohol), sugar content ($g \cdot L^{-1}$), total acidity ($g \cdot L^{-1}$ sulfuric acid), total dry extract ($g \cdot L^{-1}$), acidity volatile ($mg \cdot L^{-1}$ acetic acid) and glycerol ($g \cdot L^{-1}$). All analyses were performed by the standard methods (Tardea C., 1980): ebulliometer method for alcoholic strength; titrimetric method for total acidity; distillation method *Saunier-Cazenave* for acidity volatile; *Tabarie* method for total dry extract and volumetric method for glycerol.

**Specific Extraction of Volatile Compounds.** *Sauvignon* wines vintage from 2007, 2008 and 2011.

200 mL of wine, placed in a conical flask, were successively extracted (3 x 20 min) at 0°C with 3 x 25 mL of freshly distilled dichloromethane and then centrifuged for 15 min. The three organic extracts were pooled, dried with anhydrous sodium sulfate and concentrated to 5 mL in a Danish concentrator (45°C), then to 1 mL under a stream of nitrogen (Baek H., 1997; Campeanu G., 2001, Serot Th, 2009; Tranchant J. et al, 1995).

**Specific Extraction of Volatile Thiols.** *Sauvignon* wines harvest 2011 (Tominaga T. et al., 1998).

A volume of 500 mL of wine containing 4-methoxy-2-methyl-2-mercaptobutane as an internal standardwas brought to pH 7.0 with a sodium hydroxide solution and extracted with two successive additions of 100 mL of dichloromethane with magnetic stirring for 5 min; The organic phases were centrifuged for 5 min to break the emulsion and separated in a funnel; The organic phase obtained was then extracted with two additions of 20 mL of a p-hydroxymercurbenzoate solution 5 min; The two aqueous phases, from the extraction, were combined brought to pH 7.0 by addition of a 5% solution of hydrochloric acid; They were loaded into a strongly basic anion exchange column; The volatile thiols were released from the complex thiol-p-hydroxymercurbenzoate fixed on the column by percolating for 40 min using a cysteine solution adjusted to pH 7.0; The organic phases were collected, dried on anhydrous sodium sulfate and concentrated under nitrogen flow (Tominaga T. et al., 1998).

*GC/MS*. 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.

**Quantitative analysis of volatile compounds identified in *Sauvignon* wines vintage from 2007, 2008 and 2011 by GC/MS.** 1µL from each extract was injected into an HP 5-MS capillary column with dimensions: 30 m x 0.25 mm x 0.25 mm (film thickness). Column temperature: 30° C for 10 min., followed by temperature gradient 10° $min^{-1}$ up to 80° C, then gradient of 25° C/min. up to 250° C where stationed 10 minutes. Detector and injector temperatures are: 280° C and 250° C resp. Carrier gas is He, flow-0.5 ml $min^{-1}$. 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 $min^{-1}$.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 (Serot Th., 2001; Visan L., 2007).

**Quantitative analysis of volatile thiols identified in *Sauvignon* wines harvest 2011 by GC/MS** (T. Tominaga and ot., 1998). 2 µL from extract was injected into an HP 5-MS capillary column. The three volatile thiols were detected: 4-mercapto-4-metylpentan-2-one, 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate (Tominaga T. et al., 1998).

## RESULTS AND DISCUSSIONS

**Chemical and organoleptic analyses of wine.** The three *Sauvignon* wines from the Romanian wine region *Dealu Mare*, harvest 2007 (*Sauvignon 1)*, 2008 (*Sauvignon 2)* and 2011 (*Sauvignon 3)* were analyzed chemical and

organoleptic.

Results are presented in Table 1.

At organoleptic analysis three wines were presented as high quality, semidry; the wines showed a great finesse and pleasant, discrete, but with personality. It is however noted that *Sauvignon* 2 with a higher alcohol content, balanced and with a distinct flavor.

Table 1. The Main Physico-Chemical Parameters Analyzed wines

| Wine | total acidity ($g \cdot L^{-1}$ sulfuric acid) | sugar content ($g \cdot L^{-1}$ sugar) | alcoholic strength (vol% alcohol) | Glycerol ($g \cdot L^{-1}$) | total dry extract ($g \cdot L^{-1}$) | Volatile acidity ($mg \cdot L^{-1}$ acetic acid) |
|---|---|---|---|---|---|---|
| *Sauvignon 1* | 5.8 | 10 | 12.2 | 8 | 22 | 0.3 |
| *Sauvignon 2* | 5.4 | 12 | 13.0 | 10 | 23 | 0.4 |
| *Sauvignon 3* | 5.2 | 12 | 12.5 | 8 | 23 | 0.4 |

**Analysis of GC/MS of wines. Identification of volatile compounds.**

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.

From experience the *Sauvignon* wines is seen that the same esters are found in large quantities in all three wines, although varies from year to year: *2-hydroxy ethyl propanoate*, *ethyl acetate* and *methyl butanoate* (fig.1).

It follows that these esters participate to a greater extent in *Sauvignon* flavor. It is noteworthy that as with the esters and other volatile compound found in similar concentrations, although varies slightly depending on the year of harvest. Of aliphatic alcohols found in highest concentrations, in all the years, *3-methyl-1-butanol* (fig.2).

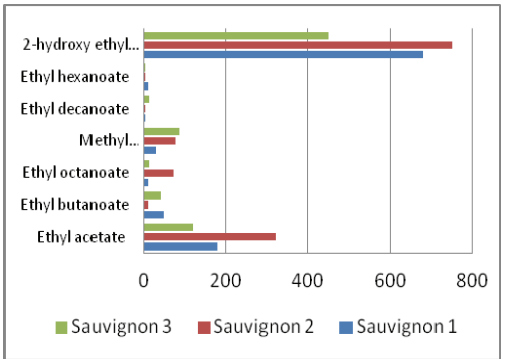


Figure 1. The main esters of a *Sauvignon* wines ($\mu g \cdot L^{-1}$)

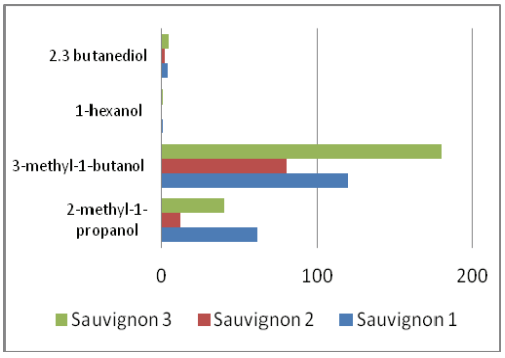


Figure 2. Concentration of aliphatic alcohols in *Sauvignon* wines ($\mu g \cdot L^{-1}$)

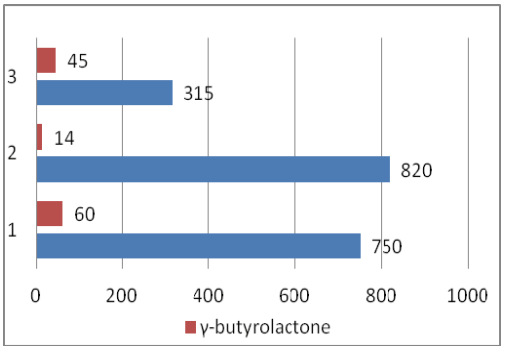


Figure 3. Concentration of aromatic alcohols and lactones in *Sauvignon* wines ($\mu g \cdot L^{-1}$)

Of terpenes *linalool* was identified only in concentrations of 1, 0.8, respectively, 1 $\mu g \cdot L^{-1}$ (fig.4).

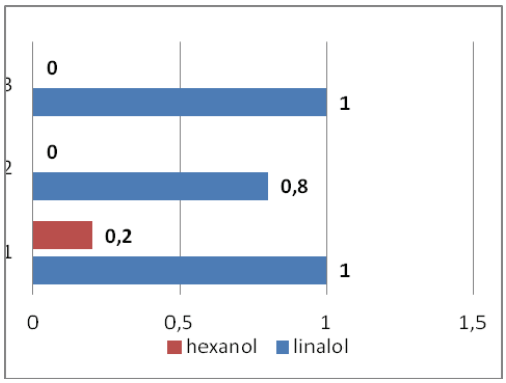


Figure 4. Concentration of terpenes in *Sauvignon* wines (µg·$L^{-1}$)

**Analysis of GC/MS of wines. Identification of volatile thiols (Sauvignon wines, harvest 2011)**

*Sauvignon* wines from the harvest of 2011 were analyzed by GC/MS, after Tominaga T. method (1998), for identification and quantitative dosing of volatile thiols. The three volatile thiols were detected:
*4-mercapto-4-metylpentan-2-one*, *3-mercaptohexan-1-ol* and *3-mercaptohexyl acetate*.

Between thiols identified the lowest threshold of perception it has *4-mercapto-4-metylpentan-2-one*, under 1 ng·$L^{-1}$ (Tominaga T, 1998) (fig.5); the compound with flavored mature bark, it was identified in low concentrations near the threshold of perception of black blueberry, but also in other wines as: *Riesling*, *Gewurztraminer* etc, in low concentration.

In Romanian *Sauvignon* wine this mercaptan was identified in 23 ng·$L^{-1}$ concentration, a concentration that demonstrating participation of this thiol at *Sauvignon* flavor. Also we identified *3-mercaptohexan-1-ol*, thiol with a grapefruit flavor, pomelo (fig.6) and *3-mercaptohexyl acetate*, made with passion fruit flavor and bark. The first thiol was found in concentration of 690 ng·$L^{-1}$, concentration that shows the participation at the *Sauvignon* flavor. *3-mercaptohexyl acetate* (fig.7), was found in a concentration of 120 ng·$L^{-1}$, well above the threshold of perception of 5 ng·$L^{-1}$.

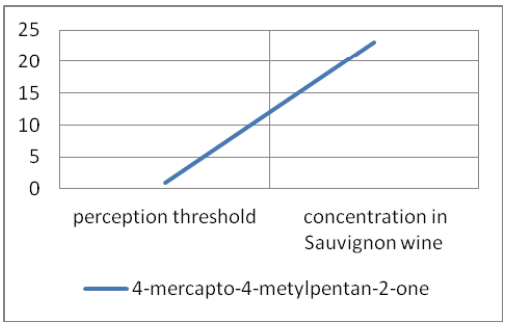


Figure 5. The threshold of Perception and concentration in *4-mercapto-4-metylpentan-2-one* from the *Sauvignon* wine (ng·$L^{-1}$)

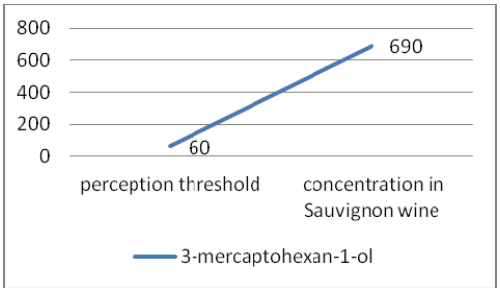


Figure 6. The threshold of Perception and concentration in *3-mercaptohexan-1-ol* from the *Sauvignon* wine (ng·$L^{-1}$)

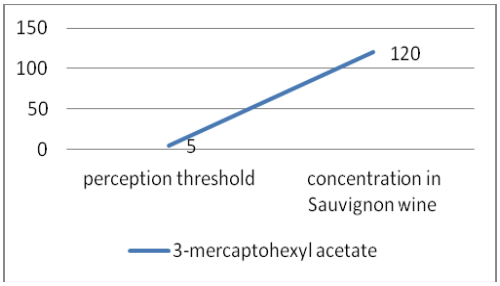


Figure 7. The threshold of Perception and concentration in *3-mercaptohexyl acetate*from the *Sauvignon* wine (ng·$L^{-1}$)

## CONCLUSIONS

The *Sauvignon* wines from the 2007, 2008 and 2011 harvest were assessed as high quality wines, balanced and smooth flavor, discrete, citrus and flower vines flavor. Among these wines is remarkable *Sauvignon* 2008 which was considerably more balanced and discreet flavor but with personality.

The content volatile compounds of the wine wasslightly varied from year to year,but the same compounds were present in concentrations similar regardless of the year of harvest;between esters the most important in defining the specific character of *Sauvignon* were identified *2-hydroxy ethyl propanoate*, *ethyl acetate* and *methyl butanoate*;It follows that these esters participate to a greater extent in *Sauvignon* flavor. It is noteworthy that as with the esters and other volatile compound found in similar concentrations, although varies slightly depending on the year of harvest; in considerable quantities are found *3-methyl-1-butanol* and terpenes were represented by *linalool*.

*Sauvignon* wines from the 2011 harvest were analyzed for identification and quantitative dosing of volatile thiols. The three volatile thiols were detected: *4-mercapto-4-metylpentan-2-one, 3-mercaptohexan-1-ol* and *3-mercaptohexyl acetate*.

Between thiols identified the lowest threshold of perception it has *4-mercapto-4-metylpentan-2-one*, under 1 $ng \cdot L^{-1}$; the compound with flavored mature bark, it was identified in low concentrations near the threshold of perception of black blueberry, but also in other wines as: *Riesling*, *Gewurztraminer* etc, in low concentration.

In romanian *Sauvignon* this mercaptan was found in concentrations of 23 $ng \cdot L^{-1}$, a concentration that demonstrates the participation of this thiol at *Sauvignon* flavor.

Also were identified *3-mercaptohexan-1-ol*, thiol with a grapefruit flavor, pomelo and *3-mercaptohexyl acetate*, made with passion fruit flavor and bark.

The first thiol was found in concentration of 690 $ng \cdot L^{-1}$, concentration that shows the participation at the *Sauvignon* flavor.

*3-mercaptohexyl acetate*, was found in a concentration of 120 $ng \cdot L^{-1}$, well above the threshold of perception of 5 $ng \cdot L^{-1}$.

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# STUDIES ON THE CHROMATIC CHARACTERISTICS OF RED WINES AND COLOR EVOLUTION DURING MATURATION

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***Abstract***

*The chromatic profile of red wines are formed by the participation of various compounds phenolic: anthocyanins, tannins, flavones and phenolic acids, anthocyanins are polyphenolic substances with the most imported role in the color of young wines. The evolution of red wine leads to changes of structures and chromatic properties of wine due to polymerization reactions, condensation and oxidation. Also the red wine color is strongly influenced by wine region of origin, the wine grape variety and vintage year. There were studied chromatic characteristics of red wines as Merlot and Feteasca Neagra from two different vineyards in terms ecopedoclimatics, Dobrogea and Moldova, the 2008 year harvest. The polyphenolic composition of wines was judged by the content in polyphenols, tannins and anthocyanins. A wine tannin structure was analyzed by their concentration in condensed tannins, astringent tannins and tannin-polysaccharide complex. Analyzes have been carried out in the wine by UV-VIS spectrometry techniques. Total content of polyphenols have been determined by IPT technique. Tannins have been determined by the Ribereau-Gayon method (1996), tannin structure after Glories (1978) method; anthocyanins were determined by the discoloration technique with $SO_2$. Color intensity was determined at $\lambda$=420 nm and $\lambda$=520 nm. The study on color of red wines analyzed during their evolution referred to the study of chromatic parameters, the content of anthocyanin monomers and polymers (Glories method).*

***Keywords****: chromatic characteristics, anthocyanin monomers and polymers*

## INTRODUCTION

In wines, although are find in low concentration compared with other components, the phenolic compounds have a very important role in the quality of wine. They influence the organoleptic characteristics of wines as color, taste, astringency, hardness, but even the feature stability of those. They have an important role in the protection of must and wines against oxidation (Bourzeix M., 1976, Mazue F., 2001).

The poliphenolic compounds have an important role in the evolution of wines during the mature time.

The evolution of red wines leads to the modification of the structure and chromatic proprieties tanks to polymerization reactions, condensation and oxidation. The content of wines in poliphenolic compounds depends of many factors, among of them the most important are from the originally region, the variety and year of harvest (Landrault N, 2001). Our studies concern the influence of the region (with different ecopedoclimatics conditions) and grape wine about the chromatic characteristics of two red quality wines, *Merlot* and *Feteasca Neagra*. Also, was studied the chromatic profile evolution of wines through the modification of structure of anthocyaninsin the mature curs of wine.

## MATERIALS AND METHODS

*Merlot* and *Feteasca Neagra* wines, from two different vineyards in terms ecopedoclimatics, Dobrogea and Moldova, the 2008 year harvest, were analyzed in terms of physico-chemical: alcoholic strength (vol% alcohol), total acidity ($g \cdot L^{-1}$ sulfuric acid), volatile acidity ($mg \cdot L^{-}$

[1]acetic acid), total dry extract ($g \cdot L^{-1}$) and glycerol ($g \cdot L^{-1}$). Based analyzes were performed by standard methods ebulliometer method for alcoholic strength; titrimetric method for total acidity; distillation method *Saunier-Cazenave* for acidity volatile; *Tabarié* method for total dry extract and volumetric method for glycerol.

The polyphenolic composition of wines was judged by the content in polyphenols, tannins and anthocyanins. Analyzes have been carried out in the wine by UV-VIS spectrometry techniques (Giusti M, 2001).

Total content of polyphenols have been determined by IPT technique ($g \cdot L^{-1}$ gallic acid) (Ribereau-Gayon J, 1978). Tannins have been determined by the Ribereau-Gayon method (1996) and tannins structure after Glories method (1978), based on the following indicators: gelatin index (for astringenttannins); HCl index (for condensedtannins); ethanol index (for the macromolecular associations tannins -polysaccharides).

These indicators was determined by spectrophotometric method at $\lambda$ = 280 nm (Glories Y, 1984). The anthocyanins were determined by the discoloration technique with $SO_2$ (Dallas C., 1994). Color intensity was determined at $\lambda$=420 nm and $\lambda$=520 nm. The study on color of red wines analyzed during their evolution referred to the study of chromatic parameters, the content of anthocyanin monomers and polymers (Glories method). Wines have been noted: $M_1$ – *Merlot Dobrogea region*; $M_2$ – *Merlot Moldova region*; $FN_1$ – *Feteasca neagra Dobrogea region*; $FN_2$ – *Feteasca neagra Moldova region.*

## RESULTS AND DISCUSSIONS

The quality red wines, *Merlot* and *Feteasca Neagra* had a good behavior in the experimental year, in both regions. The studied parameters had recorded slightly superior values in Dobrogea region, at both wines (table1).

The *Merlot* wines were characterized that been a fruitful wines, of ruby color, with a discrete aroma of berries (raspberry) andalmond, average contentin extract. *Merlot* 1 (Dobrogea region) was characterized with more personality, and *Merlot* 2 (Moldova-Cotesti region) like a wine with more finesse, delicate and great softness (fig.1).

*Feteasca Neagra* was characterized like an ample wine, with personality. A higher alcoholic strength and a good balance between the components, was recorded in the wine from Dobrogea region ($FN_1$).

Table 1. Chemical parameters of red wines *Merlot* and *Feteasca neagra*

| Wines | Chemical parameters of red wines | | | | |
|---|---|---|---|---|---|
| | alcoholic strength (vol % alcohol) | total acidity ($g \cdot L^{-1}$ sulfuric acid) | volatile acidity ($mg \cdot L^{-1} CH_3COOH$) | dry extract ($g \cdot L^{-1}$) | glycerol ($g \cdot L^{-1}$) |
| $M_1$ | 12.5 | 3.6 | 0.3 | 27.0 | 8.2 |
| $M_2$ | 12.5 | 3.4 | 0.4 | 26.2 | 8.5 |
| $FN_1$ | 13.0 | 4.3 | 0.4 | 26.5 | 8.5 |
| $FN_2$ | 12.8 | 4.2 | 0.45 | 24.2 | 8.0 |

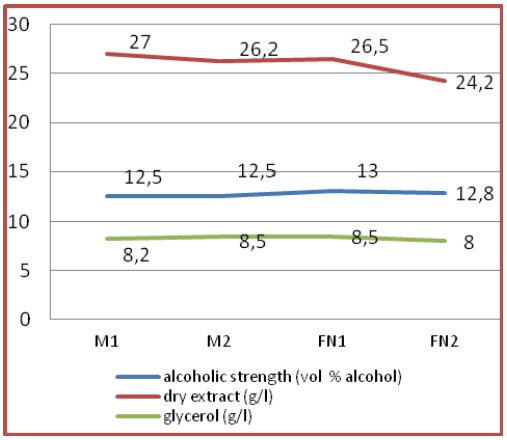



Figure 1. The main chemical parameters in red wines (*Merlot* and *Feteasca neagra)*

Content of poliphenolic compounds and tannins in wine is medium, the results showed that in both varieties a similar behavior: content in poliphenols and tannins is grater in Dobrogea region.

Both wines ($M_1$ and $FN_1$) were characterized as more astringent and with a intense color (fig. 2).

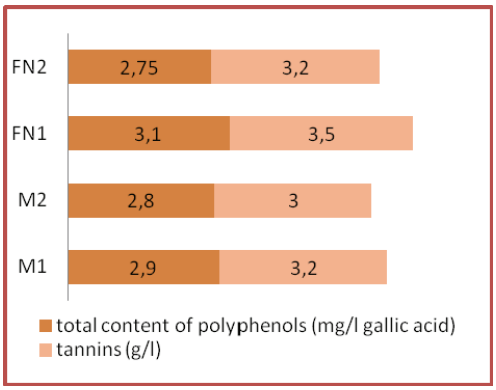



Figure 2. Total content of polyphenols and tannins in red wines (*Merlot* and *Feteasca neagra)*

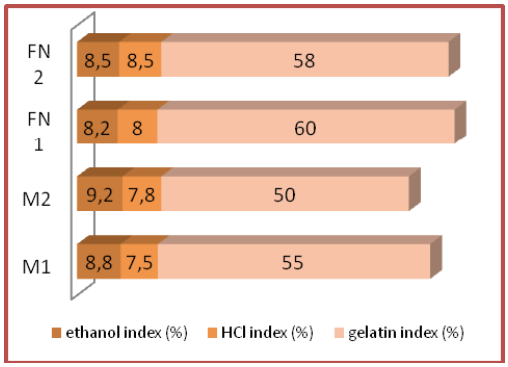



Figure 3. Ethanol index, HCl index and gelatin index

Regarding the tannins structure of wines, the results show that the *Merlot* wine has a grater ethanol index: the tannins proportion from the tannins-polysaccharides complex is higher than $M_2$ (Moldova-Cotesti region). The astringent tannins concentration is lowest than $M_2$ and $FN_2$ variants (fig.3).

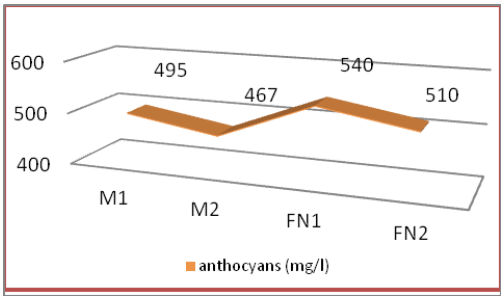



Figure 4. Anthocyanins ($mg \cdot L^{-1}$) content of red wines

The anthocyanins concentration and the intensity color from the wines (fig.4, fig.5) follows the same equation, the values been higher for Dobrogea region.

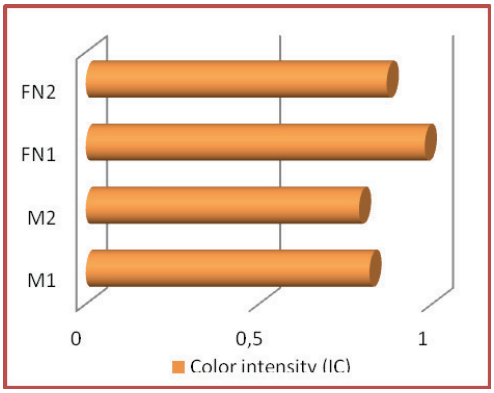



Figure 5. Color intensity (IC)

The poliphenols compounds structure is change during the wine maturation due to polymerization reactions, condensation and oxidation, leading to the change of the chromatic proprieties. As regarding anthocyanins, at the red wines color participate the anthocyanin monomers, polymersand copigmented, they change during wine maturation. Therefore, the anthocyanin monomers turns into the polymeric form.

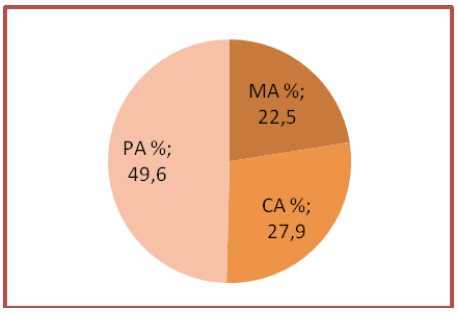



Figure 6. Percentage of anthocyanin monomers (MA%), polymers (PA%) and copigmented (CA%) in young *Merlot* wine

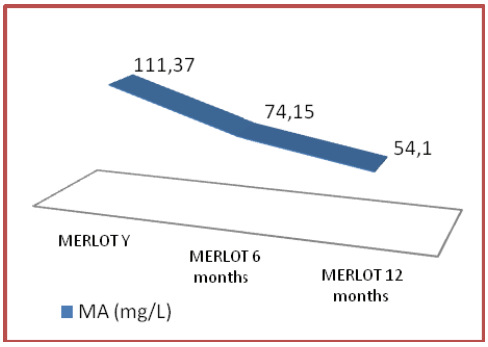
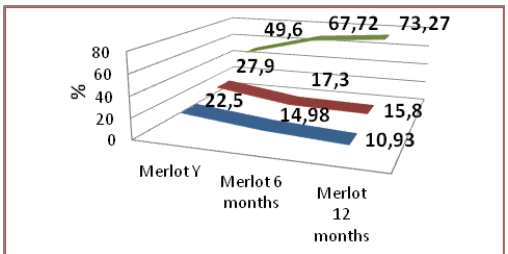



Figure 7. Evolution of anthocyanin monomers (mg·$L^{-1}$cianidin-3-glucozidă) during the *Merlot* wine maturation

If at the young *Merlot* (*Merlot Y*) wine the percent of anthocyanin monomers is 22,5% (fig.6) this records drops during the wine maturation, the value reach 10,93% (54,1 mg·$L^{-1}$ cianidin-3-glucozidă) (fig. 7 and fig. 8). In the *Feteasca Neagra* wine the percent of anthocyanin monomers is lower (19,9%) than the *Merlot* wine case (fig.9); it also records drops of anthocyanin monomers, the drop is more visible than *Merlot*: 29,8 mg·$L^{-1}$ cianidin-3-glucozidă (fig. 10, 11).




| | Merlot Y | Merlot 6 months | Merlot 12 months |
|---|---|---|---|
| MA (%) | 22,5 | 14,98 | 10,93 |
| CA (%) | 27,9 | 17,3 | 15,8 |
| PA (%) | 49,6 | 67,72 | 73,27 |

Figure 8. Evolution of anthocyanin (%) during the *Merlot* wine maturation

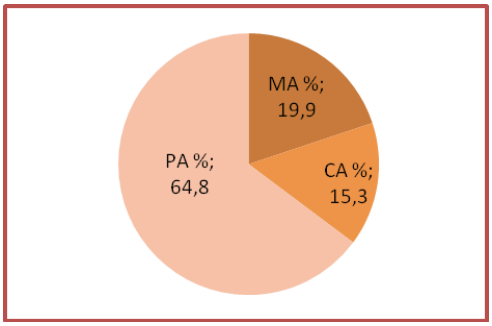



Figure 9. Percentage of anthocyanin monomers (MA%), polymers (PA%) and copigmented (CA%) in young *Feteasca neagra* wine

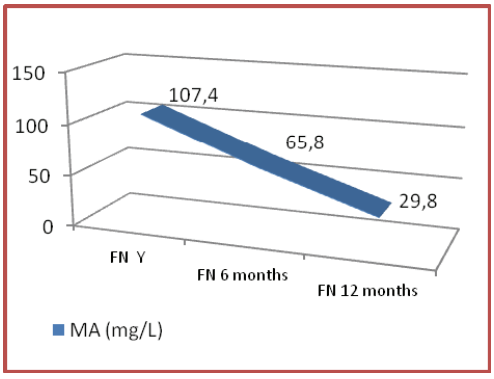
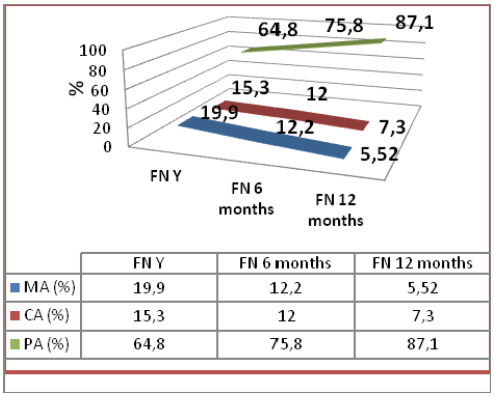



Figure 10. Evolution of anthocyanin monomers (mg·$L^{-1}$cianidin-3-glucozidă) during the *Feteasca neagra* wine maturation




| | FN Y | FN 6 months | FN 12 months |
|---|---|---|---|
| MA (%) | 19,9 | 12,2 | 5,52 |
| CA (%) | 15,3 | 12 | 7,3 |
| PA (%) | 64,8 | 75,8 | 87,1 |

Figure 11. Evolution of anthocyanin (%) during the *Feteasca neagra* wine maturation

## CONCLUSIONS

The *Merlot* and *Feteasca Neagra* wines has recorded in the study year features of superior quality.

In the case of *Merlot* wine, the variety from the Moldova (Cotesti) region was of a remarkable quality under the finesse and flavor report and the balance between components; the *Feteasca Neagra* wine has performed better in Dobrogea region ($FN_1$), the wine had a great personality.

The content of wines in poliphenolic compounds and tannins is medium, the wines had similar behavior and it is recording grater values than the Dobrogea region.

Regarding the tannins structure of wines, the results show that *Merlot* has an higher ethanol index: the tannins proportion from (tannins-polysaccharides complex is higher for the region of Moldova-Cotesti, similar situation of *Feteasca Neagra* wine.

The astringent tannins concentration is lowest than for both wines is lowest, for the wines from Moldova region.

The chromatic evolution of wines during the wine maturation, has resulted in the drop of the percent of anthocyanin monomers and the higher percent of polymers in the both wines, the drop was visible for the *Feteasca Neagra* wine.

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# STUDY OF SOME CHANGES THAT OCCUR DURING MEAT FERMENTATION

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***Abstract***

*Dry-fermented sausages and dry-cured ham, constitute one of the most representative traditional foods represents meat products, which have a high variety of flavors and textures, represent an important part of local economies, particular cultures and gastronomic heritages.*
*Purpose of this paper is to present present comparative physical, chemical and biochemical changes that occur during maturation of three products maintained under the same conditions: Plescoi sausages, Chorizo salami and Prosciutto. The drying removes 38,8 to 58,8%, while amino acids content increased between 1.87 and 2.52 times. pH variation was small because fermentation without starter was chosen.*

***Keywords:*** *meat products, ripening and drying, pH, amino acids.*

## INTRODUCTION

As shown by Brown (2003) and Bogdan (2010) demand for meat has increased and this innate hunger for animal protein, which occurs in any society, raised global demand for meat every year for the past 40 consecutive years.
The production of fermented foods is one of the oldest food processing technologies known to man (Nollet, 2006). Raw-dried meat products are frequently obtained from pork, beef or sheep meat and they are presented as minced meat or entire piece. Three products can be given as examples to cover this range of products: Plescoi sausages, Chorizo salami and Prosciutto.
Numerous articles shows physicochemical and biochemical changes that occur during maturation of meat. In dry-cured products, one of the main factors affecting final product quality is proteolytic activity, which depends on many factors, such as pH, water content, NaCl content and drying conditions (Arnau et.al. 1998; Zhao et al., 2008). Free amino acids are generated by proteolysis during the processing of dry-cured meat products and can thus serve as an indicator of maturation. They are also known to be related to the development of a particular taste, flavour or aroma like saltiness, acid taste, aged/dry-cured taste, bittertaste, etc (Sforza et.al., 2001) In this material, follow these changes for Plescoi sausages, Chorizo salami and Prosciutto, the three above mentioned meat products, which were stored in similar condition.

## MATERIALS AND METHODS

All materials were stored in a refrigerator at a temperature of $4^0$C until processing. Plescoi sausages recipes was: sheep meat 55%, beef 40%, spices 2.4%, mixture of curing 2.6%. Chorizo is found in many varieties, some with special association. Recipes in this case was: lean pork 74%, fat 14%, water 4%, salt and nitrite 2,6% and spices 4,4%.. For these two products filling was done in natural pork casings. For the third product, Prosciutto, the green hams without rind and external fat was salted using dry salt and was placed in a tray. After 6 days exudates water was removed and the ham was suspended. All products were ripened and dried by storing in the refrigerator at a temperature of $4^0$C in order to maturation
Water content was determined as SR ISO 1442:2010 and pH as SR ISO 2917:2007. For amino acids content methods of analysis were those described by Banu et al (1984).

## RESULTS AND DISCUSSIONS

Sausages are usually made from lean pork, mixtures of pork and beef, or solely beef. Some other animal species may be used depending on the type of product and geographic location like Pleşcoi sausages which are a Romanian sausage made from mutton spiced with chili peppers and garlic.
During the technological process of fermented meat products, physicochemical and bioche-

mical processes occurring. These processes are influenced by product type (chopped or whole piece), the product recipe, operations and parameters of technological process.

Among the physicochemical processes, variation of water content and pH were studied (Figure 1 and Figure 2).

As expected in all cases humidity decreased over time. In case of, minced meat products, drying was more intense because the two products were permanently suspended. Drying speed, expressed as moisture variation in time, is higher in the early days, and it was 3.2 for Plescoi sausage, and 3.45 for Chorizo after the first 6 days. After 30 days, the drying rate drops, reaching 1.3 respectively 0.95.

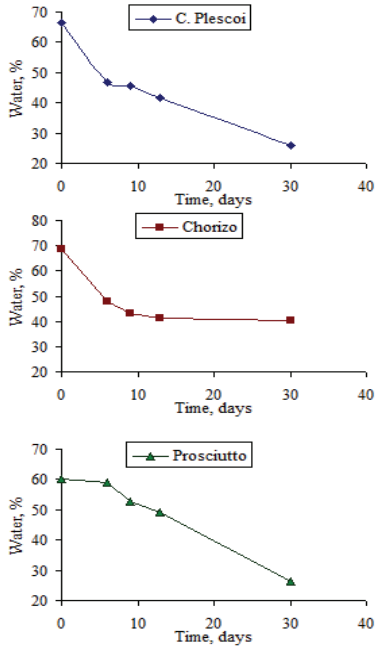


Figure 1. Time variation of moisture content for three products: Plescoi sausage, Chorizo salami and Prosciutto

Previously (Toldrá, 2004) has been pointed that the reduction of water activity is slower in those sausages containing beef because this type of meat is more resistant than pork in the process of desiccation. This was observed for Plescoi sausages just for the first six days, but a second explanation is that, for Chorizo composition meat and fat was used. This may explain why the percentage of water after 30 days is higher in Chorizo than in Plescoi sausages.

For Prosciutto initial water losses were lower because green ham was placed in the tray, in which the water exudate has accumulated under the action of salt water from the meat surface.

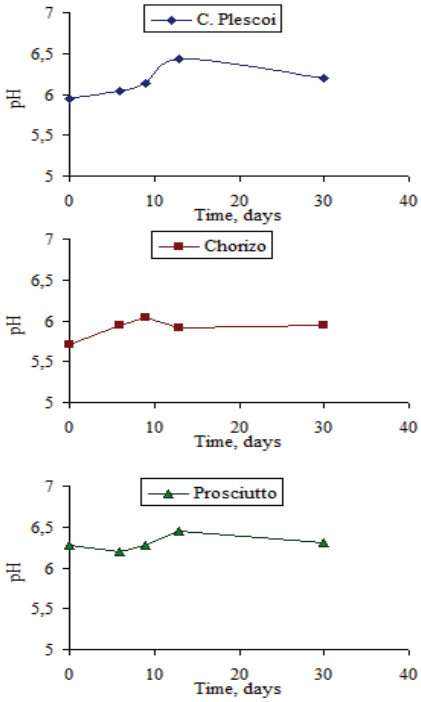


Figure 2. Time variation of pH for three products: Plescoi sausage, Chorizo salami and Prosciutto

The rate of water loss depends on pH. In case of the studied products, microbiological processes take place only under the action of spontaneous microflora.

As shown in Figure 2, the pH varies in narrow limits, and for all three products is found that in general it increases.

The pH can rise during the ripening and drying due to the buffering action of proteins as well as the enzymatic formation of nonprotein nitrogen basic compounds and ammonium ions.

Among the biochemical processes, proteolysis and lipolysis are important. In the present paper only variation in free amino acids content has been assessed. They are formed mainly by the action of enzymes in meat. Dipeptidases, aminopeptidases and carboxypeptidases acts on dipeptides, peptides (amino termini), respectively peptides (carboxy termini) to form mainly free amino acids. These substrates for enzymes

mentioned above, results from the action of other enzymes involved in proteolysis such as endopeptidase and exopeptidase. Among the aminopeptidases and carboxypeptidases, it was shown that the first act to a higher pH environment, while carboxypeptidases have an optimal activity at acidic pH (Toldrá and Flores, 1998; McDonald and Barrett, 1986).

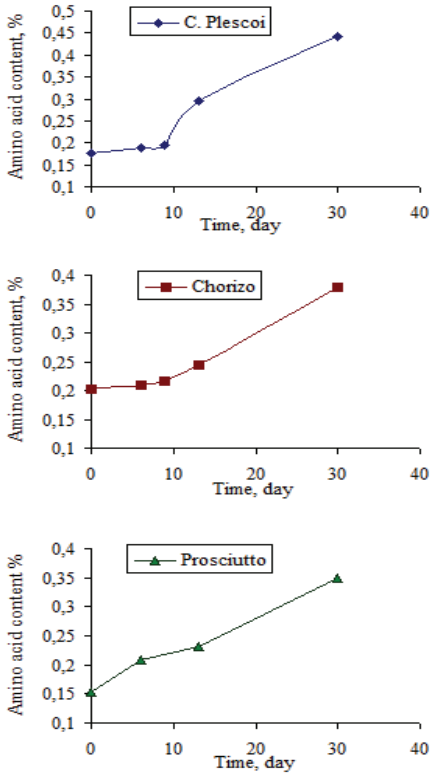


Figure 3. Time variation of amino acids content for three products: Plescoi sausage, Chorizo salami and Prosciutto

Free amino acids content increases for all three products, but in the first few days is more in Plescoi sausage and in Prosciutto than in Chorizo. This can be explained by the fact that initially Chorizo salami has a pH slightly smaller than the other two products. (Figure 3.) Rapid growth of the amino acid content is higher in sausages Plescoi because they have in composition, only minced meat (60% sheep meat and 40% beef) in comparison with Chorizo which contains 84% meat and 16% lard. The small increase in the content of amino acids for Prosciutto registers as the meat, remained whole piece.

## CONCLUSIONS

Composition varies widely due to the number of fermented product types. The major factors influencing composition of the finished product are the composition and ratio of raw materials used, processing procedures utilised, and the intensity of drying. After 30 days of ripening and drying, moisture in products that are made from lean meat is lower (25.8% in sausage Plescoi and 26.3% in Prosciutto) than in Chorizo, which additionally contains lard. The lowest value of the final pH, namely 5.94 was obtained for Chorizo. The highest content of free amino acids, 0.441%, which indicates a more intense enzymatic activity, was recorded for Plescoi sausages, which are obtained only from lean minced. These results will be compared in the future with those obtained if using starter cultures.

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# INDUSTRIAL AND ENVIRONMENTAL BIOTECHNOLOGY

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# *IN VITRO* EVALUATION OF CRUDE OIL DEGRADATION POTENTIAL OF SOME *PLEUROTUS OSTREATUS* ISOLATES

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***Abstract***

*In the last years degradation of aromatic compounds by white-rot fungi has been intensively studied. Several studies revealed the abilities of the white-rot fungus Pleurotus ostreatus to degrade a variety of polycyclic aromatic hydrocarbons in liquid culture as well as in a semi-natural substrate. In the present study, two isolates of P. ostreatus (P50, P421) and a sample of P. ostreatus collected from Chitila woods were evaluated for their ability to use crude-oil as a source of carbon. The P. ostreatus samples were inoculated in plates with crude oil (1ml) spread on the entire carbon free mineral salt medium (MSM) surface and in plates with pieces of filter paper soaked in crude oil and placed around the inoculum. Two weeks after, the culture media were completely covered by mycelium. These results indicated that both isolates of P. ostreatus as well as P. ostreatus originated from Chitila forest were able to utilize the crude oil as a source of carbon and energy in their metabolism.*

***Keywords:*** *crude-oil, Pleurotus ostreatus, mycoremediation.*

## INTRODUCTION

Polycyclic aromatic hydrocarbons, relatively abundant, may persist in the environment affecting animals and aquatic organisms due to their carcinogenic and mutagenic properties (Clemente et al, 2001; Cerniglia and Sutherland 2001). Bioremediations is a natural process that can accelerate degradation of hydrocarbons-contaminated waste into non-toxic residues by bacteria and fungi. During bioremediation, microorganisms utilize chemical contaminants in the soil as an energy source.
This process is based on microbial enzyme activities (Philip et al, 2005). Biological method provides the best solution for oil polluted environment remediation using the ability of indigenous microorganisms from soil to convert oil into harmless substances (Perelo, 2010). Different strains of bacteria in bioremediation such as *Bacillus* sp., *Pseudomonas* sp., *Micrococcus* sp., *Vibrio* sp. (Ijah and Antai, 2003) or fungal isolates of *Aspergillus* sp*., Alternaria* sp*., Penicillium* sp. and *Fusarium* sp. displayed highest ability for biodegradation of aromatic hydrocarbon-contaminated soil (Chaudhry et al., 2012). Mycoremediation, which is the use of the mushroom in the remediation of various polluted media, has demonstrated positive results, verified by scientists. *Pleurotus ostreatus, Pleurotus tuber-regium, Irpex lecteus* or *Lentinus* sp. have been used in the bioremediation of engine-oil polluted soil, crude oil contaminated soil and chemically polluted soil (Bezalel et al., 1996a; Adenipekun and Fasidi, 2005; Adenipekun, 2008; Ogbo and Okhuaya, 2008; Okparanma et al., 2011). Particularly, the white-rot fungus *Pleurotus ostreatus* have the potentials to degrade polycyclic aromatic hydrocarbons (Sack and Gunther, 1993; Vyas et al., 1994; Okparanma et al., 2011). Therefore, the goal of this study was to evaluate the potency of *Pleurotus ostreatus* for the growth on minimal salt media (MSM) with crude oil as carbon source.

## MATERIALS AND METHODS

**Inocula preparations.** A sample of *P. ostreatus* collected in 2012 from Chitila woods and two isolates of *P. ostreatus*, P50 and P421 kindly provided by dr. Ioana Tudor were used in experiments. For mycelia production a well

grown mushroom from the Chitila forest was selected and a gill portion was taken using a sterile forceps and inoculated on Potato Dextrose Agar (PDA) or 2 % malt extract broth media plates under aseptic conditions. In the case of the two isolates of *P. ostreatus*, segments of 5 mm from the culture media were taken and placed on the same media as those described above. The samples were incubated at $25^0$C for a week. The mycelia grown on the media surface were used for the following experiments.

**Evaluation of *P. ostreatus* growing potential on culture media with crude-oil added.** Crude oil used for this study was obtained from the Petroleum Refinery, Ploiesti. To evaluate the crude-oil degradation potential of *P. ostreatus*, mycelia inoculum were taken from the culture media and placed on carbon free mineral salt medium (MSM) prepared according Bhattacharya et al., 2012 (g/l: 0.5 $(NH_4)_2HPO_4$; 0.8 $KH_2PO_4$; 0.3 $MgSO_4.7H_2O$; 0.055 $CaCl_2.2H_2O$; 0.004 $ZnSO_4.6H_2O$; 0.07 $CuSO_4$; 0.2 yeast extract; 1 ml Thiamine 2mg/ml; pH 6 ). The crude oil was added to the medium surface in the following ways:

a. 1 ml crude oil spread on the entire MSM medium surface using a sterile glass baguette;
b. Sterile filter paper pieces (20x10 cm) soaked in crude oil and placed around the inoculum.

The control was prepared on 2% malt extract media without pollutant. Three replicates of each experimental variant were made for the *P. ostreatus* samples. All the Petri dishes were incubated at 27 C for 14 days. Every day, the radial growth of mycelia (in cm) was measured and extrapolated as growing percentage to culture plate diameter (9 cm).

## RESULTS AND DISCUSSIONS

In this work, a sample of *P. ostreatus* collected from Chitila woods and two isolates named P50 and P421 were used, in order to evaluate the potency of fungal pure culture samples for the growth on minimal salt media (MSM) with crude oil as carbon source. Two weeks after experiments initiation, in the samples which contained the filter paper soaked in crude oil mycelium growth of both isolates was faster (89% P50; 80% P421) (Figure 1) compared with the treatments which the crude oil was spread on the entire media surface (69% P50 and 75.5% P421 respectively) (Figure 2).

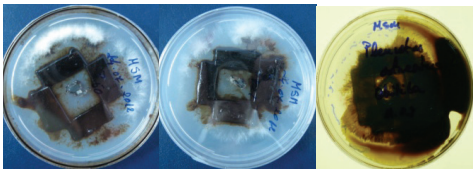

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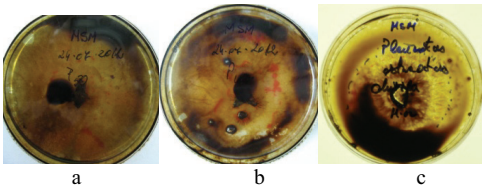
Figure 1. *P. ostreatus* mycelia growth on MSM medium with filter paper in crude oil P50 (a), P421 (b) and *P. ostreatus* originated from Chitila forest (c)



a b c

Figure 2. *P. ostreatus* mycelia growth on medium covered with oil: P50 (a), P421 (b) and *P. ostreatus* originated from Chitila forest (c)

The same situation was observed at the *P. ostreatus* mushroom collected from Chitila woods (Figures 1 and 2): the rate of mycelium growth was 78% on the filter paper with crude oil treatment and 67% in the plates with the medium surface completely covered with oil.

The observations extended for another two weeks revealed that mycelia covered completely the media surfaces treated with filter paper soaked in oil (Figure 3). Effectively, the mycelium covered the filter paper and has been extended to the plate edge.

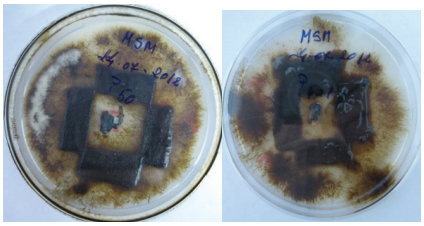


Figure 3. Mycelia growth of P50 (left) and P421 isolates (right) on filter paper with crude oil four weeks after

Assessment of fungal growth a month after, showed that both P421 and P50 mycelia almost covered the medium surface (Figure 4).

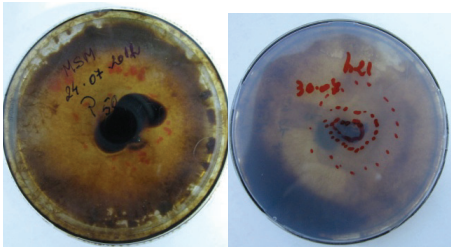


Figure 4. Mycelia growth of P50 (left) and P421(right) on crude oil covered culture media, four weeks after

These results show that the tested *P. ostreatus* samples had about the same growth potential on media supplemented with crude-oil (Figure 5). All *P. ostreatus* tested grew on carbon free minimal salt media treated with oil but the mycelium growth was considerably reduced when the oil was applied on entire surface on the medium compared to growth on media partially treated with oil.

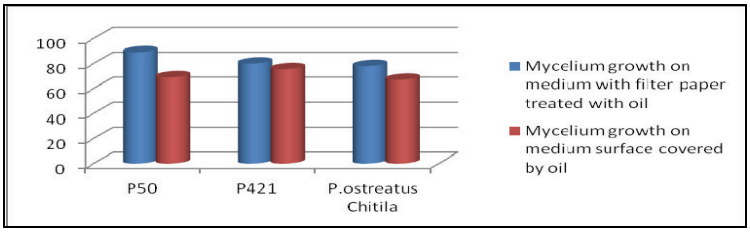



Figure 5. Mycelium growth of *P. ostreatus* on MSM medium treated with crude-oil

Apparently, in the first phase, the radial growth of mycelium was inhibited at higher concentration of oil. It has been demonstrated that the tolerance of mycelia of various species of *Pleurotus* of the pollutant which served as treatments in some studies, varied (Adedokun and Ataga, 2006). Ogbo et al. (2006) investigated the effect of different levels of spent lubricating oil (from 5 to 160%) on the growth of *P. tuber-regium*. They found that the fungus grew optimally at 98% level of contamination. However, in our experimental variants with crude oil treated culture media has been noted that developed mycelium had a dark brown colour compared with the typical white- gray colour of the *P. ostreatus.* Moeder et al. (2005) mentioned that *P. ostreatus* are the ability to eliminate the hydrocarbon pollutants accumulated from a contaminated soil to the fruit bodies. Moreover, some authors shown that *Pleurotus ostreatus* produce lacasse which it seems to be implicated in hydrocarbons degradation (Pozdnyakova et al, 2011). In mycoremediation process the fungi can degrade or transform organic contaminants to less toxic or non toxic compounds (Sasek et al, 2003). White-rot and waste –decomposing fungi are the potential candidates for the treatment of contaminated soils because of their high ability to degrade a wide range of xenobiotics in culture media or in contaminated soil (Hou et al., 2004) as well as due the hyphal penetration and the excretion of some oxidative enzymes in the polluted sites.

## CONCLUSIONS

We investigated the effect of crude oil on the growth of two isolates of *Pleurotus ostreatus*, P50 and P421 respectively and a *P. ostreatus* mushroom originated from Chitila woods for their capability to use crude-oil as a source of carbon and energy. For this purpose, the *P. ostreatus* samples were inoculated in Petri plates with crude oil applied in two different ways: spread on the entire MSM medium surface as well as in plates with pieces of filter paper soaked in oil and placed around the inocula. In the first phase, the radial growth of each isolates mycelium was inhibited on culture media treated with oil spread on the entire surface. Four weeks later, the culture media were covered by mycelium. These results indicated that samples of *P. ostreatus* tested were able to utilize the crude oil as a source of carbon in their metabolism.

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# BIODEGRADABLE MATERIALS AND ITS APPLICATIONS IN MEDICAL TOOLS DEVELOPMENT – A REVIEW

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***Abstract***

*Biopolymers are the preferred materials for biological and life science applications because of their high adaptability and compatibility with biological molecules and cells. Polymer-based biomaterials are widely used for medical applications due to the multiple physical and chemical properties of these materials. Biopolymers can be easily fabricated in various complex shapes and structures and additionally surface properties can be easily tuned. The concept of biodegradable plastics is of considerable interest with respect to solid waste accumulation and environment sustainability issues. Greater efforts have been made in developing degradable biological materials without any environmental pollution to replace oil-based traditional plastics. Among numerous kinds of degradable polymers, polylactic acid sometimes called polylactide, an aliphatic polyester and biocompatible thermoplastic, is currently a most promising and popular material with the brightest development prospect and was considered as the 'green' eco friendly material. In this review different types of available biopolymers and their usage in medical tools development will be described.*

***Keywords:*** *biopolymers, biodegradable, polylactic acid.*

## INTRODUCTION

Biomaterials are sometimes characterized as materials used to construct artificial organs, rehabilitation devices, or implants to replace natural body tissues (Bauer et al., 2013). More specific, biomaterials are materials that are used in close or direct contact with the body to augment or replace faulty materials. In general, they can be classified into living or once living materials, which fit into the division of, for example, tissue engineering, and materials that are of a synthetic origin. Such biomaterials can be defined as inorganic or organic materials that are biocompatible and can be implanted in the human body to replace or repair failing tissue. In recent years, progress in many different fields has paved the way to creating innovative biomaterials to improve existing treatments and develop new ones for a higher quality of life. Biomaterials play an important role in human health.

Biopolymers are the main type of biomaterials. According to their degradation properties, biopolymers can be further classified into biodegradable and non-biodegradable biopolymers. Many implants, such as bone substitution materials, some bone fixing materials, and dental materials, should possess long term stable performance in the body. In recent years, developments in tissue engineering, regenerative medicine and controlled drug delivery have promoted the need of new properties of biomaterials with features of biodegradation (Huayu et al., 2012). Biologically derived and synthetic biodegradable biopolymers have attracted considerable attention.

This paper reviews recent refereed published literature, particularly associated with the characteristics of biomaterials which are important to medicine.

## MATERIALS AND METHODS

With an ageing population, war and sports related injuries there is an ever-expanding requirement for hard tissue replacement such as bone. Engineered artificial scaffold biomaterials with appropriate mechanical properties, surface chemistry and surface topography are in a great demand for enhancing cell attachment, cell growth and tissue formation at such defect sites. Most of these

engineering techniques are aimed to mimick the natural organization of the bone tissues and thereby create a conductive environment for bone regeneration. As the interaction between the cells and tissues with biomaterials at the tissue–implant interface is a surface phenomenon, surface properties play a major role in determining both the biological response to implants and the material response to the physiological condition. Therefore surface engineering of biomaterials is aimed to modify the material and biological responses through changes in surface properties while still maintaining the bulk mechanical properties of the implant (Sameer and Narendra, 2009). Implants not only have to be biosafe and biostable in terms of cytotoxicity and degradation, they also have to match with the biological requirements of any structural biocompatibility. In other words, shape, inner structure and design of an implant need to be adapted to the characteristics of the tissue to be replaced (Bauer et al., 2013).

Low cost manufacturing of polymeric microdevices for transdermal and subcutaneous drug delivery is also slated to have a major impact on next generation devices for administration of biopharmaceuticals and other emerging new formulations. These devices range in complexity from simple microneedle arrays to more complicated systems incorporating micropumps, micro-reservoirs, on-board sensors, and electronic intelligence (Ochoa et al., 2012).

The different classes of materials used for the fabrication of bioimplants and bio-devices can be broadly classified as metallic materials, *polymers*, ceramics, *composites* and *natural materials*.

*Polymers* are long chain molecules consisting of large number of small repeating units known as monomers. They belong to the family of macromolecules and represent the largest class of biomaterials. Polymers can be derived either from natural sources or from synthetic organic sources (Figure 1.), (Bauer et al., 2013). Polymers can be easily manufactured to various complex shapes and structures and additionally surface properties can be easily tuned. Polymers are widely used in biomedical applications due to the range of physical and chemical properties possible with these materials (Mark, 1996).

A *composite* consists of two or more materials each with distinct physical or chemical properties. It is designed to have a combination of best characteristic of each component material. Biomedical composites are often designed to provide superior mechanical and biological compatibility.

They can be classified based on the matrix material or on the bioactivity of the composites. Some promising medical applications of biomedical composites include their use in total joint replacements, spine rods, discs, plates, dental posts, screws, ligaments and catheters (Makarov et al., 2013).

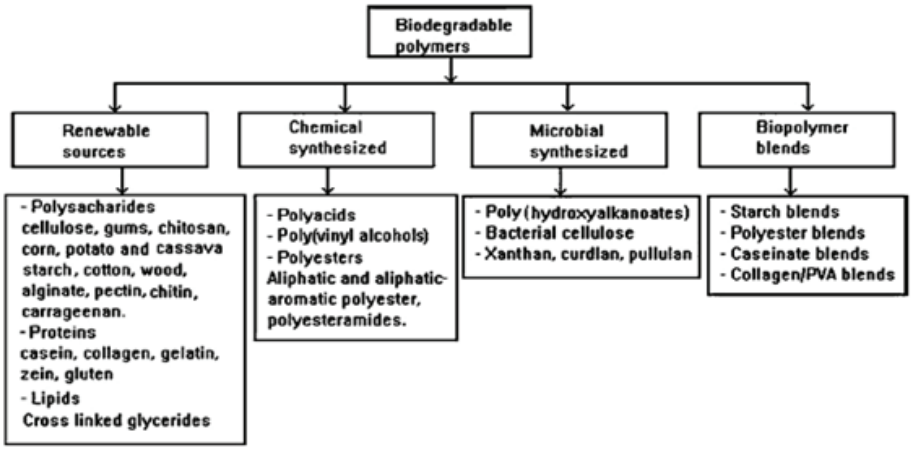


Figure 1. Clasification of biodegradable polymers and their nomenclature (Kestur et al., 2009)

*Natural polymers* such as starch, collagen, chitosan and glycosaminoglycans (chondroitin sulphate, hyaluronic acid) are the most commonly used natural materials for clinical applications (Liu et al., 1999).

Starch is a potentially useful polymer for the thermoplastic biodegradable materials because of its low cost, availability and production from renewable resources (Pal et al., 2006).

Collagen is a fibrous protein that connects and supports other bodily tissues such as skin, bone, tendons, muscles, and cartilage.

It is the wealthy available protein present in the bodies of mammals, including humans.

Chitosan is a natural biopolymer derived from crustacean and structurally similar to hyaluronic acid, a polymer component of the extracellular matrix (ECM), making it particularly attractive for tissue engineering (Foster and Butt, 2011; Mourya et al., 2011).

Glycosaminoglycan is the most abundant heteropolysaccharide present in the body. Condroitin Sulphate (CS) plays a key role in biomaterials field: it is a widely distributed glycosaminoglycan in the human body, structurally present in cartilage and other tissues such as eye, aorta, skeletal muscle, lung and brain. In biomedical applications, CS has shown *in vivo* anti-inflammatory effect. However the most commonly exploited natural polysaccharide in scaffold assembly for tissue engineering and as component for implant materials is hyaluronic acid (HA). HA, in fact, present a high capacity for lubrication, is very hydrophilic and influences several cellular functions such as migration, adhesion and proliferation (Agostino et al., 2012).

The advantages associated with these natural biomaterials can be listed as follows (Sameer and Narendra, 2009) : i) these materials being similar to the macromolecular substances, get easily recognized by the biological environment and therefore deal metabolically; ii) problems of toxicity, chronic inflammation, and lack of recognition by cells which occurs mostly with synthetic materials can be avoided; iii) these materials are biodegradable, and therefore they can be used for applications where it is desired to deliver a specific function for a temporary period of time.

Poly (vinyl alcohol) (PVOH) is generally prepared by the saponification of poly (vinyl ester) s, such as poly (vinyl acetate) (PVAc).

PVOH has gained increasing attention in the biomedical field due to its bioinertness (Yong-Woo et al., 2000). PVOH hydrogels resemble organic tissue and have a high elastic modulus even though their water content is very high.

Poly (lactic acid) (PLA), is a biodegradable, biocompatible and compostable polyester derived from renewable resources such as corn, potato, cane molasses and beet sugar. It has a bright future as an environmentally friendly thermoplastic. With the help of this green polymer industries will be able to close the carbon cycle, and their dependence on non-renewable fossil resources will be reduced considerably. Aliphatic polyesters such as PLA, derived from lactic acid (LA), 2-hydroxy-propionic acid, produced from renewable resources, has promising applications in packaging, consumer goods, fibers and in biomedicine because of its excellent mechanical properties, transparency, compostability and bio-safety.

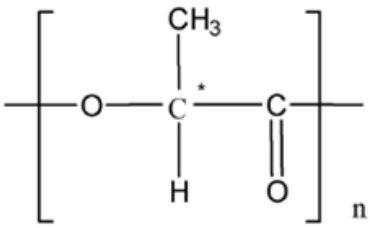


Figure 2. Structural formula of PLA, chiral molecule

Potential feedstock for the production of PLA is lactic acid (LA), which contains a chiral centre (Figure 2). LA can be produced by fermentation from renewable resources such as

milk and carbohydrates such as corn, potato, cane molasses and beet sugar (Sameer and Narendra, 2009).

Poly (hydroxyalkanoate) (PHA). Another family of polyesters being studied widely are poly (hydroxyalkanoate) s (PHAs) that occur in nature. They are produced by a wide variety of micro-organisms as an internal carbon and energy storage, as part of their survival mechanism. Bacterially synthesized PHAs have attracted attention because they can be produced from a variety of renewable resources and they are truly biodegradable and highly biocompatible thermoplastic materials (Long et al., 2006).

## RESULTS AND DISCUSSIONS

### Biopolymers with reactive groups

-*Aliphatic polyesters*, such as poly (lactic acid) (PLA), poly (glycolic acid), poly (-ε capro-lactone) (PCL) and their copolymers, have been widely investigated for biomedical application because of their biodegradability, bioresor-bability and biocompatibility. They are among the most commonly used degradable materials for the preparation of clinical devices. Aliphatic polyesters with reactive groups have attracted attention because of the demand of synthetic biopolymers with tunable properties, including features such as hydrophilicity, biodegradation rates, bioadhesion, drug/targeting moiety attachment, etc. (Lou et al., 2003).

In particular, polymeric biomaterials with properties that can be tailored by introducing functional groups, such as carboxyl, hydroxyl, amino, ketal, bromo, chloro, carbon–carbon double bonds or triple bonds, etc., are needed.

-*Polycarbonate (PCs)*. Since the last decade, polycarbonates (PCs) have attracted increasing attention in pace with their significant applications in the medical field, owing to their unique combination of biodegradability and biocompatibility. Polycarbonates have been commonly used as integral components of engineered tissues, medical devices and drug delivery systems (Feng et al., 2012).

-*Polyurethans* (PUs) are a large family of polymeric materials with an enormous diversity of chemical compositions, mechanical proper-ties, tissue-specific biocompatibility and biodegradability, with mechanical flexibility and moderate blood compatibility being their most prominent features. Because of their diversity of composition and mechanical properties, PUs are among the most extensively used synthetic polymers in biomedical applications, and remain one of the most popular groups of biomaterials applied to medical devices after half a century of use in the healthcare system (Chen et al., 2013).

-*Poly (amino acids)* is an important kind of biocompatible and biodegradable synthetic polymers and have been studied for biomedical application in many fields (Deming, 2007).

However, their application is limited because of their insolubility or pH-dependent solubility and lack of functional groups.

-*Polyphosphoesters*. PPEs with repeating phosphoester units in the backbone are attractive biocompatibile and biodegradable biomaterials because of their structural similarity to the naturally occurring nucleic acid and easy functionality as compared to conventional polyesters (Zhao et al., 2003).

### Biopolymers with responsive activities

-Due to the ability to mimick the basic response process of living systems, *stimuli-responsive polymers* have attracted increased attention. These polymers can respond to small changes (temperature, pH, photo, redox) in environ-mental stimuli with distinct transitions in phy-sical-chemical properties, including confor-mation, polarity, phase structure and chemical composition (Alarcon et al., 2005).

-*Electroactive biomaterials*. After the discovery that electrical signals can regulate cell attach-ment, proliferation and differentiation, many researchers sought to incorporate conducting polymers into biomaterials to take advantage of electrical stimuli.

- *Specific bonding biopolymers*. Alternative biodegradable platforms have been described in studies of nanoconjugate drug delivery poly-mers such as poly (l-glutamic acid) s, PLHis, polysaccharides, and PLLA, PLGA.

-*Biopolymers for tracing and bioimaging* (Bio-polymers for optical tracing and bioimaging, Biopolymers for MRI).

### Specific biomedical applications

-*Medical devices*. Synthetic biodegradable polymers have attracted considerable attention for applications in medical devices, and will play an important role in the design and

function of medical devices. The general criteria of polymer materials used for medical devices include mechanical properties and a degradation time appropriate to the medical purpose. In addition, the materials should not evoke toxic or immune responses, and they should be metabolized in the body after fulfilling their tasks. According to these requirements, various synthesized biodegradable polymers have been designed and used. Some synthesized biodegradable polymers that have been used or show potential in selected fields are summarized below.

▪ Drug-eluting stents (DES). DESs have been widely used as a default treatment for patients with coronary artery disease. Biodegradable polymers are always used as a biodegradable and bioresorbable coatings on stents to control the release of drugs. Besides being used as biodegradable coatings, biodegradable polymers are also candidate materials for fully biodegradable stents because of their suitable properties for controlled drug release and good mechanical performance to prevent stents from deforming or fracturing (Shen et al., 2012).

▪ Orthopedic devices. Orthopedic devices made from biodegradable materials have advantages over metal or nondegradable materials. They can transfer stress over time to the damaged area as it heals, allowing of the tissues, and there is no need of a second surgery to remove the implanted devices. Many commercial orthopedic fixation devices such as pins and rods for bone fracture fixation, and screws and plates for maxillofacial repair are made of PLLA, poly (glycolide) and other biodegradable polymers (Maharana et al., 2009; Mark, 1996).

▪ Disposable medical devices. In the 21$^{st}$ century, environment factors concern all manufacturing industries. Many disposable medical devices, such as syringes, injection pipes, surgical gloves, pads, etc., are usually made of non-degradable plastics, resulting in serious environmental and economic issues. PLA, poly (glycolide), poly [D,L- (lactide-co-glycolide)] and PCL are all biodegradable. Therefore, they are promising materials for use in disposable medical devices meeting environmental friendly requirements. These biodegradable polymers have been used to prepare some disposable medical devices and will likely have a widening commercial application.

▪ Other medical devices. Biodegradable polymers have also been used to prepare anastomosis rings used for intestinal resection, drug delivery devices, in situ forming implants and stents used in urology (Huayu et al., 2012; Mendez-Probst et al., 2010).

-*Tissue engineering*. Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes used to restore, maintain or improve tissue functions. The main purpose of tissue engineering is to overcome the lack of tissue donors and the immune repulsion between receptors and donors (Schmedlen et al., 2002; Kim et al., 2011).

- *Drug delivery and control release*. Biodegradable polymers, such as poly (α-malic acid), with reactive pendant carboxyl groups, can conjugate drugs (via ester or amide bonds) to form a biodegradable macromolecular prodrug to reduce the side-effects of free drugs. Drugs can be released via the degradation of biodegradable polymers (Crispim et al., 2012).

-*Gene delivery*. Gene delivery has great potential for treating various human diseases (Merdan et al., 2002). Recently, nonviral vectors have been proposed as safer alternatives to viral vectors for gene delivery. Many carriers are non-degradable and the risk arises of accumulation in the body, especially after repeated administration. A good gene carrier should be able to deliver the target gene to specific cells with high efficacy; it should also be degradable and be excreted from the body after a given time period. Recently, some research has evaluated non-degradable polymers with biodegradable polycations via hydrolysable linkers as gene carriers.

-*Bioseparation and diagnostics applications*. The development of biomedical polymers conjugated with peptide or protein domains has mostly focused on their use as bioactive materials in controlled drug delivery or tissue engineering. A new challenge arises in the development of materials for bioseparation and diagnostics applications. For these applications, materials that are biocompatible with reduced non-specific absorption and denaturation, that are able to amplify and transmit signals and

that are beneficial for high-throughput screening with enhanced sensitivity and reduced size are in great demand. To meet these demands, polymeric materials in various shapes, such as membranes, thin films, micro/nano-particles, hydrogel and micro/nano-fibers have been widely investigated (Li et al., 2004).

## CONCLUSIONS

Characterization of any material, particularly newly developed ones, is important from various standpoints, such as its utility and value addition, which may open up new areas for further development to assess the effectiveness of its processing, the effect of different environments on its properties and to find suitable areas of application.

Biodegradable biomaterials have been widely used and have greatly promoted the development of biomedical fields because of their biocompatibility and biodegradability.

Biodegradable polymers can be classified as natural or synthetic polymers according to the source.

Future integrated biorefineries based on green chemistry operations will serve as the basis for providing sustainable raw materials for biocomposite production.

The present and future look promising for the introduction of sustainable biocomposites as a replacement for traditional fossil based materials in diverse applications. A wide range of raw materials from recycled and renewable resources will be readily available for choice, together with controlled synthetic and modification routes to tailor the desired properties of the designed biocomposites (Vilaplana et al., 2010).

Once manufactured, products made from sustainable biocomposites must satisfy the requirement that no hazardous effects to the environment or to human health will be derived throughout their service life in the intended application. The development of biotechnology and medical technology has set higher requirements for biomedical materials. The development of commercially viable "green products" based on natural resources for both matrices and reinforcements for a wide range of applications is on the rise. This effort includes new pathways to produce natural polymers with better mechanical properties and thermal stability using nanotechnology and adding different natural fillers such as lignocellulosic fibers.

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# OPTIMIZATION OF *TRICHODERMA* STRAIN CULTIVATION FOR BIOCONTROL ACTIVITY

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**Abstract**
*Pathogens cause world-wide economically significant diseases in numerous agricultural, horticultural and ornamental crops. Most of the pathogens are difficult to control by conventional fungicides. Biocontrol represents an economical, environmentally friendly alternative to chemical pesticides for diseases produced by phytopathogens. Trichoderma strains have received particular attention as biocontrol agent of fungal plant pathogens. The present work is focused on the optimization of growth and sporulation of antagonistic Trichoderma T36. Assessment of microbial cultures was done by measuring the fungal colony growth on solid medium. Likewise, visual and microscopically observations were performed. Trichoderma T36 was cultured on different nutrient media and M1 medium, a Czapek-Dox medium supplemented with sodium phosphate, ammonium chloride and malt extract, was selected for further experiments. A wide range of carbon sources has been tested replacing initial source in M1 medium. The best results were obtained in media with fructose as carbon source. As expected, ammonium dihydrogen phosphate was found to be the best nitrogen source for Trichoderma T36 cultivated on M1 medium and colony diameter decreases in the following order: $NH_4H_2PO_4$<$NH_4Cl$<$NH_4NO_3$=$NaNO_3$<$KNO_2$<$KNO_3$=$NaNO_2$<urea. Fungal growth was excellent at temperatures of 26 - 37°C and pH range of4.0 – 5.5.*

***Keywords:*** *biocontrol, microbial antagonism, phytopathogens, Trichoderma*

## INTRODUCTION

Strains of *Trichoderma* can produce a great number of metabolites and because of these properties, the genus has high biotechnological potential [1; 2]. Several studies have been reported the capacity of *Trichoderma* to be used as biocontrol agent against phytopathogens, such as *Fusarium, Phytium, Rhizoctonia* and *Sclerotinia* species [3; 4;5; 6]. When planning the application of antagonistic *Trichoderma* strains for the purposes of biological control, it is very important to consider the parameters affecting the growth and sporulation [7; 8; 9]. The work is a continuance of a complex research to obtain an antagonistic *Trichoderma* and is focused on optimization of growth and sporulation of selected fungal strain.

## MATERIAL AND METHOD

### Microorganisms

A potential biocontrol agent *Trichoderma* strain T36 used in the study provides from Microbial Collection of ICECHIM. The strain isolated from forest soil was maintained on potato dextrose agar (PDA) slants medium at 4°C.

### Cultivation conditions

The microbial growth was evaluated on solid agar media in Petri plates. The compositions of tested nutrient media are:
MEA medium [10] (g/l): malt extract malt, 30; peptone, 3, agar 20, 1ml $CuSO_4$ x 5 $H_2O$ 0.5g/l; 1ml $ZnSO_4$ x 7 $H_2O$ 1g/l; pH, 5.5.
PDA medium: potato, 250, dextrose, 20, agar, 18 [10].

Czapek medium [10] (g/l): $NaNO_3$, 3, $K_2HPO_4$, 1, $MgSO_4x7H_2O$, 0.5, $FeSO_4$, 0.01; KCl, 0.5; saccharose, 30g; agar, 18.
Czapek-Dox medium [10] (g/l): $NaNO_3$, 3; $K_2HPO_4$, 1; $MgSO_4x7H_2O$, 0.5; $FeSO_4$, 0.01; KCl, 0.5; glucose, 30; agar, 18.
OM medium [11] (g/l): starch, 5; glucose, 5; peptone, 5; yeast extract, 7.5; after autoclaving, 10 ml of solution A and B and 1 ml of solution C and D. Composition of solution A g/l): $KH_2PO_4$, 5; $K_2HPO_4$, 5. Composition of solution B (g/l): $MgSO_4$, 17, NaCl, 1; $MnSO_4$, 0.7; $CuSO_4$, 0.06. Composition of solution C (g/100 ml): $FeSO_4$ x 7 $H_2O$, 0.1:, sodium citrate, 2,2; ammonium acetate, 2; sodium succinate, 3.3. Composition of solution D (mg/100 ml): biotin, 10 mg; p-aminobenzoic acid, 20; vitamin $B_{12}$, 5; calcium pantothenate, 10; pyridoxal hydrochloride, 10; nicotinamide acid, 35.
Mediul Mandels [12] (g/l): glucose, 10; urea, 0.3; $(NH_4)_2SO_4$, 1.4; $KH_2PO_4$, 2; $CaCl_2$ x $2H_2O$, 0.4; $MgSO_4$ x $7H_2O$, 0.3; peptone, 0.75; yeast extract, 0.25; $FeSO_4$ x $7H_2O$, 0.005; $MnSO_4$ x $4H_2O$, 0.0016; $ZnSO_4$ x $7H_2O$, 0.0014; $CoCl_2$ x $6H_2O$, 0.02..
Miller medium [13] (g/l): $Na_2HPO_4$, 6; $KH_2PO_4$, 3; NaCl, 0.5; agar, 15; pH, 6.5. Salts and agar are autoclaved separately and then supplemented with 2 ml of 1M $MgSO_4$ x 7 $H_2O$, 10 ml 20% glucose, 0,1 ml 1M $CaCl_2$ and 0.5 ml vitamin $B_1$.
M1 medium (g/l): Czapek-Dox medium supplemented with $NaHPO_4$, 3; $NH_4Cl$ 1; malt extract, 3.
The media were sterilized at 1 atm ($121^0$C), 20 minutes. Petri plates were inoculated with 20 µl of sporal suspension ($1,2x10^7$ conidii/ml) and incubated for 120 hours at 26±2°C. All the culturing experiments were carried out in triplicate.
In selected medium, carbon and nitrogen sources were replaced and the effects were determined. The concentration of carbon and nitrogen source was 0.2% (w/v) and 0.1% (w/v), respectively. The effect of several aminoacids and vitamins (B1 and B12) was analyzed by medium supplementation with 0.1 % (w/v) of each compound tested.

*Influence of pH values*

Fungal cultures were incubated at different pH values, from 4.0 to 9.0. OAKTON pH- meter was used.

*Influence of temperature*

Fungal cultures were incubated in static incubator Heidolph Unimax 1010 at 2°, 4°, 6°C, 10°, 16°, 22°, 26°, 30° and 37°C.

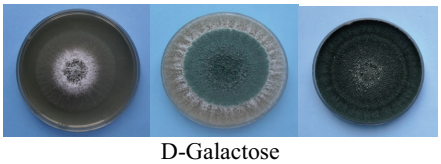
## RESULTS AND DISCUSSIONS

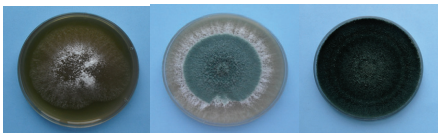
Nutritional requirements and psychological parameters of antagonistic *Trichoderma T36* were studied.
Initially, *Trichoderma* T36 was cultivated in Petri plates on different solid nutrient media. The fungal growth was measured as colony diameter. The results presented in Table 1 reveal that M1 medium offers conditions for faster and better growth of *Trichoderma* T36. The difference between media is obviously at 48 hours of cultivation, although at the end of incubation period most of tested media present similar colony diameters. According to these results, M1 medium was selected for further experiments.

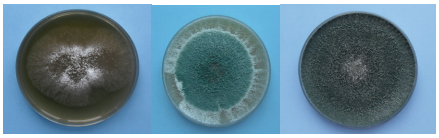
Table 1. Influence of nutrient medium on the radial growth and sporulation of *Trichoderma* T36

| Culture solid medium | Colony diameter (cm) | | Sporulation |
|---|---|---|---|
| | 48 h | 120 h | |
| MEA | 5.4 | 8.5 | +++ |
| PDA | 5.0 | 8.5 | +++ |
| Czapek | 2.4 | 6.1 | + |
| Czapek-Dox | 2.2 | 7.2 | + |
| OM | 5.4 | 8.5 | +++ |
| Mandels | 5.4 | 8.5 | +++ |
| Miller | 4.5 | 8.5 | +++ |
| M1 medium | 5.5 | 8.5 | +++ |

Based on composition of M1 medium, several carbon sources was tested for *Trichoderma* T36 growth.


D-Galactose

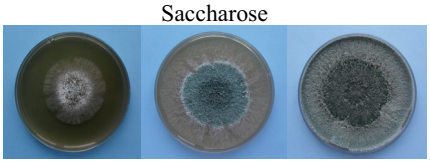

Fructose



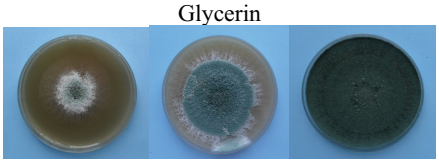
D-mannose

Lactose

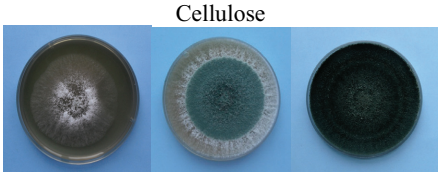
Saccharose



Glycerin



Cellulose



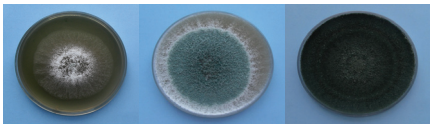
Dextrose

Glucose

Citric acid

Mannitol

Ribose



Arabinose

Figure 1. Effects of carbon source on the radial growth and sporulation of *Trichoderma* T 36; photos taken at 48, 72 and 120 hours of cultivation in Petri plates on solid M1 medium

The values of colony diameters corresponding to the photos depicted in Figure 1. are presented in Table 2.

Table 2. Influence of carbon source on the radial growth and sporulation of *Trichoderma* T36

| Carbon source | Colony diameter (cm) | | Sporulation |
|---|---|---|---|
| | 48 h | 120 h | |
| D-glucose | 5.90 | 8.5 | |
| D galactose | 5.20 | 8.5 | +++ |
| Fructose | 6.10 | 8.5 | +++ |
| Ribose | 5.95 | 8.5 | +++ |
| D mannose | 5.20 | 8.5 | +++ |
| Arabinose | 4.10 | 8.5 | +++ |
| Mannitol | 5.82 | 8.5 | +++ |
| Saccharose | 3.61 | 8.5 | ++ |
| Lactose | 4.16 | 8.5 | +++ |
| D maltose | 5.26 | 8.5 | +++ |
| Starch | 4.82 | 8.5 | +++ |
| Cellulose | 5.42 | 8.5 | +++ |
| Glycerin | 3.10 | 8.5 | + |

It can be seen that the highest value of colony diameter is obtained on the medium with fructose as carbon source.

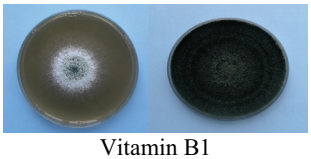
The effects of different nitrogen sources on fungal growth were studied by replacing initial nitrogen source of M1 medium with one of eight alternative nitrogen sources.

Ammonium dihydrogen phosphate was found to be the best nitrogen source for *Trichoderma* T36 growth. Colony diameter is decreasing in the following order: $NH_4H_2PO_4$< $NH_4Cl$< $NH_4NO_3$=$NaNO_3$<$KNO_2$<$KNO_3$=$NaNO_2$<urea. It is generally agreed that ammonium-nitrogen is the preferred form for microbial metabolism as it requires less energy to be assimilated. After 120 hours of cultivation the colony diameters are similar for all nitrogen sources tested

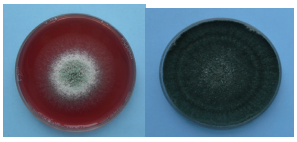
The results of the investigations dealing with the influence of the aminoacids and vitamins on fungal growth are presented in Figure 2, concluding that at 48 hours after inoculation, the highest value was obtained with valine.

Table 3. Influence of nitrogen source on the radial growth and sporulation of *Trichoderma* T36

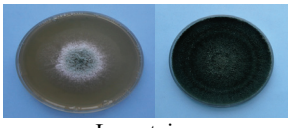
| Nitrogen source in | Colony diameter (cm) | | Sporulation |
|---|---|---|---|
| | 48 h | 120 h | |
| $NH_4H_2PO_4$ | 5.6 | 8.5 | +++ |
| $NH_4NO_3$ | 5.3 | 8.5 | ++ |
| $NaNO_2$ | 3.2 | 8.5 | ++ |
| $NaNO_3$ | 4.6 | 8.5 | ++ |
| $NH_4Cl$ | 5.4 | 8.5 | +++ |
| $KNO_2$ | 3.5 | 8.5 | ++ |
| $KNO_3$ | 3.2 | 8.5 | ++ |
| Urea | 2.8 | 8.5 | ++ |



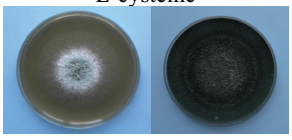
Vitamin B1



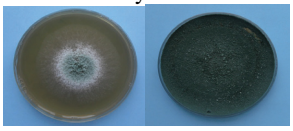
Vitamin B12



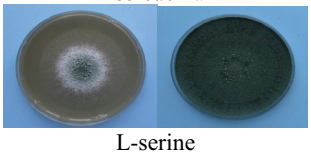
L-cysteine



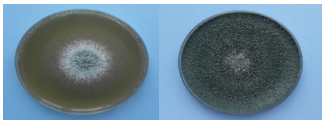
L lysine



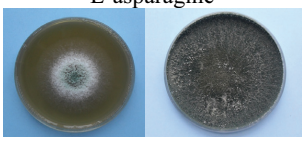
Isoleucina



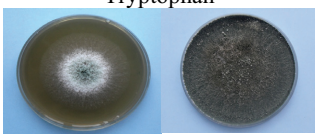
L-serine



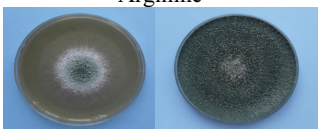
L-asparagine



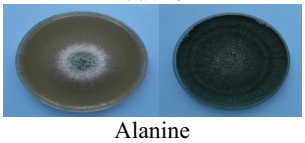
Tryptophan



Arginine



Valine



Alanine

Figure 2. Influence of aminoacids and vitamins on the radial growth and sporulation of *Trichoderma* T36

The values are decresing in following order: valine>alanine>L-lisine=isoleucine=L-serine, with colony diameter variying form 6.4 to 6.0 cm.

The next step was the investigation of physical factors affecting ***fungal growth***.

The pH values of nutrient medium belong to the most important parameters affecting microbial cultivation. As it can be shown in Figure 3, the pH values between 4.5–5.5 produce colonies larger than 6.5 cm in diameter, whereas for higher pH values the diameter did not exceed 4.2 cm. The strain grew best at pH 5.5, results in agreement with other scientific reports [14, 15].

For optimum growth, temperatures must be in a range that allows the most efficient progression of the chemical reactions necessary for growth. In this respect, the ability of *Trichoderma* T36 to grow at 2, 4, 6, 10, 16, 22, 26, 30 and 37˚C was tested (Figure 4.).

It was observed that *Trichoderma* T36 did not grow at lower temperatures, such as 2, 4 and

6˚C. As expected, the increasing temperature facilitates the growth, reaching the maximum growth and sporulation between 26 and 37˚C. This behavior is normal for mesophilic fungal strain at it has been reported [15].

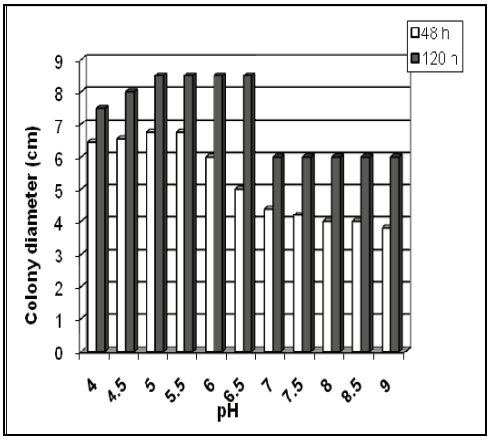



Figure 3. Influence of pH values on the radial growth and sporulation of *Trichoderma* T36

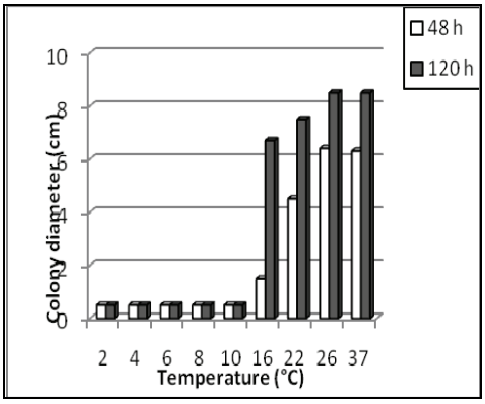



Figure 4. Influence of temperature on the radial growth and sporulation of *Trichoderma* T36

## CONCLUSIONS

The optimization study covered physical parameters (pH and temperature) and nutrients composition (carbon and nitrogen source) on a selected solid medium. The nutrient solid medium that promoted the highest growth (estimated as colony diameter) was used for the subsequent steps of the investigation. The composition of selected M1 medium was modified as regarding the nature of carbon and nitrogen source. The highest colony diameter is obtained on M1 medium with fructose. The ammonium-nitrogen salts $NH_4H_2PO_4$, $NH_4Cl$ and $NH_4NO_3$ are preferred for microbial metabolism, $NH_4H_2PO_4$ being the best nitrogen source. Among the aminoacids and vitamins tested, the best result was obtained in medium supplemented with valine. *Trichoderma* T36 has a maximum growth and sporulation between 26 and 37˚C at pH 5.5. The studied strain is a potential candidate for the biological control of plant diseases. In this respect, further researches will be dedicated to production of volatile and non-volatile metabolites [16], capable of inhibiting mycelial growth at several pathogens.

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# NOVEL FUNGAL COLLAGENASE FROM *ASPERGILLUS ORYZAE*

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***Abstract***
*The industrial processing of leather has a high environmental impact due to the heavy use of polluting chemicals in the tanning process. Microorganisms can be used in order to degrade natural fibers like fur, leather and cotton because they synthesized enzymes that can break chemical bonds in these materials. Some proteolytic enzymes, like collagenases, have a number of industrial applications in fur and leather industry and, moreover, are nontoxic and eco-friendly. This paper reports the purification and characterization of a fungal collagenase produced by an Aspergillus strain. Aspergillus oryzae Mi 156 and Aspergillus ochraceus Mi 153 were tested for their capacity to degrade native collagen. Enzymatic activity and protein content were determined using spectrophotometrical methods. The best results regarding collagenolytic activity were obtained for fungal strain Mi 156. The enzyme was separated by ammonium sulphate precipitation and analyzed for its optimum pH and temperature. The effect of some activators and inhibitors on the enzymatic activity was also tested. The results indicated 8 − 8.3 as optimum pH and 35 − 40 $^{o}C$ as optimum temperature. When using $Zn^{2+}$ as activator the collagenase activity was about 29 % higher, while for $Co^{2+}$ as inhibitor, the activity dropped down about 14 %.*

***Key words****: activators, Aspergillus oryzae, characterization, collagenase, inhibitors.*

## INTRODUCTION

The industrial processing of leather has a high environmental impact due to the heavy use of polluting chemicals in the tanning process. Microorganisms can be used in order to degrade natural fibers like fur, leather and cotton because they synthesized enzymes that can break chemical bonds in these materials. Some proteolytic enzymes, like collagenases, registered an increasing use for industrial applications in fur and leather industry because they are nontoxic and eco-friendly.

Collagenases are endopeptidases that digest native collagen in the triple helix region. Collagen is the major fibrous component of animal extracellular connective tissue. Unlike animal collagenases that split collagen in its native triple-helical conformation, bacterial collagenase is unique because it can degrade both water-insoluble native collagens and water-soluble denatured ones. It can attack almost all collagen types, and is able to make multiple cleavages within triple helical regions (Worthington C., 1988).

Different authors reported as collagenase-producing strains *Bacillus* and *Aspergillus*: *B. subtilis* (Tran and Nagano, 2002; Okamoto, 2001; Rui et al., 2009), *B. licheniformis* (Baehaki et. al, 2012), *A. fumigatus* (Reichard U. et al, 1990).

This paper reports the separation and characterization of a fungal collagenase produced by some *Aspergilus* strain. The separation of the enzyme was done by precipitation with ammonium sulphate salt in a variety of saturated degree. The characterization of purified enzyme includes: determination of optimum pH and temperature and study of the effect of some activators and inhibitors on the enzymatic activity. Enzymatic activity and protein content were determined using spectrophotometrical methods.

## MATERIALS AND METHODS

**Microorganism and growth conditions.** The fungal strains *Aspergillus oryzae* Mi 156 and *Aspergillus ochraceus* Mi 153 were obtained from the collection of Faculty of Biotechnology, University of Agronomical Sciences and Veterinary Medicine Bucharest. The inoculation was done in a basal salt

medium supplemented with 0.1% glucose and 1% sheep leather meal. Proteolitic enzymes were produced in 500 ml flasks, kept at 27°C, agitated at 150 rpm, for 15 days. The culture was centrifuged at 6000 rpm, at 4°C, for 20 minutes.

**Enzymatic assay of collagenase,** based on some classical refferences (Moore S., Stein W.H., 1948; Mandl I. *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 °C).

**Separation of collagenolytic enzyme**

The enzyme was separated by ammonium sulphate precipitation using 90% $(NH_4)_2SO_4$ saturated degree. The obtained precipitate was dissolved in 50 ml 0.05 M phosphate buffer and dialyzed against distillated water for 18 hours, at 8°C, with agitation.

**Effects of pH and temperature**

The pH and temperature effect on the enzymatic activity were studied in order to characterize the collagenase biosynthesized by selected strain. For this purpose measurements of enzymatic reaction rate were made at pH between 6.8 – 7.9 using TES buffer and between 8.0 – 8.6 with Tris-HCl buffer. The tested temperature range was 25°C – 45°C.

**Effects of various activators and inhibitors**

Effects of activators on the enzyme were investigated by adding 0,36 M $ZnCl_2$ solution in the mixture reaction in order to test the ability of $Zn^{2+}$ ion to activate the activity of collagenase biosynthesized by selected strain. 10mM EDTA and $CoCl_2$ solutions were used as inhibitors of collagenolytic activity.

**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 O.H. et al., 1951).

## RESULTS AND DISCUSSIONS

The fungal strains *Aspergillus oryzae* Mi 156 and *Aspergillus ochraceus* Mi 153 were tested regarding the collagenolytic activity (table 1).

Table 1. Collagenolytic activity of enzymatic preparate sinthesized by fungal strains

| Fungal strain | Soluble protein (mg/ml) | Collagenase | |
|---|---|---|---|
| | | Enzymatic activity (U/ml) | Specific enzymatic activity (U/mg prot) |
| Mi 153 | 0.021 | 14.56 | 693.33 |
| Mi 156 | 0.020 | 15.86 | 780.0 |

The best results were obtained for fungal strain Mi 156, which was selected in order to obtain and characterize the enzymatic preparate synthesized by cultivation in culture medium containing sheep leather as carbon source.

Total protein extract (TPE) obtained by cultivation of *Aspergillus oryzae Mi156* strain was purified by precipitation with ammonium sulphate. The obtained precipitate, which is a protein fraction that contains most of collagenase, was redissolved in 0.05 M phosphate buffer, than the solution was dialysed for removing the ammonium sulphate. Both the centrifuged culture medium (TPE) and the protein fraction (PF) obtained by precipitation with ammonium sulphate were analyzed regarding the protein content and the collagenolytic activity.

The results (table 2) indicated a moderate efficiency of enzyme separation process by precipitation with ammonium sulphate, as the obtained purification factor of the enzyme was 1.48.

Table 2. Results of precipitation with ammonium sulphate

| Mi 156 | AES (μmol/mg proteins) | Purification factor |
|---|---|---|
| TPE | 722 | 1 |
| PF | 1068 | 1.48 |

**Effects of pH and temperature**

Enzymatic activity depends on the thermodynamic activity of the hydrogen ions, that is solution pH. Most enzymes accomplish a maximum activity at certain pH value, namely optimum pH; the enzymatic activity decreases below and over this pH value.

Regarding the effect of temperature, generally the enzymatic activity increases as the temperature increases if the enzyme is stable and active during the respective temperature range.

The influence of pH on the activity of the collagenase (fig. 1a) was determined measuring the enzymatic activity by the previously described method at increasing values of pH (between 6.8 − 8.6). To test the influence of temperature on the activity of the collagenolytic enzyme, the enzyme was incubated in the reaction mixture at various temperatures (25-45°C) (fig. 1b). The remaining activity was measured as described previously.

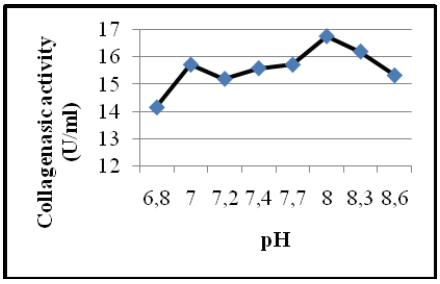


Figure 1a. Effects of pH on collagenolytic activity

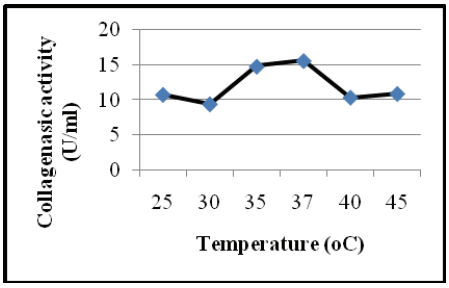


Figure 1b. Effects of temperature on collagenolytic activity

Experimental results (fig. 1a, 1b) indicated that the collagenase synthesized by the selected strain achieved maximum activity at pH 8.0 and 37°C.

**Effects of various activators and inhibitors**

An adequate activity of collagenases involves binding of the $Ca^{2+}$ and $Zn^{2+}$ ions, but only zinc ions are bounded in the catalytic situs, while calcium ions are necessary only for maintaining of the enzyme conformation (Lowry C.L. et al, 1992; Housley T.J. et al, 1993; Zhang Y. et al, 1997). Therefore, a $ZnCl_2$ solution was used in order to characterize the collagenolytic activity; calcium chloride is component of the buffer solution used in determinations.

Regarding the potential inhibitors, recent studies indicated that beside EDTA, well-known inhibitor for collagenases, also cobalt ions may inhibits this enzyme because they can be bounded by hystidin located in the catalytic situs instead zinc ions (Macartney H.W., Tschesche H., 1981).

For these reasons the sensitivity of the purified collagenase towards solution 0.36 M $ZnCl_2$ as activator and towards some inhibitors such as 10 mM EDTA and 1mM $CoCl_2$ was investigated. The activity was compared with that of the reaction that was free of the corresponding metal ions (control).

The obtained results (fig. 2) indicated that collagenolitic activity was significant increased in the presence of the tested $Zn^{2+}$ concentration, the registered values beeing 30% higher compared to control activity.

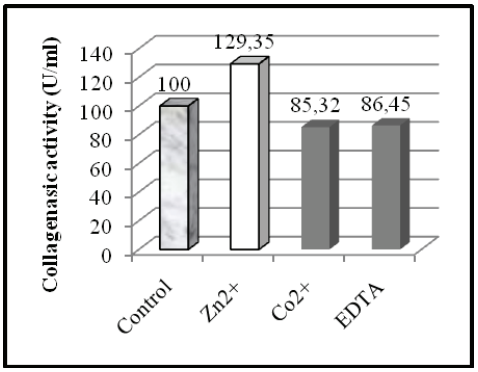


Figure 2. Effect of activators and inhibitors on collagenolytic activity

The values registered during the inhibition study revealed a decreasing of the collagenolytic activity both in the presence of EDTA and of $Co^{2+}$ ions (fig. 2). When using

$Co^{2+}$ as inhibitor, the activity of collagenase dropped down about 14 %.

## CONCLUSIONS

The best results regarding the collagenolytic activity were obtained for fungal strain Mi 156 (15.86 enzymatic units/ml).
The purification factor of the enzyme was 1.48 after the precipitation with ammonium sulphate, which indicated a moderate efficiency of enzyme purification process.
Experimental results indicated that the collagenase synthesized by the selected strain achieves maximum activity at pH 8.0 and 37°C.
The collagenolitic activity was significant increased in the presence of $Zn^{2+}$, the registered values beeing with 30% higher compare to control activity.
As a result of using $Co^{2+}$ as inhibitor, the activity of collagenase dropped down about 14 %.

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# SCREENING FOR HIGH LIPASE PRODUCING MICROORGANISMS

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***Abstract***

*The study of lipolytic enzymes has shown great scientific interest in modern biotechnology researches, due to the discovery of applications such as transesterification and ester stereospecific hydrolysis, used in oil industry, detergents and pharmaceutics. Enzyme catalysis using lipases has been intensely researched since the reaction takes place at regular pressure and 30-40$^{o}$C, achieving the necessary energy, and reducing at the same time the emergence of unwanted secondary compounds. Lipases used in biotechnology processes are usually microbial in origin and are commercially available, immobilized on different supports. They present the advantage of the possibility of both recycling the biocatalyst and also developing continuous processes. In biotechnological processes, the most exploited lipase is lipase B from Candida antarctica. Given the price of such enzymes, there is a continuous need for novel and better enzymes to be used in biocatalysis reactions. To this purpose, a series of industrial importance strains pertaining to the Microorganisms Collection of the National Institute for Chemical Pharmaceutical Research and Development ICCF Bucharest have been tested in order to determine potential lipase production. The screening was performed on 3 bacterial strains, 16 yeast strains and 14 fungi strains. The microorganisms were grown on several specific lipase inductor media. The enzymes produced by the strains which developed lipolytic activity are to be used later for biodiesel obtainment through biocatalysis.*

***Keywords:*** *enzyme, lipase, screening, transesterification.*

## INTRODUCTION

The ever increasing and developing industries all around the world have brought us to the challenging situation of needing to discover novel and better biocatalysts, to enhance the production of widely used products, such as food produce, cleaning agents, pharmaceuticals and many more. Also highly developing is the use of microbial populations for the treatment of agricultural, industrial and household wastes.

One of the most versatile groups of enzymes with biotechnological applications is represented by lipases (EC 3.1.1.3.), which carry out hydrolytic reactions in both aqueous and non-aqueous media, by performing interfacial catalysis. Lipases are carboxylesterases which catalyze the hydrolysis of long-chain triglycerides to mono-and di-glycerides, free fatty acids and glycerol (Aehle, 2007). Their catalytic activity covers esterification, trans-esterification, interesterification, acidolysis, alcoholysis and aminolysis (Joseph et al., 2008). Due to their wide area of activity, lipases are used in food and feed processing, fats and oils industry, detergents and various degreasing products, synthesis and production of fine chemicals, cosmetics and pharmaceuticals, bakery and brewing, biofuels and waste treatment (Kazlauskas et al., 1998; Sharma et al., 2001; Babu and Rao, 2007; Hasan et al., 2009; Balakrishnan et al., 2011; Kishore et al., 2011).

The main and most common source of lipases is represented by microorganisms (Gupta et al., 2004), although lipases are part of the physiology of living organisms and can be also found in plants and animals, as well as microbes (Rahnman et al., 2006). However, they are more abundantly found in bacteria, yeasts and fungi (Haki et al., 2003), which constitute nowadays the principal lipase generators for almost all processes that require them.

Given the augmentation of the demand for superior biocatalysts in industries requiring

lipases, the need of finding novel sources of lipolytic enzymes has been increasing as well.
The purpose of this study was to determine the possibility of using and enhancing microbial strains pertaining to an industrial microorganism collection for lipase production, with various applications in biocatalytic processes.

## MATERIALS AND METHODS

The strains that we have taken under consideration for our study were chosen from the Industrial Importance Microorganism Collection (IIMC) pertaining to the National Institute for Chemical Pharmaceutical Research and Development ICCF Bucharest, registered in the World Federation of Culture Collections Directory, no. 232, from the conservation vegetative stock. For our study, we have selected for the screening 3 bacterial strains, 16 yeast strains and 14 fungi strains, which we are describing below (Table 1).

Table 1. Strains that have been studied for lipase producing potential from the ICCF collection

| Strain name | Registration |
|---|---|
| *Pseudomonas aeruginosa F*– ATCC* 9027 | ICCF 90 |
| *Pseudomonas sp. no. 2* | ICCF 399 |
| *Pseudomonas sp. no. 3* | ICCF 400 |
| *Candida arborea* – CBS** 64 | ICCF 193 |
| *Candida glabrata* | ICCF 182 |
| *Candida famata* | ICCF 181 |
| *Candida robusta* | ICCF 194 |
| *Yarrowia lipolytica*– ATCC 16618 | ICCF 214 |
| *Yarrowia lipolytica* | ICCF 215 |
| *Candida boidinii*-CMBG*** 221 | ICCF 26 |
| *Candida paraffinica* | ICCF 190 |
| *Candida paraffinica* | ICCF 184-1 |
| *Candida paraffinica* | ICCF 184-2 |
| *Candida albicans*– ATCC 10231 | ICCF 91 |
| *Candida utilis*-CMGB 237 | ICCF 191 |
| *Candida guillermondii*– CMGB 229 | ICCF 183 |
| *Pichia pastoris* – CMGB 267 | ICCF 189 |
| *Candida utilis*– CMGB 237 | ICCF 263 |
| *Candida sp.* | ICCF 315 |
| *Aspergillus niger* | ICCF 250 |
| *Aspergillus awamory* | ICCF 171 |
| *Aspergillus niger* | ICCF 21 |
| *Aspergillus awamory* | ICCF 173 |
| *Aspergillus niger*– ATCC 16404 | ICCF 92 |
| *Aspergillus niger* | ICCF 164 |
| *Aspergillus awamory* | ICCF 167 |
| *Aspergillus awamory* | ICCF 165 |
| *Aspergillus oryzae* | ICCF 24 |
| *Aspergillus flavus* | ICCF 233 |
| *Aspergillus ochraceus* | ICCF 401 |
| *Aspergillus nigricans* | ICCF 402 |
| *Rhizopus stolonifer* | ICCF 223 |
| *Aspergillus versicolor* | ICCF 232 |

American Type Culture Collection
* Centraalbureau voor schimmelcultures, Baarn, Netherlands
** Center for Research, Forming and Consultancy in Microbiology, Genetics and Biotechnology – MICROGEN, University of Bucharest

In order to determine lipase production by the microorganisms taken into study, various screening media have been used.
For the selection of lipase producing bacteria nutrient agar with added 1.0%g/V Tween 80 and 0.1%g/V $CaCl_2$ was used.
For the yeast strains a selective YPG medium containing calcium chloride and Tween 80 as lipase production inductor has been used (%g/V) : Glucose 2.0; Peptone 0.5; Yeast extract 0.5; Tween 80 1.0; $CaCl_2$ 0.1; Agar 2.0; pH 6.0-6.5, 120$^o$C for 15 min sterilization.
For the lipase producing fungi the media (YPG, malt and Czapeck-Dox) supplemented with Tween 80, $CaCl_2$ and sodium deoxycholate were used. Sodium deoxycholate was added to diminish colony diameter and facilitate lipase extraction through hyphae permeabilization.
YPG (%g/V) : Glucose 2.0; Peptone 0.5; Yeast extract 0.5; Tween 80 1.0; $CaCl_2$ 0.1; Sodium deoxycholate 0.05; Agar 2.0; pH 6.0-6.5, 120$^o$C for 15 min sterilization.
Malt extract medium (%g/V): Malt extract 4.0; Tween 80 1.0; $CaCl_2$ 0.1; Sodium deoxycholate 0.05; Agar 2.0; pH 6.0-6.5, 120$^o$C for 15 min sterilization.
Czapeck-Dox (%g/V): Sucrose 3.0; $NaNO_3$ 0.33; $K_2HPO_4$ 0.1; $MgSO_4 \cdot 7H_2O$ 0.05; KCl 0.5; $FeSO_4 \cdot 7H_2O$ 0.004; $ZnSO_4 \cdot 7H_2O$ 0.008; $CaCl_2$ 0.1; Tween 80 1.0; Sodium deoxycholate 0.05; Agar 2.0; pH 6.0-6.5; 120$^o$C for 15 min sterilization.
All of the microorganisms were grown in Petri dishes, 10 cm in diameter, 24-48 hours at 26-28$^o$C.
The method of determination of lipase activity for the strains grown on agar media with inducers consists in the presence or absence of opacity areas around the colonies.
The opacity areas are a proof that the saponification reaction has taken place, being composed out of calcium soap crystals, as a

result of extracellular lipolytic enzymes on the Tween, in the presence of inducers in the medium.

Lipolytic activity of microorganisms was expressed through the ratio between the diameter of the opacity area added to the diameter of the colony and the diameter of the colony (Ionita et al., 1997), as follows:

$$Vr = \frac{R + r}{r}$$

Where:

R = opacity area diameter;

r = colony diameter;

$V_r$ = size of the opacity area.

The strains for which $V_r > 2$ are considered to be good lipase producers.

Throughout the experiments, the value of the opaque area represents an average of 5 estimations for each of the strains chosen for the screening.

## RESULTS AND DISCUSSIONS

Once the incubation period was finished, viability and presence or absence of certain opacity areas around the colonies was determined.

Table 2 shows the results obtained from the yeast strains taken under study, the type of halo formed, as well as the value of the ratio indicating lipase activity. The Petri dishes were studied after 5, 10 and 20 days of inoculation, and the table presents the general aspect at the end of the observation period, as well as the initial and final Vr values ($Vr_1$ after 5 days and $Vr_2$ after 20 days observation), if modified.

Table 2. The aspect of yeast strains on Petri dishes with agar medium, containing lipase inducers

| Crt. | Yeast name and registration no. | Aspect | $V_{r\,1}$ | $V_{r\,2}$ |
|---|---|---|---|---|
| 1 | *Candida arborea* ICCF 193 | Clarifying halo | 3 | 4.75 |
| 2 | *Candida glabrata* ICCF 182 | Semi-compact clarifying halo | 2.25 | 3.5 |
| 3 | *Candida famata* ICCF 181 | Compact wavy halo | 2.66 | 3.33 |
| 4 | *Candida robusta* ICCF 194 | Absent halo | 0 | 0 |
| 5 | *Yarrowia lipolytica* ICCF 214 | Compact matt halo | 2.6 | 2.6 |
| 6 | *Yarrowia lipolytica* ICCF 215 | Compact matt halo | 2.5 | 3 |
| 7 | *Candida boidinii* ICCF 26 | Semi-compact halo | 3.35 | 3.35 |
| 8 | *Candida paraffinica* ICCF 190 | Semi-compact halo | 3.4 | 3.4 |
| 9 | *Candida paraffinica* ICCF 184-1 | Compact matt halo | 3.8 | 3.8 |
| 10 | *Candida paraffinica* ICCF 184-2 | Compact matt halo | 2.05 | 2.05 |
| 11 | *Candida albicans* ICCF 91 | Compact diffuse halo | 2.95 | 3 |
| 12 | *Candida utilis* ICCF 191 | Clarifying halo | 0.2 | 0.2 |
| 13 | *Candida guillermondii* ICCF 183 | Diffuse crystal halo | 3.75 | 5 |
| 14 | *Pichia pastoris* ICCF 189 | Absent halo | 0 | 0 |
| 15 | *Candida utilis* ICCF 263 | Clarifying halo | 0.2 | 0.2 |
| 16 | *Candida sp.* ICCF 315 | Diffuse matt crystal halo | 2.08 | 2.08 |

The test aimed at distinguishing lipolytic activity for the yeast strains on YPG medium with Tween 80 and calcium chloride as inducers.

The halos of interest which proved lipase production on the agar media are compact matt halos.

The best results were obtained for the *Candida paraffinica*– ICCF 184-1 strain, which presented a well formed halo, 3.8 cm in diameter after 5 days.

The yeast strain *Candida famata*– ICCF 181 presented a slightly different halo, which continued its development during the observation period.

*Yarrowia lipolytica*– ICCF 215, a cell line modified for lipase production, and *Candida albicans*– ICCF 91 developed also a significantly large halo (Vr = 3 cm), which modified itself in size over the 20 days observation interval.

The strains *Yarrowia lipolytica*– ICCF 214, *Candida sp.* – ICCF 315 and *Candida paraffinica*– ICCF 184-2 also presented lipolytic activity, with halos over the 2 cm threshold value: Vr = 2.6, 2.08, and 2.05, respectively.

The clarifying halos which appear around the yeast colonies indicate the presence of

amylolytic activity (*Candida arborea* – ICCF 193, *Candida utilis*– ICCF 191, *Candida utilis*– ICCF 263)

The results observed are also presented in figure 1 (colony aspect) and figure 2 (halos highlighting lipase secretion in the agar medium).

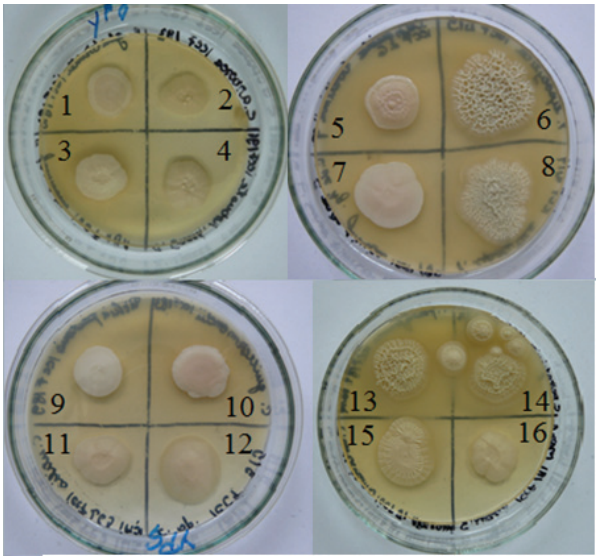


Figure 1. Yeast colonies on Petri dishes

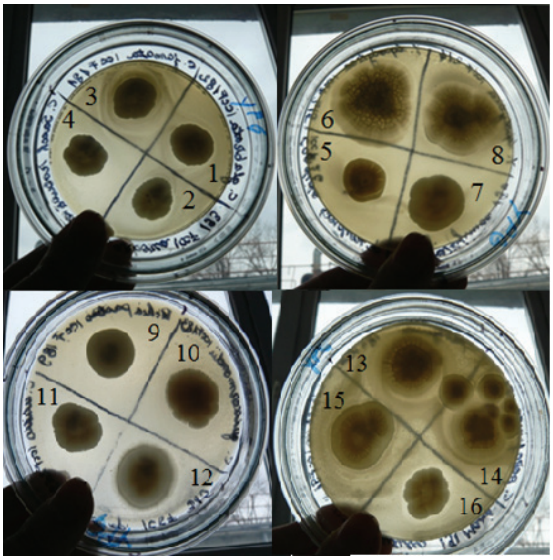


Figure 2. Presence of halos around the yeast colonies, proving lipase activity

Where:

1 = *Candida glabrata* – ICCF 182; 2 = *Candida arborea* – ICCF 193; 3 = *Candida famata* – ICCF 181; 4 = *Candida robusta* – ICCF 194; 5 = *Candida boidinii* – ICCF 26; 6 = *Yarrowia lipolytica* – ICCF 215; 7 = *Candida paraffinica* – ICCF 190; 8 = *Yarrowia lipolytica* – ICCF 214; 9 = *Pichia pastoris* – ICCF 189; 10 = *Candida guillermondii* – ICCF 183; 11 = *Candida utilis* – ICCF 263; 12 = *Candida sp.* – ICCF 315; 13 = *Candida paraffinica* – ICCF 184-1; 14 = *Candida paraffinica* – ICCF 184-2; 15 = *Candida albicans* – ICCF 91; 16 = *Candida utilis* – ICCF 191

As can also be inffered from figure 2, where the opacity halos can be clearly distinguished, the yeast strains which presented lipolytic activity were: no. 3 –*Candida famata*– ICCF 181, no. 6 –*Yarrowia lipolytica*– ICCF 215, no. 8 –*Yarrowia lipolytica*– ICCF 214, no. 12 –*Candida sp*. – ICCF 315, no. 13 –*Candida paraffinica*– ICCF 184-1, no. 14 –*Candida paraffinica*– ICCF 184-2, no. 15 –*Candida albicans*– ICCF 91,

The fungi strains have known rapid development, covering up the surface of the Petri dishes in 48 hours on the specific culture media (YPG and malt extract, respectively) and showed no presence of lipase activity under the form of specific halos (Figure 3).

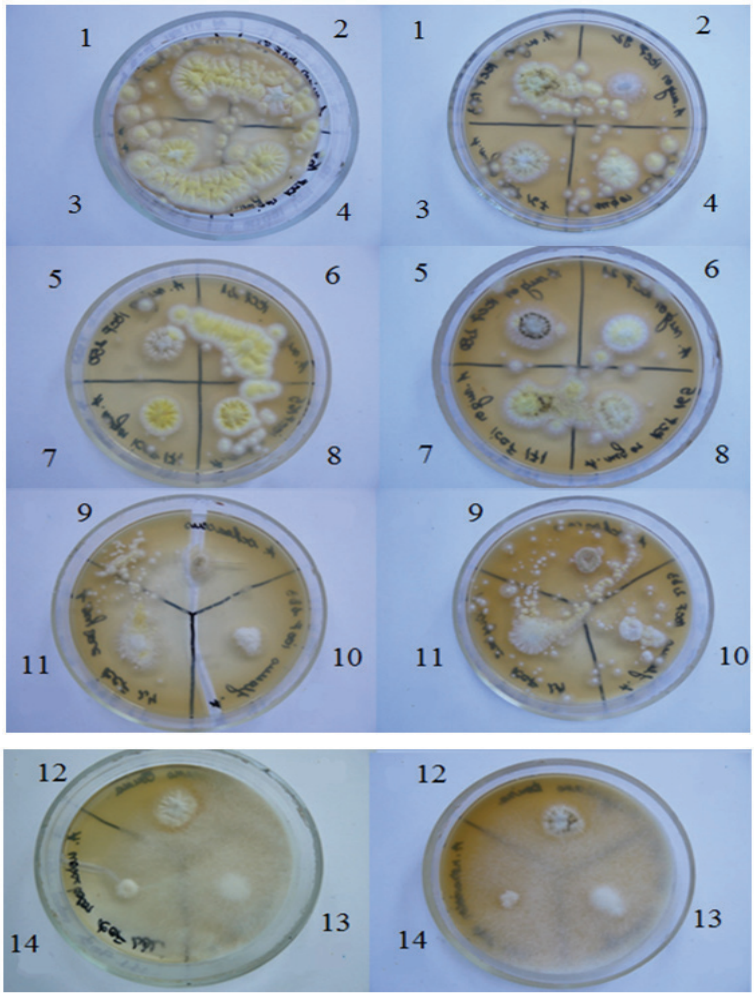


Figure 3. Aspect of fungi strains on Petri dishes containing agar media: YPG (left column) and Malt extract (right column)

Where:

1 = *Aspergillus awamory*– ICCF 173
2 = *Aspergillus niger*– ICCF 92
3 = *Aspergillus awamory*– ICCF 167
4 = *Aspergillus niger*– ICCF 164
5 = *Aspergillus niger*– ICCF 250
6 = *Aspergillus niger*– ICCF 21
7 = *Aspergillus awamory*– ICCF 171
8 = *Aspergillus awamory*– ICCF 165
9 = *Aspergillus ochraceus* ICCF 401
10 = *Aspergillus flavus*– ICCF 233
11 = *Aspergillus oryzae*– ICCF 24
12 = *Aspergillus nigricans* ICCF 402
13 = *Rhizopus stolonifer*-ICCF 223
14 = *Aspergillus versicolor*-ICCF 232

The *Pseudomonas* strains formed no specific opaque halos on the agar medium (Figure 4).

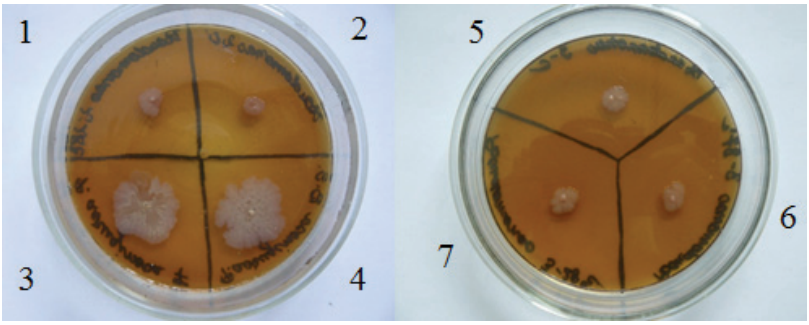


Figure 4. Appearance on Petri dishes of the Pseudomonas strains

Where:
1 = *Pseudomonas sp.* nr. 2 – ICCF 399 – grown on test tube at 28°C
2 = *Pseudomonas sp.* nr. 2 – ICCF 399 – vegetative stock strain
3 = *Pseudomonas aeruginosa* F – ICCF 85
4 = *Pseudomonas aeruginosa* F – ICCF 90
5 = *Pseudomonas sp.* nr. 3 – ICCF 400 – vegetative stock strain
6 = *Pseudomonas sp.* nr. 3 – ICCF 400 – grown on test tube at 37°C
7 = *Pseudomonas sp.* nr. 3 – ICCF 400 – grown on test tube at 28°C

## CONCLUSIONS

The aim of the study on industrial strains pertaining to the Industrial Importance Microorganism Collection (IIMC) registered to the National Institute for Chemical Pharmaceutical Research and Development ICCF Bucharest was the determination of lipase producing microorganisms, selected from several bacterial, yeast and fungi strains.

After growth on specific agar media containing lipase inducers on Petri dishes, the colonies have been observed for lipolytic activity in the form of opaque halos surrounding the cultures.

The *Pseudomonas* bacterial strains showed no presence of lipases on the medium, and neither did the fungi strains selected for the screening, the latter of which developed quickly, covering the surface of the Petri dishes in the cases of both specific growth media (YPG and malt extract) used.

Out of the 16 yeast strains considered for the screening, 7 showed lipolytic activity on the specific YPG media containing Tween 80 and calcium chloride as lipase production inducers. The yeast strains that developed opaque halos are: *Candida famata*– ICCF 193, *Yarrowia lipolytica*– ICCF 215, *Yarrowia lipolytica*– ICCF 214, *Candida sp*. – ICCF 315, *Candida paraffinica*– ICCF 184-1, *Candida paraffinica*– ICCF 184-2, *Candida albicans*– ICCF 91.

These yeast strains are going to be subjested to further analysis in order to quantify lipase production and enzymatic activity, for subsequent use in biodiesel obtainment through biocatalytic processes.

## ACKNOWLEDGEMENTS

National Institute for Chemical Pharmaceutical Research and Development ICCF Bucharest for setting at our disposal their Industrial Importance Microorganism Collection (IIMC), for the performance of the screening tests.
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# SELECTION OF YEAST STRAINS WITH ENHANCED LIPOLYTIC ACTIVITY

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***Abstract***

*In reaction to expanding markets and increasing demand for novel biocatalysts, commercial enzyme production has been continuingly growing during the last century. Although some enzymes are extracted from plants and animals, the major source of industrial enzymes consists in microorganisms. Obtainment of a new microbial enzyme begins with a screening of microorganisms for the desired activity, using specific methods of selection. Lipases or glycerol-ester hydrolases are carboxyl-esterases which catalyze hydrolytic cleaving of glycerol esters with fatty acids. Microorganisms are rich sources of lipases, especially yeast strains pertaining to the genus Candida. Yeast strains from the CMII-WFCC232 industrial interest strain collection of the National Institute for Chemical Pharmaceutical Research and Development ICCF Bucharest have been studied regarding their lipase production. Specific screening media have been used in order to stimulate lipolytic activity in the yeast strains. Viability and presence of opaque areas on Petri dishes containing fatty acid esters and lipase inducers as proof of enzyme activity have been determined. The strains which presented lyoplytic activity have later on been cultivated on specific liquid medium and enzymatic activity has been determined.*

***Keywords:*** *lipase, screening, transesterification, yeast.*

## INTRODUCTION

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are an important group of water soluble enzymes which have as main biological function the interfacial hydrolytic catalysis of lipids (Aehle, 2007).

Due to their versatility, they present vast biotechnological relevance and applications in fats and oils industries, food and feed processing, synthesis and production of fine chemicals, detergents and various degreasing products, cosmetics and pharmaceuticals, bakery and brewing, biofuels and waste treatment (Falch, 1991; Kazlauskas et al., 1998; Villeneuve et al., 2000; Sharma et al., 2001; Babu and Rao, 2007; Hasan et al., 2006; Hasan et al., 2009; Balakrishnan et al., 2011; Kishore et al., 2011).

Enzyme catalysis using lipases has been intensely researched since the reaction takes place at regular pressure and 30-40$^{o}$C, achieving the necessary energy, and reducing at the same time the emergence of unwanted secondary compounds.

At low water content, theses enzymes are also able to catalyze synthesis reactions, achieving esterification, alcoholysis, interesterification, transesterification, acidolysis, aminolysis, thiotransesterification, oximolysis, in addition to their hydrolytic activity on triglycerides (Villeneuve et al., 2000; Joseph et al., 2008).

Lipases can be found in different sources, such as plants and animals, but the most abundant source is represented by microorganisms (Haki et al., 2003; Gupta et al., 2004; Rahnman et al., 2006)

Due to their wide use as biocatalysts for numerous and also novel biotechnological applications (Jaeger and Eggert, 2002), there is a continuous search for new lipase sources and intensive studies are performed to better understand their structural biochemical characteristics and mechanisms (Sirisha et al., 2010; Fickers et al., 2011; Feng et al., 2012).

The production of the extracellular lipase is affected by various factors, such as: temperature, pH, composition of the medium, agitation and aeration (Chen et al. 1999; Gupta et al. 2004; Alonso et al. 2005).
The aim of the present paper was to obtain new microbial enzymes as a result of a screening performed on lipase producing yeasts pertaining to the CMII-WFCC232 industrial interest strain collection of the National Institute for Chemical Pharmaceutical Research and Development ICCF Bucharest, registered in the World Federation of Culture Collections Directory, no. 232.

## MATERIALS AND METHODS

The yeast strains that have been selected to undergo the screening for lipase production belong to the Industrial Importance Microorganism Collection (IIMC) pertaining to the National Institute for Chemical Pharmaceutical Research and Development ICCF Bucharest, registered in the World Federation of Culture Collections Directory no. 232. 16 yeast strains were taken from the vegetative stock and grown on specific media, with lipase activity inducers (Table 1).

Table 1. Strains that have been studied for lipase producing potential from the ICCF collection

| Strain name | Registration |
|---|---|
| *Candida arborea* – CBS* 64 | ICCF 193 |
| *Candida glabrata* | ICCF 182 |
| *Candida famata* | ICCF 181 |
| *Yarrowia lipolytica* – ATCC** 16618 | ICCF 214 |
| *Yarrowia lipolytica* | ICCF 215 |
| *Candida boidinii*-CMBG*** 221 | ICCF 26 |
| *Candida parafinica* | ICCF 190 |
| *Candida parafinica* | ICCF 184 |
| *Candida albicans* – ATCC 10231 | ICCF 91 |
| *Candida utilis*-CMGB 237 | ICCF 191 |
| *Candida guillermondii* – CMGB 229 | ICCF 183 |
| *Pichia pastoris* – CMGB 267 | ICCF 189 |
| *Candida sp.* | ICCF 315 |
| *Hansenula polymorfa*-CMGB 257 | ICCF 218 |
| *Hansenula subpelliculosa*-CMGB 261 | ICCF 187 |
| *Hansenula anomala*-CMGB 243 | ICCF 217 |

The yeast strains were grown on a specific selection medium, containing cattle suet. The cattle suet is melted down and poured on the Petri dishes, in a shallow layer, 2-3 mm thick. Once it has solidified, the specific lipase synthesis culture medium is poured on top.
The cultures are than passed on the screening medium in the form of vertical strips, using the loop.
The lipase screening medium with cattle suet contains (%g/V) : peptone 1.0; sodium chloride 0.5; calcium chloride 0.01; Tween 80 1.0; agar 2.0. The pH was corrected to 5.5 and sterilization of the medium was at 120$^{o}$C for 15 min.
The microorganism strains which showed synthesis of extracellular lipases have later on been grown on liquid culture medium, in order to determine enzyme activity.
The preinoculum obtained in agar YPG medium, containing (%g/V) : yeast extract 0.5; peptone 0.5; glucose 2.0; agar 2.0; pH 5.5, 120$^{o}$C for 15 min sterilization, after cultivation for 72 hours at 28$^{o}$C was used for inoculation of 50 mL liquid inoculum medium in 500 mL Erlenmeyer flasks.
The culture resulted after 24 hours at 28$^{o}$C (the inoculum) was used for inoculation (2% vol/vol) of 50 mL of fermentation medium in 500 mL Erlenmeyer flasks.
The inoculum and fermentation media were the same: glucose 2.0; yeast extract 1.0; peptone 0.5; ammonium sulfate 0.1; Tween 80 1.0; dipotassium phosphate 0.5; pH 7, 120$^{o}$C for 15 min sterilization.
The fermentation was conducted at 28$^{o}$C, with shaking at 250 rpm, and samples were taken every 4 hours.
PH, biomass accumulation, glucose consumption and enzyme activity were determined for each sample.
The microbial growth was determined spectrophotometrically, measuring the values of optic density at 540 nm, 1:50 dilution ratio.
Lipolytic activity from the supernatant (after biomass removal by centrifugation at 6000 rpm, for 20 min, at 4$^{o}$C) was determined using the titrimetric Willstätter method, using olive oil as a substrate, according to Iordachescu and Dumitru (1980). Superior fatty acids resulted from triglyceride hydrolysis, in the presence of microbial lipase, were extracted with organic solvents and titrimetrically dosed with a sodium hydroxide alcoholic solution. One lipase unit, $UL_{FIP}$ is the mass of the enzyme, which, under standard conditions, hydrolyses the vegetal oil and leads to the release of a microequivalent of carboxyl group per minute.

## RESULTS AND DISCUSSIONS

The yeasts strains have been observed on the cattle suet medium for lipase activity. The lipases synthesized by the microorganisms act on the lipids in the cattle suet, leading to soap formation (Figure 1).

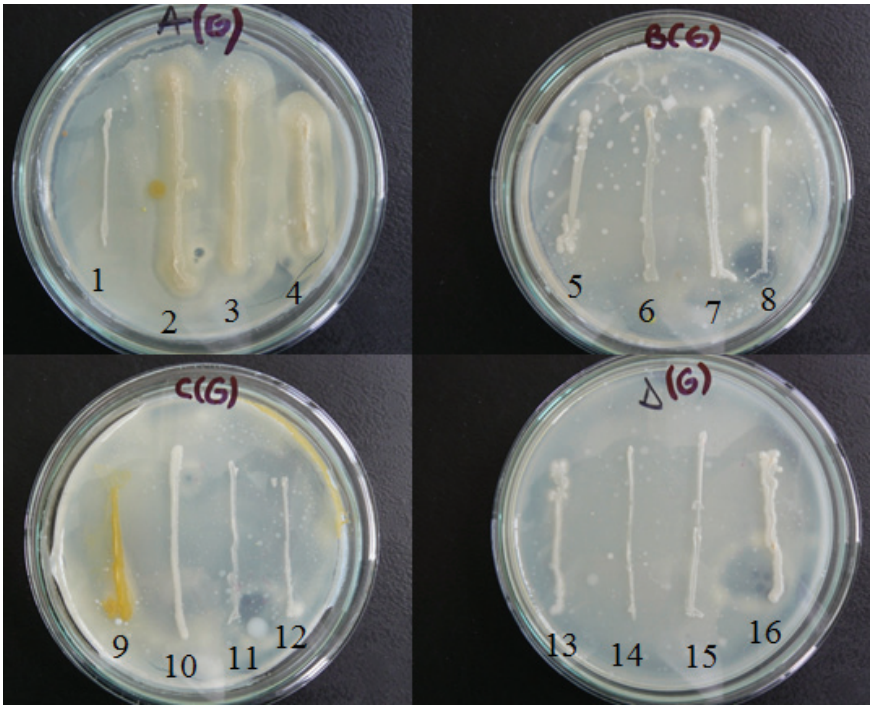


Figure 1. Lipase activity on culture medium containing cattle suet

Where:
1 = *Candida albicans* – ICCF 91
2 = *Candida parafinica* – ICCF 184
3 = *Yarrowia lipolytica* – ICCF 215
4 = *Yarrowia lipolytica* – ICCF 214
5 = *Candida parafinica* – ICCF 190
6 = *Candida boidinii* – ICCF 26
7 = *Candida famata* – ICCF 193
8 = *Candida glabrata* – ICCF 194
9 = *Hansenula anomala* ICCF 217
10 = *Hansenula subpeliculosa* ICCF 187
11 = *Hansenula polymorfa* ICCF 218
12 = *Candida arborea* ICCF 193
13 = *Candida sp.* – ICCF 315
14 = *Pichia pastoris* – ICCF 189
15 = *Candida utilis* – ICCF 191
16 = *Candida guillermondii* –ICCF 183

From the observation of the yeast strains, it can be seen that three of them showed lipolytic activity, namely *Candida paraffinica* – ICCF 184, *Yarrowia lipolytica* – ICCF 215, *Yarrowia lipolytica* – ICCF 214, and they have been further on grown on liquid fermentation medium, to determine enzyme activity.

For each of the three strains, samples were collected every 4 hours and pH, cell growth, glucose consumption and enzyme activity were determined.

The variation of the parameters and lipase yield is presented below (Figures 2 to 4).

Lipase activity was determined beginning with 16 hours of fermentation until the end of the process.

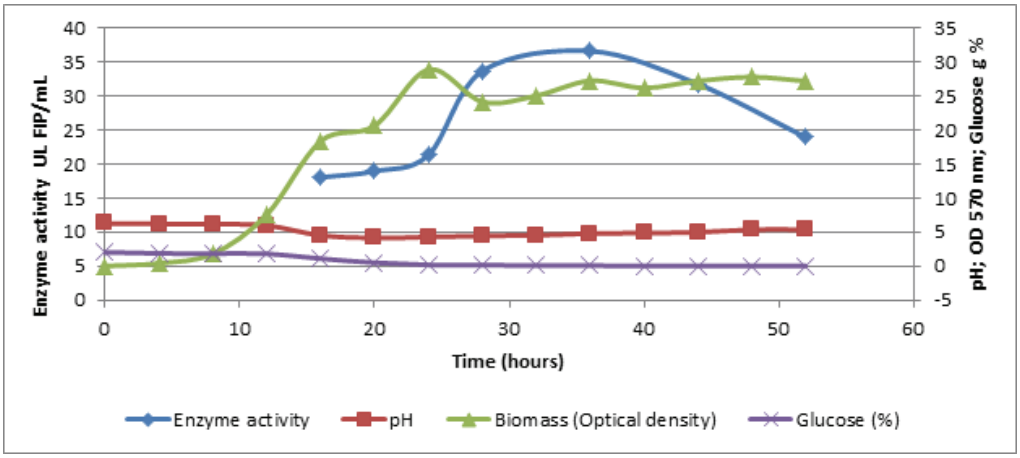


Figure 2. Variation of the main parameters during fermentation for *Yarrowia lipolytica* ICCF 214

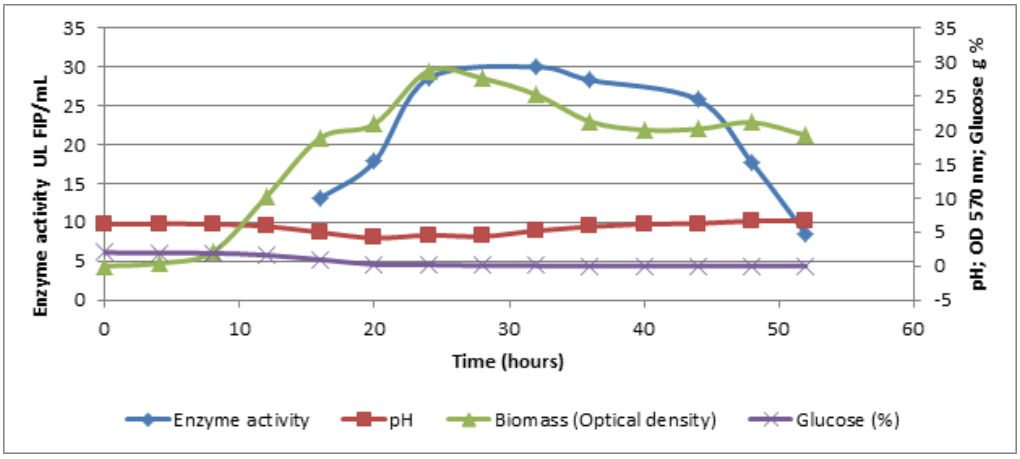


Figure 3. Variation of the main parameters during fermentation for *Yarrowia lipolytica* ICCF 215

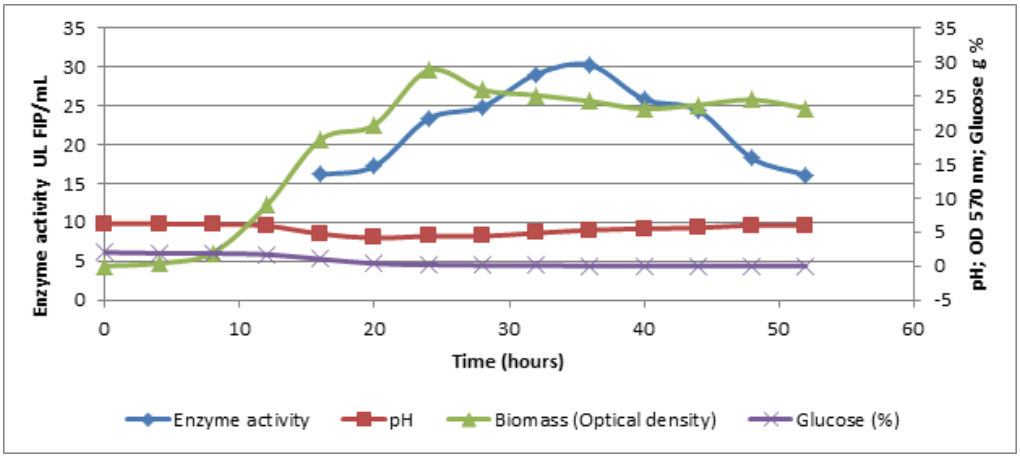


Figure 4. Variation of the main parameters during fermentation for *Candida paraffinica* ICCF 184

For all of the samples, the pH values varied during fermentation, registering a decrease from 6 to 4, between 12 and 20 process hours, coinciding with the beginning of the enzyme production.
At 24 hours, all three strains reached a maximum value for biomass growth, after which it decreased after glucose depletion.
The glucose was entirely consumed after 36 hours of fermentation.
The biosynthesis kinetics was not associated to microbial cell growth, the moment of lipase production being associated with the stationary phase.
All of the strains presented high lipolytic activities from the beginning to the end of the fermentation, with maximum values between 32 and 36 hours of cultivation. The highest activity showed *Yarrowia lipolytica* ICCF 214, with a value of 36.6 $UL_{FIP}$/mL.

## CONCLUSIONS

Out of the 16 yeast strains selected from the Industrial Importance Microorganism Collection (IIMC), three presented lipolytic activity on cattle suet medium, namely *Candida paraffinica* – ICCF 184, *Yarrowia lipolytica* – ICCF 215, *Yarrowia lipolytica* – ICCF 214, and they were selected for fermentation on liquid medium.

During fermentation pH, biomass, glucose and enzyme activity levels were monitored for all three strains.

All of them showed high enzymatic activities, throughout the fermentation, but *Yarrowia lipolytica* ICCF 214 reached the highest value, of 36.6 $UL_{FIP}$/mL, at 36 hours fermentation.

Our results are better than those presented by other authors (Brígidaa et al., 2007; Kebabci et al., 2012), the level of lipase activity for *Yarrowia lipolytica* being higher than the ones presented by the literature.

Due to its enhanced lipase production potential, this strain will be used in biodiesel production through biocatalytic methods.

## ACKNOWLEDGEMENTS

National Institute for Chemical Pharmaceutical Research and Development ICCF Bucharest for setting at our disposal their Industrial Importance Microorganism Collection (IIMC), for the performance of the screening tests.
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# STUDIES ON DIACEREIN BIODEGRADABILITY

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***Abstract***

*Diacerein (DCN) is an anti-inflammatory, non-steroid drug with an analgesic effect, used in the treatment of osteoarthritis. DCN is pharmaceutically formulated as a yellow powder in capsules for oral use and it is mainly obtained from extracts of Rheum Emodi (rhubarb) and Aloe Vera. The pollution with pharmaceutical products represents one of the main interests in environmental protection. The influence of DCN was tested on different strains of bacteria and yeasts, common species that can be found in water and soil, and play an important part in decomposing chemical pollutants. The direct action of the pharmaceutical powder evenly spread or dispersed in solid specific culture media was evaluated. In liquid media in which different DCN concentrations were added, the microbial growth and DCN metabolization were analyzed using spectrophotometric methods. On solid media, DCN bioaccumulated in the microorganism colonies, giving them an orange colour depending on their specificity. In liquid media, DCN had an inhibitory effect or was an enhancer of the pigments colour naturally secreted by some strains of bacteria.*

***Keywords:*** *bacteria, bioaccumulation, biodegradation, diacerein, yeasts.*

## INTRODUCTION

One of the most frequent articular affections, osteoarthritis (OA) is a degenerative disease, which occurs from the biochemical distruction of the synovial articular cartilage. OA is an idiopathic phenomenon, without having an initiation factor and it is associated to the aging process (80% of people above 60 years old present signs of OA) (Subhash et al., 2012). Diacerein is a new drug, used for the treatment of osteoarthritis, with an anti-inflammatory, analgesic and antipyretic action (Medhi et al., 2007). The drug is formulated for oral administration and presents few side effects (diarrhea, stomach ache, nausea, yellowish urine colour), due to the fact that is not totally absorbed by the digestive tract. The way DCN acts at a therapeutical level is based on the reconstruction of the articular cartilage, stimulating the production of TGF-β (growth factor), the proliferation of chondrocytes, collagen, proteoglycans and hyaluronan synthesis (McCalla, 2009; Subhash, 2012;). DCN ($C_{19}H_{12}O_8$, 4,5-Diacetyloxy-9,10-dioxo-2-anthracene-2-carboxylic acid) is a semi-synthetic derivative of anthraquinone, obtained from vegetal extracts of *Rheum emodi* and *Aloe vera* (Mahajan et al., 2006). The pharmacological properties of the product are given by the main active metabolite, rhein ($C_{15}H_{18}O_5$, 4,5-Dihydroxyanthraquinone-2-carboxylic acid) (Pelletier et al., 2000). The structural formulas of DCN and rhein are presented below (Fig. 1) (Rajesh et al., 2009).

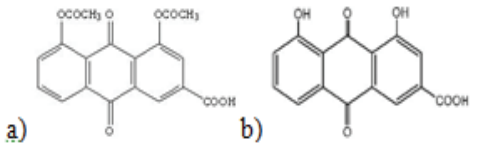


Figure 1. Structural formula of: a) DCN; b) Rhein

DCN inhibits cytokines synthesis (interleukin-1, IL-1) and metalloproteinases (collagenase, stromelysin) which are involved in articular cartilage degradation (Medhi et al., 2007).
The purpose of this paper was to evaluate the influence of DCN over some strains of bacteria

and yeasts, common species from water and soil and to study the drug's biodegradability.
The biodegradation of pharmaceutical products has a significant importance in conservation, protection and improving environmental quality.

## MATERIALS AND METHODS

A. Bacterial strains (genera *Alcaligenes, Pseudomonas, Ralstonia*)and yeast strains (genera *Candida*, *Saccharomyces*, *Yarrowia*) were provided by the National Institute for Chemical Pharmaceutical Research and Development, affiliated at the WFCC-232 Collection (see Table 1).
B. Reactives: the media ingredients were purchased from Merck, Germany; dimethylsulfoxide (DMSO) from Sigma-Aldrich; Diacerein RPH (70 mg/capsule) from Rompham Company, Romania.
C. Methods
Biological method
The bacterial and yeast strains were cultivated on solid media.
The solid culture media were: *Cantacuzino Gelose* (nutritive medium for bacteria); *King B* (%g/v): proteose-peptone 2; glycerine 1; $K_2HPO_4$ 0,15; $MgSO_4x7\ H_2O$ 0,15; Noble Agar 1,8; distilled water ad 100 ml; *YMPG* (%g/v): glucose 1; yeast extract 0,3; malt extract 0,3; peptone 0,5; Noble Agar 1,8; distilled water ad 100 ml) to which DCN was added after media sterilization (120ºC/17 minutes), dispersed on or integrated in different quantities (mg): 20, 70, 120, 165.
The liquid culture medium was *King B* (100 ml/Erlenmeyer flask) to which 1 ml stock solutions of DCN in different concentrations were added.
Stock solutions: 3,3 x $10^{-6}$M; 1,1 x $10^{-5}$M and 1,1 x $10^{-4}$M.
The Petri dishes with selected bacteria and yeast strains were incubated for 7 days (30°C/28°C, darkness), controlling the colonies growth.
The liquid media were inoculated with 4 different bacterial strains (see Table 1) using 500 ml conical flasks. These were incubated for 96 h (30°C, darkness) using a rotary agitator (220 rpm).

Analytical methods
The bacterial cells growth was monitored by optical density (OD) using a UV-Visible spectrophotometer (Jasco Corporation V 630, Japan) at $\lambda = 550$ nm.
The free cells media were spectrophotometric analysed (270-600 nm), after centrifugation (6000 rpm/12 min) and filtration (0,45 µm syringe filter).

Table 1. Bacterial strains used in the experiment

| Solid media | |
|---|---|
| Bacterial strain | Registration number |
| *Alcaligenes latus* | DSM 1123; ICCF 383 |
| *Nitrosomonas sp.* | ICCF 401 |
| *Pseudomonas aeruginosa* | ATCC 9027; ICCF 90 |
| *Pseudomonas aeruginosa* | ICCF 389 |
| *Pseudomonas fluorescens* | ICCF 392 |
| *Pseudomonas putida* | ICCF 391 |
| *Pseudomonas sp.* | ICCF 390 |
| *Pseudomonas sp.* | ICCF 399 |
| *Pseudomonas sp.* | ICCF 400 |
| *Ralstonia eutropha* | DSM 545; ICCF 384 |
| Yeast strains | Registration number |
| *Candida albicans* | ATCC 10231; ICCF 91 |
| *Candida arborea* | ICCF 193 |
| *Candida boidinii* | ICCF 26 |
| *Candida glabrata* | ICCF 182 |
| *Candida guillermondi* | ICCF 183 |
| *Candida paraffinica* | ICCF 190 |
| *Candida utilis* | ICCF 191 |
| *Pichia pastoris* | ICCF 189 |
| *Saccharomyces cerevisiae* | ICCF 225 |
| *Saccharomyces cerevisiae* | ICCF 227 |
| *Yarrowia lipolytica* | ATCC 16618; ICCF 214 |
| *Yarrowia lipolytica* | ICCF 215 |
| Liquid media | |
| *Pseudomonas fluorescens* | ICCF 392 |
| *Pseudomonas sp.* | ICCF 390 |
| *Pseudomonas sp.* | ICCF 400 |
| *Ralstonia eutropha* | DSM 545; ICCF 384 |

## RESULTS AND DISCUSSIONS

On solid media, the macroscopical aspect of microbial colonies was observed during and after the incubation and it is presented in Figures 2-5.

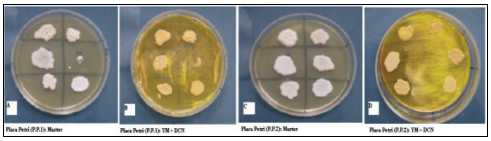

Figure 2.Yeasts on *YM* medium with DCN (165 mg)

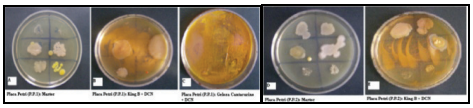

Figure 3. Bacteria on *King B* (A, B, D, E) and *Cantacuzino Gelose* (C) media with DCN (165 mg)

On solid media where on dispersed pharmaceutical powder of DCN (165 mg) was added, it was observed that bacteria and yeast strains formed yellow-orange coloured colonies (initial colour was white-beige).

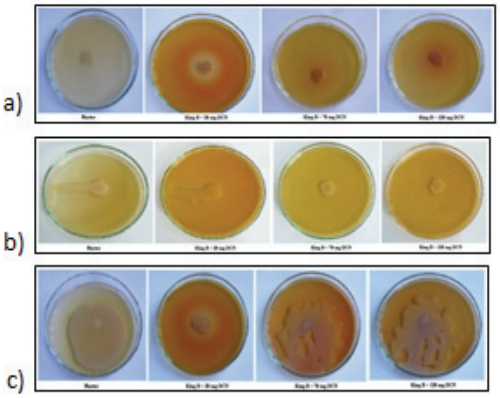


Figure 4. Bacteria on solid media with integrated DCN (20, 70, 120 mg/ Petri dish), 96 h cultivation: a) *Alcaligenes latus;* b) *Pseudomonas sp.;c) Pseudomonas putida*

On solid media in which DCN powder was integrated, besides the change of colonies colour, an accumulation of the pharmaceutical powder initially yellow coloured and then, brown-purple was noticed. Also, there was observed the formation of some pigments, possibly pyorubin (*Alcaligenes latus*) and pyoverdin (*Pseudomonas putida*), which were dependent on the added DCN quantities. On medium with the lowest amounts of DCN, clarification zones appeared (*Alcaligenes latus*; *Pseudomonas putida*) and so, the pharmaceutical powder was bioaccumulated (high concentrations in the culture medium) or it was metabolised by the microorganisms (low concentrations).

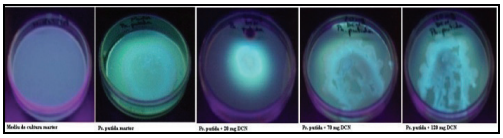

Figure 5. *Pseudomonas putida*: fluorescence on solid media with integrated DCN (0, 20, 70, 120 mg/Petri dish)

By exposure of the media with the selected strains to ultraviolet light (365 nm), DCN's noninhibitory effect on the production of the fluorescent pigment released by *Pseudomonas putida* was noticed.

In liquid inoculated with 4 selected bacterial strains, the cell growth was monitored by optical density (OD) at $\lambda$ = 550 nm (dil. 1:25). The results are presented in Figure 6.

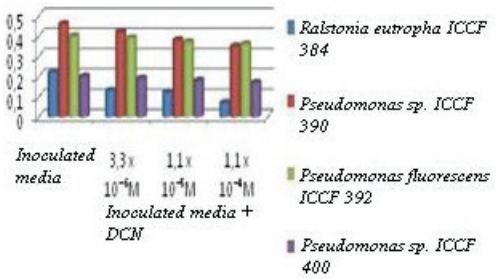


Figure 6. Dynamics of optical density (OD) values

Graphical representations of UV-Visible spectra are presented below (Figure 7-8).

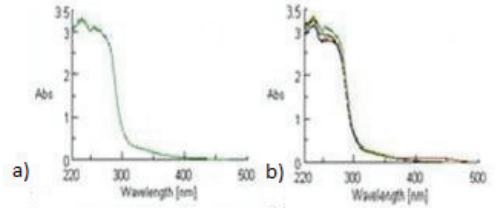


Figure 7. UV-Visible spectra of culture media before bacteria inoculation: a) initial; b) with DCN in different concentrations

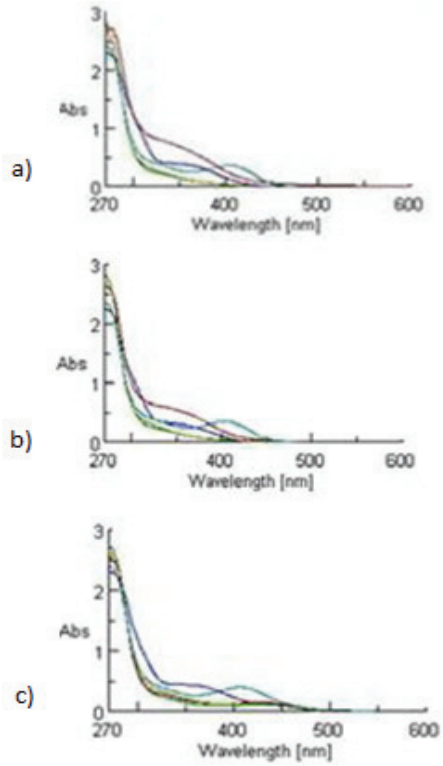


Figure 8. UV-Visible spectra of free cells media (96 h) with different concentrations of DCN ( Initial medium; *Ralstonia eutropha*; *Pseudomonas sp.*; *Pseudomonas fluorescesns* ; *Pseudomonas sp.*): a) 3,3 x $10^{-6}$M; b) 1,1 x $10^{-5}$M; c) 1,1 x $10^{-4}$M

In liquid media with DCN, the production of some new compounds was observed, which are associated with specific pigments produced by bacterial strains. The maxima of their UV-absorption were determined at the same wavelength presented in the literature: *Ralstonia eutropha* (λ = 345 nm), *Pseudomonas sp.* (λ = 340-370 nm) and *Pseudomonas fluorescenes* (λ = 408 nm) (Tourkya et al., 2011; Xiao, 1995).

## CONCLUSIONS

On solid media, DCN was bioaccumulated in the bacteria and yeast colonies and it was metabolized by *Alcaligenes latus* and *Pseudomonas putida*. In small concentrations, DCN stimulated the production of an intracellular pigment (pyorubin), which has later on been excreted in the media.
DCN inhibited the fluorescent pigment production by *Pseudomonas putida*.
In liquid media with different concentrations of DCN, a higher sensitivity was shown by *Ralstonia eutropha* and *Pseudomonas fluorescens* presented a higher resistance.

## ACKNOWLEDGEMENTS

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# GROWTH AND ACTIVITY OF CELLULASE-AMYLASE ENZYME *PENICILLIUM NALGIOVENSE* AND *ASPERGILLUS TAMARII* MOLDS ISOLATED FROM COW RUMEN FLUID

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***Abstract***

*Cow rumen fluid is a fluid rich in cellulolytic microbes that play a role to help digest food that contains high crude fiber. Two species of moulds have been isolated from the cellulolytic rumen fluid of local cattle and have identified as Penicillium nalgiovense and Aspergillus tamarii. Cellulolytic Index both of Penicillium nalgiovense and Aspergillus tamarii were 2.33 and 1.24 respectively. The purpose of this study is to characterize their growth and activities of cellulase and amylase. Research include: 1) growth of mould 2) macroscopic description, and 3) activities of cellulase and amylase enzyme using the DNS (3,5-dinitrosalicylic acid) method. Based on the growth curve is known that the logarithmic phase of growth of Penicillium nalgiovense peak occurred on day 6 with a population of 9.696 x $10^3$ cfu cells, whereas Aspergillustamarii logarithmic phase, occurred on day 5 with a population of 4.65 x $10^2$ cfu cells. Cell morphological characters of P. nalgiovense as conidium colour milky white, and spore powdery, in the early growth of Penicillium nalgiovense white colonies after a while the colonies get old is also white and conidiophores with smooth stipe. As macroscopic A.tamarii is yellow-brownish, at the beginning of growth colonies colour is a bright yellow growth after several days, color were changed to dark brownish yellow. This mold has a conidial head yellow-brown, not columnar and have ornaments that are not clear. Cellulase enzyme activity in Penicillium nalgiovense amounted to 2.420 units / ml, and amylase enzyme activity of 2.146 units / ml. Aspergillustamariii to be used for degradation of fiber for feed.*

***Keywords:*** *description, characteristic, cellulose and amylase enzymes activity, Penicilium nalgiovense, Aspergillus tamari, cow rumen fluid.*

## INTRODUCTION

Superior microorganisms which can be used as a source of enzymes as well as fermentative microorganisms can be isolated from a natural source. Natural sources are sources such as cow rumen fluid derived from the Slaughterhouse waste (RPH), because they contain different types of enzymes that can break down the most complex structure in the fodder. Jovanovic and Cuperlovic (1977) states rumen microbes can increase the nutritional value of food due to microbial protein, resulting in increasing digestibility. In addition, rumen is a source of polysaccharide degrading enzymes due to synergistic effects and interactions of complex microorganisms, mainly producers of cellulase and xylanase (Trinci et al., 1994).

Andriani (2010) isolated a fungus cellulotik of local cattle rumen fluid, and got the results that based on cellulotic index (CI), *Penicillium nalgiovense* and *Aspergillus tamarii* are molds that have the largest cellulotic ability among all aerobic fungi isolated, respectively 2.33 and 1.24. The cellulolytic potential can be utilized in the process of pre-digestion of the coarse fiber in the form of cellulose, hemicellulose and lignin, which are abundantly found in waste cellulose, so that at the time of entry into the body of the fish it is already available in a form that is more easily digested, for example in the form of poly or oligosaccharides.

Enzymes have a specific ability to degrade the material according to its kind. These

capabilities can be measured by conducting an enzyme activity test. Similarly, the use of enzymes from bovine rumen fluid microorganisms origin requires testing first, so it can be measured in its ability to degrade feedstuffs containing high crude fiber, cellulose in this case. Testing will then become the basis for the use of natural more economical enzymes as an alternative to commercial enzymes on the pre-digestion of agricultural waste in-vivo, with the goal of lowering coarse fiber, particularly cellulose agricultural waste based fish feed ingredients.

## MATERIALS AND METHODS

The tools used in this study include incubator oven, UV-Vis spectrophotometer, centrifuges, vortex, autoclave, incubator shaker, laminar air flow, balance scales, water heaters, micropipette, tip micropipette, polypropylene tubes, Eppendorf tubes, and glass tools. The sample used in this study were *Penicilium nalgiovense*, *Aspergilus tamarii* fungal isolate, PDA and SBA agar, glucose, distilled water, soluble starch, CMC (Carboxy Methyl Cellulose), phosphate buffer, 3.5-Dinitro-salisilat acid reagent (DNS).

The microbes were cultured for 7 days using the submerged fermentation method. The medium used was Potato Dextrose Agar, Sabouroud Dextrose Agar, to which has been added to roomates CMC 1% and Medium cellulose to which tetracycline has been added respectively.

After 24 hours, colonies were growth and then calculated using dillution series methods (Total Plates Count). Cellulase and amylase enzyme activitiy were carried out during in the peak of logaritmic phase (4 days). Research observed include: 1) growth of mold, 2) macroscopic of mould description, and 3) The activity of cellulase and amylase enzyme by using the DNS (3,5-dinitrosalicylic acid) method.

## RESULTS AND DISCUSSIONS

The results can be described if the macroscopic identification of *P. nalgiovense* is milk-white in color, and its spores resemble milk powder, initially white and also white after aging, but less bright. While *A.tamarii* was macroscopic yellow-brownish, at early rejuvenation bright yellow and after a few days it turned stark contrast to dark brownish yellow. Results of microscopic identification key, based on the identification of the book *Introduction of Food Borne Fungi*, indicated that *A. tamarii* colonies were yellow, conidial head yellow-brown, non-columnar head conidial yellow or brown, conidial heads had the real chocolate ornaments, while *P.nalgiovense* colony was white and conidiophores with smooth stipes.

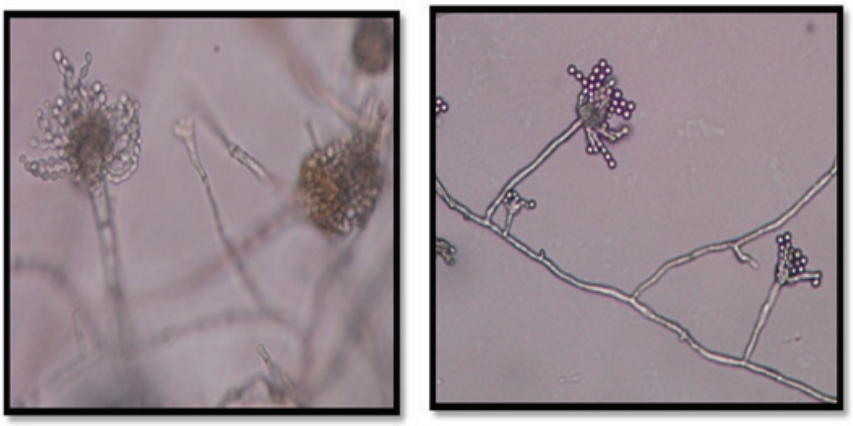


Figure 1. Macroscopic identification (left-right: *P. nalgiovense-A. tamari* 400x)

Judging from the genus of microorganisms selected and identified, the genus of microorganisms selected is known to be in accordance with the several studies previously conducted. The fungi isolates of cellulotic aerob of bovine rumen origin are among others *Aspergillus*, *Geotrichum*, *Penicillium*, *Rhizopus*, and *Trichoderma* (Ogimoto, 1981; Suhardini, 2008). However, further review to the species, no report was found stating the isolation of species *Aspergillus tamarii* and *Penicillium nalgiovense* from cow rumen fluid.

The types of microorganisms are commonly found in soil substrate, rice straw, corn leaves and plants (Moreira, 1999).

The type and amount of microorganisms in cow rumen, is very much influenced by the fodder consumed (Hungate, 1973; Ogimoto, 1981). The process liquidifying and the type of feed given to cattle feed allows entry of other microorganisms in the digestive tract along with the green fodder consumed. Based on the asumption in Ogimoto (1981), that a diversity of rumen microorganisms is very dominantly influenced by microorganisms carried through cattle feed. It is further stated that the feed follwing of microorganisms, particularly fungi, are able to survive in the conditions of the rumen, and there are some types that are functional in the digestion of cattle.

One of the requirements to be selected as microorganisms feed biodegradator is that it must be in the category of facultative aerobic microorganism; because the biodegradation process feed material will take place aerobically. The circumstance in which the microorganisms live which is crucial in energy metabolism of microorganisms is oxygen. The *Aspergillus tamarii* and *Penicillium nalgiovense* microorganisms are cow rumen fluid microorganisms that survive in aerobic culture conditions. At the time it was isolated the microorganisms was living and was capable of breeding on selective media.

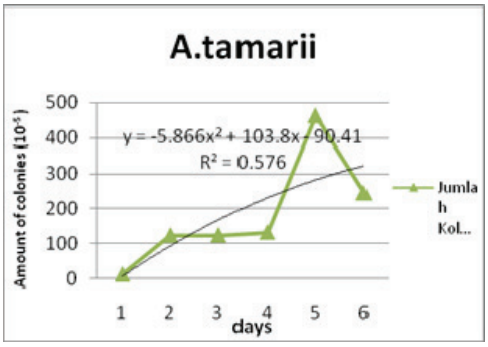
The growth curve was observed for best starter age for inoculum propagation. Mold growth curve was calculated by the method of *Total Plate Count* (TPC) (Table 1).When drawn, the mold growth curve of *P. nalgiovense* and *A. tamarii* has the same pattern as the general microbial growth curve (Figure 2).

Figure 2 showed when the peak growth of *A. tamarii* is on day 5, but on the 6th day a drastic decline is seen, until the effective time for *A.tamarii*was on day 5. In the meantime, *P. nalgiovense* experienced peak growth at day 6, as many cfu 9696.33.

Table 1. The average number of mold cells *A. tamarii* and P. nalgiovense in substrate

| *A. tamarii* | | *P. nalgiovense* |
|---|---|---|
| Days of culture | Total colonies in $10^6$ cfu | Total colonies in $10^6$cfu |
| 1 | 1.2 | 195.8 |
| 2 | 12.4 | 99.9 |
| 3 | 12.5 | 188.2 |
| 4 | 13.2 | 201 |
| 5 | 46.5 | 697.3 |
| 6 | 24.6 | 969.6 |

TPC (Total Plate Count) results done singly for both types of fungi showed that *A. tamarii* and *P. nalgiovense* have exponential phase (log phase) on day 5. Exponential time is used as the optimum treatment time and the time of the start of the fermentation process, because the log phase is a period where microbial growth occurs very rapidly (Pelczar and Chan, 1986) so a lot of microbial activity occurred at this time.Mold growth trendline indicated a relationship between time (days) and the number of spores, the longer the treatment, the more spores produced.



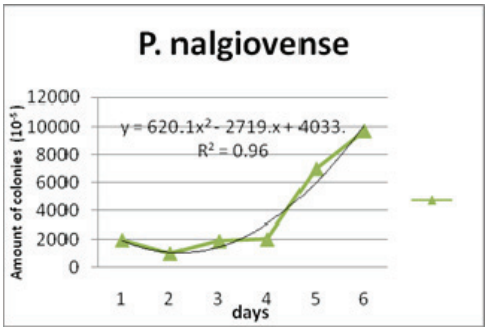



Figure 2. Growth curves of *A.tamarii* and *P.nalgiovense*

Based on enzyme activity assays, *A.tamarii* and *P.nalgiovense* fungi have cellulolytic ability / amylolytic capability measurable through the production of cellulase and amylase as secondary metabolites (Table 1). Cellulase activity of *P. nalgiovense* and *A. tamarii* increased from the first day of culture, reaching a peak on second day and its activities decreased on the third and fourth day. While the activity of amylase *P. nalgiovense* peaked at culture day 1 for 2.0165 units / ml and *A. tamarii* on day-3 of 4.110 units / ml.

These results were consistent with previous studies stating that mold of *A. tamarii* is known have amylase and glucoamylase activity (Moreira, 1999), and *P. nalgiovense* had a high cellulase activity, amounting to 0.027 units / ml (Nugroho, 2006).

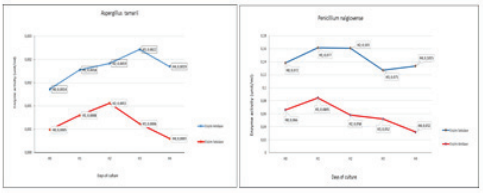


Figure 3. Activity of cellulase and amylase enzymes of *Aspergillus tamarii* fungi (left) and *Penicillium nalgiovense* (right)

## CONCLUSIONS

Based on the description, growth and cellulase and amylase enzyme activity observed, molds *Aspergillus tamarii* and *Penicillium nalgiovense* isolated from bovine rumen fluid had quite high amylase and cellulase activity and were easily cultured, making them potential to be used as crude fiber degrading microbes in cellulose waste.

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# FOOD SAFETY

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# ESSENTIAL OILS UTILIZATION IN FOOD INDUSTRY - A LITERATURE REVIEW

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***Abstract:***

*Nowadays, essential oils are used in pharmaceutical, sanitary, cosmetics, agriculture and food industry because of the biological activity, notably antibacterial, antifungal and antioxidant properties.*
*Essential oils, or (EOs) for short, are natural compounds with a complex composition that contains volatile principles present in plants such as terpenes, terpenoids, phenol-derived aromatic components and aliphatic components.*
*In food industry, it is crucial that the products to be supplied without any microbial contamination, but the biological efficacy of EOs to be used as antimicrobial agents in food system, depends on some factors as temperature, pH, appearance, load of microbial flora and the favorable environment.*
*Studies demonstrated that the antimicrobial effects of EOs confirm structural and functional damages of membrane of bacterial pathogenic, resulting the leaking of the inner cell components, and eventually leading to the cell death (Cox et al., 2000). Usually, Gram-negative bacteria are less susceptible to antimicrobials, but this doesn't mean that Gram-positive bacteria are always more sensitive.*
*Due to the fact that essential oils present compounds with antioxidant activity (phenolic compounds) it can be used in food industry as preservatives to prevent the spoilage of the products and to increase the shelf life.*

***Keywords:*** *antimicrobial activity, essential oils, food products.*

## INTRODUCTION

Lately, the consumers demand for safe and natural food products, free from synthetic chemical preservatives and minimally processed, with a longer shelf life. It's a challenge to obtain these products, and in the same time the products to be safety and healthy. Because of this situation, natural compounds with antimicrobial activity found in herbs and plants are recommended to be added, either alone or in combination.
In recent years, several researches have shown that many species and herbs which exert antimicrobial activity due to their essential oil fractions can be used in food system like antifungal, antibacterial and antioxidant agents by inhibiting the growth of pathogenic microorganisms, this way ensuring the microbiological safety of food products (Benkeblia et al., 2004; Burt, 2007, Ye Chun-Lin et al., 2013).

According to the 8th Edition of the French Pharmacopeia (1965), essential oils (EOs for short) are products of complex general composition that contain volatile principles present in plants, more or less modified during their preparation. EOs are obtained from different plant parts, such as flowers, leaves, seeds, bark, fruits and roots and are stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes. The composition of EOs from a particular species of plant can differ between harvesting seasons and between geographical sources (Burt, 2004).
EOs are liquid, volatile, limpid and rarely colored, lipid soluble and soluble in organic solvents with a generally lower density than the density of water.
The composition of EOs includes a complex mixture of several compounds. The main group is composed of terpenes and terpenoids and the other of aromatic and aliphatic constituents, all

characterized by low molecular weight (Bakkali et al., 2008).

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 phenolic in nature. Aromatic compounds occurs less frequency than the terpenes, but the antimicrobial effect of essential oils depends on the content of phenolic components (Cakir et al., 2004).

There are as well various chemical components present in plant-origin antimicrobials including, saponins and flavonoids, thiosulfinates, glucosinolates and saponins (Tajkarimi et al., 2010).

The most studied compounds of EOs are carvacrol, thymol (one of the major components of *Origanum compactum* essential oil and thyme oils) and eugenol (found in *Eugenia caryophylata*).

Essential oils are efficient against some varieties of organisms, such as bacteria, virus, fungi, protozoa, parasites, acarids, larvae and mollusks.

For the extraction of essential oils there are known several methods, such as liquid carbon dioxide or microwave, and the most commonly methods for obtaining antimicrobials from plants are SD (steam distillation) and HD (hydro distillation) methods, and alternative methods such as SFE (supercritical fluid extraction) provide higher solubility and improved mass transfer rates (Bakkali et al., 2004; Tajkarimi et al., 2010).

## MECHANISM OF ACTION

The exact mechanism of action of many natural extracts is not well known, but it has been demonstrated by studies that essential oils have bacteriostatic or bactericide activity and that the antimicrobial effects of EOs exhibit structural and functional damages of membrane of bacterial pathogenic by various antimicrobial mechanisms (Cox et al., 2000; Burt, 2007). The quantity of spices and herbs mostly used in food products is between the ranges of 0.05–0.1% (500–1000 ppm). Some spices have stronger antimicrobial activity than others and can be effective at 1000 ppm.

Most of the studies on the mechanism of action of phenolic compounds have focused on their effects on cellular membranes. It has been shown that EOs pass through the cell wall and cytoplasmic membrane, attack the different layers of polysaccharides, and phospholipid bilayer of the cell membrane, disrupt enzyme systems, and compromise the genetic material of bacteria causing the permeabiliziation of the cells (Burt et al., 2007; Arque et al., 2008). It is considered that phenolic compounds not only attack cell walls and cell membranes, but it also interfere with membrane function (electron transport, nutrient uptake, protein, nucleic acid synthesis and enzyme activity). In consequence it's supposed that phenolic compounds are responsible for the inhibition of microorganisms.

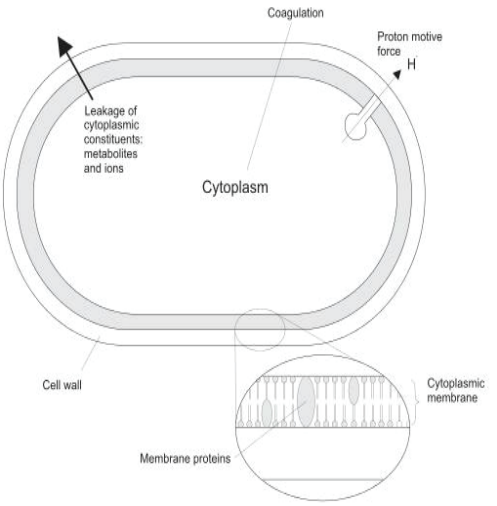


Figure 1. Locations and mechanisms in the bacterial cell thought to be sites of action for EO components: degradation of the cell wall; damage to cytoplasmic membrane; damage to membrane proteins; leakage of cell contents; coagulation of cytoplasm and depletion of the proton motive force (Burt, 2007).

Di Pasqua et al. (2007), Turina et al. (2006) showed that in bacteria, it can occur losing of ions and reduction of membrane potential by the permeabilization of the membranes. Usually, Gram-negative bacteria are less susceptible to antimicrobials, but this doesn't mean that Gram-positive bacteria are always more sensitive (Burt, 2004).

Because of the change of fluidity of membranes which become abnormally permeable due to

the use of essential oils, it results the leaking of the inner cell components, cytochrome C, calcium ions and proteins and eventually the permeabilization of mitochondrial membranes it leads to the cell death (Cox et al., 2000).

## RESULTS AND DISCUSSIONS

### Specificity of EOS *in vitro*

Most studied effect of essential oils regarding the antimicrobial have been conducted *in vitro* and it is well known that the antibacterial and antifungal activity of essential oils in food products is usually reduced compared to the *in vitro* work,due to the presence of fats, carbohydrates, proteins, salts and pH which influence the efficacity of volatile oils (Burt, 2007). The question of specificity of the different essential oils also arises.

The antimicrobial activity of essential oils is related to the chemical configuration of the components, the proportions in which they are present and to the synergism that exists between the components of volatile oils (Burt, 2004). Essential oils can be used alone or in combination with other compounds to inhibit the microorganisms, for example in a study made by Dimitrijevic et al. (2006) it was shown that essential oils of *Thymus vulgaris L., Rosmarinus officinalis L.* and *Origanum vulgare L.* in combination with sub-lethal dose of lactic acid noticeably increased the antilisterial activity against *Listeria monocytogenes*, especially of rosemary and thyme oils, but the synergistic effects of the mixtures were reduced with higher concentrations of oils. Other study demonstrated that using the checkerboard method and oregano combined with thyme had additive effects against spoilage organisms, and *Listeria* strains were more sensitive than spoilage bacteria (Gutierrez J. et al., 2004).

In a study realized by Celiktas et al. (2007) it was shown that the antimicrobial activity of the essential oils obtains from *Rosmarinus officinalis* against *Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella pneumonia, Enterococcus feacalis, Escherichia coli, Staphylococcus epidermidis, Bacillus subtilis* and *Candida albicans* differs, depending on location and seasonal variations of the plants.

The researchers Benkeblia (2004), Ye C.-L. et al. (2013) have studied the effectiveness of essential oils extracted from the *Allium* plants (garlic and onions) against food spoilage and food-borne pathogenic microorganisms and its antioxidant activity.

According to the results, the essential oils revealed a potent inhibitory antimicrobial effect against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Salmomella enteritidis,* and three fungi *Aspergillus niger, Penicillium cyclopium* and *Fusarium oxysporum.* The essential oil obtained from garlic showed the highest inhibition action and the *Allium cepa* EOs exerted a broad antimicrobial spectrum and showed a high antimicrobial action effect on *B. subtilis.* Lalitha et al. (2011) showed that the essential oil of *Allium sativum* had a maximum effect on the inhibition of all the bacteria tested, and recorded a complete inhibition in eight fungi from the tenth tested compared to control.

It has been shown that essential oils can be used against microorganisms as *Aeromonas hydrophila*, *Bacillus sp*. (Lalitha et al., 2011), *Clostridium perfrigens*, *Camphylobacter jejuni*, *Escherichia coli* (Celiktas et al., 2007, Lalitha et al., 2011), *Listeria monocytogenes* (Dimitrijevic S., 2006; Gutierrez J. et al., 2009), molds and yeast (Ye C.-L. et al., 2013), *Pseudomonas sp*. (Celiktas et al., 2007), *Salmonella sp*. and *Shigella sp*. (Bajpai V. K. et al., 2012), *Staphylococcus aureus* (Benkeblia N., 2004), *Vibrio parahaemolyticus*, Gram-positive and Gram-negative bacteria.

In consequence of these studies it can be concluded that the antimicrobial effect of essential oils depends on the location and the variations between seasons, the composition of EOs and the synergism of all molecules or reflect only those of the main molecules present at the highest levels according to gas chromatographical analysis, butit also depends on some factors as temperature, pH, appearance, load of microbial flora and the favorable environment of tested products.

### Antimicrobial activity of EOS in food products

There are differences between *in vitro* and in-food trials of plant-origin antimicrobials,

mainly because only small percentages of EOs are tolerable in food materials. Finding the most efficient essential oils depends on a number of factors such as type, effects on organoleptic properties, composition, concentration, biological properties of the antimicrobial, the target microorganism and processing and storage conditions of the targeted food product (Gutierrez et al., 2009).

Despite some positive reports regarding the application of plant-origin natural antimicrobials in food industry, two major issues are faced: odors created mostly by the high concentrations and the increased price of these materials (Proestos et al., 2008).

Studies demonstrated that EOs can be used as an alternative preservative and pathogen-control method in food materials such as meat products, fish, vegetables, rice, fruits and dairy products.

The antimicrobial effects of essential oils against various microorganisms from meat products and fish contaminated can be reduced due to the high fat content (Burt, 2004).

EOS of rosemary has antimicrobial effects against *Listeria monocytogenes* from meat products (Caraminana et al., 2008), oregano EOs can be used against *Clostridium botulinum* spores, winter savoy (*Satureja montana*) EOs in combination with other preservatives methods can be used as natural antibacterial substance to control growth of food-borne bacteria and improve quality of minced pork. Busatta et al. (2008) studied the antimicrobial activity in a fresh sausage of marjoram (*Origanum majorana L.*) essential oil against several species of bacteria. Results showed that addition of marjoram essential oil to a fresh sausage exerted a bacteriostatic effect at oil concentrations lower than the MIC, while a bactericidal effect was observed at higher oil concentrations which also caused alterations in the taste of the product.

Wild thyme (*Thymus seryllum*) EOs has a preservative effect and increase the shelf life of fresh-water fish (Oral et al., 2008). Essential oils of *Aloysia sellowii* have antimicrobial effects against a variety of Gram - positive and negative - microorganisms and two yeasts in brine shrimp (Simionatto et al., 2005).

Opposite to meat products and fish, in milk the high water content affects the application of EOs by increasing the transfer and movement of EOs toward the targeted microorganisms (Cava, Nowak et al., 2007). Abdalla et al. (2007) demonstrated that extract of mango seed kernel could inhibit the coliform growth, reduce total bacterial count and have antimicrobial activity against the *Escherichia coli* strain and in the same time extend the shelf life of pasteurized cow milk.

EOs of cardamom, cinnamon, clove inhibits the growth of yoghurt starter culture more than mint oil. In another study, it has been shown that mint oil is effective against *Salmonella enteridis* in low-fat yoghurt and cucumber salad (Burt, 2004).

Regarding to vegetables, the use of essential oils in washing water has a good effect against natural spoilage flora and food-borne pathogens, because of the low fat content of the products. In another study made by Burt (2007), it has been shown that oregano oil was effective against *Escherichia coli O157:H7* and reduced the final populations in eggplant salad.

In rice, the use of sage oil and carvacrol had a significant effect against *Bacillus cereus* (Burt, 2004).

As for fruits, the efficacy of EOs might depend of the pH of the fruits.

It was also tested the antimicrobial activity of essential oils in chocolate held at different temperatures, in dry or humidified environment and observed that lemon flavor applied to chocolate inoculated with *E. coliO157:H7* had the most inhibitory action. Plant extracts tested on chocolate show an enhanced inhibitory effect during storage at $20^0$C concluding that their application may provide protection in case of storage at the above temperature or even higher.

It has been shown that the antimicrobial activity of essential oils was diminished in food system as they appeared less efficacious when added to chocolate (Kotzekidou, 2008).

Recently, the essential oils started to be used because of their antimicrobial activity in food packaging, to edible films. Incorporating antimicrobial compounds into edible films provides a novel way to improve the safety and shelf life of ready-to-eat foods. This method of

packaging can be used on fresh and minimally processed fruit and vegetable, meat and meat products, poultry, fish, tree nuts (Du Wen-Xian et al., 2011).

In a study made by Seydim (2006) with whey protein isolate (WPI) films containing ratios of oregano, rosemary and garlic essential oils which were tested against *E. coli O157:H7*, *Staphylococcus aureus*, *Salmonella enteritidis, Listeria monocytogenes* and *Lactobacillus plantarum* it was shown that oregano essential oil was the most effective against all the microorganisms tested than those containing garlic and rosemary extracts.

Matan (2012) showed that edible films incorporated with essential oils had antimicrobial effects against some major molds (*Aspergillus flavus*, *Penicillium* sp.) and bacteria (*Staphylococcus aureus*) found on dried fish (*Decapterus maruadsi*). The results showed that film containing anise oil was the most effective against mold and the shelf life of the dried fish was extended by the used of essential oils incorporated in edible films.

## CONCLUSIONS

Essential oils contain compounds with antioxidant activity (phenolic compounds) and can be used as preservatives because it can prevent the spoilage of the products and in the same time can increase the shelf life without influencing the properties of food products. As the demand of consumers is to reduce or replace chemical preservatives with natural ones, the compounds from volatile oils can be used because it reduce the secondary effects of chemical preservatives.

In order to prevent the modification of organoleptic properties of food product, it's recommended to add a small concentration of essential oils to the products.

It has been reported that combined methods of application of the EOs with other methods such as hurdle technology and modified-atmosphere packaging improve the flavour and increase shelf life of food products (Burt, 2004).

Still, the use of EOs which has antimicrobial effects compare with the use of synthetic additives is still limited cause of the persistent flavour which can affect the sensorial quality of products, and the high costs of food products.

The use of plant extracts and essential oils in consumer goods is expected to increase in the future due to the fact that volatile oils can be considered as a natural alternative to traditional food preservatives and could be used to enhance food safety and shelf life.

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# RESEARCH ON THE CORRELATION BETWEEN PHYSICO-CHEMICAL, SENSORY ANALYSIS OF SMOOTHIE TYPE PRODUCTS AND CONSUMER PREFERENCES

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***Abstract***

*Numerous studies have documented the prevention of certain chronically diseases, e.g. hypertension, coronary heart diseases, and the risk of stroke by consuming an adequate amount of fruits and vegetables.*
*To increase the intake of phytochemicals, the food industry offers smoothies as an alternative or addition to the consumption of fresh fruits.Their production is based on the usage of the entire fruit, which is processed from pulp to puree, the seeds and the peel being removed. To develop different flavors and to obtain the appropriate texture of the final product the juice from the corresponding fruits is used as well. Smoothie products are characterized by a high concentration of nutrients and a low content of energy.*
*The aim of this study is to develop new high quality products based on mixtures of pressed and squeezed fruit without adding preservatives, stabilizers or chemical correctors of pH and acidity. For this purpose, some mixtures of several pressed fruit like apples, pears, bananas, mango, peaches, cherry, strawberries and orange, maracuja, apples or grapes juices, have been made.*
*The sensorial analysis tests have shown that a Brix degree equal with 13 and a pH smaller than 4, are the ideal values for the microbiologic stability of the newly developed products. These values have been corrected by the addition of grape juice for the samples with a low pH and a low Brix degree and by the addition of orange juice for the samples with a high pH and a high Brix degree.*
*The structure and the physico-chemical properties of the smoothies have an essential role in the increase of taste as well as the sensorial perception of the consumers. The texture is a critical attribute of quality which influences the consumer's acceptance of the smoothies, being in this way of a major interest in the development of the product.*

***Keywords:*** *fruit smoothie, sensory analysis, quality, new products development.*

## INTRODUCTION

In recent time, the demand for fresh fruit juices and smoothies with high quality level has been growingquickly.
Structure of food and its physico-chemical characteristics have a significant effect in the mouth and thus, on the sensory perception (Wilkinson, Dijksterhuis, & Minekus, 2000). Texture is a quality attribute that is critical in influencing the acceptability of raw and processed fruits so it is of primary concern in product development.
Sensory descriptive analysis is one of the most powerful, sophisticated and most extensively used tools in sensory science. Its application has steadily grown in the end of the 20th century and the beginning of the 21st. This methodology enables to measure the sensory reaction to the stimuli resulting from the consumption of a product, providing a description of the qualitative and quantitative aspects of human perception, and allowing correlations to other parameters (Lawless & Heymann, 2010; Moussaoui & Varela, 2010; Murray, Delahunty, & Baxter, 2001; Stone & Sidel, 2004).
Fruit and vegetable purees are considered as concentrated suspensions where the dispersed phase (pulp) consists of soft (deformable) insoluble particles (cell wall clusters) that are dispersed into an aqueous solution (serum) of sugars, organic acids, salts and pectic substances (Cepeda & Gomez, 2002; Qiu & Rao, 1989; Rao, 1999; Sato & Cunha, 2009)
Numerous studies have documented the prevention of certain chronical diseases, e.g. hypertension (Dauchet et al., 2007), coronary heart diseases (Dauchet, Amouyel, Hercberg, & Dallongeville, 2006; He, Nowson, Lucas, & MacGregor, 2007), and the risk of stroke

(Dauchet, Amouyel, & Dallongeville, 2005; He, Nowson, & MacGregor, 2006) by consuming an adequate amount of fruits and vegetables. These diseases are still responsible for the highest mortality rate in Western countries, such as Germany (DeStatis, 2008). Furthermore, polyphenolic compounds such as tea flavonoids (Stoner & Mukhtar, 1995), oligomeric procyanidins (Gerha¨user, 2008), and anthocyanins (Wang & Stoner, 2008) are implicated in the prevention of cancer. The daily uptake of fruits and vegetables was estimated to be lower than the recommended dietary intake (RDI), advised by the German Nutrition Society (DGE), of 650 g (250 g fruits, 400 g vegetables), especially for vegetables (Max-Rubner-Institut, 2008). To increase the consumption of fruits and vegetables, Germany started the campaign ''5 a day'' on the basis of the American one initiated by the National Cancer Institute (Rechkemmer, 2002). To increase the intake of phytochemicals, the food industry offers smoothies as an alternative or addition to the consumption of fresh fruits and vegetables. Smoothies belong to fruit juices and this term is used since 70 years, primarily in the USA and UK, recently also in Germany. Their production is based on the usage of the total fruits, which are processed to pulp or puree, partially with pieces.

Seeds and peel are removed (Qian, 2006). To create different flavours and to allow a drinkable texture, corresponding fruit juices are used. Smoothies are characterised by a high nutrient concentration with low energy content (Watzl, 2008).Smoothies are blended beverages containing fruit, fruit juice, ice, yoghurt, milk; and are a popular way of consuming fruit (Safefood, 2009). These products are typically purchased freshly prepared from juice bars or as a processed product (mildly pasteurised) from the chilled section of retail outlets. Despite worsening global economic conditions, smoothies remain a popular and convenient way of consuming fruit. In fact, the world smoothie market is projected to touch $9 billion by the year 2015 (Global Industry Analysts, 2010). This is primarily driven by rising health consciousness among consumers, on-the-go consumption, convenience, and perceived fresh like taste offered by smoothies.

The aim of this study is to develop new high quality products based on mixtures of pressed and squeezed fruit without adding preservatives, stabilizers or chemical correctors of pH and acidity. For this purpose, some mixtures of several pressed fruit like apples, pears, bananas, mango, peaches, cherry, strawberries and orange, maracuja, apples or grapes juices, have been made.

The obtained mixture were analysed in comparison with the market samples bought from Austrian and German markets (3 brands).

## MATERIALS AND METHODS

### Samples

The pressed fruit and the juices were purchased from Austria, being packed in containers of different sizes and frozen at-18$^0$C. By mixing different fruit piurees were generated 4 smoothies recipes like: peach-mango, orange-maracuja, sourcherry-banana and banana-strawberry.

### 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.

*Brix degree determination*

This method evaluates the content of total soluble sugar content, by measuring index of refraction. Index of refraction was determinated with a digital handheld Refractometer Reichert AR 200.

*Consumer acceptance and preference tests*

Before determining the final recipes, numbers of attempts tested by a small group of consumers were made.

Final tests, before production, were done on 50 consumers, from which 52% were women and 48% men with ages between 20-29 years. They were divided into two groups, the first group had as objective to evaluate the degree of

acceptance for the 4 recipes, while the second group had the objective of evaluate the suitability and the degree of perception between recipes.

Figure 1. View from acceptance and preferences tests

Scheme of testing for group 1 was: 4 products (1A, 2A, 3A, 4A) with monadic assessment.

Scheme of testing for group 2 was: 8 products presented simultaneously – pair comparative (1A-1B, 2A-2B, and 3A-3B, 4A-4B)

Sample 1B, 2B, 3B and 4B were purchased from Austrian local fruit market.

Our purpose for acceptance level was 70%, and for preference 60/40.

The acceptance threshold of 70% means that the product can be accepted in case of obtainning at least 70% of the scale of assessment. The scale used is 9 points and therefore the minimum acceptance threshold is given by a score of 6.3. If the acceptance average points is higher than 6.3, the product meets the criterion of the acceptance required in research objective.

It is similar in the case of preference test. The research objective was to set a minimum threshold 60/40, which means that the product was the favorite from at least 60% of the respondents that analyzed a pair of products (it is a product with a significantly higher preference level). In opposition, a product that obtain 40% or less at preference test, is a product with a significantly preference low level, and rejected from the research objective. In case the both analyzed pairs were obtained scores between 40-60, these show a similar preference level.

## RESULTS AND DISCUSSIONS

The obtained results showed that the values of newly developed products pH between 3.73 and 4.19, the acidity between 0. 524 g citric acid/100 g product and 0.984 g citric acid/100 g product, and Brix degree between 13.2 and 15.2, have been similar with products purchased on the market.

To reach the proposed values, recipes have been corrected by the addition of grape juice for the samples with a low pH and a low Brix degree and by the addition of orange juice for the samples with a high pH and a high Brix degree.

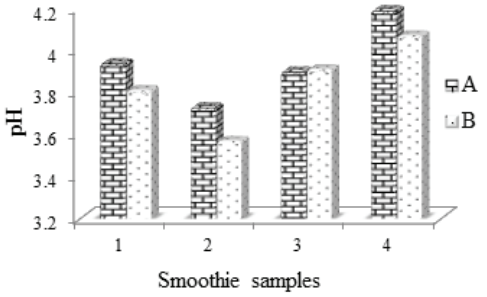


Figure 2. The graphical representation of the pH values for the smoothie samples, A-newly developed products, B-market products

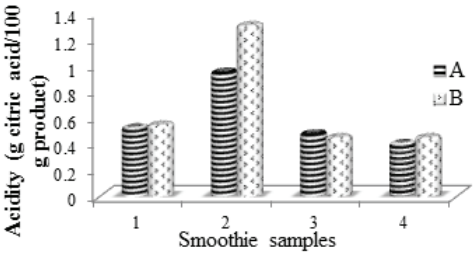


Figure 3. The graphical representation of the titratable acidity values for the smoothie samples, A-newly developed products, B-market products

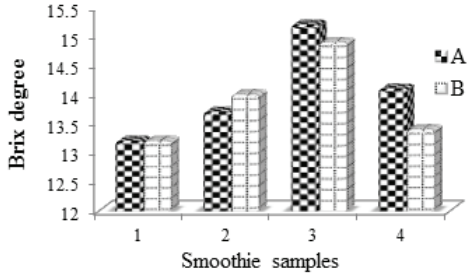


Figure 4. The graphical representation of the Brix degree values for the smoothie samples, A-newly developed products, B-market products

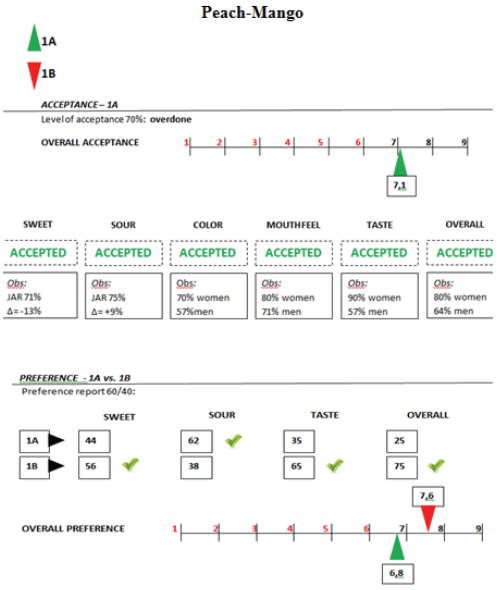


Figure 5. Acceptance and preference tests for Peach-Mango recipe

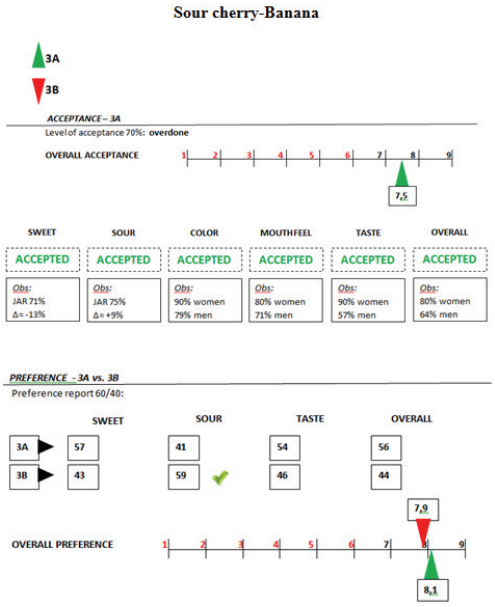


Figure 7. Acceptance and preference tests for Sour cherry-Banana recipe

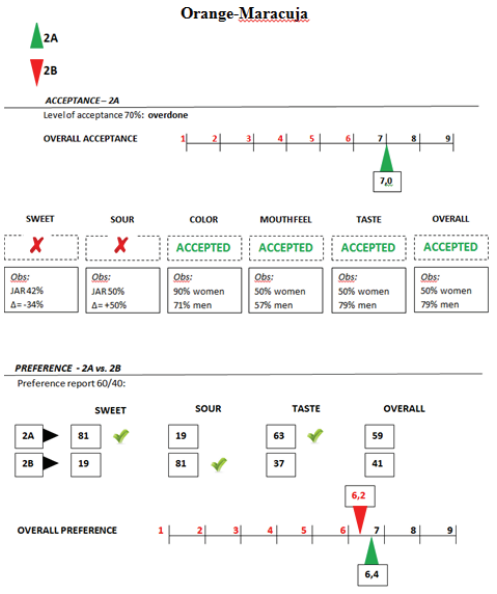


Figure 6. Acceptance and preference tests for Orange-Maracuja recipe

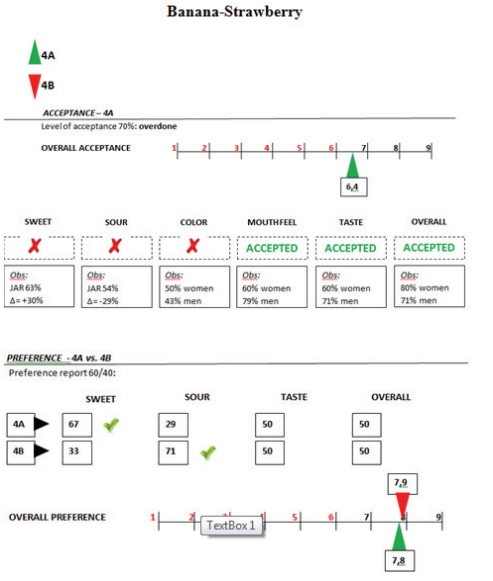


Figure 8. Acceptance and preference tests for Banana-Strawberry recipe

## CONCLUSIONS

The texture and 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.

The sensorial analysis tests have shown that a 13 Brix degree and a pH between 3.7 and 4.2 values –fit very well with the consumer acceptance and preference.

More, the acceptance and preference tests have shown that the newly developed products meet required criteria of objective research (70% acceptance level and 60/40 preference level).

In this work was developed and tested recipes for new high quality products based on mixtures of pressed and squeezed fruit without adding preservatives, stabilizers or chemical correctors of pH and acidity.

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# SELECTIVE DETERMINATION OF NITRITE IN CURED MEAT PRODUCTS USING A NONCONDUCTIVE POLYMER FILM BASED SENSOR

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***Abstract***

*For nitrite determination in cured meat, we have developed an original amperometric method based on a modified sensor surface using nonconductive polymers. The nonconductive films allow the target analyte to cross the sensor surface where an electrochemical reaction is taking place, albeit, restricting intereferences. Compared to a spectroscopic method, which requires a stringent pH control and the use of carcinogenic reagents, the amperometric method for nitrite determination is simple, rapid, and does not require dangerous reagents. The polymeric films were deposited onto the Pt and carbon paste electrodes, using cyclic voltammetry. The monomers used for nonconductive polymer film development were: 2,6-dihydroxynaphtalene (2,6-DHN), o-dianisidine (o-DIA) and 1,8-diaminonaphtalene (1,8-DAN). From all the permselective membrane that we have studied, the 2,6-DHN/Pt based sensor presents the sensibility and endurance required for developing the further studies for nitrite detection using a batch and a flow injection analyse system (FIA). Electrochemical tests have shown that 2,6-DHN/Pt is sensitive for sodium nitrite detection, it restricts potassium ferricyanide crossing, and acts as a barrier against ascorbic acid interference. Ascorbic acid is a major interferent in food products.The 2,6-DHN/Pt has a linear range (5-200μM), a correlation factor of 0.9994, and a low detection limit (2.5μM, S/N=3). Such a sensor has the ability to detect nitrite in various meat samples (e.g., hot-dogs, ham, italian salami, and canned beef).*

***Keywords****: nitrite, nonconductive polymers, modified electrodes*

## INTRODUCTION

Sodium nitrate and sodium nitrite are chemicals that are being used in food, especially to preserve meat. These chemicals are also used in other foods as preservatives, antimicrobial agents, as well as flavouring agents (to confer them their characteristic colour, texture, taste, etc). Nitrates are considered compounds of lower toxicity, representing a danger only when ingested in excessive doses or when converted to nitrites (Pinho et al., 1998). This conversion occurs at high temperatures, such as when frying cured meat at high temperatures. It is well known that the nitrite's most toxic effect is methemoglobinemia.

Nitrites combining with amines form N-nitrosoamines, which are known to be potent carcinogen compounds. Nitrite is added to cured meat in concentrations of less than 150ppm for preventing the growth of *Clostridium botulinum,* which is responsible for food poisoning. Therefore, the level of nitrite in cured meat must be kept at a minimum level. For this reason a number of substitutes were tested and it became customary to add ascorbic acid in order to reduce the amount of nitrite added.

Ascorbic acid is added in higher concentrations than nitrite because of its preservative and antioxidant properties. As we have already reported in a previous work (Badea M. et al, 2004), nitrite can be oxidized at a potential of +0,9V at a platinum electrode using an Ag/AgCl reference electrode. The high potential required causes the direct oxidation of interfering species such as ascorbic acid.

In the presence of oxygen and in media of pH lower then 5.5, oxidation of ascorbic acid leads to the formation of dehydroascorbic acid and hydrogen peroxide (Taqui & Martell, 1967), without being involved in the electrode reaction. In order to determine the nitrite from cured meat samples, we proposed a selective amperometric procedure using three different size/charge exclusion membranes: 2,6-dihydroxynaphtalene (2,6-DHN), o-dianisidine (o-DIA) and 1,8-diaminonaphtalene (1,8-DAN).

The nonconductive film obtained through the electropolymerization of 2,6-DHN showed a better stability in time when introduced into a FIA system. The electrode developed showed a good response in respect to nitrite when we compare its signals with the ones obtained using the Griess spectroscopic method.

## MATERIALS AND METHODS

All reagents were prepared from analytical reagent grade chemicals using bi-distilled water. Acetate buffer (Riedel de Haen) solution was prepared using 0.1M sodium acetate 0.5% methaphosphoric acid (Fluka) and 50µM EDTA (Sigma).

The 2,6-DHN (Fluka) was dissolved in 0.1M phosphate buffer pH7. A 0.5M stock L-ascorbic acid solution (Riedel de Haen) was performed in bi-distilled water and dilluted daily when needed. Hydrogen peroxide (Fluka) was prepared daily in acetate buffer. The titer of the hydrogen peroxide solution was verified through titration with $K_2Cr_2O_7$.

A potentiostat/galvanostat µ-AUTOLAB, type II (Ecochemie) was used for voltammetric studies. A Pt electrode (2mm) and an Ag/AgCl (3M KCl) Metrohm were used for all tests.

A four-channel Minipuls 3 Gilson peristaltic pump fitted with tygon tubing (1.52 mm id) was used for the propulsion of fluids. Also, the FIA system contains an injection valve (Rheodyne, 7725i model) and a wall jet flow cell which present a Ag/AgCl reference electrode and a gold counter electrode.

The valve loop volume was 100µL. Fittings and connectors were used to connect the different components of the manifold. The optimum flow rate was 0.6mL/minute. The detector was the same potentiostat/galvanostat used for voltammetric measurements. A diagram of the FIA manifold employed is shown in fig.1.

## RESULTS AND DISCUSSIONS

2,6-DHN was prepared accordingly to 'Materials and Methods' section. The working electrode used was carrefully cleaned onto PSA microcloth (Buehler, UK) using micropolish II with different doses (1µm $Al_2O_3$, 0,3µm $Al_2O_3$ and 0,05 µm $Al_2O_3$). After mechanical cleaning the electrodes were immerse in distilled water and ultrasonicated for ten minutes.

The first voltammogram showed two oxidation peaks which corespond to the oxidation of amino groups, indicated by the formation of the non-conductive polymer film (figure 2).

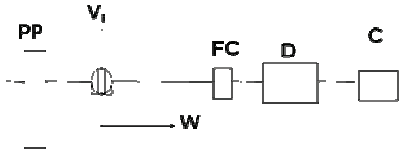



Figure 1. FIA system for nitrite determination of nitrite. PP-peristaltic pump, $V_i$ - injection valve, W-waste, FC-flow cell, D-detector, C-computer

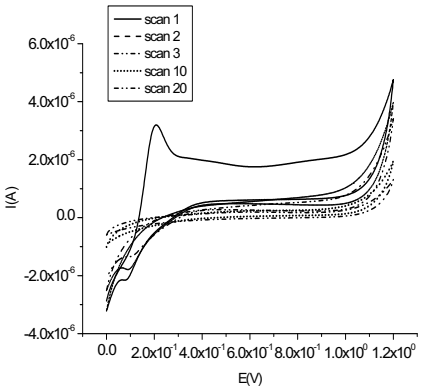



Figure 2. Electropolimerization of 2,6 - DHN onto the Pt (2mm) at a scan rate of 0.01V/s and a potential range between 0 – 1.2 V

In order to analyse the properties of the polymeric membrane, we studied its response to ascorbic acid interference (the main interferent of nitrite in canned meat) and also the size of the array formed (testing the electrode in potassium ferrycyanide). For the interference studies we investigated the voltammetric behaviour of the Pt 2mm electrode in acetate buffer pH4 and in ascorbic acid before and after the electropolymerization (figure 3).

As figures 3 and 4 show, the ascorbic acid gives an anodic peak at +0.6V when using the Pt electrode, which disappears when switching to the 2,6-DHN/Pt sensor. In conclusion, the polymer film is selective in respect to ascorbic acid. The results, demonstrate that the 2,6-DHN/Pt is selective to nitrite, restricts potassium ferrycyanide crossing, and acts like a barrier against ascorbic acid interference.

In order to study the nitrite optimum oxidation potential, we have characterized the 2,6-DHN/Pt electrochemical properties in DC amperometry. The maximum signals were

obtained via hydrodynamic voltammetry within a potential range 0.8-1V (figure 5).

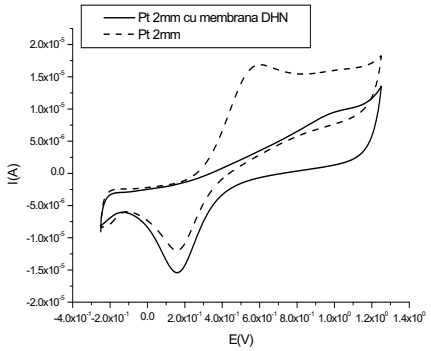



Figure 3. Influence of the interferences
a) cyclic voltammogram for Pt performed in 5mM ascorbic acid; b) cyclic voltammogram for 2,6-DHN/Pt performed in 5mM ascorbic acid. Working condition : 0.2-1.2V

The cathodic peak at 0.25V corresponds to the acetate buffer (figure 4).

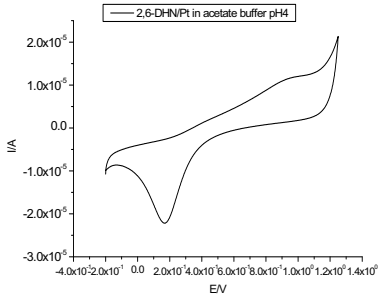



Figure 4. 2,6-DHN/Pt voltamogram obtained in acetate buffer pH4

The optimum potential is in the range of 0.9 – 0.95V.

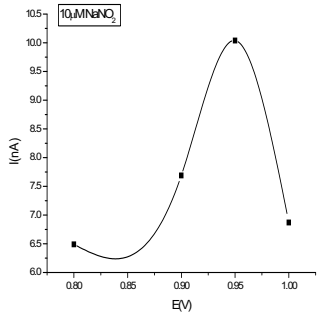
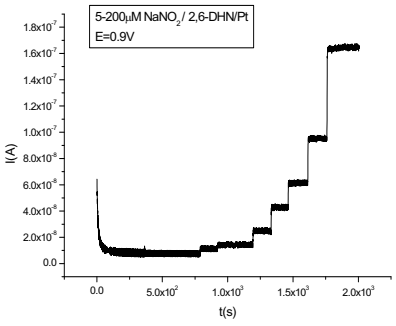



Figure 5. Hydrodynamic voltamogram for10μM nitrite. Working conditions: E=0.8 - 1V.

In figure 6 it is exemplified the aspect the DC amperogram obtained for various concentration of added nitrite at the applied potential of 0.9 V *vs* Ag/AgCl.

*A*




*B*

Figure 6. Amperograms obtained in a batch system for nitrite determination using 2,6-DHN/Pt (E=0.9V)

*Batch Procedure*

The 2,6-DHN/Pt electrode was immersed in a stirred buffer solution. After the base signal was stabilized, successive additions were performed using a stock solution of 5mM sodium nitrite in order to obtain final concentrations within the range 5-100μM nitrite. Based on the calibration graph (figure 7) obtained for the batch data, we have chosen 0.9V as the optimum potential for running further studies.

The equation $I(nA) = 1{,}15+0.19 \cdot C_{nitrite}$ (μM) is linear in the range of 5 - 200μM nitrite and has a correlation factor $r^2=0.9994$.

*Flow injection analysis (FIA)*

By using the optimal conditions mentioned, we have tested the 2,6-DHN/Pt in the FIA system presented in 'Materials and Methods' section.

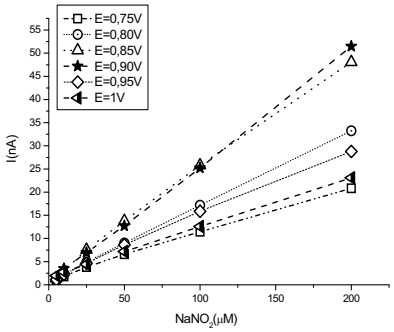



Figure 7. Calibration graphs at different applied potentials for nitrite determination in batch system.

The first studied parameter was the flow rate. In figure 8 are presented the FIA peaks recorded for flow rates varying between 0.36 and 0.67 mL/min. We have found an optimum flow rate equal to 0.6mL/min, varying the flow rate between 0.26-1.1mL/min (data not shown). FIA system showed an extended linear range of nitrite concentration up to 500 µM (Figure 9).

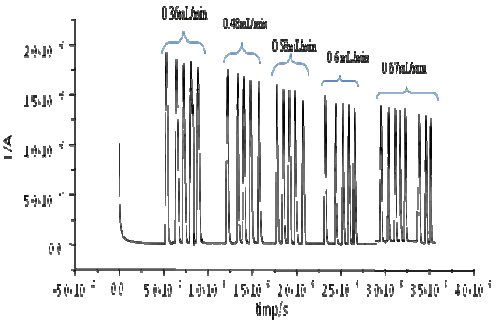



Figure 8. Influence of the flow rate on the FIA peaks recorded for injection of 100 µL of 50 µM nitrite

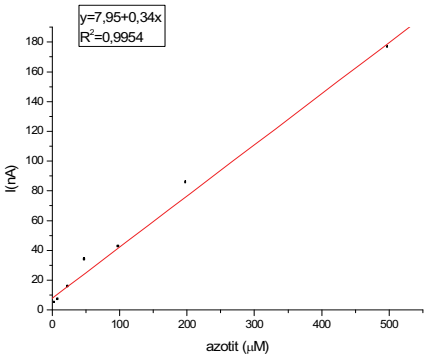



Figure 9. Calibration graph for nitrite determination using the DHN/Pt sensor in FIA system

*Determination of nitrite in real samples*

The extraction of nitrite from real meat samples was performed according to the methodology used by (Badea et al., 2004). The meat samples (hot-dogs, canned beef, Italian salami and ham) were crushed into small pieces and then blended until homogenous mixtures were obtained. The sample extracts were prepared by mixing 5g of homogenous mixture with 100mL acetate buffer. This mixture was stirred for 30minutes and then was filtered through a 150 µm filter. For further analysis, 15mL of the mixture was taken. A control was prepared similarly with the samples.

In order to evaluate the recovery of the electrochemical method, these samples were analyzed with and without spiking the meat samples with 100 ppm nitrite (table 1). The 2,6-DHN/Pt was immersed in 15mL acetate buffer and left for polarization for several minutes. Following addition of the 15mL sample, the signal was recorded.

Table 1. Recovery test for electrochemical method for nitrite determination

| Sample | Electrochemical Method | | |
|---|---|---|---|
| | nitrite (µg/g) | Spiked sample with 100 ppm nitrite (µg/g) | Recovery |
| Hot-dog | 103,6 | 247,6 | 121% |
| Canned beef | 91 | 199,5 | 104% |
| Italian Salami | 250,5 | 333,2 | 95% |
| Ham | 338 | 337,9 | 105% |

## CONCLUSIONS

In this work, we have proposed a nonconductive polymer film based sensor for determination of nitrite in cured meat samples. The interference study shows that the developed sensor presents the advantages of selectivity and reproducibility. Furthermore, introduced into a FIA system, it has great operational versatility, reduction of reagent consumption and automatic sample analysis.

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# DIOXINS AND FURANS CONTAMINATION OF FOOD AND THEIR TOXICOLOGICAL IMPLICATIONS ON THE HUMAN BODY MINI-REVIEW

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***Abstract***

*Dioxins (sum of polychlorinated dibenzo-p-dioxins - PCDDs) and furans (polychlorinated dibenzofurans - PCDFs), are polychlorinated aromatic compounds with high toxic potential, which persist in the environment. Their degradation is extremely slow, they accumulate in the human body mainly through the consumption of contaminated food (in more than 90% of cases) and can not be destroyed by cooking or metabolized by living organisms. This study aims to underline the importance of their determination through advanced analytical methods such high-resolution gas chromatography coupled with high resolution mass spectrometry (HRGC-HRMS), in order to increase the food safety.*

***Keywords:*** *dioxins, food, furans, HRGC-HRMS.*

## INTRODUCTION

Dioxins and furans are a group of chemical substances, with high toxic p otential, which persist in the environment and they accumulate in the living organisms through the food chain.
Dioxins as referred to in the Commission Regulation (EC) No 1881/2006, and replaced by 1259/2011, cover a group of 75 polychlorinated dibenzo-*p*-dioxin (PCDD) congeners and 135 polychlorinated dibenzofuran (PCDF) congeners, of which 17 are of toxicological concern. In order to be able to sum up the toxicity of these different congeners, the concept of toxic equivalency factors (TEFs) has been introduced to facilitate risk assessment and regulatory control. TEFs values were proposed first by the World Health Organisation (WHO), in 1997 in Stockholm, Sweeden. In 2005, they were updated by the same organisation (Van den Berg et al. 1998; Van den Berg et al. 2006) (Table 1).
To the compound with the highest toxicity, 2,3,7,8 - TCDD, was assigned the biggest TEF value, 1.

Dioxins (sum of polychlorinated dibenzo-*p*-dioxins (PCDDs)) and polychlorinated dibenzofurans (PCDFs) are expressed as World Health Organisation (WHO) toxic equivalent using the WHO-toxic equivalency factors (WHO-TEFs) in pg/g fat.

Table 1. TEFs proposed by WHO for PCDDs and PCDFs

| | **WHO-TEF$_{95}$** | **WHO-TEF$_{05}$** |
|---|---|---|
| **PCDDs** | | |
| 2,3,7,8-TCDD | 1 | 1 |
| 1,2,3,7,8-PeCDD | 1 | 1 |
| 1,2,3,4,7,8-HxCDD | 0.1 | 0.1 |
| 1,2,3,6,7,8-HxCDD | 0.1 | 0.1 |
| 1,2,3,7,8,9-HxCDD | 0.1 | 0.1 |
| 1,2,3,4,6,7,8-HpCDD | 0.01 | 0.01 |
| OCDD | 0.0001 | *0.0003* |
| **PCDFs** | | |
| 2,3,7,8-TCDF | 0.1 | 0.1 |
| 1,2,3,7,8-PeCDF | 0.05 | *0.03* |
| 2,3,4,7,8-PeCDF | 0.5 | *0.3* |
| 1,2,3,4,7,8-HxCDF | 0.1 | 0.1 |
| 1,2,3,6,7,8-HxCDF | 0.1 | 0.1 |
| 1,2,3,7,8,9-HxCDF | 0.1 | 0.1 |
| 2,3,4,6,7,8-HxCDF | 0.1 | 0.1 |
| 1,2,3,4,6,7,8-HpCDF | 0.01 | 0.01 |
| 1,2,3,4,7,8,9-HpCDF | 0.01 | 0.01 |
| OCDF | 0.0001 | *0.0003* |

## MATERIALS AND METHODS

For this paper, we consulted series scientific articles, between 1988 and 2012, including the European Commission Regulations, using as keywords, the terms: dioxins, furans, food.

## RESULTS AND DISCUSSIONS

### 1. FOOD CONTAMINATION

Dioxins and furans are secondary products resulted from: chemical reactions (carbo-chemical processes, wood treatment with penta chloro-phenol, paper whitening, herbicides production etc.), combustion processes (wood and wood waste, oil and coal combustions), incineration of municipal, hospital and industrial wastes, technological incineration of dangerous wastes, incineration of the plant wastes on the fields, big fires in which can be involved big quantities of materials containing chlor (for example, PVC), industrial accidents and occasional fires (Zedeck, 1998; Scialli, 2001; Schecter, 2001; McKay, 2002).

The decomposition of the dioxins and furans in the environment is extremely slow, reason why, dioxins can concentrate in the food chain, animals getting to have in their body (by bioaccumulation) much higher concentrations than plants, water or even soil. The accumulation of the dioxins in the human body, occurs mainly through the contaminated food consumption (in more than 90% of the cases). Air inhalation and absorbtion by the dermis, are considered to be minor contamination sources with dioxins and furans for the human body (Travis, 1991; Ganesh, 1995; Liem, 1999).

Dioxins and furans from foods can not be destroyed by preparation methods, can not be metabolized by the organism and can not be eliminated by faeces or urine, but contrary, they accumulate, their concentration getting higher and higher from one food to another, in body′s fat deposits.

According to the US Environmental Protection Agency, the highest dioxins and furans concentrations are found in beef (longer the animal lives, higher concentrations accumulates). Follows, milk and milk products (yogurt, cream, butter, cheese etc.), chicken meat, pork, fish and eggs. Likewise, the presence of these toxic compounds in human milk, continues to be a major risk for the breast feed babies health (Schuhmacher, 2004).

The contamination of food with dioxins and furans is a thorny problem all over the world. Unfortunately in Europe there were a lot of cases related to food products contamination. In Germany, in 1997, was detected a contamination with dioxins, of some milk, butter, beef and veal samples. The contamination source was found to be the dehydrated citrus pulp, imported from Brazil, and used in animals feed. The concentration in dioxins found in this pulp was from 20 to 100 times higher than the maximum level. After removing the contaminated citrus pulp from animals feed, the concentration of the dioxins in the milk, decreased substantially (Malisch, 2000).

Ramos et al. (1997) determined the dioxins and furans content, of some milk samples from different farms in Spain. The milk from neighboring areas with different sources of dioxins and furans contamination (waste incinerator, chemical plant, paper mill factory), presented significant concentrations of dioxins, unlike the milk taken from „control farms″ (located in rural areas, without any specific contamination sources), where the concentrations of dioxins, were below the maximum admissible limits.

Lovett et al. (1998), studied the contamination degree with dioxins and furans in eggs and poultry, from farms situated in different areas of Great Britain. Of course, the eggs and poultry from a farm situated close to a chemical waste incinerator, had very high dioxins and furans concentrations, far beyond the limits permitted by legislation. The scientists reported as well, the presence of dioxins and furans, in samples from farms located in rural areas, apparently unexposed to sources of dioxins and furans contamination.

Another very serious contamination with dioxins and furans, of poultry and derivated products, was registered in Belgium, in 1999. The contamination source was found to be a lot of recycled fat (containing a very high dioxins and furans concentration), used for obtaining feed for the chickens (Bernard, 1999). Since 1999, Belgium, has established a comprehensive program for monitoring the concentration of dioxins and furans in feed, poultry, beef and pork, using both screening

and confirmatory methods, high resolution gas-chromatography, coupled with high resolution mass spectrometry (HRGC-HRMS).

In 2000, Engwall and Hjelm reported a dioxins and furans contamination, for a batch of carrots cultivated on a land irrigated with sewage. They compared their results with a control batch, irrigated with non-contaminated water, where the presence of dioxins wasn′t detected.

Researchers from Spain and Finland (Schuhmacher, 2004), revealed through HRGC-HRMS analysis, that the breast milk from 15 mothers, living in the neighborhood of waste incinerators, had a significant higher content in dioxins and furans, expressed as a sum of dioxins (WHO-PCDD/Fs-TEQ), of 5.1 to 46.8 pg/g of fat.

In 2006, M. De Vries et al., presented the results of a study, undertaken in EU countries, that showed up that eggs obtained in organic farming system, have very high concentrations in dioxins and furans, between 0.4 and 19 pg/g fat, in many cases exceeding the maximum level, of 3 pg/g fat (Commission Regulation (EU) 1881/2006, replaced by 1259/2011).

In 2007, specialists from Hungary (Kasza et al.) reported the presence of dioxins in E-412 food additive (guar gum), imported from India, by a swiss company. German and French authorities, withdrawn from commercial the yogurts and cheeses which had in their composition guar gum from this lot.

In 2008, the Italian Ministry of Health in collaboration with the European Commission developed a comprehensive monitoring program for contamination with dioxins and furans of buffalo milk, in order to prevent the possible contamination of soft buffalo cheese. Using HRGC-HRMS, was confirmed that 25.8% of the total milk samples, weren′t consistent in terms of dioxins and furans content (Scortichini, 2009).

Marti-Cid et al., in 2008, studied the exposure degree to dioxins and furans contamination of Tarragona population (Catalonia, Spain), located near waste incinerators. In order to determine the dioxins and furans concentrations, were analyzed a series of foods (vegetables, cereals, fruits, fish, seafood, meat and meat products, eggs, milk and milk products, oils and fats), used frequently in the diet of this population. Using, HRGS-HRMS, it was found that the total intake of dioxins and furans to the overall population of the province of Tarragona was estimated to be 27.81 pg/g fat/ day. It has been revealed that animal products contribute at a rate of 78% to the dioxins and furans intake, in the diet of the population located in this area of Spain.

In 2008, was reported the contamination with dioxins and furans of pork meat, from some Irish farms. The contaminating source was found to be the feed used for feeding pigs (Tlustos, 2012).

In 2010, was detected the contamination with dioxins and furans of pork, poultry and eggs, from many German farms. The contamination source, as in most of the cases presented before, was the feed used for feeding animals, which contained oils with high dioxins and furans concentrations (Schwind, 2010).

## 2. TOXICOLOGICAL IMPLICATIONS

Dioxins and furans have many harmful effects on the human body, including immunotoxicity, hepatotoxicity, birth defects, endocrine disruption or induction of numerous enzymes. It is thought that dioxins and furans exert their effects by binding to a specific cellular protein known as the aryl hydrocarbon receptor (AhR) (Aoki, 2001), an intracellular ligand involved in the regulation of a very large number of genes (Mitrou et al., 2001).

In humans and other vertebrates, the toxic responses to dioxins and furans may include: nervous system pathology, dermal toxicity such as skin cancer or dermal lesions, including rarely, chloracne or acne (the most known case in the world, the food poisoning of the Ukrainian presidential candidate, Viktor Yushchenko, in 2004), immune deficiency by affecting the hormones involved in the immune response of the body, immunosuppression induced by direct action on T and B lymphocytes, endocrine disruptions, including diabetes and thyroid disorders, decreased pulmonary functions, carcinogenity-hepatic, genital or pulmonary cancer (especially caused by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), which was evaluated as group 1 carcinogenic to humans by International Agency for Reasearch on Cancer, in 1997), reproductive and developmental toxicity by causing spontaneous abortions, infertility and

disorders of endocrine glands, teratogenic effects such as heart defects, hydrocephalus, spina bifida (WHO, 1997; CDC, 1998; Otles, 2003; EFSA, 2012).

## 3. FOODS CONTRIBUTING TO THE DIETARY EXPOSURE

According to the 2012′s EFSA Report, seven main groups of food should be taken into account when it comes to infants, children, adolescents, adults, elderly and very elderly population groups, dietary exposure: fish and seafood products, milk and dairy products, meat and meat products, eggs and eggs products, oils and fats from animal and plant origin, foods for infants and young children and other foods, such as dietary supplements and honey.

According to the same report, in almost all adolescents, adults, elderly and very elderly populations, fish and seafood products are the main contributor to the total dietary exposure, 30.2-75%, followed by meat and meat products 8.8-34.4% or milk and dairy products 7.3-24.6%. In infant and children cases, the major contributor was milk and dairy products with 27.5-49.6%, followed by fish and seafood products 10.7-35.8% or meat and meat products 10.4-33.7%.

In Table 2, are presented the maximum levels for the dioxins and dioxin-like polychlorinated biphenyls (PCBs) content in foodstuffs:

Table 2. Maximum levels for dioxins and furans in foodstuffs (CR (EU) 1259/2011)

| **Foodstuffs** | **Sum of dioxins (WHO-PCDD/F-TEQ)** | **Sum of dioxins and dioxin-like PCBs (WHO-PCDD/F-PCBs-TEQ)** |
|---|---|---|
| (*) Meat and meat products (excluding edible offal) of the following animals:<br>bovine animals and sheep<br>poultry<br>pigs | <br>2.5 pg/g fat<br>1.75 pg/g fat<br>1.0 pg/g fat | <br>4.0 pg/g fat<br>3.0 pg/g fat<br>1.25 pg/g fat |
| Liver of terrestrial animals referred to in (*), and derived products thereof | 4.5 pg/g fat | 10.0 pg/g fat |
| Muscle meat of fish and fishery products and products thereof, with the exemption of:<br>wild caught eel<br>wild caught fresh water fish, with the exception of diadromous fish species caught in fresh water<br>fish liver and derived products<br>marine oils<br>The maximum level for crustaceans applies to muscle meat from appendages and abdomen. In case of crabs and crab-like crustaceans (*Brachyura* and *Anomura*) it applies to muscle meat from appendages. | 3.5 pg/g wet weight | 6.5 pg/g wet weight |
| Muscle meat of wild caught fresh water fish, with the exception of diadromous fish species caught in fresh water, and products thereof | 3.5 pg/g wet weight | 6.5 pg/g wet weight |
| Muscle meat of wild caught eel (*Anguilla anguilla*) and products thereof | 3.5 pg/g wet weight | 10.0 pg/g wet weight |
| Fish liver and derived products thereof with the exception of marine oils referred to (***) | - | 20.0 pg/g wet weight |
| (***) Marine oils (fish body oil, fish liver oil and oils of other marine organisms intended for human consumption) | 1.75 pg/g fat | 6.0 pg/g fat |
| Raw milk and dairy products, including butter fat | 2.5 pg/g fat | 5.5 pg/g fat |
| Hen eggs and egg products | 2.5 pg/g fat | 5.0 pg/g fat |
| Fat of the following animals:<br>bovine animals and sheep<br>poultry<br>pigs | <br>2.5 pg/g fat<br>1.75 pg/g fat<br>1.0 pg/g fat | <br>4.0 pg/g fat<br>3.0 pg/g fat<br>1.25 pg/g fat |
| Mixed animal fats | 1.5 pg/g fat | 2.5 pg/g fat |
| Vegetable oils and fats | 0.75 pg/g fat | 1.25 pg/g fat |
| Foods for infants and young children | 0.1 pg/g wet weight | 0.2 pg/g wet weight |

## CONCLUSIONS

Dioxins and furans are widespread environmentally and biologically highly toxic stable polluants, all over the world, that accumulate in the living organisms. Human exposure to these chemicals is chronic and can provoke serious health effects and diseases, such as cancers or damages for the immune system. The major sources for dioxins and furans entering in the atmosphere are incineration and combustion processes. A major pathway of exposure for humans is through the consumption of fish and seafood products as main source, followed by meat and meat products and milk and dairy products. By reducing the environmental contamination with dioxins and furans, can be diminished the bioaccumulation in the food chain and this way reduced the intake levels and of course their toxic effects.

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# THE MICROWAVES EFFECTS ON LIQUID FOODS

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***Abstract***

*The microorganisms present in food could be beneficial (for example in the fermentation's process) or could cause human health risk. For this reason, is necessary a good control of microorganisms in food. There are two kinds of factors that affect growth and survive of pathogenic microorganisms in food:*

1) *intrinsic factors such as: acidity, pH, water activity, redox potential, the presence of nutrience;*
2) *extrinsic factors: storage conditions (temperature, time and humidity) and the type of processing and packaging.*

*In our days, the traditional thermal process used for pasteurization of liquid foods is replaced with new methods: electronic pasteurization, high-pressure processing, and pulsed electric field technology.*
*In this paper we present a report about the microwaves field on different microorganisms from liquid foods (fresh milk). It was used a microwave source with 2.4 GHz frequency and max. 1500 W power, for different exposure times. It was determined the total number of germs per ml, from every samples and compared with the standard. It remarks that the microwaves effects on microorganisms depend on electric fields strength, the treatment time and the shape of the pulse wave.*

***Keywords:*** *microwave, milk, microorganisms.*

## INTRODUCTION

Food microbiology has been an interesting science to mankind for centuries and continues to deepen its roots in recent years. Microorganisms in foods continue to be a problem both to the safety and quality of food products, and hence food safety continues to be an important issue for the industry. There are several factors affecting the survival and growth of microorganisms in foods, and understanding these factors will aid in controlling microorganisms in foods. Detecting microorganisms in foods is an important issue, and there are several methods available.The industry is continuing to develop rapid methods for better detection. There are several technologies (physical, chemical, and biological) used to control or reduce microorganisms in foods. Apart from the traditional processes such as thermal processing, novel technologies such as high-pressure processing, pulsed electric field processing, irradiation, and others are also being developed.Using appropriate detection and control methods, the industry should be able toproduce a safe food supply for the current consumer (Ravishankar and Maks, 2007).

Milk is a complete and complex food, with high nutritional value. In the same time, it represents a good environment for the development of all organisms. The raw milk will always contain a lower or higher number of bacteria, according to animal health and the compliance with the hygiene rules. Microorganisms in milk causes degradation of key components: lactose, protein and fat.

The quality and quantity content of microbiota from milk differs depending on the source of contamination and is conditioned mainly by the conditions of hygiene laid down in legislation and primary treatment applied to milk. The normal microbiota of milk contains: lactic bacteria, propionic bacteria and typical yeasts.

The thermal treatment like: thermization, low temperature for long time,high temperature for short time, ultra high temperature or sterilizationareclassical methods used in our days; they can produce distortion of milk proteins or could have adverse effects on appearance, taste and nutritional value of milk.

The high temperatures offers the advantage of rapid inactivation of microorganisms and enzymes, and short exposure time provides less undesirable changes in milk quality. The conventional antibacterial treatment applied to milk should not modify sensory and physico-chemical properties of milk.

The unconventional antibacterial treatments applied on milk are: sterilization by ionizing radiation, ultrasound treatment, electropasteurization, the used of microwave etc.

By irradiating milk, besides bactericidal effect occur a number of changes in milk components: the destruction of vitamins A, E, C, B12, PP, fat oxidation (rancid and bitter taste occurs), lactose and proteins undergo modifications.

The ultrasound treatment is a method of hygienisation the milk, which does not lead to complete destruction of microorganisms. If ultrasound intensity increases, increase the destruction of bacteria, but appear negative influences on milk components.

The advantage of electropasteurization is that this hygienisation system can treat milk after packaging. The main disadvantage is the high cost of treatment that greatly limiting its application on an industrial scale.

Microwaves are nonionizing radiation, with varying electromagnetic waves of radiant energy with frequencies ranging from 300 MHz to 300 GHz. Domestic ovens operate at 2.450 MHz.

In food heating operations, microwave heating offers several distinct advantages when are compared to conventional heating methods. The advantages include speed of heating, energy saving, precise process control, and faster start-up and shutdown times. Other advantages include higher quality product in terms of taste, texture, and nutritional content.

There are two main mechanisms by which microwaves product heat in dielectric materials: ionic polarization and dipole rotation. Ionic polarization occurs when ions in solution move in response to an electric field. Kinetic energy is given up to the ions by the electric field. These ions collide withother ions, converting kinetic energy into heat. When the electric field is rotating at $2.45 \times 10^9$ Hz, numerous collisions occur, generating a great deal of heat. However, thedipole rotation mechanism is more important. It is dependent on the existence of polar molecules. The most common polar material found in foods is water.Water molecules are randomly oriented under normal conditions. In the presence of an electric field, the polar molecules line up with the field. As mentioned above, the electric field of a microwave system alternates at $2.45 \times 10^9$ Hz, so that while the molecules try to align themselves with this changing field, heat is generated. When the field is removed, the molecules return to their random orientation.

The dielectric property is most important in the microwave heating of foods. The dielectric properties of foods at microwave frequencies are related to their chemical composition. They are also highly dependent on the frequency of the applied electric field, the moisture content, temperature, and bulk density (Decareau, 1985).

Sterilization refers to the complete destruction of microbial organisms. Commercially, sterility means that all pathogenic, toxic-producing organisms and spoilage organisms have been destroyed or reduced to safe levels. Microwave sterilization operates in the temperature range of 110–130°C. Pasteurization is a gentle heat treatment usually at temperatures between 60 and 82°C. Microwave sterilization/ pasteurization has been applied to several foods including fresh pasta, bread, granola, milk, and prepared meals. The main advantage of microwave sterilization or pasteurization is the effective reduction in the time required for the heat to penetrate to the food center (Tewari, 2007).

The microwave heating of food is directly influenced by food properties regardless of their nature. Thus, density, surface area its exterior shape, size, salt content and moisture, thermal and electrical conductivity, initial temperature, heat capacity and dielectric properties form a sum of factors that determine the rate of heating, heat transfer and penetration depth of microwaves (Albert et al., 2009).

The dielectric properties of foods and their dependence on temperature, frequency and composition have a great influence on the temperature distribution developed during microwave sterilization (Ohlson and Bengtsson, 1975).

Milk as colloidal material has electrical charges likely to move under the influence of electric

field of the microwaves. The heating generated by microwave can significantly reduce the time required for pasteurization and sterilization comparative with the classic methods. As an immediate result of this, the quality for pasteurized or sterilized food by microwave increase significantly (Constantin, 2011).
The milk pasteurization by continuous microwave proved to be an effective method to achieve a satisfactory product quality in microbial and sensorial terms, free from damage caused by thermal effect (Da-Wen, 2005).

## MATERIALS AND METHODS

In this paper we made preliminary tests of microwave treatment for milk samples, to relieve the effects of unconventional methods on milk microbiota.
For milk treatment, we realized an experiment with a microwave source, defined by power 1500 W and frequency 2.4 GHz. The mean value of irradiance was 250 $mW/cm^2$ and the mean intensity of electric field was 15 V/m. Both of these operational factors were invariable during the experiment.
The time of exposure was 15, 30, 60, 90 and 120 seconds for 1 to 5 variants (V1 to V5).
The fresh cow milk sample was from Ciupa farm, Arges County. The samples were tested for chemical properties with Lactosar device and the microbiological properties were established by inoculating on nutrient media. These two kinds of tests were realized both before and after microwave exposure.
The determined chemical properties were fats, proteins, lactose and dry matter. Also, we established the freezing point before microwave exposure and after that.
The microbial categories were: yeasts and moulds (YM), determined on Compact Dry YM medium; total number of viable bacterial cells (TVC) determined on Compact Dry TC medium, lactic acid bacteria (L), determined on Lafon Lafourcade medium.
The samples were diluted from $10^{-1}$ to $10^{-3}$ and 1 ml of each appropriate dilution was inoculated on the adequate medium in three repetitions. The inoculated media were incubated at 37°C for 24 h (L) and 48 h (TVC), at 30°C for three to five days for yeasts and moulds.
The number of colonies was counted with Funke Gerber colony counter and for each sample was established the number of colony forming units per ml (CFU/ml) in terms of yeast and moulds, total viable aerobic bacteria and lactic acid bacteria.

## RESULTS AND DISCUSSIONS

The results of chemical tests are presented in Table 1. The values before exposure were in normal range for fresh cow milk. After exposure, all the samples had almost the same values. No significant differences between the milk samples before and after microwave exposure were observed.

Table 1. The chemical properties of milk samples

| | Fats (%) | Proteins (%) | Lactose (%) | Dry matter (%) | Freezing point (°C) |
|---|---|---|---|---|---|
| Before exposure | 4,0 | 3,5 | 5,1 | 9,35 | -0,599 |
| After exposure | | | | | |
| V1 (15´) | 3,85 | 3,53 | 4,9 | 9,14 | -0,582 |
| V2 (30´) | 3,94 | 3,53 | 4,9 | 9,14 | -0,582 |
| V3 (60´) | 3,94 | 3,53 | 4,9 | 9,14 | -0,582 |
| V4 (90´) | 3,94 | 3,53 | 4,9 | 9,14 | -0,582 |
| V5 (120´) | 3,94 | 3,53 | 4,9 | 9,14 | -0,582 |

Some minor differences may be registered because of irregular blending of samples, but the microwave treatment does not decrease the amount of important substances from milk.
The results of microbiological tests for fresh milk sample are presented in next figures.
In Figure 1 are presented the number of CFU/ml for yeasts and moulds (YM), on the Compact dry YM medium. The untreated sample (UT) had 600 CFU/ml of milk and V1 (after microwave irradiation over 15 seconds) had 200 CFU/ml, whereas the other variants (V2 – V5) were lack of yeasts and moulds.
The total number of viable aerobic bacterial cells (TVC) for untreated sample (UT) and microwave irradiated samples (V1 - V5) are presented in Figure 2. The number of viable cells decreased from untreated sample and V1 to other variants. The decrease was significant for V2 variant (after microwave irradiation over 30 seconds), while V3 and V4 had similar values for total bacterial cells. The smallest

value was registered for V5 variant, just 150 CFU/ml.

The lactic acid bacteria were determined on Lafon Lafourcade medium and the number of viable cells (CFU/ml) decreased significant after 30 second of irradiation (Figure 3). The values for V3, V4 and V5 registered an easy decline.

In Figure 4 are presented bacterial colonies on Compact Dry TC medium, for the untreated sample.

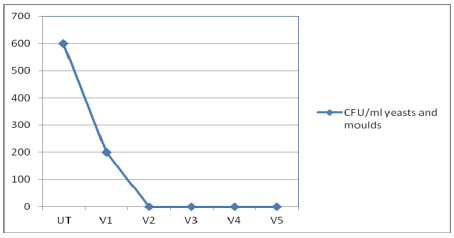


Figure 1. Yeasts and moulds in milk samples

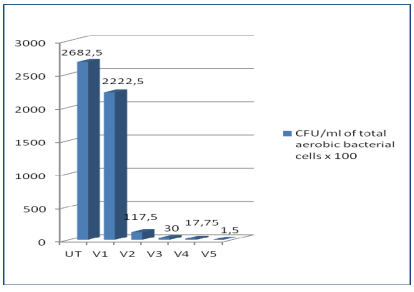


Figure 2. Total viable bacterial cells in milk samples

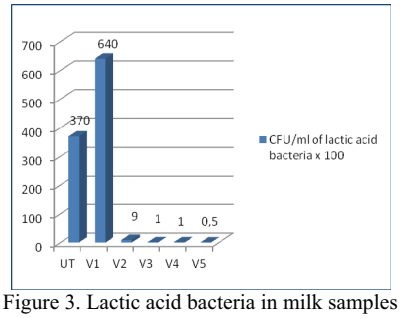


Figure 3. Lactic acid bacteria in milk samples

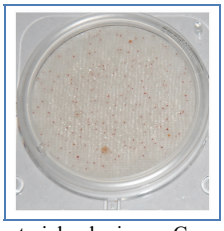

Figure 4. Bacterial colonies on Compact Dry TC

## CONCLUSIONS

The microwave irradiation may be use as unconventional method of milk sterilization.

This treatment does not produce significant alteration of chemical properties under 250 $mW/cm^2$ (irradiance) and 15 V/m (electric field intensity).

The effect of microwave irradiation on milk microbiota is obvious, in terms of decrease the number of yeasts, mould, total viable bacterial cells and lactic acid bacteria. The most significant differences were registered over 30 seconds of irradiation.For exposure time longer than 30 seconds, neither yeast nor mould cells were viable in the milk samples. The colony forming units for total bacteria decreased from $2682.5x10^2$ for untreated sample to $30x10^2$ or less for exposure time longer than 30 seconds. The decrease was registered for lactic acid bacteria too, in the same manner, for exposure time 30 seconds or more the colony forming units were 100-fold less than untreated sample.

The additional experiments with microwave irradiation are needed for the fresh milk at different time exposure, in order to obtain the sterile samples.

Considering these results, we intend to continue research with microwave irradiation for other liquid food (such grape juice).

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# PHYTOCHEMICALS, ANTIOXIDANT AND α-AMYLASE INHIBITORY ACTIVITIES OF *SMYRNIUM OLUSATRUM* L. LEAF, FLOWER AND FRUIT

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***Abstract***

*Alexanders (Smyrnium olusatrum) has long been regarded as a food and medicinal plant. The essential oil composition of S. olusatrum leaf, flower and fruit was characterized by high proportion of furanosesquiterpenes (51.66-69.35%). Furthermore, quantitative differences among plant parts were observed for the majority of oil components. Among all organs significant variability in fatty acids composition were also observed. Main constituents were found to be polyunsaturated fatty acids (37.74-49.36%).*
*The total phenolic, flavonoid and carotenoid contents varied significantly between plant parts. Flower extract exhibited the highest contents of total phenolic (48.97 mg GAE/g) and flavonoid (52.63 mg RE/g). The β-carotene and lycopene contents were in the range of 4.55-26.14 mg/100g, and 8.00-49.45/100g, respectively.*
*Methanolic extracts and essential oils of different organs were found to possess antioxidant activities, as determined by scavenging effect, chelating activity and β-carotene-linoleic acid model system. Extracts and essential oils showed a strong inhibitory activity against α-amylase. However, the level of biological activity varied according to extracts and organs.*

***Keywords:*** *Antioxidant activity, Type 2 diabetes, carotenoid, furanosesquiterpenes, polyunsaturated fatty acids, Polyphenols, Smyrnium olusatrum.*

## INTRODUCTION

The genus *Smyrnium* L. (Umbelliferae) consists of seven species widely distributed throughout the world (Mölleken et al., 1998; Mungan et al., 2011). The majority of *Smyrnium* species are known for their diuretic, depurative, aperients, carminative, restorative and stomachic properties (Mölleken et al., 1998; Khanahmadi et al., 2010). Young sprouts, plant stems and roots are consumed as vegetables, which impart a pleasant flavour similar to celery (Mölleken et al., 1998).

In Tunisia, the genus includes two species (*Smyrnium olusatrum* L. and *Smyrnium perfoliatum* (L.) Mill. ssp. *Rotundifolium* (D.C.) P.F.) which grow wild in different bioclimatic zones (Pottier-Alapetite, 1981).

*Smyrnium olusatrum* L., commonly known as Alexanders, is a stout, glabrous, celery-scented, biennial plant. Leaves are 2-3-pinnate, dark green and glossy. Flowers are yellow, shortly pedicellate and grouped in numerous umbels. Fruit is a schizocarp comprising two single-seeded mericarps, broadly ovoid and laterally compressed.

In traditional medicine, *Smyrnium olusatrum leaves are used as* antiscorbutic, fruits as stomachic and antiasthmatic, and roots are known for their aromatic, appetite stimulant, diuretic and laxative properties (Mölleken et al., 1998; Papaioannou et al., 2010).

Previous phytochemical studies on *Smyrnium olusatrum* root, stem, leaf and fruit have revealed the presence of a wide array of components such as sesquiterpene lactones, furanosesquiterpenes and phenolic acids (Mölleken et al., 1998; El-Gamal, 2001). However, there is no information regarding carotenoids, fatty acids and antioxidant activity of essential oils and extracts of different plant parts.

Therefore, the aim of this study was to determine the essential oil and fatty acids compositions, phenolic and carotenoid contents of *Smyrnium olusatrum* leaf, flower and fruit, and examine for the first time the antioxidant and the α-amylase inhibition activities of the

essential oils and methanol extracts of these plant organs.

## MATERIALS AND METHODS

### Plant material

*Smyrnium olusatrum* aerial parts were harvested from a random sample of 25-30 plants growing wild in Korbous Jebel Mountain (Cap Bon region of Tunisia, latitude 36° 47′ 18″ N; longitude 10° 35′ 14″ E, altitude 400m, rainfall 550mm/year). Leaves, flowers and immature fruits were air-dried at room temperature for two weeks.

### Essential oil extraction and GC-FID and GC-MS analyses

Three lots of 100 g of each organ type (leaves, flowers and fruits), finely ground in a mortar grinder mill, were separately hydrodistilled for 3 h using a Clevenger-type apparatus. The obtained essential oils were dried using anhydrous sodium sulphate and then stored at 4°C until analyses. The essential oil composition was determined by GC-FID and GC-MS analyses following the methods of Messaoud and Boussaid (2011). Essential oil components were identified by comparison of their retention indices determined with reference to a homologous series of $C_9$-$C_{24}$ n-alkanes and with those of authentic standards. Identification was confirmed by comparison of their mass spectra with those recorded in NIST08 and W8N08 libraries. Component relative percentages were obtained directly from GC-FID peak areas without correction factors.

### Lipid extraction, Fatty acid methyl esters (FAMEs) preparation and GC-FID analyses

Triplicate sub-samples of 10 g of each ground organ were separately extracted using the continuous Soxhlet extraction technique with petroleum ether for 3 h. Extracts were filtered and concentrated under reduced pressure and temperature.

FAMEs were prepared according to Lechevallier (1966). In a methylation tube, 0.2 ml of the concentrated extract of total lipid were saponified with 4 ml methanolic sodium hydroxide solution (0.5 M) for 15 min in a boiling water bath at 65 °C. As for transmethylation, the mixture was homogenized with 3 ml of $BF_3$ methanolic solution (14%), and the reaction was allowed to proceed for 5 min at 65 °C. Subsequently 10 ml of water were added to the mixture and FAMEs were extracted twice with 10 ml of petroleum ether.

FAMEs were analyzed by a gas chromatograph Agilent model 6980 series, equipped with HP-Innowax capillary column (30 m x 0.25 mm; 0.25 µm film thickness), FID (280 °C) and split/splitless injector (220 °C). Oven temperature was held at 150°C for 1 min, then heated to 200°C at a rate of 15°C/min, and from 200 to 250°C at 2°C/min, and held isothermally at 250°C for 10 min. Helium was the carrier gas at an initial flow rate of 1 ml/min. Split ratio was 20:1. The identification of FAMEs peaks was determined by a comparison of their relative retention times with those of FAME authentic standards. Quantification of fatty acid methyl esters, expressed as percentages, was obtained directly from the GC peak area integration.

### Polyphenols extraction

The air-dried leaf, flower and fruit were finely ground separately in a mortar grinder mill. Triplicate sub-samples of 1g of each ground organ were extracted with 20 ml of pure methanol for 24 h in a water bath shaker maintained at room temperature. The extracts were filtered using a 0.45-µm Millipore filter and stored in a brown bottle at 4°C prior to further analysis.

### Total phenolic and flavonoid contents

Total phenol contents were determined using the Folin-Ciocalteu method (Singleton and Rossi, 1965). Total phenolic contents of leaf, flower and fruit were expressed in terms of milligrams gallic acid equivalent per gram of dry weight (mg GAE/g DW). Analyses were carried out in triplicate.

Flavonoid contents of leaf, flower and fruit were estimated according to the aluminum chloride colorimetric method of Djeridane et al. (2006). Flavonoid contents were expressed as milligrams of rutin equivalent per gram of dry weight (mg RE/g DW).

### Carotenoid contents

A fine dried sample (150 mg) of each organ type (leaves, flowers and fruits) was vigorously shaken with 10 ml of acetone-hexane mixture (4:6) for 1 min and then filtered through Whatman filter paper. The absorbance of the obtained filtrate was measured at 453, 505, 645 and 663nm. β-carotene and lycopene contents were calculated according to the following equations: β-carotene (mg/100ml) = 0.216 x

A663 - 1.22 x A645 - 0.304xA505 + 0.452 xA453; lycopene (mg/100ml) = -0.0458 x A663 + 0.204 x A645 - 0.304 x A505 + 0.452 x A453, and further expressed in mg/100 g of dry weight (Nagata and Yamashita, 1992).

**Free radical-scavenging activity**

The DPPH radical scavenging activity was measured from the bleaching of purple colored methanol solution of DPPH radical according to Brand-Williams et al. (1995). Briefly, 50 µl of each methanol extract or essential oil (at different concentrations in methanol) were mixed with 1.95 ml of 60 µM DPPH radical solution and allowed to react in the dark for 30 min. The absorbance was determined at 517 nm. Radical-scavenging activity was estimated as RSA% = $[(A_0 - A_1)/A_0]$ x100, where $A_0$ is the absorbance of the control reaction and $A_1$ is the absorbance of the test sample. The antiradical activity was presented by $IC_{50}$ value, defined as the concentration of the antioxidant required to scavenge 50% of DPPH present in the test solution. Trolox was used as positive control.

**Ferrous ion chelating activity**

The ferrous ion chelating activity of different organ extracts and essential oils was measured according to Yan et al. (2006). 0.5 ml of different concentrations of extracts or essential oils in methanol were added to 0.5 ml of $FeSO_4$ solution (0.125 mM), and left for incubation at room temperature for 5 min. Then, the reaction was initiated by adding 0.5 ml of ferrozine (0.3125 mM). The mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was measured at 562 nm against a methanol blank. The ability to chelate ferrous ion was calculated using the following formula: Chelating effect (%) = [100 x ($A_C$ - $A_S/A_C$)]; where $A_C$ is the absorbance of the control and $A_S$ represents the absorbance of the tested sample. Results were expressed as $IC_{50}$ (efficient concentration corresponding to 50% ferrous iron chelating). EDTA was used as positive control.

**Inhibition of β-Carotene bleaching**

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (20 ml). 4 ml of this solution was mixed with 40 mg linoleic acid and 400 mg Tween 40. After the chloroform was removed at 40 °C under vacuum, 100 ml of oxygenated ultra-pure water was added, and then the emulsion was vigorously shaken. Aliquots (1.5 ml) of this emulsion were transferred into different test tubes containing different concentrations of organ extracts or essential oils in methanol (0.1 ml). As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm and the test emulsion was incubated in a water bath at 50 °C for 120 min, when the absorbance was measured again. In the negative control, the tested sample was substituted with ultra-pure water. β-carotene bleaching inhibition was calculated using the following equation: Inhibition (%) = $[(A_t-C_t)/(C_0-C_t)]$ x100; where $A_t$ and $C_t$ are the absorbance values measured for the test sample and control, respectively, after incubation for 120 min, and $C_0$ is the absorbance values for the control measured at zero time during the incubation. The results are expressed as $IC_{50}$ values, the concentration required to cause a 50% β-carotene bleaching inhibition. BHT was used as positive control.

**α-amylase inhibition assay**

Mathanolic extracts were concentrated to dryness. The resulted extract or essential oils were dissolved in DMSO [50% in sodium phosphate buffer (0.02 M, pH 6.9)] to give different concentrations.

α-amylase inhibition was tested by the agar disc diffusion method, according to Cha et al. (2009). Starch hydrolysis assay was performed on plates composed of 1% (w/v) starch dispersed in 1.5% agar. Sterile Whatman disc papers (6 mm) were individually placed on agar plates, and 10 µl of samples (porcine pancreatic α-amylase with or without extracts) were applied to the filter paper disc. After incubation at 37 °C for 72 h, starch plates were stained with iodine solution (5 mM $I_2$ in 3% KI) for 15 min at room temperature. Iodine was removed from the plates by washing with distilled water. Amylase activity was determined by measuring the zone diameter of hydrolysed areas around the wells. The percentage of inhibition of α-amylase was calculated using the following formula: Amylase inhibition (%) = 100 x (d0 - d1)/d0; where d0 is the diameter of the negative control, and d1 is the diameter of the tested sample.

**Statistical analysis**

For each organ type, three samples were analyzed and all assays were carried out in triplicate. Results were presented as means ± standard deviation (SD). For each measured data, quantitative differences between organs was assessed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test using the SAS v. 9.1.3 program. The level of statistical significance was set at $p \leq 0.05$.

## RESULTS AND DISCUSSIONS

**Essential oil composition**

Essential oil compositions of *S. olusatrum* leaf, flower and fruit were summarized in Table 1. Twenty three components, representing 94.51%, 95.66% and 96.32% of the total essential oil, were identified in leaves, flowers and fruits, respectively.

Significant variations in the oil composition were observed between *S. olusatrum* plant parts. Furanosesquiterpenes (65.41%) and sesquiterpene hydrocarbons (19.65%) formed the predominant fractions of leaf essential oil. The main compound was curzerene (30.98%), followed by furanoeremophil-1-one (28.61%) and germacrene D (9.02%). Flower oil showed the highest percentage of myrcene (8.23%), curzerene (42.23%), furanodiene (6.81%), germacrone (12.78%), heneicosane (1.97%) and tricosane (3.74%), and was found to be rich in furanosesquiterpenes (51.66%), monoterpene hydrocarbons (21.43%), oxygenated sesquiterpene (12.78%) and sesquiterpene hydrocarbons (9.84%).

Table 1. Chemical composition (%) of essential oils of *Smyrnium olusatrum* plant parts

| **Compounds** | **RI** | **Leaf** | **Flower** | **Fruit** |
|---|---|---|---|---|
| α-pinene | 938 | 0.31 ± 0.02$^{c}$ | 1.41 ± 0.17$^{b}$ | 1.76 ± 0.12$^{a}$ |
| β-Pinene | 978 | tr$^{b}$ | tr$^{b}$ | 0.46 ± 0.2$^{a}$ |
| Myrcene | 991 | 1.02 ± 0.11$^{c}$ | 8.23 ± 0.73$^{a}$ | 2.51 ± 0.32$^{b}$ |
| β-Phellandrene | 1010 | 4.07 ± 0.28$^{c}$ | 6.08 ± 0.92$^{b}$ | **9.89 ± 0.87$^{a}$** |
| Cis-β-Ocimene | 1040 | 0.61 ± 0.10$^{a}$ | tr$^{b}$ | tr$^{b}$ |
| Citronellyl acetate | 1356 | 0.29 ± 0.06 | tr | tr |
| β-Elemene | 1387 | 1.55 ± 0.11 | 1.4 ± 0.22 | 1.65 ± 0.28 |
| β-Caryophyllene | 1418 | 2.05 ± 0.32$^{a}$ | tr$^{b}$ | tr$^{b}$ |
| γ-Elemene | 1429 | 0.48 ± 0.14$^{a}$ | tr$^{b}$ | 0.42 ± 0.11$^{a}$ |
| α-Humulene | 1445 | 0.34 ± 0.09$^{a}$ | tr$^{b}$ | tr$^{b}$ |
| Germacrene D | 1474 | **9.04 ± 0.74$^{a}$** | 2.97 ± 0.13$^{c}$ | 3.78 ± 0.21$^{b}$ |
| **Curzerene** | **1483** | **30.98 ± 1.58$^{c}$** | **42.23 ± 1.23$^{a}$** | **36.97 ± 1.75$^{b}$** |
| â-Bisabolene | 1507 | 2.76 ± 0.22$^{a}$ | 1.89 ± 0.14$^{b}$ | 0.85 ± 0.11$^{c}$ |
| δ-Cadinene | 1516 | 0.38 ± 0.08$^{a}$ | tr$^{b}$ | tr$^{b}$ |
| Germacrene B | 1546 | 3.09 ± 0.21 | 3.58 ± 0.33 | 2.88 ± 0.17 |
| Furanodiene | 1678 | 5.49 ± 0.32$^{b}$ | 6.81 ± 0.27$^{a}$ | 5.62 ± 0.72$^{b}$ |
| Germacrone | 1682 | 2.84 ± 0.36$^{b}$ | **12.78 ± 0.82$^{a}$** | 2.76 ± 0.41$^{b}$ |
| Furanoeremophil-1-one | 1735 | **28.61 ± 1.97$^{a}$** | 2.62 ± 0.47$^{b}$ | 2.37 ± 0.29$^{b}$ |
| Alexandrofuran | 1739 | tr$^{b}$ | tr$^{b}$ | 0.57 ± 0.12$^{a}$ |
| 1-β-acetoxy-furanoeudesm-4 (15)-ene | 1761 | 0.32 ± 0.11$^{b}$ | tr$^{b}$ | **23.71 ± 0.96$^{a}$** |
| 1-β-acetoxyfuranoeudesm-3-ene | 1763 | tr | tr | 0.11 ± 0.02 |
| Heneicosane | 2101 | tr$^{b}$ | 1.97 ± 0.13$^{a}$ | tr$^{b}$ |
| Tricosane | 2302 | 0.35 ± 0.14$^{b}$ | 3.74 ± 0.24$^{a}$ | tr$^{c}$ |
| Total identified (%) | | 94.51 ± 1.27 | 95.66 ± 1.84 | 96.32 ± 1.53 |
| Monoterpene hydrocarbons (%) | | 6.36 ± 0.88$^{c}$ | 21.43 ± 1.85$^{a}$ | 14.62 ± 0.72$^{b}$ |
| Oxygenated monoterpenes (%) | | 0.29 ± 0.06 | tr | tr |
| Sesquiterpene hydrocarbons (%) | | 19.65 ± 0.93$^{a}$ | 9.84 ± 0.56$^{b}$ | 9.58 ± 0.62$^{b}$ |
| Oxygenated sesquiterpenes (%) | | 2.84 ± 0.36$^{b}$ | 12.78 ± 0.82$^{a}$ | 2.76 ± 0.41$^{b}$ |
| Furanosesquiterpenes (%) | | 65.41 ± 2.07$^{b}$ | 51.66 ± 1.88$^{c}$ | 69.35 ± 2.32$^{a}$ |

Values are given as mean ± SD; RI: retention indices relative to n-alkanes on a HP-5MS column;
tr: trace (< 0.1%).Values in each row followed by different letter are significantly different ($P < 0.05$).

A large proportion of the fruit essential oil was composed of furanosesquiterpenes (69.35%), monoterpene hydrocarbons (14.62%) and sesquiterpene hydrocarbons (9.58%). Curzerene

(36.97%), 1-β-acetoxy-furanoeudesm-4(15)-ene (23.71%) and β-phellandrene (9.89%) were detected to be the major components in the oil from fruits.

The variation of essential oil composition between *S. olusatrum* organs have been reported (Mölleken et al., 1998; Bertoli et al., 2004; Papaioannou et al., 2010). However, independently of the chemical polymorphism among organs, the essential oil composition of the species presented in our study differed from these earlier reports. This finding indicates that the essential oil composition of *S. olusatrum* is influenced by environmental and/or genetic factors.

**Fatty acid composition**

Thirteen fatty acid components, representing 91.13 to 92.16% of the total oil according to organ type, were identified (Table 2). All plant parts were found to be rich in palmitic acid (12.62-13.83), palmitoleic acid (3.87-12.19%), oleic acid (5.19-18.35%), linoleic acid (15.57-23.81%) and α-linolenic acid (22.17-26.22%). The high contents of unsaturated fatty acids, mainly linoleic and linolenic acids, in different organs could make *S. olusatrum* leaf, flower and fruit lipids an important source for a variety of healthy applications. Polyunsaturated are essential in the human diet since they cannot be synthesized by the body, and they are very important to human immumne system, to help regulate blood pressure and to alleviate cardiovascular, inflammatory, heart diseases, atherosclerosis, diabetes and other diseases (Simopoulos, 1991;Tapiero et al., 2002; Richard et al., 2009).

Significant quantitative variations in the fatty acid composition between *S. olusatrum* organs were disclosed (Table 2). Leaves contained the highest percentage of saturated fatty acids (36.12%) and the lowest amount of monounsaturated and polyunsaturated ones (17.61 and 37.74%, respectively). The highest contents of myristic acid (3.54%), palmitoleic acid (12.19%), stearic acid (7.84%), arachidic acid (2.58%), behenic acid (2.44%) and lignoceric acid (4.47%) were determined in leaves. Conversely, flowers and fruits were found to be significantly rich in oleic acid (15.81-18.35%), linoleic acid (23.14-23.81%) and α-linolenic acid (24.22-26.22%).

As far as our literature survey could as certain, there is only one report regarding the fatty acid composition of *S. olusatrum* leaf growing wild in Southern Italy (Conforti et al., 2011). This study reveals lower amounts of linoleic and linolenic acids than that observed in our work. The composition of fatty acids depends on genetic factors and geographical origin as well as on the used methodology for isolation and extraction (Primomo et al., 2002; Boschin et al., 2007).

Table 2. Fatty acid composition (%) of different *Smyrnium olusatrum* plant parts

| **Fatty acid** | **Leaf** | **Flower** | **Fruit** |
|---|---|---|---|
| Myristic acid (C14:0) | 3.54 ± 0.31$^{a}$ | 0.68 ± 0.15$^{b}$ | 0.56 ± 0.21$^{b}$ |
| Palmitic acid (C16:0) | 13.35 ± 0.98 | 13.83 ± 1.45 | 12.62 ± 1.29 |
| Palmitoleic acid (C16:1) | 12.19 ± 1.12$^{a}$ | 3.87 ± 0.22$^{c}$ | 7.12 ± 0.15$^{b}$ |
| Stearic acid (C18:0) | 7.84 ± 0.83$^{a}$ | 3.93 ± 0.25$^{b}$ | 3.52 ± 0.51$^{b}$ |
| Oleic acid (C18:1) | 5.19 ± 1.02$^{c}$ | 18.35 ± 0.93$^{a}$ | 15.81 ± 1.77$^{b}$ |
| Linoleic acid (C18:2) | 15.57 ± 1.53$^{b}$ | 23.81 ± 0.66$^{a}$ | 23.14 ± 1.03$^{a}$ |
| α-Linolenic acid (C18:3) | 22.17 ± 1.64$^{b}$ | 24.22 ± 1.35$^{ab}$ | 26.22 ± 1.88$^{a}$ |
| Arachidic acid (C20:0) | 2.58 ± 0.22$^{a}$ | 0.92 ± 0.15$^{b}$ | 0.68 ± 0.21$^{b}$ |
| Eicosenoic acid (C20:1) | 0.22 ± 0.11 | 0.15 ± 0.06 | 0.17 ± 0.04 |
| Behenic acid (C22:0) | 2.44 ± 0.34$^{a}$ | 0.82 ± 0.13$^{b}$ | 0.97 ± 0.19$^{b}$ |
| Tricosanoic acid (C23:0) | 0.14 ± 0.06 | 0.15 ± 0.03 | 0.17 ± 0.08 |
| Lignoceric acid (C24:0) | 4.47 ± 0.77$^{a}$ | 0.26 ± 0.10$^{b}$ | 0.28 ± 0.13$^{b}$ |
| Cerotic acid (C26:0) | 1.76 ± 0.23 | 1.18 ± 0.42 | 1.21 ± 0.36 |
| Total identified (%) | 91.46 ± 2.41 | 92.16 ± 2.17 | 91.13 ± 1.93 |
| Saturated Fatty Acids | 36.12 ± 1.82$^{a}$ | 21.77 ± 1.47$^{b}$ | 20.01 ± 1.71$^{b}$ |
| Monounsaturated fatty acids | 17.61 ± 1.61$^{b}$ | 22.37 ± 1.88$^{a}$ | 23.1 ± 1.01$^{a}$ |
| Polyunsaturated fatty acids | 37.74 ± 1.14$^{b}$ | 48.03 ± 1.79$^{a}$ | 49.36 ± 1.26$^{a}$ |

Values are given as mean ± SD. Values in each row followed by different letter are significantly different ($P < 0.05$).

**Phenolic and carotenoid contents**

There are considerable variations among phenolic and carotenoid contents for the three analyzed organs (Table 3). The total phenolic and flavonoid contents ranged from 31.48 to 48.97 mg GAE/g and 7.46 to 52.63 mg RE/g, respectively. Flowers contained the highest contents of total phenolic and flavonoids, followed by leaves and fruits.

A previous study on *S. olusatrum* leaf hydroalcoholic extract (Conforti et al., 2011) reported a different total phenolic content (70 mg chlorogenic acid equivalents per gram of extract) than that observed in our present study. However, there is no information regarding flowers and fruits.

To the best of our knowledge no data exist regarding the carotenoid contents in *Smyrnium olusatrum*. In our study, β-carotene and lycopene contents were determined by spectrophotometric methods. Significant differences in carotenoid concentration were also found among plant parts, and leaves exhibited the highest concentrations of β-carotene (26.14 mg/100 g DW) and lycopene (49.45 mg/100 g DW), followed by fruits and flowers (Table 3).

Phenolic and carotenoid compounds are secondary plant metabolites. The quantitative and qualitative variations of these phytochemical classes between plant parts have been largely determined. These natural products are marker of the nutritional quality of foods. Polyphenols and carotenoids are known for their antioxidant activity and possible beneficial roles in human health, protecting against oxidative damage to cells, stimulating immune function, reducing the risk of cancer and cardiovascular disease (Selappan et al., 2002; Barros et al., 2011).

Table 3. Total phenolic, flavonoid, ß-carotene and lycopene contents of *Smyrnium olusatrum* plant parts.

| Organs | Total phenols | Flavonoid | ß-carotene | Lycopene |
|---|---|---|---|---|
| **Leaf** | 33.22±1.19$^{b}$ | 22.36±1.13$^{b}$ | 26.14±1.92$^{a}$ | 49.45±2.03$^{a}$ |
| **Flower** | 48.97±1.92$^{a}$ | 52.63±1.09$^{a}$ | 4.55±0.68$^{c}$ | 8.00±0.97$^{c}$ |
| **Fruit** | 31.48±1.73$^{b}$ | 7.46±0.87$^{c}$ | 6.24±0.37$^{b}$ | 10.19±0.78$^{b}$ |

Values are given as mean ± SD. Total phenolic was expressed as mg gallic acid equivalent in 1 g of dry sample. Flavonoid was expressed as mg rutin equivalent in 1 g of dry sample. ß-carotene was expressed as mg in 100 g of dry sample. Lycopene was expressed as mg in 100 g of dry sample. Values in each column followed by different letter are significantly different ($P < 0.05$).

**Antioxidant activity**

The antioxidant activities of *S. olusatrum* extracts have never been measured. Therefore, three different in vitro assays were used for the evaluation of the antioxidant properties of methanolic extracts and essential oils of leaves, flowers and fruits. The results of scavenging activity on DPPH radicals, inhibition of β-carotene bleaching, and chelating ability are shown in Table 4. Methanolic extracts of different *S. olusatrum* parts gave statistically similar DPPH scavenging activity ($IC_{50}$ = 0.126 mg/ml for leaf, $IC_{50}$ = 0.092 mg/ml for flower, 0.138 mg/ml for fruit), while Trolox was a considerably more effective DPPH radical scavenger ($IC_{50}$ = 0.076 mg/ml).

*S. olusatrum* flower extract exhibited the highest β-carotene bleaching inhibition ($IC_{50}$= 0.105 mg/ml) and chelating ability ($IC_{50}$ = 2.84 mg/ml), than leaf and fruit methanolic extracts. However, all extracts exhibited low antioxidant activity when compared to that reported for standards BHT and EDTA ($IC_{50}$ = 0.029 and $IC_{50}$ = 0.019, respectively). The greatest antioxidant activity of flowers is in agreement with their higher phenolic and flavonoid contents in comparison to the other plant parts.

The essential oils were found to be less active than methanolic extracts. Although all oils showed no metal chelating activity, the free radical scavenging capacity, based on $IC_{50}$ values, ranged from 40.68 mg/ml (flower) to 48.32 mg/ml (leaf). Essential oils of different plant parts ($IC_{50}$ = 26.35 mg/ml for leaf, $IC_{50}$ = 20.82 mg/ml for flower, and $IC_{50}$ = 23.74 mg/ml for fruit) are also able to inhibit β-carotene bleaching. From the results, we can infer that the antioxidant effect was associated to furanosesquiterpenes which formed the predominant fractions of all essential oils.

Recent interest in these substances has been stimulated the potential health benefits arising from the antioxidant, antinociceptive, hypothermic, anti-inflammatory and antifungal activities of these furanosesquiterpenoids compounds (Amorim et al., 2009; Fraternale et al., 2011).

The antioxidant activity of essential oils cannot be attributed to the major compounds as minor compounds are likely to play a significant role in the activity, and synergistic effects being also reported (Kelen and Tepe, 2008). Phenols, but also many terpenes, notably monoterpenes, are known to exhibit antioxidant properties (Misharina et al., 2009). Accordingly, the highest antioxidant abilities of *S. olusatrum* flower essential oil were probably related to their richness in hydrocarbon monoterpenes and/or to the synergistic effect of more than one individual oil compound.

**α-Amylase inhibition**

Medicinal plants continue to play an important role in the treatment of diabetes. The inhibition of α-amylase activity, is considered to be an effective strategy for the control of diabetes by diminishing the absorption of glucose (Hara and Honda, 1990). α-amylase inhibitors have been one of the research hotspots as oral hypoglycemic agents for diabetic. As shown in Figure 1, degradation of starch by pancreatic α-amylase was inhibited by methanolic extracts and essential oils of all *S. olusatrum* organs (table 5). However, percentage inhibition varied according to type of extract (methanolic extract and essential oil) and plant parts. Flower methanolic extract (250µg/ml) displayed the highest inhibition (48.78%). The percentage inhibition of essential oil (2000µg/ml) varied between 13.41% (fruits) and 31.08% (flowers).

Table 4. Antioxidant activities of the essential oils and the methanolic extracts of *Smyrnium olusatrum* plant parts.

| | **DPPH ($IC_{50}$, mg/ml)** | **â-carotene bleaching inhibition ($IC_{50}$, mg/ml)** | **Chelating ability ($IC_{50}$, mg/ml)** |
|---|---|---|---|
| *Methanolic extract* | | | |
| Leaf | 0.126 ± 0.022$^{c}$ | 0.193 ± 0.009$^{d}$ | 4.36 ± 0.55$^{a}$ |
| Flower | 0.092 ± 0.012$^{c}$ | 0.105 ± 0.011$^{e}$ | 2.84 ± 0.37$^{c}$ |
| Fruit | 0.138 ± 0.016$^{c}$ | 0.291 ± 0.018$^{c}$ | 5.62 ± 0.21$^{b}$ |
| *Essential oil* | | | |
| Leaf | 48.32 ± 2.62$^{a}$ | 26.35 ± 2.41$^{a}$ | - |
| Flower | 40.68 ± 1.36$^{b}$ | 20.82 ± 1.75$^{b}$ | - |
| Fruit | 47.97 ± 2.44$^{a}$ | 23.74 ± 1.88$^{ab}$ | - |
| *Synthetic antioxidant* | | | |
| Trolox | 0.076 ±0.004$^{d}$ | | |
| BHT | | 0.029 ± 0.002$^{f}$ | |
| EDTA | | | 0.019 ±0.008$^{d}$ |

Values are given as mean ± SD. Values in each column followed by different letter are significantly different ($P < 0.05$).

Table 5.α-amylase inhibition essay of methanolic extract and essential oil of *Smyrnium olusatrum* organs

| | **Diameter of hydrolysed areas (mm)** | |
|---|---|---|
| Organ | Methanolic extract (250µg/ml) | Essential oil (2000µg/ml) |
| Leaves | 17.42 ± 0.83 (27.16% ± 1.22) * | 16.43 ± 0.51 (31.38% ± 0.88) |
| Flowers | 12.25 ± 0.75 (48.78% ± 1.97) | 14.82 ± 0.46 (38.08% ± 1.02) |
| Fruits | 18.10 ± 0.44 (24.32% ± 0.91) | 20.71 ± 0.38 (13.41% ± 0.73) |

Zone diameter of hydrolysed areas for the Control is 23.91±0.4 (using 1U/ml of α-amylase) and 16.24 ± 0.34 (using 0.75U/ml of α-amylase) *: Percentage of inhibition is in parenthesis

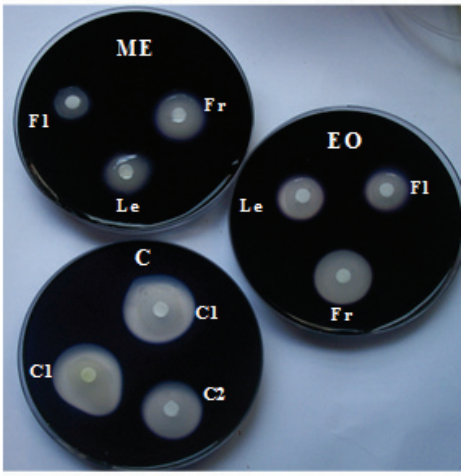


Figure 1. The inhibition of α-amylase by methanolic extracts and essential oils of *S. olusatrum* organs as detected by the agar diffusion method. C: negative control (C1:1U/ml; C2:0.75U/ml), ME: methanolic extract (Fl: flowers, Le: leaves, Fe: fruits), EO: essential oil (Fl: flowers, Le: leaves, Fe: fruits).

## CONCLUSIONS

Natural products, especially those produced by medicinal plant species, are currently under special interest due to their safety, usefulness and accessibility. This study, on *Smyrnium olusatrum* plant parts, reports appreciable amounts of several interesting phytochemicals. According to the results of this study, the essential oil and the methanolic extracts of *S. olusatrum* may be suggested as a new potential source of bioactive compounds.

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**CHARACTERIZATION AND DEVELOPMENT OF CHROMATOGRAFIC METHOD FOR SIMULTANEOUS DETERMINATION OF ARTIFICIAL SWEETENERS IN SOFT DRINK SAMPLES**

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# DECONTAMINATING MYCOTOXINS IN AGRICULTURAL PRODUCE FOLLOWING BIOREMEDIATION APPROACH

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***Abstract***

*Mycotoxins are secondary metabolites produced by certain species of fungi on grains in the field or after harvest and during transport and storage, etc. These are becoming ubiquitous pollutants in agricultural products, and represent a potential threat to human as well as animal health. Laws enacted to control the presence of mycotoxins in food and feed are increasing. Although some physical and chemical methods of detoxification are reported, bioremediation is going to be method of choice due to its environment friendly nature and low cost involved. Bioremediation is the elimination or biotransformation of mycotoxins into non-toxic metabolites by microbes such as bacteria or fungi. The genes involved in the degradation of mycotoxins by microbial activity have been reported to be cloned, and microbial trials for the treatment of contamination of agricultural products are in progress. This paper briefly describes the toxicity of mycotoxins i.e. deoxynivalenol (DON) and aflatoxin. In addition, the escalation of microbes such as bacteria and fungi, capable of detoxifying these toxins in mixed cultures or pure culture is described. However, the results obtained so far can only be used as a first step in the development of technologies and business practices, as the experiments were performed on a laboratory scale only so far. Finally, future challenges and innovative strategies for decontamination of mycotoxins by microorganisms are elaborated.*

***Keywords:*** *Bioremediation, Cereal Grains, Mycotoxins, Food Safety, Public Health.*

## INTRODUCTION

Agricultural produce are potential host for the contamination of fungi in field as well as during storage, especially in the climatic conditions of Pakistan. Some of these fungi produce secondary metabolites, mycotoxins, which are potential threat to food and feed safety. Mycotoxins may be carcinogenic, mutagenic or may cause interference to hormonal functions in the body of consumer. In advance countries with following industrialized farming systems and sophisticated food processing technologies, mycotoxins may not be a serious threat, however, in developing countries like Paksitan, mycotxin residues in agricultural produce pose potential risk and may be a cause of chronic illnesses. For example, Ochratoxin may be a cause of renal cancer, deoxynivalenol (DON) can cause human IgA nephropathy, while zeralenon may cause oestrogen in human beings (Creppy et al., 1998; Rotter et al., 1996; Price and Fenwick, 1985). Similarly, presence of these mycotoxins in animal feed may present serious problems in live stock production, their meat and milk etc. Public awareness about mycotoxins contamination is increasing day by day due to number of reasons. Firstly, world has been global village and people have access to latest information about health and environment issues. Secondly, number of sophisticated techniques for the analysis of mycotoxins have been developed, and general public is conscious about the safety of the food they are going to consume. As, prevention of the contamination of mycotoxins is not practicable, the scientist are putting much more concentration over their decontamination technologies. Though, number of physical and chemical methods have been reported for the detoxification of myctoxins in agricultural produce, biological methods are of first choice due to their cost effectiveness and environment friendly behavior (Bhatnagar et al., 1991; Park, 1993). Current study deals with latest development for microbial decontamination of mycotoxins in agricultural produce.

**PREVENTION STRATEGIES AGAINST MYCTOTOXIN CONTAMINATION.** The best pro-active approach to avoid mycotoxins contamination in agricultural produce is the adoption of good agricultural practices (GAPs), which may serve as primary defense line, followed by the good manufacturing practices (GMPs) during handling, transportation and storage etc. The further line of defense may be the practice of Hazard Analysis and Critical Control Point (HACCP) during processing and production systems. However, adoption of these strategies mainly depends upon the local environmental conditions, culture and cropping systems.This is the reason that much attention is paid by the researcher on the solution of problem by developing novel decontaminating technologies, so that the potential health risk associated with the presence of toxin could be minimized. These strategies mainly focus on the: • Inactivation of mycotoxins or their transformation in to non-toxic products • Destruction of fungal spores and mycelia, so that re-production of toxins could be avoided • Sustainability of nutritional value of foods after the application of decontamination techniques • The process should be economical, and easy to applicable

**MICROBIAL DETOXIFICATION OF MYCOTOXINS.** To meet the above mentioned criteria, researchers have paid much attention on discovery of microbes having potential to biodegrade the mycotoxins. Microorganisms are being screened from different niches capable of transforming the mycotoxins in less toxic compounds (Schatzmayr et al., 2006). This approach called bioremediation is attracting much attention due to its good results and environment friendly, as well as low cost attitude. Some microbes have already been investigated, such as *Flavobacterium aurantiacum* is capable of detoxifying aflatoxins (Ciegler et al., 1996), *Phenylobacterium sp*. capable of degrading ochratoxin (Wegst and Lingens, 1983), and *Gliocladium roseum* capable of detoxifying zearalenone (El-Sharkawy and Abul-Hajj, 1988) has been reported already. We will discuss two major examples in details in the following discussion.

**DETOXIFICATION OF DEOXYNIVALENOL (DON).** DON is a chemically stable mycotoxin, and most commonly produces on the cereal crops. A large number of strains (1285) were isolated and screened for their DON degrading capability by Volkl et al., (2004). A mix culture was found capable of transforming DON into 3-Keto-DON, and five other unknown metabolites which showed les toxicity than DON. Shima et al. (1997) also reported a microbial strain, *Agrobacterium rhizobium* E3-39, capable of transforming DON into 3-Keto-DON under aerobic conditions. The enzymes responsible for degradation were found in the cell cultures, as well as in cell free filtrate, while absent in cell extract, showing that these were extra-cellular enzymes. Six agricultural soil based strains were reported by Zhou (2008) capable of transforming more than 87% DON from culture media, while two of them were capable of complete removal of DON from culture media.Studies have shown the effectiveness of rumen cultures on the biotransformation of DON. Microbial culture from rumen fluid of dairy cow transformed DON into de-epoxy DON (Yoshizawa et al., 1983; He et al., 1992). Eubacterium strain BBSH 797 was isolated from a rumen fluid, and is most extensively studied DON transforming strain, which also formed a base for a commercial feed additive available in market (Binder et al., 2000b; Shcatzmayr et al., 2006). Microflora from chicken intestines has also shown considerable DON degradation activities (He et al., 1992). However, considerable variations have been observed depending upon the breed of chicken, individuals and intestinal regions. Though several authors reported the DON degradation activity of ruminal and chicken intestinal microflora, however Binder et al., (2000a) first time reported a pure bacterial strain, capable of degrading DON. Similarly, Awad et al., (2004, 2006), reported a *Eubacterium sp*. DSM 11798 capable of compensating adverse effects of DON in poultry. However, rumen and intestinal microflora are strictly anaerobic in their functions. So, research on aerobic microbes is still in progress.

**DETOXIFICATION OF AFLATOXIN (AFB).** Aflatoxins are mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin B1 (AFB1) is the most toxic, mutagenic and carcinogenic, posing most serious threats to animal and human health causing

huge economic losses worldwide. Now a day, much work is being carried out on microbial detoxification of AFB1. Many fungal and yeast species has been reported capable of detoxifying AFB1, such as *Pleurotus ostreatus* (Motmura et al., 2003), *Trametes versicolor* (Zjalic et al., 2006), *Rhizopus sp*., *Mucor sp*. (Varga et al., 2005), Saccharaomyces cerevisiae (Shetty and Jespersen, 2006) and *Trichoderma sp*. (Shantha, 1999) etc. Cell extracts of these fungi were capable of detoxifying AFB1, however there were number of limitations found in their practical applications i.e. long incubation time (more than 120 h) and complicated procedures of obtaining their cell extracts etc. Some bacterial species have also been reported as AFB1 decontaminating agents, such as *Lactobacillus sp*. (El-Nezami et al., 1998), *Bifidobacterium* (Peltonen et al., 2001), *Propionibacterium* (El-Nezami et al., 2000) and *Lactococcus* (Pierides et al., 2000) etc. However, it should be noted that decontamination activity was mainly due to binding of toxins with bacterial cells rather than degradation in to non-toxic metabolites. However, bacterial species like *Rhodococcus erythropolis* (Alberts et al., 2006), *Mycobacterium fluoroanthenivorans* (Hormisch et al., 2004) and *Nocardia corynebacterioides* (D′Souza et al., 1998) etc are found involved in biodegradation activity of AFB1.

## CONCLUSIONS

Elimination of mycotoxins from foods and feed still a problem worldwide, and researchers are seeking technologies which could be comercially viable and easy to apply. Microbes can be used as a absorbing agents to decrease the contamination level, as well as biodegrading agents to inactivate the mycotoxins. The main issue to understand in this matter is the mechanism of action of microbes, whether microbes actually degrade the toxin or the toxin is disappeared due to adsorption by microbes? The absorbance just decreases the bio-availability of toxins, while degradation transforms the toxins into non-toxic products. In this regard, first the resistant strains should be screened out, and then study of their mechanism of resistance to mycotoxins may be helpful to understand the biodegradation activity.

Isolation and characterization of enzymes responsible for degradation activity is still under way, and may be a technology of choice, as enzymes offer specific, irreversible, efficient and environment friendly way for the detoxification of mycotoxins. Finally, the development of a technology, which is economically feasible, easy to applicable and friendly to environment is a need of time to decontaminate mycotoxins from agricultural produce, and certainly the microbes are potential candidate for that.

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# EFFECT OF EXTRACT OF *GINKGO BILOBA* ON VEGETABLE OILS

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***Abstract***

*In this paper, we studied the effect of active components of Ginkgo Biloba extract on vegetable oils. We used a commercial product of Ginkgo Biloba extract and an alcoholic extract of Ginkgo Biloba obtained from the commercial product. Influence of active compounds from Ginkgo Biloba, both in commercial and product of alcoholic extract was determined by peroxide and TBA index of vegetable oil. The extract added has no negative effects on the oil and it's good to know that the extract of Ginkgo Biloba in ethanol can be used as an antioxidant to prolong stability of oils. The results obtained indicate that oxidative processes have been slowed down both, for the commercial product and in the case of alcoholic extract. For a better demonstration of this data has been used, the notion of protection factor. After determination of the peroxide the best values were obtained for the samples with added Ginkgo Bioba extract in ethanol with a concentration of Ginkgo Biloba by 4 mg/mL, its oxidative stability increasing with 60% in relation to initial stability of oil without added.*

***Keywords:*** *Ginkgo Biloba extract, antioxidants, vegetable oils, peroxide value.*

## INTRODUCTION

Ginkgo biloba (Ginkgoaceae) is probably the oldest species of tree known, dating back to 300 million years and it is often called the "living fossil". The female trees produce a fruit with an orange or yellow flesh surrounding a hard, tan shell containing the kernel of the seed, which is edible (Máriássyová, M., 2006). Antioxidants are found in various plant products such as fruits, vegetable, cereals, spices, teas and oils, which contain flavonoids, tannins, phenols, terpenoids and many others (Rhee, D.-Myers, J,2001; Máriássyová, M., 2006). In recent years, Ginkgo biloba is coming to the attention. Especially the leaves of Ginkgo biloba contain compounds possessing an antioxidant character.Antioxidant effects. The underlying principle behind the therapeutic action of the Ginkgo leaf extract on chronic ailments (such as neurodegenerative diseases, cardiovascular diseases and cancer) has focused on its antioxidant properties. The 2 proposed mechanisms of action are (1) directly scavenging free radicals and (2) indirectly inhibiting formation of free radicals. The Ginkgo leaf extract can scavenge reactive oxygen species (ROS) such as hydroxyl radicals ($OH^{\cdot}$), peroxyl radical ($ROO^{\cdot}$), superoxide anion radical ($O^{2-}$), nitric oxide radical ($NO^{\cdot}$), hydrogen peroxide ($H_2O_2$), and ferryl ion species (Mahady GB. 2002; DeFeudis FV, Papadopoulos V, Drieu K. 2003). Several studies assume its neuroprotective properties. Ginkgo biloba can enhance concentration, improve memory, but there was no effect observed on short memory (Byeoung-Soo, P.-Sung-Eun, L., 2000). There have been no studies aimed at the application of Ginkgo biloba extract as a potential natural antioxidant used in food industry.There have been isolated three main compounds from Ginkgo biloba with an antioxidant activity– kaempferol, quercetin and isoharmnetin (Spence, K. E.-Jane, J.4, 1999). The antioxidant activity of a ginkgo extract is determined mainly by flavonoids, which scavenge and destroy free radicals and the reactive forms of oxygen (Ellain Wojtaszek, M.-Krucynski, Z.-Kasprzak, J., 2002). The activated oxygen forms such as peroxide, hydrogen peroxide, hydroxyl radical and singlet oxygen may cause various diseases such as carcinogenesis, inflammation, atherogenesis, as well as food deterioration, for which the naturally occurring antioxidants may be effective (Rhee, D.-Myers, J, 2001). At present there are many commercially available preparations made from

Ginkgo biloba leaves, which results from the broad spectrum of its advantageous action on the human organism. The antioxidant effect is determined by the presence of flavonoids, capable of free radical scavenging (Kobus J. et al., 2009). Antioxidant potential of extracts from Ginkgo biloba leaves is comparable to that of ascorbic acid, glutathione or alphatocopherol (Kalisz O., Wolski T., Gerkowicz M., 2006).So far Gingko extracts have not been used as additives of antioxidant character in food production. Thus the aim of this paper was to assess the effect of an addition of ethanol extracts from green and yellow leaves of Ginkgo biloba (Ginkgo biloba L.) on oxidative stability of lipids in sunflower oils.

## MATERIALS AND METHODS

Ginkgo Biloba extract was purchased from Huisong Pharmaceuticals having in composition ginkgo flavonoids 24% and terpene lactones 6%. (Huisong, www.huisongpharm.com/manu.asp? id=82) The analyses were performed using sunflower oil (Spornic, manufactured by Prutul S.A.) and the following reagents: n-butanol (Chimopar), tiobarbituric acid (Merck), chloroform, glacial acetic acid, potassium iodide, sodium thiosulfate, freshly prepared starch, soya lecithin.To stabilize the sunflower oil was used the extract of Ginkgo Biloba in ethanol. To compare the antioxidant activity was used a commercial extract of Ginkgo Biloba. Concentration of the extract in oil was 40 mg of commercial extract in oil and 2 mg/mL and 4 mg/mL of the extract of Ginkgo in ethanol.Ginkgo Biloba extract was prepared according to the method developed by Lucia Zahradnikova et al., 2007, with small modification. Was used the commercial extract to do an extract in ethanol using 200 mL ethanol and 10 grams of extract.PEROXIDE VALUESPrimary oxidation products, namely hydroperoxides, were determined as peroxide values (PV) by iodometric standard procedure and expressed as meq kg-1 (Farmacopeea Romana, 1993) with small modification namely: for determination was used 15 mL glacial acetic acid, 10 mL chloroform, 1mL potassium iodide and freshly prepared starch for coloring.

**Determination of the 2-thiobarbituric acid value: direct method**. For the quantification of the end-products of lipid peroxidation, the most commonly test called a TBARS Assay (thiobarbituric acid reactive substances assay) was used according to the method provided in Standard Methods for the Analysis of Oils, Fats and Derivatives (7th Edition,2000). The reading was carried out at the 530nm and the T80 UV/VIS spectrophotometer (PG Instruments Ltd) was used.Expression of results:

$$TBA=\frac{50*(A-B)}{m}$$

A= the absorbance of the sample solution
B= the absorbance of the blank solution
m= the mass in mg of the sample
50= correction factor (A.Dieffenbacher,W.D. Pocklington)

## RESULTS AND DISCUSSIONS

The antioxidant activity of the extracts was expressed as the protection factor *PF*. The values of $PF > 1$ indicate an antioxidant activity, the value of $PF = 1$ corresponds to no antioxidant activity and the values of $PF < 1$ mean prooxidative activity.
Oils without any additives were analysed as blanks and the antioxidant efficiency was expressed as the protection factor:
$PF = IP0/IP$,
where:
*IP* is the peroxide index of oil with addition of an antioxidant and,
*IP0* is the peroxide index of oil without the addition of the antioxidant. (Ixtaina et al., 2012).
Analysing the results obtained it was observed that both extracts have antioxidant activity and increased oxidative stability of sunflower oil.

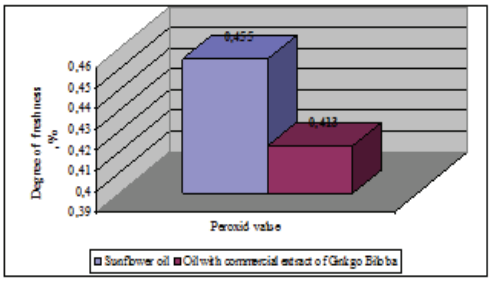


Figure 1. The values obtained from the analysis of peroxide value no oil addition and with the addition of a commercial extract of *Ginkgo Biloba*

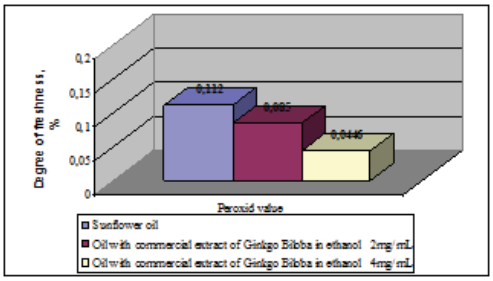


Figure 2. The values obtained from the analysis of peroxide value no oil addition and with the addition of natural Ginkgo in ethanol.

In Figure 1 and Figure 2 it can be seen that the greater stability of sunflower oil was obtained from Ginkgo Biloba extract in ethanol obtained in the laboratory at a concentration of 4 mg/mL in ethanol extract.

The addition of 40 mg of commercial extract in oil it is noted that there has been an increase in oxidative stability of oil with the addition of a commercial extract of Ginkgo with approximately 10% from the initial stability of oil without added.

When was added a quantity of 2 mg/mL of extract of Ginkgo Biloba in ethanol, it is noted that the oxidative stability of oil has increased by about 25% relative to baseline stability of oil without added, and to the addition of 4mg/mL of extract of Ginkgo Biloba in ethanol, it was noted that the oxidative stability of oil has increased by approximately 60% relative to baseline of oil stability without added.

As mentioned earlier, the protection factor (PF) for the commercial extract of Ginkgo Biloba in sunflower oil was 1.09, while for the added of 2mg/mLof extract in ethanol of Ginkgo Biloba the PF was 1,3 and for the added of 4mg/mLof extract in ethanol of Ginkgo Biloba the PF was 2,5. The results obtained can be summarized by starting that the added of 4 mg/mLof extract in ethanol of Ginkgo Biloba in sunflower oil has a better antioxidant activity.

In Figure 3 and Figure 4 it can be seen that the greater stability of sunflower oil was obtained from Ginkgo Biloba extract in ethanol obtained in the laboratory at a concentration of 4 mg/mL in ethanol extract.

At the addition of 40 mg of extract in oil it is noted that there has been a decrease in the value of the value of TBA in the oilwith addition of Ginkgo extract commercial with approximately 74% from the amount of TBA value of oil without added.

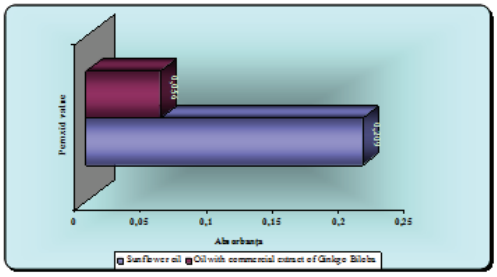


Figure 3. The values obtained as a result of determining absorbance of 2-tiobarbituric acid of the oil with no addition and with the addition of *Ginkgo Biloba* extract in ethanol.

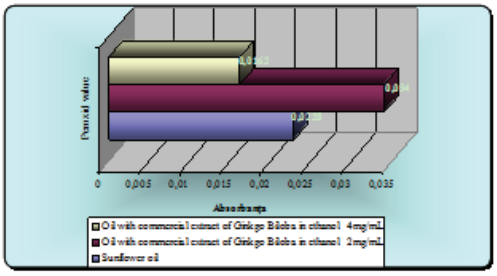


Figure 4. The values obtained as a result of determining absorbance of 2-tiobarbituric acid of the oil with no addition and with the addition of *Ginkgo Biloba* extract in ethanol in different concentration.

When was added a quantity of 2 mg/mL of extract of Ginkgo Biloba in ethanol, it is noted that there has been a decrease in the value of TBA by about 24% relative value of TBA of oil without added, and the addition of 4 mg/mL of extract of Ginkgo Biloba in ethanol, it is noted that there has been a decrease in the

value of TBA by about 61% relative the initial stability of oil without added.

As mentioned earlier, the protection factor (PF) for the value of TBA using the commercial extract of Ginkgo Biloba in sunflower oil was 3.7, while for the added of 2mg/mLof extract in ethanol of Ginkgo Biloba the PF was 0.7 and for the added of 4mg/mLof extract in ethanol of Ginkgo Biloba the PF was 1.4. The results obtained can be summarized by starting that the added of 4 mg/mLof extract in ethanol of Ginkgo Biloba in sunflower oil has a better antioxidant activity.

## CONCLUSIONS

The purpose of this work was to analyze the effects of the extracts of Ginkgo Biloba in terms of their active compounds over the sunflower oil, through two different methods: determination of hydrogen peroxide by titration and determination of TBA-standardization.

The results obtained using our methods of analysis Ginkgo Biloba extract obtained by extraction with ethanol seems to be much better in the oxidative stability of sunflower oil. In terms of results, it was observed that both extracts have antioxidant action.

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# MISCELLANEOUS

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# INTERACTION OF *ASPERGILLUS NIGER* HYPHAE AND SPORES WITHCOLLOIDAL SILVER NANOPARTICLES

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***Abstract***

*The experiments explored the interactions and antifungal properties of silver nanoparticles, against a model microorganism, Aspergillus niger. Scanning Electron Microscopy analysis were used for assessment of structural alterations done to fungal cells and hyphae, coupled with Energy Dispersive X-ray Spectroscopy (EDAX). The toxicity of the silver nanoparticles was tested using two methods: liquid exposure to the solution containing silver NPs, and spraying the NPs solution directly on the fungal culture. Analysis revealed significant cellular alteration due to the exposure to silver nanoparticles as well as effects on the growth of Aspergillus niger strain, in comparison to deionized water treatment, used at control sample. Microscopic SEM images revealed that silver nanoparticles treated hyphae were damaged on cell walls level, inducing plasmolysis, while EDAX analysis revealed strong silver depositions in the damaged areas of vegetative cells and spores walls, aspects that could be correlated with silver presence on the affected sites.*

***Keywords:*** *antimicrobial, silver, Aspergillus niger, SEM, EDAX.*

## INTRODUCTION

Nanotechnology is currently employed as a powerful tool in aiding biomedical applications regarding antimicrobial activity. As known, the smaller a particle is, the greater it is surface area to volume ratio and the higher its biological activity and chemical reactivity. Metal particles in the nanometer size range exhibit physical properties that are different from both the ion and the bulk material. This makes them exhibit remarkable properties such as increased catalytic activity due to morphologies with highly active facets (Singh et al., 2008). The use of metal nanoparticles represents a quick and straightforward way of fighting against different types of microorganisms, colloidal silver being an effective bacteria-fighting agent (Gibbs, 1999).
The interaction between silver nanoparticles, and different microorganisms, is of outmost importance as a natural process that takes place in the nanometer scale region. Silver has long been known to exhibit a strong toxicity to a wide range of micro-organisms and for this reason silver-based compounds have been used extensively in many bactericidal applications (Singh et al., 2008).
Generally, the antimicrobial mechanism of chemical agents depends on the specific binding with surface and metabolism of agents into the microorganism. As an inhibitory mechanism, it has been proposed, that the silver easily adders to the cell wall, which at the exterior has nucleophile centers and alters cell respiration process. Ionic silver strongly interacts with thiol groups of vital enzymes which lead to their inactivation (Matsumura et al., 2003; Gupta et al., 1998). Through the transmembranar transport of the silver nanoparticles inside the cell, the functions of proteins and DNA are altered, which leads to the microorganism not being able to reproduce himself anymore. Studies underlined structural changes in the cell membrane and formation of small electron-dense granules formed by silver and sulfur (Feng et al., 2000; Nover et al., 1983). The positive charge on the Ag ion is crucial for its antimicrobial activity through the electrostatic attraction between negative charged cell membrane of microorganism and positive charged nanoparticles.

## MATERIALS AND METHODS

*Aspergillus niger* (*A. niger*) was cultivated on a Czapek-Dox (2% w/v glucose, 2% w/v agar) media, at 29°C, for 14 days. The samples were kept on the agar media during the experimental procedure, in order to avoid mechanical damage that could influence the final results. After the culture was fully grown, the media containing the mature fungus was split in 2 equal halves, using a sterile spatula, one half for each method. The used colloid solution had a silver concentration of 1500 ppm, within a mix of styrenesulfonic acid and maleic anhydride. The silver nanoparticles were of colloidal shapes with an average size of 9 nm. For both methods, all samples were incubated 2 days at 29°C, with SEM and EDAX analysis being carried out after the incubation period. Before mounting, samples were washed carefully with deionized water. After stab mounting, the samples were allowed to dry in a desiccator at room temperature. For both methods, scanning electron microscopy (FEI Quanta 200) was used to observe changes in cell morphology after exposure to NPs. Also, EDAX (attached to a FEI Quanta 200 SEM) analysis was carried out to determine the deposition of silver nanoparticles on fungal cells. For liquid exposure method, the sample was fully sunken in the colloidal silver solution, thus being provided an equal coverage of the strain. For spraying method, the solution was sprayed over the media containing the mature fungus, using a pulveriser. In contrast to the previous method, this technique allows a much better O access. SEM images were taken after 2 days of exposure.

## RESULTS AND DISCUSSIONS

In this study the antifungal activity of colloidal silver against *A. niger* cells was evaluated. *A. niger* was chosen due to its ubiquitous character and aggressive growing properties. The SEM images collected demonstrated that the silver nanoparticles inhibit cell wall integrity, as silver nanoparticles present a highly reactive potential. A study carried out by Morones et al. (2005) stated that silver nanoparticles disrupt transport systems, including ion efflux. The dysfunction of ion efflux can cause rapid accumulation of silver ions, interrupting cellular processes at their lower concentrations such as metabolism and respiration by reacting with molecules (Seon Min, et al., 2009) Sulfur-containing proteins from the cell wall are likely to be preferential sites for silver nanoparticles binding. Also, following silver nanoparticles transmembranar passage process, involved in a possible DNA binding process, the nanoparticles may have a role in gene inhibition, which may result in the cell not being able to reproduce anymore. Reports on the mechanism of inhibitory action of silver ions on microorganisms show that upon Ag treatment, DNA loses its replication ability and expression of ribosomal subunit proteins as well as some other cellular proteins and enzymes essential to ATP production becomes inactivated (Pal et al., 2009).The two different methods presented slightly different action sites, as fungal hyphae were more affected by the liquid exposure method, Figure 1, while fungal spores responded more efficiently to the spraying method, Figure 2.

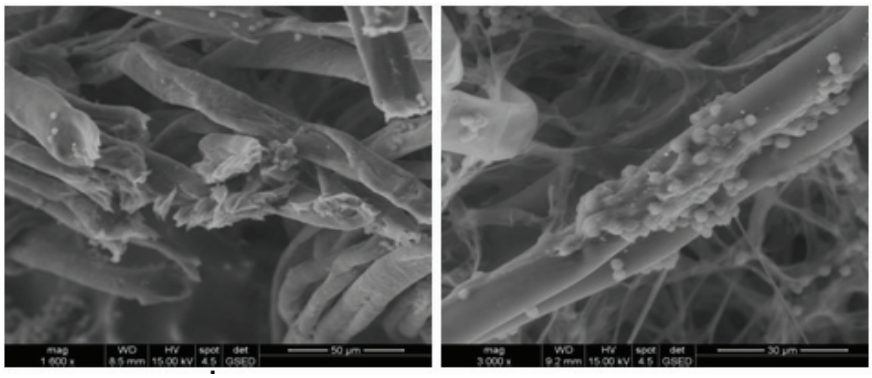

Figure 1. SEM images of sunken *A. niger* cells

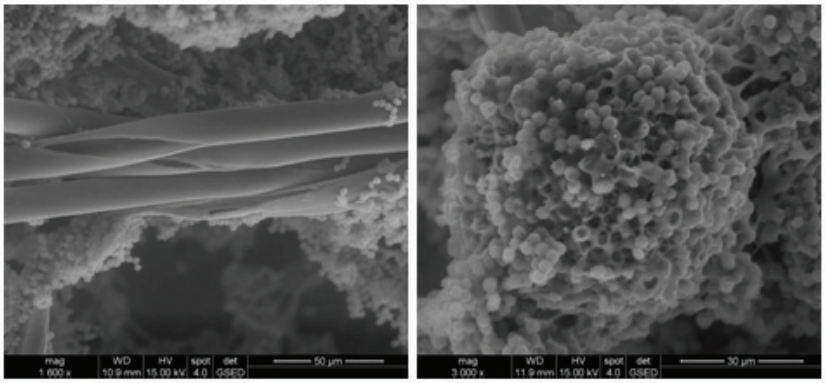

Figure 2. SEM images of sprayed *A. niger* cells

After silver treatment, microscopic images revealed that silver nanoparticles treated hyphae were damaged on hyphal walls, inducing hyphae plasmolysis, therefore cell lysis could be one of the reasons for the observed antibacterial property. For the witness samples treated with deionized water, no changes were noted varying the exposure period, Figure 3.

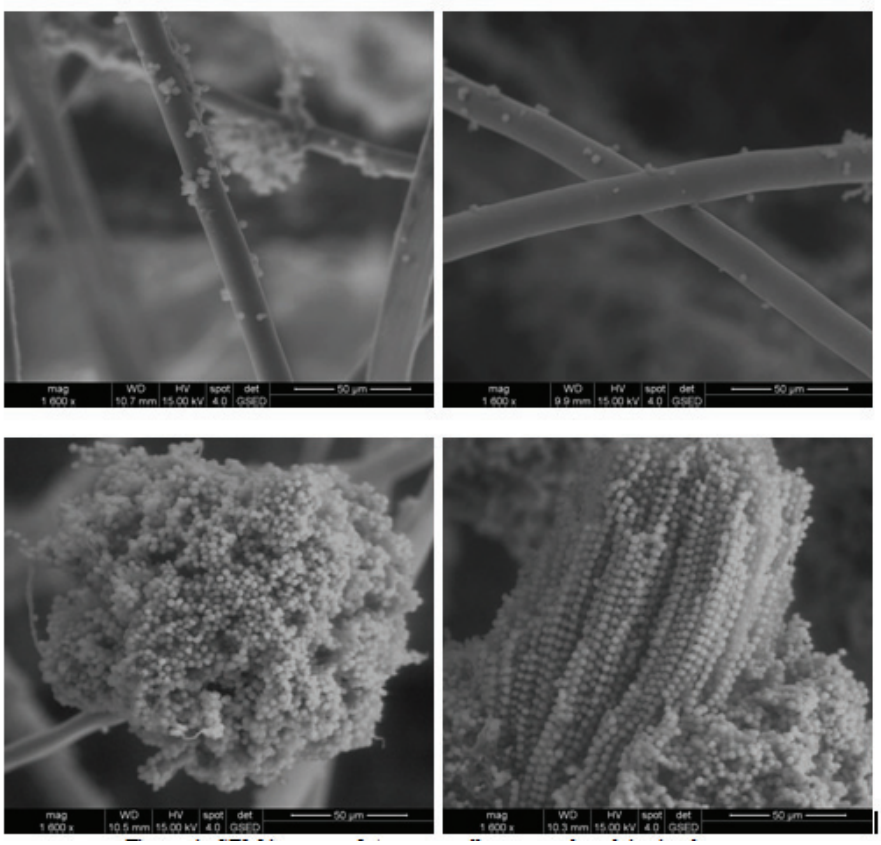

Figure 3. SEM images of *A. niger* cells exposed to deionized water

The Energy Dispersive X-Ray spectroscopy (EDAX) was used as an analytical technique used for elemental analysis and chemical characterization of the interaction between the culture and silver NPs. The analysis allowed identification of silver depositions in the damaged areas of hyphal and spores walls, Figure 4.

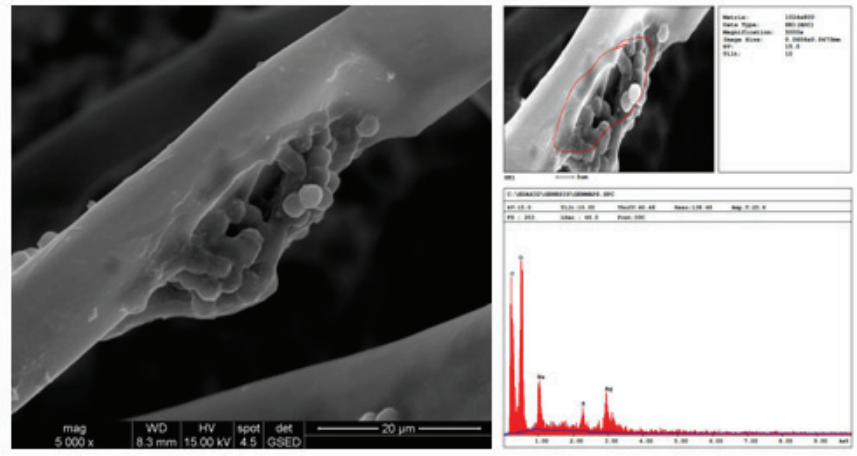


Figure 4. SEM and EDAX analysis of damaged hyphae and spores

## CONCLUSIONS

The slightly different effect of the two methods may be explained be explained by a possible formation of silver nanoparticles aggregates, which in the case of the liquid exposure method they gather at the bottom of the Petri dish where they have a better access at the inferior part of the fungus culture. On the other hand, for the spraying method, before spraying the culture, the solution was well stirred, to provide a homogeneous distribution of silver nanoparticles within the solution. Therefore, after spraying, the conidial heads were covered in a solution "capsule", this way providing a better area of contact, in comparison with the fungus hyphae. The microscopic observations revealed that the silver nanoparticle solution clearly damaged fungal hyphae and spores, while the samples treated with deionized water appeared to remain intact.

The EDAX data acquisition was made from the damage site of fungal hyphae. The area or intensity of a peak in the acquisition spectrum is proportional to the concentration of the corresponding element in the sample. The peaks specific to carbon (C – 0.277 keV) and oxygen (O – 0.523 keV) have the largest share due to the organic character of the sample. The signal-peaks characteristic to sodium (Na-1.040 keV) and sulphur (S-2.307 keV) may be generated by proteins/enzymes present in the wall of the fungus, following cell lysis. Silver nanoparticles may have a crucial role in affecting the function of membrane-bound enzymes, those involved in the respiratory chain. This process can facilitate the generation of reactive oxygen species, which in the end can lead to cell death. The signal-peak found at 2.984 keV specific to silver (Ag) demonstrates the localization of silver in the damaged sites, thus strengthening the correlation between silver effect and hyphae and spores collapsing.

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# FUNGAL STRAINS ISOLATED FROM SEVERAL CASES OF HUMAN DERMATOPHYTOSES

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***Abstract***

*Dermatophytosis has become one of the most common human infectious diseases in the world so it is of interest to dedicate more studies to its etiological agent's dermatophytes. These keratinophilic and keratinolytic filamentous fungi have the ability to invade and colonize keratinized layers of the skin and their appendages. Dermatophytes fungi group include three anamorphic genera namely Epidermophyton, Microsporum and Trichophyton. These three genera include geophilic, zoophilic and anthropophilic species. Usually these filamentous fungi are identified on the basis of conidia morphology and sometimes with specific physiological characters, such as the hair strand perforation and urea hydrolysis. The objective of present study was to isolate and to identify some filamentous dermatophytes fungi from human superficial mycoses. Isolated samples (scales, fragments of nails and subungual debris) were cultured on specific culture media during four months. After incubation time, morphological characters of cultured fungal were observed macroscopically and microscopically. The following cultural characteristics were analyzed: texture, surface and reverse color of the colony, the presence of pigmentation. Microscopic examination offered data on specific characters such as, the presence/absence of macroconidia and microconidia, theirs shape, their septa number, the presence of chlamydoconidia, spiral hyphae and nodular organs. The strains identification was completed with in vitro hair strand perforation and urea hydrolysis. The isolated microbial strains were identified as belonging to Trichophyton and Microsporum genera.*

***Keywords:*** *dermatophytosis, dermatophytes fungi, Microsporum, Trichophyton.*

## INTRODUCTION

Dermatophytosis are superficial skin infections confined to the stratum corneum, produced by filamentous fungi called dermatophytes. In a case of dermatophytosis the lesions are very characteristic and can affect different areas of the body surface. Depending on the area of the body affected by fungal infection, dermatophytosis (ringworm or *tinea*) are classified in several type, namely: *tinea barbae* (chin, mustache), *tinea capitis* (scalp, eyebrows, eyelashes), *tinea corporis* (glabrous skin), *tinea cruris* (groin), *tinea faciei* (on the face), *tinea favosa* (favus), *tinea imbricata*, *tinea manuum* (on hand), *tinea unghium* (on nails), *tinea pedis* (at legs) (Hainer, 2003; Vander Straten et al., 2003; Gupta et al., 2008).
In the dermatophytes group are included three anamorphic genera of hyaline filamentous fungi namely *Epidermophyton, Microsporum* and *Trichophyton* (Weeks, 2003; Kayzer, 2005; http://www.doctorfungus.org/). They have the ability to invade and colonize keratinized layers (Sharma et al., 2011) and to produce enzymes (keratinases), endoproteases and exoproteases (Monod, 2008). Dermatophytes include geophilic, zoophilic and anthropofilic species, with a restricted or a worldwide geographical distribution (Achterman, 2012).
Dermatophytosis can vary from acute to chronic forms, depending on many factors, including the host, species of fungus involved or lesions location on the body surface (Vermout et al., 2008, Bramono, 2012).
Dermatophytosis can be transmitted directly by contact with infected person or indirectly through contact with infected products or objects (Gupta et al., 2003).

In recent years, the risk of fungal infections has been increasing drastically so it is of interest to solve these diseases. The objective of present study was to isolate and to identify some filamentous dermatophytes fungi from human superficial mycoses. Isolated samples (scales, fragments of nails and subungual debris) were cultured on specific culture media during four months.

## MATERIALS AND METHODS

### Samples

A number of 34 samples were used in this study, represented by skin scales, hair strands, nails fragments and subungual debris. Samples used in study were collected in 2008-2009 in the Mycotic Infections laboratory of the National Institute of Research & Development for Microbiology and Immunology Cantacuzino from apparently healthy people.

### Sampling method

A classic protocol was used for samples collection (Coman and Mares, 2000; Mares and Bazgan, 2008). The samples were collected after cleaning the affected area with 70% alcohol. From the skin surface, the samples were collected by scraping the lesion from the center to its edge using a sterile scalpel. The hair samples were plucked or shave (where the hair could not be plucked) using a sterile tuck. The nail fragments were collected using a sterile scissors, while the nail bed was scraped and the subungual debris have been collected.

### Culture method

Collected samples were cultured on two solid media, potato extract medium and Sabouraud medium. After inoculation, the Petri plates were incubated at 25-30˚C, for 4 weeks. The microbial growth was monitored daily during the entire period.

### Identification methods

*Macroscopic observations*

Macroscopic observations of isolated strains in solid cultures were carried out weekly, noting the growth rate, colonies morphology and pigment formation in the culture medium.

*Microscopic observations*

The investigations were done using an Olympus BX51 microscope and consisted in direct microscopy in a drop of 10% potassium hydroxide (KOH) for skin scales examination and 20% for nails examination and in wet mounts mounted in a drop of lactophenol cotton blue and, respectively.

*Slide culture technique*

The technique was performed in a wet room made into a Petri plate by placing a fragment of sterile filter paper and moistening it with sterile distilled water. Over the filter paper a fragment of sterile glass in U-shape was placed and over which was placed a sterile microscope slide. A block of potato extract medium of about 1 $cm^2$ was placed over the slide, in the center. The medium was inoculated with a mycelium from the fungal strain thereafter covered by a cover slip. The Petri plate was incubated at 30˚C until fungal growth was observed (Figure 1) Microscopic examination was made by wet mounts prepared in a drop of lactophenol cotton blue.

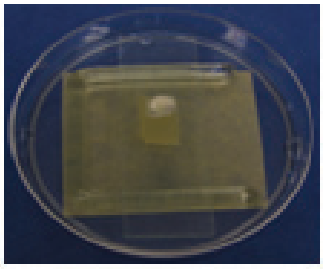

Figure 1. Slide culture technique

*Urea hydrolysis test*

The isolated fungi were incubated in urea liquid medium for 7 days at 25-30°C. The inoculated test tubes were examined daily to observe a possible color change of the culture medium in case of a positive test from straw to reddish-purple. Urea hydrolysis test was used to distinguish *Trichoplyton mentagrophytes* from *Trichophyton rubrum. Trichophyton mentagrophytes* is usually urease positive in 7 days and *Trichophyton rubrum* is usually urease negative

*In vitro hair strand perforation test*

The test was performed by placing a few hair strands fragments in a sterile Petri plate and adding 10 ml of sterile distilled water and 0.1 ml of yeast extract 10%. A piece of fungal mycelium was transferred in Petri plate and incubated at 25°C for 21 days. Periodically was done wet mounts, mounted in a drop of lactophenol cotton blue.

## RESULTS AND DISCUSSIONS

In the positive skin scraping samples, on direct microscopy (KOH), fungal elements as networks of branching fungal hyphae were observed (Figure 2). For 6 samples, the KOH test was negative and no fungal elements were relieved.

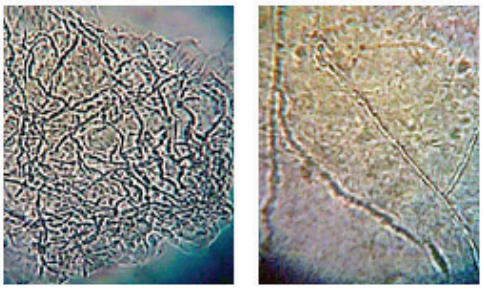

Figure 2. Skin scraping infected with septate, branching fungal hyphae

Collected samples were cultured on potato extract medium and Sabouraud medium in Petri plates. Of the 34 samples were obtained 28 fungal isolates and all were used for subsequent identification. The fungal colonies developed after 14 days of incubation were investigated through macroscopic and microscopic observations. Macroscopic observations of isolated strains offer information about growth rate, colonies morphology (color of colony obverse and reverse, shape of the edges, colony surface appearance, and texture) and pigment formation in the culture medium. Based on these informations, isolated fungi were identified as belonging to *Trichophyton* and *Microsporum* genera. Therefore, 22 strains belong to the genus *Trichophyton*, as *Trichophytoninterdigitale, Trichophyton mentagrophytes*, *Trichophyton rubrum* and *Trichophyton sp.* and 6 strains belong to the genus *Microsporum, as Microsporum canis* and *Microsporum sp.* (Figure 3).

These fungal isolates are known to be involved in the etiology of human dermatophytosis (Jackson, 2006; Mahmoudabadi and Yaghoobi, 2008).

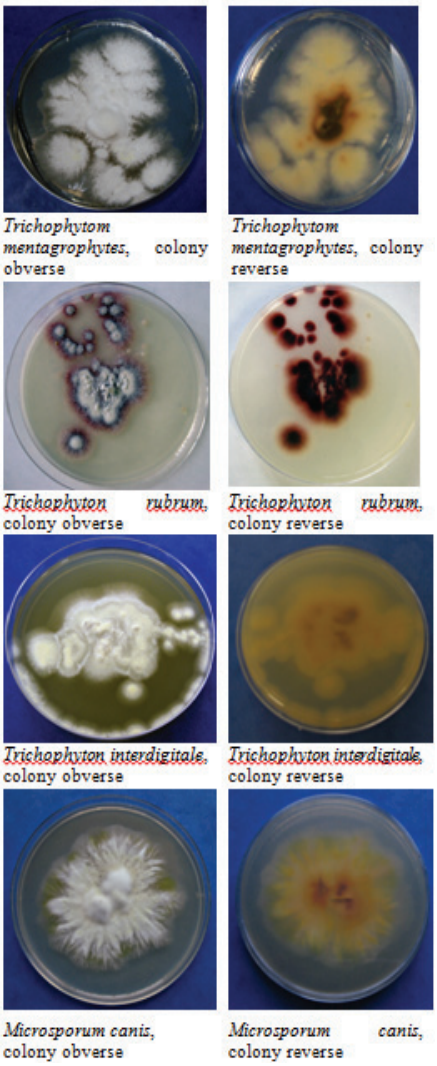


Figure 3. Macroscopic characteristics of isolated Trichophyton sp. and Microsporum sp. strains, on PDA medium at after 14 days of incubation

The preparations with lacthophenol cotton blue relieved some important characters for fungal strains identification, as shape and dimensions of macroconidia and microconidia, macroconidial septa number, presence of chlamydoconidia, spiral or raquet hyphae and nodular organs. The results of this investigation are presented in Figure 4.

Characteristic of *Trichophyton mentagrophytes* culture are: rapid growth, granular surface, flat colonies, white to cream colour on obverse, irregular edge, brownish yellow to reddish-brown on reverse.

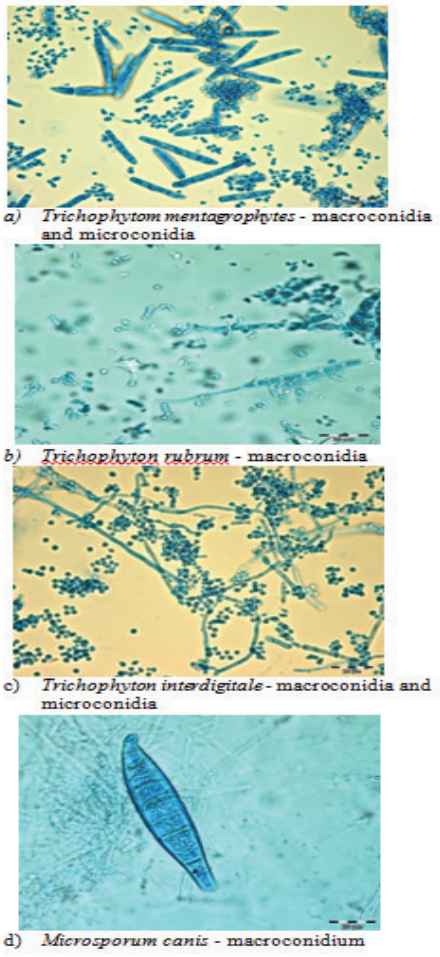


Figure 4. Microscopic characteristics of isolated Trichophyton sp. and Microsporum sp. strains in lacthophenol cotton blue-slide culture technique (400x)

Microscopic observation showed smooth macroconidia (4-6 septa), numerous spherical or pyriform, microconidia, in clusters and spiral hyphae (Figure 4a).

Characteristic of *Trichophyton rubrum* culture are**:** moderate growth, flat to slightly raised, white to cream colonies obverse, velvety with an wine red reverse. Microscopic observations showed pencil-shaped abundant macroconidia, often show an terminal appendages and pyriform microconidia (Figure 4b). Characteristic of *Trichophyton interdigitale* culture are**:** moderate growth, flat, white to cream colonies obverse, granular surface, with an yellow reverse. Microscopic observation showed few clavate macroconidia and abundant pyriform microconidia (Figure 4c).

Characteristic of *Microsporum canis* culture are**:** fast growth, flat colonies, white-yellowish surface; golden yellow colony reverse. Microscopic observation showed abundant long, rough, fusiform macroconidia and a few pyriform to clavate microconidia. (Figure 4d).

For a more precise identification, more tests were carried out such as urea hydrolysis test and *in vitro* hair strand perforation test. Urea hydrolysis test was used to distinguish *Trichoplyton mentagrophytes* from *Trichophyton rubrum*. *Trichophyton mentagrophytes* is usually urease positive in 7 days and *Trichophyton rubrum* is usually urease negative.

As it can be shown in Figure 5, the result for *Trichophyton mentagrophytes* is positive (left), the strain producing urease and breaking down urea. Meanwhile, *Trichophyton rubrum* has no hydrolytic activity against urea (right).

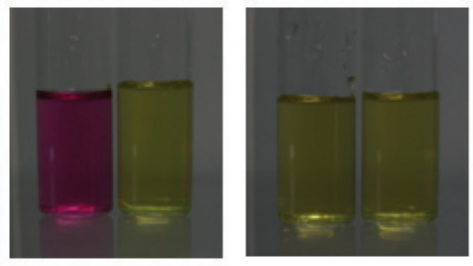

Figure 5. Urea hydrolysis test

The positive result at the *in vitro* hair strand perforation test for *Microsporum canis* is presented in Figure 6.

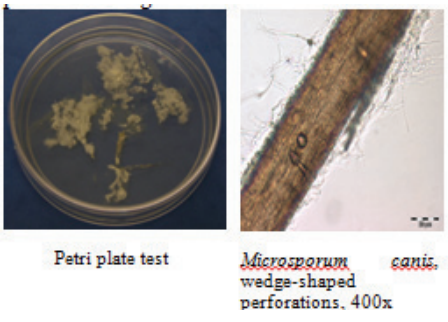


Figure 6. The in vitro hair strand perforation test

Also, *Trichophyton interdigitale*, *Trichophyton mentagrophytes* are able to perforate the hair

strand. *Trichophyton rubrum* concerning the ability to perforate the hair strand in vitro and are negative (data not shown).

The isolation and identification procedure finally provided two predominant fungal strains, namely *Trichophyton rubrum* and *Trichophyton mentagrophytes*.These results are similar to those reported by other authors (Szepietowski et al., 2002; Venkatesan, 2007; Seebacher et al., 2008; Woodfolk, 2012).

## CONCLUSIONS

In this study, several dermatophytes fungi were isolated from human infections. From the total number of samples, 82.35% were positive and the isolated strains belonged to dermatophytes fungi. The most frequently isolated strains belong to the *Trichophyton genus*, as *Trichophyton rubrum and Trichophyton mentagrophytes*; our results being in accordance with other similar studies. Of the total of 28 isolated strains *Trichophyton rubrum* reprezents 35,71%; *Trichophyton mentagrophytes* 21,43%; *Trichophyton interdigitale* 7,14%; *Trichophyton sp.* 14,29%; *Microsporum canis* 7,14% and *Microsporum sp*. 14,29%.

The practical importance of the study is that the methods used to identify the fungal strains participate in improvement of dermatophytosis diagnostic algorithm.

Also was performed the monitoring of incidence of etiologic agents involved in human dermatophytosis

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# SCREEN-PRINTED CARBON ELECTRODES MODIFIED WITH PRUSSIAN BLUE AND A NON-CONDUCTING ELECTROPOLYMERIZED FILM FOR SELECTIVE DETERMINATION OF $H_2O_2$ IN BEVERAGES

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***Abstract***

*The development of a highly selective and sensitive sensor for $H_2O_2$ in beverages such as natural juices, is described in this work. The sensor is based on the deposition of Prussian Blue (PB) onto screen-printed carbon electrodes (SPCEs) followed by the electropolymerization of a non-conducting film. Several procedures for PB deposition on the SPE electrodes were tested: electrochemical deposition (potentiostatic, cyclic voltammetry) and chemical deposition. The electrochemical and analytical properties of the SPCE/PB sensors had been evaluated and the potentiostatic method for PB deposition was selected for the further development of the $H_2O_2$ sensor. In order to develop a robust sensor for $H_2O_2$ determination in samples with complex matrix, we covered the PB layer with an electropolymerized non-conducting film with a high permselectivity for $H_2O_2$. This film is a copolymer based on 2,6-DHN (2,6-dihydroxynaphtalene) and APEA (2-(4-aminophenyl)-ethylamine).*
*The SPCE/PB/copolymer sensor demonstrated improved stability in operational conditions and excellent interference rejection properties. This sensor may be successfully applied on-field, using a portable potentiostat-galvanostat and the chronoamperometry technique, as well as in a laboratory bench flow injection analysis system with amperometric detection. The developed sensor was able to measure $H_2O_2$ in the linear range 1 $\mu$M – 500 $\mu$M (R=0.9989), with a detection limit of 0.5 $\mu$M. The SPCE/PB/copolymer sensor maintained for a long period its response for $H_2O_2$ (94% response was retained after 60 days).*

***Keywords****:screen printed carbon electrode, Prussian Blue, nonconductive copolymer, $H_2O_2$, beverages*

## INTRODUCTION

The direct amperometric detection of hydrogen peroxide at conventional electrodes is possible only at 0.6 V vs. Ag/AgCl. At this potential, the presence of easily oxidizable compounds present in real samples (ascorbic acid, bilirubin, uric acid, etc.) can easily interfere in the measurement, being oxidized at the electrode together with hydrogen peroxide. For this reason the detection of $H_2O_2$ at potentials around 0 using electrodes modified with electrochemical modifiers, such as Prussian Blue (PB), has enormous advantages and applications in many fields [1-3].

The first sensors for hydrogen peroxide based on PB modified glassy carbon electrode were reported by Karyakin et al [4]. The PB deposited on these sensors has an electrocatalytic effect on the reduction of $H_2O_2$, allowing its detection at potentials close to 0 V, thus making possible the coupling with oxidase enzymes while avoiding or reducing the electrochemical interference.
Screen-printed electrodes are frequently used in analytical applications because of their unique properties such as small size, low detection limit, fast response, high reproducibility, etc. [5].
Screen-printed carbon electrodes (SPCEs) are devices that are produced by printing different inks on various types of plastic or ceramic

substrates. The composition of the inks used for printing on the electrodes determines the selectivity and sensitivity required for each sensor development. Screen-printed electrodes are inexpensive, simple to prepare, versatile and suitable for the mass-production of disposable electrodes [6].

The aim of this work was to develop a simple, robust and portable sensor based on PB and a non-conducting copolymer which are deposed on a SPCE for hydrogen peroxide determination in beverages (commercial juices).

## MATERIALS AND METHODS

### Apparatus

Electrochemical measurements were carried out using a μ Autolab type III potentiostat/galvanostat computer controlled by the GPES software, as well as a portable PalmSens potentiostat/ galvanostat controlled via the PalmSensPC software.

The flow injection analysis system consisted from a four-channel Minipuls 3 Gilson peristaltic pump fitted with tygon tubing (1.52 mm id) used for the propulsion of fluids, an injection valve (Rheodyne, 7725i model) and a flow cell special for SPCE from Dropsens, Spain. The valve loop volume was 100μL. Fittings and connectors were used to connect the different components of the manifold. The optimum flow rate was 0.36 mL/minute. The detector was the same potentiostat/galvanostat used for voltammetric measurements.

### Electrodes

Screen-printed carbon electrodes (SPCEs) model DRP-110 purchased from DropSens (Spain) were used for electrochemical measurements. In this case the electrochemical cell is composed by a graphite working electrode (d = 4mm), a graphite auxiliary electrode and a silver pseudoreference electrode, with silver electric contacts deposed on a ceramic substrate.

### Reagents

All chemicals from commercial sources were of analytical grade. Iron chloride ($FeCl_3$), potassium ferricyanide $K_3[Fe(CN)_6]$,HCl 37%, sodium chloride, hydrogen peroxide (30%), were purchased from Sigma-Aldrich. AOT (Dioctyl sulfo-succinate sodium salt) was from Carlo Erba. The monomers 2,6-dihydroxynapthalene (2,6-DHN) and 4-(2-aminoethyl)aniline (APEA) were from Aldrich and respectively Fluka. Double-distilled water was used throughout.

2,6-DHN and APEA were dissolved in 0.1M phosphate buffer pH 7.4. Hydrogen peroxide (Fluka) was prepared daily in phosphate buffer, pH 6.5.

### Modification of SPCE with Prussian Blue

Three procedures were investigated for PB film formation on the working electrode of screen printed carbon electrodes in order to prepare sensitive and robust PB sensors for $H_2O_2$ determination. The tested procedure were based on: chemical, galvanostatic and cyclic voltammetry based deposition.

Prior to Prussian Blue modification, the SPCEs were pretreated in the presence of 50 mM phosphate buffer in 0.1 M KCl, pH 7.4, by applying the potential of + 1.7 V versus Ag/AgCl for 3 minute. For the *chemical deposition* of PB films, two solutions were prepared. Solution 1: 100 mM $K_3[Fe(CN)_6$ in 10 mM HCl. Solution 2: 100 mM $FeCl_3$ in 10 mM HCl. Prussian Blue modification of SPCE was then accomplished by placing 5 μl of precursor solution 1 and 5 μl of precursor solution 2 onto the working electrode area [7]. The solution was left onto the electrode for 10 min and then rinsed with a few millilitres of 10 mM HCl. The electrodes were then left 90 min in the oven at 100° C to obtain a more stable and active layer of PB.

For electrochemical deposition of PB the galvanostatic and cyclic voltammetry techniques were tested. The *galvanostatic deposition* was made in a mixture (solution 3) of 2.5 mM $K_3[Fe(CN)_6$,], 2.5 mM $FeCl_3$ and 1 mM AOT prepared in 100 mM KCl and 100 mM HCl solution by applying the potential of 0.4V for 40 sec [1]. After a gentle rinsing with water, the sensor was placed in a solution of 100 mM KCl in 100 mM HCl and a number of 20 cycles, between - 0.2 and 0.4 V, at a scan rate of 50 mV/s, was run. The *cyclic voltammetrydeposition* was carried out in the solution 3 (20 cycles, between - 0.2 and +0.4 V, at a scan rate of 50 mV/s) [14].

The deposition of the PB film was confirmed by performing cyclic voltammetry in 50 mM phosphate buffer, pH 7.4.

The PB modified electrodes were stored dry at room temperature in the darkness.

**Electrodeposition of non-conducting films on SCPE/PB**

The SPCE/PB sensors were covered with a non-conducting copolymer electrodeposited using the cyclic voltammetry technique. The copolymer was synthesized from a solution of 0.9 mM 2,6-DHN and 10 mM APEA by cycling for 10 – 20 times the potential from + 0.2 V to +1.1 V with a scan rate of 5 – 10 mV/sec.

## RESULTS AND DISCUSSIONS

### SPCE / PB sensors characterization

After the preparation, the SPCEs modified with PB (SPCE / PB) where characterized by cyclic voltammetry, in 0.1 M KCl prepared 0.1M HCl, in order to prove the PB film formation.

Figure 1 shows the specific voltammograms of the SPCE / PB sensors prepared according to the procedures described in 'Materials and methods' section. Two characteristic peak couples corresponding to conversion of high and low spin ions appear with formal potentials of 0.10 V and 0.80 V. The oxidation and reduction peaks centred at 0.10 V are much narrower and well shaped for the PB sensors obtained via electrochemical deposition compared with those obtained via chemical deposition. However, the SPCE / PB obtained by cyclic voltammetry showed the lowest oxidation and reduction peaks, and also some other experiments led to the conclusion that the PB film was too thin and fragile. For this reason, the most important further studies were done on the SPCE / PB sensors obtained by chemical and galvanostatic deposition.

The influence of the thermal stabilization by keeping the electrodes at 100° for 90 min was also studied. No evident differences between the treated and nontreated PB electrodes were observed regarding the response of the electrodes in KCl, phosphate buffer or for $H_2O_2$, but the operational stability was greater improved for the electrodes stabilized via the thermal treatment.

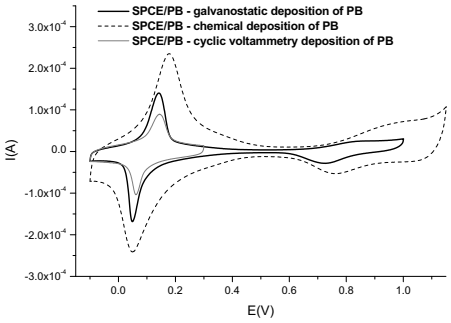


Figure 1. Influence of the deposition method of PB on the cyclic voltammograms recorded for the SPCE / PB sensors (Electrolyte 0.1 M KCl in 0.1M HCl; 50 mV/s)

The effect of potential scan rate on the oxidation ($I_{ox}$) and reduction peak ($I_{red}$) currents was studied for the redox couple present around 0.10 V. Plotting the $I_{ox}$ and $I_{red}$vs. square root of scan rate showed a linear relationship (figure 2), the result indicating a diffusion limited process. This behaviour was observed for all the three tested methods for PB deposition.

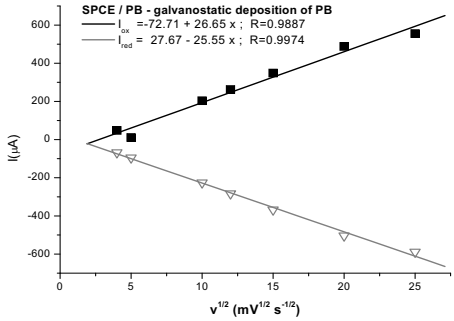


Figure 2. Variation of $I_{ox}$ and $I_{red}$vs. square root of scan rate in electrolyte solution (0.1M KCl, 0.1M HCl)

The pH value of the electrolyte solution is an important parameter for $H_2O_2$ determination using the PB modified SPCEs. The stability and sensitivity of the PB sensor may be affected by the hydroxide ions which can break the Fe-(CN)-Fe bonds, but also by the protons which may block the electrochemical reactivity of PB [8].

The pH influence on the electrochemical determination of 1 mM $H_2O_2$ using the SPCE / PB was studied at pH ranging from 6-7.4 (figure 3). For all the tested electrodes the highest reduction peaks were obtained for the

pH 6.5. For all the following studies, as optimum electrolyte solution, was used the 50 mM phosphate buffer, pH 6.5.

The selection of the optimum working potential to be applied when measuring $H_2O_2$ was studied for the next electrochemical techniques: chronoamperometry, amperometry in stirred solution and amperometry in flow injection system (FIA). Potentials ranging from -100 mV to +200 mV were applied in presence of a selected concentration of hydrogen peroxide (100µM). The highest signal was obtained for the potentials of -50 mV when working in chronoamperometry and of -100 mV in amperometry (for both, stirred solutions and FIA). The lowest background noise and the highest signal for $H_2O_2$ measurement was recorded for the PB sensor prepared via the galvanostatic procedure.

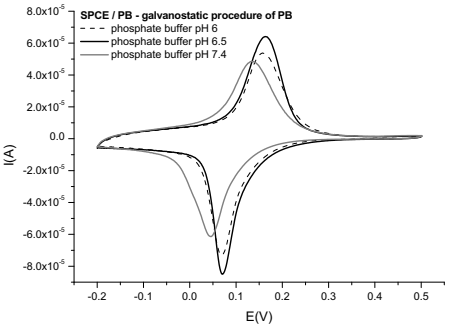


Figure 3. Influence of electrolyte solution pH on the response of SPCE / PB to 1mM $H_2O_2$

Regarding the hydrogen peroxide determination, the amperometry under stirring technique gave the best results for the SPCE / PB sensor prepared via galvanostatic method in rapport with the background noise and with the response time. Even that the sensitivity recorded with sensor prepared by chemical deposition of PB is higher than that obtained for that obtained via electrochemical procedures, the linear range of concentration for PI shorter (data not shown).

Cronoamperometry technique is very advantageous due to the fact that the screen printed electrodes allow working with a very low volume of sample (100 µL), the determination is fast and reproducible, and does not require a time for working electrode polarization. Also this electrochemical technique may be applied for on-field measurements using a portable detector.

According to the measurement results, the linear range was from 1 µM to 100 µM $H_2O_2$ with the linear correlation of 0.9967 for the sensor obtained by chemical deposition of PB and, respectively, of 0.9985 for the sensor prepared by galvanostatic deposition.

The sensitivity of the galvanostatic prepared sensors was with 50 % higher than obtained via the chemical deposition (figure 4). For both type of sensors the detection limit was 0.5 µM $H_2O_2$.

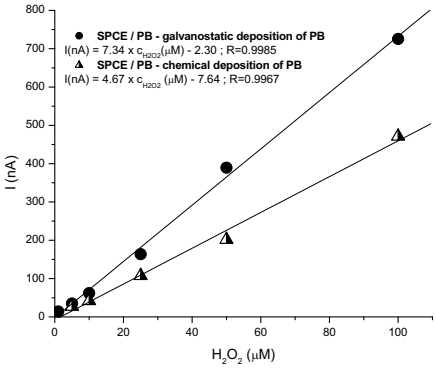


Figure 4. Calibration plots for detection of $H_2O_2$, using chronoamperometry technique (E = -50 mV/s)

**Characterization of SPCE / PB / copolymer sensors**

The PB exhibits excellent catalytic activity for the electroreduction of hydrogen peroxide, but the operational stability of PB is still a matter of concern in real samples with complex matrix, such as the food samples. In order to protect the PB layer, the coverage with non-conducting films as the poly(*o*-aminophenol) [1] or with ionomers as Nafion [9] was reported.

In this work we report the use, for the first time, of the non-conducting copolymer electrosynthesized from a mixture of 2,6-DHN and APEA for protection of the PB layer. The copolymerization was performed via the cyclic voltammetry technique by cycling the potential from + 0.2 V to +1.1 V. In figure 5 one can observe that the irreversible oxidation peak at the +0.65 V present in the first three cycles disappears in the following cycles. The oxidation peak current decrease proves the

formation of a non-conducting film on the surface of the SPCE / PB sensor.

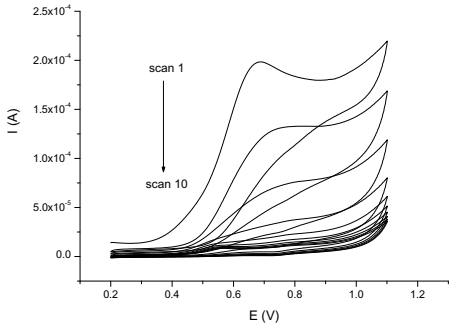


Figure 5. Cyclic voltammograms recorded during the formation of the copolymer poly(DHN – APEA) on the SPCE / PB prepared via the galvanostatic method (10 mM APEA; 0.9 mM 2,6-DHN; 10 mV/s; 10 cycles)

The optimization of the copolymer formation related to the hydrogen peroxide determination was performed studying the influence of the scan rate and number of cycles.

In figure 6, one can observe the influence of the number of cycles used for the copolymer electrodeposition on the calibration graphs obtained by chronoamperometry. A higher number of cycles led to a higher thickness of the copolymer layer and consequently to a more difficult diffusion of $H_2O_2$ across it.

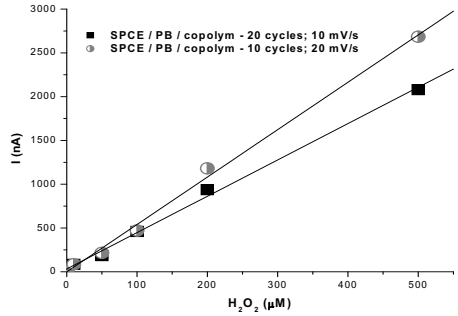


Figure 6. Influence of the number of cycles used for copolymer electrodeposition on chronoamperometric calibration graphs (E = -0.1 V)

In Figure 7 the influence of the scan rate used during the copolymer electrodeposition process on the $H_2O_2$ determination in chronoamperometry is presented. The scan rate has a major influence on the film porosity. A low scan rate, as 5 mV/s, lead to a copolymer film less porous, which acts as a barrier especially for higher concentration of $H_2O_2$.

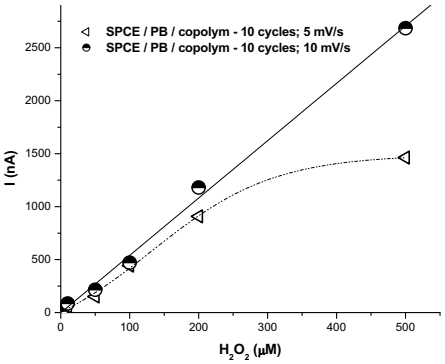


Figure 7. Influence of the scan rate used for copolymer electrodeposition on chronoamperometric calibration graphs (E = -0.1 V)

By comparing figures 4 and 6, it can be concluded that an important feature achieved by the copolymer electrodeposition on the PB layer was the extending of the linear concentration range up to 500 μM in cronoamperometry.

The optimum conditions for copolymer electrodeposition were: scan rate = 10 mV/s; number of cycles = 10; potential range = 0.2 V - 1.1 V.

Another important characteristic of the SPCE / PB / copolymer sensors is represented by a higher operational stability even in flow injection analysis conditions. In figures 8 is presented the FIA amperogram recorded for the PB sensor covered with the poly(DHN – APEA) copolymer.

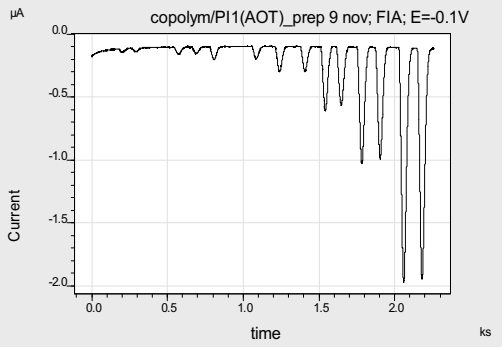


Figure 8. FIA amperogram recorded for the SPCE/PB/copolymer sensor ($v_{inj}$=100 μL; flow rate=0.36mL/min; H2O2 concentration injected in duplicate = 2; 5; 10; 20; 50; 100; 200 μM)

Also, in FIA conditions the sensitivity for $H_2O_2$ detection was much higher comparing with those obtained in tcronoamperometric conditions.
The possible interference of compounds present in beverages, such as ascorbic acid and glucose, was tested. Glucose showed no interference in $H_2O_2$ determination with SPCE/PB/copolymer sensor. Ascorbic acid may interfere, giving a false negative signal, only if the ascorbic acid concentration is much higher than of $H_2O_2$.
The SPCE/PB/copolymer sensor maintained for a long period its response for $H_2O_2$ (94% response was retained after 60 days)

**Real sample analysis**
Sometimes, for the aseptic packaging of natural fruit juices, hydrogen peroxide is used as a chemical agent for sterilization. However, the hydrogen peroxide residues in higher concentration are irritative for the skin and may affect the human health.
The developed SPCE/PB/copolymer sensor was applied for the fast and simple determination of $H_2O_2$ in several commercial fruit juices. The sample treatment consisted only in dilution with 50 mM phospahate buffer, pH=6.5.
In table 1 is presented the $H_2O_2$ concentration determined in the tested samples with the SPCE /PB/copolymer sensor by chronoamperometry.

Table 1. $H_2O_2$ concentration in several commercial fruit juices

| sample | $H_2O_2$ (μM) |
|---|---|
| Orange juice (Granini) | 3.12 |
| Peach juice (Prigat) | n.d.* |
| Apple Juice (Granini) | n.d. |
| Orange pulpy juice (Cappy) | n.d. |
| Orange juice (Fanta) | 2.48 |

*n.d. = not detectable

The results demonstrated that the level of hydrogen peroxide concentration used in tested juices preservation is very low.

## CONCLUSIONS

In this work, it was developed a robust and cost-effective sensor based on SPCE modified with PB and an electropolymerized non-conducting film for $H_2O_2$ determination. The experimental results showed that the copolymer film remarkable improves the operational stability of the PB sensor. The SPCE/PB/ copolymer sensor has an excellent electrocatalytic activity for the reduction of $H_2O_2$, with a broad linear range from 1μM to 500 μM, and a detection limit of 0.5 μM. Furthermore, introduced into a FIA system, the sensor proved a great operational stability. The developed sensor was successfully applied to the determination of $H_2O_2$ in commercial juices.

## ACKNOWLEDGEMENTS

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# SUPPORTING STUDENTS FOR A BIOTECH CAREER

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***Abstract***

*The biotech high-education in Romanian has started relative recently, the first accredited college being registered in 1996 in Bucharest, in the University of Agronomical Sciences and Veterinary Medicine. Since then, more than 1000 license students have graduated in different specializations as Agricultural Biotechnology, Industrial Biotechnology and Veterinary Biotechnology. Recently, during the implementation of a structural funds project for human resources (POSDRU/109/2.1/G/81570) has been conducted a survey on how the biotech graduates have been placed on the labour market. The statistics shows that less than 27% from the graduates have found jobs in biotech fields, such as scientific research and education, food and beverages production, environmental protection, including biofuel production, pharmaceutical products, instrumentation and suppliers.*
*In this regard, educators, professionals and counsellors, on regional level, have started to take important measures to develop and implement activities to increase the absorption level of the biotech graduates.*
*Aside the curricula improvement, an important step it has been the counselling of 100 students, between 20 and 30 years old about self-knowledge, neuro-linguistic programming, CV content, letter of intention writing, as well as about behaviour during an interview. Also, it has been underlined the importance of the continuous learning. More than 93% from the counselled students has found it very useful.*
*Further, a network of 12 economical biotech operators have been developed and 38% of the counselled students have been involved in internships in fields as agriculture and food production, pharmaceutical industry, food safety and consumer protection, hygiene products design and biotech research.*
*As preliminary result, all the graduated counselled students have decided to continue their studies in master courses, and 27% has already been employed in biotech field.*
*The program will continue in the next years, with the aim to increase the employability for the biotech graduates.*

***Keywords:*** *biotech career, counselling, internship.*

## INTRODUCTION

By definition, biotechnology means the use of advances in life science to create products and services for our world (Frierman-Hunt G, 2008). Although biotechnology industries make many different products from vaccines to seeds to specialized equipment, many of the job duties and titles are similar across the industry. To understand the different job functions, the jobs can be grouped into five areas: research and development (which includes research and development, laboratory support and technician jobs); manufacturing and services; quality and regulatory affairs; sales and technical support; and administration and management.
The biotech high-education in Romanian has started relative recently, the first accredited college being registered in 1996 in Bucharest, in the University of Agronomical Sciences and Veterinary Medicine having different specializations such as Agricultural Biotechnology, Industrial Biotechnology and Veterinary Biotechnology. In the mean time, the faculty leads to Master of Science degree in the fields of „Biotechnologies and Food Safety", „Biotechnologies in Environment Protection", as well as „Modern Applications of Biotechnology in Agriculture" (http://en.usamv.ro/faculty-of-biotechnologies).
Generally talking, Biotechnology is a growing industry and supposes to offer excellent opportunities, pay and benefits. These opportunities are available for people with a background in biological science with good laboratory and computer science skills (Frierman-Hunt G, 2008). In the past last years, the biotech graduates have faced difficult problems related to their

integration in the labour market in biotech fields (Matei, 2012). The causes are multiples and relate to biotech jobs market in Romania, student motivation, career counselling, don′t forgetting the last years of economical crisis.
In 2011 our team have started to implement a structural funds project, financed by POSDRU (HRDSOP-Human Resources Development Sectorial and Operational Program) in the Bucharest-Ilfov region. The main objective of the project is to increase the biotech graduates employability developing counselling services and implementing good quality internships. The project financing lasts two years, but there are undertaken measures to sustain its activities after the end of EU funding. As mains activities, the project developed a survey on biotech graduated employability in the labour market, as well as organizing career counselling sessions for students and creating a partnership with economical agents for internships development.

## MATERIALS AND METHODS

The project is run by a multi-actor team, consisting in teachers from biotechnology high education field (UASMV Bucharest, Faculty of Biotechnologies), researchers from the same field (CBM Biotehgen), human resources and counselling professionals, former and actual students, stakeholders from biotech sector (research institutes, food production and food safety, pharmaceuticals, cosmetics, environmental protection).

**1. Labour market insertion survey**

The target group for the survey consists of Biotechnology graduates between 2007 and 2011 in the region Bucharest-Ilfov. They questions addressed to their professional background and how they have passed the transition from school to active life on the market. The questionnaire consisted in eight questions batteries with answers on choice. The answers have been processed by tools of classical mathematical statistics.
The questionnaire has been send to the graduates via e-mails existent in the Faculty of Biotechnologies database, as well as by socialisation website (Facebook) and groups created along the years by former and actual students from the biotechnology field.

**2. Information, awareness and career counselling**

During March–May 2012 time frame and following the results′s survey the team has conducted a virulent campaign of biotech students information and awareness regarding the importance of their involvement in counselling sessions for their career development and in real and qualitative internships.
The information and awareness tools were oral communication in front of the classes, distribution of flyers and brochures, electronic messages via e-mails and socialisation networks, as well as the use of the campus visibility spots.
Between April 2012 and June 2012, 100 students (22 Master students and 78 licence students) have been involved in counselling sessions, where they have learned about self-knowledge, neuro-linguistic programming, CV content, letter of intention writing, as well as about behaviour during an interview. Also, it has been underlined the importance of the continuous learning.

**3. Preparing and developing biotech internships**

Having in the team teachers from biotech high education and stakeholders from the field, it has been created a partners network with economic operators from the biotech fields. The economic operators by their legal representative or operational representatives, have been contacted via e-mail, phone or in person. Considering the sectors were the faculty members and former students have been employed, the dialogue have been conducted in the research field, food industry, pharmaceutical industry, food safety authorities and laboratories, environmental protection.

## RESULTS AND DISCUSSIONS

**1. Labour market insertion survey**

We have received feedbacks from around 10% of the graduated students, from which 84% are living and working in Romania. The answers have come from licence graduates (54%) and from Master courses graduates (46%).
The statistical results show the following facts:
-67% from the licence students have follow further Master courses;
-50% from the graduates have followed other types of professional training by their own

funds, the main reason being the career advancement;
- 96% consider that they have a high level knowledge in the biotech field, while only 19% stated relevant knowledge in complementary fields;
- from the graduates sample, 40% have been already employed in the labour market, 29% were following other type or studies, mainly Master courses and 31% were in job hunting;
-regarding the way of finding a job, 14% graduate consider that the university network is the most important;
- very interesting fact, only 50% from them have been interested to find a job during the study, while the other half started the job hunting only after the graduation;
-from the employed graduates, 93% have a single job, while 7% have parallel jobs;
- 58% from the employed graduates have contracts on indeterminate time, while the rest have contract on determinate time;
- only for 35% of graduates the stated that the employers takes into account the licence or dissertation paper field and the marks obtained during the school;
- for the employer final decision, 37% considered that the university reputation has been important, while 44% consider that their personality was the most important;
-33% from the graduates consider that the Master courses are relevant for their employability, while only 10% consider useful doctoral and post-doctoral training;
-38% from the graduates considered that the biotech job offer in Romanian labour market is very limited.

**2. Information, awareness and career counselling**

During the conducted campaign regarding the importance of the biotech students′ involvement in counselling sessions and internships have been distribute around 350 flyers and 250 brochures. More than 200 students from licence and master courses have been informed and awarded about the opportunities given by the implementing project to get involved in counselling activities and to apply for an internship.

As a result of the awareness campaign, 100 students, between 20 and 30 years old, got involved in counselling sessions with professional from human resources (Mari Net 21 SRL). They have received a brochure regarding their possible professional biotech pathway and a mini-guide for the young employees. They have learned about self-knowledge, neuro-linguistic programming, CV content, letter of intention writing, as well as about behaviour during an interview.

Regarding the satisfaction grade, more than 93% from the counselled students considered that the sessions were very interesting and useful, as well as the received materials (table 1).

Table 1. Statistic on the students′ satisfaction grade after the counselling sessions

| **Quality indicator*** | **Very week** | **Weak** | **Good** | **Very good** | **Excellent** |
|---|---|---|---|---|---|
| The training content | 0 | 0 | 7.5 | 41.9 | 50.6 |
| Lecturer effort for the material accessibility | 0 | 0 | 1.1 | 21.5 | 77.4 |
| Lecturer efficiency in teaching | 0 | 0 | 3.2 | 29.0 | 67.8 |
| Lecturer expression clarity | 0 | 0 | 3.2 | 21.5 | 75.3 |
| Lecturer ability for alternative explanation, when applied | 0 | 0 | 3.2 | 34.4 | 62.4 |
| Used examples and illustration | 0 | 0 | 15.1 | 38.7 | 46.2 |
| Students′ encouragement to express themselves | 0 | 0 | 4.3 | 33.3 | 62.4 |
| Overall assessment | 0 | 0 | 8.6 | 38.7 | 52.7 |

*results are given in %*

As a special remark, all the counselled students from the licence level have decided to continue their further studies in Master courses.

**3. Preparing and developing biotech internships**

Having in the team teachers from biotech high education and stakeholders from the field, it has been created a partners network with 12 economic operators from the biotech fields, respectively agriculture and food and beverages production (Angst Buftea, VelPitar Bucharest, United Romanian Breweries Bereprod-Tuborg), pharmaceutical industry (Medica Group, Slavia Pharma, LabormedPharma), food

safety and consumer protection (DSVSA Bucharest, S.C. ICA Research & Development S.R.L.), hygiene products design (Evic Romania – Bio High Tech SRL) and biotech research (NARDI Fundulea, NICPRI Bucharest, Institut of Food Bioresources Bucharest).
Finally, 38% from the counselled students have been involved in 3 weeks of internships implemented with the help of the 12 economic operators, totalising 3420 internship hours.
When choosing the internship sector, the first choice of the students it was the pharmaceutical industry, being motivated by the higher jobs opportunities, as well as the higher salaries in the field. The second choice was the food industry, which in Romania is well developed and still offers job positions of high interest.
In a foreseeable manner, the last students' options were towards the national research institutes, because of the actual low financing opportunities.
After the internship a strong relation have been developed between the students and the hosts, and by March 2013, 27% of them have been employed in the existent network or in very similar companies related to the existent network.

## CONCLUSIONS

The statistical survey shows that about 40% from the 2006-2011 biotech graduates have found a job, while 29% were following other type or studies; less than 25% from the graduates have found jobs in biotech fields, such as scientific research and education, food and beverages production, environmental protecttion, including biofuel production, pharmaceutical products, instrumentation and suppliers.

In this regard, more effort should be done to create the partnerships between university, research institutes and economic operators leading to the increase of the absorption rate of biotech graduates into the labour market and to counsel the students from their early study years in finding a job.

Regarding the counselling sessions, more than 93% from the counselled students considered the training very interesting and useful, as well as the received materials. All the counselled students from the licence level have decided to continue their further studies in Master courses.

From the 38 students involved in internships, in less than one year 27% have been already employed in the existent network or in very similar companies related to the existent network. The others are continuing their studies.

It has been demonstrated that the internships firstly, give experience to the student. Secondly, the company gets to "look over" a prospective employee. Employers prefer to hire people they know over strangers. Thirdly, internships count as job experience. Listing an internship and the skills used in it on a resume will help get a job.

After meetings involving the project team, students and former students, as well as stakeholders, it has been concluded that the practical competences acquired during an internship have a huge impact on finding an adequate job in the biotech field.

The project activities, counselling and internships, will last one more year with EU funding. The team has already foreseen some measures to assure the activities continuation, by enlarging the partnership with economical operators and by proposing on the university level the establishment of a counselling bureau for the students, as well as for their parents.

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