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SUMMARY

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PRELIMINARY STUDIES ON *IN VITRO* BEHAVIOR OF VARIOUS SOMATIC EXPLANTS FROM SOME CULTIVATED *AMARANTHUS* GENOTYPES

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Abstract

The recent renewal of interest in underutilized crops of nutritional and economic potential for the agriculture of the future stimulated the research in Amaranthus sp. cultivation and breeding. In vitro systems have important practical applications not only for rapid breeding of this rediscovered crop but also for producing cell biomass to be used as source of phytochemicals of practical interest. The response of explants from hypocotyl, root and cotyledon node of three varieties of Amaranthus species (Amaranthus cruentus"Amont", Amaranthus hypochondriacus"Intense Purple"and Amaranthus ssp."Plenitude") were recorded, upon their cultivation "in vitro" on media supplemented with different combinations of auxins and cytokinins.Our experimental results pointed out that the explant type, the auxin supplement and the genotype were the most important factors in callus initiation. Calluses were induced most frequently on Murashige&Skoog-MS (1962) basal medium with 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D),whereas the root development occurred in the presence of NAA 1-2 mg/l(α-naphthaleneacetic acid). Proliferative callus derived from cotyledons and hypocotyls of the studied Amaranthus species were transferred to MS media enriched with hydolyzated casein, NAA and kinetin in order to compare their morphogenetic capacity for plant regeneration.

Key words**:** *Amaranthus species, callus initiation -tissue culture*

INTRODUCTION

The nowadays renewal of interest in underexploited crops resulted in intensive efforts made for identification / cultivation and quality evaluation of many plant species that for a long time enjoyed only a local importance. A suitable plant alternative crop must have reduced requirements for inputs (especially water and energy-intensive fertilizers), must withstand adverse environmental conditions and have a high growth rate, a higher energy efficiency and produce substances that can be used in energy, food and industry [5].

In this context, the attention focused mainly on three ancient *Amaranthu*s species: *Amaranthus caudatus* (L), *Amaranthus hypochondriacus* (L) and *Amaranthus cruentus* (L) which are at present cultivated worldwide because of their exceptional nutritional value of both seeds and leaves. Additionally, the species are widespread ornamentals and also have a potential as forage crops and as sources of red food colorants, of antioxidant compounds and of other valuable phytochemicals such as α-amylase trypsin inhibitors and other active compounds with important uses in medicine. Besides, this re-discovered crop has some agricultural advantages and noted ability to grow successfully in adverse environmental conditions, such as high irradiance, temperature and drought. [2, 3, 10].

"Grain amaranth" is a name commonly used for certain lines of at least three species of the family Amaranthaceae, viz. *A. hypochondriacus* A., *A. caudatus* L. and *A. cruentus* L. Though centuries ago grain amaranth was a staple food in Aztec and other Mexican Indian diets, only in the 1970s some research reports revealed the nutritional value of these tiny grains.

The seed contain about 17-19% (of seed dry eight) high quality protein (5% lysine and 4% sulfur-containing amino acids) and 63% easily digestible carbohydrates, as compared to more traditional crops that have an average of approx.10% proteins. In *Amaranthus* 50% of the total seed proteins at maturity are globulin and albumin [6,9].

Some varieties of *Amaranthus* species (AMA 5, V2, AMA 18, VOP, etc..), were recorded as containing different concentrations of vitamin C, nitrogen and minerals [7]. Extensive scientific research was lately conducted on the biology, ecology, biomass accumulation, and harvest quality Amaranthus species (*Amaranthus cruentus* L., *A. hypochondriacus* L., *A. Caudatus* L.). The relatively high content of essential aminoacids in Amaranthus recommended it as a possible substitute for meat. Thus, the use of Amaranthus grain may have implications both in food intended for human consumption and in the diet for certain special categories of consumers. Starting with the 8th decade of the twentieth century, Amaranthus species began to be exploited commercially, but its market is still limited to people allergic to gluten products, local traditional medicine and regional restaurants. In the relevant scientific literature there were published results on the cultivation technologies, elements of culture, acclimatization methods, the chemical composition, oil production and its composition [6].

The underexploited crops, including amaranth, offer a special challenge for the use of *in vitro* approaches, because extensive efforts are required from the plant breeders to select and improve this plant material.

Currently, there are not much published reports on the tissue culture of *Amaranthus* sp.,but among the practical applications of tissue culture in amaranth we shell mention micropropagation of selected genotypes and their subsequent exploitation, rescue of the genetic variation or inducing new variation, phytoremediation studies and using the cell biomass to obtain phytochemicals of practical interest.

Previous studies conducted by H. Flores et al. (1982) and A. Bennici et al. (1992) [1, 4] on several species and varieties of the genus *Amaranthus* showed its potential with regard to dedifferentiation and morphogenetic processes *in vitro,* with emphasis on age dependant competence of explanted tissues and the cytokinin / auxin ratio in the culture medium

With this background, the aim of the present study was to evaluate the growth and morphogenetic responses of various types of explants from three varieties of Amaranthus species cultivated *"in vitro": Amaranthus cruentus* "Amont", *Amaranthus hypochondriacus* "Intense Purple" and *Amaranthus* ssp."Plenitude".

MATERIAL AND METHOD

The seeds belonging to three varieties of *Amaranthus* species (*Amaranthus cruentus* "Amont", *Amaranthus hypochondriacus* "Intense Purple" and *Amaranthus* ssp. "Plenitude" were germinated "*in vitro*", in aseptic conditions, on the basal medium of Murashige-Skoog (1962), having half strength as regards the concentration of macro and microelements, 3% sucrose , 0.8% Agar Noble, pH 5,8, without the addition of hormones (Fig. 1).

For successful aseptic cultivation of explants in "*in vitro*" conditions, an important role is played by the chemical composition of the culture media. Preferential depletion of some elements leads to symptoms of deficiency or toxicity, sometimes with necrosis of the inoculum.

The basal medium Murashige - Skoog (MS), rich in nitrogen meets the nutritional requirements of explants cultured *in vitro* for most species [8]. This was also the optimal formulation for the initiation and for the transfer of *"in vitro"* cultures in *Amaranth*, following the supplementation of the culture medium with several types and combinations of phytohormones (Table 1).

a)

b)

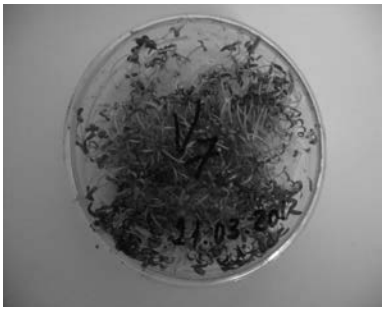

Fig. 1. *Amaranthus* seeds germinated in controlled laboratory conditions:
a.) in the Erlenmeyer flask;
b.) in Petri dishes (photo date: 3/29/2012)

Plant hormones were added before sterilization of the media by autoclaving, which was performed for 20 minutes at 121 °C, and pH correction was made to values of 5.8 to 6.

Table 1. Experimental versions of the culture medium used for in vitro culture initiation and establishment from *Amaranth* tissues

	Growth regulators				Nutritive Supliment
Variants	Auxins– mg/l			Citokinins – mg/l	CH mg/l
	NAA	2,4-D	IAA	Kin	
V1	2	-	-	1	-
V2	1	0,5	-	0.5	-
V3	2	-	-	1	200
V4	1	0,5	-	0.5	200
E7	-	-	1.8	0.022	-

Legend: NAA = acid α –naphthalene-acetic; 2,4-D = acid 2,4-dichlorophenoxyacetic ; IAA acid indolil acetic; Kin = chinetin; CH = casein hydrolyzate of.

Three types of explants (hypocotyl, cotyledon node and roots) were placed on the surface of the culture media (Variants 1 and 2) distributed in 5 cm in diameter Petri plates (containing 5 ml of sterile autoclaved culture medium variants solidified with 8 g/l agar) and the incubation was performed in the growth chamber, at 25± 2 °C, under a 16/8 h photoperiod, with a light intensity of 3000 lux. The periodical transfers on fresh culture media were performed at 3 week intervals.

The mean increasing of callus biomass / petri dish and the other evaluated morphogenetic processes (hypertrophy, appearance of adventitious roots) were recorded at intervals of 3 weeks for 3 months, depending on the type of explant and on the *Amaranthus* genotype from which explants originated.

Three different combinations of citokinins and auxins were used in the first phase of the initiation and establishment of callus cultures (V1, V2 and E7). Callus development was evident at 2 weeks after inoculation on the inductive culture media. First developed on the cut edges, callus covered gradually the whole explant over periodic transfers. The proliferative capacity of the callus cultures increased following the periodic transfers on fresh culture media, every 3 weeks for 3 months (Table 2).

Table 2. Comparative effect of the hormonal supplement of the media variants used to initiate and establish "in vitro" cultures from explants from the three genotypes of *Amaranthus* sp..

Recipe	*Amaranthus* sp. genotypes		
	Amaranthus cruentus **"**Amont*"*	***Amaranthus hypochondriacus*** **"**Intense Purple"	***Amaranthus ssp."***Plenitude"
V1	58.33%	92.15%	90.07%
V2	**66.07%**	**100%**	**100%**
E7	64.7%	**100%**	88.36

Legend: V1 (MS-1962 medium,supplemented with: 20 g / l sucrose, 7 g / l agar, 2.0 mg / l NAA and 1.0 mg / l Kin); V2 (MS-1962 medium,supplemented with: 30 g / l sucrose, 8 g / l agar, 1.0 mg / l NAA + 0.5 mg / l and 2,4-D 0.5 mg / l Kin); E7= 1,8 mg/ L IAA+0.022 mg/ l Kin

Calli developed on these variants were yellow-green, partly loose, with morphogenesis expressed by developing adventitious roots in small numbers (to *Amaranthus cruentus* **"**Amont" genotype) in average number (to *Amaranthus* ssp "Plenitude") and in large numbers (to *Amaranthus hypochondriacus* "Intense Purple").

After 3 months of ”in vitro” culture, the recorded results according to the genotype and to the type of the explant were as follows:

I. For the genotype *Amaranthus cruentus* “Amont”:

- from the root explants were obtained: 64% hypertrophied explants, 60% thereof with callus and 46% with adventitious roots;
- from the explants of hypocotyl were obtained: 100% explants were hypertrophied: 62.5% thereof with callus and 34.37% with adventitious roots;
- from the cotyledon node explants were obtained: 88.33% hypertrophied explants, of which 50.0% with callus and 46.91% with adventitious roots.

II. For the genotype *Amaranthus hypochondriacus* “Intense Purple” :

- from the root explants were obtained 100% the hypertrophied explants: 53% thereof with callus and 61.53% with adventives roots;
- from the hypocotyl explants were obtained 100% hypertrophied explants: 100% thereof with morphogenetic callus and without adventitious roots;
- and from the cotyledonary node explants were obtained 100% hypertrophied explants, 100% thereof with callus and 13.6% with adventitious roots.

III. For the genotype *Amaranthus* ssp "Plenitude":

- from the root explants resulted: 100% hypertrophied explants of which: 50.79% with callus and 59.52% with adventitious roots;
- from of hypocotyl explants resulted 96% hypertrophied explants of which: 71% with callus and 26% with adventitious roots;
- and from the cotyledon node inoculated explants resulted 54.48% hypertrophied explants of which: 84.56% with callus and 19.51% with adventitious roots.

Calluses developed from inoculated hypocotyl fragments on hormone supplements medium variants V1 and V2, were yellow-green, friable and expressed morphogenesis by developing adventitious roots, though in a fewer number than in the roots explants (Fig.2).

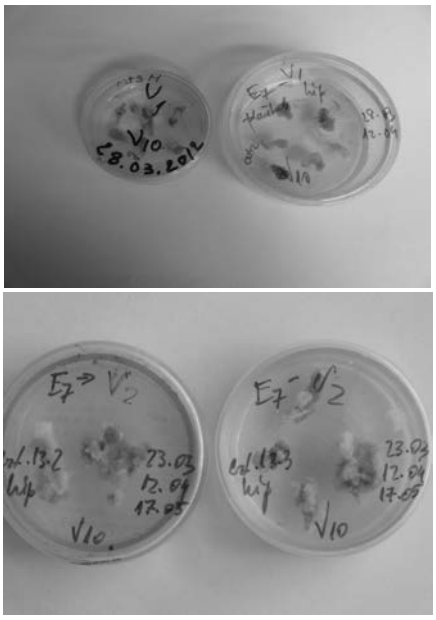

Fig. 2. The influence of explant type on morphogenesis induction in inoculated *Amaranthus* ssp "Plenitude" hypocotyl type of explants: a.) V1 (MS medium,supplemented with: 20 g / l sucrose, 7 g / l agar, 2.0 mg / l NAA and 1.0 mg / l Kin) b.) V2 (MS medium,supplemented with: 30 g / l sucrose, 8 g / l agar, 1.0 mg / l NAA + 0.5 mg / l and 2,4-D 0.5 mg / l Kin).

Calluses developed from the cotyledon node inoculated explants on variants V1, V2 and E7 hormone supplements medium, were yellow-green, loose, had a low rate of the morphogenetic development and formed adventitious roots in a smaller number of inoculated explants (Fig.3).

During the regular transfers performed every 3 weeks for 3 months on media variants V1, V2 and E7, in the presence of moderate concentrations of auxins and citokinins, a 100% multiplication rate was recorded on the V2 and E7 variants (Table 2). Development of multiple shoots from each apex was lower on V1, which consisted in a combined hormonal supplement of naphthyl acetic acid and kinetin.

By transferring the callus culture developed "in vitro" from hypocotyl and cotyledon node explants on the variants V2,V3, V4 and E7 (Table 3), the callus biomass increased every 3 weeks averaging close values, measured by the number of viable proliferative explants / culture dish.

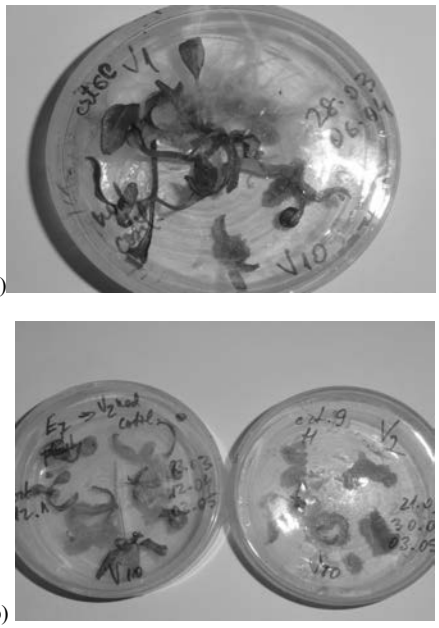

Fig. 3. The influence of explant type (cotyledon node) on morphogenesis induction in *Amaranthus* ssp "Plenitude"; a.) V1 (MS medium supplemented with: 20 g / l sucrose, 7 g / l agar, 2.0 mg / l NAA and 1.0 mg / l Kin) b.) V2 (MS medium supplemented with: 30 g / l sucrose, 8 g / l agar, 1.0 mg / l NAA + 0.5 mg / l and 2,4-D 0.5 mg / l Kin) and E7 (MS medium supplemented with: 30 g / l sucrose, 8 g / l agar, 1.8 mg / l IAA and 0.022 mg / l Kin)

Table 3. Compared effect of the phytohormone combinations (V2, V3, V4 and E7) on callus development after 60 days since the initiation of the experiment

Recipe	*Amaranthus* sp. genotypes		
	Amaranthus cruentus "Amont"	*Amaranthus hypochondriacus* "Intense Purple"	*Amaranthus ssp."*Plenitude"
V2	89.04%	91.66%	81.91%
V3	84.61%	77.7%	87.06%
V4	91.66%	90.12%	91.83%
E7	_	_	94.28%

Legend: V2= (MS medium,supplemented with: 30 g / l sucrose, 8 g / l agar, 1.0 mg / l NAA + 0.5 mg / l and 2,4-D 0.5 mg / l Kin); V3= NAA 2 mg / L + 1 mg / L Kin + 200 mg / L hidrolizat; V4= NAA 1 mg/ L+2,4 D-0,5 mg/ L +Kin- 0,5 mg/ L +200 mg/ L casein hydrolyzate: E7= 1.8 mg/ L IAA+0.022 mg/ l Kin.

Superior results were recorded on the variant V4, with values of calluses /culture vessel ranging from 90.12% to 91.83% for all the tested genotypes, and on variant E7 (94.28%), but only for root explants from *Amaranthus ssp."*Plenitude" genotype.

CONCLUSIONS

The underexploited crops, including amaranth, offer a special challenge for the use of *in vitro* approaches for micropropagation of selected genotypes and their subsequent exploitation, rescue of the genetic variation or inducing new variation, phytoremediation studies and using the cell biomass to obtain phytochemicals of practical interest.

Our observations on the effect of phytohormones on the evolution of *Amaranthus* sp. explants in *"in vitro"* culture conditions after 3 weeks since inoculation led to conclude that the phytohormones such as the auxins NAA, 2,4-D and IAA had a stimulating effect on callus development and morphogenesis expressed by the development of adventitious roots.

Thus, callus induction, callus growth and organogenetic processes expressed by root development were achieved under the effect of moderate concentrations of these auxins, alone or in combination with low concentrations of citokinins (kinetin).

Therefore, our experimental data reveal the potential of somatic explants of *Amaranthus sp.* to develop *in vitro* long-term, continuous callus cultures, under the effect of optimal hormone concentrations, which alongside the genotype and the type of explants represent important factors that influence the obtaining of cell biomass in reliable quantities, to be used for practical purposes.

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BIOLOGICAL CHARACTERIZATION OF SOME MICROORGANISMS OF BIOTECHNOLOGICAL INTEREST

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Abstract

Successful use of biological insecticides depends on their "active substance" quality, which ensures the biological efficacy and performance of bioproducts in field conditions: the active substance have to be virulent and to have ecological competences (epizootiological qualities, multiplication capacity, spreading and persistence in habitats and target pests populations).These parameters are objective criteria for selection of microorganisms that can be successfully included in biological control programs of pests. In this paper are presented results of some laboratory tests aimed to estimate biological parameters specific for entomopathogenic fungi, including the spores germination and spores production on culture media and insects. The biological material used in this study consisted in five Beauveria bassiana entomopathogenic biotypes, isolated from insects belonging to the ord. Coleoptera, Hymenoptera and Diptera in natural epizootic outbreaks, during the years 2008-2010. The results led to the following conclusions: (i) B. bassiana strains having different origins in terms of habitat and host insect exhibit different biological qualities, (ii) the evaluation of test insects mortality induced by B. bassiana doesn't provides a complete pathological characterization of fungal isolates; to assess the biological control potential of B. bassiana isolates, the virulence have to be correlated with the biological cycle of the fungus and the spores production on the insect cuticle; (iii) the quantification of spore production on insect is also necessary to further evaluate the fungal strains potential to naturally increase of virulence.

Key words**:** *entomopathogenic fungi, biotypes, Beauveria bassiana*

INTRODUCTION

Agricultural production is still the main source of food for humankind. Population explosion has determined the increase of agricultural production in response to growing food requirements. The growth in human population around the world affects all people through its impact on the economy and environment. In many cases it was made an irrational exploitation of natural resources leading to ecological imbalance. One of the biggest ecological challenges facing plant protectionists is the development of environmentally friendly alternatives to the extensive use of chemical pesticides for crop diseases and pests control. Improvements in crop management systems can have an important influence on product quality as well.

Uses of synthetic pesticides led to undesirable effects on non-target organisms sharing the ecosystem and decrease the loss of efficacy due to adaptation of pest agents. Moreover, the toxicity of their residues affects the environment and people health too (Keller&Brenner, 2005; NRC, 1996; Robinson et. al, 1980).

Research on biological pest control methods can make an important contribution to reducing the use of chemicals for crop protection. (Butt&colab, 2001; Gerhardson, 2002; Lacely&colab., 2001).

Beauveria bassiana (Bals.) Vuill. is an enthomopathogenic fungus that grows naturally in soils (Steinhaus, 1956). It is being used as a biological insecticide all over the world in order to control pests such beetles, whiteflies, aphids, mealybugs, grasshoppers etc.

B.bassiana strains vary in their host ranges, having, generally, a wide host range. *B.bassiana* strains isolated from different hosts inhabiting different geographical regions differ in theirs ecological capacities.

Ecological competences of different strains (epizootiological qualities, multiplication capacity, spreading and persistence in habitats and target pests populations) are objective criteria for selection of those which can be successfully included in biological control programs of pests (Andrei, 1999).
Several strains of the entomopathogenic fungi (including 5 strains of *B. bassiana* presented in this experiment) have been isolated in order to use them for biological insecticides production at the Research-Development Institute for Plant Protection Bucharest.
This work aimed at selecting *B. bassiana* strains favourable growth, sporulation, viability and infectivity responses on different culture media.

MATERIAL AND METHOD

Five strains of *B. bassiana* were used as biological material in this study. All strains were isolated from natural outbreaks, purified and stored on sterilized potato dextrose agar (PDA) slants, at 4^0C (Table 1).

Table 1. *Beauveria bassiana* strains isolated from natural epizootic outbreaks during the period 2008-2010

Fungal strain	Insect		
	Name and order	Isolated from/at	Natural outbreaks
BbIt	The European spruce bark beetle (*Ips typographus*) Coleoptera	Iacobeni / Sept. 2010	Spruce trunk in a coniferous forest
BbAl1	East Asian sawfly (*Aprocerus leucopoda*) Hymenoptera	Şoldăneşti, / June 2010	Elm leaf in a deciduous forest
BbBrodoc		O.S. Brodoc / July 2011	
BbCr	Leaf beetle (*Chryptocephalus* sp.) Coleoptera	Cap Kaliakra / Aug. 2009	Sunflower crop
BbSc	Fungus gnats (*Sciara* sp.) Diptera	Topoloveni, Aug. 2008	Pot with wet soil

The strains used in the experiment were grown on plates using three different culture media (Sabouraud, Czapek-Agar and PDA) as monosporal cultures, and incubated at 25^0C, under dark conditions. For each strain were prepared separately nine Petri dishes, three plates for each type of culture media (three replications).

The colonies were measured daily during 14 days. Macroscopic observations were made at 10 and 15 days after inoculation and were analyzed the average growth of colonies, their appearance, the sporulation, the emergence of sporulating structures and pigmentation for every strain and for each of the three media.
The experiment aimed also the virulence of these five strains. Insects from order Coleoptera and Lepidoptera (2^{nd} and 3^{rd} larval stage) were selected for this purpose: *Galleria mellonella* L., *Plodia interpunctella* (Hübner) and *Tenebrio molitor* L. The artificial inoculation was made by spraying on insects the *B.bassiana* conidial suspensions. The larval mortality was analyzed after 5 and 10 days. The sporulation rates of each strain on death test insects were analyzed after 30 days using serial dilution method for counting fungal spores.

RESULTS AND DISCUSSIONS

The influence of solid media on the development of the *B. bassiana* strains is presented in Table 2-7.
The growth rate was monitored daily by measuring the colony diameter.
The highest mycelial growth rate of *B. bassiana* colonies cultivated on Czapek medium was observed at BbAl strain (Fig. 1). The average fungal colonies diameter was 39.67 mm. The lowest growth rate was recorded for BbIt strain, with an average diameter of 33.33 mm colony growth after 14 days.

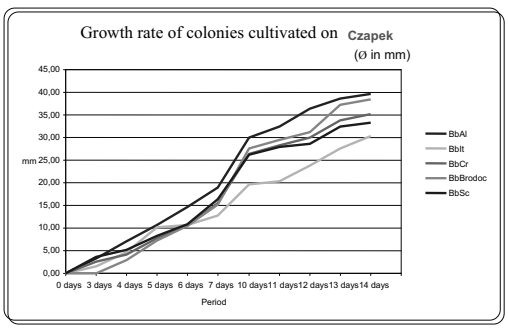

Fig. 1. Growth rate of colonies cultivated on Czapek medium

On Sabouraud medium, the highest mycelial growth rate of colonies was observed at BbCr strain. The average fungal colonies diameter

was 32.23 mm. The lowest growth rate was recorded for BbSc strain, with an average diameter of 19.11 mm colony growth after 14 days (Fig 2).

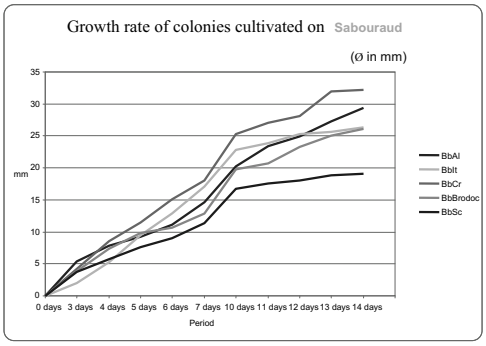

Fig. 2. Growth rate of colonies cultivated on Sabouraud medium

The highest mycelial growth rate of *B. bassiana* colonies on PDA medium was observed at BbCr strain. The average fungal colonies diameter was 45.50 mm. The lowest growth rate was recorded for BbBrodoc strain, with an average diameter of 29.78 mm colony growth after 14 days (Fig 3).

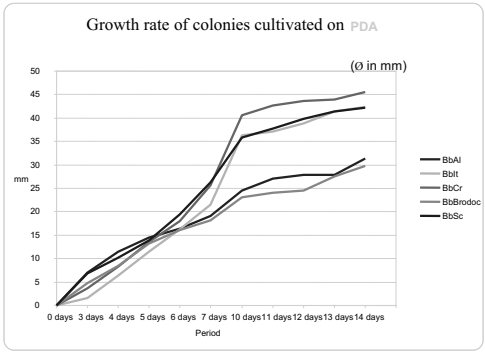

Fig. 3. Growth rate of colonies cultivated on PDA medium

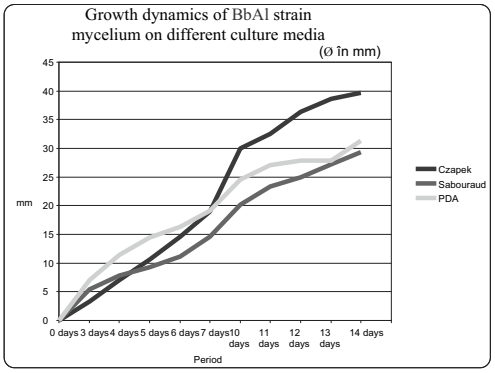

Fig. 4. Growth dynamics of BbAl strain mycelium on different culture media

The highest increase in BbAl mycelial colony diameter was recorded on those cultivated on Czapek medium (39.67 mm), followed by those on PDA (31.39 mm) and Sabouraud (29.33 mm) (Fig 4).

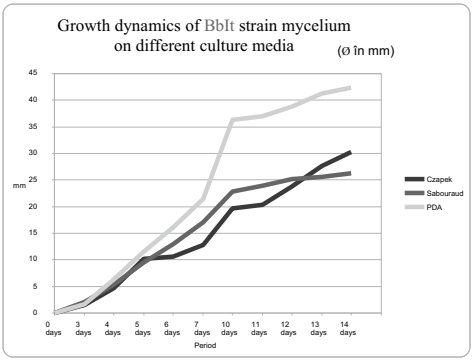

Fig 5. Growth dynamics of BbIt strain mycelium on different culture media

The highest increase in BbIt mycelial colony diameter was recorded on those cultivated on PDA medium (42.33 mm), followed by those on Czapek (30.33 mm) and Sabouraud (26.29 mm) (Fig. 5).

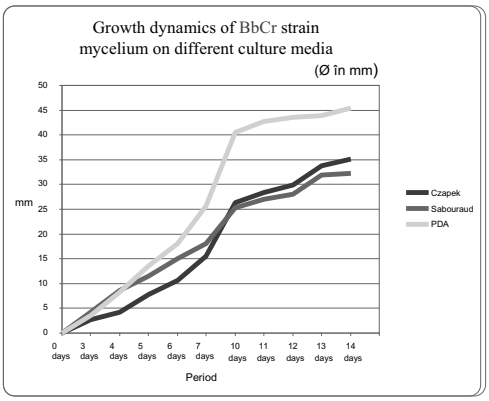

Fig. 6. Growth dynamics of BbCr strain mycelium on different culture media

The highest increase in BbCr mycelial colony diameter was recorded on those cultivated on PDA medium (45.50 mm), followed by those on Czapek (35.13 mm) and Sabouraud (32.23 mm) (Fig 6).

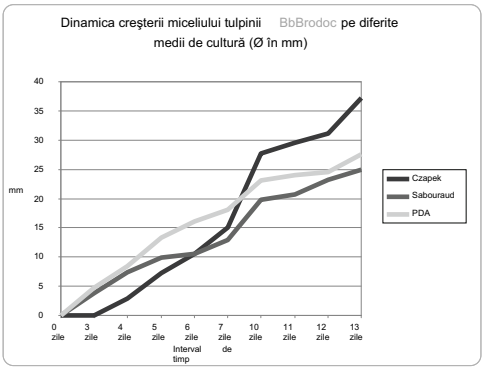

Fig. 7. Growth dynamics of BbBrodoc strain mycelium on different culture media

The highest increase in BbBrodoc mycelial colony diameter was recorded on those cultivated on Czapek medium (38.50 mm), followed by those on PDA (29.78 mm) and Sabouraud (26.11 mm) (Fig 7).

The highest increase in BbSc mycelial colony diameter was recorded on those cultivated on PDA medium (42.19 mm), followed by those on Czapek (33.33 mm) and Sabouraud (19.11 mm) (Fig 8).

The influence of culture media in viability test of conidia of entomopathogenic fungi was experimented on *Lecanicillium lecanii*, *Beauveria bassiana* and *Paecilomyces fumosoroseus* isolates (Francisco et al., 2006).

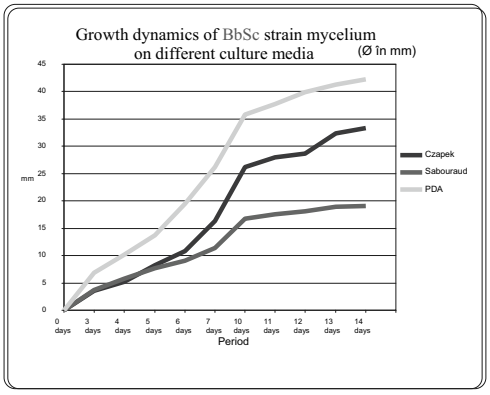

Fig. 8. Growth dynamics of BbSc strain mycelium on different culture media

The tests were performed on microscope slides containing one of the culture media: agar-water, minimal medium, potato-dextrose-agar, potato-dextrose-1% yeast extract agar, sabouraud-dextrose-yeast extract-agar and complete medium. The culture media influenced the germination of the species studied, verifying within and inter specific variations. *B. bassiana* development was also tested in different liquid cultures, resulting in different yield of blastospores. In peptone-glucose, the yeald of blastospores was four-fold higher than in glucose-peptone-yeast extract (Bidochka et al., 2004).

Rombach (2006) tested several simple liquid media for *B. basiana* submerged conidiation. The results proved maximum yields of conidia in medium consisting of sucrose-yeast extract and basal salts and maximum yields of hyphal bodies in a sucrose-yeast-extract medium. More than that, Kmitowa (1979) studied the effect of the quantitative gradient of nitrogenous compounds added to the culture medium has on the growth and pathogenicity of over 30 *B. bassiana* strains. The author reported that among the *B. bassiana* strains under study some formed poorly growing and weakly sporulating colonies while others grew rapidly and proliferously forming dense and sporulating colonies. Biomass of some strains exceeded many times that of the others. The pathogenicity of these strains was not correlated with their rates of growth.

Colonial morphology of *B.bassiana* biotypes is presented in Photo 1-8 and Table 2-6.

BbAl strain growth and sporulation

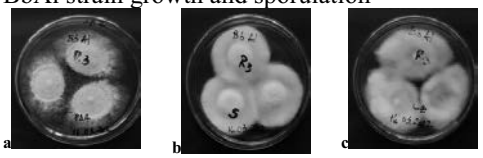

Photo 1. Mature colonies of BbAl strain grown on three different culture media: a. PDA; b. Sabouraud; c. Czapek

Table 2. Colonial morphology of BbAl strain grown on different culture media

Colony morphology (10 days after inoculation)	BbAl strain on culture media		
	Czapek	Sabouraud	PDA
Shape	circular	circular, raised	circular
Elevation	flat	hemispheric, fluffy mycelia	convex
Margin	regular edge	entire	entire
Opacity	translucent	opac	opac
Sporulation	unsporulated	unsporulated	sporulated

BbIt strain growth and sporulation

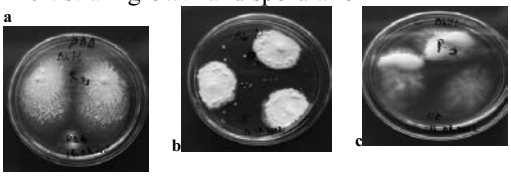

Photo 2. Mature colonies of BbIt strain grown on three different culture media: a. PDA; b. Sabouraud; c. Czapek

Table 3. Colonial morphology of BbIt strain grown on different culture media

Colony morphology (10 days after inoculation)	BbIt strain on culture media		
	Czapek	Sabouraud	PDA
Shape	circular	circular	circular
Elevation	flat	crateriform	flat
Margin	filiform	curled	regular edge
Opacity	translucent	opac	translucent and 1/3 opac
Sporulation	sporulated	sporulated (2/3 started from center)	sporulated (1/3 started from center)

BbCr strain growth and sporulation

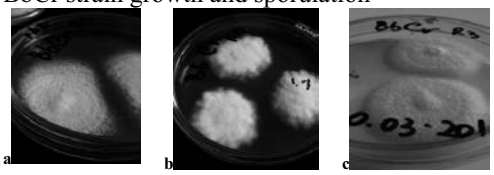

Photo 3. Mature colonies of BbCr strain grown on three different culture media: a. PDA; b. Sabouraud; c. Czapek

Table 4. Colonial morphology of BbCr strain grown on different culture media

Colony morphology (10 days after inoculation)	BbCr strain on culture media		
	Czapek	Sabouraud	PDA
Shape	circular	circular	circular
Elevation	flat	crateriform	flat
Margin	regular	curled	regular
Opacity	translucent	opac	translucent in growing area and opac in area with sporulation
Sporulation	sporulated	sporulated	sporulated

BbBrodoc strain growth and sporulation

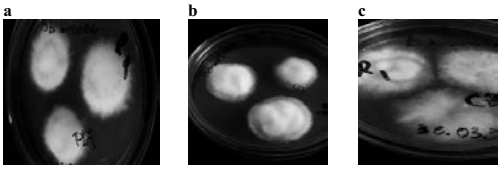

Photo 4. Mature colonies of BbBrodoc strain grown on three different culture media: a. PDA; b. Sabouraud; c. Czapek

Table 5. Colonial morphology of BbBrodoc strain grown on different culture media

Colony morphology (10 days after inoculation)	BbBrodoc strain on culture media		
	Czapek	Sabouraud	PDA
Shape	circular	circular, gibbous	circular, slightly bulging
Elevation	plat	hemispheric fluffy mycelia	flat
Margin	filiform	entire	regular
Opacity	translucent	opac	opac
Sporulation	unsporulated	unsporulated	unsporulated

BbSc strain growth and sporulation

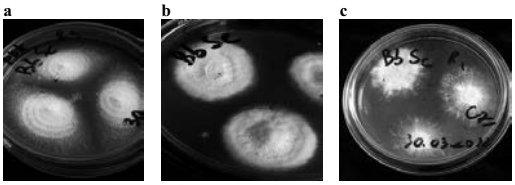

Photo 5. Mature colonies of BbSc strain grown on three different culture media: a. PDA; b. Sabouraud; c. Czapek

Culture media differentially influenced the growth, colony character and sporulation of *B.bassiana* tested strains. The results reported by Barnes G.L. (Barnes et al., 1975) proved

that *B. bassiana* growth and sporulation are influenced by various peptone sources in culture.

Table 6. Colonial morphology of BbSc strain grown on different culture media

Colony morphology (10 days after inoculation)	BbSc strain on culture media		
	Czapek	Sabouraud	PDA
Shape	circular	circular, gibbous	circular, slightly bulging
Elevation	plat	hemispheric, fluffy mycelia	flat
Margin	filiform	entire	regular
Opacity	translucent	opac	opac
Sporulation	sporulated	sporulated	sporulated in concentric circles

Photo 6. Fungal coremia of BbSc strain grown on Sabouraud

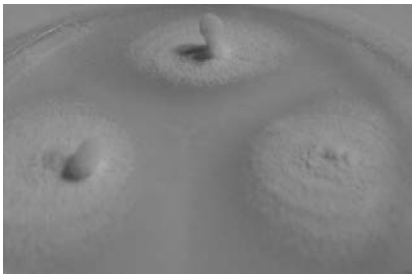

Photo 7. Fungal coremia of BbSc strain grown on PDA

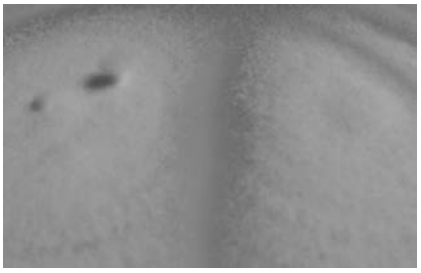

Photo 8. Fungal coremia of BbSc strain grown on Czapek

Pathogenicity tests performed in laboratory conditions and quantified in external sporulation on test-insects (Photo 9) proved that the saprophytic development of *B. bassiana* biotypes vary from one to another strain (Table 7).

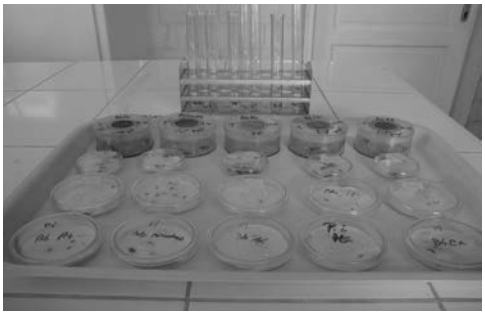

Photo 9. Pathogenicity test on insects

From all tested strains, BbSc strain recorded the largest amount of spores, both on *G. mellonella* and *P. interpunctella* larvae.

Table 7. *B. bassiana* strains spore production on test insects

B. bassiana strains	test insects	
	Galleria mellonella	*Plodia interpunctella*
	spore concentration ml^{-1}	spore concentration ml^{-1}
BbCr	0.525	1.175
BbSc	0.825	10.9
BbIt	0.425	0.975
BbBrodoc		0.675
BbAl		0.55

CONCLUSIONS

The results of this work show that BbAl and BbCr strains are recommended for further investigation in order to determine the most favourable substrate for mass production of bioformulated products.

Statistically, PDA environment has provided the best carbon source for vegetative development *B.bassiana* strains.

B. bassiana tested strains, regardless of their origin, has biotechnological potential, related to the ability to degrade various synthetic nutritive substrates and to keep the pathogenicity against insects belonging to different orders.

Spores production tests proved different capacities of *B. bassiana* biotypes to efficiently exploit the nutritive substratum.

B. bassiana strains with different origins in terms of habitat and host insect exhibits different biological qualities.

The evaluation of test insects mortality induced by *B. bassiana* doesn't provides a complete pathological characterization of fungal isolates; to assess the biological control potential of *B. bassiana* isolates, the virulence have to be correlated with the biological cycle of the fungus and the spores production on the insect cuticle.

The quantification of spore production on insect is necessary to further evaluate the fungal strains potential to naturally increase of virulence.

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ISOLATION AND IDENTIFICATION OF LOCAL WINE YEAST STRAINS FROM DEALURILE BUJORULUI VINEYARD

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Abstract

In the last years it has been given a high importance to promote on the market the Romanian wines with strong indigenous character related mainly to their sensorial profiles (aroma and taste). The wide use of commercial starter cultures, mainly applied to reduce the risk of spoilage and unpredictable changes of wine flavour*, can ensure a balanced* wine flavour*, but it may also cause a loss of characteristic aroma and* flavour *determinants. This work belongs to a wider project in which, the goal is to demonstrate how the local yeast strains produce volatile compounds and fermentation metabolites which finally will modulate the variety characters on sensorial level. During 2009 -2010, from hundreds of yeast strains isolated from DealurileBujorului vineyard,9 yeast strains have been kept for their fermentative power. Classical and molecular identification of these strains have been performed. The classical identification has followed mainly the oenological practices and classification. The results show that these strains belong to the specie Saccharomyces cerevisiae under different subspecies groups (according to technological classification): S. ellipsoideus, S.bayanus, S.rosei, S.oviformis. By the use of molecular tools (ITS amplification), all the strains belongs undoubtedly to Saccharomyces cerevisiae group, but the instraspecific polymorphism is important. All the strains will be subject of fermentative and flavour development tests on local grapes variety.*

Key words**:** *Dealurile Bujorului vineyard, local yeast strains, Saccharomyces cerevisiae, autochthonous wine.*

INTRODUCTION

It is well known that Romania is situated in top 10 wine producers in the world, with an average production of 5.5 mil hl/year. Almost 1 mil hl goes to the export. Taken into account that the Romanian wines have to compete with well know wines coming from other traditional wine-making countries (France, Italy, Spain, etc) or more recent producers as USA, Argentina or South Africa, there is a special need to find solutions to be competitive on the market. In the last years it has been given a high importance to promote on the market the Romanian wines with strong indigenous character related mainly to their sensorial profiles (aroma and taste) [3,8,9]. The distinctive flavour of wine depends on many variables as grape variety, viticultural practices, soil composition, which will influence the sensory descriptive analyses. In the same time the influence of the local yeast strain should be taken into account [9, 10].

The wide use of commercial starter cultures, mainly applied to reduce the risk of spoilage and unpredictable changes of wine flavour, can ensure a balanced wine flavour, but it may also cause a loss of characteristic aroma and flavour determinants.

This work belongs to a wider project in which, the goal is to demonstrate how the local yeast strains produce volatile compounds and fermentation metabolites which finally will modulate the variety characters on sensorial level.

The work of valuable wine yeast isolation undergoes a few steps (isolation, purification, identification, laboratory testing and pilot-scale testing for their oenological characters). In this paper we will describe the identification step for strains isolated from a relative cool climate vineyard from Moldavia region, Romania.

Also, in the very last years, from different vineyard of colder climate (Northern Europe) have been isolated, aside strains of *S.cerevisiae*, different hybrids belonging to *S.uvarum, S. pastorianus*or*S.kudriavzevii* [4, 11, 12].

MATERIAL AND METHOD

The origin of the yeast isolation is situated in DealurileBujorului vineyard (Galati county), belonging to the Moldavian winemaking areal, specific for the production of white wines of high quality under a relative cooler climate.

Sampling and yeast isolation. The sampling has been performed during 2 harvest years, respectively 2009 and 2010. The grape berries have been harvested in September and October with pedicel. For each grape variety have been harvested samples from 3 points of the plot, from different levels of the rope: ground, middle and top.

The samples have been prepared in two ways. For the first series the berries have been harvested in aseptic conditions and introduced in an Erlenmeyer with sterile distillate water in order to collect the yeast from the grape surfaces. After 24 hours of shaking, from the suspension, plates with YEPD have been inoculated and cultivated during 48–72 hours at 27^{o}C.

For the second series, the berries have been crushed in aseptic conditions and the natural fermentation has occurred. During the fermentation, three moments for sampling were chosen: beginning, middle and final phase of the fermentation. After applying Domerq dilutions, the samples have been inoculated on YEPD.

Every isolated colony has been passed in slant on GYP medium and pure cultures have been obtained which were the identification subjects.

Yeast identification and characterization.

Two approaches have been followed for the identification: classical morphological and a molecular approach, by the use of ITS-RFLP.

In the case of classical identification there have been examined morphological and cultural aspects (shape and cell dimension of the cell) of each strain on liquid media after 3 days cultivation on GYP media at 27^{o}C and on solid media YEPD; also, the pseudomycelia formation on potato-agar media after 12 cultivation days have been observed.

For the molecular approach, the strains of oenological interest have been cultivated on GYP at 27^{o}C during 24 horus/160rpm. The biomass has been collected and the DNA extraction has been performed according to an adapted method of Hoffman and Winston (1987) consisting of grinding the yeast with glass beads, phenol-chloroform extraction, and isopropanol precipitation [6]. The DNA has be conserved at -20^{o}C until the PCR analysis.

For the PCR amplification has been the used the following ITS primers:

ITS 1 (5'-TCCGTAGGTGAACCTGCGG –3') and

ITS 4 (5'-TCCTCCGCTTATTGATATGC-3').

The amplification parameters have been the following: denaturation at 94°C/1.5 min; hybridization at 55^{o}C/1.5 min; elongation at 72^{o}C/2 min with a final elongation of 10 min.

Crude PCR products were digested with *Hinf*I, *Hae*III, *Hha*I (Promega) at 37^{o}C during/hours and the ITS sequences were determined from the digested fragments.

All the DNA products have been visualized by UV on gel electrophoresis run at 90 V during 60 minutes.

RESULTS AND DISCUSSIONS

During 2009 -2010, from hundreds of yeast strains isolated from Dealurile Bujorului vineyard (Galati county), 9 yeast strains (MD6 to MD14) have been kept for their oenological characteristics, respectively the fermentative power and alcoholic production (data not shown). Classical and molecular identification of these strains have been performed. The classical identification has followed mainly the oenological practices and classification.

According to these criteria, all the 9 strains belong to the specie *Saccharomyces cerevisiae*s howing under the microsopoval toround large globosetoellipsoidalyeastcells, size of 3-8 µm to 5-10 µm,with multilateral budding [1,7].

According to the technological criteria (old classification), including killer profile [8], these 9 strains belong different subspecies groups: *S. ellipsoideus* (MD7, MD9, MD10),

S.bayanus(MD8, MD13), *S.rosei*(MD 11, MD 12), *S.oviformis* (MD6, MD 14).

The molecular characterization by PCR-ITS RFLP techniques was performed on the 5.8S-ITS region. The principle is to use a minimum number of restriction enzymes in order to obtain the maximum identification for strains, species or genders.

For the DNA extraction, the concentration and the purity of the DNA were adequate for all the strains (table 1).

Table 1. DNA quantification and purity

No.	Strain code	Conc. ng/ul	260/280	260/230
1.	MD6	176,7	1,55	0,48
2.	MD7	232,0	1,63	0,64
3.	MD8	249,4	1,68	0,74
4.	MD9	286,8	1,73	0,81
5.	MD10	113,5	1,38	0,35
6.	MD11	170,5	1,45	0,42
7.	MD12	213,3	1,55	0,53
8.	MD13	173,4	1,68	0,63
9.	MD14	105,7	1,35	0,33

The fragments obtained by *Hha*I digestion are shown in Fig. 1.

Fig.1. Fragments of yeast DNA after *Hha*I digestion (against 100 bp ladder) (MD 6 to MD9)

After *Hha*I digestion, all the 9 strains have shown two fragments of 385 bp, respectively of 365 bp and no difference could be noticed. Consequently, has been applied digestion with other restriction enzyme, respectively *Hae* III.

After the digestion with *Hae* III (Fig. 2) all the strains showed two common fragments (of 320 bp and 230 bp). The difference appeared for three strains (MD6, MD11 and MD14) which have amplified a 165 bp fragment, while the rest of the strains had amplified two other fragments (180 bp and 150 bp). That was a proof of an intraspecific polymorphism among the *S.cerevisiae* strains.

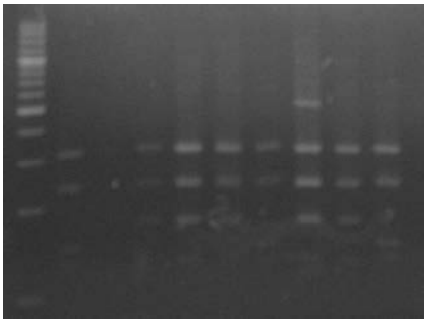

Fig.2. Fragments of yeast DNA after *Hae* III digestion (against 100 bp ladder) (MD 6 to MD9)

Regarding the *Hinf* I digestion, all the strains have showed the same fragments, respectively 365bp and 155 bp and no intraspecific aspects have been found (Fig. 3).

All the obtained fragments are summarized in Table 2.

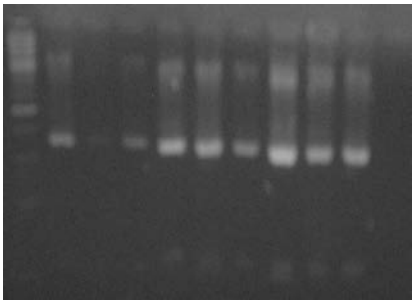

Fig.3. Fragments of yeast DNA after *Hinf*I digestion (against 100 bp ladder) (MD 6 to MD9)

According to the results, all the strains belong to *Saccharomyces cerevisiae* group from a molecular point of view, while from technological point of view they have different species characteristics. It should be taken into account that some of the isolated strains may be hybrids belonging to *S.uvarums* or *S.kudriavzevii*, which should be proven by advanced molecular techniques (flow cytometry, microstaellite loci selection and analysis) [4,5,12]. These yeast hybrids may

appear by horizontal transfer event or by classical hybridization. According to Erny, 2012 both types of hybridization have taken place in the *Saccharomyces* genus.

Table 2. Fragments size (bp) obtained after digestion with different restriction enzymes of DNA yeast strains

No.	Yeast strain	*Hha*I (*Cfo*I)	*Hae*III	*Hinf*I
1.	MD6	385+365	320+230+165	365+155
2.	MD7	385+365	320+230+180+150	365+155
3.	MD8	385+365	320+230+180+150	365+155
4.	MD9	385+365	320+230+180+150	365+155
5.	MD10	385+365	320+230+180+150	365+155
6.	MD11	385+365	320+230+165	365+155
7.	MD12	385+365	320+230+180+150	365+155
8.	MD13	385+365	320+230+180+150	365+155
9.	MD14	385+365	320+230+165	365+155

As described in literature [5,11] these hybrids usually have been isolated from winery running in cold climate, known as cryophilic strains.

CONCLUSIONS

During 2009-2010 have been isolated hundred yeast strains from Dealurile Bujorului vineyard According to the Hoffman and Winston (1987) protocol sufficient DNA quantities have been extracted from 9 yeast strains isolated from Dealurile Bujorului vineyard.

The identification has followed classical protocols (morphological characters), compared to the molecular characterization by PCR-ITS RFLP techniques.

The results show that these strains belong to the specie *Saccharomyces cerevisiae* under different subspecies groups (according to technological classification): *S. ellipsoideus, S.bayanus, S.rosei, S.oviformis*. By the use of molecular tools (ITS amplification), all the strains belongs undoubtedly to *Saccharomyces cerevisiae* group, but the instraspecific polymorphism high [1,2]. Moreover, it should be taken into account that some of the isolated strains are hybrids belonging to *S.pastorianus* or *S.kudriavzevii* [4], which should be proven by advanced molecular techniques (flow cytometry, microstaellite loci selection and analysis).

The adapted PCR-ITS RFLP employed method is a rapid and relative simple method to be used for the yeast isolated form vineyards and wineries. It can be used as a routinely, feasible and sensible method which can replace successfully the morphological identification for a high number of yeast strains in a short time.

We consider our identification work as a small but important step in the characterisation of our local wine yeast biodiversity as part of the world wide effort for the biodiversity conservation and valorisation.

Further, all these strains will be subject of fermentative and flavour development tests on local grapes variety.

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THE INFLUENCE OF GROWTH REGULATORS CONCENTRATIONS ON *IN VITRO* MICROPROPAGATION OF *RIBES RUBRUM* SPECIES

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Abstract

In vitro cultures have found in a short time many practical applications, including genetic improvement of crop species, the multiplication of valuable genotypes, free virus propagation of existent vegetal stocks and conservation of genetic resources. In the present study we aimed to undertake a basic and simple protocol for in vitro micropropagation of Ribes rubrum considering the influence of various concentrations of growth regulators. There were used six variants (M1 ÷ M6) of half-strength MS medium supplemented with growth regulators, as follows: M1 (BA 0.2 mg/l and IBA 0.01 mg/), M2 (BA 0.4 mg/l, IBA 0.02 mg/l and GA_3 0.1 mg/l), M3 (BA 0.4 mg/l, IBA 0.02 mg/ and GA_3 0.2 mg/l), M4 (BA 0.15 mg/l, IBA 0.015 mg/l and GA_3 0.15 mg/l), M5 (BA 0.5 mg/l) and M6 (BA 1 mg/l). The best medium for obtaining micro shoots was half-strength M3 medium. Therefore, the highest rate of in vitro shoot proliferation and elongation was obtained by using combination BA/IBA/GA_3 as growth regulators.

Key words**:** *growth regulators, micro propagation, Ribes rubrum, medium*

INTRODUCTION

Ribes is a genus of about 150 species of flowering plants native throughout the temperate regions of the Northern Hemisphere. *Ribes rubrum* (red currant) is a rare species from the spontaneous flora, valuable for its medicinal properties and commercial uses in pharmaceutical and food industry due to his high antioxidant potential, provided by the high content of ascorbic acid [1]. Besides this, offers the possibility to exploit the entire bush, constituted from leaves, fruits, seeds, buds and flowers.

In vitro multiplication efficiency in various species is widely dependent on composition of culture medium, growth regulators and genotype [2, 3, 4].

Usually, gibberellic acid promotes shoot elongation and reduced the callus and roots formation as well as the development of shoots [5].

Studies on *in vitro* currant culture are limited in comparison to other fruit woody species. One of this studies was represented by red currant cultivar *Detvan*. The objective of this study was to develop a multiplication protocol using two different variants of medium containing various concentrations of BAP, IBA and GA_3 [6]. Other study, describe the obtainig of rudimentary plantlets from fertlized ovules collected from old berries of red currant cultured on Miller's medium [7].

However, they were indicated some successfull data with regard to propagation of *Ribes* genus, but the number of newly formed plantlets usually did not exceed 3–4 shoots per original explant [5].

Application of *in vitro* micro propagation of axillary bud has been recorded in great number of blackberry and black currant cultivars. With regard to black currant micro propagation, fourteen black currant cultivars have been studied from a range of geographical origins, using medium supplemented with BA [7, 8, 9].

In vitro micro propagation requires an extraordinary deal of experiments on optimization of both black and red currant cultivar [10].
This study was conducted to establish an efficient method of micropropagation of red currant plantlets.

MATERIAL AND METHOD

Branches of red currant with dormant buds were cut and placed in distilled water under laboratory conditions, at room temperature. After the activation of buds, standard sterilization procedure was applied of the bud surface. The buds were isolated with size ranging from 0.3 - 0.8 cm and placed on Murashige and Skoog [11] nutrient medium with following hormone concentrations: M1(BA 0.2 mg/l and IBA 0.01 mg/), M2(BA 0.4 mg/l, IBA 0.02 mg/l and GA_3 0.1 mg/l), M3 (BA 0.4 mg/l, IBA 0.02 mg/ and GA_3 0.2 mg/l), M4 (BA 0.15 mg/l, IBA 0.015 mg/l and GA_3 0.15 mg/l), M5 (BA 0.5 mg/l) and M6 (BA 1 mg/l). The medium consisted of half-strength MS basal salts and vitamins supplemented with 3% sucrose, and solidified with 0.7 % agar. The pH of MS medium was adjusted to 5.7 using 0.1 N NaOH.
The study was conducted on 40 samples for each variant of medium, inoculating one explant; the tubes were closed and kept in climate chamber at a temperature between 23-25° C and a photoperiod of 16 hours (fluorescent lights).

RESULTS AND DISCUSSIONS

In this experiment were significant differences noticed on plant height and number of shoots (Table 3).
Culture medium containing different concentrations of growth regulators present different values of multiplication in four weeks after starting experiment. Placing the explants for three weeks led to a high rather percentage incidence of microbial infection, which induced a large increase in highly infected cultures, especially in case of M1and M2 (Table 1).

Table 1. Obtainment of aseptic culture – rosette formation

Variants	% of infected cultures	Induced rosette (%)
M1	60.08	39.08
M2	54.55	45.45
M3	26.42	73.58
M4	32	68
M5	55	45
M6	85	15

Upon establishment of aseptic culture, were collected growth peaks and inoculated on MS multiplication medium with different concentrations of growth regulators listed in Table 2. The explants were placed on the multiplication medium and after four weeks, following parameters were monitored: number of shoots and plant height.

Table 2. Hormonal content of medium used in multiplication of *Ribes rubrum* explants

Variants	Macroelements NH_4NO_3 / KNO_3	BA mg/l	IBA mg/l	GA_3 mg/l
M1	MS/2	0.2	0.01	-
M2	MS/2	0.4	0.02	0.1
M3	MS/2	0.4	0.02	0.2
M4	MS/2	0.15	0.02	0.15
M5	MS/2	0.5	-	-
M6	MS/2	1	-	-

There is a significant difference in response of the different explants inoculated on the better selected medium. The best medium for obtaining micro shoots was half-strength M3 medium with regard to plant height. Also, in this case, there were rising many and highest shoots with plantlets having green leaves and firm consistency (Table 3).
Usually, MS medium supplemented with higher concentration of BA stimulates formation of shoots [12]. Therefore, in case of M5 medium, plant height was similar with the results obtaining using the combination BA/IBA/GA_3 (M3). At the opposite pole, we find that a great concentration of BA inhibits the growth of *in vitro* newly plantlets.
According to Figure 1, we can observe a significant influenced shoot proliferation and average number of shoots in case of M5 medium. However, the best results in both plant height and shoot proliferation we obtained in case of M3 medium.

Table 3. Mean values for plant height (PH), assessed at different growth regulators concentrations and number of shoots (NS), after four weeks from cultivation

Treatments	Means	
	PH (cm)	NS
M1	0.51	1.5
M2	0.54	1.24
M3	0.65	1.5
M4	0.62	1.00
M5	0.64	2
M6	0.40	1.6

With regard to M3 and M5 variants of medium, we can say that increasing BA concentration up to 1 mg/l increase and average values of plant height and number of apices.

The difference between mean values for plant height in case of M3 and M5 treatments is 0.01cm, insignificant. However, considering the range of variation in the concentration of BA, 0.1mg/l would induce an increase in height of 0.01 cm.

With regard to the concentration of IBA, we can not say that influenced positively or negatively *in vitro* multiplication of red currant explants. One explanation could be that it was found in much lower concentrations compared with the others growth regulators.

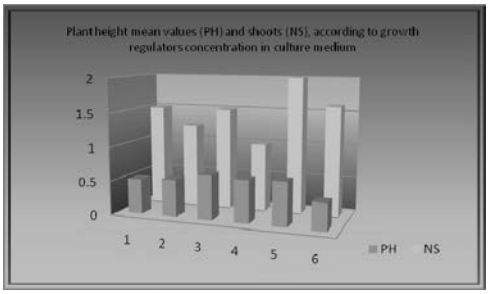

Fig. 1. Plant height mean values (PH) and number of shoots (NS), according to growth regulators concentration in culture medium

To compare the different treatments it was calculated the standard deviation and coefficient of variance within each treatment with regard to plant height and number of shoots (Table 4 and 5).

The calculation formulas used, were:

- The average (μ),

$$\mu = \frac{\sum_{x=i}^{x=n} xi}{n};$$

- Standard deviation (**SD**),

$$SD = \sqrt{\frac{\sum_{x=i}^{n} (xi - \mu)^2}{n}};$$

- Coefficient of variance (**CV**) %,

$$CV = \frac{S}{\bar{x}} \times 100.$$

With regard to CV in Table 4, we can speak about the M6 and M5 variants of medium like a homogenous population of plant height with a high degree of representation. The highest percentage is reached when the M4 medium, being below 45%, having an average with low significance level and a heterogeneous population.

Table 4. Standard deviation and coefficient of variance according to PH, in different variants of medium

The values of standard deviation (SD) and coefficient of variance (CV) according to plant height						
Variants of medium	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**
Average	0.524	0.527	0.634	0.641	0.644	0.408
Standard deviation (SD)	0.13	0.14	0.18	0.25	0.12	0.03
Coefficient of variance (CV) %	25.41	27.18	28.55	39.35	18.87	8.08

According to CV from table 5, we can observe a homogeneous population with a significant degree of representation, present in M6 medium.

At the opposite pole are M3 and M5 variants of medium, having a CV between 43 – 45%. In this case, the average is not representative.

Table 5. Standard deviation and coefficient of variance according to NS, in different variants of medium

The values of standard deviation (SD) and coefficient of variance (CV) according to number of shoots						
Variants of medium	M1	M2	M3	M4	M5	M6
Average	1.533	1.238	1.564	1.06	2	1.62
Standard deviation (SD)	0.52	0.42	0.7	0.23	0.87	0.69
Coefficient of variance (CV) %	34.03	34.4	45.31	22.49	43.64	4.34

The plantlets obtained after one month, were shaped and transferred to a rooting medium: MS (with 1/5 macro) supplemented with 0.01 mg/l BA and kept in darkness for five days. Then, they were transferred to light for three weeks. After this period, the process of rooting was not visible.

CONCLUSIONS

From the results, we can say that plant growth regulators clearly affects shoot development from buds explants cultivated *in vitro* and shoot multiplication.

Growth regulators concentration influenced accumulation of biomass (fresh weight) of *Ribes rubrum* plantlets propagated *in vitro*. The presence of BA 0.4 mg/l, IBA 0.02 mg/ and GA_3 0.2 mg/l in the culture medium was the most efficient treatment for increasing height and fresh weight of plants *in vitro* cultured.

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BIO-BASED COMPOSITE USE IN FERTILIZATION OF PETUNIA AND CARNATION CULTURE

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Abstract

Bio-based composite were used in NPK biodegradable fertilizers. The composition of fertilizers contains 0%, 5%, 10%, 15%, 20% starch from wood flour and the form of them were sticks. For the plant test there were used Petunia hybrid and Dyanthus caryophyllus in 8 cm diameter pots filled with 1:1 peat and garden soil. At every pot was used 0.5 g fertilizer stick. The biodegradable process of sticks determines the slow release of fertilizers in the pot. During vegetation period there were made some biometrical determinations (length, number of shoots) and agrochemical analysis at substrates and plants. Comparison between petunia and carnation development show that petunia answer better at fertilization process. From the point of view of experimental variants the best results were at variants with with 15% and 20% wood flour.

Key words: *biocompost, slow release fertilizer, wood flour*

INTRODUCTION

During the last years the use of vegetal-derived materials (wood flour, plant fibres, reprocessed cotton) has shown continuous growth [2, 4]. The drivers for this trend are the cost savings, weight reduction and recyclability: in the last years cost efficient technologies has been developed to manufacture vegetal based composite as a result. The forest biomass represents an abundant, renewable, no-food competition and low cost resource that can play an alternative role to petro-resources. The production and use of forest biomass energy is greenhouse neutral while the expansion of plantation forestry is a positive benefit to greenhouse gas reduction through increasing the forests as a carbon sink. Wood fibres can be used as natural fillers to replace synthetic and glass fibres in composites production [3, 1]. Loading of wood fibres is limited by difficult compatibility with hydrophobic polymers [5]. Research activity was devoted to the production of composites based on wood fibres with biodegradable polymeric matrices (polylactic acid, polycaprolactone, polyhydroxyalkanoates, etc.) and with polypropylene. A high fibre content will be achieved by increasing toughness of polymeric matrices. Materials developed for applications in agriculture were enriched with active substances respectivelly fertilizers.

MATERIAL AND METHOD

Experience was organized in seven variants of 10 repetitions. Experimental scheme is presented in Table 1.

Table 1. Experimental scheme

No.	Variant	Specification
1.	Ct unf.	Unfertilized
2.	Ct	Vilmorin*Sticks
3.	V1	Starch - 50 % NKP fertilizer
4.	V2	Starch - 5 % FT400 (wood flour) - 50 % NKP fertilizer
5.	V3	Starch - 10 % FT400 (wood flour) - 50 % NKP fertilizer
6.	V4	Starch - 15 % FT400 (wood flour) - 50 % NKP fertilizer
7.	V5	Starch - 20 % FT400 (wood flour) - 50 % NKP fertilizer

*N:P:K – 10:6:7

Before mounting the experience fertilizers were analyzed to determine pH, the amount of available K in the form of total NP (Table 2).

Table 2. Fertilizers analyses

No.	Fertilizer	pH	Total content %		
			N	P	K
1.	S-50-NKP	5.48	5.66	9.75	8.19
2.	S-5-WF-50 NKP	5.31	5.62	9.40	8.19
3.	S-10-WF-50-NKP	4.91	5.95	9.75	8.34
4.	S-15-WF-50 NKP	5.02	5.60	9.00	8.34
5.	S-20-WF-50-NKP	4.96	5.90	9.75	8.48

For the experiment were made *Petunia* and *Dianthus* transplants and when they have reached 5 cm tall they were transplanted into pots of about 8cm diameter and for substrat was used a mixture of celery soil and peat in 1:1 ratio, analysis of this substrate is shown in Table 3.

Table 3. The analyze regarding the culture substratum before transplanting

No.	Specif.	pH	Soluble salts CE	Soluble form content, ppm			
				N-NH4	N-NO3	P-PO4	K
1.	Substratum	7.58	0.58	9.56	256.6	50.0	355

When transplanting in pots was introduced one bar of fertilizer which was weighed prior to the fertilizer put in experience to be similar in weight. During the vegetation, biometric observations were made on plant growth and development: plant height, number of shoots, number of flowers. Also periodically weeks away when the plant samples were collected and passed substrates corresponding to that.

Biometric measurements analysis was performed using the line. Substrate tests were performed in soluble form, extract distilled water in 1:5 ratio and pH in 1:2.5 ratio. pH determination has been made with potentiometers. Conductivity soluble salts, ammoniacal nitrogen and phosphorus colorimetrically and potassium flamfotometric. Methods are standardized.

RESULTS AND DISCUSSIONS

Petunia

Measurements of the petunia plant height varied during the experiment according to added fertilizer. If initial planting their height ranged from 5.1 cm (control unfertilized) and 6.15 cm (V2) in the second period of measurement plants reached 11.33 cm (V5). Later plants have developed very well reaching 20.22 cm at V5 at 24 May and 36.50 cm in variant V3 at 7 June. In terms of alternatives are found if initial second harvest period (17 May) Variants V4 and V5 have been the greatest heights of petunias on 24 May the best alternative was a V3 and V5 height of 18.56 cm with a height of 20.22 cm. Then the only option at 31 May V5 recorded the highest height of 28.71 cm, followed by variant V2 with 27.29 cm 7 June. A week to record heights was variants V3 and V4 with 36.50 cm to 35.17 cm.

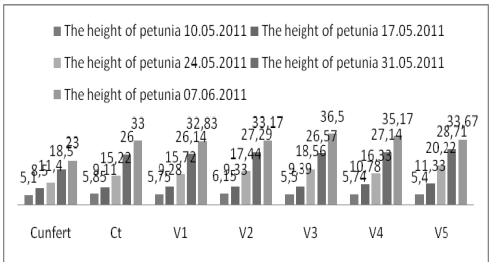

Fig. 1. The evolution of height of petunia plants during vegetation period

Examining variants fertilized versus unfertilized control can be seen that the latter had a downturn because it has available the required amount of N, P, K development.

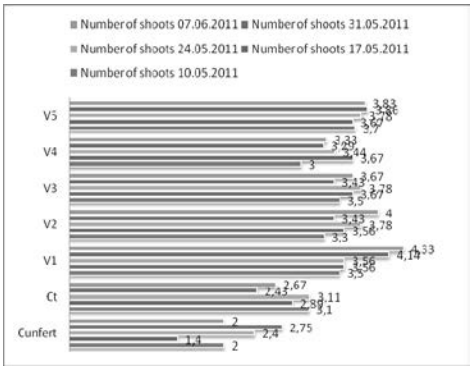

Fig. 2. Evolution of number of shoots during vegetation period

Record number of shoots (Fig. 2) also varied depending on experimental variants that fertilizers applied. If the original versions had a similar number of shoots after the first week after planting the number of shoots varied very little reaching 3.67 in the second week 24 May the number of shoots increased also least almost equal among them being the largest number of 3.78 shoots the variants V2, V3 and V5. In last week's record number of shoots reached 4.33 and 4.00 on a variant to variant V2. Examination of the experimental variants clearly shows that unfertilized variant is slower. Control fertilized with Vilmorin has fared worse than the variants fertilized with bars containing flour starch and sawdust. After applying the fertilizer sticks and watering after the first week they were so scattered in the soil were not found in tests conducted at periodic substrates experiences. These analyzes were performed with analysis of plants.

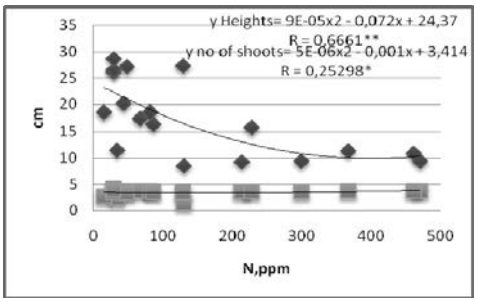

Fig. 3. The correlation between nitrogen content of substratum and the development of Petunia plants

To see the influence of main nutrients on plant growth and development has been the interpretation of statistical correlations between N use and plant height, N the number of shoots from experimental variants (Fig. 3), P and plant height and number of shoots (Fig. 4) and K correlated with plant height and number of shoots (Fig. 5). It is known that nitrogen is the main element that directly influences plant growth, correlation analysis of this element with the main biometric characteristics of plants (Fig. 3) shows that this factor influenced significantly distinct plant height and number of shoots significantly only.

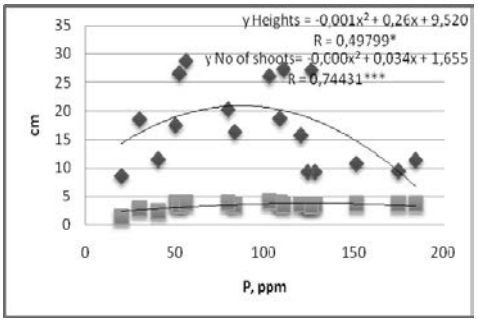

Fig. 4. The correlation between phosphorus content of substratum and the development of Petunia plants

Phosphorus is the second nutrient cofactor in plant growth and development influenced plant growth in height but less intensely significant number of shoots produced (Fig. 4).

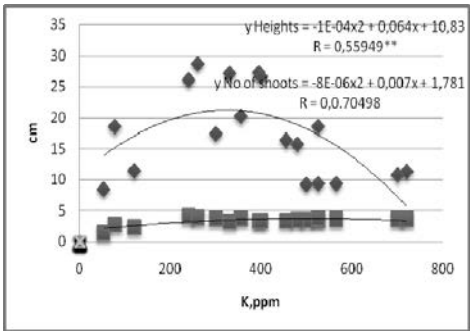

Fig. 5. The correlation between potassium content of substratum and the development of Petunia plants

Potassium content provided distinct substrates significantly increased plant height and shoot emergence significantly intense in petunias. To see the influence of the flour content of wood flour on growth and development of Petunia

plants were performed correlations between biometric measurements and percentage of wood flour (Fig. 6, 7).

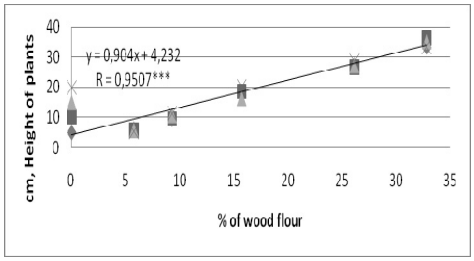

Fig. 6. The correlation between wood flour and the height of plants

If wood flour contents influence on growth in height of plants is very significant 0.9507 coefficient obtained when the number of shoots and the correlation coefficient is just significant 0.70922.

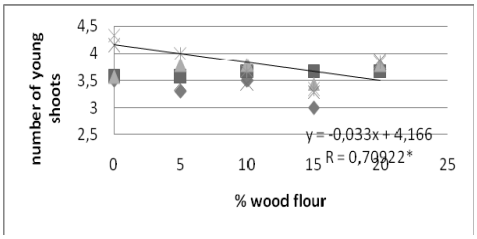

Fig. 7. The correlation between wood flour and the number of shoots Dianthus

Dianthus were planted and after emergence and development have been cropped so as to experience start from the same plant height. After entering the bar with fertilizer plants have evolved and weekly samples were collected from plants and substrate.

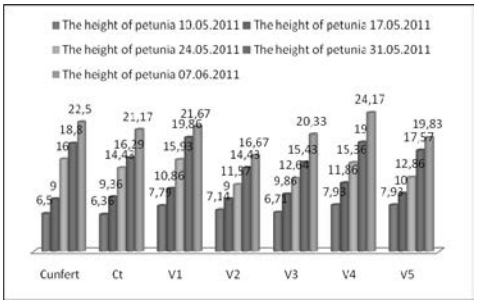

Fig. 8. The heights of Dianthus during vegetation periods

Height (Fig. 8) increased dianthus whichever experimental biggest increase was registered version 5 which is composed of 20% wood flour. Examination of average height variation can be observed that the control had fertilized most average height of 31.00 cm and between variants fertilized variant 5 with 20% wood flour had a height of 26.00 cm. Variant who presented the poor results, worse even than the control was unfertilized variant 3 with 10% flour wood flour. Because unfertilized control reached heights close to the fertilized variants can be said dianthus were not affected by fertilization.

In the case of shoots (Fig. 9) the number of them were stronger in five variants with 4.67 shoots from 6 June and version 4 with a number of 5.00 shoots in the end.

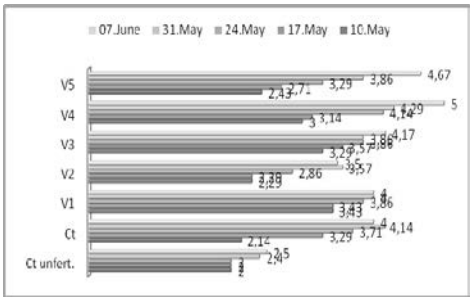

Fig. 9. Number of dianthus shoots during vegetation period

Examining the average number of shoots obtained in the experimental variants shows that unfertilized control recorded the lowest number between 2.71 and 10% wood flour V3 variants showed the highest number of shoots of 4.86. Every week they collected samples were analyzed agrochemical substrate. If the initial pH decreased from 7.58 to 6.65, pH gradually returned to baseline. To see the influence of fertilizers on plant growth and development of dianthus they started to make correlations between elements N, P, K and plant height and number of shoots (Fig. 10, 11, 12). If substrate nitrogen content influence significantly distinct the shoots and dianthus insignificant increase in height.

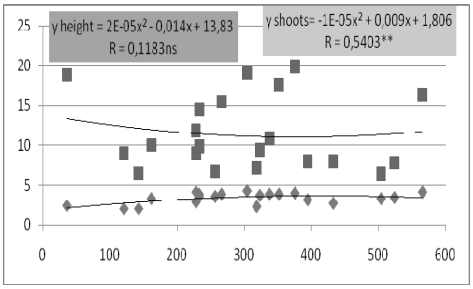

Fig. 10. The correlation between nitrogen content of substratum and the development of Dianthus plants

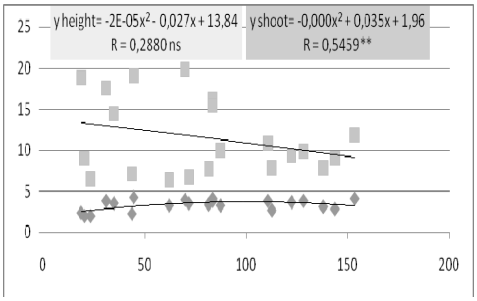

Fig. 11. The correlation between phosphorus content of substratum and the development of Dianthus plants

The phosphorus correlations performed (Fig. 11) shows that the number of shoots was statistically significantly distinct compared with plant height where correlation is obtained unsignificantly statistically assured.

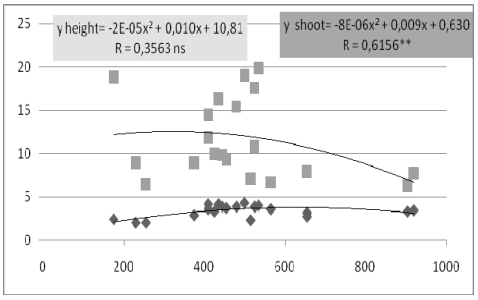

Fig. 12. The correlation between potassium content of substratum and the development of Dianthus plants

Potassium, an element which was released slowly during the growing season significantly affects plant growth in height and distinctly significant number of shoots.

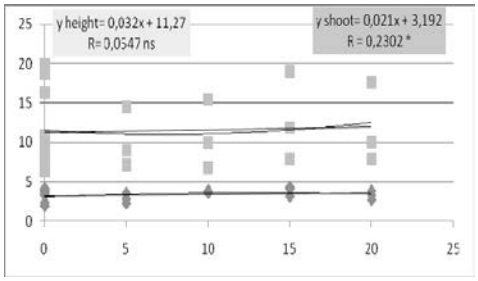

Fig. 13. The correlation between the wood flour content from fertilizers and the development of plants

CONCLUSIONS

1. The height of plants increased during the vegetation period, variants determined the best results are 15% flour V4 and V5 with 20% wood flour.
2. Because unfertilized control from dianthus plants reached heights close to fertilized variants can be said that carnations were not affected by fertilization.
3. The number of shoots increased to a lesser extent with good results and variants are variants fertilized V1 to V5, insignificant differences between them are small;
4. Growing media analysis showed that variants fertilized with manure had experienced:
a. pH decreased immediately after application of fertilyzers and during vegetation period grew;
b. Although ammoniac N fertilizers was predominant during the vegetation period it was transformed into N-NO3 and consummated;
c. Phosphorus and potassium in fertilizer during the experiment provided a high content of culture substrate;
5. Statistical analysis at petunia plants showed significantly distinct influence of nitrogen and potassium in plant growth and development in height and number of shoots was influenced by P and K in the substrate;
6. In the presence of different concentrations of wood flour correlation made between the two indicators which also resulted in a coefficient is insignificant, so dianthus species is less influenced by the presence of wood flour into fertilizer;
7. In the case of petunia plants the presence of wood flour influenced intense significantly the heights of plants and only a positively influence of the development of shoots.

8.The use of bio based composite in the preparation of fertilizers have no negative influence, a nontoxic one so the plants have a good development during vegetation period.

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THE SEMI-SOLID STATE CULTIVATION OF EDIBLE MUSHROOMS ON AGRICULTURAL ORGANIC WASTES

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Abstract

The main aim of this work was focused on testing new practical procedures in order to optimize the efficiency of edible mushroom cultivating by enhancing their fruit body formation during the semi-solid state cultivation on winery and fruit processing wastes. In this respect, the culture media for fungal growing were mainly prepared from winery and apple wastes mixed with relative small amounts of wheat and rye bran (15-30% w/w) as well as germinated barley seeds (3-5% w/w). After their steam sterilization at 121°C, 1.1 atm., for 15 min. they were transferred aseptically inside of 250 ml work volume flasks. These culture media were aseptically inoculated by using 10-20 ml suspension of fungal pellets collected from submerged cultures of the pure strains of Lentinula edodes (Berkeley) Pegler and Pleurotus ostreatus (Jacquin ex Fries) Kummer. After inoculation with mushroom pellets, the semi-solid cultivation was set up for each one of the tested mushroom species following the main parameters of culture media: constant temperature 23°C, pH level 5.7–6.0 units, relative humidity level of culture media 30-50%. During a period of semi-solid cultivation lasting between 30-50 days, the fungal mycelia were developed inside the culture media in the first 10-20 days and after that during the following 20-30 days the first mushroom primordia were emerged and developed the mushroom fruit bodies. In this final stage, the final mushroom fruit bodies were harvested and weighted, all results showing the percentage of 40-50% relative to the whole weight of culture media. Poplar, beech and birch sawdust were used as control samples for the tested culture media.

Keywords: *edible mushrooms, semi-solid state cultivation, apple and winery wastes*

INTRODUCTION

The agricultural works as well as the industrial activities related to grape and apple processing have generally been matched by a huge formation of wide range of cellulosic wastes that cause serious environmental pollution effects if they are allowed to accumulate in the environment or much worse they are burned on the soil [1, 2].
The solid substrate fermentation of plant wastes from agro-food industry is one of the challenging and technically demanding biotechnology that is known so far [3-5].
The major group of fungi which are able to degrade lignocellulose is represented by the edible mushrooms of Basidiomycetes Class [6-9].
The main aim of this work was to find out the best biotechnology of recycling the winery and apple wastes by using them as a growing source for edible mushrooms and, last but not least, to protect the environment [9-12].
Taking into consideration that most of the edible mushrooms species requires a specific micro-environment including complex nutrients, the influence of physical and chemical factors upon fungal biomass production and mushroom fruit bodies formation were studied by testing new biotechnological procedures [7-9].

MATERIAL AND METHOD

According to the main purposes of this work, two fungal species of Basidiomycetes group, namely *Lentinula edodes* (Berkeley) Pegler (folk name: Shiitake) as well as *Pleurotus ostreatus* (Jacquin ex Fries) Kummer (folk name: Oyster Mushroom) were used as pure

mushroom cultures isolated from the natural environment and now being preserved in the local collection of the University of Pitesti.
The stock cultures were maintained on malt-extract agar (MEA) slants (20% malt extract, 2% yeast extract, 20% agar-agar). Slants were incubated at 25°C for 120-168 h and stored at 4°C. The pure mushroom cultures were expanded by growing in 250-ml flasks containing 100 ml of liquid malt-extract medium at 23°C on rotary shaker incubators at 110 rev. min $^{-1}$ for 72-120 h. To prepare the inoculum for the spawn cultures of *L. edodes* and *P. ostreatus* the pure mushroom cultures were inoculated into 100 ml of liquid malt-yeast extract culture medium with 3-5% (v/v) and then maintained at 23-25°C in 250 ml rotary shake flasks.
After 10–12 d of incubation the fungal cultures were inoculated aseptically into glass vessels containing sterilized liquid culture media in order to produce the spawn necessary for the inoculation of 10 kg plastic bags filled with compost made of winery and apple wastes.
These compost variants were mixed with other needed natural ingredients in order to improve the enzymatic activity of mushroom mycelia and convert the cellulose content of winery and apple wastes into protein biomass. The best compositions of five compost variants are presented in Table 1.

Table 1. The composition of five compost variants used in mushroom culture cycles

Compost variants	Compost composition
S1	Winery and apple wastes (1:1)
S2	Winery wastes + wheat bran (9:1)
S3	Winery wastes and rye bran (9:1)
S4	Apple wastes and wheat bran (9:1)
S5	Apple wastes + rye bran (9:1)
Control	Poplar, beech and birch sawdust (1:1:1)

In this way, the whole bags filled with compost were steam sterilized at 121^{o}C, 1.1 atm., for 30 min. In the next stage, all the sterilized bags were inoculated with liquid mycelia, and then, all inoculated bags were transferred into the growing chambers for incubation. After a time period of 10-15 d, on the surface of sterilized plastic bags filled with compost, the first buttons of mushroom fruit bodies emerged. During a period of 20-30 d there were harvested between 1.5–3.5 kg of mushroom fruit bodies per 10 kg compost of one bag [10-14].

RESULTS AND DISCUSSIONS

To increase the specific processes of winery and apple wastes bioconversion into protein of fungal biomass, there were performed experiments to grow the mushroom species of *P. ostreatus* and *L. edodes* on the previous mentioned variants of culture substrata (see Table 1). During the mushroom growing cycles the specific rates of cellulose biodegradation were determined using the direct method of biomass weighing the results being expressed as percentage of dry weight (d.w.) before and after their cultivation [14, 15]. The registered data are presented in Table 2 and Table 3.

Table 2. The rate of cellulose degradation during the growing cycle of *P. ostreatus*

Variants of culture substrata	Before cultivation (g% d.w.)	After cultivation (g% d.w.)
S1	2,7-2,9	0,9
S2	2,5-2,8	0,7
S3	2,3-2,5	0,4
S4	2,5 -2,7	0,8
S5	2,5-2,7	0,7
(Control	3,0	1,5

Table 3. The rate of cellulose degradation during the growing cycle of *L. edodes*

Variants of culture substrata	Before cultivation (g% d.w.)	After cultivation (g% d.w.)
S1	2,6-2,7	0,5
S2	2,3-2,5	0,4
S3	2,3-2,5	0,5
S4	2,5 -2,7	0,7
S5	2,7-2,9	0,5
Control	3,0	1,4

The registered data revealed that by applying this biotechnology, the winery and apple wastes could be recycled as useful raw materials for mushroom compost preparation in order to get significant mushroom production.
In this respect, the final fruit body production of the cultivation of these two mushroom species was registered as being between 20–28 kg relative to 100 kg of composts made of apple and winery wastes. In order to determine the evolution of the total nitrogen content in the

fungal biomass there were collected samples at precise time intervals of 50 h and they were analyzed by using Kjeldahl method. The registered results concerning the evolution of total nitrogen content in *P. ostreatus* biomass are presented in figure 1 and the data regarding *L. edodes* biomass could be seen in figure 2.

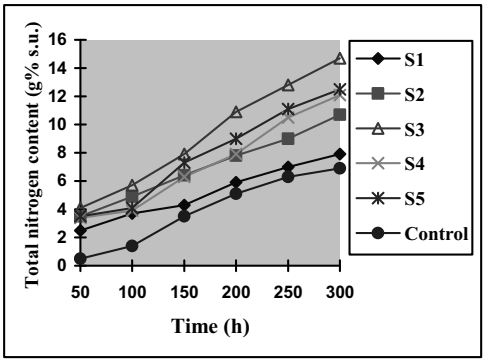

Fig. 1. The evolution of total nitrogen content in *P. ostreatus* biomass

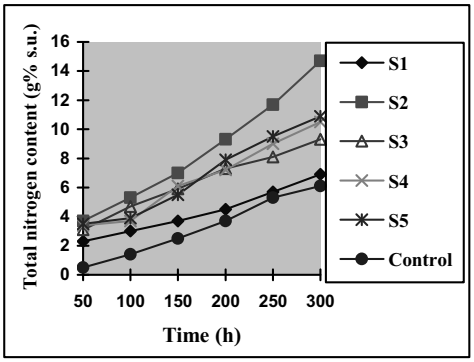

Fig. 2. The evolution of total nitrogen content in *L. edodes* biomass

According to the registered results of the performed experiments the optimal laboratory-scale biotechnology for edible mushroom cultivation on composts made of marc of grapes and apples was established.

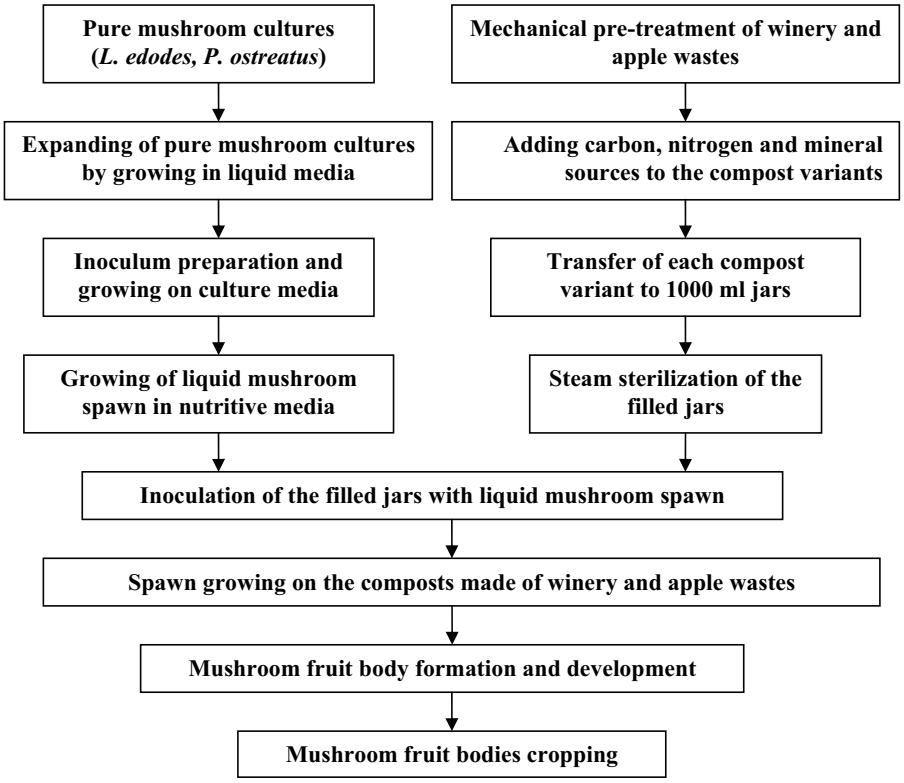

Fig. 3. Scheme of laboratory-scale biotechnology for edible mushroom production by recycling winery and apple wastes

As it is shown in figure 3, two technological flows were carried out simultaneously until the first common stages of the inoculation of composts with liquid mushroom spawn followed by the mushroom fruit body formation.

The whole period of mushroom growing from the inoculation to the fruit body formation lasted between 30–60 d, depending on each fungal species used in experiments.

During the whole period of fruit body formation, the culture parameters were set up and maintained at the following levels, depending on each mushroom species: air temperature, 15–170C; the air flow volume, 5–6m3/h; air flow speed, 0.2–0.3 m/s; the relative moisture content, 80–85%, light intensity, 500–1,000 luces for 8–10 h/d. The final fruit body production of these mushroom species used in experiments was registered between 1.5 – 2.8 kg relative to 10 kg of composts made of winery and apple wastes.

CONCLUSIONS

1. The registered data revealed that by applying this biotechnology, the winery and apple wastes could be recycled as useful raw materials for culture compost preparation to get edible mushrooms

2. By applying this biotechnology, the winery and apple wastes could be recycled as useful raw materials for mushroom compost preparation in order to get significant mushroom fruit body production and protect the natural environment surrounding apple juice factories as well as wine making industrial plants.

3. The fruit body productions of these two mushroom species were registered as being between 20–28 kg relative to 100 kg of composts made of vineyard and apple wastes.

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RESEARCHES REGARDING THE IMAGE ANALYSIS IN WHEAT QUALITY EVALUATION

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Abstract

There have been analyzed the main quality parameters of 27 wheat samples from Romanian crops, of the years 2010 and 2011, namely: *Humidity (%), Hectolitric mass (kg / hl), Falling number (sec), Protein content (%), Wet gluten content (%) and Gluten Index. Afterwards, the wheat samples were ground on the Chopen pilot-type mill and the alveografic parameters were determined from the resulting flour: Resistance (P, mm), Extensibility (L, mm), Mechanical Work (W, 10E-4J), Elasticity index (Ie,%), Gluten Extensibility index and the P/L report. The wheat samples taken for analysis were scanned using a commercial scanner, at a resolution of 200 dpi. The obtained results were examined with a specific software, used for the analysis of ImageJ image. For each image we determined the color histogram with the specific parameters: R, G, B, Brightness and Fractal Dimension. Our results have shown highly significant correlations between the color histogram parameters (Brightness, R, G, B) and the alveografic parameters, namely: Resistance (-0.64< r <– 0.72), Mechanical work (- .62 < r < -0.64) and the P/L ratio (-0.62 < r < -0.68). No significant correlations were found between color parameters of the image analysis and the physical and chemical parameters of the wheat. Models of multiple regression have been described for the prediction of alveografic parameters P, W and P/L, based on the color parameters. The results suggest an interesting potential for including the image analysis in a coherent assessment procedure of the wheat, but in order to validate this conclusion, there are necessary further experimental researches.*

Key words: alveograph, image analysis, wheat crops quality

INTRODUCTION

Wheat grain color can vary from light yellow to brown red. The main factors which determine the color are some flavonoid pigments (tricine) and carotenoids (xanthophylls such as lutein).
The analysis of color based on digital images of wheat grains has been used in various studies. *Neuman et al*. (1989) have shown that the method can be used to distinguish certain varieties of Canadian wheat [1].
Klepacka et al. (2002) have shown the existence of a significant relationship between the wheat bran shades of gray of and ferulic acid content of these (r = -0.65). They also found a correlation between the level of gray color grains and the degree of extraction (r = 0.74) [2].

Manickavasagan et al. (2008) studied the potential of the wheat image analysis (using monochrome images based on the shades of gray) to provide information which allow the discrimination of different classes of wheat, to automation of industrial processes [3]. Newton's experiments and confirmed by the Young-Helmholtz theory, demonstrated that the human eye retina contains three types of cone receptors, each being sensitive to a certain range of light waves. These receptors are: Long or Red receptors (sensitive to red light with long wavelengths, in the range 500 nm-700nm), the Green or Middle receptors (sensitive to green light having medium wavelengths, 450nm-630nm) and Short or Blue receptors (sensitive to blue light with short wavelengths, in the range 400nm- 500 nm) [4,5].
In practice, the description of any color in the visible spectrum consists of its noting,

representation or specification, through three numerical color parameters, which define a set of tristimulus values. A tristimulus value expresses, directly or indirectly, the extent to which primary RGB colors combine to form a new color. Implicitly, it expresses the characteristics of color stimuli, which are sensitive to LMS wavelengths, corresponding to the primary color components (RGB).
The scanners read the amounts of light reflected by a RGB image and convert them in the tristimulus values (digital), and monitors receive tristimulus values (digital) and convert them in RGB light, visible on the fluorescent screen.
The RGB color model can be implemented in different ways. The range of colors which can be described using this model is determined, dimensionally, by the number of bits used to describe each color component. The most common mode of implementation, used since 2006 for computer monitors, uses 24-bit color and 8 bit color / pixel or 256 digital levels / channel (28 = 256), which is why the number of colors that can be represented based on this model is limited to 256R x 256G x256B = 16.7 million colors, about the number that can be distingushed by human eye [5].
Since several studies have shown significant correlations between the levels of certain compounds in wheat grain and its color, we decided to investigate the relationship between the color of wheat grains and their technological quality, in the Romanian wheat samples .

MATERIAL AND METHOD

27 Romanian wheat samples were analyzed to determine the physical and chemical parameters listed in Table 1.

Table 1. Methods of analysis used for analyzing the quality parameters of wheat

Quality parameter	Analysis method
Hectolitric mass (MH, kg/hl)	STAS 6123/2-73
Moisture (M, %)	SR ISO 712/1999
Protein content (P, %)	ICC 159-95 (NIR method, Perten Inframatic 8600)
Wet gluten (WG,%)	SR ISO 21415-2:2007
Gluten Index (GI)	ICC 155-94
Falling number (FN, s)	SR ISO 3093:2005

Wheat samples were scanned at a resolution of 200 dpi, using a commercial scanner, to obtain digital images. Digital images were analyzed using a specialized software, ImageJ, developed by the National Institutes of Health (U.S.).
We used the options of the program which assess the way the image colors are constructed, starting from the three primary colors, Red, Green and Blue. Basically, the program made for each image a specific histogram for each color. The x coordinate axis contains the possible values for each primary color (between 0 and 255), and the y coordinate axis contains the number of identified pixels. Thus, the program calculates average quantities of red, green and blue and the associated standard deviations (Photo 1).

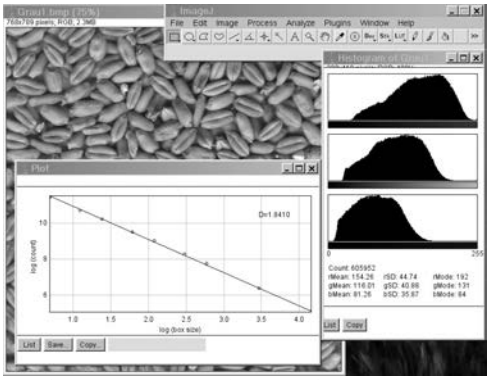

Photo 1. Image of a wheat sample, according to, Software Image J

Each sample was grounded in a Chopen type pilot mill, to obtain the corresponding flours. For each flour we determined alveografic parameters, according to ISO 27971:2008, Resistance (P, mm), Extensibility (L, mm), Extensibility Index (G), Mechanical work (W, 10E-4 J), P / L Ratio, and Elasticity index (Ie,%). Also, we used the options to assess the fractal dimensions of the analyzed images.
The results were interpreted statistically, using a specific software, StatSoft, Inc.. (2004),STATISTICS (Data Analysis Software System), version 7, www.statsoft.com).

RESULTS AND DISCUSSIONS

The results obtained by determining the quality parameters for the 27 wheat samples are shown in Table 2, using the estimates of variability:

the arithmetic average (X), the standard deviation (sx) and the coefficient of variation (CV).

Table 2. The estimates of variability for the quality parameters of wheat samples

Quality parameters	$X \pm s_x$	CV (%)
MH (kg/hl)	75.115 ± 1.680	2.236
U (%)	11.667 ± 0.850	7.285
P (%)	12.733 ± 1.160	9.133
GU (%)	27.000 ± 5.440	20.139
GI	71.604 ± 22.190	30.993
FN (s)	246.286 ± 90.420	36.715
P (mm)	65.632 ± 24.260	36.965
L (mm)	63.947 ± 24.280	37.964
G	17.575 ± 3.280	18.662
W (10^{-4} J)	137.895 ± 66.350	48.117
P/L	1.208 ± 0.730	60.460
Ie (%)	36.235 ± 21.350	58.912

Table 2 shows that the wheat samples were characterized by a high variability of the rheological parameters (P, L, G, W, G, P / L and Ie), and also of the physical and chemical parameters expressing the enzymatic activity (FN , GI). The parameter Wet gluten content (GU) was characterized by an average variability, while the parameters Hectolitric Mass (MH), Humidity (U) and Protein content (P) were characterized by low values of variability.

The results obtained by determining the color parameters and the fractal dimensions (D), for the 27 wheat samples are shown in Table 3. The analyzed color parameters were: Brightness (M), Average content of red (R), Average content of green (G) and Average content of blue (B).

Table 3. The estimates of variability for the color parameters and fractal dimension of the wheat samples

Quality parameters	$X \pm s_x$	CV (%)
M	112.506 ± 4.572	4.063
R	148.332 ± 5.207	3.510
G	111.381 ± 4.650	9.133
B	77.381 ± 4.448	5.748
D	1.836 ± 0.035	1.901

From Table 3 we can see that, unlike the variability of wheat quality parameters, the variability of wheat color parameters is several times lower.

This can be interpreted as the determinants of color of the wheat kernels (probably genetic factors, dependent on variety), are relatively little influenced by environmental factors. The highest variability affects shades of green of the images, and the lowest variability appears in the shades of red.

Table 4 presents the values of correlation coefficients between the physico-chemical and the image parameters of wheat samples. In Table 4 we can see that the only physico-chemical parameters which correlate with image parameters we used, are Hectolitric Mass and Protein content.

Hectolitric Mass increases distinct significantly, as luminosity (M) decreases, for the wheat sample analyzed.

Also, the parameter Hectolitric Mass decreases as the Content of red, green and blue images decreases in the analyzed images.

This fact allows us to model the parameter Hectolitric Mass due to the color parameters, according to the regression model below:

$$\mathbf{MH = 89.099 – (0.018 \cdot R) – (0.673 \cdot G) – (0.335 \cdot B) + (0.796 \cdot M)}$$

The regression model explains about 45% of the Hectolitric Mass variability ($r = 0.673$, $r2=0453$, $F=4.22$, $p<0.008$).

There was no significant correlation between the shape of grains, evaluated by fractal dimension, and the Hectolitric Mass. Table 4 shows that the sample of wheat, characterized (by image analysis) by a smaller amount of red, had a significantly higher protein content.

There have been no significant correlations between the image parameters and the other quality parameters of wheat, namely: Humidity, Wet gluten content, Gluten index and Falling number.

Table 5 presents the coefficients of correlation between the image parameters and the alveografic parameters for the wheat samples.

The table shows that the alveografic parameters Resistance (P) and Mechanical work (W) are significantly correlated with the image parameters.

The alveografic Resistance decreased distinct significantly, as the brightness of wheat was higher, respectively as the images that had a higher content of green and blue.

It is interesting the correlation of this parameter with the values of Fractal dimension. The Fractal dimension measures the complexity of shape of the analyzed structure, its value being

higher as the structure has a more complex shape.

Table 4. The correlation coefficients between quality physico - chemical parameters and and image parameters, for the wheat samples

Perechi		r	Perechi		r
U	MH	-0,13	GI	P	-0,27
	GU	-0,28		FN	-0.08
	GI	0,27		M	-0,22
	P	-0,09		D	0,28
	FN	0.00		R	-0,00
	M	0,06		G	-0,22
	D	0,09		B	-0,35
	R	0,01	P	FN	-0.01
	G	0,06		M	-0,30
	B	0,07		D	0,30
MH	GU	0,29		R	**-0,44**
	GI	0,12		G	-0,31
	P	0,28		B	-0,14
	FN	0.15	FN	M	-0.10
	M	**-0,57**		D	-0.01
	D	-0,04		R	-0.04
	R	**-0,39**		G	-0.13
	G	**-0,59**		B	-0.15
	B	**-0,64**	M	D	**-0,60**
GU	GI	**-0,51**		R	**0,86**
	P	**0,90**		G	**1,00**
	FN	0.10		B	**0,95**
	M	-0,23	D	R	**-0,55**
	D	0,22		G	**-0,58**
	R	-0,34		B	**-0,48**
	G	-0,23	R	G	**0,85**
	B	-0,11		B	**0,68**
			G	B	**0.95**

(n = 27, where, if r = 0.38-0.48 when p <0.05, r = 0.49-0.60, when p <0.01, r = 0.61-1.00, then p <0.001)

Table 5. The coefficients of correlation between the alveografic parameters and the image parameters for the wheat samples

Pairs		r	Pairs		r
P	M	**-0.67**	W	M	**-0.69**
	D	**0.52**		D	**0.51**
	R	-0.43		R	**-0.56**
	G	**-0.65**		G	**-0.68**
	B	**-0.69**		B	**-0.61**
L	M	-0.03	P/L	M	-0.41
	D	0.10		D	0.21
	R	-0.09		R	-0.18
	G	-0.06		G	-0.40
	B	0.07		B	**-0.53**
G	M	-0.02	Ie	M	-0.24
	D	0.13		D	0.42
	R	-0.08		R	-0.27
	G	-0.04		G	-0.23
	B	0.09		B	-0.10

(n = 20, where, if r = 0.42-0.54 when p <0.05, r = 0.55-0.65, when p <0.01, r = 0.66-1.00, then p <0.001).

As the analyzed wheat kernels were deviated from a smooth aspect, the alveografic parameter Resistance increased. This may be partly the result of a higher presence of shrunken kernel grains, as shown by the images in photo 2. These images show grains that had the highest (1879, above) respectively the lowest Fractal dimension (1764, below).

We can find in the literature opinions that flours obtained from the shrunken kernel grains have higher quality characteristics. This might be because, at the beginning of ripening, grains accumulate a higher amount of proteins that generate gluten. Drought leads to shrunkening of kernels, hurry the postmaturation of these proteins and prevent migration in the grain of other non proteinaceous components [6]. However, increasing the quality of gluten in flours obtained from shrunken kernel grains is decompensated by a sharp decrease of the degree of extraction of flour.

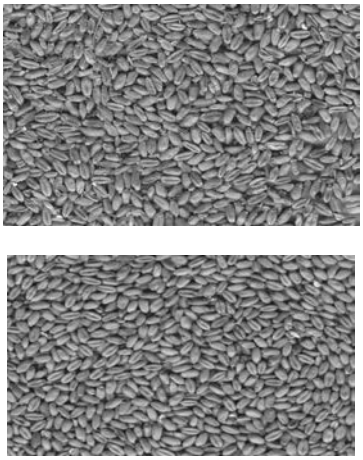

Photo 2. The image of wheat with the highest (down) and the smallest (up) fractal dimension

The best image predictors for the alveografic parameter Resistance, using the specific algorithm for the forward steepwise regression were Fractal dimension (D) and Content of blue (B).

Multiple regression model that includes these image parameters is shown in photo 3. It explains 52.5% of the variability of the parameter alveografic Resistance (r = 0.725, r2 = 0.525, F = 9.39, p <0.018):

Table 5 shows that all image parameters correlated with the alveografic parameter Mechanical work (W).

The alveografic Mechanical work had a very significantly lower value as the image brightness was higher. Also, its value decreased

as the amount of red, green and blue were higher.

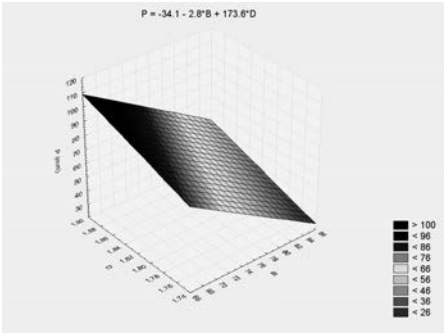

Photo 3. Multiple regression between the alveografic resistance (P) and the image parameters Fractal Dimension (D) and Blue content (B)

The best predictor for the alveografic parameter W was brightness, which explains about 48% of the variability in this parameter, as seen from the regression equation shown in Figure 1 (r = 0.69, r2 = 0.48, F = 15.94, p <0.0008).

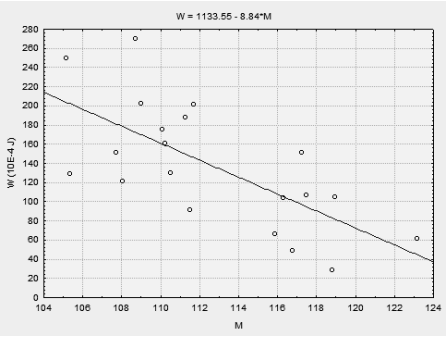

Fig. 1. Regression between alveografic Mechanical Work (W) and brightness (M) of wheat grains

Even in this case, we could notice the significant correlation of the Fractal dimension (D) with the value of the alveografic parameter W (r = 0.51). Perhaps the explanation is also offered throughout the shrunken kernels, which determined the increasing of the Fractal dimension of wheat. The increasing of the Fractal dimension of various biological structures (cells, cell organelles, etc.) has been associated in many studies with different deviations from their normal status. Thus, the correlation of Fractal dimension of the grains with the increasing of baking quality of wheat may suggest a similar phenomenon, of deviation from the variety normality, due to the action of certain environmental factors or plant growing factors.

The parameter P/L, which quantifies the relationship between Resistance and Extensibility of dough, had a significantly higher value as the content of Blue images was lower.

We notice that the alveografic parameters related to dough extensibility (Extensibility L, Gluten swelling index and Elasticity index of gluten G Ie) were not significantly correlated with image parameters.

CONCLUSIONS

Our results show that there are important prerequisites for the evaluation of wheat quality parameters such as Hectolitric mass, Resistance, and Work on behalf of the alveografic image parameters: Brightness, Fractal dimension and Contents in the primary colors (Red, Green, Blue).

We believe that our results can be an interesting research direction, in order to use optical methods for rapid assessment of wheat quality.

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DEFENSE RESPONSE INDUCED IN TOMATO PLANTS (*LYCOPERSICON ESCULENTUM*) BY MICROBIAL INFECTIONS

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Abstract

Most of the microorganisms associated with plants are pathogens so plants generally respond to pathogen infections by using their defensive system. Plant pathogen-related (PR) proteins including peroxidase, β-1,3-glucanase, phenylalanine ammonia-lyase and chitinase are proteins that are induced as result of pathogen infections. Chitinases and β-1,3-glucanase can degrade the cell walls of some plant pathogens and that may play a role in the host plant's defensive system. In our experiments we used Bacillus licheniformis designated as Bw, that shown antagonistic activities against various fungal pathogens including Rhizoctonia solani that cause Damping–Off of tomato. Soil application on tomato seedling roots (var. Marmade) of R. solani alone and B. licheniformis in combination with R. solani induced a significant increase in the activities of β-1,3-glucanase and phenylalanine ammonia-lyase (PAL). Peroxidase activity in both fungal and bacterial treatments was also significantly stimulated. In contrast, peroxidase activity was reduced upon inoculation of plants with a mixture of B. licheniformis- R. solani. Results indicated that PR proteins were induced in all treatments with pathogenic or non-pathogenic microorganisms.

Key words: *Bacillus licheniformis,* β-1,3-glucanase, *Lycopersicum esculentum,* peroxidase, phenylalanine ammonia-lyase, *Rhizoctonia solani*.

INTRODUCTION

Plant defence related enzymes, including PR-proteins, are generally induced by plant pathogens or by mechanical injuries. Treatment with various types of biotic and abiotic elicitors leads to increased activities of peroxidase (PO), PAL, PPO, β-1,3-glucanase and chitinase. Peroxidases have been implicated in a number of physiological functions that may contribute to systemic resistance induction, including phenol oxidation, lignification [18] and in deposition of phenolic compounds into plant cell walls during interaction [10]. Enzymes like β-1,3-glucanase and chitinase have been reported to induce systemic resistance and differential expression of defense related genes by pathogen agents and other elicitors, in several crops [20], [12], [13]. This defense proteins have the potential to hydrolyse the major components of fungal cell walls like chitin and β-1,3-glucans respectively [11]. PAL plays an important role in the biosynthesis phenolic phythoalexins [7] and like PO and PPO catalyse the last step in the biosynthesis of lignin or other oxidative phenols. Phenolic compounds may be fungitoxic and may increase the mechanical strength of the host cell wall. In the last decades, many studies have been carried out on the antagonistic capacity of several species of fungi and bacteria (*Trichoderma, Pseudomonas, Bacillus*). Soil application of *P. fluorescens* increased the level of enzymes involved in the phenyl propanoid pathway and pathogenesis related proteins (PR- proteins) in response to *Fusarium oxysporum* f. sp.*lycopersici* causing wilt of tomato and *Colletotrichum capsici* causing fruit rot of pepper [18]. *Bacillus subtilis* is known to control diseases caused by the fungi such as *Rhizoctonia solani* and *Fusarium solani* [1].

In the present study we have evaluated *Bacillus licheniformis* Bw for its ability to control *Rhizoctonia solani* which is the causal agent of damping off of tomato seedlings. Induction of defense-related enzymes in tomato plants was investigated after soil application of different treatments.

MATERIALS AND METHODS

Microbial strains and culture conditions. *Bacillus licheniformis* BW strain, selected in previous experiment for its antifungal activity was used in this work. The fungal pathogen *Rhizoctonia solani* was kindly provided by dr. Maria Oprea from Institute for Plant Protection- Bucharest. The isolates were maintained on slants of potato-dextrose-agar (PDA) (*R. solani*) and nutrient agar (NB) (*B. licheniformis*).

Plant materials and experimental treatments. Tomato seeds (*Lycopersicum esculentum* cv. Marmande), after sterilization and drying, were grown in pots containing sterilized soil (500 g /pot). 14 days after, uniform seedling were selected and transplanted to pots divided into 5 groups (15 seedlings/ group) and treated as the following:

- Plants without any treatments witch serve as healthy control;
- Plants infected with *Bacillus licheniformis* (antagonistic control);
- Plants infected with *Rhizoctonia solani* (infected control);
- Plants infected firstly with *Rhizoctonia solani* at the time of transplanting, and than with *Bacillus licheniformis* Bw suspension.

Induction of defense responses. For the pots inoculated with *R. solani* alone, discs (5 mm) of the pathogen were cut from the edge of 3-days-old culture and incorporated in the root zone of the each tomato seedling. Bacterial inoculum containing $9x10^9$ cfu / ml was used as biocontrol agent against *R. solani*. Plants samples were collected 10 days after treatments application. Fresh plant samples were homogenizated with liquid nitrogen and stored at – 18°C.

Enzymatic assays

Plant tissue (0,5 g) was crushed in a mortar under liquid nitrogen. Soluble proteins were extracted in 50 mM potassium phosphate buffer (pH 7,0. 0,1 mM EDTA, 4 % polyvinylpirolidone (PVP) and 0,2 mM ascorbic acid. After centrifugation at 10000 x for 10 min. at 4°C, the supernatant was used for enzime activity assay.

Estimation of peroxidase (PO) activity. For PO extraction sodium acetate buffer 0,2M (pH 6.5) containing 1% PVP, 15 mM/l β-mercaptoethanol and 0.25% Triton x100 was used.

The reaction mixture contained enzyme extract 0,1 ml, 30 mM guaiacol, 1 ml sodium acetate buffer 0.1 M (pH 5.4), and 0.05 H_2O_2 3 %. Absorption was measured at 470 nm for 5 min at intervals of 60s. Enzyme activity was expressed in units/ ml enzyme. One unit of enzymatic activity represents the changes in D.O. with 0.1 units/ min.

Estimation of β-1,3-glucanase activity. Leaf samples (0,5 g) were extracted with 2 ml sodium acetate buffer 0.05 M (pH 5.0) and centrifugated at 12000 g for 15 min at 4°C. The activity of β-1,3-glucanase was determined by measuring the release of reducing sugars using laminarin dinitrosalicylate method [17]. The reaction mixture contained 62.5 µl laminarin 4% and 62.5 µl enzyme extract was incubated at 40°C for 30 min. The reaction was stopped by adding 375 µl dinitrosalicylic acid reagent (preparated by adding 300 ml NaOH 4,5% to 880 ml containing 8,8 g of dinitrosalicylic acid and 22.5 g K Na tartrate) with subsequent heating for 5 min in a boiling water bath. The resulting solution was dilued with 4,5 ml distilled water and vortexed. Absorption was measured at 500 nm. Enzyme activity was expressed as 1 nmol of reducing substances/ min/ ml enzyme.

Estimation of phenylalanine-ammonia-lyase (PAL) activity. Activity of PAL was evaluated according to the method of Assis et al. (2001) [2]. 0,4 ml enzyme extract was incubated with 0.5 ml of borate buffer 0.1 M (pH 8.8) and 1ml L-phenylalanine (20 mM) for 30 min at 37 °C. The reaction was stopped with 1ml HCl 1N. The assay mixture was

extracted with 3ml toluene by vortexing for 30 sec. The absorbance of toluene phase containing trans- cinnamic acid was measured at 290nm. The amount of trans- cinnamic acid formed was calculated using its extinction coefficient of 9630 M^{-1} [8]. One unit is defined as the formation of µmol trans-cinnamic acid / min/ ml enzyme.

Isolation of total RNA

Total RNA from both treated and untreated tomato cultures were isolated using SV Total RNA Isolation System kitt (Promega Corporation, USA) according to the manufacturer's instructions.

RT-PCR (Reverse Transcription- PCR) assay

For RT-PCR, first strand cDNA synthesis and PCR reactions were performed using the Access RT-PCR System kitt (Promega Corporation, USA) according to the manufacturer's indications. For cDNA amplification, 1 µg of RNA was added to 50 µl PCR mixture. PCR amplification was performed using peroxidase specific primers:

Forward: 5` - ATA CTT GCC CGA ACG TCA CCA GC -3`

Reverse: 5`- CAT CCA ATG ACA AAG CAG TCG TGG-3`

The mixtures were amplified with the following cycling parameters: 45°C for 3 min at first cycle, 94°C for 2 min, 1 cycle, 94°C for 30 sec., 60°C for 1 min; 68 °C - 2 min for 45 cycles with a final extinction at 68°C, 7 min. The RT-PCR products was determined by 1,2 % (w/v) agarose gel electrophoresis.

RESULTS AND DISCUSSIONS

Change in activities of peroxidase, β-1,3-glucanase**,** and phenylalanine ammonia lyase was recorded 10 days after treatments application.

Peroxidase (PO) activity. The production of reactive oxygen species is one of the earliest cellular responses following pathogen recognition [9]. As shown in figure 1, in contrast with results obtained from control sample (13,25 U/ml), infection by *Rhizoctonia solani* produced an increased levels in peroxidase activity in tomato leaves (28,35 U/ml). Significantly increased levels in PO activity (37,74 U/ml) were recorded in the plant inoculated with *Rhizoctonia solani* and *Bacillus licheniformis* (fig. 1). High levels of PO activity in tomato leaves is an important element of desease resistance mechanism which are involved directly or indirectly in restriction of pathogen spreading.

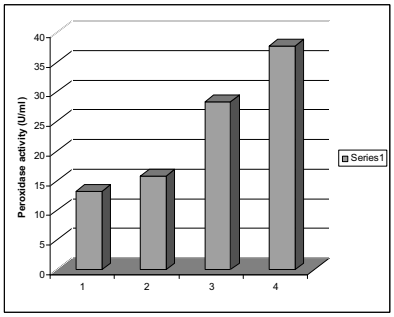

Fig. 1. Induction of peroxidase (PO) activity in the leaves of tomato: (1) Control; (2) Plants infected with *Bacillus licheniformis*; (3) Plants infected with *Rhizoctonia solani*; (4) Plants infected with *Rhizoctonia solani* + *Bacillus licheniformis*.

Induction of β-1,3-glucanase activity. Plant β-1,3-glucanase are pathogenesis- related proteins which are implicated in plant defense responses against pathogen infection or other stimuli [6], [15], [20]. Induction of β-1,3-glucanase in response to various pathogen and their elicitors has been well investigated in plants. In this study β-1,3-glucanase activity was significantly increased both in the harvested pods of plant infected with *Rhizoctonia solani* alone and *R. solani* in combination with *Bacillus licheniformis*. This activity was higher in the treated plants with *R. solani* (3,988 U/ml) (fig. 2). Plant β-1,3-glucanase can directly inhibit the fungal growth *in vitro* by catalyzing the hydrolysis of β-1,3-glucan which is a major component of the cell walls of many pathogenic fungi.

Induction of phenylalanine ammonia - lyase (PAL) activity. Soil aplication of *B. licheniformis* as biocontrol agent and *B.licheniformis* - *R. solani* in combination caused plants to synthesize PAL .This activity ranged from 0,3572 U/ml (*B. licheniformis*) to 0,1570 U/ml (*B. licheniformis* - *R. solani*) respectivly. Phenylalanine ammonia-lyase (PAL) catalyses the first step in the biosynthesis of phenylpropanoids, which form a wide variety of plant secondary products.

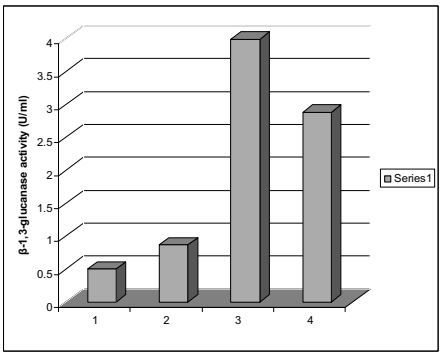

Fig. 2. Induction of β-1,3-glucanase activity in the leaves of tomato: (1) Control; (2) Plants infected with *Bacillus licheniformis*; (3) Plants infected with *Rhizoctonia solani*; (4) Plants infected with *Rhizoctonia solani* + *Bacillus licheniformis*.

The transcription of PAL is regulated in response to various factors that induce the accumulation of flavonoids, lignin and compounds thought to be involved in plant defence reactions [3]. Lignin is the major product of phenylpropanoid metabolism and deposition of lignin to reinforce plant cell in a induncible defense mechanism used for protection against pathogen invasion [14]. Phenylpropanoids are also known to act as molecular signals during recognition processes in the interaction between some soil born bacteria and their host plants [16]. PAL activity is often correlated with changes in the rate of acumulation of phenylpropanoids. The induction of PAL is influenced by a variety of stimuli including plant hormones, mechanical injuries, light and disease which can be involved in PAL inactivation system [4], [21]. In our experiments the activity of PAL was increased significantly in healthy plants (control) and in plants treated whit *R. solani* alone (fig. 3). The time required to activate the defense mechanisms is important for the supression of the pathogen. It seems that the activity of PAL reached a maximum levels on the first days after treatments and once time the PAL activity decreased dramaticaly.

Determination of mRNA transcript level by reverse transcription (RT)- PCR. To investigate the transcriptional expression of the *POX* gene in tissues treated with different types of biotic stresses, we isolated total RNA from leaf tissues and carried out RT-PCR analysis using the specific PCR primers. As shown in figure 4, the RT-PCR products from tomato treated plants were relieved that *POX* gene expression was highly induced in plants subjected to various types of treatment: *B. licheniformis* (lane 2), *R. solani* (lane 3) and *R. solani* in combination with *B. licheniformis* (lane 4).

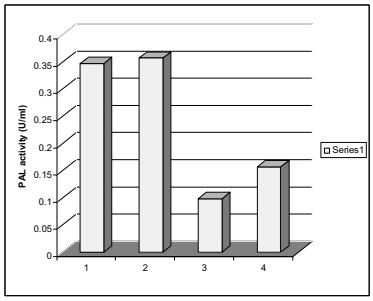

Fig. 3. Induction of phenylalanine ammonia - lyase (PAL) activity: (1) Control; (2) Plants infected with *Bacillus licheniformis*; (3) Plants infected with *Rhizoctonia solani*; (4) Plants infected with *Rhizoctonia solani* + *Bacillus licheniformis*.

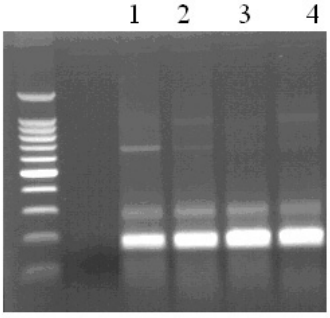

Fig. 4. Expression of transcript level for *POX* gene in tomato plants: (1) Control; (2) Plants infected with *Rhizoctonia solani*; (3) Plants infected with *Bacillus licheniformis*; (4) Plants infected with *Rhizoctonia solani* + *Bacillus licheniformis*.

CONCLUSIONS

In the present study, soil application of *Bacillus licheniformis* as a biocontrol agent against *Rhizoctonia solani* increased the activity of defense related enzymes in tomato seedlings 10 days after treatments application. Plant treatments with *Rhizoctonia solani* or *Rhizoctonia solani* in combination with *Bacillus licheniformis* produced significantly increased levels in peroxidase (PO) and β-1,3-

glucanase activities in tomato leaves. Peroxidases, which generate H_2 O_2 and oxidize phenols, are important in lignin biosynthesis. The expression of *POX* gene in tissues treated with different types of biotic stresses, was investigated by RT-PCR analysis using the specific PCR primers. RT-PCR products from tomato treated plants were relieved that *POX* gene expression was highly induced. However, in all cases there was a strong correlation between induction of mRNA activity and enzyme activity.

Phenylalanine ammonia-lyase (PAL) activity was positively correlated with the plants infected by *Bacillus licheniformis*. However, the highest levels in PAL activity was recorded in the control plants.

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RESEARCH ON BIOCOMPOSITE COMPOST INFLUENCE ON THE DEVELOPMENT OF HYBRID TOMATO SEEDLINGS "SYMPATHIE" F1 (FAMILY *SOLANACEAE*)

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Abstract

Biodegradable composting materials production is growing because of the environmental protection requirements which are becoming more and more restrictive regarding the plastics obtained from petroleum resources. Safety requirements must be met if the compost product is designed and used for agriculture, so far, the harmful effects of solid waste was estimated based mainly on its chemical composition. Phytotoxicity is described as an intoxication of living plants when certain substances which are present in the environment, are accumulated in plant tissue. Plant material analyzed was the hybrid tomato seedlings "SYMPATHIE" F1 (family Solanaceae), resulting from seed germination in greenhouse multiplier conditions.Were tested two types of compost: compost Control: only biowaste and test compost: biowaste + composite (10% PLA / Ecoflex (50/50)-Rettenmaier15%) in three experimental series, representing three proportions of mixtures S1 (25%), S2 (50%) and S3 (75%).The behavior of seedlings in various mixtures of compost before transplantation was considered. Different components of mixtures and experimental variant blends themselves were analyzed to see the content of nutrients, in soluble forms which could be available to plants during the vegetation.After two months of agrochemical analyses performed weekly on tomato seedlings, the results showed that the cultivated version of the compost sample P 25% (25% compost from V2-variant and 75% Profesional substrate of brown peat and dark peat), recorded high supply of N (53,20ppm), P (307,80 ppm) and K (5600 ppm) which will be used for transplanting in the field or greenhouse lower consumption of nutrients for plant growth and development.The findings obtained from experimental samples were compared with values recorded in tomato seedlings that were grown in parallel only in Professional Substrate mixture of peat and peat-dark brown 75% 25%, as control.

Key words**:** *biocomposite, compost, phytotoxicity, tomato seedlings*

INTRODUCTION

Compost has a high content of organic matter and helps to recover the intensive properties of the soil which were lost or damaged during use. Although compost is not considered a fertilizer it contains nutrients that could enhance plant growth. When used in combination with fertilizers, compost acts as a plant fertilizer that provides nutrients needed for a longer period than in the case of the application of fertilizers alone [1, 2].

Materials used in obtaining PLA based Biocomposites that are rich in carbon, are usually dried material, e.g. sawdust, cardboard, dried leaves, straw, branches and other woody or fibrous materials that are slowly disintegrated.

Mineral nutrition of tomato has some peculiarities that must be taken after fertilization. Thus, the specific consumption varies depending on the culture system, variety, production, supply degree of soil minerals [3].

MATERIAL AND METHOD

In preparing the test solution to determine the effect of substrate on growth and development of tomato seedlings from "SYMPATHIE"F1 hybrid (*Solanaceae* family*)*, the following were taken into account: biological features of the hybrid analysis and the components of the biocomposite versions.

The compost samples were provided by OWS beneficiary.

Simpathie F1 is a tomato hybrid with undetermined growth, suitable for fresh consumption. The hybrid is very adaptable in various cultivation conditions due to excellent resistance to a range of diseases. Also, it has good resistance to storage and clusters are uniform and vigorous.

Tomatoes seedlings used for initiation of the experiment were obtained previously by seeding and sub-cultivating procedure, and when they reached the height of 5 cm and the second leaf grew out of the soil, they were transferred in vegetation pots with a diameter of 7 cm (Fig. 1).

Fig. 1. The tomato seedlings "SYMPATHIE" F1 when before they were transferred in vegetation pots with a diameter of 7cm, on March 12, 2012

The experiments were performed between March 12 – April 27, 2012 for tomato plants "SYMPATHIE" F1 in three experimental series, representing three mixture proportions 25% (S1), 50% (S2) and 75% (S3) from work versions as explained in Table 1.

The Control substrate used was 100% peat KAKKILÄ (Finland) DSM 110 (Dark Seed Mix) that contains: "starter" NPK fertilizers 14-16-18+mE, pH=5.5-5.9.

For every experimental series (S1, S2 and S3) of the two compost versions (Version 1-Control compost and Version 2-Test compost) ten seedlings for each sample (one vegetation pot = one repetition per sample), one seedling per pot, for all the six samples (V1-V6) were provided.

Further results of plant growth and development were compared with Control (MT) version represented by 100% KAKKILÄ DSM 110 peat substrate. These were also provided with ten seedlings, one seedling per vegetation pot.

Table 1. Definition of samples/ substrate versions used in the experiments

Biocomposite samples versions	Work samples	Sample
Version 1 Control compost (only biowaste at start)	V1 - C 25%	S1 (25% Control compost)+ (75%) KAKKILÄ DSM 110 substrate
	V2 - C 50%	S2 (50% Control compost)+ (50%) KAKKILÄ DSM 110 substrate
	V3 - C 75%	S3 (75% Control compost)+ (25%) KAKKILÄ DSM 110 substrate
Version 2 Test compost: Biowaste + 10% PLA/Ecoflex(50/50)-Rettenmaier15% at start	V4 - P 25%	S1 (25% Test compost)+(75%) KAKKILÄ DSM 110 substrate
	V5 - P 25%	S2 (50% Test compost)+(50%) KAKKILÄ DSM 110 substrate
	V6 - P 75%	S3 (75% Test compost)+(25%) KAKKILÄ DSM 110 substrate
Control	MT	100% KAKKILÄ DSM 110 peat substrate (0% Biocomposite samples versions)

For each 7 cm diameter vegetation pot were used 20 grams/ each pot of mixture in the set proportions for the three experimental series: S1, S2 and S3 for both compost versions, noting that the control used each time was KAKKILÄ peat (Finland) DSM 110 (Dark Seed Mix).

RESULTS AND DISCUSSIONS

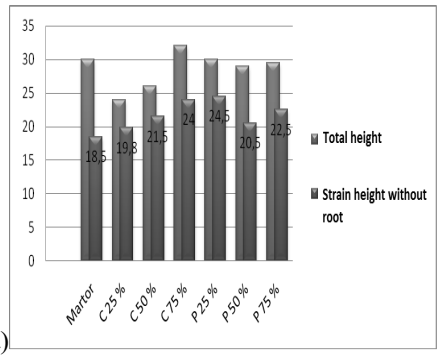
During vegetation period, the plants' height was measured weekly in and the plants' shoots were numbered. Also agrochemical analyses were performed weekly on the substrates and plants, to determine the status of the fertilizer supply.

For testing the seedlings behavior in different compost mixtures , right before transplantation both experimental versions mixture

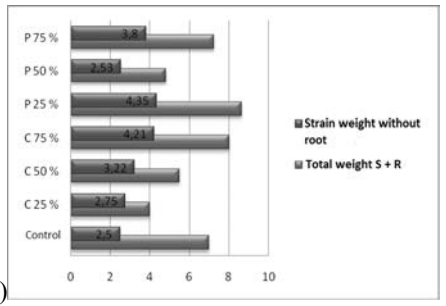
components and the peat mixtures themselves were analyzed, to determine the nutritive elements content, in soluble forms that are available to plants during vegetation period. The results are presented in Table 2.

After transplantation, the tomatoes were left for two weeks for acclimatization (Fig. 2) and then collected weekly for analysis. Thus, the first analysis was made on April 10, 2012, when some biometric measurements of the plant height and weight, and agrochemical analyses to the substrate and plants were made (Fig. 3).

Fig. 2. Picture from March 30, 2012 (after ten days from the start of the experiment) - culture in the greenhouse multiplier (UASVM Bucharest)



a)



b)

Fig. 3. Results of tomato seedlings "SYMPATHIE" F1 biometric measurements on April 10, 2012 in experimental versions: a.) height variation; b.) weight variation

In the same time with the biometric measurements on April 10, 2012, agrochemical analyses were performed for the experimental versions substrates (Table 3). Analyzed tomato seedlings presented high quantities of N, P and K which created the conditions to obtain a high supply of plant and achieve high yields while transplanting in the field or greenhouse (Table 4).

Table 2. The analysis of the components and substrate mixtures on March 22, 2012

Version code	Version name	pH	Soluble salts %	Concentration, ppm			
				N-NH4	N-NO3	P-PO4	K
1.P	**Testing compost**	7.3	1.450	68.832	163.400	58.050	320
2.T	**Peat**	7.0	0.375	27.724	147.250	109.687	50
3.Ct	**Control compost**	7.1	1.736	59.272	427.500	106.312	480
4.V1	**C 25%**	7.1	0.375	27.724	118.750	38.475	50
5.V2	**C 50%**	7.1	0.346	9.560	121.600	24.050	105
6.V3	**C 75%**	7.0	0.332	8.604	115.900	26.075	275
7.V4	**P 25%**	6.9	0.245	6.692	68.400	25.312	245
8.V5	**P 50%**	7.0	0.375	16.252	77.900	25.312	355
9.V6	**P 75%**	6.9	0.231	11.472	94.050	14.175	160

* P = test compost
C = compost biowaste, control

Table 3. Tomato soil analysis on April 10, 2012

Version code	Version name	pH	Soluble salts %	Concentration, ppm			
				N-NH4	N-NO3	P-PO4	K
1.Ct	**Control**	6.9	0.2601	17.208	36.100	8.438	70
2.V1	**C 25 %**	7.0	0.2601	25.812	33.250	15.863	290
3.V2	**C 50 %**	7.3	0.2745	25.812	12.428	16.538	330
4.V3	**C 75 %**	7.4	0.3381	24.856	24.700	12.150	400
5.V4	**P 25 %**	7.2	0.3265	28.680	24.700	27.675	360
7.V5	**P 50 %**	7.2	0.4190	29.636	36.100	14.175	465
8.V6	**P 75 %**	7.2	0.3468	32.504	48.450	24.638	345

Table 4. Tomato plant analysis on April 10, 2012

Version code	Version name	Concentration, ppm		
		N-NO3	P-PO4	K
1.Ct	**Control**	216.60	70.20	4960
2.V1	**C 25 %**	425.60	303.75	4420
3.V2	**C 50 %**	273.60	337.50	4540
4.V3	**C 75 %**	277.40	324.00	5400
5.V4	**P 25 %**	395.20	375.30	5800
7.V5	**P 50 %**	406.60	382.05	4100
8.V6	**P 75 %**	444.60	441.45	5100

The second time of analysis, on April 24, 2012, biometric measurements on plant height and on tomato seedlings weight were developed; the results are presented in Fig. 4.

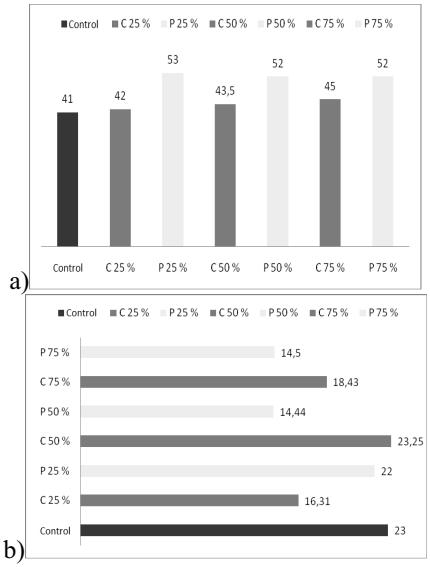


Fig. 4. The increase in tomato seedlings ”SYMPATHIE” F1 biometric measurements evaluation on April 24, 2012 in experimental versions: a.) height variation; b.) weight variation

On April 24, 2012 agrochemical analyses for experimental versions substrates and tomato seedlings were performed (Table 5 and Table 6). The potassium appeared to be the only element that increased significantly, almost doubled in the second time of analysis.

In Fig. 5, tomatoes seedlings are depicted after 40 days from the start of the experiments.

Fig. 5. Pictures from April 20, 2012 (after 40 days from the start of the experiments) -cultivated in multiplier greenhouse (UASVM Bucharest)

Table 5. Tomato soil analysis on April 24, 2012

Version code	Version name	pH	Soluble salts %	Concentration, ppm			
				N-NH4	N-NO3	P-PO4	K
1.Ct	**Control**	6.1	0.2312	9.560	5.70	4.388	920
2.V1	**C 25 %**	5.9	0.3323	10.516	13.30	16.538	1175
3.V2	**C 50 %**	5.8	0.3843	17.208	15.20	9.788	1025
4.V3	**C 75 %**	6.0	0.4046	21.032	16.15	9.450	1350
5.V4	**P 25 %**	5.9	0.4190	21.988	15.20	15.863	970
7.V5	**P 50 %**	6.0	0.3034	19.120	23.75	17.550	1175
8.V6	**P 75 %**	5.9	0.3612	19.120	18.05	12.825	1050

Table 6. Tomato plant analysis on April 24, 2012

Version code	Version name	Concentration, ppm		
		N-NO3	P-PO4	K
1.Ct	**Control**	26.60	438.75	160
2.V1	**C 25 %**	38.00	513.00	860
3.V2	**C 50 %**	68.40	459.00	980
4.V3	**C 75 %**	34.20	355.05	1540
5.V4	**P 25 %**	26.60	533.25	920
7.V5	**P 50 %**	53.20	625.05	860
8.V6	**P 75 %**	26.60	560.25	1024

The plant analysis from the experimental versions showed a good supply of nutritive elements assured by the transplantation high quantity productions.

The last time of analysis was on May 3, 2012. The tomato seedlings in experimental versions showed similar growth rates in terms of heights (Fig. 6).

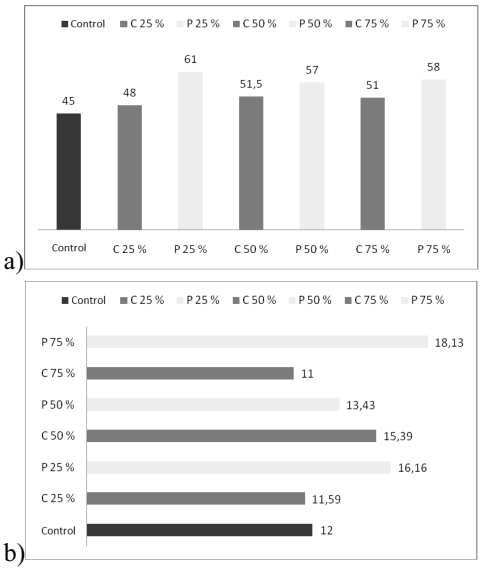


Fig. 6. The increase in tomato seedlings "SYMPATHIE" F1 biometric measurements evaluated on May 3, 2012 in experimental versions: a.) height variation; b.) weight variation

Generally, the testing compost versions recorded higher accumulations of fresh substance comparative with control compost versions.

Tomato seedlings weight (Fig. 6 b) oscillated between 11g in version C75% to 18.13g in version P75%. In general, the tomato seedlings who grew in testing compost versions recorded higher accumulations of fresh substance comparative with the tomato seedlings that grew in control compost versions.

Agrochemical analysis of the substrate (Table 7) from the experimental versions showed that the pH increased oscillating between 6.4 and 6.7, the soluble salts content remained high characterizing weak saline substrates, the nitrogen supply was high, and the phosphorus content decreased characterizing low supply .

The statistical interpretation of the relation between the height of tomato seedlings and the nitrogen content of the substrate, based on mathematical regression, showed a statistically significant linear correlation with the coefficient R=0.90332 (Fig. 7).

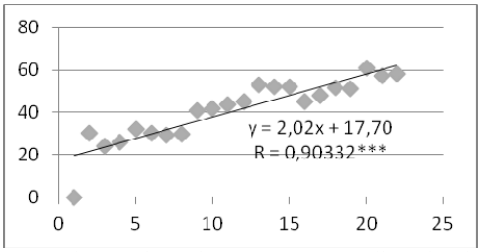


Fig. 7. The correlation between tomato seedlings height and the nitrogen content (N) of the substrate

Also, between the N content from the substrate and the N content from the tomato seedlings there is a statistically significant correlation, with the coefficient R = 0.77588 (Fig. 8).

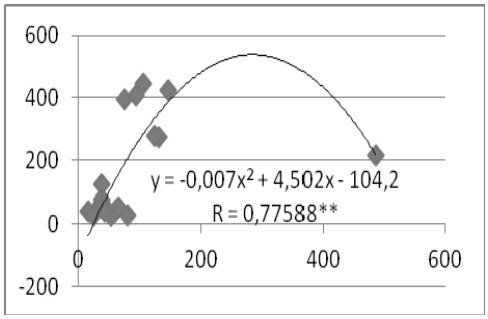


Fig. 8. The correlation between N content from the substrate and N content from the tomato seedlings

Table 7. Tomato soil analysis on May 3, 2012

No.	Version	pH	Soluble salts %	Concentration, ppm			
				N-NH4	N-NO3	P-PO4	K
1.Ct	Control	6.5	0.3179	4.780	7.6	3.713	20
2.V1	C 25 %	6.6	0.7225	21.032	20.9	5.738	320
3.V2	C 50 %	6.7	0.4624	17.208	20.9	5.400	95
4.V3	C 75 %	6.6	0.4335	18.164	14.25	7.763	340
5.V4	P 25 %	6.7	0.5491	19.120	15.2	9.450	190
7.V5	P 50 %	6.5	0.4335	30.592	29.45	8.100	260
8.V6	P 75 %	6.4	0.6647	27.724	18.05	8.775	190

CONCLUSIONS

The testing compost versions recorded higher accumulations of fresh substance compared with control versions;

Agrochemical analyses of the substrates from experimental versions showed that the pH initially decreased at about 5.8 – 6.1 and then increased oscillating between 6.4 and 6.7;

Soluble salts content from the substrates experimented on remained high characterizing weak saline substrates;

Nitrogen supply of the substrates was high, phosphorus content decreased during vegetation characterizing low supplied substrates on this element, while potassium from the substrates was very high because of the slow release of this element from the compost;

Tomato seedlings agrochemical analyses showed that these had a high supply in N, P and K which would in turn provide a lower consumption of nutrients for plant growth and development when transplanting in the field or greenhouse;

The statistical interpretation by analysis of variance on seedling height showed that the versions made with test compost (P25%, P50%, P75%) had significant results and in the matter of plant weight, all versions had statistically insignificant results;

Taking into account the composition of the six experimental samples (V1-V6), it is a clear conclusion that as general tendency, the phytotoxicity is lower for the sample based on PLA Biocomposite (Biowaste + 10% PLA/Ecoflex(50/50)-Rettenmaier15% at start =V4-V6) in comparison with the Control compost Biocomposite (only biowaste at start =V1-V3) and control KAKKILÄ peat (Finland) DSM 110.

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ECOLOGICAL PRODUCTS FOR AGRICULTURE

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Abstract

This paper aims to reduce the environmental pollution caused by fertilizer use in agriculture by chemical synthesis, by identifying suitable technologies and biological products to be used to fertilize soil in Romania. An alternative to limiting pollution from synthetic fertilizers and pesticides is to use in agriculture some products based on bacteria, bacterial consortia or biomass with plant or animal origin. The microbial fertilisers shall be characterized in terms of agrochemical efficiency.

Key words: *bacterial fertilisers, agrochemical efficiency*

INTRODUCTION

Fertile soil contains several hundred million microorganisms/gram (table 1). The most numerous are bacteria (unicellular), followed in decreasing order of actinomycetes (specialized groups there are species of bacteria that produce antibiotics), fungi, soil algae, cyanobacteria (blue-green algae that are actually photosynthetic microorganisms that can add small amounts of carbon in soil and can cause trouble on the golf lawn adorned with lawn areas) and soil protozoa (unicellular organisms in the soil with organic material role in the decomposition)

Table 1. Types of microorganisms from the soil [1]

Group of microorganism	Number of germs / gram soil
Bacteria	100.000.000 – 1.000.000.000
Fungi	100.000-1.000.000
Algae and cyanobacteria	1.000- protozoa 1.000.000
Protozoa	1000-100.000

One of macronutrients – nitrogen, is essential for all living organisms for the synthesis of proteins, nucleic acids and other compounds that contain nitrogen. Relative independence of leguminous plants from the soil available nitrogen has great ecological importance because it reduces the required nitrogen mineral fertilizers in crop vegetables prevents accumulation of nitrogen compounds in groundwater and ensures the accumulation of high concentrations of protein in beans. Symbiotic association of bacteria of the genera R*hizobium* and *Bradyrhizobium* and leguminous plants, soil building each year with 90×10^6 metric tons of nitrogen fixed amount is double that obtained by industrial processes [1-5]. According to studies undertaken by several researchers, symbiotic nitrogen fixation productivity in grain and leguminous plants and the feed is presented in Table 2 and Table 3

Table 2. Symbiotic fixation in grain [1]

Vegeta-bles type	Average level $kg \bullet ha^{-1} \bullet year^{-1}$	Maximum level, $kg \bullet ha^{-1} \bullet year^{-1}$
beans	210	552
peas	65	80
soy	60	100
lentil	100	114

The relationship between vegetables and bacteria (*Rhizobium* or *Bradyrhizobium*) can be presented as a symbiotic process that takes place for the benefit of both parties that:

- bacteria receive nutrients from the plant (carbohydrate);
- plant receives its nitrogen needs from bacteria.

Nodule-forming bacteria have specific hosts, such as specific types of plants are node specific species of bacteria (Table 4).

Table 3. Symbiotic fixation in fodder vegetables [1]

Vegetables type	Average level kg• ha^{-1} •year-1	Maximum level kg• ha^{-1}•$year^{-1}$
Lucerne	184	465
Melilotus alba	183	-
Clover	183	673
Lotus corniculatus	116	-
Vetch	73	93

Table 4. Types of plant which make nodules with bacteria

Bacteria which form nodules	Types of plants
Rhizobium meliloti	lucerne, clover
Rhizobium leguminosarum	peas
Rhizobium trifolii	white or red clover
Rhizobium phaseoli	dried beans
Rhizobium viceae	vetch
Rhizobium loti	clover
Bradyrhizobium japonicum	soybeans, peanuts

MATERIALS AND METHODS

The influence of microbial fertilizers on the germination of crop plants

Experiments were conducted in green house at SC ECOAGRICOLA SRL in 3 repetitions, each repetition using the 40 seeds each. Seeds of *Cucumis sativus*, *Daucus carota, Beta vulgaris*, *Solanum lycopersicum, Capsicum annum, Triticum aestivum, Pisum sativum* and *Vicia faba* were immersed for 10 minutes in:
a) solution of *Rhizobium* inocula, or in
b) solution of compost, or in
c) water (as witness)
After immersion, the seeds were dried in the shade, and then were planted in plastic pots. The substrate used in experiment was washed sand. Culture vessels were kept in the greenhouse and were watered as often as was necessary. Seedlings obtained were collected, were washed under running water and were dried with filter paper were then weighed with a Sartorius balance type digital. Counting seedlings were made every day.

The influence of microbial fertilizers on the development of crop plants

Experiments were conducted in randomized blocks in three repetitions in period: autumn 2 until next summer. Soil plots had pH = 5.64 and initial nutrient content of soil is shown in table 5.

Table 5. Initial nutrient content of the soil

C_{total} %	N_{total} %	P_{total} %	K_{total}, %
1.03	0.108	0.00158	1.85

Experimental variants included soil fertilization with fertilizer mixed and/or with mixed fertilization+bacterial inocula or organic fertilizer (compost)+bacterial inocula, according to Table 6, and as test plant was used *Glycine max* (soybean)*)*

Table 6. Experimental fertilization variants for soil cultivated with *Glycine max*

Ferti-liza-tion vari-ant	Quantity of active ingredient per hectare (fertilization of basis) (NPK)	Crop	Quantity of active ingredient per hectare (spring fertilization) (NPK)
V1	80:60:30	*Glycine max*.	100:60:30
V2	80:60:30	*Glycine max.*	60:60:30
V3	80:60:30	*Glycine max.*	30:60:30
V4	80:60:30	*Glycine max.*	0:60:30
V5	80:60:30	*Glycine max.*	Inocula*+ 60:60:30
V6	80:60:30	*Glycine max.*	Inocula+ 30:30:30
V7	80:60:30	*Glycine max*	Compost+ 60:60:30
V8	80:60:30	*Glycine max.*	Compost+ 30:60:30
V9	80:60:30	*Glycine max*	Inocula+ 0:0:0
V10	80:60:30	*Glycine max.*	Compost +0:0:30
V11	80:60:30	*Glycine max.*	Compost + inocula + 30:60:30

* Inocula:=strains of *Rhyzobium sp.* + *Bradyhrzobium sp.*

Bio fertilizer (inocula) was incorporated into the soil with seeding; time of use was 1:10 (1 gram to 10 grams of inoculated seeds). One third of the total amount of nitrogen and all phosphorus and potassium were applied in autumn. The remainder of nitrogen (2/3) was applied after 20 days and 35 days respectively after sowing. For soybeans has adopted an area of 40x20 cm, corresponding to a population of about 375.000 plants•hectare^{-1}. Plant height, number of nodules, nodule mass was determined by a range between (14÷60) days from sowing.

RESULTS AND DISCUSSIONS

INFLUENCE OF MICROBIAL FERTILISERS REGARDING SEED GERMINATION

Significant differences were obtained between the two treatments in terms of percentage of germination for most species (figure1-8) treatment with bio fertilizer solution indicated a large number of seeds germinated. Inoculated microbial seed treatment with *Rhyzobium* type can increase the percentage of germination of leguminous plants type *Cucumis sativus, Daucus carota*, *Beta vulgaris, Solanum lycopersicum, Capsicum annum, Tritium aestivum, Pisum sativum, and Vicia faba*, and furthers the plant normally developed.

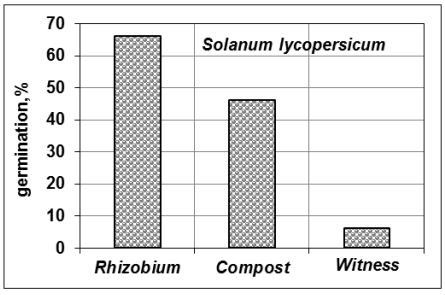

Fig. 1. Experimental variants influence on seed germination of *Solanum lycopersicum*

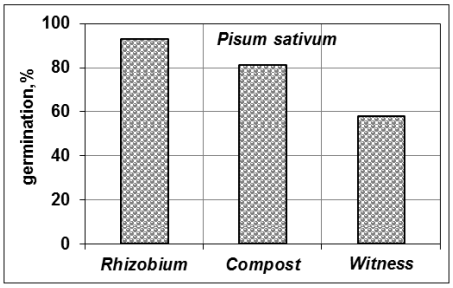

Fig. 2. Experimental variants influence on seed germination of *Pisum sativum*

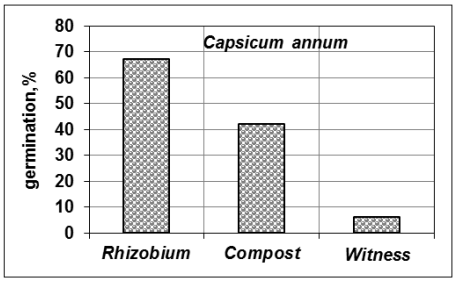

Fig. 3. Experimental variants influence on seed germination of *Capsicum annum*

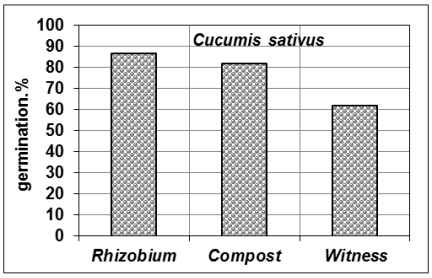

Fig. 4. Experimental variants influence on seed germination of *Cucumis sativus*

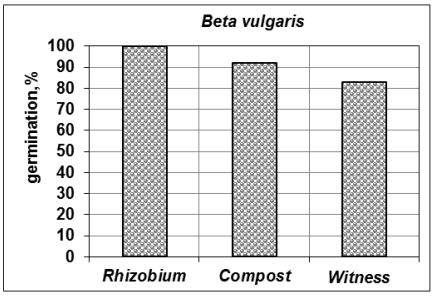

Fig. 5. Experimental variants influence on seed germination of *Beta vulgaris*

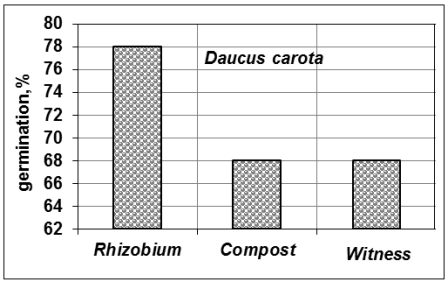

Fig. 6. Experimental variants influence on seed germination of *Daucus carota*

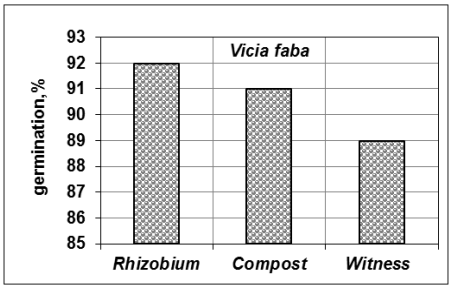

Fig. 7. Experimental variants influence on seed germination of *Vicia faba*

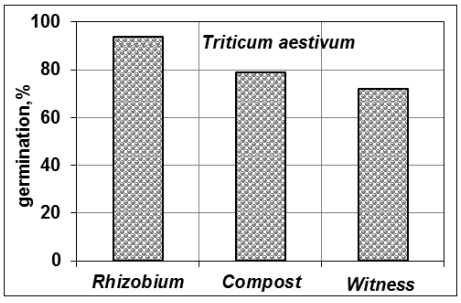

Fig. 8. Experimental variants influence on seed germination of *Triticum aestivum*

INFLUENCE OF MICROBIAL FERTILISERS REGARDING THE DEVELOPMENT OF CROP PLANTS

After two weeks of sowing, the average height of *Glycine max* plants ranged from 12 to 15.7 cm. Highest plants were obtained for variant V1 (100:60:30). After 28 and 42 days after sowing, after harvest values are obtained for both large and variant V1 to V11 version (Fig. 9-12)

Influence of fertilization variant to the number of nodules formed

After 28 days of seeding, the numbers of nodules on *Glycine max* ranging from 15.3 to 35.3. Higher values are obtained in variant V6 and V5, as a result of nitrogen fixing capacity by microorganisms (figure 13).

After six weeks after sowing, the average number of nodes varies from 19.3 to 50, in this case the largest number of nodes is recorded for variant V6 (figure 14)

After 56 days from sowing, significant differences occur in cases where inorganic fertilizers are applied without inoculated or in the presence of inocula and compost (figure 15). These differences can be attributed to development of nitrogen fixing ability by microorganisms.

Fertilizer type influence regarding the mass of nodules formed

After 28 days, nodules formed mass range from 93.3 to 283.3 mg, the highest value being obtained for the V6 version, probably because nitrogen-fixing microorganisms used as inocula (figure 16).

After six weeks of sowing, the average mass formed nodules ranging from 121.7 to 400 mg, the highest values being obtained for variant V6 and V5. These values show that under the influence of inocula, nodules formed mass increases significantly compared to the version that uses high doses of nitrogen fertilization with inorganic sources (figure 17), obtaining similar results after 56 days (figure 18).

Fertilization type influence regarding biomass production of *Glycine max*

After 28 days, the amount of biomass obtained range from 4782 to 9375 $t \bullet ha^{-1}$, and in this case the highest values are obtained for fertilizer variant V1 (100:60:30) or V5 (I +60:60:30 where I=inocula) and V7 (C +60:60:30 where C=compost) (figure 19). The results show that small doses of fertilizer applied with inoculated compost can get the same biomass production and variant V1 (100:60:30).

After 42 days from sowing, most production is obtained in the fertilization variant V5 (I+60:60:30) production that exceeds that obtained in the variant V1 (100:60:30) (figure 20), similar results were obtained 56 days after sowing (Fig. 21).

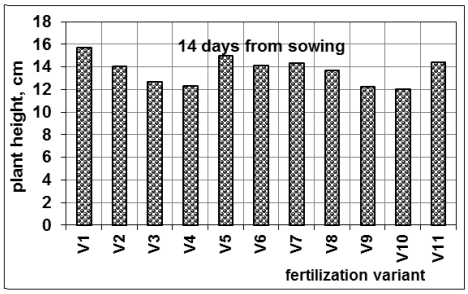

Fig. 9. Influence the types of fertilization regarding *Glycine max* plant height after 14 days from sowing.

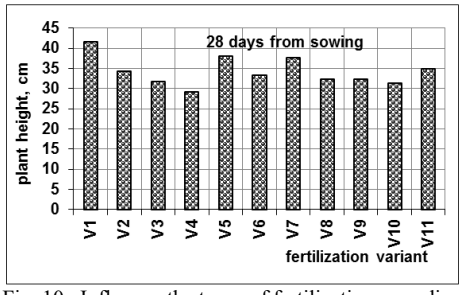

Fig. 10. Influence the types of fertilization regarding *Glycine max* plant height after 28 days from sowing.

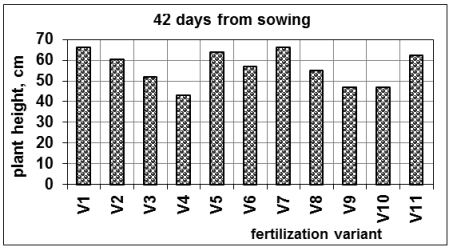

Fig. 11. Influence the types of fertilization regarding *Glycine max* plant height after 42 days from sowing.

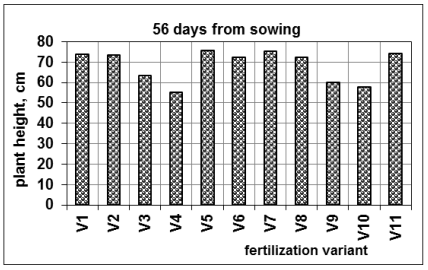

Fig. 12. Influence the types of fertilization regarding *Glycine max* plant height after 56 days from sowing.

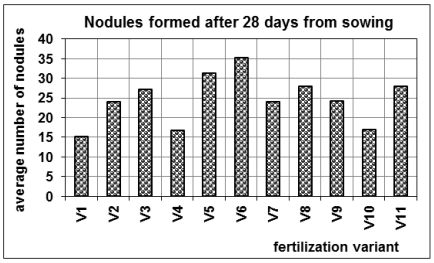

Fig. 13. Influence of fertilization variants on the number of nodules formed on roots of *Glycine max.* after 28 days from sowing.

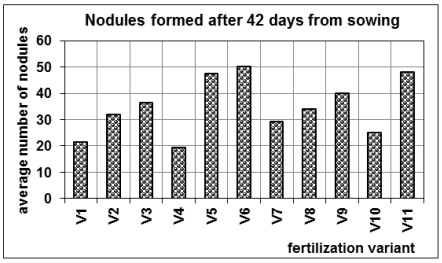

Fig. 14. Influence of fertilization variants on the number of nodules formed on roots of *Glycine max.* after 42 days from sowing.

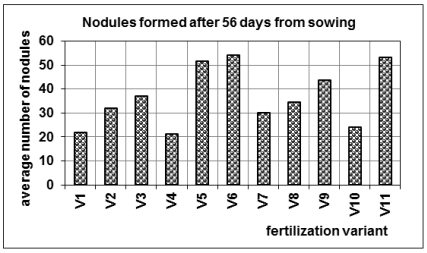

Fig. 15. Influence of fertilization variants on the number of nodules formed on roots of *Glycine max.* after 56 days from sowing

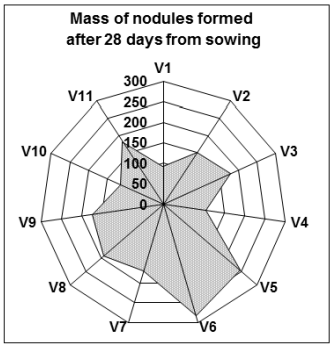

Fig. 16. Influence of fertilization type regarding the mass of nodules formed on roots of *Glycine max* after 28 days from sowing

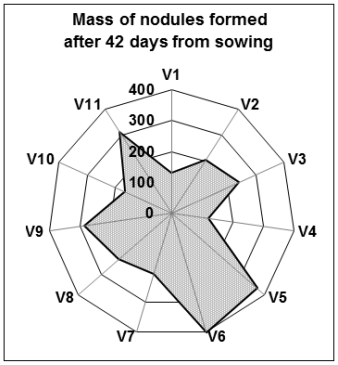

Fig. 17. Influence of fertilization type regarding the mass of nodules formed on roots of *Glycine max.* after 42 days from sowing.

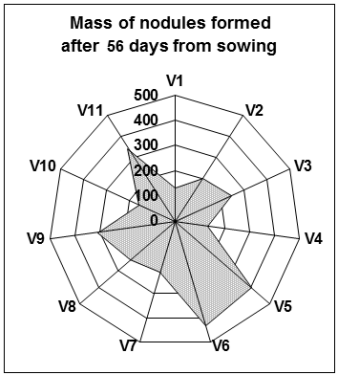

Fig. 18. Influence of fertilization type regarding the mass of nodules formed on roots of *Glycine max.* after 56 days from sowing.

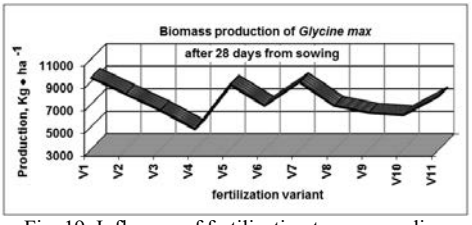

Fig. 19. Influence of fertilization types regarding biomass production of *Glycine max* after 28 days from sowing

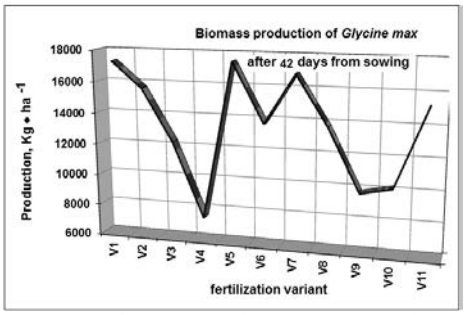

Fig. 20. Influence of fertilization types regarding biomass production of *Glycine max* after 42 days from sowing

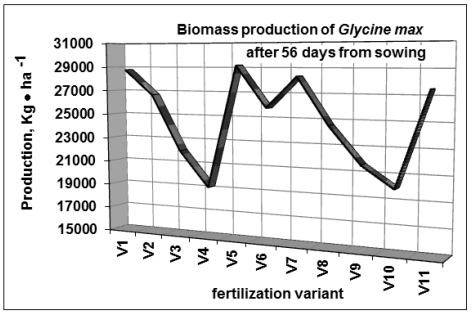

Fig. 21. I Influence of fertilization types regarding biomass production of *Glycine max* after 56 days from sowing

CONCLUSIONS

• Experiments performed in order to establish the effects of microbial inocula regarding germination of crop plants such as *Cucumis sativus, Daucus carota, Beta vulgaris, Solanum lycopersicum, Capsicum annum, Triticum aestivum, Pisum sativum and Vicia faba* have shown that treating seeds with Rhyzobium sp. inocula can increase germination percentages, favouring obtaining the normally developed plants.

• Monitoring crop demonstration of *Glycine max* (soybean) made from a complex pattern of fertilization has been used as fertilizer material combinations inoculated with Rhizobium, compost and NPK fertilizer type, demonstrated that:

- one after 42 and that after 56 days from sowing height of plants fertilized spring mix inoculated microbial + compost + NPK with the low concentration of nitrogen was compared with the control group (which was made only chemical fertilization and the

amount of active substance (kg / hectare) was 100:60:30);
- average number of nodules formed on roots of *Glycine max* is maximum after 42 and 56 days from sowing for the version microbial inocula + NPK of fertilization with nitrogen and phosphorus in low concentrations. This conclusion is valid in terms of mass of nodules.
- after 42 or 52 days after sowing, the highest biomass production per hectare of Glycine max is obtained for fertilization variant which include bacterial inocula variant + NPK with an average amount of nitrogen;
In conclusion, it can be stated that microbial organic fertilizer to replace chemical fertilizers at least some of the following reasons:
• the rising cost of fertilizers decreases their accessibility for small farmers;
• in 2020 the estimated world production of 321 million tons of grain•year^{-1}, nutrient requirements would be at about 28.8 million tons•year^{-1}, chemical synthesis is possible only to a quantity of about 21.6 million tons thus creating a deficit of about 7.2 million tons of soil nutrients [6-7] .

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SCREENING OF ANTAGONISTIC *TRICHODERMA* FOR BIOCONTROL ACTIVITIES ON PHYTOPATHOGENS

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Key words *: biocontrol, dual solid culture, microbial antagonism, phytopathogen, Trichoderma,*

Abstract

Biological control, the use of specific microorganisms that interfere with plant pathogens and pests, is a nature-friendly and ecological approach to overcome the problems caused by standard chemical methods of plant protection. Trichoderma species are known as biocontrol agents against plant phytopathogens. This finding is a consequence of several key factors, such as faster metabolic rates, anti-microbial metabolites and physiological conformation. In this study, in vitro potential of selected isolates strains of Trichoderma were evaluated against well known and virulent phytopathogens, such as Rhizoctonia solani, Fusarium graminearum, Sclerotinia sclerotiorum, Botrytis allii, Botryris cinerea and Pythium debaryanum. The selection of bioantagonistic microorganisms was carried out using dual culture method observing the pathogen growth inhibition by biocontrol agent. The inhibition extent varies from strain to strain. Trichoderma T27 strain presented the highest bioantagonistic activity and the most sensitive pathogens were Fusarium graminearum, Botryris cinerea and Pythium debaryanum . The optical microscopy observations revealed changes of morphological characters at pathogens due to biocontrol agent activity. Full exploitation of the biocontrol potential of Trichoderma spp. could easily provide growth enhancement of domestic plants, green house plants, and agricultural crops.

INTRODUCTION

Plant pathogens produce significant losses to agricultural products. Traditional chemical control methods are not absolutely efficient to minimize these effects. Biological control of plant pathogens can be highly effective especially with potentials of antagonists on pathogenic fungi. *Trichoderma* spp. is the most widely studied biocontrol agents (BCAs) against plant pathogens. The genus comprises a great number of fungal strains that act as biological control agents [1, 2, 3]. In the present study, in vitro potential of four selected isolates strains of *Trichoderma* were evaluated against well known and virulent phytopathogens, such as *Rhizoctonia solani, Fusarium graminearum, Sclerotinia sclerotiorum, Botrytis allii, Botryris cinerea and Pythium debaryanum.* The selection of bioantagonistic microorganisms was carried out using dual culture method observing the pathogen growth inhibition by biocontrol agent.

MATERIAL AND METHOD

Microorganisms

Potential biocontrol agents *Trichoderma* strains (T27, T36, T57, T83) were cultured on solid PDA medium.

Phytopathogen strains: *Rhizoctonia solani, Fusarium graminearum, Sclerotinia sclerotiorum, Botrytis allii, Botryris cinerea* and *Pythium debaryanum*. Strains were grown for 7 days on potato dextrose agar (PDA) at 28^{o}C.

Dual solid culture assay

The antimicrobial capacity of the selected strains was evaluated by dual solid culture assay in Petri plates. Agar dextrose-potato-agar medium was inoculated with culture solid disks from biocontrol agent and from fungal pathogen. After 3-5 incubation days, fungal growth was evaluated by measuring the clear inhibition zone around the disks.

The formula to determine the antagonism level is:

$\mathbf{X = i_A/i_B \times e_B/e_A}$

where i = inner radius; e = outer radius; A = fungus test; B = fungus antagonist; X < 1 – antagonism prezent; X > 1 – antagonism absent.

Morphological analysis

The effect of biocontrol action against pathogens was observed using optic microscope Olympus BX 51 (40x photos).

RESULTS AND DISCUSSIONS

In vitro potential of selected isolates strains of *Trichoderma* were evaluated against virulent phytopathogens, such as *Rhizoctonia solani, Fusarium graminearum, Sclerotinia sclerotiorum, Botrytis allii, Botryris cinerea* and *Pythium debaryanum*. Among the four isolated strains only Trichoderma T 36 and T 27 were been of interest as potential biocontrol agents. These strains were evaluated by dual solid culture method (Table 1.).

Table 1. Antagonistic activity of *Trichoderma* strains evaluated by dual solid culture method

Biocontrol	Phytopathogen	Antagonism evaluation (X)*
Trichoderma T36	*Rhizoctonia solani,*	0.30
	Fusarium graminearum	0.53
	Sclerotinia sclerotiorum	0.31
	Botrytis allii	0.17
	Botryris cinerea	0.27
	Pythium debaryanum	0.19
T*richoderma* T27	*Rhizoctonia solani,*	0.46
	Fusarium graminearum	**1.05**
	Sclerotinia sclerotiorum	0.38
	Botrytis allii	0.23
	Botryris cinerea	**1.07**
	Pythium debaryanum	**1.01**

* Each value is an average of five replicates.

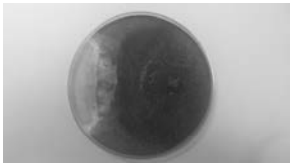
From the results of dual culture assay it was found that *Trichoderma* T 27 inhibited pathogen more than the other tested strain.

Trichoderma T 27 was particularly very active against *Fusarium graminearum* (X=1.05*), Botryris cinerea (X*=1.07) and *Pythium debaryanum* (X=1.01). For both *Trichoderma* strains, *Botrytis allii* presented higher resistance to growth inhibition. In this case, the antagonism level expressed as X was 0.17 for *Trichoderma* T36, and 0.23 for *Trichoderma* T27, respectively.

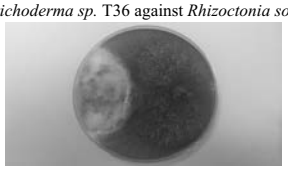
The visual observations of solid dual cultures in Petri places are presented in Fig. 1 and Fig. 2.

From Fig. 1 it can be seen that *Trichoderm*a T36 presents higher inhibition against *Fusarium* strain (Fig.1 b).

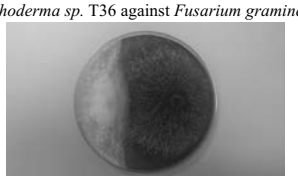
The photographs presented in Fig 1 and 2 were taken 5 days after inoculation.



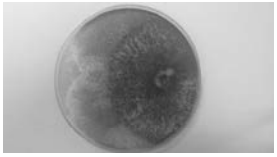
a)*Trichoderma sp.* T36 against *Rhizoctonia solani*



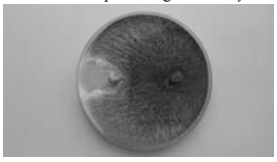
b) *Trichoderma sp.* T36 against *Fusarium graminearum*



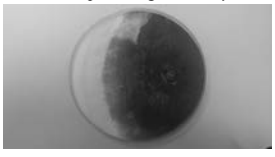
c) *Trichoderma sp.* T36 against *Sclerotinia sclerotiorum*



d) *Trichoderma sp.* T36 against *Botrytis alli*



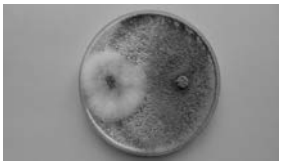
e) *Trichoderma sp*. T36 against *Botrytis cinerea*



f) *Trichoderma sp*. T36 against *Phytium debaryanum*

Fig. 1. Performance of *Trichoderma* T36 in confrontation as assays on PDA with pathogens

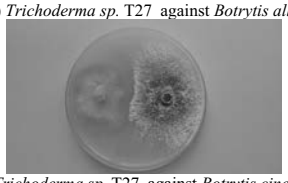
a)*Trichoderma sp. T27* against *Rhizoctonia solani*



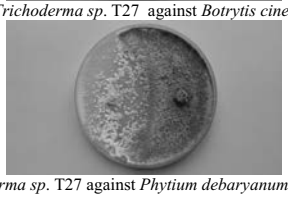
b) *Trichoderma sp.* T27 against *Fusarium graminearum*

c) *Trichoderma sp.T* 27 against *Sclerotinia sclerotiorum*

d) *Trichoderma sp.* T27 against *Botrytis allii*



e) *Trichoderma sp*. T27 against *Botrytis cinerea*

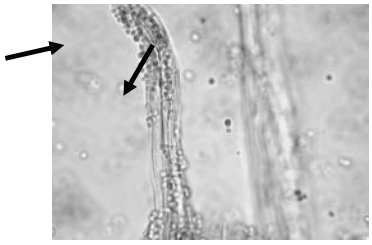


f) *Trichoderma sp*. T27 against *Phytium debaryanum*

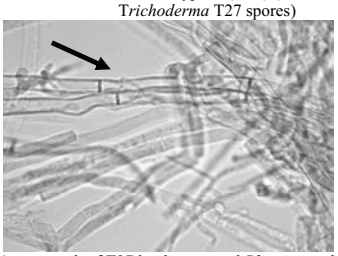
Fig. 2. Performance of *Trichoderma* T27 in confrontation as assays on PDA with pathogens

Fig. 3 presents morphological aspects of microbial cultures taken from contact zone between biocontrol agent and pathogen.

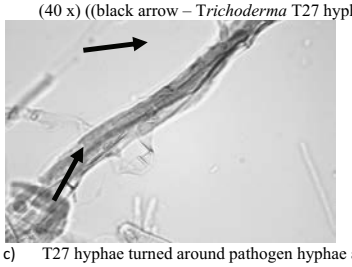
The toxic action of *Trichoderma* was evident in various alterations of the hyphal structure of *Rhizoctonia solani* grown together with *Trichoderma*, similar to the effects described in other studies [4, 5].



a) agglomeration of *Trichoderm*a T27 spores around *Rhizoctonia solani* hyphae (40 x) (black arrow – T*richoderma* T27 spores)



b) growth of T27 hyphae around *Rhizoctoni*a hyphae (40 x) ((black arrow – T*richoderma* T27 hyphae)



c) T27 hyphae turned around pathogen hyphae and hyphal shrinking (40 x) (black arrow – T*richoderma* T27 hyphae)

Fig. 3. Morphologicals characteristics between *Trichoderma* T27 and *Rhizoctonia solani*

CONCLUSIONS

This study demonstrated the efficacy of *Trichoderma T27* in controlling several virulent pathogens, such as *Fusarium graminearum* (X=1.05*), Botryris cinerea (X*=1.07) and *Pythium debaryanum* (X=1.01).

The result implied that the extent of inhibition by the fungi provides the use of potential antagonists capable of controlling the pathogenicity for sustainable agriculture.

ACKNOWLEDGMENTS

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NEW SCREENING METHODS FOR EVALUATION OF *FUSARIUM* SPORULATION INHIBITION BY *BACILLUS* BIOCONTROL STRAINS

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Abstract

In vitro antagonistic activity of microorganisms is the first approach in the selection of biocontrol microbial strains. The method usually used in this direction is the double culture technique. This method has been improved, in time, in order to be more relevant for the inhibition of some pathogenic growth. However, pathogenic growth reduction is not sufficient in agricultural systems. The vegetative growth inhibition must be complemented by suppression of resistant and/or spreading forms of the pathogens so that the probability of pathogen dissemination will be reduced. Due to these requirements we propose new screening methods to determine the sporulation inhibition, using Fusarium species as a model. Within this study we demonstrated the functionality of these new methods using Bacillus biocontrol strains. The methods are based on the quantification of spores formation, spores dissemination and germination when growing the fungus together with the bacterial biocontrol strains.

Key words: *sporulation inhibition, biocontrol*

INTRODUCTION

In the agricultural field diseases suppression is an important step in crop production. Chemical pesticides were found to have the largest inhibition effect against phyto-pathogenic microorganisms [4]. There are many chemical fungicides available to control the important crop diseases caused by different phyto-pathogenic fungi [1]. However, chemical pesticides are not ideal for long term application due to the concerns of exposure risks, residual persistence and tolerance of pathogenic fungi [8]. Biological treatments that reduce fungal and spore formation could be an alternative to chemical pesticides, with benefits for the environment.

For the biological control of phytopathogenic fungi, microbial biocontrol agents and spices are cited for their antifungal and antitoxigenic activities [2, 3].

For the selection of biocontrol alternatives there are numerous analytical and cultural methods described for the detection and quantification of fungal inhibition [5, 6]. Fungal inhibition methods must relay on mycelial growth suppression, sporulation inhibition and mycotoxins reduction.

Since pathogenic growth reduction is not sufficient for plant diseases suppression in agricultural systems, the vegetative growth inhibition must be complemented by suppression of resistant and/or spreading forms of the pathogens so that the probability of pathogen dissemination will be reduced. Due to these requirements we propose new screening methods to determine the sporulation inhibition, using *Fusarium* species as a model. Within this study we demonstrated the efficacy of these new methods using *Bacillus* biocontrol strains. These methods are based on the quantification of spores formation, spores dissemination and germination when growing the fungus together with the bacterial biocontrol strains.

MATERIALS AND METHODS

Fungal material

The phytopathogenic fungi concerned in *Fusarium graminearum* DSM 4527 strain and *Fusarium oxysporum* f.sp.*radicis lycopersici* ZUM 2407 strain. Fungi were routinely grown on Potato-Dextrose-Agar (PDA) for maintenance.

To obtain fungal spore suspension the fungi were multiplied in Czapek-Dox broth medium at 25°C and 150rpm shaking for 5 days.

The fungal broth culture was then filtered through four cheesecloth layers in order to harvest only the spore suspension, which was adjusted to a concentration of 10^6 spores /ml, revealed by counting on Bürker-Türk chamber.

Bacterial inoculums

Three bacterial strains were previously selected for their antagonist activity against several phytopathogenic fungi [7, 9].

Bacterial strains used in this study were Romanian isolates of *Bacillus pumilus* OS15 strain, *Bacillus amyloliquefaciens* OS17 and BW strains. The origin of this strain is presented in table 1.

Bacteria were routinely grown on Luria Bertani medium at 28°C.

To obtain the bacterial cell suspension, 48h old broth cultures were centrifuged at 3750rpm, for 20minutes, at 10ºC and pelet was resuspended in saline phosphate buffer up to the desired concentration.

The microbial load of the bacterial suspension was spectrophoto-metrically evaluated at 10^8cfu/ml when the optical density reached the absorbance of 1 at 600nm wavelength.

Table 1. Biocontrol bacterial strains used in the experiments

Strain	Source	Provenience
Bacillus pumilus OS15	Isolated from onion rhizosphere	RDIPP - Bucharest collection
Bacillus amyloliquefacien OS17	Isolated from onion rhizosphere	RDIPP - Bucharest collection
Bacillus amyloliquefacien BW	Isolated from soil	Faculty of Biotechnology Bucharest Collection

Bacterial inoculum, consisting of filtered supernatant, was obtained from bacterial fresh cultures, where broth cultures were centrifuged and the resulted supernatant was filtered through a 0.22µm membrane (Millex®GP) so that no bacterial cell would be in the final liquid.

In situ evaluation of *Fusarium graminearum* spores disemination inhibition by biocontrol bacterial strains

Spores inhibition test was evaluated *in situ* on wheat straw leftovers which is one of the natural substrates for *F. graminearum* development in the agricultural environments. The experiment was made in situ Roux plate (20cm/16cm/5cm). The growth media concerned in grinded wheat straw moistened with saline phosphate buffer at 3:10 w/v rate.

Plates were inoculated with 4 ml of fungal spore suspension (1×10^6 spores /ml concentration), linearly distributed at 4 cm from the end of the plate. The bacterial treatment consisted in 8ml inoculum of 10^8cfu/ml, linearly distributed and parallel to the fungal inoculum at 8cm distance from it. Plates were incubated at room temperature in daylight conditions, for one week, when spore traps were installed inside the Roux pates. The spore traps consisted in sterile glass slides, loaded with100µl PDA, and placed obliquely, as in figure 1. After spore traps installation, the plates were incubated for other 3 days on previously mentioned conditions.

At the same time a negative control was prepared only with *Fusarium graminearum* inoculum and chemical control with thiophanate methyl to compare the bacterial treatments with a standardized fungicide, Topsin 500SC, in 0.25% concentration and the same dose as the biological treatments.

To compare the bacterial treatments with a standardized fungicide we prepared a chemical control with Topsin 500SC (thiophanate methyl) in 0.25% concentration and with the same dose as the biological treatments.

At the same time a negative control was prepared only with *Fusarium graminearum* inoculum.

Treatment ability to inhibit spore formation and propagation was evaluated by microscopic analysis of the spore traps.

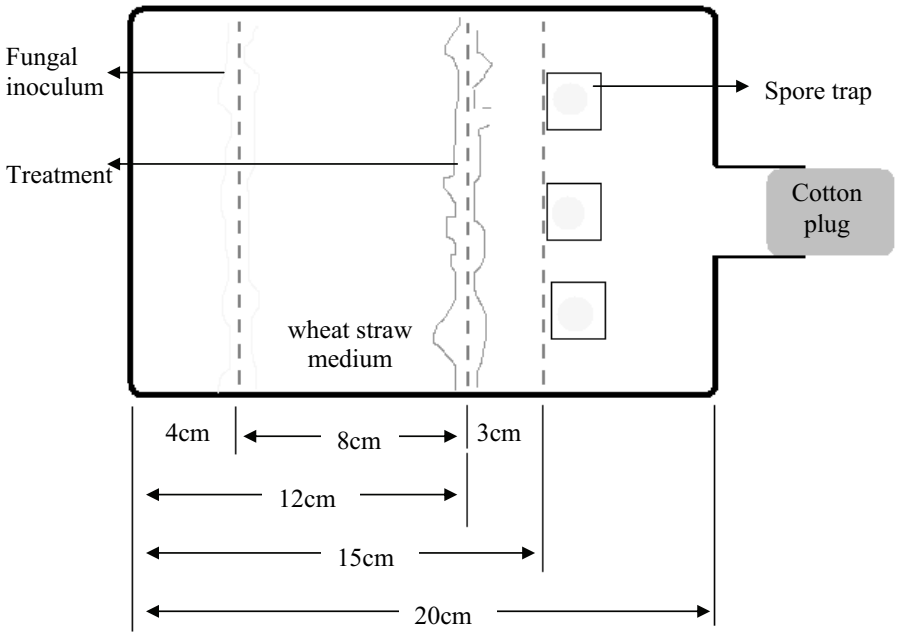

Figure 1. Schematic presentation of the Roux plates prepared for *in situ* evaluation of *Fusarium graminearum* inhibition into spores formation and propagation.

***In vitro* evaluation of *Fusarium oxysporum* f.sp. *radicis lycopersici* sporulation inhibition by biocontrol bacterial strains**

Test was performed on LB broth medium, 10ml/sample. Fungal inoculum consisted in two micelial plugs, 6mm in diameter, colected from fungal growth with the same age. Bacterial treatment concerned in filtred supernatant from bacterial fresh cultures obtained in LB broth medium. Chemical control consisted of thiophanate methyl treatment with Topsin 500SC at 0,14% final concentration. As a negative control we used untreated samples incubated in the same condition with the other experimental variants. Samples were incubated at 28ºC with 150rpm shaking for 5 days. Spores counting was made after three and five days of incubation. Spores concentration was evaluated on Bürker-Türk chamber. The test was performed three times, each sample having three replicates.

***In vitro* evaluation of the ability to delay spore germination of *Fusarium oxysporum* f.sp. *radicis lycopersici* spores using biocontrol bacterial strains**

The experiment was carried out on sterile glass slides loaded with 100µl of PDA medium. Slides were inoculated with 10µl spores suspension of *Fusarium oxysporum* f.sp. *radicis lycopersici* in 2×10^6spors/ml concentration. This led to a load of 2×10^4 spores/slide. Biological treatments consisted in application of 20µl filtered supernatant from bacterial fresh cultures. Slides were preserved in sterile Petri plates and incubated for 3 hours at room temperature.

Samples visualization was performed with an optical microscope using the 40X objective. The growth of the fungal filaments generated by the spore germination was precisely determined by processing the images captured from the microscope with APS Assess 2.0 soft. In this way the hyphal growth from the spore germination has been measured and compared with those from the untreated control. This appreciation is possible because of the identical conditions of images collection: same microscope (MC1, IOR), distances from the sample, magnification, camera (Model GN-B100/SA/W5, Gen Security) and attributes of the captured images.

RESULTS AND DISCUSSION

In situ evaluation of *Fusarium graminearum* spores dissemination inhibition by biocontrol bacterial strains

Treatment evaluation of spore inhibition formation and propagation was evaluated by microscopic analysis of the spore traps (photo1).

Spore formation and propagation detected in the negative control were estimated to be above all treated samples. In this experimental variant spores were evaluated as having significantly higher size compared with treated variants. In the chemical control the disseminated spore number was considerably smaller than the negative control (three times smaller). Biological treatments with BW and OS15 registered spore number/ traps was smaller than in chemical control and compared with the negative control were 10X and 4X respectively smaller than in non-treated control variant. OS17 treatment reduced spore dissemination by 2X comparing with the non-treated control variant. Results of the spore dissemination are presented in table 2.

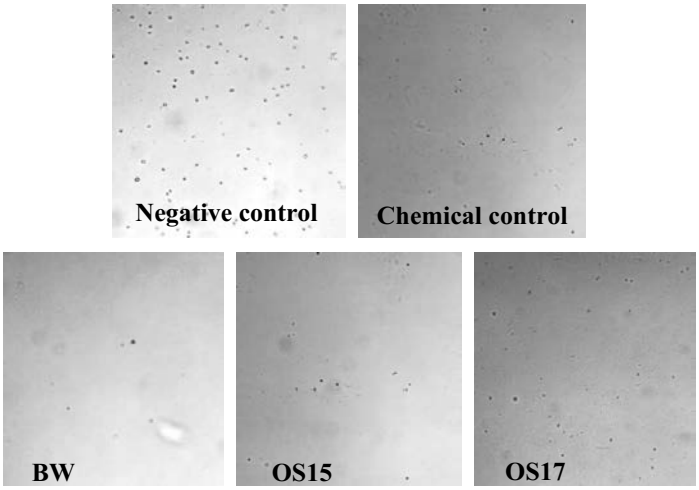

Photo 1. Image captures from the spore traps

Table 2. The inhibition of *Fusarium graminearum* DSM4527 spore dissemination

Experimental variants	Spore load / trap with 100µl PDA medium
Negative control	2.4×10^6 spores
Chemical control	7.8×10^5 spores
Bacillus amyloliquefacien BW treatment	2.5×10^5 spores
Bacillus pumilus OS15 treatment	6.3×10^5 spores
Bacillus amyloliquefacien OS17 treatment	1.2×10^6 spores

General view of the Roux plates is revealed in photo 2. The visual appreciation of the biological treatments showed a reduced mycelial density than in controls.

In vitro evaluation of *Fusarium oxysporum* f.sp. *radicis lycopersici* sporulation inhibition by biocontrol bacterial strains

According to the representation in table 3, the most efficient treatment in sporulation inhibition was found in the chemical control.

The inhibitory effect of the bacterial filtered supernatant on fungal sporulation revealed that biological treatment with BW strain had similar results with the thiophanate methyl treatment regarding spores inhibition. All treatments reduced sporulation compared to untreated control where an average sporulation of 3.2×10^7 was found after 5 days of incubation.

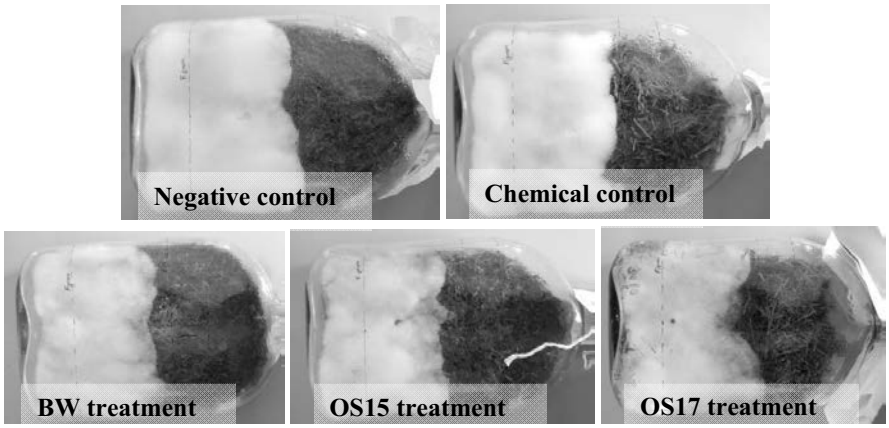

Photo 2. General view from the *in situ* evaluation of *Fusarium graminearum* spores inhibition

Table 3. The inhibition of *Fusarium oxysporum* f.sp. *radicis lycopersici* ZUM 2407 spores formation

Experimental variant	*Fusarium* spore concentration (spores/ml)	
	Three day incubation	Five days incubation
Bacillus amyloliquefaciens BW	$1{,}6 \times 10^5$	$1{,}7 \times 10^5$
Bacillus pumilus OS15	$5{,}2 \times 10^5$	$1{,}2 \times 10^6$
Bacillus amyloliquefaciens OS17	$1{,}0 \times 10^5$	$2{,}3 \times 10^5$
Chemical treatment	$1{,}1 \times 10^5$	$1{,}3 \times 10^5$
Negative control (Forl)	$5{,}9 \times 10^6$	$3{,}2 \times 10^7$

The inhibitory effect on sporulation increases the efficacy of the treatments, since the sporulation reduction has an additional impact on the fungus capacity of propagation.

***In vitro* evaluation of the ability to delay spore germination of *Fusarium oxysporum* f.sp. *radicis lycopersici* spores using biocontrol bacterial strains**

Compared with the control all treatments reduced spores germination (see photo 3). The most efficient was *B. pumilus* OS15 treatment that reduced spores germination by 90.9% compared with the untreated control, followed by the *B. amyloliquefaciens* OS17 and BW treatments which reduced spores germination by 82.8% and 81.8% respectively.

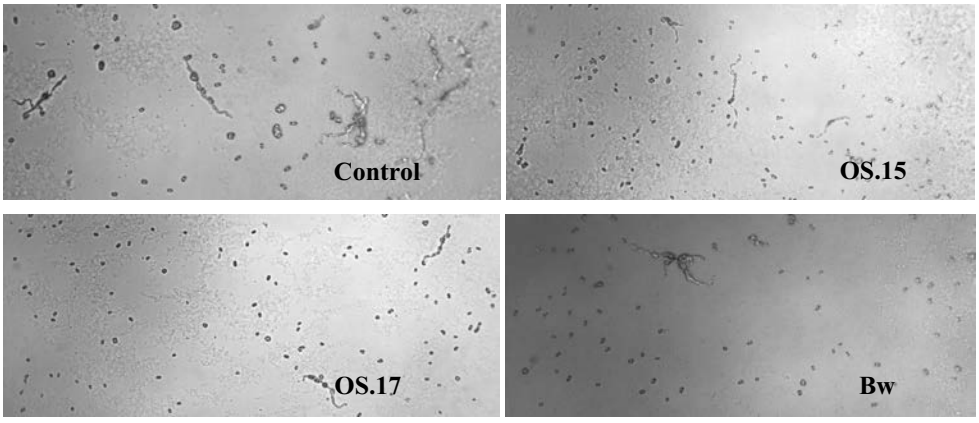

Photo 3. Delayed germination of *Fusarium oxysporum* f.sp. *radicis lycopersici* ZUM 2407 spores induced by the treatments with bacterial filtered supernatant

CONCLUSIONS

Spores dissemination inhibition assay is an innovative method for the evaluation of *Fusarium* propagation.

The bacterial treatment efficacy into inhibiting spores spreading and reducing spores germination revealed that tested *Bacillus* strains are capable to diminish *Fusarium* disease dissemination.

The tested bacterial strains were able to reduce *Fusarium* sporulation, the results obtained in the biological treatment with BW strain being comparable to those from the chemical control.

Considering the antifungal properties of the tested *Bacillus* strains (OS15 of *B. pumilus*, OS17 and BW of *B. amyloliquefaciens*) from others studies and the sporulation inhibition capacity we can say that these strains are efficient biocontrol agents that can suppress phytopathogenic fungi disease.

The antifungic activity and plant growth promotion proved by these strains in other studies correlated with the results revealed through this paper offers great prospects in plant protection technologies for these biocontrol strains.

Research performed on these bacterial strains proved that they are competitive rhizobacteria which can be successfully used to improve alternative agricultural systems by reducing the incidence of the phytopathogenic fungi attack.

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THE EFFECT OF THE *FUSARIUM* SP. ATTACK ON THE QUALITY PARAMETERS OF ROMANIAN WHEAT

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Abstract

*There have been analyzed 102 samples of Romanian wheat, coming from the crops of the years 2007-2011, in order to assess the influence of the Fusarium sp. attack on the main quality parameters. In this respect, there have been analyzed the following physical and chemical parameters: Hectolitre mass (kg/hl), Moisture (%), Protein content (%), Wet gluten content (%), Falling Number (sec), Gluten deformation (mm), Gluten Index, as well as the Content of kernels attacked by Fusarium (%). The Fusarium attack ranged from 0.1% to 4.5% and was characterized by very high variability. The evaluation of Fusarium attack was made through the Spearman statistical test, which showed its significant influence on the amylase activity of wheat (expressed by the parameter Falling Number, r = -0.22 *) and on the proteolytic activity (expressed by the Gluten deformation, r = -0.28 *).*

Key words**:** *crops quality, Fusarium sp., wheat*

INTRODUCTION

In Romania and in Europe wheat is the most important cereal crop, being grown on an area of about 2 million hectares per year. Most wheat varieties grown in Romania are susceptible to the attack of the following *Fusarium* species: *F. culmorum, F. graminearum* and *F. avenaceum*. Other species reported in Romania are *F. verticillioides, F. oxysporum* and *F. poae*. These species can cause significant crop losses, but the main problem is related to the contamination by mycotoxins (deoxynivalenol, zearalenone, fumonisin, etc.) of the food chains which are based on cereals. Regarding the effect of *Fusarium* attack on the wheat quality, Wang *et al.* (2005) have shown that the addition of a liquid contaminated with *F. culmorum* to the flour, can significantly decrease the Falling Number parameter [1].

Nightingale et al. (1999) have shown through the electron microscopy analysis of wheat grain infected with *Fusarium* sp. that there exist definite zones of endosperm protein degradation. The proteolytic activity caused by *Fusarium* sp. is inhibited by chloromercuribenzoate, and not by the p-trypsin inhibitor from soy, or by the iodoacetic acid, suggesting that the protease in *Fusarium* is an alkaline protease. The rheology studies of dough prepared from the flour obtained from wheat attacked by *Fusarium* showed a decrease of dough consistency and its resistance to extension. Also, the bread made from this flour is characterized by a small volume [2].

Wang et al. (2005) studied protease of *Fusarium culmorum* and identified the optimal range of pH between 6 and 8 and an optimum temperature of 50^0C for the proteasic activity. Protease is still active in the pH range 4.5 to 8.5 and in the range of temperatures 10^0C to 100^0C [3].

Eggert et al. (2011) showed that the *F. graminearum* protease hydrolyzes better the glutenins than the gliadins [4]. *Papoušková et al*. (2011) also showed that the intensity of *Fusarium* attack is related to the deterioration

of dough rheological qualities, evaluated with mixolab. They found a worsening of the mixolabic parameters which are responsible to some extent for the protein quality, especially for the fraction of starch from flour [5]. Considering the importance of the milling and baking industry products to the Romanian people nutrition, we considered useful to undertake a study regarding the effect of *Fusarium* sp. attack on the quality of Romanian wheat, a study based on data collected by us in the last five years.

MATERIAL AND METHOD

We analyzed 102 wheat samples from the crops of the years 2007 to 2011, taken from the Southern Romania, in order to assess the effect of *Fusarium* attack on the baking quality of wheat. For each wheat sample we determined the physical and chemical parameters listed in Table 1, according to the appropriate standards.

Table 1. Methods of analysis used for analyzing the quality parameters of wheat

Quality parameter	Analysis method
Hectolitre mass (HLM, kg/hl)	SR EN ISO 7971-3:2010
Moisture (M, %)	SR EN ISO 712:2010
Protein content (P, %)	ICC 159-95 (NIR method, Perten Inframatic 8600)
Wet gluten (WG, %)	SR EN ISO 21415-2:2008
Gluten deformation (GD, mm)	SR 90:2007
Gluten Index (GI)	ICC 155-94
Falling Number (FN, sec)	SR EN ISO 3093:2010

The attack by *Fusarium* sp. has been estimated using the parameter Content of kernels attacked by *Fusarium*, according to the method described by ISO 7970 and expressed in percentage by weight. Additionally, we determined for the analyzed samples the Content of kernels attacked by *Eurygaster* sp. (KAE, %), determined according to ISO 7970. The results were interpreted statistically, using a specific software, StatSoft, Inc.. (2004), STATISTICS (Data Analysis Software System), version 7, www.statsoft.com).

RESULTS AND DISCUSSIONS

The results obtained by determining the quality parameters for the 102 analyzed wheat samples are presented in Table 2, using the following variability estimates: the arithmetic mean (X), the standard deviation (Sx) and the coefficient of variation (CV).

Table 2. Estimates of variability for the wheat samples

Quality Parameters	$X \pm s_x$	CV (%)
HLM (kg/hl)	75.684 ± 2.968	3.921
M (%)	12.753 ± 0.871	6.830
P (%)	13.924 ± 2.004	14.392
WG (%)	30.065 ± 6.586	21.906
GD (mm)	9.396 ± 7.308	77.778
GI	41.344 ± 25.221	61.003
FN (sec)	315.147 ± 81.929	26.000
KAF (%)	0.520 ± 0.746	143.461
KAE (%)	2.021 ± 2.706	133.89

In Table 2 we can see that the heterogeneity regarding the areas and the years of wheat samples induced a significant increase of the coefficients of variability of certain quality parameters. Thus, while for the parameters for Hectolitre mass, Moisture, Protein content and even Wet gluten content, the variability coefficients can be considered as being normal, for the parameters describing the enzymatic activity (Falling Number, Gluten deformation and Gluten Index) the values of the coefficients of variability were high and very high.

Special cases were represented by the percentage of kernels attacked by *Fusarium*, where the coefficients of variability exceeded by almost 50% the average value and the percentage of kernels attacked by *Eurigaster* sp. (34%).

Given the excessive variability of some quality parameters, especially for the Content of kernels attacked by *Fusarium*, we had apply a series of statistical tests, in order to check the normality of the distribution. Thus, we studied the links between variables, taking into consideration nonparametric statistical methods.

Table 3 presents the results of the test Kolomorogov-Smirnov (KS), in order to check the normal distribution of the quality parameters of wheat samples (where n - number of analyzed samples; max.D - specific estimator of KS test for the difference between the two distributions, p - probability of transgression for the values of D estimator, corresponding to the following significance levels: 0.05 - significant, 0.01 - very significant, distinct significant 0.001).

Table 3. *Kolomorogov-Smirnov* test (normality of data distribution), for the quality parameters of wheat

Parameter	max D	K-S - p
HLM (kg/hl)	0.102	p > 0.20
M (%)	0.132	p < 0.10
FN (sec)	0.112	p < 0.20
P (%)	0.090	p > 0.20
WG (%)	0.107	p > 0.20
GD (mm)	**0.217**	**p < 0.01**
KAF (%)	**0.287**	**p < 0.01**
KAE (%)	**0.239**	**p < 0.01**
GI	0.122	p < 0.15

Table 3 shows that distribution of the values of Gluten deformation, Content of kernels attacked by *Fusarium* and Content of kernels attacked by *Eurygaster* sp. differ significantly from the normal distribution curve. Other quality parameters of wheat are characterized by values that are not significantly different from the theoretical distribution. Given the results for these three parameters we consider arithmetical averages and standard deviations (Table 2) as being unrepresentative in terms of central tendency. Most suitable are other estimators of central tendency, such as median and module (Table 4).

Table 4. Median and module of wheat quality parameters, which were not normally distributed

Parameter	Median	Module
GD (mm)	6.5	4
KAF (%)	0.2	0.1
KAE (%)	1.0	0.2

Table 5 presents the Spearman correlation coefficients, which describe the relationships between the quality parameters of wheat.

Table 5 shows that **the Percentage of kernels attacked by *Fusarium* does not significantly influence the Hectolitre mass and the Protein content**, ie **Gluten**, in wheat. Also, the percentage of kernels attacked by *Fusarium* is not significantly correlated with Moisture of grains.

The percentage of kernels attacked by *Fusarium* significantly affects the amylase activity of wheat, measured by the Falling Number parameter. Basically, **as the percentage of kernels damaged by *Fusarium* grows, the Falling Number decreases** with the same amount (the amylase activity increases). This phenomenon is likely the result of the action of the enzymatic equipment, rich in amylase, with which fungus attacks the substrate (wheat kernel endosperm). Although, some studies in the literature have suggested that the Falling Number method would not be appropriate for evaluating the quality of wheat *Fusarium* attack (*Wang et al.*, 2008), our results show a minor but significant impact of the attack of *Fusarium* on this parameter [6]. Approximately 5% of the variability of Falling Number is due to the *Fusarium* attack, according to the results of our study ($r^2 = 4.84$).

The percentage of kernels attacked by *Fusarium* was significantly negatively correlated with the Gluten deformation of wheat. Apparently, our results suggest that *Fusarium* attack decreases the proteolytic activity expressed by the Gluten deformation. Results are in conflict with some observations in the literature, describing the effects of attack by *Fusarium* sp. on the proteolytic activity of wheat. Our results (the decreasing of the proteolytic activity with the increasing of the Percentage of kernels attacked by *Fusarium*), support the hypothesis of ecological relationships between *Fusarium* sp. and the insects, whose attack is responsible to a large extent of the proteolytic activity of wheat (*Eurygaster* sp.).

Literature refers to entomopathology cases caused by *Fusarium* sp. and *Beauvaria bassiana* at species of the genus *Eurygaster*. Thus, Assaf *et al.* (2011) quoting Ali (1995) and Mohamad (2000), referr to a series of studies which show a mortality of 80% to *E. integriceps*, induced by the mentioned species of fungi [7].

In a literature review, *Sandhu et al.* (2012) considered the *Fusarium* and *Aspergillus* fungi as facultative pathogens and general (nonspecific) pathogens of insects [8].

Table 5. *Spearman* correlation coefficients, between the quality parameters and their significance

Pairs	*HLM*	*M*	*FN*	*P*	*WG*	*GD*	*KAF*	*GI*	*KAE*
HLM	1.00								
M	-0.12	1.00							
FN	0.30	-0.34	1.00						
P	-0.40	-0.31	-0.04	1.00					
WG	-0.28	-0.28	-0.11	0.93	1.00				
GD	-0.19	-0.30	0.24	0.28	0.14	1.00			
KAF	**0.01**	**0.004**	**-0.22**	**-0.001**	**0.07**	**-0.28**	**1.00**		
GI	0.24	-0.15	0.05	-0.29	-0.24	-0.46	0.10	1.00	
KAE	-0.37	-0.22	-0.02	0.44	0.31	0.73	**0.305**	-0.36	1.00

Basically, at least partially attack of *Fusarium* induces a reduction of wheat bug attack (*Eurygaster* sp.), a phenomenon that is expressed through a reduction in proteolytic activity of wheat.

The result of *Spearman* correlation test showed that between the Percentage of kernels attacked by *Fusarium* and the Percentage of kernels damaged by wheat bug, there is a significant negative correlation (r = -0.305 *). Practically, the higher the degree of *Fusarium* attack, the lower the degree of bug attack. We also note that the coefficient of correlation between the two parameters is very close to the coefficient of correlation between parameters Content of kernels attacked by *Fusarium* and Gluten deformation (r = -0.280 *).

We believe that in light of the literature, the existence of correlations is justifying the assumption of **a fungus - insect interaction**, which is highlighted in values of proteolytic activity of wheat, expressed by the Gluten deformation.

CONCLUSIONS

Our results show that the *Fusarium* attack, expressed by Content of kernels attacked by *Fusarium* parameter, did not significantly affect the Falling Number, Moisture, Protein content and Gluten Index of wheat. The *Fusarium* attack causes a minor decrease of the parameter Falling Number, as a consequence of the amylase brought by the fungus in the wheat endosperm. *Fusarium* attack caused a decrease in proteolytic activity in wheat samples, probably due to entomopathological effect of fungus on insects of the genus *Eurygaster* sp.

Our hypothesis is confirmed by the existence of a significant correlation between the percentage of kernels attacked by *Fusarium* and the percentage of kernels damaged by wheat bug.

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PRELIMINARY STUDY OF LACTIC ACID PRODUCTION FROM INULIN HYDROLYSATES USING *LACTOBACILLUS ACIDOPHILUS* LA-5

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Abstract

Lactic acid is widely used in the chemical industry, food industry, cosmetics, and pharmaceuticals. The main objective of the present work was chemical and enzymatic inulin hydrolysis from inulin rich feedstock (dahlia and Jerusalem artichoke) followed by hydrolysates fermentation to lactic acid using Lactobacillus acidophilus LA-5. Chemical hydrolysis of Jerusalem artichoke and dahlia flour was performed with concentrated H_2SO_4 for one hour at 100°C at different pH of the medium, and the amount of reducing sugars obtained varied between 0.10 to 0.51 g fructose/g dry weight flour for Jerusalem artichoke and between 0.099 to 0.448 g fructose/g dry weight flour for dahlia. The enzymatic hydrolysis was performed by varying the substrate concentration and the pH, and the amount of reducing sugars produced was between 0.439 to 0.56 g fructose/g dry weight flour for Jerusalem artichoke and between 0.435 to 0.522 g fructose/g dry weight flour for dahlia flour. The hydrolyzed substrates were then fermented with a commercial culture of Lactobacillus acidophilus LA-5 for 96 hours. Lactic acid production was monitored and recorded every 24 hours, measuring the pH and total acidity of the samples. The obtained results were then analyzed using Principal Component Analysis. An optimum number of 4 principal components are enough to explain 87.1% of data variation for calibration and 69.8% for validation. PC-1 is given by the acidity evolution, correlated with pH drop during fermentation. The lactic acid accumulation was maximum in the third day of fermentation (after 48 hours) for both dahlia and Jerusalem artichoke.

Key words: *acid hydrolysis, enzymatic hydrolysis, fructose, lactic acid fermentation, reducing sugars.*

INTRODUCTION

Inulin is a natural storage polymer found in the tubers of Jerusalem artichoke, dahlia, in roots of chicory, burdock, murnong, yacon, salsify, in bulbs of onion, leek, garlic, camas, in cereals such as rye, barley, etc [19].

Inulin is a polydisperse fructan that ranges in its degree of polymerization from 2 to 60, or higher [2].

The fructosyl units in inulin are linked by β (2-1) linkages with the polymer chains terminating in glucose residues. The tubers of Jerusalem artichoke, dahlia and chicory contain important amounts of inulin, those being most widely used natural sources of inulin. Van Loo determined the inulin content from Jerusalem artichoke tuber between 14-19% of fresh weight [19].

A variety of fractionated inulin can be produced from native inulin by physical, chemical or enzymatic processes, leading to products with a defined range of degree of polymerization and specific properties [13].

The acid hydrolysis of inulin has been investigated using sulphuric or hydrochloric acid [1, 12, 3].

Glibowski and Bukowska concluded that inulin chemical stability decreases in an acidic environment at pH under 4 due to the heating time and temperature increase and in a neutral and alkaline environment inulin is chemically stable independently of pH, heating time and temperature [6].

Inulin is hydrolysed by enzymes known as inulinases. Inulinases are classified into endo- and exoinulinases, depending on their mode of action. Endoinulinases (2,1-β-D-fructan fructanohydrolase; EC 3.2.1.7) hydrolyze

inulin by breaking the bonds between fructose units that are located away from the ends of the polymer network, to produce oligosaccharides. Exo-inulinases (β-D-fructohydrolase; EC 3.2.1.80), split terminal fructose units in sucrose, raffinose and inulin to liberate fructose [18, 11, 15].

The inulinases are produced by moulds, yeasts and bacteria. *A. niger* and *Kluyveromyces marxianus* are the most widely used for inulinase production. Among fungi, some well-known sources of these enzymes include *A. niger*, *Aspergillus ficuum*, *Chrysosporium pannorum* and *Penicillium purpurogenum*. Among yeasts, the best-known producers are *Kluyveromyces marxianus*, *Candida kefyr*, *Debaryomyces cantarellii* and *Pichia polymorpha*. These yeasts appear to produce only exo-inulinases whereas most inulin-hydrolyzing molds produce both endo- and exo-inulinases [16].

Szambelan and Nowak conducted the enzymatic hydrolysis with commercial inulinase (0.06 $mg \cdot g^{-1}$ and 0.12 $mg \cdot g^{-1}$ sugars) at pH 5.0 and with invertase (1.79 $mg \cdot g^{-1}$ and 17.90 $mg \cdot g^{-1}$ sugars) at the same pH. The results were best after 72 hours of enzymatic hydrolysis with inulinase dose of 0.12 $mg \cdot g^{-1}$ sugars and invertase dose of 17.90 $mg \cdot g^{-1}$ sugars [17].

Lactic acid (2-hydroxypropionic acid or 2-hydroxypropanoic acid), have applications in food, pharmaceutical, textile, leather, and other chemical industries. Lactic acid has gained many other industrial applications like biodegradable plastic production. Lactic acid is considered generally recognized as safe (GRAS) for use as food additives by the regulatory agencies like FDA in USA. In food industry, lactic acid is used as acidulant, flavoring or buffering agent or inhibitor of bacterial spoilage in a wide variety of processed foods, such as candy, breads and bakery products, soft drinks, soups, sherbets, dairy products, beer, jams and jellies, mayonnaise, and processed eggs, often in conjunction with other acidulants [8]. Besides high product specificity, as it produces a desired optically pure L-(+)- or D-(−)-lactic acid, the biotechnological production of lactic acid offers several advantages compared to chemical synthesis like low cost of substrates, low production temperature, and low energy consumption [7].

Until now, only a few studies have been made on lactic acid production from inulin rich feedstock, especially from Jerusalem artichoke tubers.

This study is focused on lactic acid production by a commercial culture of *Lactobacillus acidophilus* from acid and enzymatic inulin hydrolysates.

MATERIALS AND METHODS

Materials

The inulin rich feedstock used in this study was dahlia and Jerusalem artichoke flour. Dahlia flour was produced in the laboratory by cleaning and cutting the roots followed by freeze-drying using Alpha 1-4 LD Plus lyophilizator (Martin Christ, Germany) and finally grinding with VC2011 grinder (Victronic, PRC).

Jerusalem artichoke (*Helianthus tuberosus* L.) flour was kindly delivered to us by the Romanian company S.C. Hofigal Export-Import S.A.

Hydrolysis and fermentation

For chemical and enzymatic hydrolysis 2% and 6% (w/v) dahlia and Jerusalem artichoke flour were prepared by mixing with distilled water. The pH was measured using a pH meter S20 (Mettler Toledo, USA). The pH of the dahlia and Jerusalem artichoke flours were subsequently adjusted to 2, 4, and 6 using various concentrations of sulphuric acid.

For chemical hydrolysis, the solutions were adjusted to a pH value of 2 and 4 and then heated at 100 ± 2°C for one hour. For reducing sugars analysis, and inoculation with lactobacilli, the samples were cooled at 25°C and neutralized at pH = 6.0...6.1 using NaOH solution.

The enzymatic hydrolysis was conducted with commercial inulinase from *Aspergillus niger* (Novozyme A/S, Denmark). For enzymatic hydrolysis, the solutions were adjusted to a pH of 4 and 6, and then 0.7 ml/g substrate of commercial inulinase was added. The enzyme parameters were studied and found to be optimum at 60°C and 90 hours. After that, the

enzyme was inactivated by boiling the solutions at 100 ± 2°C for 10 minutes. The pH was adjusted at 6.0...6.1 after cooling at 25°C, using NaOH solution. All the hydrolysates were then pasteurized at 80°C for 30 minutes using Stericell 111 oven (MMM, Germany) and then cooled at 25°C.

The produced hydrolysates were coded according to Table 1.

Table 1. Hydrolysates codes used in the study

Code of the sample	Flour type	Hydrolysis type	Flour concentration (%)	Hydrolysate pH
Tha2-2	Jerusalem artichoke	acid	2	2
Tha2-4	Jerusalem artichoke	acid	2	4
Tha6-2	Jerusalem artichoke	acid	6	2
Tha6-4	Jerusalem artichoke	acid	6	4
The2-4	Jerusalem artichoke	enzymatic	2	4
The2-6	Jerusalem artichoke	enzymatic	2	6
The6-4	Jerusalem artichoke	enzymatic	6	4
The6-6	Jerusalem artichoke	enzymatic	6	6
Dha 2-2	dahlia	acid	2	2
Dha 2-4	dahlia	acid	2	4
Dha 6-2	dahlia	acid	6	2
Dha 6-4	dahlia	acid	6	4
Dhe 2-4	dahlia	enzymatic	2	4
Dhe 2-6	dahlia	enzymatic	2	6
Dhe 6-4	dahlia	enzymatic	6	4
Dhe 6-6	dahlia	enzymatic	6	6

The hydrolysates were then inoculated with 1% commercial culture of *Lactobacillus acidophilus* LA-5 (kindly provided by Christian Hansen, Denmark) and immediately incubated at 37°C using BF 4000 incubator (Binder, Germany). The fermentations were conducted in 100 ml flat bottom flasks. Lactic fermented samples were taken every 24 hours and analyzed for pH, reducing sugars and acidity.

Physico-chemical analysis

As analytical methods, Romanian standard determinations have been used for titrable acidity (g lactic acid/100 g product) and pH. The DNS (3, 5-dinitrosalicylic acid) method [10] was used for the quantitative analysis of the reducing sugar in inulin solutions. Adequately diluted samples were reacted with DNS acid. The intensity of developed color was measured using a 6505 UV-VIS spectrophotometer (Jenway, UK). Fructose was used for the establishment of a standard curve.

All the chemicals were of analytical grade.

Statistical analysis

Principal component analysis (PCA) is a variable reduction procedure. It is useful on the obtained data on a number of variables (possibly a large number of variables), when there is some redundancy in those variables. With minimal additional effort PCA provides a roadmap for how to reduce a complex data set to a lower dimension to reveal the sometimes hidden, simplified structure that often underlie it [14].

The experimental results were analyzed using Principal Component Analysis (PCA) with full cross-validation. Visualization of the results of PCA is usually achieved by plotting pairs of the first few PCs.

Principal Component Analysis was assessed using the Unscrambler X 10.1 software version from CAMO Software AS (Oslo, Norway). A principal component analysis (PCA) with full cross-validation was carried out in order to evaluate the influence of the studied parameters (type of carbon source, pH, and type of hydrolysis) on lactic acid production.

RESULTS AND DISCUSSION

After the production of chemical and enzymatic hydrolysates from Jerusalem artichoke and dahlia flour, the reducing sugars amounts were determined and the results are as follows.

The amount of reducing sugars obtained by enzymatic hydrolysis of Jerusalem artichoke flour were 0.44…0.56 g fructose/g d.w. flour.

Szambelan and Nowak [17] produced 166.32 g/kg reducing sugars when hydrolyzed with commercial inulinase the Jerusalem artichoke mashed tubers. Also, they produced 95.77 g/kg reducing sugars when treated the same mash tubers with commercial invertase.

The acid hydrolysis produced a high amount of reducing sugars only at pH of 2, the values being between 0.48...0.51 g fructose/g d.w. flour. At pH = 4 the acid hydrolysis of Jerusalem artichoke flour is not recommended, because the values obtained for reducing sugars are very low, between 0.10...0.11 g fructose/g d.w. flour.

Szambelan and Nowak [17], by doing the acid hydrolysis in the same conditions (pH=2, 100°C) produced 196.93 g/kg reducing sugars, and Glibowsky and Bukowska [6] an amount of 80g/100g, their determinations being performed on pure inulin.

The enzymatic hydrolysis of dahlia flour produced an amount of 0.43...0.52 g fructose/g d.w. flour, while the acid hydrolysis at pH = 2 liberated an amount of 0.40...0.45 g fructose/g d.w. flour.

At pH = 4 the amount of reducing sugars from dahlia hydrolysates is low, between 0.1...0.15 g fructose/g d.w. flour, comparable with those obtained for Jerusalem artichoke flour. The stability of inulin at high temperature and pH higher than 3, in the case of acidic hydrolysates, is the main cause for the production of the low amounts of reducing sugars [6].

Lactobacillus acidophilus is a homolactic bacterium, and is proved to be able to use the fructose as carbon source for lactic acid production [9].

The pH variations and the total acidity drop were measured every 24 hours. All the obtained data were analyzed using PCA analysis.

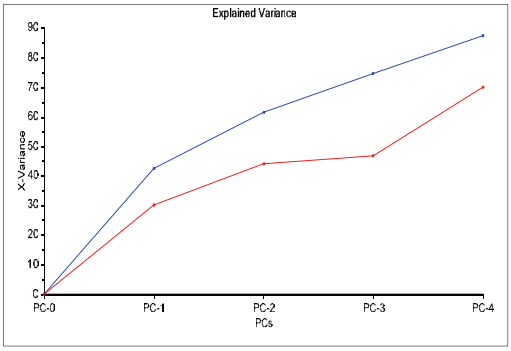

Fig. 1. The variance for the calibration and validation data.

As it can be seen from figure 1, 4 PCs are enough to explain over 87% of data variation and almost 70% for data validation.

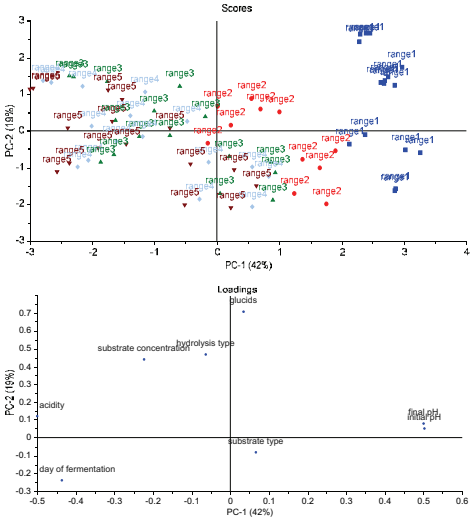

Fig. 2. Scores and loadings plots for PC-1 and PC-2 (range 1 – after 0 hours of fermentation, range 2 – after 24 hours of fermentation, range 3 – after 48 hours of fermentation, range 4 – after 72 hours of fermentation and range 5 – after 96 hours of fermentation).

In Fig. 2 the loadings plot and the scores plot are presented. The first PC (which explains 42% of data variation) is given by the acidity, pH and the day of fermentation of the samples. In this figure the samples were grouped after the day of fermentation. The total acidity of the samples is highly correlated with day of fermentation. After 24 hour of lactic acid fermentation, all the fermented substrates had a drop in pH values, around 5.00, especially for dahlia hydrolysates that had a decrease of pH around 4.00…4.50. Jerusalem artichoke acid and enzymatic hydrolysates with 2% concentration (The2-4, Tha2-4 and Tha2-2) had a slow decrease in pH, even after 48 hour of fermentation, due probably to the low concentration of substrate.

After 48 hours of fermentation, the studied inulin hydrolysates had a decrease of pH values until 4.50…3.70. Also the acidity slowly decreased after 48 hours of fermentation.

The second PC, which describes 19% of data variation, is given by fructose content of the samples.

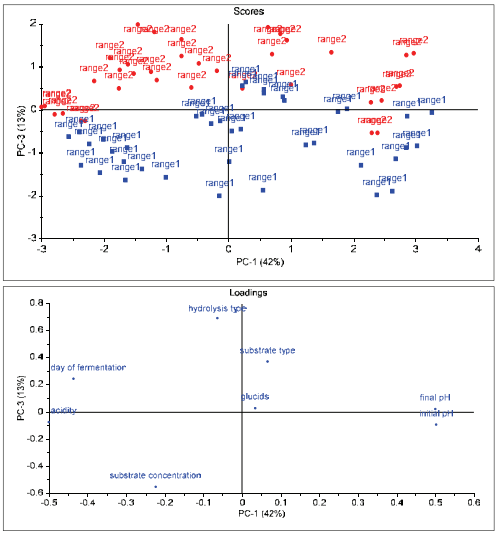

Fig. 3. Scores and loadings plots for PC-1 and PC-3 (range 1 – acidic hydrolysis, range 2 – enzymatic hydrolysis).

PC-3 explains 13% of data variation and is given by hydrolysis type: acidic or enzymatic. As it can be seen from figure 3, the hydrolysates obtained by enzymatic hydrolysis have higher concentrations in fructose (reducing sugars) and higher acidity after fermentation. The drop in pH of the samples during fermentation was faster.

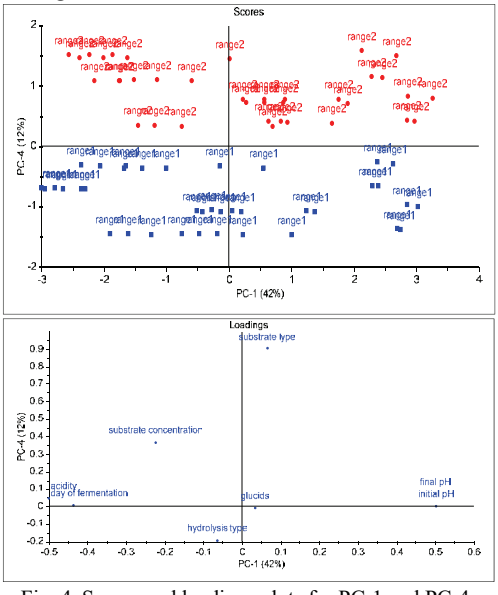

Fig. 4. Scores and loadings plots for PC-1 and PC-4 (range 1 – dahlia flour, range 2 – Jerusalem artichoke flour).

From figure 4 it can be observed that the PC-4 (which explains 12% of the data variation) is given by the type of the carbon source used in experiments. The dahlia tubers produced higher concentrations in lactic acid than Jerusalem artichoke. After 48 hours of fermentation, the 6% dahlia acid hydrolysates at pH=4 produced 6.3 g/l lactic acid, 2% dahlia acid hydrolysates at pH=4 produced 5.6 g/l, and 6% Jerusalem artichoke acid hydrolysates at pH=4 produced 5.4 g/l.

Regarding the conversion yield of reducing sugars to lactic acid, the values are presented in table 2.

Table 2. Jerusalem artichoke and dahlia conversion yield of reducing sugars to lactic acid.

Hydrolysate code	$Y_{P/S}$ at 24 h	$Y_{P/S}$ at 48 h
Tha2-2	0.505	0.609
Tha2-4	0.781	0.893
Tha6-2	0.508	0.631
Tha6-4	0.787	0.961
The2-4	0.438	0.627
The2-6	0.537	0.714
The6-4	0.542	0.717
The6-6	0.517	0.712
Dha 2-2	0.519	0.687
Dha 2-4	0.856	0.985
Dha 6-2	0.514	0.676
Dha 6-4	0.829	0.974
Dhe 2-4	0.530	0.741
Dhe 2-6	0.497	0.809
Dhe 6-4	0.544	0.821
Dhe 6-6	0.541	0.761

In the case of Jerusalem artichoke hydrolysates, the best conversion yield was obtained after 48 hours of fermentation for the 6% Jerusalem artichoke acid hydrolysates of pH = 4 (Tha6-4), followed by 2% Jerusalem artichoke acid hydrolysates of pH = 4 (Tha2-4), which had a very low initial amount of reducing sugars.

The hydrolysates with large amounts of reducing sugars had the lowest conversion yield after 48 hour of fermentation. This might be due to catabolic repression of the reducing sugars on the fermentative bacteria used in the study. For dahlia, after 24 hours of lactic acid fermentation, the conversion yield is best for 2% and 6% dahlia acid hydrolysates at pH=4 (Dha2-4 and Dha6-4) because of the low initial content in reducing sugars. After 48 hours of fermentation the conversion yield for Dha2-4 and Dha6-4 were the highest. This is due to the fact that the initial load of reducing sugars was lower. In the case of 2% and 6%

dahlia acid hydrolysates at pH=2 (Dha2-2 and Dha6-2), the conversion yields after 48 hours of fermentation were lower due to the fact that a good quantity of reducing sugars were not consumed by lactic bacteria. The obtained results are comparable with those obtained by Ge [5], who used *Aspergillus niger* SL-09 for inulinase production and *Lactobacillus* G-02 for lactic acid production. After 36 hours of fed-batch fermentation, the inulin bioconversion produced 120.5 g/l of lactic acid.

CONCLUSIONS

Lactic acid production from natural inulin rich substrates (dahlia and Jerusalem artichoke) was studied, using different methods of hydrolysis. The highest values of reducing sugars were obtained for the enzymatic hydrolysates and chemical hydrolysates at pH 2 of Jerusalem artichoke and dahlia flour. In the case of pH=4, the acid hydrolysates had the smallest amount of reducing sugars.

The pH=4 acidic hydrolysates after fermentation had the highest conversion yield. Dahlia yields for fermented acid hydrolysates at pH=4 (0.985 and 0.974) were better than the yields for Jerusalem artichoke (0.961 and 0.893) and better than all the other yields of fermented hydrolysates.

The lactic acid accumulation was maximum in the third day of fermentation (after 48 hours) for both dahlia and Jerusalem artichoke substrates.

Dahlia and Jerusalem artichoke roots can be successfully used as natural substrates for lactic acid bioproduction.

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COMPARATIVE STUDY OF THE ANTIOXIDANTS CONTENT IN SOME BERRIES FRUITS

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Abstract

Berries fruits contain many different chemical compounds with different biological and pharmacological capacities and properties. So, these fruits are a source of bioactive molecules with antioxidant activity such as phenolic compounds, flavonoids, anthocyanins, ascorbic acid, that are important to human nutrition. Antioxidants neutralize free radicals, which cause oxidative damage to lipids, proteins, and nucleic acids and thus protect the organism. Research studies carried out on this topic indicated that natural antioxidants are involved in protection against many deseases: cancer, cardiovascular deseases, osteoporosis. The objective of this research was to determine and compare the antioxidants content offered by six berries fruits species: raspberry (Rubus idaeus), blackberry (Rubus fructicosus), strawberry (Fragaria ananassa), chokeberry (Aronia melanocarpa), blackcurrant (Ribes nigrum), sea-buckthorn (Hippophaea rhamnoides). The evaluation involved determination of total phenols, anthocyanins and flavonoids, using spectrophotometrical methods. Higher total phenol content levels were obtained in chokeberries and in blackcurrants.

Key words*: antioxidants, berries, polyphenols, anthocyanins, flavonoids, ascorbic acid.*

INTRODUCTION

Berries fruits contain many different chemical compounds with different biological and pharmacological capacities and properties. These fruits are a source of bioactive compounds with antioxidant activity such as phenolic compounds (flavonoids, anthocyanins) and ascorbic acid, that are important to human nutrition.

Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species involved in producing of oxidative stress, leading to cellular damage. Oxidative stress has been linked to cancer, aging, atherosclerosis, ischemic injury, inflammation and neurodegenerative diseases [Sies, 1997].

Anthocyanins are powerful antioxidants that give blueberries and strawberries their vibrant color. Anthocyanins may contribute to a smoother blood flow, leading to lower risk of high blood pressure. They have benefits for heart health and also for brain health and could reduce the risk for cancer and potentially help people live longer [Buhler and Miranda, 2000].

Flavonoids are polyphenolic compounds that have been identified in fruits, vegetables and beverages (tea, coffee, beer, wine and fruit drinks). The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health; they have been reported to have antiviral, anti-allergic, anti-inflammatory, antitumor and antioxidant activities [Wrolstad, 2001].

The objective of this research was to evaluate and to compare the level of several antioxidants in six berries fruits species: raspberry (*Rubus idaeus*), blackberry (*Rubus fructicosus*), strawberry (*Fragaria ananassa*), chokeberry (*Aronia melanocarpa*), blackcurrant (*Ribes nigrum*), sea-buckthorn (*Hippophaea rhamnoides*).

MATERIAL AND METHODS

Samples. Six berries fruits species: raspberry (*Rubus idaeus*), blackberry (*Rubus fructicosus*), strawberry (*Fragaria ananassa*), chokeberry (*Aronia melanocarpa*), blackcurrant (*Ribes nigrum*), sea-buckthorn (*Hippophaea rhamnoides*) were purchased from the local

supermarket. The determinations were performed in triplicate, using frozen fruits. The extractions were conducted according to the protocol used for each determination. Total phenols, anthocyanins and flavonoids were analysed using spectrophotometrical methods; the measurements were achieved with a UV/Visible ThermoSpectronic Helios spectrophotometer.

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. Total phenols values were expressed in terms of gallic acid equivalent, which is a common reference compound. **Anthocyanins content** was measured using the pH-differential method (Versari A. *et al.,* 2007). Each plants extracts dissolved in methanol were mixed with 2% HCl (pH 0.6), respectively with citrate buffer (pH 3.5). The absorbance was measured at 520 nm. The results were expressed as cyanidin-3-glucoside equivalents.

Flavonoid content was determined using a colorimetric method based on the reaction with aluminium chloride (Nickavar *et al.,* 2006). Briefly, 0.5 ml solution of each methanol plant extracts were mixed with 5 ml 10% sodium acetate, 2 ml 2,5% aluminium chloride and distilled water and left at room temperature for 45 minutes. The absorbance of the reaction mixture was measured at 420 nm. Total flavonoids content were calculated using a calibration curve and were expressed as quercetin equivalents.

The results were finally reported to the calculated dry matter of each sample.

Statistical analysis was performed using statistical package ANOVA.

RESULTS AND DISCUSSIONS

The biochemical analysis of the total polyphenols content revealed high values, especially for chokeberries and blackcurrant (table 1). A considerable amount of total polyphenols was found in strawberries, which is a positive information because these berries are the most prevalent on the market.

Table 1. Total polyphenols content

	Total polyphenols galic acid equiv. mg/100g	Standard deviation	Relative standard deviation %
Chokeberry	7791.6	695.5	8.93
Raspberry	1728.6	75.3	4.36
Blackberry	3340.2	134.2	4.02
Strawberry	4501.1	207.6	4.61
Blackcurrant	6339.1	312.8	4.93
Sea-buckthorn	2890.6	119.6	4.14

Regarding the level of anthocyanins, the highest one was found in blackcurrants, while the lowest one was in strawberries (table 2). This analysis didn't involve sea-buckthorn because the dark violet-blue color of anthocyanins is not one of their characteristics. The relative standard deviation values varied a lot among the triplicates, may be due to the fact that frozen fruits were subjected to analysis.

Table 2. Anthocyanins content

	Anthocyanins cyanidin-3-glucoside equiv. mg/100g	Standard deviation	Relative standard deviation %
Chokeberry	386.2	7.4	1.92
Raspberry	109	12	11.01
Blackberry	266.9	21.3	7.98
Strawberry	91.3	3.4	3.72
Blackcurrant	520	14.1	2.71

The total flavonoids content was rather high in all the analyzed samples (table 3). The high value was obtained for chokeberries. The level of flavonoids in sea-buckthorn is similar to that reported by Eccleston et al. (2002) who found flavonoid a content of 1182 mg/L sea-buckthorn juice.

Table 3. Total flavonoids content

	Flavonoides quercetin equiv. mg/100g	Standard deviation	Relative standard deviation %
Chokeberry	1793.3	23.2	1.29
Raspberry	536.6	25.7	4.79
Blackberry	591.2	11	1.86
Strawberry	662.8	31.7	4.78
Blackcurrant	1288.4	58.3	4.52
Sea-buckthorn	1379.9	45.1	3.27

A comparative analysis of phenolic antioxidants for the studied berries highlighted chokeberries and blackcurrants as the richest sources (fig. 1). The level of flavonoids was up to 50% from total phenolic content. The highest one was obtained for sea-buckthorn (47.7%) and the lowest one for strawberries (14.7%). The anthocyanins level was not more than 10% reported to the total phenolic compounds, ranging from 8.2% for blackcurrants and only 2% for strawberries.

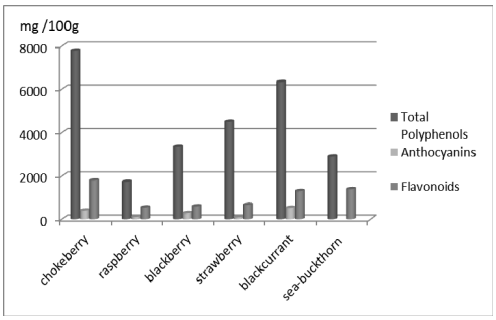

Fig. 1. Level of phenolics in berries

The high content of phenolic compounds found in the analyzed berries recommends them as excellent source of natural antioxidants with potential medicinal benefits.

CONCLUSIONS

The present study revealed the high content of phenolic compounds in several berries from Romania. Due to its considerable amount of phenolics, black chokeberries may be considered another important source of antioxidants, comparable with classical ones as blackcurrants and sea-buckthorn.

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BIOTECHNOLOGY OF SUBMERGED FERMENTATION TO PRODUCE NUTRITIVE MYCELIAL BIOMASS THROUGH CONTROLLED CULTIVATION OF EDIBLE AND MEDICINAL MUSHROOMS

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Abstract

The main aim of this research work was focused on the establishment of the best food biotechnology in order to be applied in the leading and controlling of submerged fermentation by using three edible and medicinal mushroom species Ganoderma lucidum (Curt. Fr.) P. Karst and Lentinula edodes (Berkeley) Pegler that were grown on different substrata containing grain wastes as main constituents. The experiments were carried out by cultivating these mushroom species under controlled conditions inside the culture vessel of a modern laboratory-scale bioreactor designed at the highest food quality standards. The submerged fermentation was set up in the following conditions: temperature,25- 27° C; agitation speed, 100-120 rev. min $^{-1}$; pH level, 5.7 – 6.5 units; dissolved oxygen tension within the range of 30% - 70%. During the period of controlled submerged fermentation lasting from 120 to 170 h, the mycelial biomass of fungal pellets was developed inside the broth. At the end of the culture cycles, the fungal pellets were harvested by extracting them from the culture vessel of the bioreactor and separating them from the broth by slow vacuum filtration. Pellet size, the hairy length of pellets, and the free mycelia fraction in the total biomass were microscopically investigated and the chemical composition of fungal biomass was analysed to determine and compare the protein and reduced sugar contents.

Key words: *biotechnology, biomass, submerged cultivation, edible and medicinal mushrooms*

INTRODUCTION

Submerged cultivation in liquid media of mushroom mycelium is a promising method which can be used in novel biotechnological processes for obtaining pharmaceutical substances of antitumor, antiviral and immuno-modulating actions from fungal biomass and cultural liquids as well as for the production of liquid spawn [1, 2].

The researches made for getting nutritive supplements from the biomass of *Ganoderma lucidum* species (Reishi) have shown that the nutritive value of its mycelia is owned to the huge protein content, carbohydrates and mineral salts. *Lentinula edodes* species (Shiitake) is a good source of proteins, carbohydrates (especially polysaccharides) and mineral elements with beneficial effects on human nutrition [3-5].

The main purpose of this work consists in the application of biotechnology for continuous cultivation of medicinal mushrooms by submerged fermentation in agro-food industry which has a couple of effects by solving the ecological problems generated by the accumulation of plant wastes in agro-food industry through biological means to valorise them without pollutant effects as well as getting fungal biomass with high nutritive value which can be used to prepare functional food [5-7].

The continuous cultivation of medicinal mushrooms was applied using the submerged fermentation of different natural by-products of agro-food industry that provided a fast growth as well as high biomass productivity of the investigated strains [7].

MATERIAL AND METHOD

Ganoderma lucidum (Reishi) and *Lentinula edodes* (Shiitake) were used as pure mushroom strains. The stock cultures were maintained on malt-extract agar (MEA) slants, incubated at 25°C for 5-7 d and then stored at 4°C. The seed cultures were grown in 250-ml flasks containing 100 mL of MEA medium at 23°C on

rotary shaker incubators at 150 rev min^{-1} for 7 d [7-10].
The fungal cultures were achieved by inoculating 100 ml of culture medium using 3-5% (v/v) of the seed culture and then cultivated at 23-25°C in rotary shake flasks of 250 ml. The experiments were conducted under the following conditions: temperature, 25°C; agitation speed, 120-180 rev min^{-1}; initial pH, 4.5–5.5. After 10–12 d of incubation the fungal cultures were ready to be inoculated aseptically into the glass vessel of laboratory-scale bioreactor (Fig. 1).

Fig. 1 – Laboratoy-scale bioreactor for submerged cultivation of edible and medicinal mushrooms

For the fungal growing in this bioreactor special culture media were prepared by using liquid nutritive broth, having the following composition: 15% cellulose powder, 5% wheat bran, 3% malt extract, 0.5% yeast extract, 0.5% peptone, 0.3% powder of natural argillaceous materials.
After the steam sterilization at 121°C, 1.1 atm., for 15 min. this nutritive broth was transferred aseptically inside of the culture vessel of a laboratory scale bioreactor [12-14].
This culture medium was aseptically inoculated with activated spores of *Ganoderma lucidum* and *Lentinula edodes* species. After inoculation into the bioreactor vessel, a slow constant flow of nutritive liquid broth was maintained inside the nutritive culture medium by recycling it and adding from time to time a fresh new one.
The submerged fermentation was set up at the following parameters: constant temperature, 23°C; agitation speed, 80-100 rev. min^{-1}; pH level, 5.7–6.0 units; dissolved oxygen tension within the range of 30-70%.
After a period of submerged fermentation lasting up to 120 h, small fungal pellets were developed inside the broth [10-12].

RESULTS AND DISCUSSIONS

Fermentation process was carried out by inoculating the growing medium volume (10,000 ml) with secondary mycelium inside the culture vessel of the laboratory-scale bioreactor (Fig. 1).
The whole process of growing lasts for a single cycle between 5-7 days in case of *L. edodes* and between 3 to 5 days for *G. lucidum*.
The strains of these fungal species were characterized by morphological stability, manifested by its ability to maintain the phenotypic and taxonomic identity.
The biomass collected after each one of these cultivation processes by submerged fermentation was dehydrated in order to be preserved for long time as mycelia powder. The biomass samples of *L. edodes* as well as *G. lucidum* are shown in figures 2, 3.

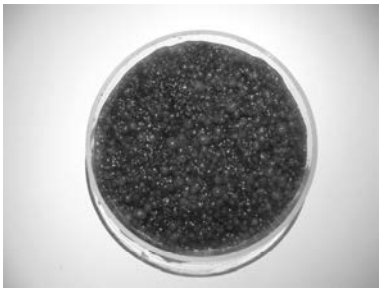

Fig. 2. Fungal pellets of *L. edodes* biomass

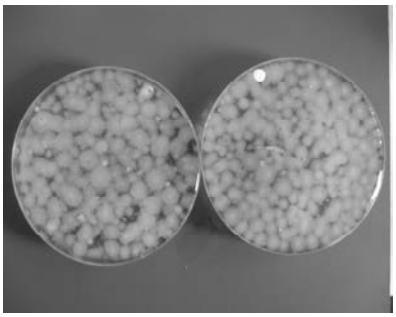

Fig. 3. Biomass of *G. lucidum* as fungal pellets

Observations on morphological and physiological characters of these two tested species of fungi were made after each culture cycle, highlighting the following aspects:

► sphere-shaped structure of fungal pellets, sometimes elongated, irregular with various sizes (from 2 to 5 mm in diameter), reddish-brown colour of *L. edodes* (Fig. 4).

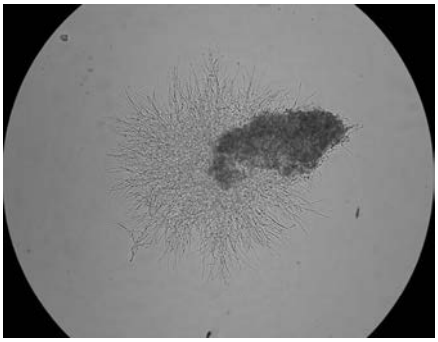

Fig. 4. Microscopical image of *L. edodes* fungal pellet (X100)

► globular structures of fungal pellets, irregular with diameters of 4 up to 7 mm or mycelia congestion, which have developed specific hyphae of *G. lucidum* (Fig. 5).

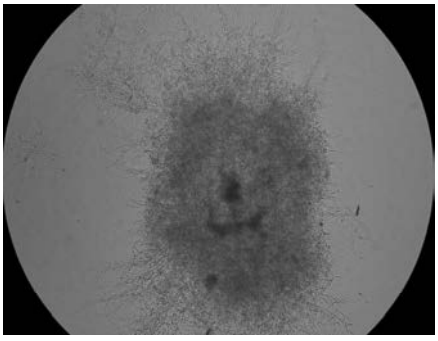

Fig. 5. Microscopical image of *L. edodes* fungal pellet (X100)

The experiments were carried out in three repetitions. Samples for analysis were collected at the end of the fermentation process, when pellets formed specific shapes and characteristic sizes.

For this purpose, fungal biomass was washed repeatedly with double distilled water in a sieve with 2 mm diameter eye, to remove the remained bran in each culture medium.

Biochemical analyses of fungal biomass samples obtained by submerged cultivation of edible and medicinal mushrooms were carried out separately for solid fraction and extract fluid remaining after the separation of fungal biomass by pressing and filtering [15-17].

The percentage distribution of solid substrate and liquid fraction in the preliminary samples of fungal biomass are shown in table 1, together with the percentage of substance remaining (which was measured for dry matter content) and the percentage level of liquid fraction (resulted after pressing the solid substrate).

Table 1. Percentage distribution of solid substrate and liquid fraction in the samples of fungal biomass

Mushroom species	Total liquid volume of per sample (ml)	Total biomass weight per sample (g)	Water content after separation (%)
L. edodes	83	5.81	83.35
L. edodes	105	7.83	82.50
L. edodes	95	7.75	82.15
L. edodes	80	5.70	79.55
G. lucidum	75	7.95	83.70
G. lucidum	115	6.70	82.95
G. lucidum	97	5.45	80.75
G. lucidum	110	6.30	77.70

Also, the most obvious sensory characteristics (color, odor, consistency) were evaluated and presented at this stage of biosynthesis taking into consideration that they are very important in the prospective view of fungal biomass using as raw matarials for nutraceuticals producing [18-23].

In each experimental variant the amount of fresh biomass mycelia was determined. Percentage amount of dry biomass was determined by dehydration obtained at a temperature of 70° C, until constant weight.

The total protein content was determined by biuret method, whose principle is similar to the Lowry method, this method being recommended for protein content ranging from 0.5 to 20 mg/100 mg on each sample [15-17].

In addition, this method requires only one sample incubation period (20 min) and using them is eliminated interference with various chemical agents (ammonium salts, for example).

The principle method is based on reaction that takes place between copper salts and compounds with two or more peptides in the composition in alkali, which results in a red-purple complex, whose absorbance is read in a

spectrophotometer in the visible domain (λ - 550 nm).
In tables 2 and 3 are presented the amounts of fresh and dry biomass as well as the protein contents for each fungal species and variants of culture media.

Table 2. Fresh and dry biomass and protein content of *L. edodes* grown by submerged fermentation

Mushroom culture variants	Fresh biomass (g)	Dry biomass (%)	Total proteins (g % d.w.)
I	20.30	5.23	0.55
II	23.95	6.10	0.53
III	22.27	4.79	0.73
IV	20.10	4.21	0.49
Control	4.7	0.5	0.2

Table 3. Fresh and dry biomass and protein content of *G. lucidum* grown by submerged fermentation

Mushroom culture variants	Fresh biomass (g)	Dry biomass (%)	Total proteins (g % d.w.)
I	25.94	9.03	0.67
II	22.45	10.70	0.55
III	23.47	9.95	0.73
IV	21.97	9.15	0.51
Control	5.9	0.7	0.3

According to registered data, using wheat bran strains the growth of *G. lucidum* biomass was favoured, while the barley bran led to increased growth of *L. edodes* mycelium and *G. lucidum* as well.
In contrast, dry matter content is significantly higher when using barley bran for both species used. Protein accumulation is more intense when using barley bran compared with those of wheat and rye, at both species of mushrooms.
The sugar content of dried mushroom pellets collected after the biotechnological experiments was determined by using Dubois method.
The mushroom extracts were prepared by immersion of dried pellets inside a solution of NaOH pH 9, in the ratio 1:5.
All dispersed solutions containing the dried pellets were maintained 24 h at a precise temperature of 25^{o}C, in full darkness, with continuous homogenization to avoid the oxidation reactions.
After the removal of solid residues by filtration the samples were analyzed by the previous mention method [15-17.
The nitrogen content of mushroom pellets was analyzed by Kjeldahl method.
All registered results are related to dry weight of mushroom pellets that were collected at the end of each biotechnological culture cycle (Table 4).

Table 4. The sugar and total nitrogen contents of dried mushroom pellets

Mushroom species	Culture variant	Sugar content (mg/ml)	Kjeldahl nitrogen (%)
L. edodes	I	5.15	6.30
L. edodes	II	4.93	5.35
L. edodes	III	4.50	5.70
L. edodes	IV	4.35	5.75
	Control	0.55	0.30
G. lucidum	I	4.95	5.95
G. lucidum	II	5.05	6.15
G. lucidum	III	5.55	6.53
G. lucidum	IV	4.70	5.05
	Control	0.45	0.35

Comparing all registered data, it could be noticed that the correlation between the dry weight of mushroom pellets and their sugar and nitrogen contents is kept at a balanced ratio for each tested mushroom species.
From these mushroom species that were tested in biotechnological experiments *G. lucidum* – culture variant III showed the best values concerning the sugar and total nitrogen content. In order, on the very next places, *L. edodes* – culture variant I and *G. lucidum* - culture variant II could be mentioned from these points of view.
These registered results concerning the sugar and total nitrogen contents had higher values than those obtained by other researchers [15-18].
The nitrogen content in fungal biomass is a key factor for assessing its nutraceutical potential, but the assessing of differential protein nitrogen compounds requires additional investigations.

CONCLUSIONS

1. The grain by-products used as substrata for growing the fungal species *L. edodes* and *G. lucidum* by controlled submerged fermentation showed optimal effects on the mycelia development in order to get high nutritive biomass.
2. The dry matter content of fungal biomass produced by submerged fermentation of barley bran was higher for both tested species.
3. The protein accumulation is more intense when using barley bran compared with those of wheat and rye, at both fungal species.

4. The correlation between the dry weight of mushroom pellets and their sugar and nitrogen contents is kept at a balanced ratio for each tested mushroom species.
5. *G. lucidum* - culture variant III showed the best values of sugar and total nitrogen contents, being followed by *L. edodes* – culture variant I.

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VARIABILITY OF POLYPHENOLIC COMPOSITION OF QUALITY RED WINES DEPENDING ON THE ORIGIN OF RAW MATERIAL AND WINEMAKING CONDITIONS

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Abstract

Our studies refers to the influence of geographical origin of the raw material and the conditions of maceration and fermentation on the composition and extraction of polyphenolic complex at two of the most important varieties of high quality red wines from Romania, Cabernet Sauvignon and Feteasca Neagra; varieties come from different wine regions, Dealu Mare and Murfatlar, harvest of 2010. As regards the oenological practices, our studies aimed three variants of maceration and fermentation of the materials from the mentioned varieties, 5, 10 and 18 day. Analyzes have been carried out in the extracts from epicarp and in the resulted wine by UV-VIS spectrometry techniques. Total content of polyphenols have been determined by IPT technique. Tannins have been determined by the Ribereau-Gayon si Stonestreet method (1996), anthocyanins by dilution technique of the extract with a large amount of solvent (methanol) in strongly acidic conditions and in case of wines was used the discolouration technique with SO_2. Color intensity and shade were determined at λ=420 nm and λ=520 nm. The results of the analyzes have shown significant differences concerning the polyphenolic complex content determined both of the origin of the raw materials as well as the technique used for maceration.

Keywords: *Polyphenolic complex, color intensity, tannins, total polyphenols index, Feteasca Neagra.*

INTRODUCTION

Obtaining a high quality red wine depends on a variety of factors, assembled in a multicriterial system influencing the accumulation of sugars and polyphenolic compounds as well as their subsequent evolution [2]. Raw material characteristics depend on the environmental factors (soil, climate and microclimate - terroir) and the potential of the variety. Further, cultivation techniques and oenological practices are important in obtaining a quality red wine, with an optimal polyphenolic complex [3].

Our studies refers to the influence of these factors, geographical origin of the raw material and the conditions of maceration and fermentation on the composition and extraction of polyphenolic complex at two of the most important varieties of high quality red wines from Romania, *Cabernet Sauvignon* and *Feteasca Neagra*; varieties come from different wine regions, Dealu Mare and Murfatlar, harvest of 2010.

MATERIAL AND METHOD

To establish the influence of the ecosystem and vineyard and winemaking condition on polyphenolic composition of quality red wines, two varieties of black grapes were studied, *Cabernet Sauvignon* and *Feteasca Neagra*, which are varieties that occupy the largest area in Romanian vineyards to obtain high quality red wines. The vineyard ecosystems studied are two wine-growing areas in southern Romania, specialized in obtaining of quality red wines: Dealu Mare and Murfatlar. Raw materials are coming from the harvest of 2010.

Grapes raw material for wine were harvested randomly from a few plots, harvesting being taken at full maturity of the grapes.

Cabernet Sauvignon and *Feteasca Neagra* wine was analyzed in terms of physico-chemical: alcoholic strength (vol% alcohol), total acidity (g/l sulfuric acid), volatile acidity (g/l acetic acid), total dry extract (g/l) and glycerol (g/l).

Total content of polyphenols have been determined by IPT technique (g/l gallic acid),

tannins (g/l) have been determined by the Ribereau-Gayon si Stonestreet method (1996), anthocyanins (mg/l) by dilution technique of the extract with a large amount of solvent (methanol) in strongly acidic conditions and in case of wines was used the discolouration technique with SO_2. Color intensity and shade were determined at λ=420 nm and λ=520 nm [1].

Tannins from wines were characterised by the use of following indices: gelatin index (astringente tannins, least condensed), HCl index (condensed tannins) and ethanol index (tannins-polysaccharides complex). Wines have been noted: V1 – *Cabernet Sauvignon Dealu Mare*; V2 - *Cabernet Sauvignon Murfatlar*; V3 – *Feteasca neagra Dealu Mare*; V4 – *Feteasca neagra Murfatlar*.

RESULTS AND DISCUSSIONS

Observations and analysis demonstrated the influence of ecosystem vineyard and and winemaking condition on the quality red wines. The results showed differences between wine quality parameters: alcoholic strength and content of the glycerol, greater at Dealu Mare compared with the values recorded for Murfatlar.

Table 1. Chemical parameters of red wines (*Cabernet Sauvignon* and *Feteasca neagra)*

Wines	Chemical parameters of red wines				
	alcoholic strength, vol % alcohol	total acidity, g/l sulfuric acid	volatile acidity, mg/l CH_3COOH	dry extract, g/l	glycerol g/l
V_1	12,5	3,5	0,6	30,8	7,8
V_2	12,0	3,2	0,4	31,2	7,5
V_3	13,0	3,3	0,3	30,2	9,5
V_4	12,5	3,0	0,3	29,4	8,0

Chromatic compounds accumulation is done in parallel and in a positive relationship with the accumulation of sugars. Often, high concentrations of characteristic phenolic compounds of the variety are reached before a high concentration of sugars, but only under a year when climatic factors have allowed [4].

Analyses showed that in case of *Cabernet Sauvignon* and *Feteasca Neagra* wines, the amount of phenolic compounds and tannins content was lower than in wine center Dealu Mare. The anthocyanins concentration followed the same curve, higher accumulation occurring in Dealu Mare center, the difference being quite large between the center Murfatlar.

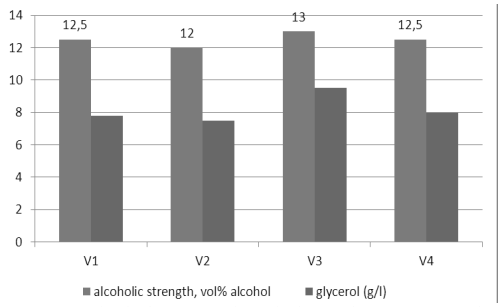

Fig. 1. Alcoholic strength and glycerol content of red wines (*Cabernet Sauvignon* and *Feteasca neagra)*

The results of our experiments showed that both vineyard ecosystems and winemaking conditions have a major influence on the technological potential of the variety and in the end on the quality of the wine. Significant differences were recorded on color characteristics of wines of two varieties analyzed.

Table 2. Polyphenols content of red wines (*Cabernet Sauvignon* and *Feteasca neagra)*

Wines	total content of polyphenols mg/l gallic acid	tannins g/l	anthocyans mg/l	intensity color wines
V_1	3,30	3,7	580	1,150
V_2	3,00	3,5	520	1,050
V_3	2,75	3,3	380	0,900
V_4	2,20	2,9	370	0,870

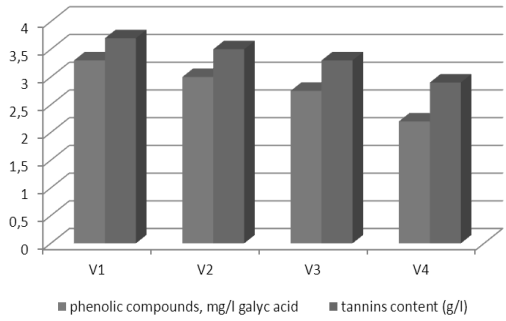

Fig. 2. Polyphenols and tannins content of red wines (*Cabernet Sauvignon* and *Feteasca neagra)*

Table 3. Indices of tannins (*Cabernet Sauvignon* and *Feteasca neagra* wines*)*

Wines	gelatin index	HCl index	ethanol index
V_1	67	8,5	8,1
V_2	62	8,0	7,9
V_3	58	6,3	8,6
V_4	55	6,8	8,5

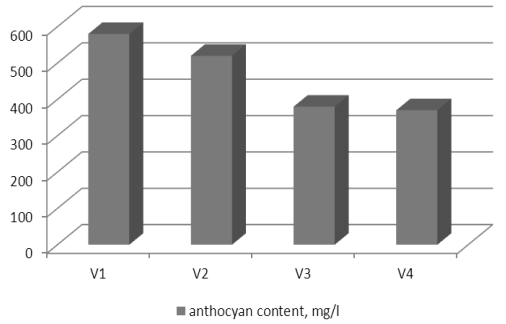

Fig. 3. Anthocyans content of red wines (*Cabernet Sauvignon* and *Feteasca neagra)*

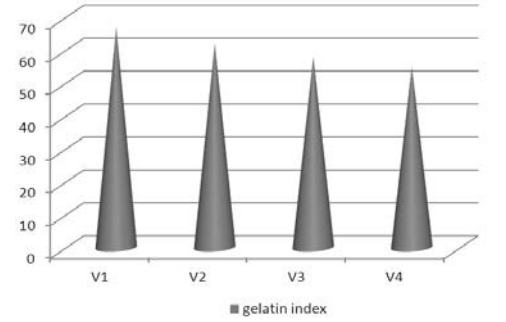

Fig. 4. Gelatin index

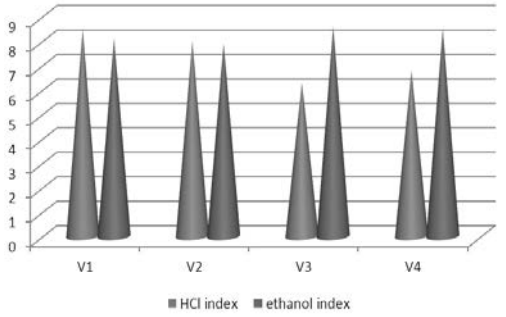

Fig. 5. HCl and ethanol index

Table 4. Influence of the extraction method on the content in polyphenols (*Cabernet Sauvignon* and *Feteasca neagra* wines*)*

Variant	CS 5	CS 10	CS 18	FN 5	FN 10	FN 18
Total content of polyphenols mg/l gallic acid	1,55	3,00	3,80	1,50	2,70	3,00
Color intensity (IC)	0,600	1,100	1,200	0,420	0,850	0,900

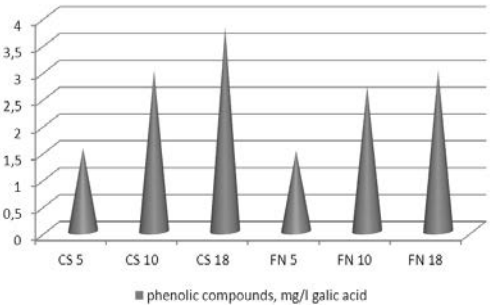

Fig. 6. Variant influence over the content in polyphenols extraction

CONCLUSIONS

Polyphenols content of red wine depends on the vineyard and winery conditions; *Cabernet Sauvignon* and *Feteasca neagra* wines have recorded higher values in the wine-growing Dealu Mare.

As regards tannins, have registered high levels of astringent tannins in the wine-growing Dealu Mare.

Each black grape variety has a different technological potential of phenolic compounds and behaves differently from color extraction; great value of content in phenolic compounds were recorded in version 2 (10 days of maceration).

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A NEW BIOTECHNOLOGICAL MEDIUM FOR BIOTRANSFORMATION OF SUBSTRATES WITH DIFFERENT WATER-SOLUBILITY

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Abstract

Reverse micellar system (RMS) provide an excellent medium for nonaqueous biocatalytic studies, being used for enzymatic conversion of aqua-low-solubility reactants. RMS is characterized by hydration degree, w_0, defined as the molar ratio of water and surfactant. This parameter induce most of the structural and physico-chemical properties of RMS, being more important even then the absolute quantity of water or surfactant in the system. The reaction of alcohol oxidase from Hansenula polymorpha solubilized in AOT-isooctane reverse micelles was used as a small scale model, both for experimental study and theoretical discussions. The potential of the new reaction medium for biotransformation-catalyzed alcohol oxidase was evaluated using substrates with different hydrophilic / hydrophobic balance. The efficiency of the bioconversion of aliphatic alcohols in RMS has been analyzed.

Key words**:** *reverse micelles, biotransformation, alcohol oxidase, AOT*

INTRODUCTION

One of the nanostructured medium with scientific and biotechnological applicative potential is the reverse micellar system (RMS), a representing of the colloidal chemistry [1], based on the self-assembling capacity of amphiphilic molecules of surfactants in organic solvent (min 95%). The resulting spherical or ellipsoidal shape surfactant aggregates are thus closely packed globules where the polar head group of the surfactant molecules occupies the interior of the aggregates whereas the hydrophobic tails extend into the bulk apolar solvent, with water encapsulated in compartments [2].
They are characterized by the hydration degree, w_0, defined as the molar ratio of water and surfactant [3]. This parameter induce most of the structural and physico-chemical properties of RM, being more important even then the absolute quantity of water or surfactant in the system.

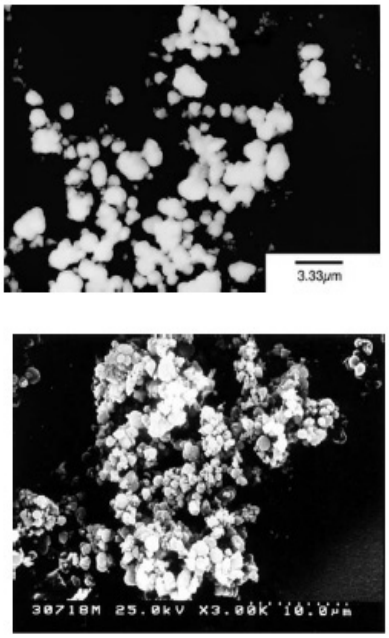


Fig.1. Electron microscope images of reverse micelles

RM media are able to solubilize proper solution, the main driving forces responsible for the solute distribution between the organized assembly and the organic medium being considered to be mainly hydrophobic effects and hydrogen bond interactions. However,

other effects such as chemical and electrostatic interactions must be considered when charges or zwitterionic molecules are involved. In these supramolecular systems, a solute can be located in a variety of microenvironments namely the surrounding organic solvent, the water pool or at the micellar interface [4]. To know the location of molecular probes in the aggregates, can give information about their residing place in biological systems.

RMS provide an excellent medium for nonaqueous biocatalytic studies, being used for enzymatic conversion of aqua-low-solubility reactants or to improve the unfavorable thermodynamic yield by shifting the reaction equilibrium due to the decrease of water content. They have two important characteristics: first, are a cell membrane-mimetic medium [5] and, second, are a macroscopically pseudo-homogeneous and optical transparence system [6], so all spectroscopic techniques can be applied.

In the present studies, we are focusing on the oxidation activity of alcohol oxidase **(**EC 1.1.3.13, alcohol:oxygen oxidoreductase) in a reverse micellar system of sodium bis(2-ethylhexyl) sulfosuccinate (AOT), in isooctane. Alcohol oxidase, a peroxisomal enzyme, plays a major role in the metabolism of methanol resulting in the formation of formaldehyde and has a significant practical role in analytical determination of alcohols and yielding aldehydes, hydrogen peroxide, and various heterologous proteins [7]. The main aim of the study is to evaluate the potential of the enzymatic oxidation of aliphatic alcohols in a AOT-isooctane reverse micelles.

MATERIAL AND METHOD

Reverse Micelles Preparation

Reverse micelles were prepared by injection of different reactants into 50 mM AOT-isooctane stock solution, followed by gentle shaking, until a completely transparent solution was formed.

Enzyme assay

The standard procedure for alcohol oxidase assay was performed according to Janssen and Ruelius [8]. The initial rate of reaction was recorded at 415 nm.

Experimental conditions

The variation of the hydration degree was obtained by changing the water volume and maintaining constant the AOT concentration. The overall enzyme concentration was kept constant through the whole range of w_0 – values.

RESULTS AND DISCUSSIONS

Certain enzymes, especially oxidoreductases, have the potential for the biotransformation of hydrophobic compounds as substrates. However, the poor solubility of such compounds in water is a critical problem for industrial applications. To overcome this problem, enzymatic reactions of alcohol oxidase in a non-conventional medium, such as reverse micelles, have been tested.

Substrates susceptible to the conversion catalyzed by alcohol oxidase are differentiated by their degree of polarity which is materialized in the case of AOT-isooctane reverse micelles through different miscibility of the substrates between the two major phases of the reaction environment: polar aqueous microenvironment and apolar organic phase.

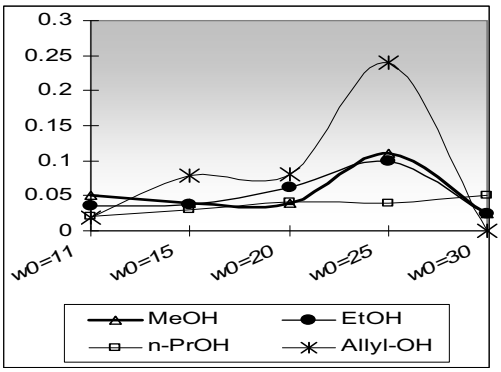
$$S_{solvent} \rightleftharpoons S_{interface} \rightleftharpoons S_{water\text{-}pool}$$

The catalytic activity of the studied enzyme, in the presence of various substrates, was performed in the range of w0 = 11-30, taking into the account that hydration degree is considering the defining parameter for the caracteristics of the reverse micellar environment [9], [10].

Methanol is characterized by immiscibility in the organic phase of reverse micellar system, i.e. isooctane, and therefore it can be considered that this substrate is accumulated only in the intramicellar aqueous cavity (the solubility of methanol in isooctane being negligible).

Experimental data not presented show that the dependence of alcohol oxidase activity vs the concentration of methanol was a Michaelis-Menten type and the catalytic activity, in the range of w_0 = 11 to 30, has a maximum value at w_0 =25 (Fig.2 A).

A.



B.

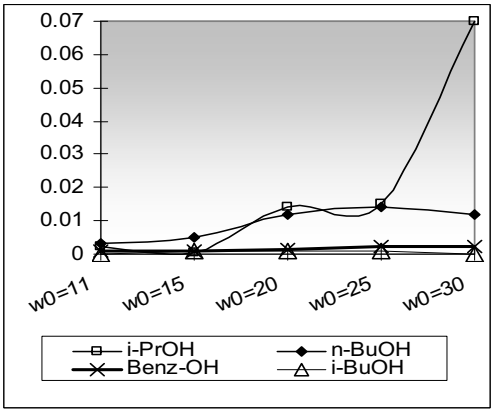


Fig. 2. Catalytic activity of alcohol oxidase in the presence of different substrates (in the range of w_0 = 11-30)

Generally, the dependence of enzyme activity vs.hydration degree was determined to be a Gaussian curve, which implies an optimum w0, where the enzyme presents maximum activity in reverse micelles environment [11].

The upturn in the curves is described either by changes in the aqueous microenvironment [12], or conformational distortion of the enzyme molecule at low values of the degree of hydration [13]. The downward of the curve is determined by variation of intramicellar pH or local dilution of reactants [14].

Unlike methanol, miscible only in the aqueous phase, other substrates, such as aliphatic alcohols with 2 or 3 atoms of carbon (ethanol, n-propanol, i-propanol) or unsaturated alcohols, such as allyl alcohol (3 propene-1-ol), are characterized by miscibility both in aqueous environment and isooctane phase. In this case, the substrates will be distributed between the two phases of RMS, according to the individual partition coefficient of each substrate.

Despite this difference, for ethanol and allyl alcohol, biotransformation profile vs w_0 (w_0 =11–30) is Gaussian, similar with those for methanol (Fig. 2A).

The hydrofobicity of enzymatic substrates is increasing for aliphatic alcohols with 4 atoms of carbon (n-butanol and i-butanol) or with aromatic ring (benzyl alcohol). Thus, their miscibility in the apolar phase versus the miscibility in the polar phase of reverse micelles is also increasing.

Even if n-propanol, i-propanol, n-butanol, i-butanol and benzyl alcohol have a good miscibility in the organic phase of RM medium, the biotransformation yield is very poor, respecting the well-known substrate specificity of alcohol oxidase in aqueous medium.

In the case of alcohols with 3 or more atoms of carbon, branched or unbranched, the biotransformation profile is no longer Gaussian (Fig. 2B).

CONCLUSIONS

From biotechnological point of view, it has demonstrated that AOT-isooctane reverse micelles with hydration degree in the range of w0=10-30 is a suitable media for enzymatic conversion of alcohols with alcohol oxidase, in a large range of their water solubility (from 100% to moderate).

The most important avantage of this new medium is the increasing of the range of substrates which are compatible with the enzymatic reaction and the performance of the reaction in a pseudo-homogeneous water-apolar solvent medium.

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OPTIMISATION OF CULTURE MEDIUM TO OBTAIN MAXIMAL INCREASE OF THE EXTRACELLULAR STARCH DEGRADATION ACTIVITY BY THE ENZYMES AS PRODUCTS RELEASED OF THE *ACIDIPHILIUM* GENUS

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Abstract

Acidophilic heterotrophic bacteria belonging to the Acidiphilium genus adapted frequently to the environmental concentration of toxic compounds developing protective mechanisms of resistance which are much diversified in those bacteria. The aim of the present paper was the extracellular enzymes production from the acidophilic heterotrophic bacteria belonging to Acidiphilium populations which hydrolysis the starch in the presence of $CaCl_2$ or/and NaCl solutions. These bacterial populations were isolated from acid waters and mine sediments containing high concentrations of heavy metals collected from Baia (Tulcea County) and Roşia Poieni (Alba County). In the culture medium, the optimum of the substrate concentration represented by starch was 0.2 % in the presence of mixed $CaCl_2$-NaCl solutions. The most important physic-chemical parameter that influence extracellular enzymatic activities of the Acidiphilium genus are the contact time between bacterial populations and culture medium with metallic ions solutions. Also, an extended exposure of Acidiphilium populations in the GYE medium with different starch concentrations and 0.1% $CaCl_2$ induce intense starch degradation by extracellular enzymes in acidic conditions.

Key words**:** *starch, acidophilic bacteria, extracellular enzymes, metallic ions*

INTRODUCTION

The research activities on the microbial ecology of acidic extreme environments provide a basis for the coal processing technologies have been developed. The pollution carbon compounds has determined an increasing of the knowledge body interest regarding the microbial resistance to carbon compounds; therefore the coal mining activities using acidophilic heterotrophic bacteria in biotechnology is important to environmental quality improvement [1, 4, 13, 26]. The capacity of the acidophilic heterotrophic bacteria capacity to convert a wide range of synthesis organic compounds into inorganic products plays an important role in the development of bioremediation technologies [20, 27, 32].

The oxidative degradation degree of organic contaminants and the obtained results within a bioremediation program contribute to a complex knowledge on the different processes which affect spreading organic substances in polluted environments [2, 15, 28].

The recent studies have shown that these bacteria can be used both for remediation of contaminated environments, by reducing the concentration of carbon compounds, as well as in biosorbtion of metallic ions from waste dumps.

The use of acidophilic heterotrophic bacteria to remove the metallic ions from polluting environments is an unconventional method, economic approach and perspective for their decontamination [19, 24, 31].

The acidophilic heterotrophic bacteria are important factors in the mineralization process of the organic substances from soil. They are involved in the solubilization of organic mineral resources from land, facilitating their use by other organisms.

It is essential to know the optimal physical-chemical conditions in which the acidophilic

bacteria are able to produce extracellular hydrolytic enzymes, in order to understand their distribution in the nature and their implication in the ecosystem [6, 22, 23].
The starch degradation by the extracellular enzymatic activity is influenced by the physiological characteristics of the acidophilic heterotrophic bacteria, the physical-chemical factors in the medium, the nutritive medium composition, all of them being important elements in the control activity of the microbiological removal process of carbon compounds from waste waters and coal sludge [6, 10, 22].
The study on metabolism of the acidophilic heterotrophic bacteria shows a great importance due to their biotechnological potential to the adaptation ways to increased values of acidity, and a theoretical importance for the knowledge and explanation of biodiversity in extreme environment [8, 16, 35].
In this context by their activities, acidophilic heterotrophic bacteria have a significant role both in the nature as well as the assessment of the anthropogenic impact on environment. These bacteria take part in the aggregation process of the soil through the ability to secrete viscous and sticky substances. In this way, they influence both the physical and the chemical structure of the soil [7, 16, 29].
It is considered that chemical and physiological reactions involved in the extracellular enzymatic activities depend on the physiological conditions of the bacterial cells and the chemical valence of the metallic ions that come in contact with them and act as cofactors. That is, the starch degrading enzymes are metalloenzymes, because they need a metallic ion for their catalytic activity as well for the settle of active conformation of the protein [5, 9, 25, 34].
The metallic ions act upon the physiological processes and they are essential for the bacterial growth. All of these are strongly influenced by the physical-chemical factors of the surrounding environment. Through their activities, bacteria belonging to the *Acidiphilium* genus lead to changes in the values of the pH and the oxidation-reduction potential [10, 18, 31].
An essential characteristic of these metalloenzymes responsible with the starch degradation, produced by the *Acidiphilium* genus, is their high thermodynamic stability when a deposit compound is formed between metallic ions and side protein [11, 17, 30].
Metallic ions can affect the catalytic function of the enzyme by acting through: (1) substrate binding and/or enzyme cofactors; (2) activation of enzyme-substrate complex; (3) induce certain changes in the conformation of the enzyme; (4) contribution to some oxidation-reduction reactions, as enzyme cofactors [3, 21, 33].
This study shows how the metallic ions, such as Ca^{2+} ($CaCl_2$) and Na^{+} (NaCl), acting as cofactors, affects the extracellular enzymatic activity of acidophilic heterotrophic bacteria belonging to the *Acidiphilium* genus in the starch degradation process.

MATERIALS AND METHODS

1. The growth of bacterial cultures

In order to obtain *Acidiphilium* populations have recolted waters and sediments mining from Baia (Tulcea county) and Rosia Poieni (Alba county), both containing high concentrations of metallic ions.
The bacterial strains belonging to the *Acidiphilium* genus have been isolated on selective GYE medium with pH 3.0, that have the glucose as source of carbon and energy.
The populations of heterotrophic bacteria tabulated in the *Acidiphilium* genus was observed after the incubation for 21 days at 28^0C on the liquid GYE medium by the modifying of medium composition correlated to the decreasing of the initial pH value of the organic medium [14].
Acidophilic heterotrophic bacteria have been represented by populations of *Acidiphilium* sp. isolated from the Rosia Poieni area noted P_4 (photo 1) and *Acidiphilium* sp. isolated from the Baia area noted P_7 (photo 2).
Regarding the obtaining populations of acidophilic heterotrophic bacteria were used for this study isolated colonies on agarized selective culture media, following the dynamics of the physiological activity in organic media specific to the *Acidiphilium* genus (photo 1-2).

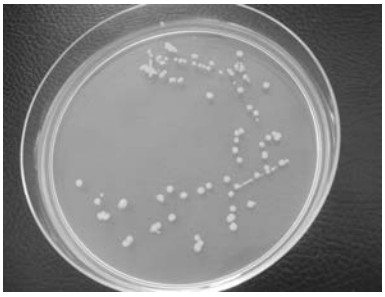

Photo 1. White colonies of *Acidiphilium* sp. isolated from mining effluents of the Rosia Poieni area

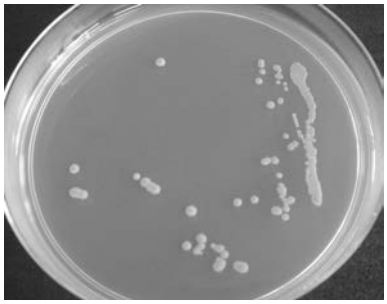

Photo 2. Red colonies of *Acidiphilium* sp. isolated from mining effluents of the Baia area

2. Shake flask experiments

The bacterial cultures have been grown in 100 ml Erlenmeyer glasses containing 50 ml growth medium and 5 ml inoculums (bacterial culture of 7 days old). These cultures were incubated up to 7, 14 and finally 21 days at 28^0C, under continuous agitation (150rpm). The bacterial density in the culture media was measured spectrophotometrically, by monitoring the optical density at 660nm for up to 21 days.

3. Extracellular starch degrading assays of bacterial cultures

The test of *Acidiphilium* populations for their capacity to produce extracellular starch degrading enzymes was made by using nine types of GYE medium: (1) medium with 0.1% NaCl; (2) medium with 0.1% $CaCl_2$; (3) medium with 0.1% NaCl and 0.1% $CaCl_2$; (4) medium with 0.1% starch and 0.1% NaCl; (5) medium with 0.1% starch and 0.1% $CaCl_2$; (6) medium with 0.1% starch, 0.1% NaCl and 0.1% $CaCl_2$; (7) medium with 0.2% starch and 0.1% NaCl; (8) medium with 0.2% starch and 0.1% $CaCl_2$; (9) medium with 0.2% starch, 0.1% NaCl and 0.1% $CaCl_2$.

Starch was selected as substrate, carbon and energy source.

The starch hydrolytic activity was assessed through spectrophotometer determination at 580 nm of the compounds formed between starch and iodine.

Extracellular starch degrading enzyme activity was evaluated by Wohlgemuth method [12].

RESULTS AND DISCUSSIONS

The results referring to the influence of mixed $CaCl_2$-NaCl solutions on the growth of the *Acidiphilium* populations, cultivated in GYE medium with starch as source of carbon and energy at 28^0C in continuous agitation conditions, are represented in figures 1-3.

Comparative studies made on the *Acidiphilium* populations show that those isolated from mining effluents of the Rosia Poieni area has a growth and a extracellular starch degrading enzyme activity which is more intense than the bacterial population isolated from mining effluents of the Baia area in same cultivation conditions (fig. 1-3).

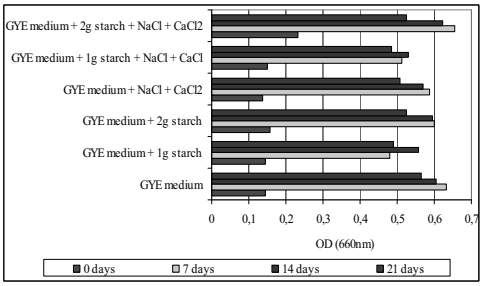

Fig.1. The bacterial density of the P_4 population in GYE medium with 0.1% NaCl, 0.1% $CaCl_2$ and different concentrations of starch

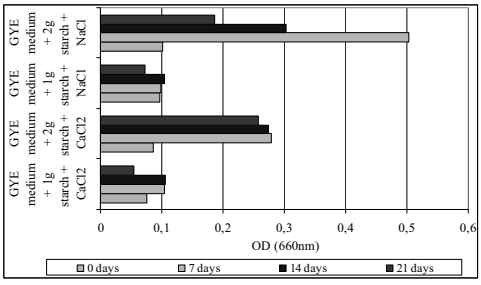

Fig.2. The bacterial density of the P_4 population in GYE medium with different concentrations of starch and 0.1% $CaCl_2$, respectively 0.1% NaCl

On the other hand, the *Acidiphilium* population P_4 is more sensitive at substrate variations because at the starch concentration between 0.1% - 0.2% the bacterial density is lower than in the case of the bacterial population isolated from Baia (fig. 2-3).

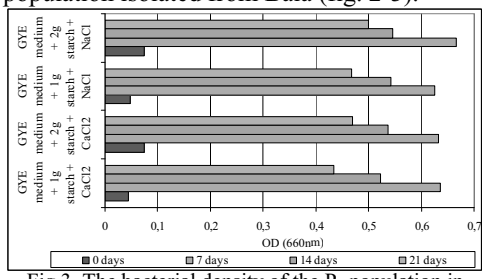

Fig.3. The bacterial density of the P_7 population in GYE medium with different concentrations of starch and 0.1% $CaCl_2$, respectively 0.1% NaCl

The analyses about the influence of mixed $CaCl_2$-NaCl solutions on the extracellular starch degradation enzymatic activity of bacterial populations, cultivated in GYE medium with different substrate concentrations at a temperature of 28^0C, are illustrated in figures 4 - 9. The compared data within this study pointed out that the two bacterial populations present the same optimum growth conditions, but the *Acidiphilium* P_4 population is more efficiently at decreasing the starch concentration of the culture medium than the *Acidiphilium* P_7 population.

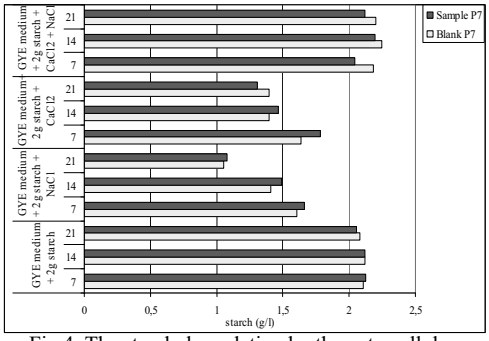

Fig.4. The starch degradation by the extracellular enzymatic activity of the P_7 population in GYE medium with 0.1% NaCl, 0.1% $CaCl_2$ and 2g/l starch

The comparative studies regarding the influence of mixed $CaCl_2$-NaCl solutions on the growth and the extracellular enzymatic activity is developed with a maximum intensity up to 7 and 21 days of incubation periods at the same substrate concentration.

The starch concentration of 2.0 g/l is optimum for the growth and the extracellular starch degradation enzymatic activity of the two bacterial populations from the *Acidiphilium* genus (fig. 4-6).

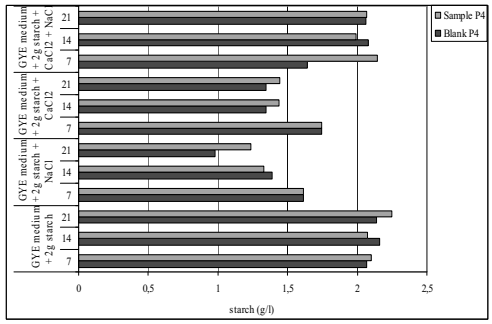

Fig.5. The starch degradation of the extracellular enzymatic activity of the P_4 population in GYE medium with 0.1% NaCl, 0.1% $CaCl_2$ and 2g/l starch

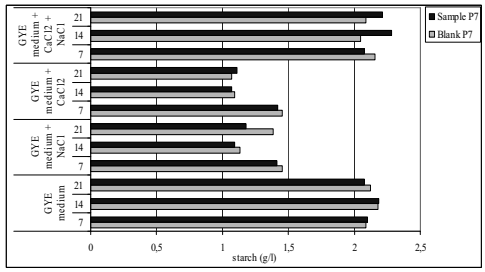

Fig.6. The starch degradation of the extracellular enzymatic activity of the P_7 population in GYE medium with 0.1% NaCl and 0.1% $CaCl_2$

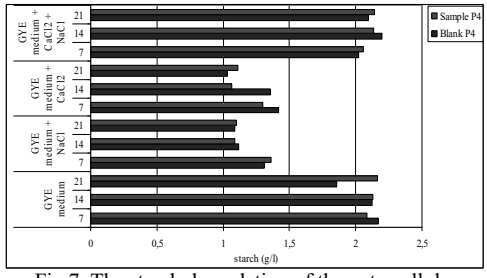

Fig.7. The starch degradation of the extracellular enzymatic activity of the P_4 population in GYE medium with 0.1% NaCl and 0.1% $CaCl_2$

The continuous agitation conditions determine a higher extracellular starch degrading enzyme activity of *Acidiphilium* populations correlated to the increasing of bacterial density in the culture medium with different substrate concentrations of starch. Also,

lowering the starch concentration to 1.0 g/l determined a reduction of the bacterial density and the hydrolytic activity at 70-80% of the values obtained at an optimum substrate concentration (fig. 1-9).

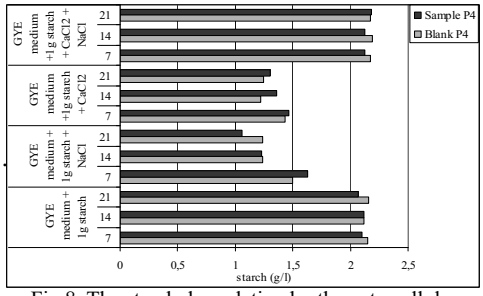

Fig.8. The starch degradation by the extracellular enzymatic activity of the P_4 population in GYE medium with 0.1% NaCl, 0.1% $CaCl_2$ and 1g/l starch

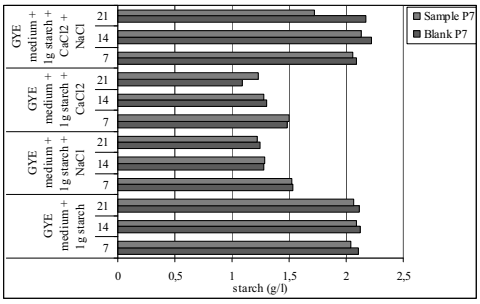

Fig.9. The starch degradation by the extracellular enzymatic activity of the P_7 population in GYE medium with 0.1% NaCl, 0.1% $CaCl_2$ and 1g/l starch

The results obtained lead to the increase of the knowledge body of the previous studies about the growth and the extracellular enzymatic activity of the heterotrophic bacterial populations from the *Acidiphilium* genus in which the extreme limits of temperature were established between 20^0C and 37^0C.

Regarding the optimum values of physical-chemical parameters is necessary in laboratory experiments that test the extracellular enzymatic activity of the bacterial cultures tabulated in the *Acidiphilium* genus to take place in the presence of raised concentrations of metallic ions in the polluted environment conditions [3, 14, 23, 34].

CONCLUSIONS

The experimental results in which were compared the effects of the $CaCl_2$ and NaCl solutions on the acidophilic heterotrophic bacteria, isolated from the mining effluents at Rosia Poieni and Baia areas, revealed a significant increase of the bacterial density and extracellular hydrolytic activity in GYE medium with 0.1% $CaCl_2$ and 0.1% NaCl at a concentration value of the substrate is established at 2g/l starch.

The comparative results regarding the influence of mixed NaCl-$CaCl_2$ solutions on the growth and the extracellular starch degradation enzyme activity in the same experimental conditions revealed the fact that they present a maximum intensity of the two populations cultivated at optimum physic-chemical parameters, such as pH=3.0 and temperature of 28^0C.

Future studies will be focused on identifying a range of the metallic ions concentrations where *Acidiphilium* populations present their highest extracellular enzymatic activities, as well as on the implementation of these results in the reduction of pollution with carbon compounds produced from industrial activity.

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COLONIZATION AND DEGRADATION OF POLYETHYLENE COMPOSITES BY FUNGAL STRAINS ISOLATED

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Key words*: biodegradation, biofilm, composites, low density polyethylene, SEM*

Abstract

Polyethylene is one of the most inert plastic materials characterized by a high molecular weight, complex three dimensional structures and hydrophobic nature. These features interfere with its availability to microorganisms and cause serious environmental problems. One of the viable alternatives to accelerate the attack of microorganisms to LDPE is the addition of natural polymers; like starch, wood flour or cellulose fibers to guarantee at least a partial biodegradation. Wood flour is considered an excellent fillers for LDPE because of their low density, low cost, high strength, desirable fiber aspect ratio, flexibility during processing and biodegradability. The objective of this study is to investigate polyethylene based composites biodegradability. Several fungal strains were isolated after exposure period to soil burial tests. Strains from existing microbial collection were also tested. Polyethylene degradation ability of microorganisms was evaluated by weight loss and scanning electron microscopic (SEM) study of plastic strips after 3 months incubation in pure shake culture conditions. SEM analysis showed a thick network of fungal hyphae forming a biofilm on the surface of the plastic pieces. The colonization extent varied from strain to strain. There was observed a small difference but no significant in the weight of polyethylene composites before and after incubation with microorganisms. Strains tested are capable to form an adherent biofilm on the surface of LDPE composites. It is a slow process but these experiments give the evidences of biodegradation of LDPE composites.

INTRODUCTION

Polyethylene (PE) occupies an important position representing the majority of thermoplastics currently used as food packaging materials. It can be classified as high density and low density polyethylenes (HDPE and LDPE). LDPE is characterized by good toughness, resistance to chemicals, flexibility and clarity. The high consumption of these polymers leads to negative environmental impact through the accumulation and disposal of plastic wastes. Degradation of waste plastics through ability of microorganisms to use it as carbon and energy source becomes a viable alternatives to deal with such problems [1, 2]. Biodegradation of polyethylene materials could be enhanced by the following approaches, (a) to exploit the microorganisms in degrading polyethylene and (b) to develop artificial polymers susceptible to biodegradation. The suitable microbial strains for biological degradation of polymeric materials could be selected from existing microbial collection or obtained after isolation from waste contaminated soil. The mixture of the conventional plastic with the biodegradable polymer is believed to produce a type of plastic material with an improved biodegradability. Natural polymers, such as starch and lignocellulosic fibers from agroindustrial or agricultural residues are good fillers for thermoplastics [3]. The objective of this study is to investigate biodegradability of polyethylene based composites using several microbial strains either isolated from plastic samples buried in soil, either from existing microbial collection. The biodegradation experiments were performed using composites containing LPDE, wood flour and glycerol. The

extent of biodegradation was evaluated by comparing the initial and final dry weights of polyethylene before and after incubation with microorganism. Morphological aspects were revealed by scanning electron microscopic (SEM) analysis.

MATERIAL AND METHOD

Polymeric substrates

Blend films with 75% LPDE, 15% wood flour and 10% glycerol were obtained by baking a mixture of components, and mixing on a Brabender Plastograph, followed by calendering and extrusion as films or sheets. The films were cut into pieces 2 cm x 2 cm and sterilized at UV light for 10 minutes. Each film was then aseptically transferred and individually placed into sterile medium.

Microorganism and growth conditions

Aspergillus niger 105 and *Phanerochaete chrysosporium* belong to Microbial Collection of INCDCP-ICECHIM. The isolated strains were: *Fusarium sp.* (strain 2, 6 and 9), *Aspergillus sp (*strain 5*), Penicillium sp. (*strain 7*).* All strains were maintained at 4^{o}C in tub test with dextrose-agar-potato medium. The composition of ¼ diluted Sabouraud medium was as follows (g/l): 2.5 peptone; 40,0 D+glucose; . pH medium 6.0. The medium was autoclaved at 121^{o} C for 20 minute. After inoculation, the cultures were carried out in 300 ml Erlenmayer flasks containing 50 ml of the liquid medium, on a rotary shaker at 160 rpm and 28◦C for 3 months. Experiments were performed in duplicate. The microbial culture without polymeric samples was used as control. After incubation, the pieces of polymer were taken out from the culture and repeatedly rinsed with distilled water, dried at 35^{o} C and use for evaluation of biodegradation efficiency.

Morphological analysis (Scanning electron microscopy)

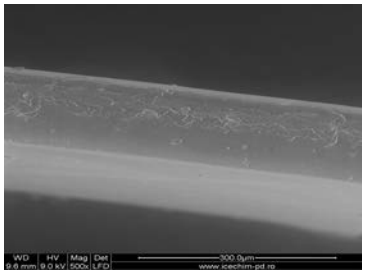
The observations of the film surfaces were performed with scanning electron microscope FEI-QUANTA 200. For SEM analysis, the film samples were dried and placed on metallic support, aluminum standard stub. The samples were performed at 10-15kV and 50-120 Pa using a Large-Field detector. Micrographs of the samples were taken at different magnifications to identify changes on the surface during the degradation process.

Determination of weight loss

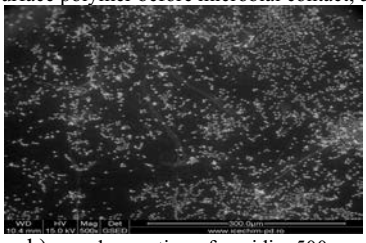
Recovered plastic samples were analyzed for degradation by weight loss before and after microbial treatment. The percentage weight loss of the inoculated plastic samples is given by the formula % Weight loss = (final weight – initial weight)/ initial weight x 100.

RESULTS AND DISCUSSIONS

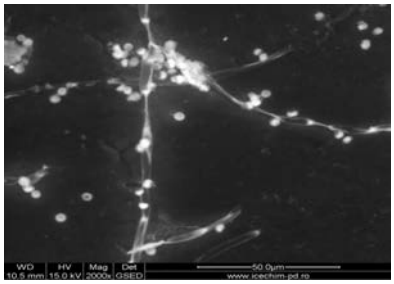
In previous paper, LDPE sample buried in soil for 3 months was used as source for isolation of fungal microorganisms [4]. The present tests were carried out with isolated strains: *Fusarium sp.* (strain 2, 6 and 9), *Aspergillus sp (*strain 5*), Penicillium sp. (*strain 7). Also, strains from microbial collection such as *Aspergillus niger* 105 [5] and *Phanerochaete chrysosporium* were tested. Morphological studies of the fungi and blend surface structure were carried out by scanning electron microscopy (SEM). The SEM micrographs of polymer composites are shown in Fig. 1-5.



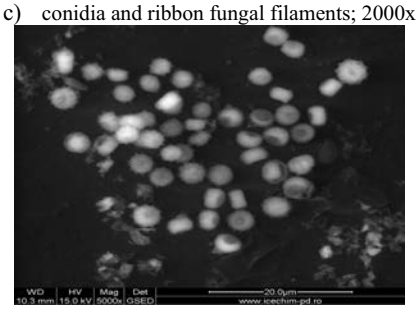
a) surface polymer before microbial contact; 500x



b) agglomeration of conidia; 500x



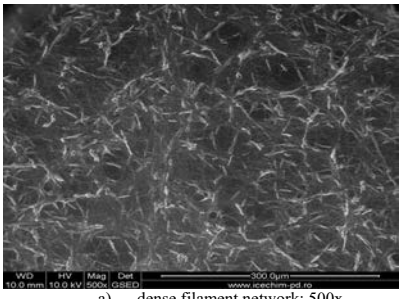
c) conidia and ribbon fungal filaments; 2000x



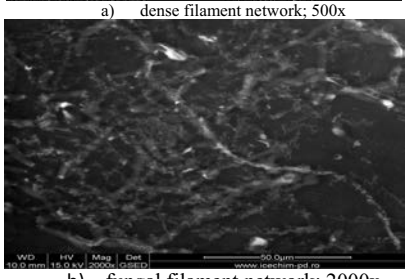
d) rare and disparate conidia; 5000x

Fig. 1. SEM micrographs of plastic film. *Aspergillus niger* 105 (b, c) and isolated *Aspergillus* sp. 5 (d) growing on polymer surface

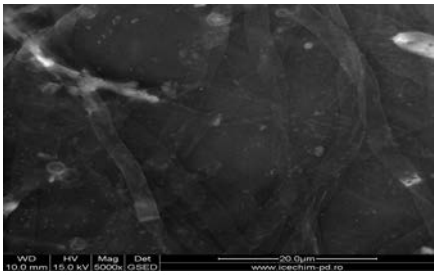
Strai*n Aspergillus niger* 105 was capable to grow and develop a dense filaments network on surface of film composite. The soil isolated strain *Aspergillus* sp. 5 presented a lower ability to produce a microbial biofilm.



a) dense filament network; 500x



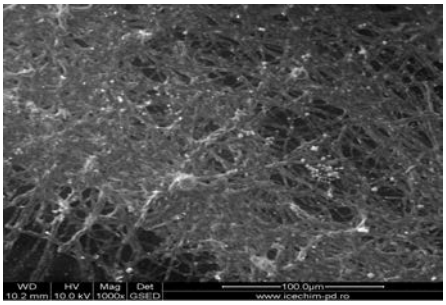
b) fungal filament network; 2000x



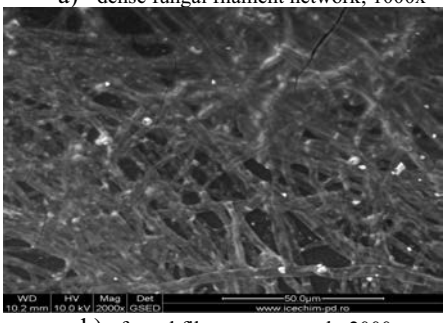
c) ribbon fungal filaments; 5000x

Fig. 2. SEM micrographs of plastic film. *Fusarium s*p. strain 2 (a) and strain 6 (b; c) growing on polymer surface

Fusarium strain 2 and 6 were isolated from the samples soil buried. The fungal networks developed on polymeric surface were less dense as comparative with *Aspergillu*s 105.



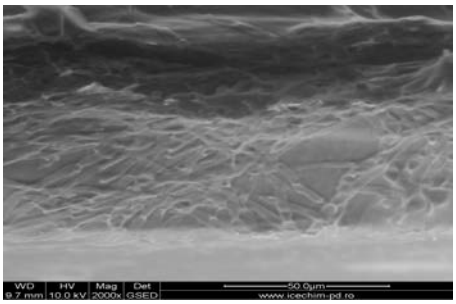
a) dense fungal filament network; 1000x



b) fungal filaments network; 2000x

Fig. 3. SEM micrographs of plastic film. *Penicillium s*p. strain 6 growing on polymer surface

More surface agglomerations of filaments could be seen in the case of *Penicilium* (Fig. 3). Examination of *Phanerochaete chrysosporium* grown on polymer surface is presented in Figure 4.



a) dense network of ribbon filaments

Fig. 4. SEM micrographs of plastic film. *Phanerochaete chrysosporium* growing on polymer surface

The incubation period was not enough to highlight the effect of microorganism towards a material polymeric containing polyethylene. However, the SEM micrographs indicate a microbial adhesion and biofilm formation on the polymer surface films. Biofilm formation depends on microbial origin, and there are microorganisms able to form a biofilm on polymer surface. This phenomenon is considered to be a preliminary step in polymer biodegradation, the biological attack of polymer begins with the colonization of microorganism on polymer film surface. After three months of incubation with microorganism, expected phenomena such as exfoliation, peeling and holes in the film structure were observed on a small scale. The weight loss is used as a quantitative measure for polymer degradation. Results of experiments are presented in Table 1.

Table 1. Weight loss of polymeric samples after incubation with microbial strains

Microorganism	Weight loss (%)*
Aspergillus niger 105	5.95
Phanerochaete chrysosporium	4.25
Fusarium sp strain 2	4.05
Fusarium sp strain 6	4.00
Fusarium sp strain 9	3.90
Aspergillus sp strain 5	3.85
Penicillium sp. strain 7	2.70

* - average of three determinations

As it can be seen, the values of weight loss are relative low and not so relevant for biodegradation process. The highest decrease of weight was obtained after incubation with *A. niger 105*. The activity of *Aspergillus sp.* in biodegradation of polyethylene has been confirmed by many reports [6, 7].

CONCLUSIONS

In this study, we have evaluated the degradation of plastic by measuring its weight loss and observing the film surface by SEM. The weight loss of polymeric samples after microbial incubation was not significant because of the high percentage of resistant polyethylene in composites. According to SEM micrographs it might be assumed that *Aspergillus niger* 105 is capable to produce a thin and dense filaments network on polymer surface. The weight loss results corroborated with those from SEM analysis suggest the beginning of a slow process of polymer biodegradation.

ACKNOWLEDGMENTS

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ENZYMATIC BIOPROCESSING OF VEGETABLE OILS FOR THE PRODUCTION OF BIODIESEL

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Abstract

Biodiesel is a renewable, alternative fuel for diesel engines, that has captured the attention of the whole world, as it can be used both alone and mixed with diesel for unmodified diesel engines. It is easily obtained from common raw materials, as well as wastes. Biodiesel obtained through biotechnological procedures (biocatalysis) is of superior quality to chemical synthesis biodiesel. The use of purified lipases, such as pig pancreas lipase, Thermomyces lanuginosus lipase or lipase B from Candida antarctica as a biocatalyst for biodiesel obtainment has shown great results and the optimum control parameters have been studied. The production of biodiesel from vegetable oils using different lipases has been investigated. Results have shown that the type of lipase, reaction media and operational parameters (reaction time, temperature, lipase load, alcohol:oil molar ratio and water concentration) have influenced biodiesel yield. In order to establish the best composition and process conditions, an optimization procedure has been carried out. The enzymatic transesterification was performed in an organic solvent-containing system, in agitated flasks, at various temperatures (40-50^{o}C) and for different periods of time (10-14 hours). Also, variations of the alcohol:oil molar ratios, enzyme concentrations and added water percent were studied. A statistic evaluation of the results was performed, for the proper optimization of the process parameters in regard to conversion. Under optimal operating conditions, the fatty acid methyl esters (biodiesel) yields were >90%.

Key words: *biocatalysis, biodiesel, lipase, transesterification*

INTRODUCTION

Fatty acid methyl esters (FAME), commonly known as biodiesel, have received great attention during recent years, due to concerning depletion of fossil fuels, oil price increase and biodiesel benefits towards the environment.

Biodiesel can be produced from various animal and plant fats, by transesterification with methanol [4, 10]. Biodiesel obtained through biotechnological procedures (biocatalysis) is of superior quality to chemical synthesis biodiesel [16] and presents many advantages over diesel fuel.

The most important are its renewability, biodegradability [18], the emissions of toxic compounds at lower levels [22], and its higher combustion efficiency [5].

Industrial scale production of biodiesel continues to be limited due to undesired by-products obtainment and their hard collection, glycerol recovery, inorganic salts and water, wastewater treatment, and the energy requirement [11]. In order to overcome these impediments, research activities regarding enzymatic catalysis have been carried out [3, 7, 20].

For the production of biofuels, one of the most reported enzyme groups is represented by lipases [12].

The use of purified lipases, such as pig pancreas lipase, Thermomyces lanuginosus lipase or lipase B from Candida antarctica as a biocatalyst for biodiesel obtainment has shown great results and the optimum control parameters have been studied [13, 23, 24].

The process of enzymatic transesterification presents certain advantages over chemical transesterification, along with its environmental benefits [6, 14].

Lipases can catalyze a variety of transesterification and esterification reactions relatively efficiently under mild conditions and in non-aqueous environments [2, 21, 9].

The type of lipase, reaction systems and operational parameters (lipase load, reaction time, temperature and alcohol:oil molar ratio) have a great influence on biodiesel yield [8]. Regarding alcohol to oil molar ratio, the stoichiometric equation requires 3 moles of alcohol and one of triglycerine for the obtainment of 3 fatty acid methyl ester moles and 1 mole of glycerol.

Higher molar ratios would lead to higher biodiesel yields. The use of solvents has proven to be necessary to maintain the miscibility between the methanol and triglicerides with the purpose of forming a monophasic system [17].

The water content is also an important parameter [1, 15], and seems to be the subject of dispute. The effect of water in the system depends on the enzyme, immobilization support and the medium (with or without solvent). Probably the main disadvantage in biocatalytic biodiesel obtainment is the cost of the enzyme.

Enzymes present different capacities to maintain their activity after recovery and repeated use, probably due to catalyst inactivation in the oil phase, the type of carrier used immobilization or enzyme sensitivity to long-term exposure [17, 19].

The main purpose of this paper was to better understand the relationship between reaction variables (time, temperature, enzyme concentration, substrate molar ratio and added water content) and process response (conversion in mass percentages) in order to optimize biodiesel biosynthesis.

MATERIAL AND METHOD

The substrates used during the enzymatic catalyzed experiments consisted of Olina palm oil, commercially available on the market, and methanol from the National Institute for Chemical-Pharmaceutical Research and Development, Bucharest.

As biocatalyst, pig pancreas lipase (PPL) from Sigma-Aldrich (22,7 U/mg) was employed.

6 mL of n-hexane (Merck Chemical Co. Darmstadt, Germany) were added to the reaction mixture, in order to permit a better solubilization of the mixture and to facilitate enzymatic biosynthesis.

For the optimization of the biodiesel obtainment process, an optimization methodology was employed to determine the interaction of different factors, optimizing one or more experimental responses. To this purpose, a Hadamard experimental matrix has been developed, with elements corresponding to 2 levels of the key factors, -1 and +1. The matrix was built by circular permutation starting from a basic generator, the factors of last experiences being always taken as level -1.

We therefore developed a matrix with 22 experiments and 5 key process parameters at 2 variation levels (chosen as minimum and maximum). Oil to biodiesel conversion was considered as response factor (Table 1).

The matrix was build based on the variation of the 5 essential parameters, for which maximum and minimum levels were chosen. The 5 parameters were: time (x_1), temperature (x_2), enzyme (x_3) (% from weight of oil), alcohol to oil molar ratio (x_4) and water (x_5) (% of oil weight) (Table 1).

The reaction mixture contained palm oil (2 g) to which 4 portions of methanol were added throughout the process at specific time intervals in order to avoid enzyme inactivation, 6mL *n*-hexane (Merck Chemical Co. Darmstadt, Germany), water (5% and 15% weight of oil) and enzyme, PPL – 45% and 55% weight of oil. The system was stirred (250 rpm) at 40 and 50^{o}C and for 10 and 14 hours. The molar ratios used were 3:1 and 5:1 methanol to oil.

For the obtainment of biodiesel at laboratory level, a Heidolph Unimax 1010 reactor with a stirring unit and Heidolph Inkubator 1000 was used. The samples were vortexed with a Vortex Heidolph Reax Top, for 10 seconds, at the beginning of the experiment and after each methanol aliquot was added.

The sample analysis was performed by injecting a 1mm^3 aliquot in split less mode into a Hewlett Packard 6890 gas chromatograph (Avondale, PA, USA) equipped with a flame-ionization detector (FID), and a CP-Select CB for FAME 50m x 0.25mm x 0.25µm Varian capillary column.

Table 1. Experimental matrix for the optimization of biodiesel obtainment technology at laboratory level

Experiment no.	Factors					Response: (Y_i)
	X_1	X_2	X_3	X_4	X_5	
1	-	-	-	-	-	Y_1
2	-	-	-	-	+	Y_2
3	+	-	-	-	-	Y_3
4	-	+	-	-	-	Y_4
5	-	-	+	-	-	Y_5
6	-	-	-	+	-	Y_6
7	-	-	-	+	+	Y_7
8	+	-	-	-	+	Y_8
9	+	+	-	-	-	Y_9
10	-	+	+	-	-	Y_{10}
11	-	-	+	+	-	Y_{11}
12	-	-	+	+	+	Y_{12}
13	+	-	-	+	+	Y_{13}
14	+	+	-	-	+	Y_{14}
15	+	+	+	-	-	Y_{15}
16	-	+	+	+	-	Y_{16}
17	-	+	+	+	+	Y_{17}
18	+	-	+	+	+	Y_{18}
19	+	+	-	+	+	Y_{19}
20	+	+	+	-	+	Y_{20}
21	+	+	+	+	-	Y_{21}
22	+	+	+	+	+	Y_{22}

RESULTS AND DISSCUSSIONS

The purpose of the experiments was the study of biodiesel obtainment and the optimization of the process.

The process has been designed using a matrix with 22 experiments to evaluate the effects of five key factors: temperature, time, enzyme concentration, alcohol:oil molar ratio and water concentration. These factors showed a significant influence on biodiesel production, each of them evaluated at two variation levels (Table 2).

As it can be observed, experiment no. 8 had the highest yield (98.5646% conversion) after 14 hours, at 40^{o}C, 45% enzyme concentration, 3:1 alcohol to oil molar ratio and 15% water. The lowest rate of conversion was registered for experiment no. 6 (10 hours reaction time, 40^{o}C, 45% enzyme concentration, 5:1 molar ratio and 5% water).

From the obtained results, a classification of the factors with a significant influence on the process response was made, according to linear coefficients (Table 3):

$$b_0 = \sum \frac{y_i}{N} \qquad b_i = \sum \frac{x_i y_i}{N}$$

Where:

b_0, b_i = linear coefficients

x_i = independent variables

y_i = process response (conversion %)

Thus, $b_i > 0$ represents a positive influence and $b_i < 0$, a negative influence, obtaining the

linear objective polynomial function of the form:

$Y=b_0 + b_1X_1 + b_2X_2+...+b_kX_k = 39.43796 + (-4.58145)\ X_1 + (-8.17159)\ X_2 + (0.515073)\ X_3 + 0.617654\ X_4 + 8.552937\ X_5$

It can thus be observed that enzyme concentration (x_3) (% from weight of oil), alcohol to oil molar ratio (x_4) and water content (x_5) (% weight of oil) had a positive influence on the bioprocess response, while time (x_1) and temperature (x_2), had a negative influence.

Table 2. Biodiesel conversion according to the Hadamard experimental matrix

Experiment no.	Factors					Conversion (%)
	Time (hours) X_1	Temperature (°C) X_2	Enzyme (%) X_3	Alcohol:oil molar ratio X_4	Water (%) X_5	
1	10	40	45	3:1	5	39.8601
2	10	40	45	3:1	15	67.4214
3	14	40	45	3:1	5	41.2884
4	10	50	45	3:1	5	13.7727
5	10	40	55	3:1	5	98.5646
6	10	40	45	5:1	5	12.18309
7	10	40	45	5:1	15	41.475
8	14	40	45	3:1	15	31.6088
9	14	50	45	3:1	5	14.9689
10	10	50	55	3:1	5	19.0209
11	10	40	55	5:1	5	87.8042
12	10	40	55	5:1	15	32.7987
13	14	40	45	5:1	15	18.7578
14	14	50	45	3:1	15	14.9156
15	14	50	55	3:1	5	19.768
16	10	50	55	5:1	5	29.4874
17	10	50	55	5:1	15	79.9573
18	14	40	55	5:1	15	51.943
19	14	50	45	5:1	15	28.2946
20	14	50	55	3:1	15	18.6713
21	14	50	55	5:1	5	78.685
22	14	50	55	5:1	15	26.3884

Table 3. Influence of significant factors

b0	**b1**	**b2**	**b3**	**b4**	**b5**
39.43796	-4.58145	-8.17159	0.515073	0.617654	8.552937

CONCLUSIONS

The purpose of this experiment was to achieve the biodiesel process optimization through the use of an experimental factorial plan represented by a Hadamard matrix. By circular permutation of 5 key process parameters, at two variation levels, the significance of their effect was evaluated according to biodiesel conversion yield.

The highest conversion yield was 98.57% after 10 hours, at 40^{o}C, 55% enzyme concentration, 3:1 alcohol to oil molar ratio and 5% water.

According to the determined linear coefficients, enzyme (x_3), alcohol to oil molar ratio (x_4) and water (x_5) had a positive influence on the bioprocess response, while time (x_1) and temperature (x_2) presented a negative influence.

In accordance to the optimization method, in order to obtain a better settlement of the optimal regions, a new experimental plan will be established in which the variable factors will be alcohol to oil molar ratio and water, the rest of the factors remaining unchanged.

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STUDIES ON BIODEGRADATION OF TANNED LEATHER

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Abstract

Wastes from the leather industry as well as insoluble and hard-to-degrade animal proteins are currently eliminated by incineration, method with high ecological disadvantages. In order to avoid these inconvenients, microbiological and/or enzymatic methods are examined. Leather degradation was performed in two trials: one enzymatic-assisted and the other microbial-assisted. Enzymatic hydrolysis of collagen, the representative protein of leather, was performed with collagenase type IA, with six enzyme concentrations, for up to 10 days. The hydrolysis yield was evaluated by free amino acids determination. Microbial hydrolysis involved 35 bacterial strains belonging to various genera (Streptomyces, Bacillus, Pseudomonas, Serratia etc), screened for their ability of growth in minimal medium containing bovine leather, ovine leather or wool as unique carbon or nitrogen sources. Three of these strains: Bacillus licheniformis ATCC 14580, Pseudomonas fluorescens ATCC 13525 and a new bacterial isolate, designated BN7, were able to grow in the presence of treated leather or wool, visible differences being observed after 10 days of incubation. The best results were obtained with the strain BN7, the level of free amino acids and of the extracellular proteins (as a measure of organic substrate degradation) was rather high (2.22 µmoles amino acids and 61 µg protein respectively). Amino acids release and extracellular protein synthesis indicates both collagenase and keratinase activity. The degradation of tanned leather was examined microscopically: significant disorganization of leather fibres was observed.

Key words: *bacterial strains, collagenase, keratinase, leather degradation, wool*

INTRODUCTION

Leather industries as well as meat industries are important generators of insoluble and hard-to-degrade animal proteins, which are converted in waste with high potential of environmental pollution. Usually, waste from these industries are eliminated by incineration, method with high ecological and sanitary disadvantages [Suzuki et al., 2006]. In order to avoid these inconvenients, microbiological and/or enzymatic methods are examined. It is well known that proteolytic enzymes constitute an important group of commercial enzymes; nevertheless their use in experiments for bioprocessing industrial waste containing animal proteins is well documented. Several experiments were carried out on enzymatic hydrolysis of collagen, the most representative protein of leather waste: the hydrolysis was carried out by collagenases, enzymes that can hydrolyze both native and denaturated collagens [Olde Damnic et al., 1995, Zerdani et al., 2004]. Other experiments are focused on keratinolytic enzymes useful for degradation of fibrous insoluble proteins in the form of feathers, hair, nails, horn etc, available as agroindustrial by-products [Brandelli, 2008]. In the last ten years, a lot of articles regarding microoorganisms able to degrade various types of animal proteins have been published [Brandelli, 2008; Suzuki et al., 2006; Kansoh et al., 2009; Riffel et al., 2003; Gousterova et al., 2005]. It was shown that strains of *Streptomyces* isolated from keratinaceous materials enriched soil were able to degrade human hair, feathers, silk and wool [(Riffel et al., 2003; Kansoh et al., 2009]. Moreover, among *Bacillus* and *Pseudomonas* genera various strains with highly proteolytic activities were identified, potentially useful for biodegradation of insoluble animal proteins [Cai et al., 2008; Tork et al., 2010; Agrahari and Wadhwa, 2010]. Most of the microorganisms isolated from soil presented reduced collagenase activity [Zerdani et al.,

2004] but significant keratinolytic action was detected among some bacterial isolates [Riffel and Brandelli, 2006; Agrahari and Wadhwa, 2010].

This study presents the action of collagenase type IA against sheep and bovine leather, as well as the capability of some mesophilic bacterial strains to synthesize hydrolytic enzymes with collagenolytic and/or keratinolyic activities.

MATERIAL AND METHODS

Culture medium for collagenolytic/ keratinolytic enzymes production contained the following constituents (g/L): NaCl 1.0; $CaCl_2$ 0.05; KH_2PO_4 0.7; sucrose 3; $MgSO_4$ 0.91; K_2HPO_4 2.38, and sheep or bovine leather, and wool 6.0; pH 7.2 [Cai and Zheng, 2009].

Microorganism. 27 strains of *Streptomyces* isolated from compost and seven bacterial strains (*Bacillus subtilis* ATCC6633, *Bacillus licheniformis* ATCC 14580, *Bacillus amyloliquefaciens* BW, *Bacillus spp.* OS15, *Bacillus spp.* OS17, *Pseudomonas fluorescens* ATCC 13525, *Serratia spp.*S1) from the collection of Faculty of Biotechnology, Bucharest, Romania, and a new isolate from soil (designated BN7) were used in experiments.

Free amino acids released by the extent of collagen breakdown were determined using a modification of the Moore and Stein (1948) colorimetric ninhydrin method, by transferring 0.2 ml of sample to test tubes containing 2.0 ml of ninhydrin-ethylene glycol monoethyl ether mixture. After boiling for 30 minutes in a water bath and after cooling, the samples were diluted with 10 ml of 50% n-propanol. The absorbance was determined at 600 nm 15 minutes later. An L-leucine standard curve was used to determine micromoles amino acid equivalent to leucine liberated.

Total soluble protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

Enzyme-assisted leather degradation test was performed using a 2 mg/ml stock solution of bacterial collagenase type IA (Sigma-Aldrich Chemicals Company). The experiments were carried out with 2 cm^2 sheep leather samples in 50 mM Tris-HCl buffer pH 7.4 and six IA collagenase concentration levels ranging from 0.05 to 0.6 mg/ml, at 37^oC, with shaking (150 rpm), for 10 days. Released amino acids determination was carried out at 48, 72, 96 and 240 hours of reaction.

Microbial-assisted leather degradation test was performed by cultivation in submerged conditions, at 28^oC, with shaking (120 rpm) for 10 days. Preliminary screening of microorganisms for proteolytic activity was performed on milk agar plates [Riffel and Brandelli, 2006]. Biochemical determinations consisted in total soluble protein and released amino acids assays carried out at the end of cultivation period.

RESULTS AND DISCUSSIONS

In vitro degradation trial using collagenase (type IA) solution proved the hydrolitic potential when breaking down the collagen from the tanned leather. Because the degradation behaviour is depending on enzyme concentration, six collagenase concentration levels (0.05, 0.1, 0.175, 0.25, 0.3 and 0.6 mg/ml) were tested in order to find out the minimum necessary amount for relevant diggestion of the tanned leather. For a longer reaction time (ten days) significant reduction in the degradation of collagen was observed when using 0.1 mg/ml, but for shorter evaluation period (four days) an increased enzyme concentration was nedeed (at least 0.25 mg/ml) (Fig. 1).

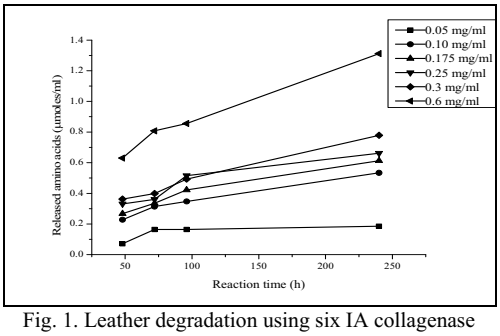

Fig. 1. Leather degradation using six IA collagenase concentrations

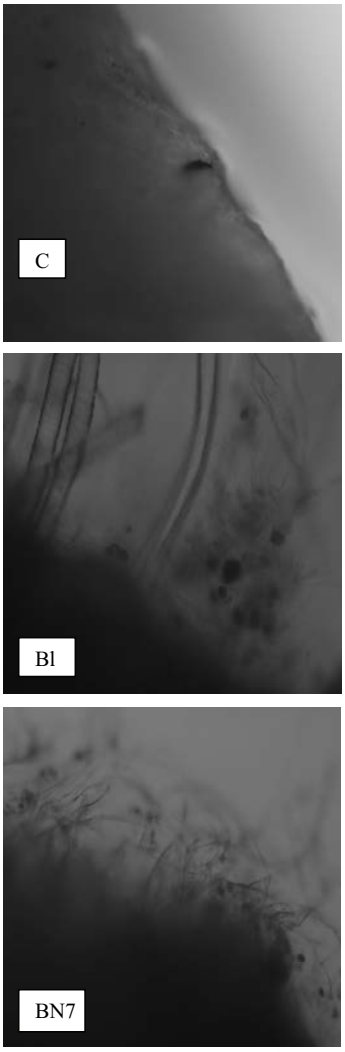

Photo 1. Microscopic examination of sheep pelts: C = control (untreated pelt); Bl = incubation with *.B.licheniformis ATCC14580* for 10 days; BN7 = incubation with the bacterial isolate BN7 for 10 days.

The screening test showed that from the seven bacterial tested strains only the two collection bacteria (*Bacillus licheniformis* ATCC 14580 and *Pseudomonas fluorescens* ATCC 13525) and the new isolate BN7 were able to grow in minimal medium containing leather as sole carbon and nitrogen source. Among streptomycetes, only five strains presented a relatively poor growth in the same medium, but their collagenolytic activity was much reduced. On milk agar plates, the strain designated BN7 produced clear zone of hydrolysis.

The microscopic examination of sheep or bovine leather fragments incubated with selected bacteria showed significant differences from control. In the control, the edges of the leather fragments were intact, and the aspect was compact (photo 1). After 10 days of incubation with the selected bacteria, the margins structure of sheep leather was modified: it has become more disorganized and both globular and fibrous fragments were released in medium.

The highest degradation, both of sheep leather and of wool samples was observed when the new isolated strain BN7 was used, suggesting the biosynthesis of collagenolytic as well as of keratinolytic enzymes (photo 2).

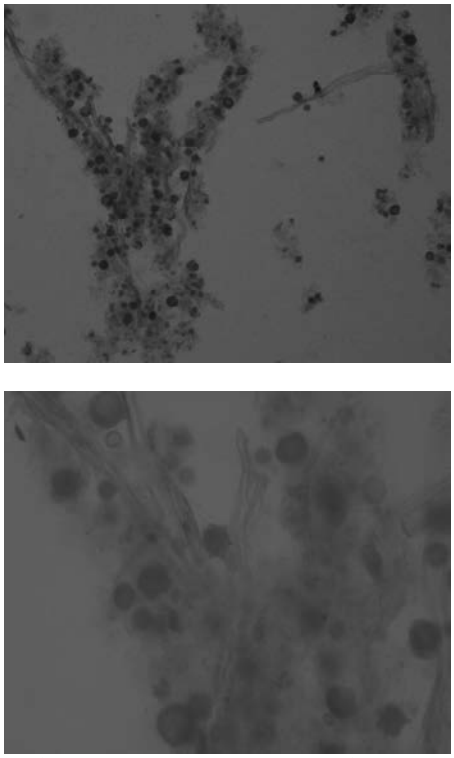

Photo 2. Microscopic aspect (10x and 40x) of degradation fragments resulted after incubation of sheep leather fragments with the bacterial isolate BN7 for 10 days

Similar aspect were observed on bovine pelts treated with the same bacterial strains: the disoganization of the margins and the release

of small fragments are the main actions of the degradative bacteria (photo 3).

Photo 3. Microscopic aspects of bovine pelts incubated for 10 days with the bacterial isolate BN7 (middle and down) comparative with the untreated sample (up)

Biochemical analysis confirmed the microscopic aspects showing high amount of released animo acids after 10 days of cultivation for all the three tested strains, with higher values for bacterial isolate BN7 (Fig. 2). Degradation of sheep leather was, at least, more than twice accelerated than the degradation of bovine leather.

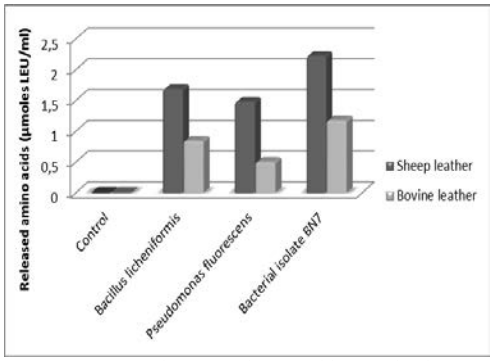

Fig. 2. Degradation of sheep and bovine leather expressed as released amino acids after 10 days of cultivation

Total soluble protein concentration was almost similar for all the tested strains, when acting both on sheep and bovine leather, meaning that enzyme synthesys was quite close (Fig. 3). Though the results obtained for the amino acids released by the action of proteolytic enzymes were totally different. An explanation might be the inhibition produced by the presence of several compounds used for leather processing and acting different, according to the enzyme microbial source.

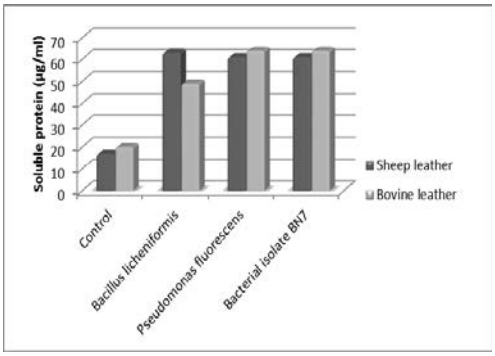

Fig. 3. Degradation of sheep and bovine leather expressed as total soluble protein concentration after 10 days of cultivation

These results suggest that the selected bacteria, and especially *Bacillus licheniformis ATCC14580* and the new isolated strain BN7 are able to produce increased levels of hydrolytic enzymes, active both on leather and wool.

CONCLUSIONS

The tested strains *Bacillus licheniformis* ATCC 14580, *Pseudomonas fluorescens* ATCC 13525 and the new isolate BN7 can be used for improvement of waste leather biodegradation process. Collagenase activity may be considered as a measure of this degradation process.

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STUDY OF HIGH EFFICIENCY SYSTEMS TO RACTOPMINE DETECTION IN ENVIRONMENTAL SAMPLES

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Abstract

The concern about the effects of growth promoter drugs exposure, such as ractopamine, has mobilized the scientific community in the search for methods to detect this compound in different matrices. The ractopamine is a β-adrenergic agonist largely used in intensive farm. Studies demonstrates that 95% of the quantity of this ingested drug are excreted in the first 3 days in swine and 55% in cattle are excreted in feces, while 10% and 45% respectively are excreted in the urine, being detected until 2 weeks after the treatment. The present study aimed to propose the development of ractopamine extraction, cleaning and detection methodology to environmental samples (water and wastewater) by a biographical evaluation about it determination in different matrices. For the cleaning and extraction/purification different techniques must be tested, for example the immunoaffinity collumn (IAC) to waters and an association of extraction liquid-liquid (LLE) followed by IAC to wastewaters, because they are complex samples. For the detection, a methodology that uses high performance liquid chromatography with detection by fluorescence (HPLC/FLD) was proposed, because it is less expensive when compared to other high efficiency systems, as liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS). However, the challenge consists in establishing of a method that achieves the validation rules and it is economically feasible to application not just for research, but in industry and government control departments, mainly in developing countries.

Key words**:** *ractopamine, chromatographic method, environmental samples*

INTRODUCTION

According to data from Abipecs - Brazilian Association of Producers and Exporters of Swine, the world production in 2011 was 101.127 thousand tons in carcass weight equivalent. Brazil is the 4th largest producer (3.227 thousand tons), behind United States, European Union and China, and is also the 4th largest world's exporter [1].

The beneficial effects on the carcass growth and composition, with the use of the β-adrenergic agonists, have been largely proven, resulting in the expansion of the muscle mass, jointly with the reduction of the fatty accumulation.

Due to the new intensive farming ways, the use of veterinarian drugs in animal containment systems has been presented an exponential growth in recent years. The use of growth promoter drugs, as the ractopamine (RAC) raises a range of concerns related to safety and toxicity [3], [14], [29].

These substances are not allowed in many countries, in example of Europe Community members and China, which forbid the use of these substances to zootechnical applications. The concern with the possible effects of prolonged exposure to RAC has been mobilized the scientific community in the search of methodologies development to the search of this compound in foods of animal origin.

The use of highly sensitive methodologies has been indispensable to reach the efficient control of these substances, but there is a lack of analytic methods to detect these compounds [20]. Among the utilized methods to RAC detection, evidence the high performance chromatographic methodologies, usually from the following matrices: animal carcass (meat and viscera), blood and urine [33], [34], [39], [40], [41], [42], [46],[50].

However, methodologies that propose the extraction, cleaning and RAC detection in complex samples, whereby is the case of the environmental samples (water and wastewater) are still scarce. Furthermore, the small concentration of the compounds present in the matrices can conduct alterations in the involved stages of the process [34].

Wherefore, the greatest challenge involves the search of high performance methodologies that presents financial and technical viability, not just for the research, but also to comply with the needs of the industries and governmental organs of control.

The present study revises the utilization of the RAC β_2-adrenergic agonist as animal growth promoter, the regulation of its use as an additive in animal feed, the presence of this compound in environmental samples as well. Ultimately, an evaluation of the methodologies for extraction, cleaning and detection in different matrices was searched, particularly to chromatographic systems, aiming the proposition of a methodology to be applied mainly for emergent countries to the environmental samples.

Ractopamine

The β-adrenergic agonists or β-agonists are substances of analogous structure to hormones called catecholamine (adrenalin and noradrenalin). These compounds are phenyl β-ethanolamine with different substituent in the aromatic ring and in the terminal amine group [4]. The use was previously authorized for therapeutic purposes (humans and veterinarians), mainly as anti-asthmatics, bronchodilators and tocolytics, generalizing as nutrient repartitioning agents from 1998 [34].

They are usually applied in livestock as additives, mostly due to anabolic effects exercised when high doses are administered. They act as animal metabolism modifiers, changing the nutrients partition diverting and promoting the growth and the lean tissue accretion and reduction the fat tenor in termination swine carcass [4], [7]. Among these compounds, the RAC has been studied with more interest for the swine farming.

Among these compounds, the RAC has been studied with more interest for the swine farming. It is a compound that belongs to the phenylethanolamines group, characterized by the presence of aromatic ring, side chain of the ethanolamine and aliphatic nitrogen [4], [13], [14], [43], with solubility in polar solvents. The RAC, (4-hydroxy-α-[[[3-(4-hydroxyphenyl)-1-methylpropyl] amino] methyl] benzenemethanol ($C_{18}H_{23}NO_3$-HCl), that contains two chiral centers, exists as a mixture of four stereoisomers – RR, RS, SR and SS. The chemical structure is represented in the Fig. 1 (A) [43].

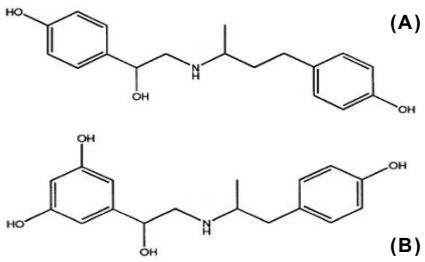

Fig. 1. Chemical structure of Racopamine (A) and Fenoterol (B). Adapted of [43]

The RAC production occurs in an only stage process, where the drug volume is produced as an aqueous solution containing between 3 and 20% of ractopamine hydrochloride. Its solubility in water varies with the pH, so the more the pH is, the more the solubility will be. It is a nonvolatile solid, with low endothermal at 180ºC and exothermal at 188ºC, coinciding with the decomposition [30].

Metabolism

The β-adrenergic agonists are largely utilized by inhalatory administration for human medicine, however, in veterinarian medicine, especially as animal growth promoter, it occurs mainly by oral administration. The low stomach pH favors the formation of a cation in the aliphatic amine, whiles the neutral nature of the duodenum, jejunum and ileum, promotes the reduction of the extension of the ionization and increases the passive absorption through the intestinal mucous [43].

The effects assigned to the RAC look like being related mostly the increase of the lipolytic activity and the inhibition of the lipogenesis, since this compound inhibits the binding of the insulin in the adrenergic receptor of the

adipocytes, and then, antagonizes the insulin action, reducing the synthesis and fat deposition [17].

The elimination of these compounds or their metabolites in the organism depends basically on the administration way. When the administration is realized by parenteral via, the renal excretion will occur, while, by oral via the previous metabolism is present in most cases [35]. Studies demonstrate that 95% of the amount of RAC that is ingested is excreted in the first 3 days: about 90% in swine and 55% in cattle are excreted in feces and between 10% and 45% in urine, respectively [13]. During the treatment, the RAC amounts in the urine vary from 44 to 473 $mg.mL^{-1}$, being detected until two weeks after the terminus of the treatment [45].

Regulation law

The regulation of drugs residues in food derived from animals is an integral component of food security programs worldwide [23]. Nowadays, different β-agonists substances are authorized for growth promoter utilization.

The RAC is allowed for the use as feed additive in 26 countries, such as the United States (where was developed by Eli Lilly®), Canada and Brazil, with the goal to promote de growth of the swine and cattle. Therefore, its use is forbidden in China and other 159 countries, including the ones members of European Union, due to the worries with its safety and toxicity [13], [14]. Its usage as additive in feed is controlled by Food and Agriculture Organization (FAO) by the *Códex Allimentarius* Commission. For the administration in the animals for the food production, the acceptable daily ingestion dosage is 0.1 $\mu g.kg^{-1}$ compared to body weight [51].

There are several documents that describe the side effects of β-adrenergic agonist in human's health, such as food poisoning related to liver residues, cardiovascular and central nervous disease [20], [25], [51].

The prohibition of the use of β-agonist as growth promoters in the European Union is regulated by the Directive 96/22/EC [8] altered by the Directive 2008/97/EC [10]. The control and monitoring must refer to the Directive 96/23/EC [9], in which the β- agonist belongs to the substances of Group A (the forbidden ones and that should be monitored). The performance of the analytical methods of monitoring as well as the interpretation of the results needs to attend the Commission Decision nº 2002/657/EC [11].

Spit of allowed use of ractopamine in Brazil, the Brazilian government with the productive sector has developed the "National Plan for Control of Residues and Contaminants" which includes the Program for Swine Production Free of RAC (Split System) in order to ensure a safe product and free RAC [6]. In the Santa Catarina State, the procedures for official examination of the self control applied in the productive chain for swine ractopamine free is regulated by the Statement Service – Nº 016/2011/GEDSA [36].

Environmental samples

The main sources of pharmaceuticals in aquatic environments are related to chemicals and pharmaceuticals residues from industrial production plants, besides the excretion (in the metabolized and non-metabolized shapes) of human beings and animals [54]. β-adrenergic agonist have been related for causing adverse effects in aquatic systems, as well as hypoactivity in *Daphnias* when exposed to high amount of RAC [12].

The presence of RAC and other β-agonists was investigated in animal feed and its drinking water, both matrices used as vehicle of drugs administration by farmers and veterinarian [20]. In this study, three samples resulted to be positive, two for RAC and one for hydroxymethylclenbuterol, in animal drinking water samples analyzed. Its presence and other veterinarian drugs was also investigated in wastewater originate from farm (swine and cattle) and subjacent underground waters indicating that this ones are inclined to contamination by these drugs in the ponds of residue water treatment [2]. The occurrence and distribution of pharmaceutical products, including RAC, in hospital wastewater and superficial waters before de flush point were investigated by [54]. In this case, although the composed studies have been detected in lower concentrations, in the superficial waters there is

potential risk to the health of human beings and animals.

Shao et al. [37] investigated the occurrence of 76 pharmaceutical drugs, including many β-agonists in slaughterhouse wastewater (influent and effluent) and a receiving river. Clenbuterol was founded at high amounts (~11 $ng.L^{-1}$) in the slaughterhouse wastewater plant located in the Tongzhou District, indicating the clenbuterol use from pig farms in that region. The high concentration of metoprolol (β-blocker) found in the outfall of swine farming suggests that this drug is illicitly used as growth promoter at this farm. The residual concentrations of drugs in the effluent from farms can be used as indicator of veterinary drug use at such venues.

High performance analytical methods for rac analysis

The analytical methods used to monitor veterinarian drugs are essential in the animal and human health protection, control of consumer exposure to drugs, reduction of chemicals impact in environment and support to the execution of laws and regulations that facilitate the international trade of food products of animal origin [23].

The immunochemical screening methods, such as radio and enzyme immunoassays [38], [41], [49] are very sensitive but the cross-reactivity properties of these tests with structural analogs of the controlled residues preclude a non ambiguous identification of the compounds in the complex matrix of a biological sample [48].

The demand of the best possible method has been a constant concern of many research groups. Thereby, a strategy perfectly defined can be observed: in one hand, there is the development of methodologies capable to determine the biggest possible number of multiresidues substances and, particularly in this case, the β-adrenergics [26]; in other hand, there is the utilization of quick detection methodologies, followed by compounds identification and posterior confirmation, always using spectrometric methods [19].

The composition complexity of the matrices and the small quantity of xenobiotics that are normally present ($ng.kg^{-1}$), sometimes modifies the process that conduces the evaluation of residues in general, and the β-adrenergic, specially, in an expensive process. In the area of substances which must have controlled use, like the β-adrenergic agonists, there is a continuous trend to tests development to detect illegal substances residues in trace levels. These methods involve more intensive samples preparation to make the detection of low concentrations residues possible. Sampling stages, pretreatment and, above all, extraction and/or purification have been showing to be of big importance to residues analysis of β-agonists, independent of the chromatographic method to be utilized [34].

Sampling

The sample integrity must be kept, preserving its physical characteristics and chemical composition [52]. The sample has to be representative and big enough to allow an appropriate analysis, the repetition and respective tests confirmation [34]. Due to the complex nature of biological matrices, the stages of sample preparation are the most important part that integrates the bioanalytical methods [27].

Samples for drug screening belonging to Group A [9] have to include matrices like plasma/serum, urine, feces, water, animal food, bile and thyroid gland [21]. When it is investigating the occurrence of β-agonists in the environment, the main points to be considered must include the effluents from farming of animals (swine and cattle), slaughterhouses and hospitals, besides the discharge points of these effluents (rivers) and groundwaters [2], [37], [54].

One of the main problems in biological samples is related to the instability of compounds and metabolites present in this kind of samples. The stability can be affected by the storage temperature, sample pH, anticoagulants and cycles of freezing/defrosting [27]. The time between the collect and analysis is a critical factor to be considered, once the substance instability or the matrix constituents can take it to significant mistakes in the analysis result [11]. The stability to effluent samples, was studied by [24] which were initially filtered with a 1.2 μm filter and divided in six subsamples of 2 L each. Four samples were

spiked with a mixture of β-agonists (including the fenoterol, that shows a structure close to the RAC (figure 1-B) and β-blockers (250 $ng.L^{-1}$ of each compound), storage at 4 ^{o}C in amber glass bottles, which were extracted and analyzed at 24h, 2, 4, and 7 days after the spiking, respectively; the authors observed that there were no alteration in the analytes.

Extraction/Cleanup and Purification

The preparation step of the sample is essential to get trustable results and, maintenance of device performance. Moreover, this procedure is slow and expensive, existing the need of a very efficient cleaning method when a high transference tax is required [40].

The liquid samples (blood, plasma, serum, bile, milk, water) are processed more easily once the present residues are found distributed with more homogeneity [21]. Centrifugation or filtration to eliminate the suspended substances, and eventual dilution are the main stages of pre-treatment to liquid samples. The homogenization is a very important step, mainly about the results accuracy. [34].

Different procedures are necessary to prepare the β2-adrenergic agonists samples to posterior chromatographic determination, the methodologies most commonly employed to extraction and cleaning and/or purification are: liquid-liquid extraction (LLE) and solid phase extraction (SPE) and a variant of this last, immunoaffinity column (IAC).

Liquid-Liquid Extraction

LLE was one of the first techniques used for preparing of biological samples [27], and one of the more classical extraction/purification. For the class of β2-adrenergic agonists are several studies with this process. For RAC, LLE has been used by several authors in different biological matrices, such as animal feed [14], [20], [18], tissue [33], [39], [40], [42] and urine [33], [41].

Solid Phase Extraction

SPE is one of the tools most often used for the extraction and/or pre-concentration of analytes present in complex matrices [32]. This technique has been preferred because it is a rapid method requires low volume of organic solvents, a low risk of contamination and can be used in online system system [52]. The retention and elution are thus faster, and the latter is even possible with a smaller amount of solvent than in the classic SPE. To the analysis of β2-agonists, SPE can be the only method in extraction/purification step or in association [34], such as ELL [33], [42] or IAC [22].

To extract and cleanup veterinary drugs, including β_2-agonists and β-blockers from environmental samples (water and wastewater), has been commonly employed technique of SPE individually [20], [37], [54] by online system [2] and in association LLE [24].

Immunoaffinity Collumn

IAC provides a selective method to isolate and concentrate analytes from complexes matrices. It is particularly advantageous when low detections levels in the $\mu g.kg^{-1}$ to $ng.kg^{-1}$ are required for banned substances, particularly when using less selective HPLC based detection systems [21]. This technique has a wide range of applications for a variety of chemical classes, including β-agonists in different matrices, such as: ractopamine in urine, liver, muscle and kidney of food animals [44]; clenbuterol from bovine pelage [15], urine [16], [31] liver and muscle [22]; salbutamol from human plasma [28].

Detection

The use of GC-MS confirmatory analyzes is recommended for β-agonists. But the methodology for β-agonists requires sample derivation, because of their high polarity and low volatility, which is a time-consuming, tediousness, laborious and expensive process. Also, quantitative results are significantly affected by sample purity when GC–MS is used for this analysis [20]. Therefore, GC methods are not the most suitable for the β-agonists screening in environmental samples [24].

Several studies report the use of LC/MS or LC-MS/MS for the analysis of β-agonists in environmental samples [2], [20], [37], [54]. Spite of the high sensitivity in the confirmation of residues in complex samples, these methods are much expensive yet.

Despite HPLC use to be very widespread, but any research study of RAC by HPLC was

found during this review. It is able to separate highly complex mixtures of compounds with different molecular weights, as well as several matrices with different polarities and acid-base properties. The most used detectors to the RAC determination are shown in Table 1.

Table 1. HPLC methods for RAC analysis

Detection	Column	Mobile phase	Matrice	Clean up	Author
HPLC/EL	C_{18}	Ammonium phosphate buffer 0,05M pH 4,5: acetonitrile (79:21,5)	Serum, plasma	SPE	[46]
HPLC/FL	C_{18}	Acetonitrile: water: acetic acid (280:720:20, v/v/v/) and 1.08 g 1-octanesulfonic acid/L	Urine, liver, muscle, kidney	SPE, IAC	[40]
HPLC/FL	C_{18}	Acetonitrile: water: acetic acid (280:720:20, v/v/v/) and 1.08 g 1-octanesulfonic acid/L	Tissue and urine	SPE	[39]
HPLC/FL	C_{18}	0.005 M sodium octanesulfonate in a mixture of water: acetonitrile: acetic acid (71:27:2).	Urine	SPE	[44]
HPLC/FL	C_{18}	5 mM sodium octanesulfonate in 2% acetic acid in water/acetonitrile (72:28, v/v)	Urine	SPE	[41]
HPLC/FL and LC–MS/MS (to confirmate)	C_{18} C_{16}	Acetonitrile: deionized water: glacial acetic acid (320:680:20, *v/v/v)*and 0.87 g of 1-pentanesulfonic acid, 0mM ammonium acetate buffer, pH 4.5: acetonitrile, gradiente flow	Muscle tissue	LLE, SPE	[42]
HPLC - UV/DAD	C_{18}	0.017 M phosphoric acid brought to pH 2.8 with diethylamine: acetonitrile-water (80:20, v/v), gradient flow	Commercial feed	SPE	[6]
HPLC/FL	C_{18}	Acetonitrile: deionized water: glacial acetic acid (320:680:20, *v/v/v)*and 0.87 g of 1-pentanesulfonic acid	Liver and muscle tissues	LLE SPE	[47]
HPLC/FL	C_{18}	Acetonitrile: deionized water: glacial acetic acid (320:680:20, *v/v/v)*and 0.87 g of 1-pentanesulfonic acid	Animal feed	LLE	[49]
HPLC/FL	C_{18}	Water: acetonitrile (80:20, v/v) with 2 mL of acetic acid and 0,7 g of pentanosulfônic acid/L	Tissue, urine, serum	LLE SPE	[33]
HPLC/FL	-	Water solution (containing 2% acetic acid and 0.087% pentanesulfonste sodium salt): acetonitrile (80:20, v/v)	Tissue	SPE MIPs	[50]
HPLC/UV	C_{8}	Acetonitrile: sodium acetate buffer 0.1 $M.L^{-1}$ pH 5.0 (25:75, v/v)	Ractopamine raw material and Ractosuin® product	SPE	[14]
HPLC/UV	C_{18}	Acetonitrile: monobasic sodium phosphate buffer 0.1M L^{-1} pH 7,6 (26:76, v/v)	Vitamin Mineral Complex and Ractosuin® product	LLE	[14]
HPLC/UV	C_{18}	Acetonitrile: ammonium formiate buffer 0,02 M pH 3,0 (20:80, v/v)	Animal feed	LLE	[14]

CONCLUSIONS

Through this work a general methodology that involves the steps of extraction, cleanup/ purification and detection of RAC for environmental samples is proposed. The care of the pretreatment of the samples are indispensable, the extraction right after the collect and storage becomes impractical and the validity of the analytical results depends totally on the integrity of the interest compounds. After homogenization, the samples must be prepared with the addition of a solution of extraction (LLE). For the extraction of RAC it is believed that there is no necessity of the enzymatic hydrolysis, then the acid hydrolysis should be tested, since RAC has a pKa = 9.4 and compounds similar studies were successful, making the method less expensive in time and economic view point. In order to the steps cleanup and purification filtration is suggested (at least for the samples of wastewater *in nature* and post treatment) followed by application to the immunoaffinity column with bound specific antibodies. The test with commercial RAC-IAC is here suggested, since they provide specificity and are for single use (a procedure that avoids cross-contamination) as well as providing a chromatogram with less interference which shows greater selectivity. The eluent of choice for marketed IAC's is the methane, however, one must carry out a comparative test with buffer solution of glycine, in the study of [40] provided cleaner chromatograms when compared to methanol by coelution of fluorescent compounds.

The HPLC detection is proposed to be the high efficiency equipment easier accessibility. The fluorescence detector was nominated for its performance and for being the most widespread in the literature for RAC. No entanto, cabe aqui ressaltar nenhum estudo com o uso de HPLC para detecção de RAC em amostras ambientais foi encontrado durante a presente revisão.

This review showed itself essential for allowing the proposal of developing a methodology for

fast and high sensitivity for complex matrices such as environmental samples. However, the challenge consists in establishing of a method that achieves the validation rules and it is economically feasible to application not just for applied research, but in industry and government control departments, mainly in developing countries.

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RESEARCH REGARDING THE SENSORY CHARACTERISTICS OF SOME VEGETABLES

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Abstract

Fruit and vegetables quality is an extremely complex problem, difficult to describe objective. Although the consumer is unable to assess the nutritional quality of a fruit or vegetable, however he is able to make a statement on the sensory aspects such as shape, color, texture, juiciness, firmness, taste and aroma. Consumers often complain about the quality of fruit and vegetables, which are offered through commercial distribution systems. The main complaint relates to taste bad and sometimes its absence. However, when it comes to defining exactly what "taste", the answers obtained are not clear and, in general, are quite divergent. Researches try to define some connections between quantitative and analytical determinations type hedonic sensory analysis. They were directed to analyze the quality of vegetables in two distinct directions, namely the analysis of physicochemical and biochemical characteristics that contribute to the scientific definition of taste: pH, acidity, total carbohydrate content, firmness and sensory analysis on issues such as shape, color, texture, juiciness, firmness, flavor and aroma.

***Key* words:** sensorial determination, cucumber, tomato, pepper

INTRODUCTION

Sensory analysis measures how foods interact with the human senses (taste, smell, sight, touch) and how they are perceived properties of foods.

Basics of sensory analysis were established in the United States during and after the Second World War, when the government wanted to improve the quality of food provided for the army. It is recognized that, even if food is highly nutritious, they can not be consumed due to poor perception of sensory properties [14]. Sensory methods have been developed, mainly for economic reasons, because they allow to establish acceptance values for any food.

Sensory analysis is a multidisciplinary subject. In recent years, contributions from different scientific fields such as psychology, physics, chemistry, neuroscience and statistics allowed to increase considerably the potential of this analytical tool.

Four basic methods of sensory evaluation are generally used:

- Methods of analysis are discriminatory and determining differences between samples.
- Descriptive tests can determine the nature and intensity attributes in a given sample.
- Hedonic tests that allow obtaining information on the preference and acceptance of products.
- Tests for determining threshold sensitivity of a given stimulus or compound [11, 13].

Sensory differences are made in pairs for comparison, where tasters are asked to taste two or more samples and then to mark on a paper test some attributes such as color, taste (aroma, acidity, sweetness, bitter), odor and an overall assessment of the food. In all tests, tasters are allowed to comment, so that they could explain, sometimes, their choice [8, 12].

Hedonic tests play an important role in assessing consumer acceptance of food. Overall, more than 20 people involved in hedonic tests. They communicate their feelings such as like or dislike the statements, evaluation sheets [3]. Obtaining data are usually qualitative.

Hedonic testing is popular because it can be done by inexperienced people and experts [4]. Samples are generally one by one to each

topic and are asked to decide how much he / she likes or dislikes. Decision is given by a mark on a scale [6] and so the subject is able to express their own perception of quality [16].
Hedonic scale with nine stages is different, ranging from "bad" to "excellent". These words are placed on a graphic scale. Many different types of scale can be used [9]. Hedonic assessment scores are converted to and treated by rank analysis or analysis of variance.
Hedonic scale tests are used for consumption [5, 10]. Very important are the characteristics of subjects, respectively tasters can be no margin of acceptance or waiting their influence results [1].
Quality fruit and vegetables is an extremely complex, difficult to describe objectively. The consumer is unable to assess the nutritional quality of a certain fruit and vegetables, yet he is able to make a statement on the sensory aspects such as shape, color, texture, juiciness, firmness, flavor and aroma.
Sensory properties of fruits and vegetables include flavor (taste and aroma), texture and appearance. Taste and flavor are considered more important than texture, because they reflect some internal qualities, that sensory quality [7].
However, it has been shown that what people really have reflected about their taste and smell with their nose papilla reflects only 20-40% of taste perception [15]. It is believed that scent plays a more important than taste in determining that the overall assessment of fruit quality. This is easy to prove that it is difficult to identify flavor if airflow through the nose is restricted [2].
In recent decades, horticultural research priorities were established on cultivars of vegetables or fruit to be cultivated to achieve higher performance, high resistance to disease and to transport and deliver a higher shelf such that a low perishability.
Some sensory aspects were also considered that the product appearance (shape, color). These parameters are undoubtedly useful, because they are easy to be assessed on the sorting lines.
Consumers complain more quality fruit and vegetables, which are offered by commercial systems of food distribution. The main concern of the complaint is bad taste and even its absence.
In recent years, fruit and vegetable industry was present, using automatic quality assessment, by measuring firmness, juicy, total acidity and sugar and also pH. Unfortunately, the main quality factors such as smell and flavor are not measurable with currently available analytical devices.

MATERIAL AND METHODS

For conducted research there were compared two cultivars of cucumber type Farbio (Luxury F1 and Pasalimo F1 cultivars) and three cultivation type cornichon (Rytm F1, Zeina F1 and Renato F1) grown in three known vegetable areas: Matca, Pucheni and Periş. Type of culture is in greenhouses, growing period was March to May 2012, harvesting was done on 8 May 2012.
After collection, samples were brought for testing to laboratories Veterinary Medicine Bucharest. Samples were washed and prepared for testing.
Each taster had distributed - one cucumber sample which was cut into several portions on the long and so we have medium samples; they were placed on a plastic plate that had sample number.
For each taster and for each sample were specific questionnaires that included some preliminary instructions as well: You will receive samples of vegetables or fruit, you are asked to appreciate the smell, taste and texture, please appreciate the intensity of each sample without compare them and fill in the table that containing more points on the color, smell, taste, firmness and an overall assessment of the vegetables.
The assessment was performed with a scale of 1-9, respectively from "very weak" to "very hard" for each sense separately. These notes have been taken into account and using a star-chart was made interpretation of results.

RESULTS AND DISCUSSIONS

The results of Farbio cucumbers type sensory analysis shows that location Pucheni determined to obtain better quality fruit, in the judgment of tasters majoring in color, odor, flavor and sweetness, characteristics that have established an overall assessment point 6.58.

Table 1. Results of Farbio cucumbers sensory analysis

Specif.	Color	Odor	Flavor	Sweetness	Acidic taste	Bitter taste	Overall
Farbio Matca, Pasalimo F1	5,12	4,59	4,72	4,94	2,29	1,82	5,41
Farbio Pucheni, Luxury F1	6,12	5,32	5,25	5,48	2,00	0,82	6,58

Cucumbers from Matca, Pasalimo, had a more acidic taste including bitter parts so overall was around 5.41 value (Table 1).

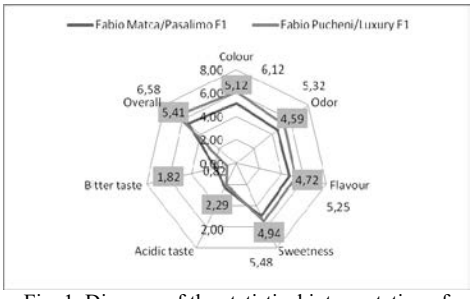

Fig. 1. Diagram of the statistical interpretation of Farbio type cucumbers

From the statistical interpretation of the diagram (Fig. 1) can be seen that there is overlap of characteristics taken in assessing cucumbers but higher notes were obtained from the cultivar Luxury F1 from Pucheni area.

Table 2. Results of cornichon cucumbers sensory analysis

Specification	Color	Odor	Flavo	weetness	Acidic taste	Bitter taste	Overall
Cornichon Matca/Rytm F1	6	4,75	5,05	4,75	2	2,4	5,75
Cornichon Pucheni/ Zeina F1	6,55	5,15	6,55	6,3	2,05	1,9	7,25
Cornichon Periş/Renato F1	7,4	6,2	6,9	6,9	2,5	2,3	7,4

Sensory analysis was conducted also to the cultivars cornichon cucumbers which were harvested at physiological maturity of three locations: Pucheni, Matca and Periş.

Overall of cucumbers shows that the highest value of 7.4 was obtained at the Periş, at Renato F1 cultivar, followed by Pucheni, Zeina F1 cultivar with 7.25 value and the last was cornichon from Matca, cultivar Rytm F1.

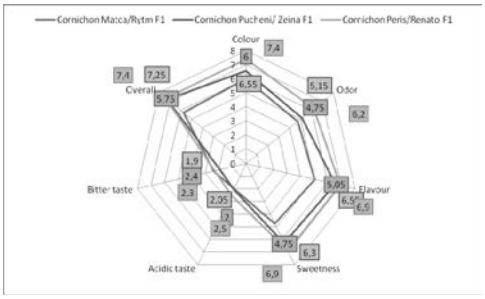

Fig. 2. Diagram of the statistical interpretation of cornichon type cucumbers

In terms of color, the best cultivar was Renato F1 with 7.4 note followed by Zeina F1 with 6.55 note (Table 2). The same trend is observed in terms of smell and taste and the best cultivar was also Renato F1 followed by Zeina F1.

Statistical analysis based on the diagram shows that are balanced in terms of sensory characteristics were Renato F1 and Rytm F1 cultivars from Periş and Matca, and Zeina F1 cultivar is unbalanced in terms of taste.

CONCLUSIONS

Sensory analyzes performed on cucumbers grown in greenhouses and solariums in our south have revealed the following:

Analysis of the results of Farbio type cucumbers sensory analysis shows that location Pucheni determined to obtain better quality fruit, in the judgment of tasters majoring in color, odor, sweetness and flavor, characteristics that have established an overall assessment point: 6.58. Cucumbers from Matca, Pasalimo, had a more acidic taste taste including bitter parts so general appreciation was around 5.41 value.

Statistical interpretation of the diagram can be seen that there is overlap of characteristics

taken in assessing cucumbers but higher grades were obtained from the cultivar Luxury F1 from Pucheni.

Overall of the cornichon cucumbers type shows that the highest value of 7.4 was obtained at the Periş, Renato F1 cultivar, followed by Pucheni, Zeina F1 cultivar with 7.25 and the last Rytm F1 cornichon cultivar from Matca area.

Statistical analysis based on the diagram shows that are balanced in terms of sensory characteristics were Renato and Rytm F1 cultivars, from Periş and Matca areas, and Zeina F1 cultivar is unbalanced from the point of view of taste.

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PRELIMINARY RESEARCH TO DEVELOP ACTIVE PACKAGING FOR BAKERY PRODUCTS USING ESSENTIAL OILS

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Abstract

In this research the aim was to evaluate the antifungal properties of aromatic plant essential oils in vapour phase, against various fungal strains that causes bakery products alteration. The vapours of clove oil, oregano and white thyme oil were tested against five fungal strains (Aspergillus flavus, Aspergillus oryzae, Aspergillus brasiliensis, Fusarium culmorum, Fusarium graminearum) in a closed system using disc volatilization method which identifies the antifungal potential of essential oil vapours. The results obtained were expressed as minimum inhibitory concentration (MIC) in ppm in a 365.64 cm^3 volume Petri dish. Were evaluated nine different concentration of these essential oils (50, 100, 200, 400, 600, 800, 1000, 1500, 2000 ppm) obtained by dilution in a 10% DMSO solution. The best result after seven days of exposure, were shown by oregano oil against all tested fungi with a MIC of 1000 ppm, especially against Fusarium spp., which presented no growth in the area above the disc with oil and no sporulation activity in rest of the Petri dish. Aspergillus ssp. was more resistant to the action of oregano vapours and gives a MIC of 1500 ppm. The vapour phase of clove and white thyme oils were more active at a MIC of 1500 ppm against all fungal strains while at 1000 ppm induced low mycelia growth, influenced the germination of spores which caused immature spores. Vapours of clove oil affected mainly Fusarium spp. by total inhibition of growth in the area of action and in rest affected mycelia development. White thyme oil had a low impact against Aspergillus spp. under study and the most efficient vapours concentration was of 2000 ppm. Main morphological changes observed under light microscopy on fungal strains grown in atmosphere of essential oils were disrupted cell structure, considerable alterations in hyphae, reduced number of conidiospores and loss of pigmentation. In conclusion, tested essential oils were effective in vapour phase leading to important alterations in fungal structure and sporulation process. This work gave us the opportunity to put the base of a protective active atmosphere using essential oils like natural antifungal agents, which could extend the shelf life of packaged bakery products.

Key words: *antifungal activity, essential oils, light microscopy, vapour phase.*

INTRODUCTION

In bakery industry, bread occupies a unique position both in production and utilization as compared to other bakery products. Bread is the most important commercial product of wheat flour consumed as staple food by most of the wheat-eating people. In Romania annual bread consumption is in the region of 97 kg per person, a third more than the European average, twice than Danes and almost seven times over the consumption in UK [8]. Consumption of bread in Romania is so high because bread is considered a basic food and also because of the national tradition, the people budget and living standards.

Since, bread is an important part of our daily diet ways and means should be explored to improve the quality and shelf life. Mould spoilage is the most common form of deterioration of bread. At the present time mould spoilage of bread is prevented by addition of chemical preservatives such as propionic, sorbis and acetic acids and their salts [11]. Since the 1980's the bread industry has been working to reduce the number of additives and so called synthetic preservatives in a genuine effort to make bread as natural and fresh as possible [7]. In the last few decades a great interest has emerged for natural preservation of food products using plant extract and essentials oils. Essential oils are volatile oily liquids obtained from

different plant parts and widely used as food flavours [4]. The antimicrobial activity of essential oils has been studied and demonstrated for years against a number of microorganisms, by different test methods such as direct contact antimicrobial assays, diffusion assays or dilution methods [4] [10]. The most interesting area of application for essential oils consist of their incorporation directly into the packaging material, coated onto polymer surface, or immobilized to polymers [3], resulting an antimicrobial packaging which preserves, in a natural manner, different food products. The use of essential oils and their chemical compounds, categorized as flavourings by the European Union [1] [2] and as GRAS (Generally Recognized as Safe) by the US Food and Drug Administration, in food preservation is a attractive opinion for "green" food products.

This research paper has two aims. First, to evaluate the antifungal activity of essential oils in vapour phase at different concentration against food spoilage and toxigenic fungi. This aim is important in identification of minimum inhibitory concentration (MIC) of volatile vapours in order to be used in future researches in active packaging of bread. Second, to identify the changes and abnormalities caused by essential oils against fungal cell by microscopic methods.

MATERIAL AND METHOD

Fungal strains and growth media

In this study were used five toxigenic fungal strains involved in grain contamination and spoilage and decay of food products especially bakery products. The fungal strains selected for the assessment of antifungal activity of volatile vapours of essential oils were as fallows: *Fusarium culmorum* 46 and *Fusarium graminearum* 96 from I.C.D.A Fundulea, *Aspergillus flavus* and *Aspergillus oryzae* from C.B.A.B Biotehnol, *Aspergillus brasiliensis* (niger) ATCC 16404 from MicroBioLogics, SUA.

Fungal strains were maintained on Potato Dextrose Agar (Biokar Diagnostics) at 4^0C and used as stock cultures. Spore suspensions were prepared in a 10 ml solution of NaCl 1% (w/v) containing 5% Tween 80 (w/v) from fresh stock cultures grown on PDA Petri dish for 7 days at 25^0C. Spore suspension obtained was diluted in sterile Peptone Physiological Serum to a concentration of 10^5spores/ml. Spore suspension was counted using plate agar method on PDA culture medium.

Essential oils and work concentrations

Based on preliminary researches on the antifungal activity of seven essential oils, three essential oils were chosen due to their active inhibition against presented fungal strains.

The essential oils of white thyme oil (*Thymus vulgaris),* oregano oil (*Thymus capitatus),* and clove bud oil (*Eugenia caryophyllata*) obtained by steam distilation, purchased from Sigma Aldrich, Germany, were the oils selected for this research. Essential oils quality parameters (Chemical Abstract Service, CAS, number, appearance, color, purity, odor, and density at -20^0C and refraction index at -20^0C) were described in an accompanying technical report.

In order to obtain work concentrations of 200, 400, 600, 800, 1000, 1500 and 2000 ppm, the essential oils were diluted in a 10% DMSO (Dimethyl sulfoxide, Sigma Aldrich, Germany) sterile medium solution based on their density.

The work concentration prepared were mixed for total solubilization at 180 rpm for 10 minutes and kept at room temperature until subsequent use.

Antimicrobial assay [12]

In vitro antifungal activity of volatile vapours of essential oils was evaluated by micro atmosphere method, a modified disc diffusion method at seven different concentrations (200 – 2000 ppm).

This method allowed us to determine the MIC (minimum inhibitory concentration) of essential oils vapour phase. The MIC is defined as the lowest concentration of the compound that inhibits growth of a microorganism after a specified incubation period [13].

In brief, 15 ml of warm PDA medium was poured into a 90 mm sterile plastic Petri dish (volume recorded with the medium poured into the dish of 365.64 cm^3) and after

solidification the medium in the dish was inoculated with 100 µl of spore suspension (10^5spores/ml) of the microorganism under study. In the cover of the Petri dish was cast a thin layer sterile medium on which was placed a 6 mm diameter paper disc (Whatman, no 1) with 10 µl of different essential oils concentration. The dishes were then sealed using sterile laboratory Parafilm to avoid evaporation of the essential oils, fallowed by incubation at 25^0C for seven days.

After incubation, the minimum inhibitory concentrations (MICs) were recorded based on the inhibitory activity against fungal growth. The MIC was defined as the lowest concentration witch made clearly visible inhibition zones. Blank discs with and without 10 % DMSO solution served as negative control.

Microscopic observation of morphological changes

Fungal strains grown on nutritive medium (PDA) treated with essential oils vapours were evaluated for any morphological changes using a light microscope (Olympus U-CMAD 3). Representative samples were taken from the surface of the fungal colony, from the margin next to the inhibition zone due to essential oils activity. The sample was placed on a clean microscopic slide and viewed on the microscope for any abnormalities. The important changes in the fungal microscopic appearance were noted and commented.

RESULTS AND DISCUSSIONS

Results obtained from the antimicrobial assay of volatile vapours of essential oils after seven days of exposure are summarized in Table 1. As was expected, all the essential oils tested expressed antifungal activity in vapour phase but with a highest concentration than in direct contact method (results are not shown in this article). Notable inhibition areas due to volatile vapours of essential oils were presented for higher concentration, the main concentrations selected were 1000, 1500 and 2000 ppm.

Table 1. MIC (ppm) of essential oils in vapour phase against toxigenic fungal strains

Essential oils	Fungal strains under study				
	F. c.	*F. g.*	*A. b.*	*A. f*	*A. o.*
Clove bud	1500	1500	1500	1500	1500
White thyme	1500	1500	2000	2000	2000
Oregano	1000	1000	1500	1500	1500

MIC – minimum inhibitory concentration; F.c. – *Fusarium culmorum*; F.g. – *Fusarium graminearum*; A. b. – *Aspergillus brasiliensis*; A. f. – *Aspergillus flavus*; A. o. – *Aspergillus oryzae*

The vapour of essential oil of oregano has showed an inhibition activity for all the fungal strains tested at 1000 ppm, especially for Fusarium spp. In this case, Fusarium spp. presented no growth in the area above the disc with oregano oil and no sporulation in the rest of the plate (Photo 1.).

In terms of susceptibility expressed by Aspergillus spp. at oregano oil vapours, the MIC who gave better result of inhibition was 1500 ppm, which limited the growth of the mycelium and development of fungal spores. *Aspergillus oryzae* and *Aspergillus flavus* growth were inhibited by vapour phase of oregano oil at 1500 ppm concentration.

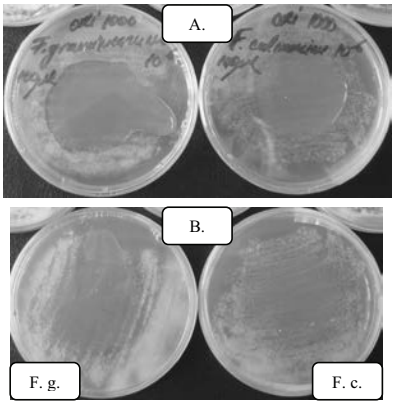

Photo 1. Vapour phase activity of oregano oil at 1000 ppm against Fusarium ssp.
F. g. – *Fusarium graminearum*; F. c. – *Fusarium culmorum*; A – front side of the plates; B - reverse side of the plates.

Growth of these strains was observed only on the margins of the Petri dish were the vapour did not have a direct diffusion, just mycelium development and no sporulation action. *Aspergillus brasiliensis* presented a low

development in the area above the oregano essential oil, but a good sporulation in the rest of the Petri dish. Clove bud essential oil expressed the same MIC against fungal strains tested as oregano oil, but Fusarium ssp. were more sensitive to 1500 ppm concentration of clove oil that 1000 ppm by oregano oil. At 1500 ppm concentration, clove bud oil inhibited fungal growth in the area above the disc and in the rest of the Petri dish there were visible affected mycelia, no sporulation and discoloration of the fungal mass.

The less effective in vapour phase against tested fungal strains was white thyme essential oil. MIC of thyme oil was of 2000 ppm in case of Aspergillus spp., from which *Aspergillus oryzae* was the most susceptible to the activity of the vapours, presenting no growth in the area above the disc and no sporulation in the rest of the Petri dish. Spore germinations of Fusarium *culmorum* and *Fusarium graminearum* were completely inhibited by the volatile vapours of thyme essential oil at 1500 ppm concentration (Photo. 2).

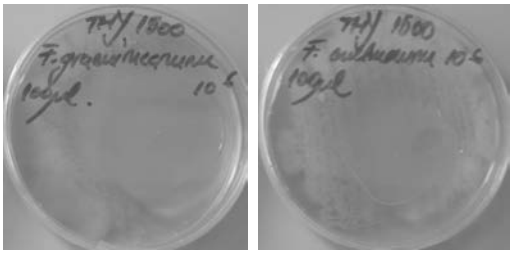

Photo 2. Vapour phase activity of 1500 ppm concentration of thyme oil against Fusarium spp.

Fusarium spp. were more sensible to the action of essential oils vapours, situation presented in the case of all tested essential oils but more predominant to oregano and thyme oil. *Aspergillus brasiliensis* showed more resistance to essential oils activity, and have grown in all the Petri dish after 10 days of exposure (data not showed).

Morphological alteration of fungal strains

Observation of fungal strains under the light microscope at 200, 400 and 500 X magnification after exposure to essential oils vapours showed some morphological abnormalities. Fungal cells exposed to clove oil vapour underwent considerable morphological abnormalities in comparison with the control. Control cells of *Aspergillus brasiliensis* showed regular growing hyphae with homogenous cytoplasm and visible conidiation on a large conidial head (Photo no. 2 – A) while the mycelia trated with clove oil vapours presented lack of sporulation, loss of cytoplasm content and distorted development of hyphae (Photo 3 – B).

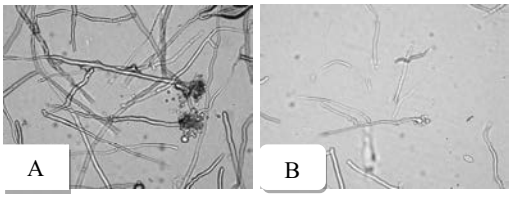

Photo 3. Light microscopy (100 X) of *A. brasiliensis* mycelium growing on PDA in clove bud oil atmosphere during 7 days of incubation at 25 – 28 °C. A – Normal conidial head of *A. brasiliensis*, development of vesicle on conidiophores, conidia clearly visible; B – treated with clove oil vapours, showing clear reduction in conidiation, disrupted hyphae integrity.

The same changes, caused by clove bud oil and oregano oil, in fungal cellular structure was observed also in microscopic preparations of *Aspergillus oryzae* and *Aspergillus flavus* (figure no. 4). The fungal cells were affected by the active vapours of clove oil by presenting reduction in conidiation, unspecific development of conidiophores and shorter hyphae.

The essential oils clearly caused reduction in conidial heads of *Aspergillus flavus*, with distorted presence of conidiophores (Photo 4 - B), decreased conidiation (lack of sporulation), visible loss of cytoplasm content, abnormal development of hyphae (Fig. 4. - C). Thyme essential oils caused the same abnormalities to Aspergillus spp., mainly reduction in conidial head.

In the case of Fusarium spp., vapour of essential oils tested act against macroconidia formulation, reduce chalmydosopores development, lack of pigmentation of hyphae and loss of structure (Photo 5).

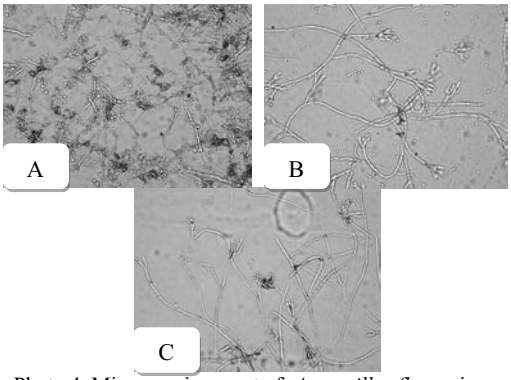

Photo 4. Microscopic aspect of *Aspergillus flavus* in control (A) and after exposure to clove (B) and oregano (C) essential oils

It is clear from these results that the vapour treatement not only alters the cell dimension and overall morphology, but has an important impact also on the surface of the cells.

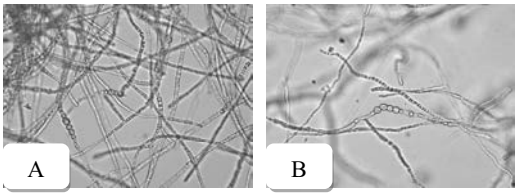

Photo. 5. Morphological changes induced by oregano oil vapours against *Fusarium graminearum*. A – Control sample present normal mycelia and chalmydospores. B – Treated mycelia present a significant reduction in hyphae and chalmydospores formation.

CONCLUSIONS

The investigations on antifungal activity of vapour phase of three essential oils against toxigenic fungi showed that by the micro atmosphere method is necessary a higher concentration of essential oils than in direct contact method, in order to express a good antifungal activity. All the tested essential oils inhibited the growth of fungal strains but at a higher concentration as presented in Table no. 1 in which are given the values of MIC's for each fungal strain. Although the MIC of essential oils was between 1000 – 2000 ppm, this concentration has a sensorial print that is not unpleasant. Fusarium spp. were more sensitive to the action of esssential oils vapours than Aspergillus spp, presenting low development of mycelia and lack of sporulation in all the Petri dish not only in the impregnated essential oil disc. The essential oils tested expressed a suppressing effect on the fungal spore development, fact presented by light microscopy.

The observations of light microscopy showed that the main morphological changes caused by tested essential oils on Aspergillus spp. were associated with the degeneration of fungal hyphae and in the sporulation process. In terms of morphological structure of Fusarium spp, the treatment with essential oils presented a reduction in chlamydospores formation and loss of structure of hyphae. Different behavior of the fungal strains in the essential oil atmosphere may be determined by the different chemical composition and concentration of phenol compounds in each type of essential oil utilized in this study.

In conclusion, our results indicate that essential oils could find a practical and applicable use in the inhibition of mould growth. Therefore, using volatile vapour of essential oils as an antifungal agent may provide a useful application in active packaging of different food products especially bakery products.

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EVALUATING SOME SENSORIAL, PHYSIC-CHEMICAL AND MICROBIOLOGICAL CHARACTERISTICS OF PEAS PRESERVED BY USUAL METHODS

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Abstract

In the present study were evaluated some sensorial, physic-chemical and microbiological characteristics of frozen and canned peas, when compared with fresh peas. The sensorial analysis consisted in aspect, aroma and texture evaluation; the physic-chemical parameters analysed were: pH and water activity and the microbiological parameter was total plate count. All samples were purchased from the market, fresh peas in pod, frozen peas packed in plastic bags, in air atmosphere and canned peas packed in glass recipients, in water. Sensorial analysis was made by untrained panellists. All samples were smashed into a Stomacher before analysing from the physic-chemical and microbiological point of view. pH was determined using a INOLAB 720 WTW series pH-meter equipped with a Sen Tix Sp Spear immersion electrode and water activity was analysed using a Novasina LabMaster AW device. Total plate count was evaluated using SR ISO 4833. Results showed that fresh peas had the highest scores for aspect and texture and the most tasteful sample was canned pea. Most acid samples were those of canned peas and most basic ones were those of frozen peas. Water activity registered the highest value for frozen peas and the lowest for fresh peas. Total plate count had the lowest values for canned peas, followed by fresh and frozen peas.

Keywords: *canning, freezing, pea, total plate count, water activity*

INTRODUCTION

Fruits and vegetables are live tissues harvested at various stages of growth and development, have tender texture, contain high moisture content (60% - 95%) and water activity, lose water to the surrounding atmosphere, and continue respiration, which produces heat and water at the expense of food reserve, carbohydrates, proteins, lipids, etc., which were otherwise replaced by photosynthates and nutrients supplied by the plant before harvest [6].

Postharvest period begins at the separation of plant organ used as food from the medium of its immediate growth or production, and ends when it enters the process of preparation for final consumption of further preservation [5].

Fruits and vegetables are consumed in fresh, minimally processed, and processed forms (canned, frozen, dried, preserves, and fermented products). Raw material quality influences the quality of processed fruit and vegetable products as quality can only at best be maintained and not improved by processing [1].

Quality attributes normally used for raw materials, as well as for final products, are physical (size, firmness, presence or absence of seeds, etc.), compositional (natural sugars and volatiles), nutritional (vitamins, antioxidants, and functional components), and sensory (colour, texture, taste, flavour, and odour) [3, 4, 7, 8].

Quality evaluation consists of measurement of appearance, texture, flavour, nutritive value, and safety of the produce. Safety aspects need to be considered first before all other quality attributes [6].

Green garden peas are eaten before reaching physiological ripeness, fresh or preserved. Peas are rich in carbohydrates (12.5 to 14%), proteins (6 to 8.4%), lipids (0.6%), fibres (6%) and mineral substances (over 0.9%). Total moisture value is 74 to 76% and energetic value is 780 – 790 kcal/kg (960 kcal/kg according to Mincu, I. et all, 1984) [2].

MATERIAL AND METHOD

We evaluated some sensorial, physic-chemical and microbiological characteristics of frozen and canned peas, when compared with fresh peas. The sensorial analysis consisted in aspect, aroma and texture evaluation; the physic-chemical parameters analysed were: pH and water activity and the microbiological parameter was total plate count.

All samples were purchased from the market, fresh peas in pod, frozen peas packed in plastic bags, in air atmosphere and canned peas packed in glass recipients, in water.

Sensorial analysis was made by untrained panellists.

All samples were smashed into a Stomacher before analysing from the physic-chemical and microbiological point of view.

pH was determined using a INOLAB 720 WTW series pH-meter equipped with a Sen Tix Sp Spear immersion electrode and water activity was analysed using a Novasina LabMaster AW device. Total plate count was evaluated using SR ISO 4833.

RESULTS AND DISCUSSIONS

Sensorial characteristics

Arithmetic and weighted averages of the three sensory parameters analysed for pea samples are presented in table 1.

Table 1. Arithmetic and weighted averages of the three sensory parameters analysed for pea samples

Sample	Panellist name	Quality indicators			Weighted average	Final rating
		Aspect	Taste – aroma	Texture		
Fresh pea	Chiriţă Elena	4	4	3	3.65	3.965
	Voinea Andreea	4	5	5	4.85	
	Cuc Cristiana	4	5	5	4.85	
	Marin Gabriela	5	5	4	4.65	
	Popa Anamaria	5	4	5	4.5	
	Grădinaru Adriana	5	4	5	4.5	
	Neacşu Dragoş	4	4	5	4.35	
	Dobrea Nicuşor	5	4	4	4.15	
	Năstase Raluca - Andreea	5	4	4	4.15	
	Hergan Vasilica	5	4	5	4.5	
Arithmetic average		**4.6**	**4.3**	**4.5**	-	**-**
Frozen pea	Chiriţă Elena	4	3	4	3.5	3.63
	Voinea Andreea	5	5	5	5	
	Cuc Cristiana	5	4	5	4.5	
	Marin Gabriela	5	4	4	4.15	
	Popa Anamaria	5	4	4	4.15	
	Grădinaru Adriana	4	5	5	4.85	
	Neacşu Dragoş	4	2	4	3	
	Dobrea Nicuşor	4	4	4	4	
	Năstase Raluca - Andreea	5	2	4	3.15	
	Hergan Vasilica	4	4	2	3.3	
Arithmetic average		**4.5**	**3.7**	**4.1**	-	**-**
Canned pea	Chiriţă Elena	3	3	4	3.35	3.5
	Voinea Andreea	4	5	5	4.85	
	Cuc Cristiana	4	5	5	4.85	
	Marin Gabriela	4	4	3	3.65	
	Popa Anamaria	4	3	4	3.5	
	Grădinaru Adriana	4	5	4	4.5	
	Neacşu Dragoş	3	3	2	2.65	
	Dobrea Nicuşor	4	4	3	3.65	
	Năstase Raluca – Andreea	4	4	4	4	
	Hergan Vasilica	4	4	3	3.65	
Arithmetic average		**3.8**	**4**	**3.7**	-	-

It can be observed that the higher value of the final rating was registered for fresh pea and the lowest for canned pea samples. From the aspect point of view, fresh and frozen pea samples had very similar values (4.6 and 4.5), while canned pea have a lower value (3.8). Regarding taste and aroma, the highest value was obtained for fresh pea (4.3), followed by canned pea (4.0) and frozen pea, with the lowest value (3.7). Best texture was registered, as expected, for

fresh pea (4.5), followed closely by frozen pea (4.1). Canned pea samples had the lowest value of arithmetic average for texture (3.7).
All above observations can be explained by the fact that thermic treatment during freezing and canning technologies is responsible of some chemical and textural modifications in green vegetables, as a result of partial breaking of the cell wall. Thermic treatment induces chlorophyll degradation, leading to processed green vegetables that have a different colour compared with raw material.

Physic-chemical characteristics

The pH and water activity values for pea samples analysed during experiments are presented in figure 1 and 2.
By analysing figure 1, it can be observed that frozen pea have the higher pH value (7.04), followed by fresh pea (6.67) and canned pea, with the lowest value (5.77). Water activity, as it can be seen in figure 2, have very similar values for frozen and canned pea samples (0.978 and 0.977). Fresh pea samples had the lowest water activity value (0.967).

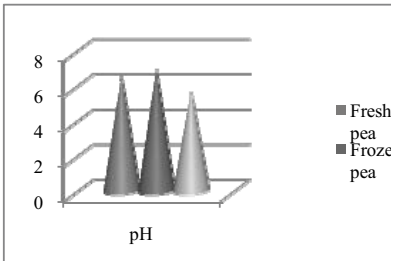

Fig.1. pH values for pea samples analysed

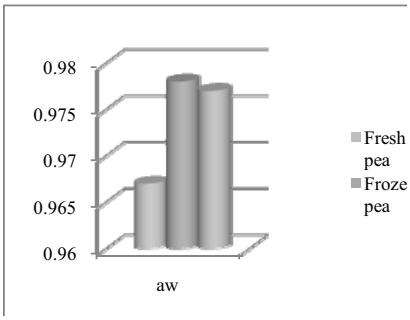

Fig.2. Water activity values for pea samples analysed

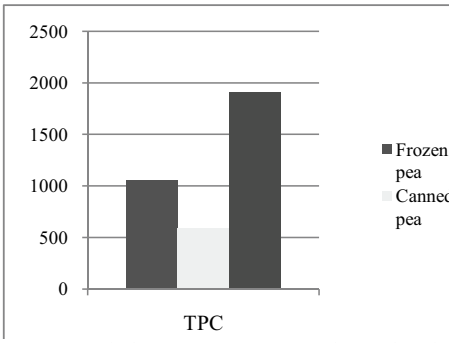

Fig. 3. Total plate count for pea samples analysed

Microbiological characteristics

Figure 3 presents total plate count for pea samples. It can be observed that the lowest microbial load was registered for canned pea samples, which confirms the efficacy of thermal sterilisation during canning technology. Frozen pea samples had a total plate count value lower than fresh pea samples. This can be explained by the fact that preliminary treatments during freezing technology have microbiostatic and sometimes even microbicide effect.

CONCLUSIONS

From the analyses performed in this study can be drawn the following conclusions:
- the highest value of the final sensorial rating was registered for fresh pea and the lowest one for canned pea samples. This can be explained by the fact that thermic treatment during freezing and canning technologies is responsible of some chemical and textural modifications in green vegetables, as a result of partial breaking of the cell wall;
- frozen pea have the highest pH value and canned pea the lowest;
- water activity values varied in very narrow limits for all pea samples analysed;
- the lowest total plate count value was obtained for canned pea, which confirms the efficacy of thermal sterilisation during canning technology. Frozen pea samples had a total plate count value lower than fresh pea samples and this can be explained by the fact that preliminary treatments during freezing technology have microbiostatic and sometimes even microbicide effect.

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ORGANIC VERSUS CONVENTIONAL FOOD PRODUCTS: PHYSICO - CHEMICAL AND MICROBIOLOGICAL COMPARATIVE ANALYSIS

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Abstract

The importance of organic agriculture has increased in recent years. Therefore, many studies have been done, trying to analyze the differences between organic and conventional food products, especially in terms of chemical composition, microbiological and sensory quality characteristics. Some studies have reported higher values of nutrients in organic foods compared to conventional ones. Development of results is difficult and generalization of findings must be made with great caution. In this respect, the present study meets current requirements in oriented food, free of pollutants and healthy, try aiming to highlight the advantages of organic products. Materials have been used, such as sheep cheese, tomatoes, peppers and cucumbers, to compare the quality of product with its conventional organic one. All samples were analyzed from physico - chemical and microbiological point of view. The results obtained have shown that organic products have a higher degree of safety correlating microbiological, physico - chemical and heavy metal content. Therefore is recommended to increase the production and consumption of organic food.

Key words: *descriptive analysis, organic and conventional, vegetables, cheese*

INTRODUCTION

Numerous studies, done both at a national and international level, have concluded that ecologically obtained products are not inferior to those obtained in a conventional system, from the quality point of view under all its forms: technological quality, visual and taste quality, agronomical quality, nutritional quality, sanitary quality, ecological quality, ethical quality, global. [2, 3]

AFNOR, France, gives the following definition to quality:"*quality is the ensemble of measurable or un-measurable characteristics of a product or service that confers the ability to satisfy the expressed or implied needs of its user*''. [2, 3]

The consumer appreciates the quality of a product by appearance, taste, smell, color, shelf life, behavior during processing, etc., in consequence, by some specific physical, chemical, and technological characteristics. [4, 5]

In ecological agriculture we emphasize the hygienic and biological quality of the product that is intended to commercial use. [5]

In ecological agricultural systems we try to obtain optimal productions, not maximal productions that are most commonly obtained with the abusive usage of resources and have, as a consequence, the degradation of the environment.

Thereby, through the practice of ecological agriculture we are looking to obtain high quality agricultural products, without the usage of residues and pesticides, with a balanced content of nutrients (proteins, fats, and carbohydrates), organic acids, vitamins, and mineral salts. [5, 6]

Following the revision of more than 150 articles, more exactly 162, which were the subjects of studies that investigated the content in nutrients of ecological and conventional products, the American Society for Nutrition suggests that ecological products are comparable to conventional products, on 10 out of 13 categories of analyzed nutrients,

significant differences being recorded at the production methods. [1]

MATERIAL AND METHOD

Experimental research and analysis regarding the determination of physico – chemical characteristics were conducted microbiological analysis for ecological and conventional samples within the laboratories of the National Institute for Research and Development for Alimentary Bio-Resources IBA Bucharest, and in the laboratories of the Faculty of Biotechnology within the University of Agronomic Science and Veterinary Medicine Bucharest.

Within the experiments conducted we used as work mater products from the dairy area such as sheep salty cheese samples from both categories: ecological cheese and conventional cheese. From the vegetable range we chose tomatoes, cucumbers, and peppers, ecological and conventional samples. These samples were chosen considering that they are representative for the Romanian market.

The salty cheese is obtained out of integral sheep milk obtained in ecological and classical processing conditions, on the production lines of an acknowledged and certified company (S.C. Asinature SRL Sibiu). This assures on one hand all the conditions for ecological processing, and on the other hand the necessary conditions for comparing the samples.

From a technological point of view, the analyzed cheese samples represent maturated salty cheese made out of integral pasteurized sheep milk (ecological and conventional), with addition of selected lactic acid bacteria cultures, preserved in salty acidified whey.

The tomatoes, cucumber and peppers were obtained by the Research and Development Society for Vegetable Growth Bacau, in both ecological and conventional systems. This Society is recognized both national and international and certified for research for the production realized in biological agriculture conditions and the modern technologies for obtaining vegetables and plants in ecological and conventional systems. The obtaining of vegetables in ecological systems was realized in accordance with the European and Romanian legislations in use.

The determined physico – chemical characteristics that reflect an important part of the elements that define the quality of ecological and conventional samples are: dry matter content, water content, water activity index, pH value, acidity, protean substance content, fat content, salt content, dry soluble substance content. [8, 9, 10, 17]

The comparative characterization of ecological and conventional samples, from a micro-biological point of view, was realized by solving the following indices: the total plate count (TPC), yeast and mold number, number of bacteria *E-coli/coliforms,* presence or absence of *Salmonella* and the number of *Enterobacteriaceae*.

RESULTS AND DISCUSSIONS

The experimental results were analyzed through the comparison between the ones of the ecological and the conventional samples, and reported to the standard recommended values, the specific literature, and the technological requirements imposed by the certified products used in the experiments.

The physico – chemical analysis and microbiological results for the ecological and conventional salty sheep cheese samples (BTE and BTC) are described in the following article.

The median values described in the following article illustrates the arithmetic mean of the obtained values, following the multitude of determinations, successive and in parallel realized for the same group of products (ecological and conventional), for each indicator or characteristic separately.

The mean values of the physico – chemical characteristics determined for the ecological and conventional salty cheese samples are described in Table 1.

Table 1. The results of the physico – chemical analysis of conventional BTC and ecological BTE salty sheep cheese

Sample	Dry matter content. (%)	Humidity content (%)	a_w	pH at 25°C	Acidity (°T)	Protein content (%)	Fats (%)	Fats content / dry matter.* (%)	Salt (%)
BTC	56.36	43.65	0.96	4.90	164.0	20.81	29.40	52.17	2.00
BTE	58.44	41.57	0.96	4.66	205.5	20.87	30.83	52.76	1.93

Note: a_w – water activity index

The experimental samples were coded as follows: the conventional salty sheep cheese as BTC and the ecological salty sheep cheese as BTE.

The mean values determined for the dry matter and humidity content were 56.36% and 43.65% for conventional samples, and 58.44% and 41.57% for ecological samples.

As described in the graph in Fig 1 the mean values of dry matter content as well as the mean values of water content, for both conventional and ecological cheese samples fit the recommended guidelines. The dry matter content is higher than the minimum of 45%, and the humidity is below the maximum limit of 55%.

Comparing the average results obtained for both cheese groups, we realize that the ecological salty cheese have a greater dry matter content with about 3.75 than the conventional one.

Moreover, the water content is smaller with about 4.76% at the ecological samples than the conventional ones. The difference between the water content of the two sample groups, is also reflected in the observed differences regarding the water activity in both samples.

As described in Fig. 1 the mean value of the water activity index a_w is smaller for ecological cheese.

As the recommended value for water activity index is 0.96 we concluded that both samples have a water activity that is appropriate, from a technological and preserving point of views.

For the acidity, on the basis of the mean pH and acidity determined values, has been concluded that both sample groups have pH and acidity values within the recommended values, respectively the acidity is above 150 Thörner degrees (205.5˚T for ecological samples and 164.0˚T for conventional samples).

As described in Fig 1 the mean values determined for acidity are 25.3% larger in the case of ecological cheese compared with the conventional cheese.

The protein and fat content have recommended values in the case of salty sheep cheese of minimum 16% and 50% (to dry matter).

The mean values of both determined characteristics for ecological and conventional cheese samples are above the minimum recommended values. Indeed the protein content is 20.87% for ecological cheese, and 20.81% in the case of conventional cheese, both values being larger than the minimum allowed value of 16%. Similarly, the fat content reported to the dry matter is in both cases greater than the minimum allowed limit of 50%. Also in this case the ecological cheese has a higher fat content than the conventional one with 1.13%.

The mean values determined for the salt content for both ecological and conventional samples were well below the maximum allowed value of 4%.

From the salt content point of view, at the ecological cheese samples the mean values of this indicator were smaller compared with the conventional samples (with approx. 3.5%).

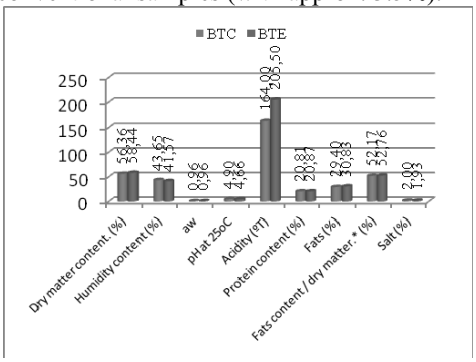

Fig. 1. The mean values determined for physico – chemical characteristics of ecological and conventional cheese samples

The evaluation of the results of analysis and measurements for all microbiological indicators was done in correlation with the imposed or recommended values for salt matured cheese made out of pasteurized and unpasteurized milk, foreseen in the CE Rulebook no. 2073/2005 and in the national standards SR ISO 7954/2001: Microbiology. The general directives for counting yeast and mold. The technique of counting colonies at 25˚C; SR ISO 5541/1-94: Milk and Dairy products. The establishment of the number of *Coliform* bacteria, Part 1: the method for counting colonies at 30˚C; SR12824/2001: the microbiology of food and forage. Horizontal method for counting the *Salmonella* germs.

Some microbiological criteria were established in the Regulation CE no. 2073/2005 that provides a guideline regarding the acceptable characteristic of dairy products and obtaining processes. It also provides rules of manipulation and distribution of these products that have to be reexamined and, by case revised or completed in order to keep up with the evolution in the food safety and the microbiology of the food products.

Following the realization of the tests and experimental determinations, the data in Table 2 was obtained.

Table 2. The results of microbiological analysis on the salty sheep cheese processed in conventional system BTC and in ecological system BTE

Sample	TPC (CFU/g)	Yeast and Mold (CFU/g)	*E. coli* (CFU/g)	Coliforms (CFU/g)	*Salmonella* (CFU/g)	*Enterobacteriaceae* (CFU/g)
BTC	57.500	15.050	-	0.15	-	0.35
BTE	39.000	11.250	-	4.9	-	2.25

From comparing the mean values obtained for the total number of aerobic mesophilic germs, has been concluded that the germs load is smaller in the case of ecological cheese in comparison with the conventional cheese (Fig.2). Indeed the mean value TPC is 39.0 CFU/g for ecological cheese and 57.5 CFU/g for conventional cheese i.e. the microbiological load is 32.17% smaller in the case of ecological cheese.

The smaller values of the total plate count (TPC), yeast and molds for ecological salty cheese in comparison with the conventional one has been evidenced. This fact is explained by a development media that is improper for microorganisms, which causes better preservation of the product.

The same comparative situation exists in the case of the load of yeast and molds. The number of yeast and molds that was determined following the analysis of the samples was: in the case of ecological samples 11.25CFU/g and in the case of conventional samples 15.05CFU/g.

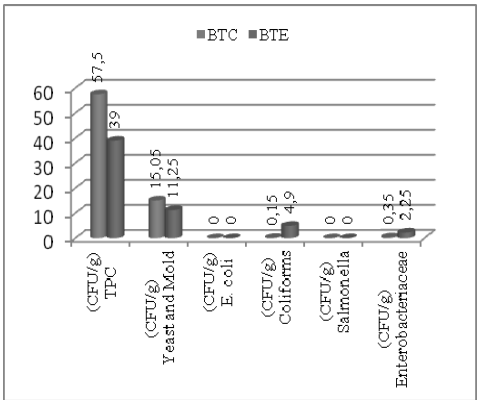

Fig. 2. The microbiological load for ecological and conventional cheese samples

The total number of coliforms resulted from the analysis is 4.9 CFU/g for ecological cheese samples and 0.19 CFU/g for conventional cheese samples. As described in the graph from Fig. 2, for both ecological and conventional samples the number of coliforms is below the maximum allowed limit of 10 CFU/g, as it is foreseen in the legislation.

From the performed analysis we found out that Salmonella is absent in both cheese sample cases (Fig.2).

In this way, the yeast and mold load is 25.24% lower in the case of ecological salty cheese in comparison with conventional cheese.

In both cases the yeast and mold number is a lot smaller than the maximum allowed limit of 1000 CFU/g foreseen in the European Regulation no. 2073/2005.

The contamination with *Escherichia coli* is absent for both ecological and conventional cheese, thus fulfilling the microbiological

norm of absent foreseen in the national and European standards (Fig.2).

For the microbiological indicator *Enterobacteriaceae*, mean values of 2.25 CFU/g resulted in the case of ecological cheese and 0.35 CFU/g in the case of conventional cheese (Fig. 2).

In Table 3 the mean values resulted from the determination of the quality of ecological and conventional vegetable samples (tomatoes, cucumbers and peppers cultivated ecologically and conventionally) are described.

Table 3. The results of physico – chemical analysis on tomatoes, cucumbers and cayenne obtained conventionally MFQ, KBV, DRA and ecologically HAY, QAF, EAI

Sample	Dry matter content (%)	Humidity content (%)	pH	a_w	Acidity (ml NaOH)	Soluble dry matter content (ºR, at 20 ºC)
Eco. tomatoes	5.21	94.79	4.11	1.00	0.36	5.60 ºR
Conv. tomatoes	4.90	91.62	4.70	0.91	0.47	5.20 ºR
Eco. cucumbers	4.65	95.35	5.52	1.00	0.05	3.90 ºR
Conv. cucumbers	3.68	96.32	5.56	0.99	0.08	1.50 ºR
Eco. peppers	5.50	94.50	5.88	1.00	0.14	3.05 ºR
Conv. peppers	3.98	96.02	5.78	1.00	0.17	3.00 ºR

Note: A_w- water activity index

The dry matter content of the vegetables has a direct influence on their preservation ability. In our case, by analyzing the mean values determined for the dry matter content, we observe that the ecological samples have a greater dry matter content than the conventional samples, and in conclusion a longer preservation period (Fig 3).

Comparing the mean results for both tomato samples, we realize that the ecological tomatoes have greater water content, having 3.46% more water than the ecological ones.

For the cucumber and peppers the mean value for humidity is higher in conventional samples than in ecological ones (see Fig. 3).

A pH value between 0 and 7, shows an acidic pH, a pH value of 7 is a neutral value, a pH value between 7 and 14 shows an alkaline pH.

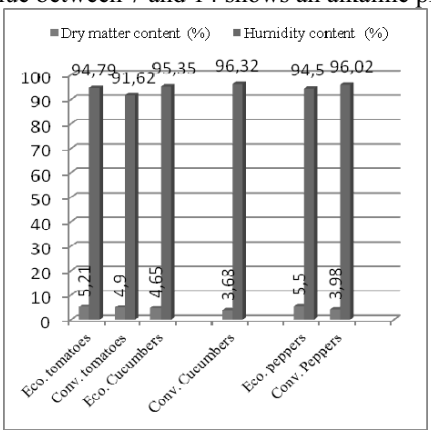

Fig. 3. The mean values of the dry matter and humidity content for the ecological and conventional vegetables samples

In our case the ecological and conventional cucumber samples and ecological and conventional peppers samples display pH values between 5 and 6 which mean that they are slightly acidic. The mean value of the pH for ecological and conventional tomato samples are below 5 and it is safe to state that we have an acidic pH value (Fig 4).

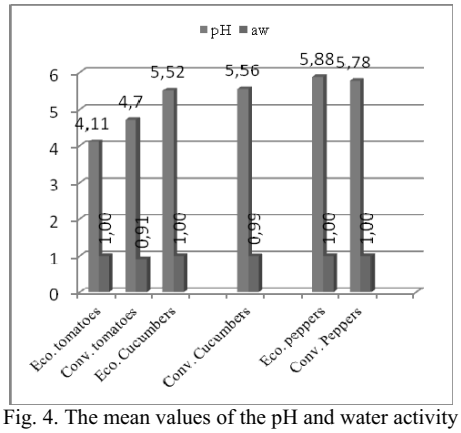

Fig. 4. The mean values of the pH and water activity for the ecological and conventional vegetables samples

The median value of the water activity index a_w for ecological tomato and cucumber samples is 9.1% for tomatoes and 0.3% for cucumbers, greater than the value of the same physico - chemical indicator of the

conventional tomato and cucumber samples. The pepper ecological and conventional samples have the same water activity value of 0.997 (Fig. 4).

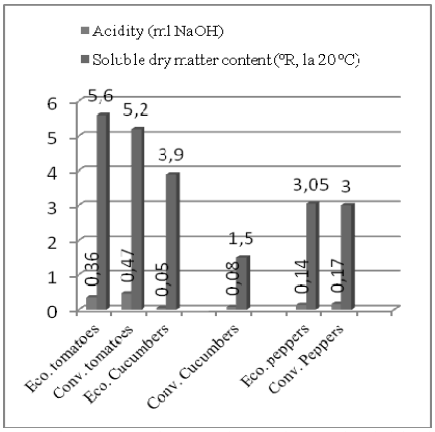

Fig. 5. The mean values of the acidity and soluble dry matter content for the ecological and conventional vegetables samples

The acidity of the conventional tomato samples is about 30.5% greater in comparison with the ecological and conventional cucumber and pepper samples. The tomatoes presented high acidity values, which in fact was expected (Fig. 5).

The dry soluble matter in the ecological vegetable samples was greater than the one in the conventional one proving that ecological vegetables have a greater enzyme, ascorbic acid, carotene, mineral substances, and vitamins content (see Fig 5).

All these physico – chemical characteristics are important because they influence the texture of the vegetable, their nutrient value, their aspect, and microorganism activity.

The microbiological indicators determined in the case of ecological and conventional tomato, cucumber and pepper samples, were the following ones: TPC, yeast and molds Y+M, *Escherichia coli* and *Enterobacteriaceae.*

The evaluation of the obtained results for all mentioned microbiological indicators was correlated with the values foreseen in the CE Regulation no. 2073/2005, with the last modifications.

The results of experimental determination for the vegetable samples (ecological and conventional peppers, cucumber and tomato) are given in Table 4.

Table 4. The results of microbiological analysis of vegetables in ecological and conventional samples

Sample	Microbiological indicators			
	TPC (CFU/g)	Yeasts and molds (CFU/g)	*Escherichia coli* (CFU/g)	*Enterobacteriaceae* (CFU/g)
Eco. Tomatoes	0	0	0	-
Conv. Tomatoes	0	0	0	-
Eco. Cucumber	17	750	0	-
Conv. Cucumbers	2400	5450	141.5	-
Eco. Peppers	16	2	0	-
Conv. Peppers	2	0	0	-

While analyzing the results described in table 4 we observed that the microbiological load in the tomato samples is absent. In the case of ecological and conventional cucumbers regarding the germs load, medium values of 16 CFU/g were obtained for ecological samples and 2 CFU/g for conventional samples. For yeast and mold contamination 2 CFU/g values were obtained. Overall it can say that the vegetables are edible, from a microbiological point of view.

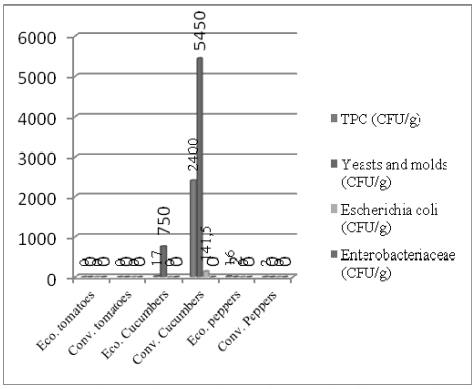

Fig. 6. The medium values obtained following the determination of the microbiological contamination of ecological and conventional vegetables

In the case of cucumber samples the germ load is smaller for ecological samples (17 CFU/g) while for conventional samples the mean value of the germ load is 2400 CFU/g.

Regarding the yeast and mold content of ecological cucumbers (having a value of 750 CFU/g) and conventional cucumbers (with a value of 5450 CFU/g) the obtained values are well above the maximum allowed limit.
Regarding the contamination with *Escherichia coli,* only one sample of conventional cucumber where found having a load of 141.5 CFU/g, in all other samples this bacteria being absent.
All six vegetable samples (tomatoes, cucumbers and peppers, ecological and conventional samples) did not show any trace of contamination with the *Enterobacteriaceae*.

CONCLUSIONS

The obtained results suggest that ecologically obtained products and the ones obtained conventionally can be comparable even though there are significant differences between their production methods.
Analyzing from a physico - chemical characteristics and a microbiological load point of view, the mean values determined for ecological samples comparative with the conventional ones, the ecological samples of cheese, tomatoes cucumbers and peppers demonstrated a bit better quality than the conventional ones. Further research will be carry out on analyzing above mentioned samples from a heavy metal contamination and sensorial point of view in order to see if other requirements for food have similar behavior.

AKNOWLEDGEMENTS

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COMPARING SOME CHARACTERISTICS OF FRESH, FROZEN AND CANNED STRAWBERRIES

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Abstract

We evaluated sensorial, physic-chemical and microbiological characteristics of fresh, frozen and canned strawberries. The sensorial analysis consisted in aspect, aroma and texture evaluation; the physic-chemical parameters analysed were: pH and water activity and the microbiological parameters were yeasts and moulds. All samples were purchased from the market, frozen strawberries packed in plastic bags, in air atmosphere and canned strawberries packed in metal recipients, in sugar syrup. Sensorial analysis was made by untrained panellists. All samples were smashed into a Stomacher before analysing from the physic-chemical and microbiological point of view. pH was determined using a INOLAB 720 WTW series pH-meter equipped with a Sen Tix Sp Spear immersion electrode and water activity was analysed using a Novasina LabMaster AW device. Yeasts and moulds number was evaluated using SR ISO 7954. From the sensorial point of view, fresh strawberries had the highest scores for aspect and texture and the most tasteful sample was canned strawberries due to sugar syrup. Most acid samples were those of frozen strawberries and most basic ones were those of canned strawberries. Water activity registered the highest value for fresh strawberries and for canned and frozen samples values were very similar. Yeasts and moulds had the lowest values for canned strawberries, followed by frozen and fresh strawberries.

Keywords**:** *canning, freezing, strawberries, yeasts and moulds, water activity*

INTRODUCTION

Fruits are an important source of vitamins, minerals, fibres, enzymes, aromatic volatile substances, and, beside vegetables, they have an essential role in human metabolism. Therefore, fruits and vegetables represent 20 – 25% of food intake and they are one of the standard of living indicators [1].

Berries are wide appreciated due to their taste, specific aroma and particular nutritive qualities. The issue of berries conservation is often approached worldwide from the preserving organoleptic properties and chemical composition of fresh fruit point of view.

Strawberries have many health benefits. Historically strawberries have been believed to have medicinal benefits in easing diarrhea, digestive upsets and gout. Furthermore, the fruit juice has been used externally to counteract sunburn, skin blemishes, and discolored teeth [3].

Strawberries are most known for being eaten raw due to the fact that they are highly perishable. In general, strawberries have a maximum storage life of between 5 to 7 days at a temperature of 0°C and 95% relative humidity [3]. Processing of strawberries occurs due to the fact that strawberries are highly perishable. Products such as juice, jam, syrup and wine are all made from strawberries. The leaves of the strawberry plant have also been used in blended herbal teas. The most common forms of processed strawberries are frozen [2].

MATERIAL AND METHOD

In order to evaluate sensorial, physic-chemical and microbiological characteristics of fresh, frozen and canned strawberries, the following indicators were used: aspect, aroma, texture, pH, water activity, yeasts and moulds.

All samples were purchased from the market, frozen strawberries packed in plastic bags, in air atmosphere and canned strawberries packed in metal recipients, in sugar syrup.

Sensorial analysis was made by untrained panellists.

All samples were smashed into a Stomacher before analysing from the physic-chemical and microbiological point of view.
pH was determined using a INOLAB 720 WTW series pH-meter equipped with a Sen Tix Sp Spear immersion electrode and water activity was analysed using a Novasina LabMaster AW device.
Yeasts and moulds number was evaluated using SR ISO 7954.

RESULTS AND DISCUSSIONS

Sensorial characteristics

Regarding the evolution of organoleptic characteristics, in the wake of analyses performed by the ten panellists, after calculating arithmetic average of the results, we observed the following:
- The most appreciated aspect, with an average of 3.7, was registered by the fresh strawberries, followed by the canned strawberries, with an average of 3.1; frozen strawberries had the lowest average – 2.5 (Table 1);

Table 1. Appreciating aspect for analysed samples

Sample	Aspect										Average
Fresh strawberries	3	5	4	4	3	4	3	4	4	3	**3.7**
Frozen strawberries	2	2	4	3	2	2	2	3	3	2	**2.5**
Canned strawberries	2	3	2	4	4	3	3	4	4	2	**3.1**

- The most intense aroma was registered by the canned strawberries, followed by the fresh and the frozen ones. This can be explained by the fact that canned strawberries were preserved in light sugar syrup (Table 2);
- The higher average for texture was registered by the fresh strawberries (3.2) and the lowest value by the frozen ones (1.6) (Table 3).

Table 2. Appreciating aroma for analysed samples

Sample	Aroma										Average
Fresh strawberries	4	5	2	3	3	4	2	3	3	4	**3.3**
Frozen strawberries	2	2	4	1	2	2	1	2	2	2	**2.0**
Canned strawberries	4	4	5	4	4	5	3	4	4	5	**4.2**

The strawberries form and size, as well as possible imperfections and colour are factors related to strawberries aspect and have a relatively great influence on consumer's first impression.

Table 3. Appreciating texture for analysed samples

Sample	Aroma										Average
Fresh strawberries	4	4	3	2	4	2	4	2	4	3	**3.2**
Frozen strawberries	1	1	1	2	2	1	1	4	1	2	**1.6**
Canned strawberries	1	2	2	2	3	2	2	3	2	2	**2.1**

Considerations regarding aspect are also useful in products calibration and rating, concurring to evenness assurance and facilitating buy and sale operations.
Strawberries aesthetic properties are referring especially to their aspect as an important factor for consumer's first impression. Aesthetic message can be sent to the consumer directly by the product or indirectly by the package. Particular contributions to an appealing aspect have strawberries colour, form and symmetry and also package characteristics.
Gustatory properties are induced by the chemical characteristics of foods, respectively foods volatile components. Taste is an expression of chemical sensitivity and is decisive for assessing and selecting foods and also for creating psycho-physiological conditions favourable for ingestion.
Aroma is a complex gustatory-olfactory characteristic, particular for foods. It is a sensation generated by the properties of some substances that stimulates taste or/and flavour. Knowing aroma forming mechanisms is very important in catering for blending aroma components and also for creating the conditions that generate it during technological processes.
The importance of texture as a general factor of acceptability for a particular food group varies greatly from case to case and according to this criterion, strawberries are classified as products that texture is important.
Considering that aroma has the higher rate in strawberries choice (50%), and that appearance and texture matter in proportion of 35% and 15%, after calculating the weighted average of the three sensory parameters analysed for strawberry samples, we obtained the results presented in Table 4.
By analysing table 4 we can observe that the highest final sensory rating was registered by fresh strawberries (3.52) and the lowest by frozen strawberries (2.11). That can be explained by the fact that both freezing and canning (during thermal treatment) leads to

changes of aspect, aroma and texture of strawberries. Canned strawberries samples had a value of final sensory rating higher than those of frozen strawberries (3.4 to 2.11) because were preserved in light sugar syrup.

Table 4. Weighted average of the three sensory parameters analysed for strawberry samples

Panellist number	Fresh strawberries				Frozen strawberries				Canned strawberries			
	A[1]	T-A[2]	T[3]	WA[4]	A[1]	T-A[2]	T[3]	WA[4]	A[1]	T-A[2]	T[3]	WA[4]
1	5	5	4	**4.85**	2	2	1	**1.85**	3	4	2	**3.35**
2	4	4	2	**3.70**	4	4	4	**4.00**	2	5	3	**3.65**
3	3	3	2	**2.85**	2	2	1	**1.85**	4	4	2	**3.70**
4	4	4	4	**4.00**	2	2	2	**2.00**	3	5	3	**4.00**
5	3	2	2	**2.35**	2	1	2	**1.50**	3	3	2	**2.85**
6	4	3	3	**3.35**	3	2	1	**2.20**	4	4	2	**3.70**
7	4	3	4	**3.50**	3	2	1	**2.20**	4	4	2	**3.70**
8	3	4	4	**3.65**	2	2	1	**1.85**	2	5	1	**3.35**
9	4	3	4	**3.50**	3	1	1	**1.70**	4	4	2	**3.70**
10	3	4	3	**3.50**	2	2	2	**2.00**	2	4	2	**2.00**
Final sensory rating	**3.52**				**2.11**				**3.4**			

[1]A = aspect;
[2]T – A = taste – aroma;
[3]T = texture;
[4]WA = weighted average.

Physic-chemical characteristics

pH values for strawberries samples varied within 3,65 and 3,81 (fig. 1).

Water activity in strawberry fruits is higher for canned strawberries compared with fresh and frozen ones due to the light sugar syrup in which they were preserved (fig. 2).

Microbiological characteristics

The highest number of yeasts and moulds was registered for fresh strawberries samples (fig. 3). Yeasts and moulds colonies developed on Petri plates inoculated with canned strawberries suspension were outnumbered by the colonies developed on plates inoculated with frozen strawberries suspension (for yeasts $3.5x10^2$ compared with $1.3x10^3$ and for moulds $1.1x10^2$ compared with $2.7x10^3$).

The fact that canned strawberries had the lowest microbial load give proof of the efficacy of thermal sterilisation on canning technological flow.

Also, the outnumbered colonies of yeasts and moulds for fresh strawberries samples when compared with frozen ones demonstrate the microbiostatic and sometimes even microbicide effect of preliminary treatments applied on frozen technological flow.

CONCLUSIONS

From the sensorial point of view, fresh strawberries had the highest scores for aspect and texture and the most tasteful sample was canned strawberries due to sugar syrup.

Most acid samples were those of frozen strawberries and most basic ones were those of canned strawberries. Water activity registered the highest value for fresh strawberries and for canned and frozen samples values were very similar.

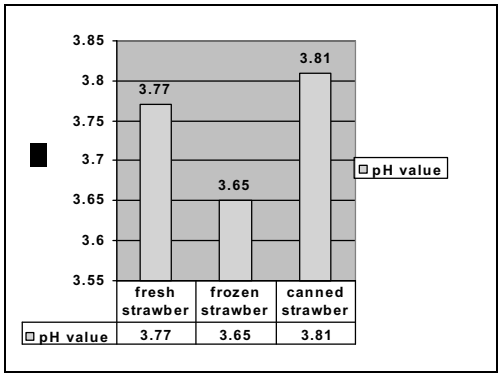

Fig.1. pH values for strawberry samples analysed

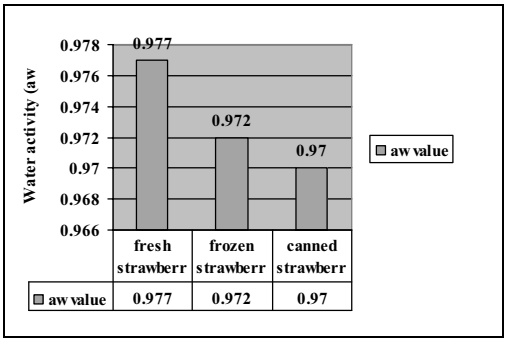

Fig.2. Water activity values for strawberry samples analysed

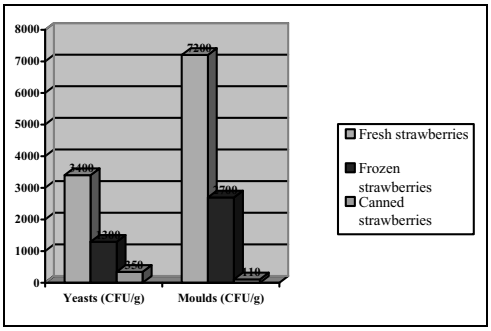

Fig. 3. Yeasts and moulds colony formatting units for strawberry samples analysed

Yeasts and moulds had the lowest values for canned strawberries, followed by frozen and fresh strawberries.

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PREVALENT *SALMONELLA* SEROTYPES IN SOME ROMANIAN POULTRY FARMS

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Abstract

The European Centre for Disease Prevention and Control and the European Food Safety Authority devote considerable resources to develop tools and recommendations to improve epidemiological investigation of food-borne outbreaks. One of the most important food-borne agents monitored are Salmonella spp. and in order to reduce risk to public health, several policy actions were designed to control salmonella infections. Present study aimed to investigate the prevalence of Salmonella serotypes in seven poultry holdings located in Giurgiu County, Romania. More than three thousand biological samples have been submitted to bacteriological exam. Briefly, samples were pre-enriched in buffered peptone water followed by enrichment in Modified Rappaport-Vassiliadis, Enrichment broth and Selenite Cystine Broth. Resulting cultures have been plated onto XLD, Istrate Meitert and Wilson-Blair agar plates, incubated at 37°C for 24h. Presumptive Salmonella isolates have been biochemically and serologically confirmed. Between 2008 and 2010 were identified and characterized Salmonella serotypes (525 isolates) as follow: S. Virchow (182 strains), S. Enteritidis (10 strains), S. Tallahassee (3 strains), S. Infantis (273 strains), S. Tennessee (9 strains), S. Mbandaka (8 strains), S. Newport (9 strains), S. Amsterdam (2 strains), S. Salamae (1 strain), S. Kottbus (2 strains), S. Glostrup (1 strain), S. Livingstone (2 strains), S. Isangi (1 strain), S. Hadar (19 strains), and S. Thompson (3 strains). The highest prevalence in 2008 registered S. Virchow (182/199) and in 2009-2010 S. Infantis (140/164 and 124/162 respectively). These results show the heterogeneity of Salmonella strains circulating in poultry farms and the competition of serotypes in different years. Also, the high number of isolates with zoonotic potential (264 strains S. Infantis, 10 strains S. Enteritidis) requires increased attention in food security.

Key words**:** *poultry salmonellosis, salmonella incidence, salmonella surveillance*

INTRODUCTION

In 1880, Eberth and Koch revealed in people died of typhoid fever a bacillus which they considered the etiologic agent of this disease [11]. In 1885, Theobald Smith discover the type bacterium (*Salmonella enterica* var. *Choleraesuis*), and denominate the genus and bacteria after Daniel Elmer Salmon, an American veterinary pathologist. The genus *Salmonella* was finally formally adopted in 1900 by J. Lignières [10].

The genus *Salmonella* contains two species (*S. enterica* and *S. bongori*) and several subspecies biochemical and genomic differentiated [6].

The prevalence and involvement in human infections of specific *Salmonella* serotypes differs from a geographical region to other. In *Salmonella* food poisoning are mainly involved *Salmonella* Enteritidis and *Salmonella* Typhimurium, and more rarelly *Salmonella* Infantis, *Salmonella* Virchow and *Salmonella* Hadar [12]. The dominant *Salmonella* serotypes in different Romanian regions vary from year to year and continuous monitoring of serotypes can supply valuable data in *Salmonella* control.

In accordance with Regulation (EC) No 2160/2003 *Salmonella* control programmes aim at reaching the *Salmonella* reduction target set by Regulations (EC) No 1003/2005, No 1168/2006 and No 646/2007 covering in breeding flocks the serotypes *S.* Enteritidis*, S.* Typhimurium*, S.* Infantis*, S.* Virchow and *S.* Hadar, and in laying hen flocks, chickens and

turkeys the serotypes *S.* Enteritidis and *S.* Typhimurium [8].
In the Annual Report on Zoonoses and Food-borne Outbreaks in the European Union for 2010, the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) shows that *Salmonella* cases in humans fell by almost 9%. In European Union, the most frequently reported causes of the food-borne outbreaks were *Salmonella* (31% of all outbreaks), Norwalk-like viruses (15%) and *Campylobacter* (9%). The most important food sources in the outbreaks were eggs and egg products, mixed and buffet meals and vegetables and derived products [9].
In poultry, paratyphoid *Salmonellas* rarely produce clinical signs, usually chickens are asymptomatic carriers of bacteria. Clinical signs are usually seen in poultry less than two weeks of age, are non-specific (e.g. diarrhoea, listless, fluffed up feathers) and similar for all serotypes. The bacteria colonise the intestinal tract and occasionally the reproductive tract of carrier fowls. This situation can cause contamination of carcasses and eggs designed for human consumption [6].
The present study aimed to investigate the prevalence of *Salmonella* serotypes in seven poultry holdings located in Giurgiu County, Romania, in the period 2008 - 2010.

MATERIAL AND METHOD

From seven layer and broiler holdings located in Giurgiu County were collected: 1,532 samples in 2008, 833 samples in 2009 and 741 samples in 2010.
Isolation, identification and serotyping of *Salmonella* spp. has been carried out using standardized methods [1, 2, 3, 4, 5, 12]. Briefly, samples were pre-enriched in buffered peptone water followed by enrichment in Modified Rappaport-Vassiliadis, Enrichment broth and Selenite Cystine Broth. Resulting cultures have been plated onto XLD, Istrate Meitert and Wilson-Blair agar plates, incubated at 37°C for 24h. Presumptive *Salmonella* isolates have been biochemically and serologically confirmed.
The populations of layers and broilers in all investigated holding were 8,860,612 subjects in 2008, 8,852,478 subjects in 2009, and 7,104,428 subjects in 2010.

RESULTS AND DISCUSSIONS

In 2008, the positive samples were 12.88% (199/1532), but almost all strains were isolate in 3 holdings (192/199). One holding was *Salmonella*-free in 2008, and in two holding were isolated only one strain.
In 2009, the number of positive samples was lower than in 2008, but the proportion of positive samples per total samples collected increased to 19.69% (164/833). Three holdings were *Salmonella*-free in 2009; all of them with a low prevalence of *Salmonella* in 2008, but the holding *Salmonella*-free in 2008 have been 18.48% (7/18) of samples positive.
A similar situation was noticed in 2010, when 21.86% (162/741) of samples were positive. One holding maintained the *Salmonella*-free status from 2009 to 2010. Two negative holdings in 2009 have been re-contaminated, but the proportion of positive samples was still low (table 1).
In our study the paratyphoid *Salmonella* contamination covered 85.72% holdings in 2008, 57.12% holdings in 2009 and 85.72% holdings in 2010. These data are higher than those reported in the whole Romania or in EU. In Romania the prevalence of *Salmonella* in breeding flocks of *Gallus gallus* (all types of breeding flocks, flock-based data) has been 2.9% in 2008, 1.5% in 2009 and 12.8% in 2010. In EU the prevalence has been 1.8 in 2008, 2.7 in 2009 and 2.0 in 2010 [7, 13, 14].
Despite the established prevention programs, the holdings with high level of contamination (holdings 1, 2 and 7) maintained their increased level of contamination during the entire period of study (fig. 1).

Table 1. Proportion of *Salmonella* spp. positive samples in seven poultry holdings during the period 2008-2010, in Giurgiu County

Poultry holding	2008			2009			2010		
	No. samples collected	Positive samples		No. samples collected	Positive samples		No. samples collected	Positive samples	
		No.	%		No.	%		No.	%
1	448	101	22.54	206	67	32.52	149	48	32.21
2	204	61	29.90	124	55	44.35	130	95	73.08
3	195	1	0.51	127	0	0.00	109	3	2.75
4	241	1	0.41	179	0	0.00	117	6	5.13
5	269	5	1.86	98	0	0.00	141	0	0.00
6	58	0	0.00	38	7	18.42	90	8	8.89
7	117	30	25.64	61	35	57.38	5	2	40.00
Total	**1532**	**199**	**12.99**	**833**	**164**	**19.69**	**741**	**162**	**21.86**

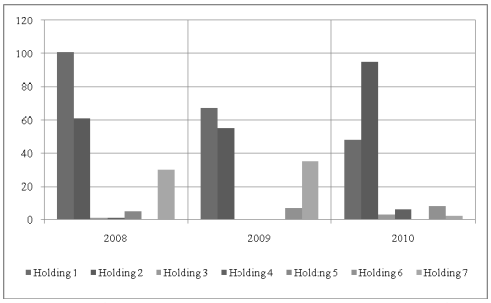

Fig.1.Evolution of *Salmonella* spp. positive samples in seven poultry holdings during the period 2008-2010, in Giurgiu County

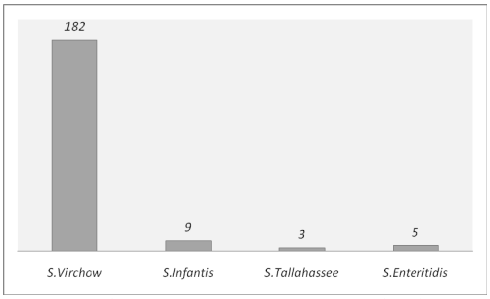

Fig. 2. Number of *Salmonella* serotypes isolated in seven poultry holdings in 2008, in Giurgiu County

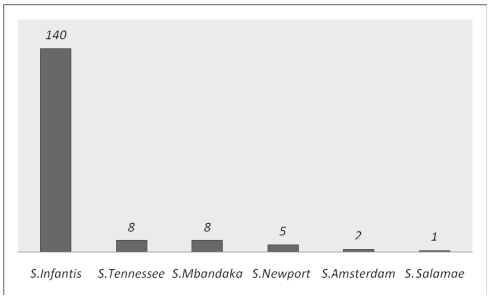

Fig. 3. Number of *Salmonella* serotypes isolated in seven poultry holdings in 2009, in Giurgiu County

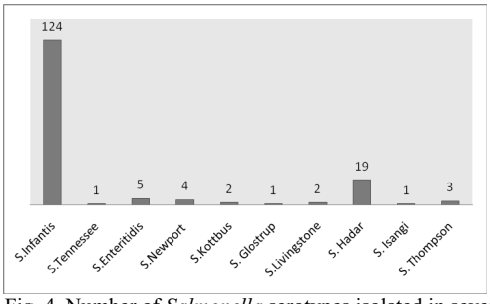

Fig. 4. Number of *Salmonella* serotypes isolated in seven poultry holdings in 2010, in Giurgiu County

Between 2008 and 2010 were identified and characterized *Salmonella* serotypes (525 isolates) as follow:

- *S.* Virchow: 182 strains;
- *S.* Enteritidis: 10 strains;
- *S.* Tallahassee: 3 strains;
- *S.* Infantis: 273 strains;
- *S.* Tennessee: 9 strains;
- *S.* Mbandaka: 8 strains;
- *S.* Newport: 9 strains;
- *S.* Amsterdam: 2 strains;
- *S.* Salamae: 1 strain;
- *S.* Kottbus: 2 strains;
- *S.* Glostrup: 1 strain;
- *S.* Livingstone: 2 strains;
- *S.* Isangi: 1 strain;
- *S.* Hadar: 19 strains;
- *S.* Thompson: 3 strains.

The highest prevalence in 2008 was registered *S.* Virchow (182/199) and in 2009-2010 *S.* Infantis (140/164 and 124/162 respectively (figures 2-4).

In EU, the prevalence of the serovars *S.* Enteritidis, *S.* Typhimurium, *S.* Infantis, *S.* Virchow and *S.* Hadar in adult breeding flocks was 1.3% in 2008, 1.2% in 2009, and 0.7% in 2010[13]. In our study, these serotypes have the following prevalence: 12.92 (196/1532) in 2008, 16.80% (140/833) in 2009, and 19.97% (148/741) in 2010.

An interesting situation was noticed in 2010, when Romania reported the highest prevalence (12.5%) of positive flocks with serovars other than *S.* Enteritidis, *S.* Typhimurium, *S.* Infantis, *S.* Virchow and *S.* Hadar. This has been correlated with data from Giurgiu County, when the serotypes *S.* Tennessee, *S.* Newport, *S.* Kottbus, *S.* Glostrup, *S.* Livingstone, *S.* Isangi, *S.* Thompson covered 8.64% (14/162) of the isolated in 2010. Furthermore, *S.* Typhimurium and *S.* Virchow were not isolated, and *S.* Enteritidis had a prevalence of 3.09% (5/162). In EU, the most common of the targeted serovars in breeding flocks was *S.* Enteritidis [13].

CONCLUSION

The heterogeneity of *Salmonella* strains circulating in poultry farms and the competition of serotypes in different years are high. The high number of isolates with zoonotic potential (264 strains *S.* Infantis, 10 strains *S.* Enteritidis) requires increased attention in food security.

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QUALITY ATTRIBUTES OF FRESH-CUT LETTUCE TREATED WITH COLD PLASMA

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Abstract

Cold plasma is a novel method that has proved to be capable as a sanitizing process due to its antimicrobial effects. This study aims to highlight the optimization of this technique for maintaining freshness and safety of fresh-cut lettuce without using any other chemical preservatives. The used atmospheric pressure plasma jet is driven by a radio frequency generator (27.12 MHz) with argon as working gas. The jet has been used at different operating powers in order to evaluate the optimal process parameters that do not affect the product quality. The quality of the lettuce leaves was assessed by optical methods such as chlorophyll fluorescence imaging analysis, fluorescence spectroscopy and colour measurement before and after plasma treatment, and also during the storage period. Depending on the applied process parameters, the effects of the cold plasma treatment on the quality of lettuce leaves can be controlled. However, the treatment conditions have to be adapted to each type of commodity.

Key words: *chlorophyll fluorescence imaging analysis, cold plasma, fluorescence spectroscopy, lettuce*

INTRODUCTION

Nowadays, consumers have been demanding more than ever both convenience and high quality ready-to-eat products. Production and consumption of minimally processed vegetables has increased in many countries in recent years [21].

As a consequence, the exploration of new technologies to preserve the quality of fresh-cut commodities and to extend their shelf life, without using any additives is highly desirable [1, 4]. Fresh produce is more susceptible to disease organisms because of the increased respiration rate after harvesting [24].

The main spoilage mechanisms affecting the shelf-life of the fresh-cut products are oxidation phenomena, such as browning, degradation and oxidation of pigments, due to the enzymatic activity of the cut leaves [6, 23].

Water loss, softening, translucency or surface dehydration may also occur as a result of their processing, conditioning and storage [23, 27].

In the context of latest foodborne outbreaks associated with these type of commodities, due to the presence of the pathogenic microorganism (*e.g. Escherichia coli* O157:H7) [10, 16], food scientists and food engineers turned their attention to novel decontamination and sterilization methods, suitable for application to fresh-cut products and different from the traditional ones [5, 8], which are based on a thermal treatment or are using different chemical sanitizers in order to inactivate foodborne microorganisms.

These innovative preservation methods have to maintain the requested characteristics of the food products as long as possible and must be effective in terms of food safety and should be economically profitable.

Cold plasma treatment is an emerging process technology for the sterilization and decontamination of different food products. Its application has a potential for the treatment of fresh produce and fresh-cut fruits and vegetables [9, 19].
Plasma is composed of gas molecules which have been dissociated by an energy input. It is constituted of particles in permanent interaction, such as photons, electrons, ions, atoms, free radicals and excited or non-excited molecules [5, 20].
As a dry, non-thermal process, cold plasma is representing an interesting and flexible sanitizing method that uses electricity and a carrier gas, such as air, nitrogen, argon or oxygen [7, 14, 22].
Recently, interactions of reactive species immanent in cold plasma and secondary plant metabolites of lamb's lettuce were investigated [11].
A correlation between applied treatment time and leaf surface morphology has been found. Secondly, a considerable reduction of phenolic acids in case of the exposed lamb's lettuce leaves was observed.
Consequently quality parameters must be considered when evaluating new treatment techniques.
While the antimicrobial effectiveness of cold plasma has successfully been demonstrated in previous reports of various research groups [12, 13, 15, 17, 26, 28], according to our knowledge, there is limited information available related to the influence of cold plasma treatment on the freshness and viability of fresh-cut vegetables.
Therefore, the objective of this study was to evaluate the impact of plasma treatment on the quality and viability as well as the effect of storage on the treated samples of fresh-cut lamb's lettuce leaves using chlorophyll fluorescence, fluorescence spectroscopy and colour measurement.

MATERIAL AND METHOD

Plant material

Being a main ingredient for many ready-to-eat salads, fresh-cut lamb's lettuce (*Valerianella olitoria* Poll.), was chosen as a model leafy vegetable for these experimental activities, in order to characterise quality attributes of the plasma treated samples.
Lamb's lettuces grown in a patch at Leibniz Institute for Agricultural Engineering Potsdam-Bornim, Germany, without any preservatives added and free from pesticides, were harvested right before the beginning of the experiments. Leaves about 7 cm length and 2 cm width were cut right from the ground using a gardening scissors.
Leaves were washed with cold tap water and dried at room temperature. The leaves were chosen randomly and all tests were conducted in triplicate.

Cold plasma treatment and storage conditions

The used equipment was an atmospheric pressure plasma jet (APPJ) driven by a radio frequency (RF) generator (27.12 MHz) with argon as working gas (Photo 1).
The plasma device consisted of a ceramic nozzle equipped with two electrodes: the inner needle electrode, placed in the centre of the nozzle and a grounded ring electrode, placed near the outlet surrounding edge of the nozzle.
The configuration of the device also includes a power supply, represented by the RF generator and the matching unit, and a gas supply.

Photo 1. Set-up of the atmospheric pressure plasma jet.

Fresh-cut lamb's lettuce leaves were fitted into transparent acrylic glass sample holders right before the treatment.
The used distance between the tip of the plasma jet and the samples was set to 2 cm, and the plasma treatment was applied for 1, 2, 3 and 4 min. Untreated plasma samples were noted as 0

min samples. Experiments were conducted at room temperature.
Plasma treated samples and also control samples were air packed by hand in transparent plastic bags (Roth, Germany).
The wall thickness of the bags was 70 μm and the bags had a length of 19 cm and a width of 13.5 cm with a volume of 390 ml. Air packaging consisted of sealing the bags with their wire without eliminating air. Packed samples were stored in the dark at 5°C for 4 days.

Chlorophyll fluorescence imaging analysis

Chlorophyll fluorescence imaging analysis has been adopted as a fast and non-invasive method [25] to determine stress effects in lamb's lettuce leaves after different plasma treatments This method was used to describe the physiological status of treated leaves immediately after the plasma treatment and during the storage period in relation to untreated control samples.
Leaf chlorophyll fluorescence measurements were conducted on the top of each sample, the exact plasma treated area being analysed. The method was used to correlate the dynamics of the physiological effects of the plant tissue with the applied external treatment.
The used device was a FluorCAM fluorescence imaging system (640MF, PSI, Brno, Czech Republic). Right after the samples were taken out from the storage conditions, before measuring chlorophyll fluorescence parameters, the leaves were pre-darkened for about 5 min, according to the used protocol.
The maximum chlorophyll fluorescence signal (F_m) and the minimum chlorophyll fluorescence signal (F_0) were measured. Using these two parameters, the maximum PSII photochemical efficiency was calculated, according to the ratio: $F_v/F_m = (F_m - F_0)/F_m$. The raw data were analysed using the manufacturer's software package (FluorCAM 6, PSI, Brno, Czech Republic).

Fluorescence spectroscopy assessment

Fluorescence measurements were conducted using a PerkinElmer LS55 fluorescence spectrometer (Rodgau-Jügesheim, Germany) equipped with a pulsed xenon lamp and a red-sensitive photomultiplier (R928). The excitation wavelengths and the corresponding parameters that were used are given in table 1. The illuminated area was 10 mm high and 3 mm wide.

Table 1. Parameter settings of fluorescence emission spectra measurements

Excitation (nm)	Emission (nm)	Emission Slit (nm)	Low pass filter (nm)
280	300-500	10	290
470	500-800	10	515
490	550-800	10	515

In order to assess the samples by using the fluorescence spectroscopy, each lettuce leaf had to be cut out using a cork borer of 14 mm diameter. Metal cuvettes were filled with transparent gel plates so that the sample leaves could be placed on top of them, right under a quartz glass disks. Subsequently, the sample holders were closed and the fluorescence spectra of the leaves were recorded.

Colour measurement

For the colour measurements the Hunter Lab-system was used. The values of L (light), a (redness) and b (yellowness) were taken at three spots of each leaf using a CR-300 Minolta Chroma Meter (Minolta Camera Co., Ltd., Japan) with illuminant D65 and 8 mm diameter measuring area and 0° viewing angle.
Three random areas of the treated leaf surface were measured for each sample at day 0 before the plasma treatment (L_0, a_0, b_0) and immediately after the treatment and also each day during the storage period (L, a and b). L (lightness, from 0 for black to 100 for white), a (redness) and b (yellowness) colour readings were recorded. From the measured values of L, a and b two colour terms were calculated: total colour differences, expressed as:
$\Delta E = [(L - L_0)^2 + (a - a_0)^2]^{1/2}$
and the difference in chroma between the samples, expressed as
$\Delta C = (a^2 + b^2)^{1/2} - (a_0^2 - b_0^2)^{1/2}$.
This colour index was used because it detects colour changes similar to the differences perceptible by human eye [2]. Each sample was measured three times, the given results being the average of the assessed measurements.

Data analysis

All plasma treatments were performed in triplicate. Chlorophyll fluorescence measurements and spectral data were firstly exported using programs of the manufacturers. All the given results represent the average of the assessed measurements along with the standard deviations.

RESULTS AND DISCUSSIONS

Effect of plasma treatment on photochemical efficiency

Photosynthesis is a complex physiological process that occurs in green plants based on different biophysical and biochemical reactions [25]. Chlorophyll fluorescence was measured in order to assess the response of the fresh-cut lamb's lettuce leaves to different process parameters of plasma treatment, in order to quantify the produce quality.

According to figure 1, which indicates the response of the plant tissue to the applied external stress, the level of maximum photochemical efficiency (F_v/F_m) was significantly affected by direct plasma treatment at 2 cm distance between plasma jet tip to the leaf surface.

Extended treatment durations for 2 min, 3 min, and 4 min led to similar inhibition of the metabolic activity of the tested samples. In contrast, 1 min plasma treatment showed a moderate alteration of F_v/F_m.

For all assessed samples, the initial inhibition of photochemical efficiency was maintained during the entire storage period, without significant variations during the determined period of time (Fig. 2).

These results indicate that chosen treatment conditions led to irreversible damage on the metabolic active plant system after 1 min duration of plasma treatment.

Hence, this quantification is valuable for further investigations, were shorter treatments and higher distances between plasma tip and sample surface can be applied to avoid inhibition of the metabolic activity of the plant.

Effect of plasma treatment on fluorescence emission spectra

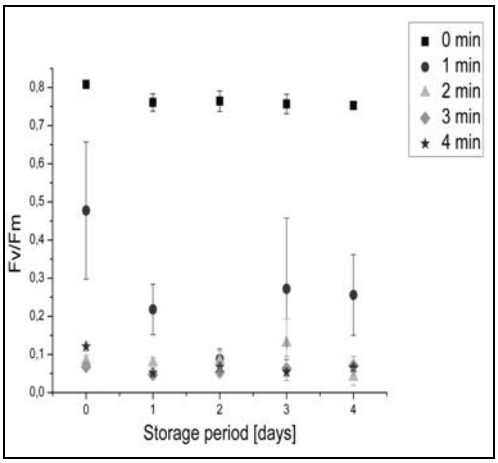

Fig. 1: Maximum photochemical efficiency of lamb's lettuce leaves after different plasma treatment times and subsequent storage.

In this study, fluorescence emission measurements were used as a potential indicator of the internal quality of plasma treated lettuce.

The fluorescence of photosynthetic pigments was assessed in order to evaluate quality changes of lamb's lettuce leaves according to the different plasma treatments that were applied. Fluorescence emission spectra during the storage period for each plasma treatment are presented in Fig. 3.

In the case of the untreated plasma samples (Fig. 3, A) there can be seen that during the storage period, the fluorescence intensity is decreasing, as a result of the degradation of chlorophyll. Plasma treated leaves however, showed different intensities of fluorescence emission as a response to the duration of the treatment.

After 1 min plasma treatment, the fluorescence intensity can be compared to that of untreated leaves, but with increasing treatment time, the fluorescence intensity was inversely proportional to the duration of the related plasma treatment.

This result can indicate that a decline of the fluorescence rate, irrespective of excitation wave and emission wavelength hence may suggest a decrease of the chlorophyll content. Grzegorzewski et al. (2011), however, reported that due to the plasma's highly reactive species, a disintegration of cell membranes may take

place, followed by a release of cellular components.
These component may than lead to different results, were the fluorescence intensities of plasma treated samples will have higher values than those of the control samples. Hence, because experimental results on the impact of plasma treatment on physiological activity of fresh-cut vegetables are sparse, further studies are needed, to complete a correlation between the antimicrobial effect of this method and its effects on food products.

Effect of plasma treatment on colour

The colour of fresh-cut lamb's lettuce leaves was affected by the direct (distance of 2 cm) plasma treatment (Fig. 4). Considerable total colour differences were found on day 0 for all plasma treated samples. Immediately after the plasma treatment, the 4 min treated samples showed the most notable colour difference.
During the storage period, untreated lettuce leaves recorded a more pronounced colour variation with respect to day 0 than the treated samples.
During the storage period, the variation in colour was mostly due to changes in green-red (a) values, being more sensitive over storage time.

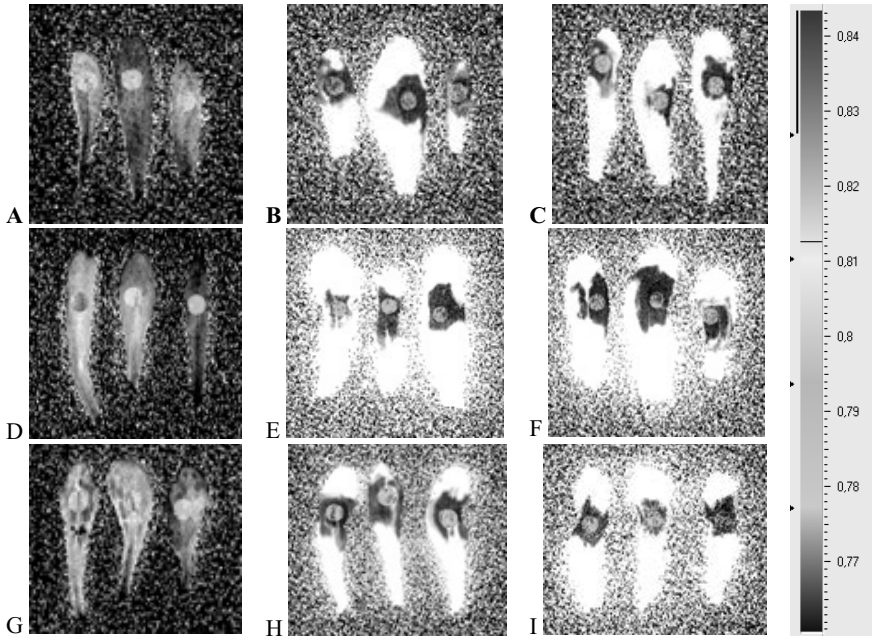

Fig. 2: Alteration of the F_v/F_m ratio on day 0 for the 0, 2 and 4 min plasma treatment (A, B, C), on day 2 for the 0, 1 and 3 min plasma treatment (E, F, G) and on day 4 for the 0, 1 and 2 min plasma treatment.

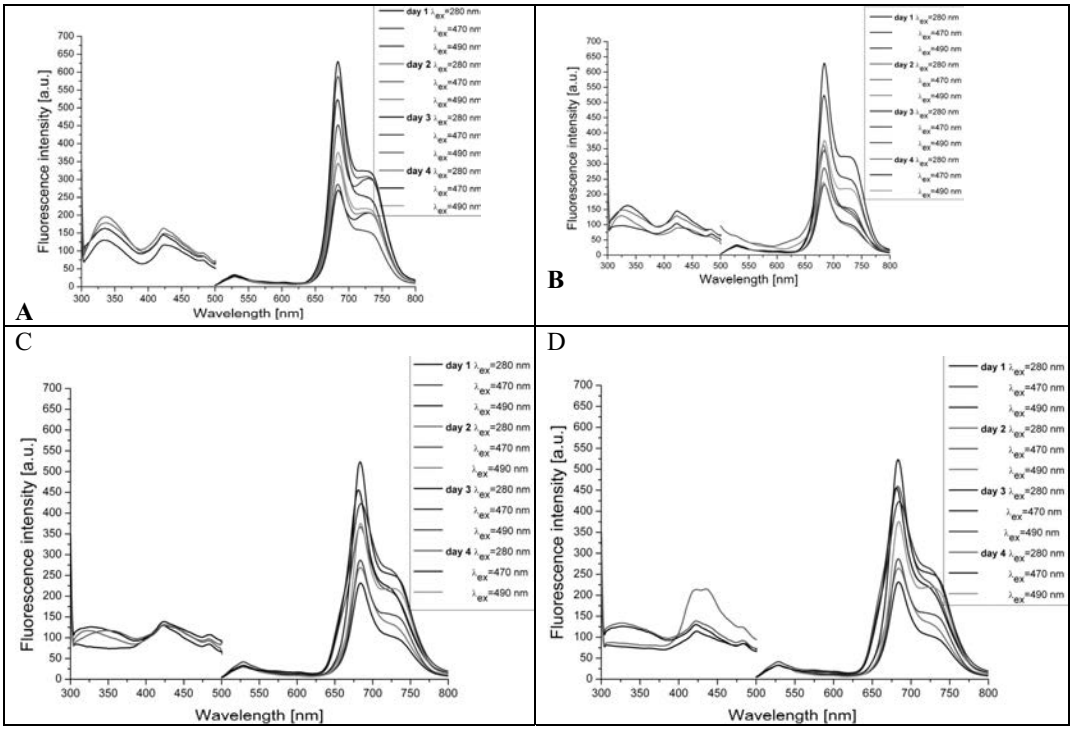

Fig. 3: Fluorescence of lamb's lettuce after 0 min (A), 1 min (B), 3 min (C) and 4 min (D) plasma treatment.

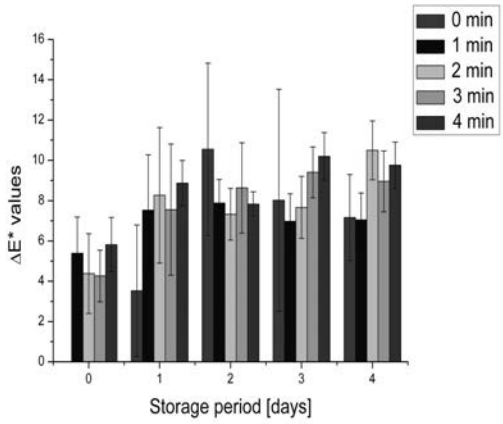

Fig. 4: Total colour differences during storage period for control and 2 cm plasma treated samples.

On the contrary, *L* and Δ*C* values (results not shown) proved to be more uniform during the tested period. As browning can be expressed by the a-value, our results are in agreement with the findings of Lonchamp et al. (2009), regarding the browning development of the leaves during the storage period, whereas in our case, the loss of green colour being more evident for the untreated samples.

CONCLUSIONS

As Perni et al. (2008) noted, the adjustment of process parameters of a plasma device can modify its effects on the treated sample. More importantly, when food products are treated and especially in the case of fresh-cut commodities, which rapidly lose their quality mainly because of degradation processes (respiration, senescence, natural microbial flora), an assessment of the final product quality is needed.

A direct plasma treatment, with a 2 cm distance from the treated sample, which was applied for 1 min, presented good results in terms of final quality attributes that were assessed immediately after the treatment but also during the storage period. Depending on the applied process parameters, the quality of the lettuce leaves was less affected by the cold plasma. However, the treatment conditions have to be adapted to each type of commodity.

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IN VITRO EVALUATION OF THE RELATIONSHIPS BETWEEN SOME FUNGAL PATHOGENS OF BLACK CURRANT CROP AND SOME SAPROPHYTIC FUNGI

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Abstract

Blackcurrant crop is affected by fungal pathogens, such as Mycosphaerella grossulariae, Drepanopeziza ribis (Kleb.)v. Hohn., Alternaria tenuissima (Kunze) Wiltshire, Sphaerotheca mors-uvae (Schw.) Berk, Cronartium ribicola Dietr., Fusarium oxysporum E.F. Sm. Swingle, Botrytis cinerea Pers. In this study has been evaluated the in vitro antagonistic effect of some fungal saprophytic isolates towards some of these phythopathogens in order to establish which of them could be used as biological control agents of black currant crop. In all the cases has been observed the in vitro antagonistic effect of the strain of the fungus Trichoderma spp.

Key words: *blackcurrant crop, biological control agents, in vitro antagonism, phytopathogenic fungi, Trichoderma spp*

INTRODUCTION

Black currant (*Ribes nigrum* L.) is high appreciated for alimentary and therapeutic value of its fruits. Young leaves and buds are also used for obtain alimentary supplements and phytopharmaceutical products, like medicinal teas and gemoderivatives [1, 9]. The antimicrobial activity of some *Ribes nigrum* essential oils and of some gemoderivatives containing buds extracts of *R. nigrum* has been demonstrated [7].
Since the quality of these products used to obtain phytopharmaceutical products may be impaired by various pathogens, especially fungi both in vegetation period of culture, and in the storage period, the establishment of biological control measures of these pathogens it is necessary.
Blackcurrant crop is affected by pathogens, like viruses and fungi [8, 4]. In 2009-2012, in experimental plots of S.C. Hofigal S.A. were identified the fungal pathogens *Mycosphaerella grossulariae, Drepanopeziza ribis (Kleb.)v. Hohn., Alternaria tenuissima (Kunze) Wiltshire, Sphaerotheca mors-uvae (Schw.) Berk, Cronartium ribicola Dietr., Fusarium oxysporum E.F. Sm. Swingle*. These phytopathogens could alter the quality of the vegetable organs like leaves or fruits which are used as a raw-material for obtaining phytopharmaceutical products.
Our work aimed to screen *in vitro* activity of some saprophytic fungi against some phytopathogens isolated from experimental plots of S.C. Hofigal S.A. from Bucharest in 2010-2011 in view to establish which of them could be used as biological control agents. Biological control using such antagonistic fungi is an alternative, safety for humans and non-polluting method for control the diseases produced by phytophathogens [2].

MATERIAL AND METHOD

Biological material was represented by isolates of phytopathogenic and saprophytic fungi. The

two strains of pathogenic fungi isolated from blackcurrant crop from experimental plots of S.C. Hofigal S.A: one strain of *Botrytis cinerea* isolated from mature fruits in July 2011 and one strain of *Fusarium oxysporum* isolated from leaves in April 2010.

The saprophytic fungi of which activity against both phytopathogens was evaluated were *Trichothecium roseum* Link, *Epicoccum nigrum* Link, *Chaetomium globosum* isolated from leaves of blackcurrant in 2010 and one strain of *Trichoderma viride* isolated in 2010 from wheat grains, County Constanţa provenance.

The *in vitro* testing was performed in the laboratory at Research-Development Institute for Plant Protection Bucharest. The dual test assay [5] was used for *in vitro* screening of the relationships of pathogenic and saprophytic fungi. Each variant had 3 replicates. The control was the variant whereby in the same Petri plate, two colonies of the same pathogenic fungus were inoculated. In each variant two fungi, one pathogenic fungus and one antagonistic fungus were inoculated at equal distance one from another (3 mm distance) and from equal distance from the centre of Petri plates containing PDA (potato-dextrose-agar) medium.

Macroscopic aspect of the contact line was evaluated by the method described by Ana Hulea [3].

The type of interaction was assessed by using the x ratio between of the internal radial growth (i) and external radial growth of the pathogenic fungus (noted A) and the saprophytic fungus (noted B). This is the method used by Romanian to evaluate the degree of antagonism [11]. Formula used was x = iA/iBxeB/eA, whereby iA = internal radius of the colony of the phytopathogenic fungus, iB = nternal radius of the colony of the antagonistic fungus, eB = external radius of antagonistic fungus, eA = external radius of the colony of the phytopathogenic fungus. The ratio x>1 indicates the lack of antagonism between the two fungi, x<indicates a phenomenon of varying degrees of antagonism, the power antagonism being in variants whereby x value is close upon 0 than 1.Value of x=1 indicates no mutual interaction and an indifferently relationship.

In order to obtain more information about the antagonism it was considered only external and internal radial growth of the pathogen and inhibition percent (I %) was calculated by the formula: I % = (eA-iA/eA)x100 [6,12]. A grow category scale from 0 to 4 was used in order to establish the degree of inhibition, where 0= no growth inhibition, 1=1-25% growth inhibition; 2 = 26-50% growth inhibition; 3 = 51-75% growth inhibition, 4 = 76-100% growth inhibition, according to Živković Svetlana [12].

The observations and biometric measurements were taken at 3 and 6 days after inoculation. Photos were taken at 6 day after inoculation.

RESULTS AND DISCUSSIONS

Botrytis cinerea is known to produce grey mould on fruits [4], and flower blight in blackcurrant crop [11]. *Fusarium oxysporum* is also known to produce a vascular wilt disease on plants.

The aspect of contact area between the pathogen and antagonistic fungus which are grown in the same petri dish depends on the growing rate of the colony [3].

In control variants (the same phytophathogenic fungus placed face to face), contact line was straight, sign of uniform growth of both colonies placed in the same Petri plates (fig. 1).

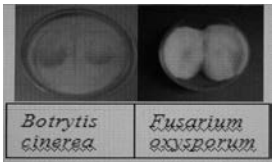

Fig. 1 Macroscopic *in vitro* aspects of the colonies of *Botrytis cinerea* and *Fusarium oxysporum* isolates on PDA medium

In the variants whereby it was tested the effect of the presence of the fungi *T.roseum*, C. *globosum* and *E. nigrum* towards fungus *B. cinerea*, the contact area was a curve, with concavity oriented towards the colony of the saprophytic fungus which had a slower growth (Fig.2).

Only in the variant whereby was tested the relationship between *B. cinerea* and *T. viride*, the aspect of the contact area was a nearly straight and higher line than the colonies of the two fungi, due to the intermingling of the

hyphae of both fungi (Fig. 2B). In portion of the colony of the strain of *B. cinerea* oriented towards those of the fungus *T. viride* an area of very weakly developed mycelium was observed. After three days to inoculation, in variant *B. cinerea*/*C. globosum*, at the contact zone between the colonies of the fungi a very narrow zone of inhibition has been observed (Fig. 2C).

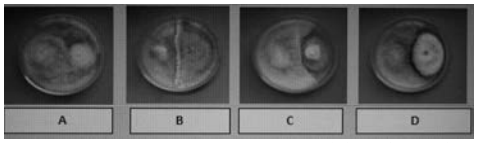

Fig. 2 Macroscopic *in vitro* aspects of the relationship between phytopathogen *Botrytis cinerea* and *Trichothecium roseum* (A), *Trichoderma viride*(B), *Chaetomium globosum* (C) and *Epicoccum nigrum* (D), on PDA medium

In variants whereby was tested the action of the saprophytic fungi towards the strain of *F. oxysporum* the contact line observed after 6 days was a line, with the exception of the variant whereby the influence of *Trichoderma viride* towards *F. oxysporum* was tested. In this case, the contact zone was a curve with concavity oriented towards the phytophatogen *F. oxysporum*, *T. viride* being the fast growth fungus (Fig. 3B). This aspect has been directly correlated with the values of the coefficient x, in this case the relationship was the antagonism.

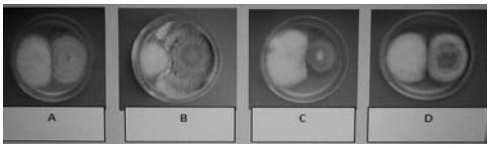

Fig. 3 Macroscopic *in vitro* aspects of the relationship between phytopathogen *Fusarium oxysporum* and *Trichothecium roseum* (A), *Trichoderma viride*(B), *Chaetomium globosum* (C) and *Epicoccum nigrum* (D), on PDA medium

Analysis of x coefficient value showed that only *T. viride* manifested a considerable antagonistic activity against the fungus *B. cinerea*. The fungus *T. roseum* had not manifested any antagonistic effect and the strains of *C. globosum* and *E. nigrum* showed a very slight action of inhibition towards *B. cinerea* at 3 days after inoculation, and then no antagonistic effect (Table 1, Table 2).

Table 1. The *in vitro* relationships between *Botrytis cinerea* and some saprophytic fungi on PDA medium, expressed by the x coefficient, calculated at 3 and 6 days, after Jouan et al. (1964)

Variant	at 3 days		at 6 days	
	x	relationship	x	relationship
B.cinerea/T. roseum	1.099	NA	1.958	NA
B. cinerea/T. viride	0.805	A	0.625	A
B.cinerea/ C. globosum	0.894	A	1.841	NA
B. cinerea/E. nigrum	0.939	A	1.261	NA
B. cinerea/B. cinerea	1.000	I	1.000	I

A – antagonistic, I- indifferently, NA- nonantagonistic

Table 2. The *in vitro* relationships between *Botrytis cinerea* and some saprophytic fungi on PDA medium, expressed by inhibition percent (I%) and category of growth inhibition on scale (0-4)

Variant	at 3 days		at 6 days	
	I%	category	I%	category
B. cinerea/T. viride	29.82	2	39.66	2
B.cinerea/ C. globosum	10.52	1	0	0
B. cinerea/E. nigrum	12.27	1	0	0
B. cinerea/B. cinerea	0.000	0	0.000	0

Towards the fungus *F. oxysporum*, the fungi which showed antagonism, in range of the degree of antagonism appreciated by x coefficient and inhibition percent were: *T. viride*, *T. roseum*, *E. nigrum* [table 3, table 4]. The most powerful antagonistic action manifested the strain of *T. viridae*. In this case the average of coefficient x at 6 days was 0.410 (Table 3) and inhibition percent was 57.3 (Table 4). The isolate of *C. globosum* showed no antagonistic effect. The other fungi showed only a slight antagonistic action (Table 3, Table 4).

Table 3 The *in vitro* relationships between *Fusarium oxysporum* and some saprophytic fungi on PDA medium, expressed by the x coefficient, calculated at 3 and 6 days, after Jouan et al. (1964)

Variant	at 3 days		at 6 days	
	x	relationship	x	relationship
F. oxysporum/T. roseum	0.881	A	0.829	A
F. oxysporum/T. viride	0.729	A	0.410	A
F. oxysporum/ C. globosum	0.935	A	1.435	NA
F. oxysporum/E. nigrum	0.890	A	0.892	A
F.oxysporum/F. oxysporum	1.000	I	1.000	I

A – antagonistic, I- indifferently, NA- nonantagonistic

Table 4 The *in vitro* relationships between *Fusarium oxysporum* and some saprophytic fungi on PDA medium, expressed by inhibition percent (I%) and category of growth inhibition on scale (0-4)

Variant	at 3 days		at 6 days	
	I%	category	I%	category
F. oxysporum/T. roseum	14.66	1	36	2
F. oxysporum/T. viride	24.33	1	57.3	3
F. oxysporum/ C. globosum	6.40	1	0	0
F. oxysporum/E. nigrum	5.60	1	28.99	2
F.oxysporum/F. oxysporum	0.00	0	0.00	0

CONCLUSIONS

Against the strain of fungus *Botrytis cinerea* isolated from blackurrant, only the strain of *Trichoderma viride* showed *in vitro* antagonism. The strains of *Trichothecium roseum*, *Chaetomium globosum* or *Epicoccum* nigrum have no antagonistic effect.

Against the fungus *Fusarium oxysporum*, the fungi which manifested antagonism, in range of the degree of their antagonistic action, were *T. viride*, *T. roseum* and *Epicoccum nigrum*. The fungus *C. globosum* was no antagonistic effect against *F. oxysporum*.

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RISK FACTORS FOR HUMAN GIARDIASIS IN TWO ROMANIAN COUNTIES

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Abstract

The hereby paper analyses the risk factors most often associated with the appearance of the disease produced by the flagellate protozoan Giardia lamblia in human population: gender, age and patient's place of origin. Applying the Student t-test in order to verify the hypothesies Ho (null hypothesis-the averages of the two paired series do not differ significantly) and Ha (the alternative hypothesis-the averages of the two paired series differ significantly) leads to the acceptance or rejection of one of them. The observational data had been recorded during 2009-2010 years on to the County hospitals of Tulcea and Vâlcea counties in Romania, the survey being based on more than 5000 human subjects: adults and children of both sexes. More such studies like ours should be carried out in Romania in order to have a better understanding of the epidemiology on parasitic diseases which affect the human population of both sexes, all ages and all life environments.

Key words**:** *human giardiasis, risk factors, test t-Student*

INTRODUCTION

Giardiasis is a very common parasitose, with universal distribution, caused by the protozoan flagellate *Giardia lamblia* sin. *Giardia intestinalis*. From clinically, it may be asymptomatic or may occur accompanied by digestive or extra-digestive symptoms. In humans, the disease occurs in both sexes and all ages [8]. We tried to align global trends pursued [1-7] in the typical epidemiological studies of parasitic infectious diseases.
Objectives we have set for this study included: investigation of groups of patients, adult and children's hospitals from Tulcea and Vâlcea Counties, throughout the years 2009 and 2010 (January-December), in order to identify a possible infestation with *Giardia lamblia*; establishing the incidence of infection, among adults and children, according to major factors: sex (M, F), age (children-adults), living environment (urban-rural), watching and recording seasonal changes; assessing the significance of risk factors on the degree of infestation with *Giardia lamblia*, both children and adults.

MATERIAL AND METHOD

The database prepared for the hereby work gathered 691 patients, adults and children from County Hospital Vâlcea (2009), and also 4326 adult and pediatric patients in the County Hospital Tulcea (2009, 2010). In both locations, human subjects have made coproparasitological analysis, which revealed in some of them, the presence of *Giardia lamblia* cysts. Student *t*-test [12] was applied in order to verify the two statistical hypotheses: Ho (null hypothesis) - the averages of both series are not significantly different pairs; Ha (alternative hypothesis) - the averages of two series pairs differ significantly.
We considered two categories of sex (male and female), two major categories of age (children and adults), and two major categories of living environment (urban, rural – where we had this information) of the host sample. The Student *t*-test for paired data was used to indicate a real symmetry of infestation in each subject – host

for *Giardia lamblia*. For simplicity, both for unpaired and paired t tests, we counted 12 items (can be considered sub-samples), corresponding to the 12 months of the year (e.g. item = total number of children with cysts in January, 2009).

We have compared the number of patients belonging to: both gender (male and female) and both age groups (children, adults). Admission of one of the hypotheses was based upon: a significance level α of 0.05, the number of freedom degrees and the value of "p" (probability of event is not due to chance, is less than or equal to the level of particular significance).

RESULTS AND DISCUSSIONS

a. Vâlcea County Hospital (2009)

The Student *t*-test for unpaired (independent) observations showed that there was a significant difference ($p < 0.0001$, $\alpha = 0.05$) between the number of children and adults infected with *Giardia lamblia* cysts. It was also compared, by Student *t*-test for paired observations, the number of patients in the two areas of life (urban and rural) in the two age groups – children and adults in each month of 2009 in which there were infestation with *Giardia lamblia*. The null hypothesis (averages of two series pairs "adult-children" are not significantly different) was rejected. Comparison of variable's series for each category (child / adult) showed a significant "p" (0.001 and 0.000).

The Student *t*-test for unpaired observations showed that there was a significant difference ($p = 0.0027$) between the number of male and female patients infected with *Giardia lamblia* cysts.

The Student *t*-test for paired observations compared the number of patients in the two sexes (male and female) belonging to the two age groups – children and adults, in each month of 2009 when the disease occurred with *Giardia lamblia*. The null hypothesis (the averages of both series are not significantly different pairs) was either accepted or rejected, based on gender. Comparison of variable's series for each category (child / adult) showed a insignificant "p" (about M sex) / significant (in relation to F sex) (0612 and 0000). On the other hand, analysis by Student *t*-test for paired observations, showed a significant difference between *Giardia lamblia* infestation, for the two categories of sex ($p = 0.000 < 0.05$). Therefore, the null hypothesis, stated above, was rejected.

Based on statistics, we found that the incidence of *Giardia lamblia* protozoan disease reported for adults and children, is higher in urban (50.38%) than rural (49.62%) environment in Vâlcea County. However, the statistical Student *t*-test for unpaired observations, indicated a significant difference ($p = 0.92$) in the case of *Giardia* positive cases detected in the two administrative categories. On the other hand, analysis by Student *t*-test for paired observations, showed a significant difference between infestation with *Giardia lamblia*, in the two types of living environments "urban areas" ($p = 0.902$).

Incidence of *Giardia lamblia* infestation reported to the whole sample of Vâlcea County (prevalence 56.58%) is higher in the adult population (68.54%) and among females (61.63%).

b. Tulcea County Hospital (2009, 2010)

Using Student's t test statistic for unpaired observations (independent), has shown a significant difference ($p < 0.0001$) between the number of children and adults infected with *Giardia lamblia* cysts in each year studied (2009, 2010) to T.C.H. To indicate a possible symmetry of *Giardia lamblia* infestation, we also applied Student *t*-test, but this time for paired data. Thus, we compared the number of patients in the two sexes (male and female) of the two age groups – children and adults, in each month of 2009 when the disease occurred with *Giardia lamblia*. The null hypothesis (the averages of both paired series are not significantly different) was rejected, because the comparison of variable's series for each category (child / adult) showed in both cases a significant "p" (0.001 and 0.002 in 2009, 0.000 and 0.000 in 2010).

By comparing the average number of patients infected with *Giardia lamblia* in two major categories of sex (male and female), Student's *t*-test for unpaired observations showed that there is an insignificant difference between the number of male and female patients infected

with *Giardia lamblia* cysts (p = 0.67 in 2009, p = 0.53 in 2010). On the other hand, analysis by Student *t*-test for paired observations, showed no significant difference between infestation with *Giardia lamblia*, in the two categories of sex (p = 0.553 for 2009, p = 0.334 in 2010).

Incidence of *Giardia lamblia* infestation reported to the whole sample of 2009 (overall prevalence = 13.97%) is higher among children (85.9%) and among females (52.79%). Incidence of *Giardia lamblia* infestation reported to the whole sample of 2010 (overall prevalence = 13.35%) is higher among children (84.96%) and among males (52.45%).

The results of our research in 2009 regarding a sample of adults and children from Vâlcea County Hospital (prevalence approx. 57%) are fit with [10] in the incidence higher among women (approx. 62%), respectively among adults (approx. 69%); with those of other authors [4], meaning that the most positive cases were reported in age groups 1-4 years and 25-44 years, and with the view that the environment urban-rural life not significantly affect the appearance of *Giardia* [5].

From the hereby study, about risk factors correlated with the appearance of *Giardia* in Tulcea County (2009, 2010), we can conclude the following informations: incidence in both years was found to be higher in children than in adults (approx. 85%); females are more prone to illness in 2009, compared to males in 2010, maintaining the same percentage of both sexes (approx. 53%), at the average prevalence of approx. 13%, in each year of study.

CONCLUSIONS

Analyzing the results of studies conducted on *Giardia lamblia* in different regions of the globe, we can say that the risk factors most often associated with this protozoan appearance are: age, sex, place of origin of patient, level of hygiene, foods consumed, seasonal, family relationship, sexual orientation, traveling abroad etc. On the other hand, as shown by the different results obtained in each of the two Romanian Hospitals, we see that there is no unanimity of opinion in the specialty literature.

We belive that in Romania, several studies such as those made by us, would be very useful for better understanding of the epidemiology of parasitic diseases affecting the human population of both sexes, all ages and from all life environments.

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A STUDY OF STOCHASTIC MODEL TO PREDICT THE GROWTH EVOLUTION OF FOOD BORN MOULDS

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Abstract

Over the last few decades stochastic study in biology has acquired global dimension. The prevention of the food product contamination with toxinogenic moulds is an actuality problem of microbiology. This means that it is necessary to have precise diagnostic methods in order to predict and describe in detail the dynamic of the alteration. Considering all these facts, we tried to develop in our paper a stochastic model to answer to this question. First, we have established some conclusions regarding the growth model; for this, we have made appropriate graphics corresponding evolution strains of Penicillium crysogenum and Fusarium graminearum during the 14 days. Secondly, we have examined the phenomenon of growth as a non-homogeneous Poisson process involving periodic variations in the occurrence rate and we have obtained the probability density function of the time intervals of growth.

Keywords**:** *food born moulds, random variable, stochastic model.*

INTRODUCTION

It is known that health is the top priority for all of us and that a balanced life that includes a proper diet leads to this goal. Starting from this consideration to justify growing interest of many researchers in biology, agriculture, medicine and food to ensure a healthy diet to include foods that meet certain nutritional parameters and are of course unaltered. Prevent food contamination with moulds toxin is thus a continuing concern of specialists in the fields mentioned above.
In [1] state that accurate methods are required to indicate detailed description of the dynamics of food spoilage. Deterministic mathematical modelling is a technique that we used for to present the growth and evolution of food born moulds is described in [4].
First, there have been isolated and identified the main species involved in the alteration of food products of intermediary activity. The strains have been screened for their toxinogenic activity by on-plate method and the toxins are quantified by Elisa Immunologic tests. From this collection four strains (Fusarium Graminearum MI 107 and MI 113 and Penicillium crysogenum MI 210) have been studied for their growth and mycotoxin (DON and OTA) production from a predictive point of view, depending on the environmental condition.
Since when analyzing the evolution mycelia growth rate are involved and random physical aspect, we intend to study this problem by applying stochastic modeling.

MATERIAL AND METHOD

2.1. Microorganisms

The *P.chrysogenum MI 210* and *F.graminearum MI 113* strains, established as potential producers of ochratoxin, respectively DON, were considered for this experiment to

watch and compare the rate of their growth in various conditions of temperature.

Before use, all strains were activated by successive passages on the average PDA for 7 days of culture at 27 ° C. The spores were harvested in a solution of water physiologic sterile (9 g / l of NaCl) going from the two strains of *Penicillium sp*. and *Fusarium sp*., through the scraping light area of colonies with a Pasteur pipettes.

The inoculation was done in the center of boxes with Petri Czapek-Dox in duplicate for each strain taken in work. On environment Czapek-Dox, it tried to follow mycelia growth (growth in cm.), for a strain of *Penicillium crysogenum (MI 210)* and one *of Fusarium graminearum (F. graminearum MI113).*

The samples have been studied in triplicate, under different temperature conditions (4° C, 12° C, 16° C, 20 ° C, 26° C, 30°C, 33° C, 36° C)

2.2. Deterministic modelling

In order to establish some conclusions regarding the growth model to determine which estimates best the studied situation, are made appropriate graphics corresponding to the development strains of *Penicillium crysogenum* and *Fusarium graminearum* during the 14 days that measurements were made, at certain temperatures. It is originally indicated, in accordance with graphics obtained, mathematical curves that estimates best studied phenomenon, namely primary model. It made such a development for *Penicillium crysogenum*, at temperature T = 30 °, as indicated rate of growth within 24 hours. In the next step is verified the growth model's plausible for the species *Pencillium crysogenum*.

Because the analysis period, the evolution phenomenon presents a continuous growth, the empirical points curve presents a form that can be estimated depending logarithmical function, the model that can be used for the evolution of the phenomenon is an approximation of the form:

$$y_t = f(t) + u_t \quad (1)$$

where:

y_t = the recorded values during the period examined phenomenon

$f(t)$ = the trend component that can be described with a logarithmical functions: $Y_t = f(t) = a + b * \ln t$ u_t = the residual variable.

2.3. Stochastic modelling

The Poisson process is considered the easiest of discontinuous Markov processes. Due to the wide applicability in areas such as biology, physics, technology, telecommunications, transportation, and so on, this process has a special place in probability theory.

It considers the following postulates for Poisson processes:

1) For the process $X(t)$ probability to have a change in time $(t, t+\Delta t)$ is $\lambda t + 0(\Delta t)$ where a given positive constant is λ.

2) The probability to occur over a change in $(t, t+\Delta t)$ is $0(\Delta t)$.

3) The probability to have been no change in the time $(t, t+\Delta t)$ is $1-\lambda t - 0(\Delta t)$.

These probabilities are independent of system states [2].

Let us note:

$$p_x(t) = P(X(t) = x), x \in N \quad (2)$$

Then,

$$p_x(t+\Delta t) = P(X(t+\Delta t) = x) =$$
$$(1-\lambda\Delta t)\, p_x(t) + \lambda\, p_{x-1}(t)\Delta t + 0(\Delta t) \Rightarrow$$
$$p_x(t+\Delta t) - p_x(t) = -\lambda\Delta t p_x(t) + \lambda p_{x-1}(t)\Delta t + 0(\Delta t) \quad (3)$$

If we divide this last relationship to Δt and then move to limit $\Delta t \to 0$ follows:

$$\frac{dp_x}{dt} = -\lambda p_x(t) + \lambda p_{x-1}(t), x \geq 1 \quad (4)$$

if $x = 0$, $p_{x-1}(t) = 0$ $\frac{dp_0}{dt} = -\lambda p_0(t)$ (5)

Equations (4) and (5) are the equations that characterize the Poisson process [3]. Their resolution is based on the initial condition:

$$p_x(0) = \begin{cases} 1, x = 0 \\ 0, x \in N^* \end{cases} \quad (6)$$

The expression:

$$p_x(t) = \frac{(\lambda t)^x}{x!} e^{-\lambda t} \quad (7)$$

is Poisson distribution, and it gives the probability that at time $t \geq 0$, the system to be

in state *x*. Relation (7) can be interpreted as the probability that changes occur within the length *t*.

The number of events in an interval of length *t* is Poisson distributed with mean and variance

$$E\left(X\left(t\right)\right)=Var\left(X\left(t\right)\right)=\lambda\cdot t \qquad (8)$$

A *random graph* is obtained by starting with a set of *n* vertices and adding edges between them at random [1]. A random graph G_t for a stochastic process $X\left(t\right)$ has not only a time dimension, but also a spatial dimension. At any snapshot at time *t*, one observes a realization of a random graph G_t

RESULTS AND DISCUSSIONS

The development strains of *Penicillium crysogenum* and *Fusarium graminearum* is a continuous process. At T = 26 ° C, the first determination of Penicillium crysogenum-day 1 to day 13-a, period in which the effect of increasing strain-linear growth. Before use, all strains were activated by successive passages on the average PDA for 7 days of culture at 27 ° C. The spores were harvested in a solution of water physiologic sterile (9 g / l of NaCl) going from the two strains of *Penicillium sp*. and *Fusarium sp*., through the scrapeing light area of colonies with a Pasteur pipettes. The analysis of the results and the determination the type of curve after which the moulds growth in the initial experience, it has been made both for *Penicilium crysogenu*m as well as for *Fusarium graminearum* with package programs Eviews.

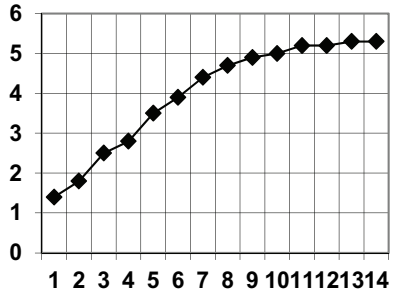

Fig. 1. The growth (cm) of *Penicillium crysogenum*

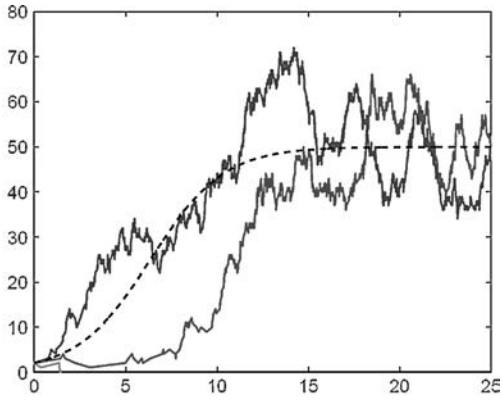

Fig. 2. The growth (mm) of *Penicillium crysogenum* - random Graph

The second measurement for Penicillium chrysogenum, from day 2 to day 12-a period in which the effect of increasing strains

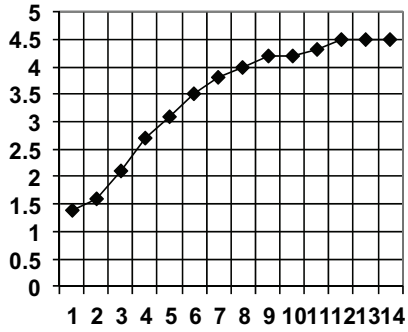

Fig. 3. The growth (cm) of *Penicillium crysogenum*

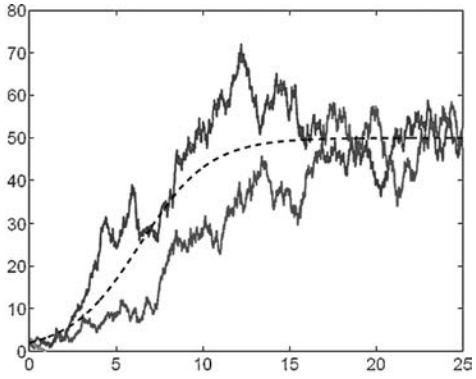

Fig. 4. The growth (mm) of *Penicillium crysogenum* - random Graph

CONCLUSIONS

In this paper, we have established some conclusions regarding the growth model of food born moulds. We have made appropriate graphics corresponding evolution strains of Penicillium crysogenum and Fusarium graminearum during the 14 days and we have examined the phenomenon of growth as a non-homogeneous Poisson process involving periodic variations in the occurrence rate and we have obtained the probability density function of the time intervals of growth. If we denoted $X(t)$ the growth of food moulds at time *t*, then, the probability distribution of this process is a Poisson distribution.

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