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AGRICULTURAL BIOTECHNOLOGY

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MIXED MICROBIAL AND THERMAL DEGRADATION OF AGRICULTURAL DERIVED PLANT WASTES

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Abstract

Agricultural and food industries produce a lot of waste biomass which needs to be disposed of. In recent years it has been understood how these biomasses can be recovered and transformed in order to produce organic derivative products with high added value to be reintroduced on the market. A non-exhaustive list of these biotechnological products includes protein hydrolysates, consisting of bioactive peptides and amino acids, growth-stimulating fertilizers for plants and nutritional additives for animal feed. The recovery and transformation of the protein component into protein hydrolysates gives great added value to the waste biomass and is a process that is generally performed chemically. This approach results however unfavourable because of the possible production of toxic side-products, and the high level of energy required, which makes this process eco-unfriendly. In the light of all these limitations, we have designed and developed a mixed procedure, applicable to plant-derived wastes, based on a microbial preliminary degradation, followed by a mild thermic treatment to produce protein hydrolysates from agriculture waste biomass.

Key words*: Chemical Hydrolysis, Circular Economy, Microbial Hydrolysis, Protein Hydrolysates, Waste Biomasses.*

INTRODUCTION

The production of protein hydrolysates from waste biomass represents one of the main challenges of *Circular Economy,* which sees the transformation of waste into a resource. Protein hydrolysates are bio-based chemicals with high added value. Depending on the biomass used for their production, they can be equipped with high bio stimulating, hormonal and fertilizing capacities and therefore produce significant qualitative-quantitative improvement of agricultural crops, without causing problems concerning environmental pollution. The hydrolysates of plant origin do not have any toxicity for the plant and humans; they are in line with European directives (EU Directive 91/676) which aim at a consistent reduction in the use of chemical products in agriculture, and at the same time promote the reduction in organic matter content of the soils. They are also marketable in areas that do not bear the use of products currently on the market that are generally prepared from animal waste. Protein hydrolysates are made up of polypeptides, oligopeptides, and amino acids and are produced from protein sources through various hydrolysis processes (McCarthy et al., 2013; Schaafsma, 2009). Protein hydrolysates can be obtained through acid, basic, enzymatic or mixed protein digestion. Enzymatic hydrolysis is carried out both through the use of microorganisms and the use of purified enzymes. According to the production method used, hydrolysates with different characteristics can be obtained. On the one hand, chemical hydrolysis is more aggressive and acts irregularly leading to the formation of greater quantities of single amino

acids, many of which are not bioavailable (Celus et al., 2007; McCarthy et al., 2013). On the other hand, enzymatic hydrolysis, conducted at lower temperatures, is less aggressive and leads to the formation of some free amino acids and many dipeptides, polypeptides and bioavailable peptides (Korhonen & Pihlanto, 2006; Schaafsma, 2009).

Protein hydrolysates find various applications in the medical, nutritional and agro-food fields. In the medical field, protein hydrolysates are used for parenteral nutrition in states of serious malnutrition following surgery, in diseases that compromise the digestion and intestinal absorption of food, in animal cell cultures for the production of monoclonal antibodies, therapeutic proteins, therapeutic drugs, and vaccines (Ganglberger et al., 2005; Heidemann et al., 2004; Lee et al., 2008; Mazurkova et al., 2008; Pasupuleti & Braun, 2008; Tripathi et al., 2009). Protein hydrolysates are also used in the treatment of patients with diseases such as Phenylketonuria, a disease affecting the amino acid metabolism, which prevents the conversion of phenylalanine into tyrosine, due to the absence or deficiency of the *phenylalanine hydroxylase* enzyme. This causes the accumulation of phenyl pyruvic acid in the blood, which can lead to serious neurological damage. The use of phenylalanine-free hydrolysates is therefore a viable alternative for infants with this type of enzyme defect, bringing beneficial effects on physical growth and mental development (Acosta et al., 1998; Berry et al., 1976; McCarthy et al., 2013). In the nutritional field, whey proteins, soy proteins, and bovine collagen are used to produce new generation sport products thanks to the hypoallergenic nature, peculiar rheological properties and better digestibility of the hydrolysates obtained (Bequette et al., 1998; Cordoba et al., 2005; Gilbert et al., 2008). In the agro-industrial sector, protein hydrolysates have found widespread application in recent years as biostimulants and fertilizers, as they improve the absorption and assimilation of nutrients (e.g. nitric nitrogen and iron), tolerance to environmental stress (salinity, drought, extreme temperatures) and product quality. In this light, by-products of agriculture-derived waste can be effectively recovered and reused without causing any adverse effect on human and animal health or on the environment (Corte et al., 2014; da Silva, 2018; Luziatelli et al., 2015; Mihalache et al., 2014; Planques et al., 2012).

This work aims at developing a mixed hydrolysis method designed for soy-derived vegetable biomass, based on a first phase of microbial fermentation and a second phase of heat treatment under alkaline conditions, in order to produce protein hydrolysates with high-added value. More specifically, an attempt was made to understand whether the first hydrolysis step due to fermentative processes by microorganisms known for their proteolytic activity could increase the success of the subsequent phase of thermal hydrolysis carried out in alkaline conditions, thus reducing exposure times at high temperatures and consequently the preparation costs.

MATERIALS AND METHODS

Sample preparation

The biomass consists of soy waste resulting from its processing.

The soy waste was mechanically shredded to form a homogeneous powder. The soy powder was suspended in deionized water and incubated for 1 hour at 80°C. During the incubation, the sample was repeatedly shaken to favour the extraction of proteins. At the end of the incubation time, the sample was centrifuged at 16000 x g at 4°C for 15 minutes and the soluble part collected. This solution was further centrifuged at 16000 x g for 15 minutes and the supernatant containing the total extract of solubilized proteins was quantified by the Bradford method (Bradford, 1976) using the Coomassie Brilliant Blue dye and measuring the absorbance at 595 nm.

1st phase: Microbial fermentation

The biomass extract of interest was subject to microbial fermentation in a mixed consortium culture or with pure cultures known for their proteolytic activity. Five different types of inocula were used: self-digestion with soy microbiota, sourdough, bacterial and yeast mix, *Aureobasidium pullulans* and *Debaryomyces hansenii*.

Each inoculum was used to set up two hydrolysis conditions, one lasting 1 day (Group A) and the other lasting 3 days (Group B).

Determination of the degree of hydrolysis after fermentation

The degree of hydrolysis achieved was assessed primarily by proteomic analysis based on polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE). The samples were diluted in the sample buffer (0.5 M Tris-HCl pH 6.8, 10% SDS (w / v), 25 mM DTT) according to Laemmli protocol (Laemmli, 1970). The samples were incubated at 95°C for 5 minutes, loaded into the gel and subject to a constant electrophoretic run at 40 mA. The run was carried out using molecular weight standard as a reference. The running buffer used was Tris 0.025 M/0.192 M glycine containing 0.1% SDS (w/v). The degree of hydrolysis was also assessed by estimating the concentration of free amino acids in solution through the Ninhydrin assay (Rosen, 1957) and by making a comparison with the concentration of free amino acids found in the starting extract. The test is carried out using 2,2-dihydroxy-1,3-dioxyhydrindene (ninhydrin) supplied by Sigma-Aldrich which, added to the protein hydrolysate, interacts with the primary amines giving a blue-violet colour with absorption at 570 nm.

2nd phase: Thermal hydrolysis under alkaline conditions

The samples corresponding to each fermentation period were subject to alkaline hydrolysis with KOH 1 N and then incubated at 80°C for 15 hours. Subsequently, each sample was centrifuged at 4500 x g for 15 minutes at a temperature of 4°C and the phase corresponding to the hydrolysate was recovered and neutralized with H_2SO_4 18 M. Again, for these samples, the degree of hydrolysis was evaluated by SDS-PAGE and the concentration of free amino acids assessed by Ninhydrin assay, as previously described.

RESULTS AND DISCUSSIONS

In the first phase of microbial fermentation, which was carried out for 1 and 3 days respectively, five different inocula, consisting of single bacterial species or consortia of bacteria and yeasts (Table 1), were used in order to understand which one could give the best degree of hydrolysis based on the type of inoculum and fermentation time.

Table 1. Different types of inocula used in this study

Sample	Composition
1	Self-digestion with Vegetable Biomass Microbiota
2	Vegetable Biomass + Sourdough
3	Vegetable Biomass + Bacteria and Yeast mix
4	Vegetable Biomass + *Aureobasidium pullulans*
5	Vegetable Biomass + *Debaryomyces hansenii*
6	Ctrl (Vegetable Biomass)

At the end of the fermentation process, the degree of hydrolysis of the biomass was assessed for each inoculum and for each time used and compared with that of the control (sample **6**). Table 2 shows the percentage values of the protein concentration obtained by the Bradford assay compared to the protein concentration of the starting biomass extract.

Table 2. Percentage values of the Protein Concentration compared to the Ctrl, after 1 day (Group A) and 3 days (Group B) of fermentation

Sample	Group A	Group B
1	30.95%	25.00%
2	51.24%	16.81%
3	33.0%	10.62%
4	34.62%	20.24%
5	87.14%	15.09%
6	87.28%	

The results show how the fermentation significantly reduces the total protein concentration with the formation of free amino-acids. This is more apparent after 72 hours of treatment (Group B).

In particular, the protein concentration of the control is about 87% of that in the starting biomass extract, while in all of the samples undergone fermentation, the protein content is reduced to 10-25% of the initial value after 3 days. Sample 3 (bacteria and yeast mix) results the best inoculum, with its protein content decreased to 33% after a 24 h incubation and becoming almost 10% after 72 hours. Conversely, sample 5 does not provide a significant degradation in one day (protein content similar to the control), but it later performs a good hydrolysis, in line with that of the other samples.

These results were confirmed by the protein profile obtained by SDS-PAGE and Coomassie Blue Staining. The proteomic analysis carried out through SDS-PAGE has provided

information on the degradation profile of the proteins constituting the biomass.
After 1 day of fermentation (Figure 1A), there is no high degree of hydrolysis, while after 3 days (Figure 1B) a satisfactory level of hydrolysis is obtained.

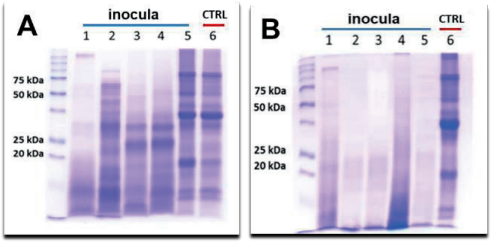

Figure 1. SDS-PAGE of fermented samples after 1 day (A) and 3 days (B)

Each of the microbial hydrolysis described above was then subject to thermal hydrolysis under alkaline conditions. In particular, thermal hydrolysis was carried out at 80°C overnight in the presence of KOH 1N. Also, in this case, the degree of hydrolysis was assessed by SDS-PAGE followed by Coomassie Blue Staining. The results in Figure 2 show that while the one-day fermentation alone does not produce a high level of hydrolysis (Figure 1A), the double passage produces a satisfactory level of hydrolysis with the formation of low molecular weight polypeptides (Figure 2A).
At the same time, the prolonged fermentation for three days produces by itself a satisfactory degree of hydrolysis (Figure 1B), which is however completed by the basic hydrolysis under mild working conditions (Figure 2B).

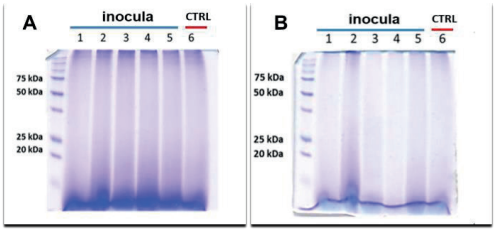

Figure 2. SDS-PAGE of 1 day (A) and 3 days (B) fermented samples treated with thermal hydrolysis under alkaline conditions

The same samples were also analysed employing the Ninhydrin assay, to obtain an estimate of the free amino acids in solution before and after the basic hydrolysis. In fact, ninhydrin is a reagent that interacts with the primary amines of amino acids leading to the formation of a complex that absorbs at 570 nm. The graph shown in Figure 3 shows how, following the microbial degradation, the concentration of free amino acids is higher compared to the control, especially after 3 days of fermentation and in particular for sample 3 composed of a mixture of bacteria and yeasts (left side of the graph).
Following thermal hydrolysis, however, after 1 day of fermentation the concentration of free amino acids is similar to that of the control; conversely, after 3 days, there is an increase in the concentration of free amino acids and again in this case sample 3 delivers the best performance (right side of the graph).

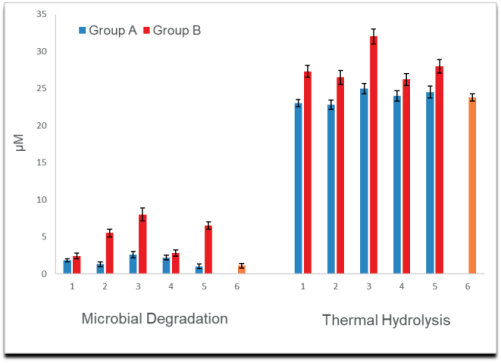

Figure 3. The amino acid concentration obtained using the Ninhydrin assay after microbial hydrolysis (left side of the graph) and mixed hydrolysis (right side of the graph)

CONCLUSIONS

The present work demonstrates the effectiveness of a mixed method of hydrolysis of the protein component of agro-food waste biomass, where soy was chosen as proof of principle.
In general, most of the protein hydrolysates are produced through thermal hydrolysis under acidic or alkaline conditions at high temperatures, usually above 100°C.
This approach is not only extremely aggressive, but it also leads to the formation of a high quantity of free amino acids which are mostly dextrorotatory and therefore not assimilable. Moreover, thermal hydrolysis is extremely disadvantageous from an economic point of view as a result of the high energy required.
Therefore, this study shows how the pre-

treatment of waste biomass with microorganisms, naturally known for their hydrolytic capacities, performs a crucial step in the process that leads to the formation of protein hydrolysates.

The action of microorganisms and in particular of the mixture consisting of consortia of bacteria and yeasts has proven effective in initiating the degradation process of the protein component of the concerned biomass promoting the subsequent passage of thermal hydrolysis, which was conducted at lower temperatures compared to the canonical one.

In this way, it would be possible to obtain not only savings in terms of costs and energy but also a higher quality product.

In fact, the hydrolysates obtained through this process will be subject to follow-up studies to evaluate their effectiveness as bio-fertilizers and bio-stimulants to be reintroduced in agriculture.

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INFLUENCE OF AGROINFILTRATION CONDITIONS ON THE TRANSIENT GREEN FLUORESCENT PROTEIN EXPRESSION IN *Nicotiana rustica* L. PLANTS

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Abstract

Transient gene expression in plants allows accumulation of large amount of recombinant proteins within a very short time. We optimized the protocol of transient green fluorescent protein (GFP) expression system in Aztec tobacco (Nicotiana rustica L.) plants. For transient transformation, the optimal optical density of the agrobacteria suspension for plant tissues infiltration was determined. The pSPV2303 genetic construct, contained the gfp gene from A. victoria under double 35S CaMV promoter, used for this purpose. The agrobacteria suspension was grown to OD = 1.0 initial optical density at wavelength 600 nm and the plants were infiltrated with the following final concentrations: 0.2; 0.4; 0.8; 1.0; 1.5; 2.0 without and with the addition of 4'-hydroxy-3',5'-dimethoxyacetophenone (acetosyringone) at 150 μM concentration. Spectrofluorometric and protein analysis were used for green fluorescent protein (GFP) detection. The highest level of expression obtained was using the OD = 1.0 agrobacteria optical density and there was not a detrimental effect on plant tissues.

Key words: *acetosyringone, agrobacteria optical density, green fluorescent protein (GFP), Nicotiana rustica L., transient transformation.*

INTRODUCTION

The heterologous gene expression system, mediated by agrobacteria in intact plant leaves, is a fast and productive method for analyzing gene expression. It can produce many heterologous proteins without the need to create stable transformants, which is difficult for many plant species. Furthermore, transient gene expression is not biased by position effect, which often occurs in stable transformation (Lee & Yang, 2006).

The heterologous gene expression system offers several advantages over stable expression. One of them is simplicity of execution. The expression of transient genes can be analyzed directly post agroinfiltration after several days (Kapila et al., 1997), subsequently the expression level decreases rapidly. This can be explained by the initiation of local silencing of RNA that blocks transgene expression (Johansen & Carrington, 2001; Voinnet et al., 2003) and is one of the main mechanisms of cell protection against foreign nucleic acids (Dougherty & Parks, 1995; Lindbo et al., 1993; Baulcombe, 2004; Tenllado et al., 2004). Specific viral proteins capable of inhibiting the mechanism of RNA silencing have been found in many plant viruses (Roth et al., 2004).

Transient expression is a common method for assessing the capacity of plant cells to produce a particular protein and⁄or to test various expression cassette arrangements (Sainsbury et al., 2009).

MATERIALS AND METHODS

Genetic construct

We used a pSPV2303 construct containing the *gfp* gene isolated from *A. victoria* encoding a Green Fluorescent Protein (GFP) under the control of a dual CaMV 35S promoter, a 5' untranslated (5'UTR) region from the Tobacco Mosaic Virus (TMV) and the CaMV 35S

terminator together with the polyadenylation signal and the 3'- untranslated region (3'UTR). The overnight culture of the genetic constructs were grown in LB medium (Bertani, 1951) supplemented with 50 mg/l rifampicin, 25 mg/l gentamicin, and 100 mg/l kanamycin.

Plant agroinfiltration

Plants of Aztec tobacco (*Nicotiana rustica* L.) were grown under greenhouse conditions at 25/18ºC and 16/8 photoperiod (day/night, respectively). Leaves of the middle tier of 4-week plants were used for infiltration.

To study the effect of different bacterial concentrations on the expression of *gfp* gene Aztec tobacco plants (*N. rustica* L.) transformed transiently. *Agrobacterium* overnight culture was grown to optical density of suspension OD_{600} = 1.0 and resuspended in infiltration buffer (10 mM $MgSO_4$, pH 5.6-5.8) with a final optical density of OD_{600} = 0.2; 0.4; 0.8; 1.0; 1.5; 2.0 without and with the addition of acetosyringone at 150 µM concentration. Plant infiltration was performed with a syringe (Sambrook et al., 1989).

Spectrofluorimetric analysis

The expression of the *gfp* gene was detected in the 7th day after infiltration visually and by spectrofluorometric analysis on a fluorescence spectrofluorometer "Fluorate-02-Panorama" (excitation at a wavelength of 395 nm, emission at 509 nm).

Protein analysis

Water-soluble proteins were isolated in extraction buffer (PBS), consisting of the following elements: 80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 100 mM NaCl, pH 7-7.5 (Sambrook et al., 1989). Plant tissues of *N. rustica* L., in which *gfp* gene expression was detected, were triturated with extraction buffer in a pre-chilled mortar (+4ºC) at a 3:1 (v/w) ratio (300 µl of buffer per 100 mg of tissue), after which the suspension was precipitated (+4°C, 14000 rpm, 30 minutes). The supernatant was used to determine protein concentration by Bradford protein assay (Bradford, 1976) and for separation of proteins in polyacrylamide gel under native and denaturing conditions.

All statistical analyzes were performed in Microsoft Office Excel, determining the mean and standard deviation for each experiment. For the statistical processing of the spectrofluorometric analysis, the average value was determined through the logarithmic transformation of the spectrofluorometer parameters.

RESULTS AND DISCUSSIONS

This study was conducted to develop an effective system of transient transformation for heterologous protein synthesis in Aztec tobacco (*N. rustica* L.) tissue.

Genetic construct and plant agroinfiltration

We tested six different concentrations of bacterial suspension with pSPV2303 vector (Figure 1) and the effect of acetosyringone on heterologous protein expression.

The fluorescence of the GFP protein (Figure 2) was detected 7 days after infiltration. Tissues of plants infiltrated with *Agrobacterium tumefaciens* GV3101 strain and tissues that were not infiltrated were used as a negative control.

Figure 1. Schematic representation of the pSPV2303 vector T-DNA

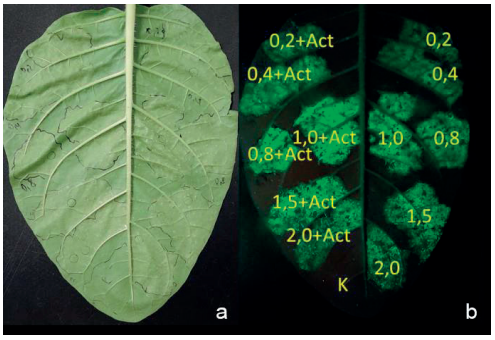

Figure 2. Transient expression of the *gfp* gene: a) leaf 7 days after infiltration (day light); b) leaf 7 days after infiltration (ultraviolet light)

Spectrofluorometric analysis

Scholz et al. (2000) showed a positive correlation between the level of fluorescence and the accumulation of recombinant GFP protein, as level of the *gfp* gene expression and explained the non-linear nature of this process, which is confirmed by our experiments between the spectrofluorometric analysis of GFP fluorescence and the determination of water-soluble protein concentration level.

Leuzinger et al. (2013) showed that low concentration of OD_{600} = 0.12, demonstrated

Agrobacterium strain balances between maximum delivery of gene construct without causing tissue necrosis and cell death. We showed that the *gfp* gene expression at low bacterial concentration, OD_{600} = 0.2, was the poorest (Table 1) and equaled 0.48 ± 0.03. Shamloul et al. (2014) used a bacterial suspension of 0.5 optical density at 600 nm wavelength.

Table 1. Fluorescent level of GFP protein in *N. rustica* L. tissue (relative units)

	Mean	-Cl	+Cl
OD_{600} = 0.2	0.48	0.03	0.03
OD_{600} = 0.2+Act150 µM	0.48	0.02	0.02
OD_{600} = 0.4	0.55	0.01	0.02
OD_{600} = 0.4+Act150 µM	0.55	0.02	0.02
OD_{600} = 0.8	0.99	0.04	0.04
OD_{600} = 0.8+Act150 µM	1.00	0.03	0.03
Kgv3101	0.02	0.00	0.01
K leaf tissue	0.01	0.00	0.01
OD_{600} = 1.0	1.02	0.04	0.04
OD_{600} = 1.0+Act150 µM	1.02	0.02	0.02
OD_{600} = 1.5	0.99	0.03	0.03
OD_{600} = 1.5+Act150 µM	0.99	0.04	0.04
OD_{600} = 2.0	0.61	0.03	0.04
OD_{600} = 2.0+Act150 µM	0.61	0.01	0.01

In our experiments we showed that the *gfp* gene expression increased depending on the increasing of suspension optical density. When we used OD_{600} = 0.4 fluorescence level was 0.55 (+0.02; -0.01), and was higher than at OD_{600} = 0.2. Spectrofluorometric analysis showed (Figure 3) that the highest level of the *gfp* gene was showed when the optical density of suspension OD_{600} = 0.8; 1.0; 1.5 were used and equaled 1.02 ± 0.04; 0.99 ± 0.04; 0.99 ± 0.03, respectively. But at the maximum bacterial suspension concentration, what we used in our experiments (OD_{600} = 2.0) for agroinfiltration, the protein GFP fluorescence level in the tissues was 0.61 (+0.04; -0.03), which is less than when was used the bacterial suspension with OD_{600} = 1.5; 1.0; 0.8. Shamloul et al. (2014) showed that none of the tested concentration of acetosyringone induced a significant increase in GFP fluorescence or protein production compared with control, where induction media contained no acetosyringone for *Nicotiana benthamiana* L. This was also confirmed by our experiments on *N. rustica* L.

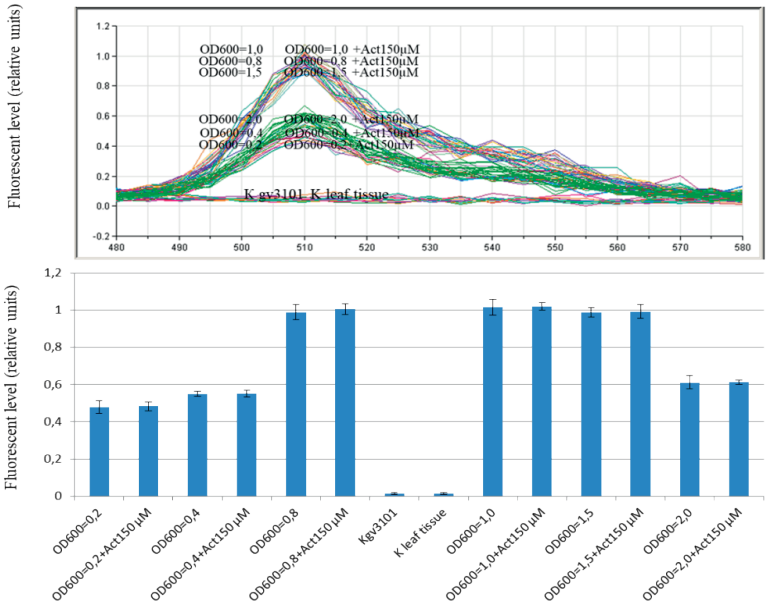

Figure 3. Spectrofluorometric analysis of GFP expression

Protein analysis.

Concentration of total water-soluble proteins (µg/ml) in *N. rustica* L. tissue (Figure 4), showed that the protein concentration (Table 2) during transient expression of the *gfp* gene was different depending on the optical density of bacterial suspension what was used for agroinfiltration.

Indicators of protein analysis are completely identical to spectrofluorometric, where the GFP protein had the highest expression level when the OD_{600} = 0.8, 1.0 or 1.5 were used, and the lowest when the bacterial concentration of OD_{600} = 0.2 was used.

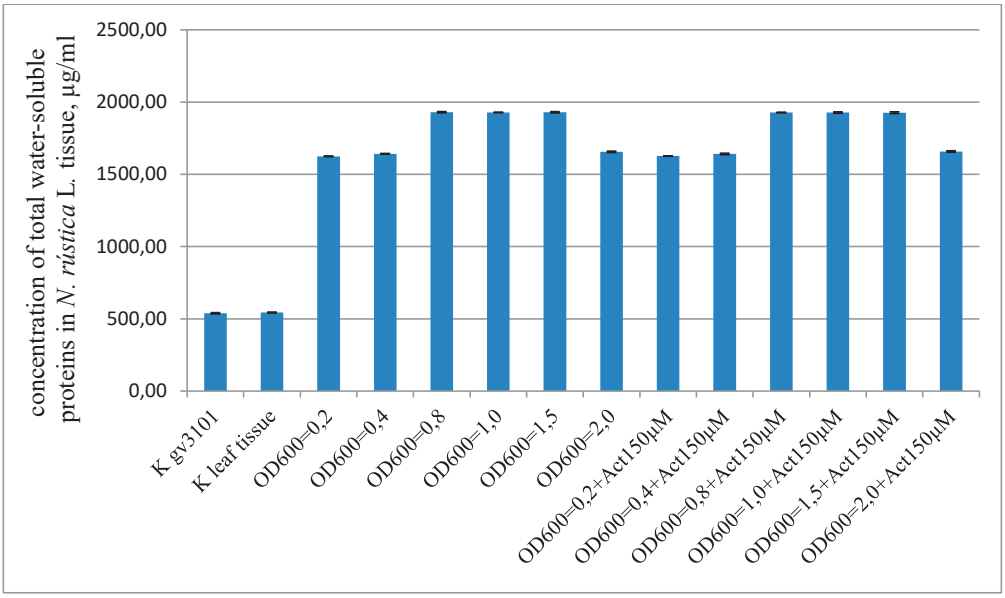

Figure 4. Concentration of total water-soluble proteins in *N. rustica* L. tissue, µg/ml

Table 2. Concentration of total water-soluble proteins in *N. rustica* L. tissue, µg/ml

	Mean	± Deviation
K gv3101	538.33	4.73
K leaf tissue	543.67	3.21
OD_{600} = 0.2	1624.33	2.08
OD_{600} = 0.4	1642.33	2.31
OD_{600} = 0.8	1930.00	4.36
OD_{600} = 1.0	1928.33	1.53
OD_{600} = 1.5	1929.67	4.51
OD_{600} = 2.0	1655.67	4.93
OD_{600} = 0.2+Act150 µM	1626.67	1.53
OD_{600} = 0.4+Act150 µM	1641.00	5.29
OD_{600} = 0.8+Act150 µM	1927.67	2.52
OD_{600} = 1.0+Act150 µM	1927.33	4.93
OD_{600} = 1.5+Act150 µM	1926.67	6.11
OD_{600} = 2.0+Act150 µM	1658.00	5.29

To identify the GFP reporter protein, we further performed polyacrylamide gel electrophoresis to separate water-soluble proteins in the presence of sodium dodecyl sulfate under denaturing (with mercaptoethanol) and non-denaturing separation conditions (Figure 5).

Protein extracts were mixed with the sample buffer and applied to the wells with and without boiling denaturation.

The presence of the GFP reporter protein was observed immediately after electrophoresis by illuminating the gel with ultraviolet light using a hand lamp.

Detection in ultraviolet light (Figure 5a) showed the functional activity of the GFP reporter protein. Other proteins were visualized (Figure 5b) by staining the gel with a Coomassie reagent (Blakesly et al., 1977).

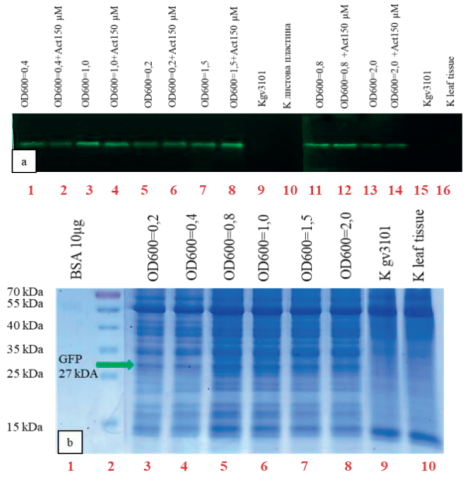

Figure 5. Electrophoretic separation of proteins in polyacrylamide gel: a) non-denaturation conditions (detection of GFP protein in ultraviolet light); b) denaturation conditions: 1-8; 11-14 - protein extracts of *N. rustica* L. tissue in which the *gfp* gene is transiently expressed under the control of different bacterial concentrations; 9,10; 15,16 - extracts of *N. rustica* L. tissue control plants infiltrated with *A. tumefaciens* GV3101 strain and uninfiltrated tissue; b) denaturing conditions: 1 - standard BSA (concentration 10 µg); 2 - a molecular weight marker; 3-8 - protein extracts of *N. rustica* L. tissue in which the *gfp* gene is transiently expressed under the control of different bacterial concentrations (the GFP protein is indicated by an arrow); 9, 10 - extracts of *N. rustica* L. tissue control plants infiltrated with *A. tumefaciens* GV3101 strain and uninfiltrated tissue

Aztec tobacco infiltrated by created construct with different optical density of suspension and recombinant GFP protein resulting from transient expression has the same molecular weight as standard GFP (Figure 5b).

Indicators of protein electrophoretic analysis are completely in line with spectrofluorometric and protein analysis, where the highest accumulation of recombinant GFP protein was when used bacterial suspension of 0.8, 1.0, 1.5 optical density at 600 nm wavelength

CONCLUSIONS

In our experiments we determined that the level of heterologous genes expression depends on the concentration of bacterial suspension, and has a positive character due to its increase from 0.2 to 0.4, then goes to the plateau, using 0.8; 1.0; 1.5 and declines at 2.0. An increase in the level of fluorescence coincides with an increase in the concentration of total water-soluble proteins in the Aztec tobacco tissues, which is confirmed by the study of other authors. The use of bacterial suspension concentrations of 0.8-1.0-1.5 leads to the highest expression of *gfp* gene in healthy plant tissues.

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ESTABLISHMENT OF SOME CULTIVATION STAGES AT VEGETATION HOUSE LEVEL IN ECOLOGICAL SYSTEM ON THREE ROMANIAN TOMATO VARIETIES

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Abstract

The aim of our research was to establish the effect of some cultivation stages in ecological system in which are use the LED light on biological material of three Romanian tomato varieties. For this purpose, young tomatoes (Lycopersicon esculentum Mill.) plants have been grown in protected crop space (vegetation house), by daily supplementing natural light for short periods of time with light provided by high power LED panels with red, blue and white monochromatic light. In order to carry out the integrated studies of the cultivation method in the ecological system, products accepted as natural and biodegradable and approved in Romania, for fertilization and control of the attacks of diseases and pests, were used . The tested varieties were selected with indefinite habitus and special forms of fruits, namely: Sonia de Buzău, Hera and Coralina. The recorded differences in the increase of the stems' length (average values) recorded, after 50 days from the beginning of the experiments, have revealed to us that the results are differentiated according to genotype, spectrum and exposure time to the used additional light. Plants of the variety "Sonia de Buzău" exposed daily under LED light recorded high values in most variants, followed by those of the variety Hera and Coralina. The results obtained after the application of additional treatments with LED light, were dynamically analysed, evaluated by statistical calculation and compared with the values obtained by the Control plants maintained only under the effect of natural light and which were treated with the fertilization and phytosanitary protection products.

Key words*: agroecosystem, artificial light, morphological assessments, tomatoes.*

INTRODUCTION

Light Emitting Diode (LEDs) were developed after 1962 and for many years they have been used only in signs or light displays due to the low light emitted and the restricted colour palette. LEDs are semiconductor diodes that have the property of converting electricity into light. The conversion operation is done cold, which gives a much better light efficiency than incandescent lamps (Davis & Burns, 2016; Morrow, 2008).

Among the many advantages of LED light sources the following are worth to be mentioned: high light efficiency (over 60 lm/W); low absorption powers, on the order of watts; long lifespan (because they have no filament or hot electrodes) of tens of thousands of hours; small in terms of size; resistant to weather elements and chemical agents; they have their own dispersion lens, so they can be used in projectors without the addition of optical systems; offer a wide range of colours, but also white light with different colour temperatures (mainly cold white); they have extremely short reaction times (fractions of milliseconds) and many more (Brazaityte et al., 2009).

The light emitted by a LED is usually monochromatic, and the color of the emitted light depends on the composition and the state of the LED properties.

The luminescent diodes can be manufactured to emit on all wavelengths of the visible spectrum, from red lighting (620 nm to 750 nm) to blue-violet lighting (380 nm to 490 nm). Because the luminous flux of a single LED is small, it is

necessary to use more LEDs concentrated on a single support. Their number varies from 3 to hundreds - depending on the destination of this light source (Gómez & Mitchell, 2015; Morrow, 2008; Kim et al., 2005).

The effect of light emitted by LEDs with different spectra has been studied in a significant number of crop species both from the point of view of stimulation on seed germination (Montagnoli, 2018; Enache & Livadariu, 2016; Gómez & Mitchell, 2015; Hernández & Kubota 2014; Yorio et al., 2001), but also on the growth of plants at different stages of development (Davis & Burns, 2016; Gómez & Mitchell, 2015). The research carried out by Cope & Bugbee (2013) aimed to study the photobiological effects of three types of white LEDs (warm, neutral and cold), in combination with blue light in different percentages (11%, 19% and 28%) on the growth and development of radish, soybean and wheat plants.

Another aspect studied was the action of light emitted by blue LEDs in the treatment of microbiological cultures in order to increase the percentage of inactivation and to inhibit the viability of pathogenic microorganism colonies (Wang et al., 2017; Popa et al., 2008).

Taking into account the results of the aforementioned research studies, the purpose of the researches carried out in this paper was to analyse the morphological response of 3 varieties of tomato lines (Coralina, Hera and Sonia de Buzau) selected from Romanian tomato species, under the conditions of supplementing the natural illumination with light from LEDs of red, blue and white colours, in the protected culture space represented by the vegetation house of USAMV Bucharest.

MATERIALS AND METHODS

Biological material

In the experiments, seedlings from three approved tomato lines from Vegetable Research and Development Station Buzău, Romania (VRDS Buzău): Sonia de Buzău, Hera and Coralina were used. The productive characteristics of the analyzed tomato varieties differ in color and shape of the fruits but also by the habit of growing of the plants.

According to the description presented in the "General Catalog of Varieties and Hybrids of Vegetables" by VRDS Buzău, Romania, the **Sonia de Buzău** variety, approved in 2014, is of early type, it forms vigorous plants, with indeterminate growth of the stems. The fruits are of the cherry cocktail type, with a cordiform shape and concentrated ripening of the fruit in bunches, with an average weight of 38.7 g/fruit. Sonia de Buzău variety is suitable for cultivation both in protected systems (greenhouse, solar) and also in open fields (https://www.madr.ro/docs/cercetare/Rezultate_activitate_de_cercetare/SCDL_Buzau.pdf).

Regarding the main characteristics of the **Hera** variety, it is noteworthy in particular the elongated form of the fruits, similar to the Capia pepper, with a weight of 160-220 g/fruit. This variety is quite adaptable and can be grown in protected areas and also in fields (https://www.buzau.net/stiri-buzau/hera).

The variety **Coralina**, obtained at VRDS Buzău, Romania, and approved in 2009, is of early type, with indeterminate growth of the stems. The fruits are of the cherry type, with a round shape and an average weight of 19.7 g/fruit. The crop can be set up in protected areas and open field, with a production potential of about 2.5 kg / plant, being able to easily obtain over 60 t/ha (https://www.madr.ro/docs/cercetare/Rezultate_activitate_de_cercetare/SCDL_Buzau.pdf).

Three experiments were carried out: Experience I/Sonia de Buzău variety, Experience II/Hera variety and Experience III/Coralina variety. Three LED panels with different colours (red, blue and white) were used in the experiments. Each type of LED panel was suspended at a height of 1 meter above the three types of tomato seedlings.

The experiments took place in the UASVM Bucharest Vegetation House. Each of the 3 VEGETA model LED panels were made by S.C. ELECTROMAGNETICA S.A., Bucharest, Romania, with the dimensions of: 385 mm x 264 mm x 169 mm and provided an additional lighting of 10,000 lx.

The electrical and optical manufacturing parameters for the VEGETA model devices with red, blue and white LEDs placed at the panel level were as follows:

- nominal frequency = 50 Hz;
- nominal voltage = 230 V;
- rated power = 77 W ± 10%;
- power factor> 0.95;

- degree of protection IP66;
- luminous flux: red = min. 700 lm; blue = min. 1000 lm; white-neutral = min. 7900 lm;
- LED spectral domain: red = 660 nm; blue = 456 nm;

The white and neutral LED spectral range: warm, neutral, cold; colour rendering index > 80.

Devices built with LED (red, blue and white) placed in groups (sub-panels) mounted in parallel, have been adapted to the suspension support system for additional light coverage of a larger exposure surface (Figure 1).

Figure 1. Installation aspect of devices used in the lighting of tomato seedlings, with monochrome LED light in the vegetation house of USAMV Bucharest

Applied working methods

The seedlings provided by VRDS Buzău, Romania, were obtained after germination and cultivation for 45 days in alveolar pallets in the protected greenhouse. Immediately after receiving them, the tomato plants of the 3 varieties were transplanted into 15 x 15 x 15 cm square pots (1 plant/pot) which were then placed in support trays, at a density of 15 plants/m². Each pot was filled with 750 ml substrate consisting of Kekkila DSM 2 W peat, which is a light peat (well ground), pre-fertilized with a basic fertilizer (NPK 14-16-18), with the pH adjusted to 5.5/5.9 values.

The fertilization of the tomato seedlings was carried out 2-3 days after transplantation, using with Florovit plant regenerator (foliar sprays) with NPK (7-5-6); this treatment being repeated 3 times at intervals of 1 week.

Starting with the second week, foliar fertilization with Lumbreco organic fertilizer based on organic biohumus extract, has been applied for the rich source of nutrients and the growth stimulating effect.

In order to prevent the onset of pest attacks specific to tomato plants, 2 products recommended as non-toxic and environmentally friendly were selected and applied: Rock Effect (product range Natura, by producer AGRO CS) and PIPERCIP microemulsion insecticide (by AMIA International producer).

Statistical analysis

The bifactorial type experiments with tomatoes were located according to the method of subdivided plots in 3 repetitions, within each experience being tested the influence of artificial lighting using LEDs on the growth dynamics of tomato plants belonging to the three varieties tested (Sonia de Buzău, Hera and Coralina).

The experimental factors considered in the study were the following:

Factor A - the colour of the light emitted by the applied LEDs with 3 graduations: a1 - blue light; a2 - red light; and a3 - white light.

Factor B - duration of lighting treatment with 4 graduations: b1 - 0 minutes (Control variant); b2 - 15 minutes; b3 - 30 minutes; and b4 - 45 minutes.

After exposure to the selected period (at 15, 30 and 45 minutes) under the LED device, we continued to grow the tomato plants in natural light for about 14 hours/day (between sunrise and sunset), this being the period corresponding to day light for July-August, when the experiments took place, in the southern part of Romania (Bucharest). The natural day light, was supplemented for the experimental variants, after 2 weeks from the moment when tomato plants were transplanted into 15 x 15 x 15 cm square pots (1 plant/pot), with light from LEDs of red, blue and white colours, in vegetation house of USAMV Bucharest, each of them in combinations with 3 variants of exposion (15, 30 and 45 minutes).

As a result of the combination of the two experimental factors, for each of the 3 experiences: Experience I/Sonia de Buzău variety, Experience II/Hera variety and Experience III/Coralina variety), 12 experimental variants, interpreting the experimental results obtained by method of analysis of variance.

In the experimental scheme, we simbolize the Control sample with "b1" and was represented

by natural day light, for all combinations, between the variants of factor A, as follows:
✓ a1b1 - 0 minutes at blue LED lighting;
✓ a2b1 - 0 minutes at red LED lighting;
✓ and a3b1 - 0 minutes at white LED lighting.
Significance of differences between different experimental variants was performed based on the analysis of the variant (ANOVA).
The results were expressed as mean values and their specific standard errors, using MS Excel software. To determine the significance of the differences at P≤5%, compared to the Control sample, the t test was used.

RESULTS AND DISCUSSIONS

Experience I - Sonia de Buzău

The first evaluation of the stem length in tomato plants was made on 16.07.2019, after the application of 5 treatments, with monochrome LED light within 1 week for plants in the experimental variants. Compared to the average values obtained in experience I, taken as a Control in which the height of the tomato plants (Figure 2) determined on the 16.07.2019 was, on average, 49.18 cm, between the 12 sample variants (in which 9 is experimental and 3 is control) tested in the experiment of the tomato variety Sonia de Buzău, recorded differences of the values of this biometric parameter that ranged from -20.98 cm to 28.19 cm (Table 1) so:
- statistically very significant negative differences (ooo) in the case of experimental variants a1b2 and a1b3;
- distinctly significant negative (oo) in the variant a1b4;
- significantly negative (o) to a2b4 and a3b2;
- insignificant in the experimental variants a2b2, a2b3, a3b3 and a3b4;
- differences that became very significant positive (***) in the variants a1b1, a2b1, a3b1, in which case the plant stem elongate as the result of the effect of the natural light that the tomato plants cultivated under greenhouse conditions had at their disposal.
After approximately one week of vegetation, the determinations made on the plants showed the same variability compared to the Control sample (a1b1, a2b1 and a3b1) of the experience so that, on 25.07.2019, the differences related to the size of the plants were between -2.72 cm and 22.95 cm, with statistical assurance very significant negative (ooo) for experimental variants a1b2, a1b3 and a1b4, insignificant (NS) for variants a2b3, a2b4, a3b2, a3b3 and a3b4, significant positive (*) for variant a1b1, distinctly significant positive at variant significantly positive (***) in experimental variants a2b1 and a3b1.

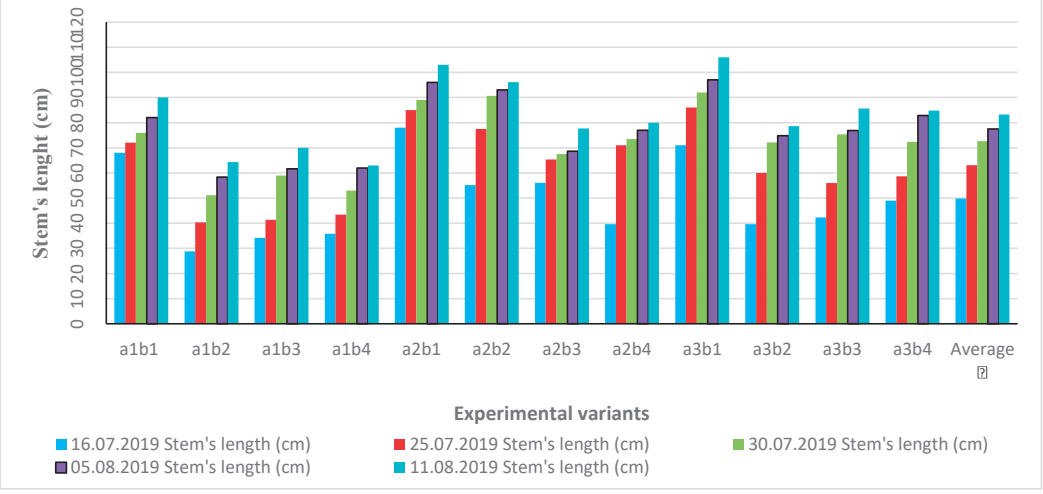

Figure 2. The values of the measured stem's length (cm), tomatoes plant- **Sonia de Buzău** variety

Table 1. The values of the statistically assured differences calculated for **Sonia de Buzău** variety

Experimental variants	Date of experimental determination									
	16.07.2019		25.07.2019		30.07.2019		05.08.2019		11.08.2019	
	Dif. (cm)	Significance level	Dif. (cm)	Significance level	Dif. (cm)	Significance level	Dif. (cm)	Significance level	Dif. (cm)	Significance level
a1b1	18.19	***	8.95	*	3.37	NS	4.49	NS	6.73	NS
a1b2	-20.98	ooo	-22.72	ooo	-21.53	ooo	-19.18	ooo	-18.94	Ooo
a1b3	-15.64	ooo	-21.72	ooo	-13.63	oo	-15.84	ooo	-13.27	Oo
a1b4	-13.98	oo	-19.62	ooo	-19.63	ooo	-15.51	ooo	-20.27	Ooo
a2b1	28.19	***	21.95	***	16.37	***	18.49	***	19.73	***
a2b2	5.36	NS	14.45	**	18.04	***	15.49	***	12.73	**
a2b3	6.49	NS	2.28	NS	-5.13	NS	-8.84	o	-5.60	NS
a2b4	-10.14	o	7,95	NS	0.87	NS	-0.51	NS	-3.27	NS
a3b1	21.19	***	22.95	***	19.37	***	19.49	***	22.73	***
a3b2	-10.21	o	-3.05	NS	0.07	NS	-2.68	NS	-4.60	NS
a3b3	-7.48	NS	-7.05	NS	2.7	NS	-0.68	NS	2.4	NS
a3b4	-0.81	NS	-4.38	NS	-0.30	NS	5.32	NS	1.56	NS
Average, %	**49.81**		**63.05**		**72.63**		**77.51**		**83.27**	
$DL_{5\%} = 8.02$; $DL_{1\%} = 11.61$; $DL_{0.1\%} = 14.87$										

***, **, *, NS indicate statistical significance at the DL5%; DL1% and DL0.1% level and nonsignificant, respectively

As the tomato plants advanced in the vegetation stage, the evolution of this biometric parameter was approximately similar being registered with the average of the experience differences between -21.53 cm and 19.37 cm in the case of the determinations made on 30.07.2019, between -19.18 cm and 19.49 cm on 5.08.2019, respectively between -20.27 cm and 22.73 cm following the determinations made on 11.08.2019.

These directions were provided, from a statistical point of view, from the very significant negative (ooo) to the variants a2b2 and a1b4, to the very significant positive (***) to the experimental variants a2b1 and a3b1, the tomato plants belonging to these variants showing an accentuated phenomenon elongation throughout the entire vegetation period.

Experience II - Hera variety

The tomato plants belonging to the Hera variety (Figure 3) have registered compared to the average of the experience differences in the height of the stems between -13.29 cm and 19.71 cm at the date of the first biometric determinations (16.07.2019), the differences being (Table 2) very significant negative (ooo) in the variant experimental a3b3, distinctly significantly negative (oo) in the case of a1b3 variant, significantly negative (o) in the variants a1b2, a2b2 and a2b3, insignificant (NS) in a1b4, a2b3, a3b1, a3b2 and a3b4 and very significantly positive (***) in the a1b1 and variants a2b1, in the case of these experimental variants, the tomato plants showing a marked elongation of the stalk.

As of 25.07.2019, (Table 2) it is observed that in most experimental varieties the phenomenon of plant elongation, irrespective of the color of the light or the duration of plant illumination, was observed, the differences recorded compared to the average of the experience taken as a Control sample, sweeping between 1.04 cm and 28.71 cm, with statistical assurance from insignificant positive (NS) in the case of experimental variant a3b3, to very significant positive (***) in experimental variants a1b1, a1b4, a2b1 and a3b1.

After another week of vegetation (30.07.2019), the biometric determinations revealed a lower variability between the 9 experimental variants, are taken in the study regarding the size of the plants, the comparative differences with the 3 Control samples (a1b1, a2b1 and a3b1), of the experience being between -10.65 cm and 12.85 cm, statistically insured from very significantly negative (ooo) to variant a2b4, to very significant positive (***) for experimental variants a1b1 and ab4.

Following the determinations made in the first decade of August (5.08.2019-11.08.2019) the same variability is observed, in the case of the same experimental variants being recorded very significant positive differences (***), differences with respect to the Control sample that ranged from -10.77 cm and 12.23 cm.

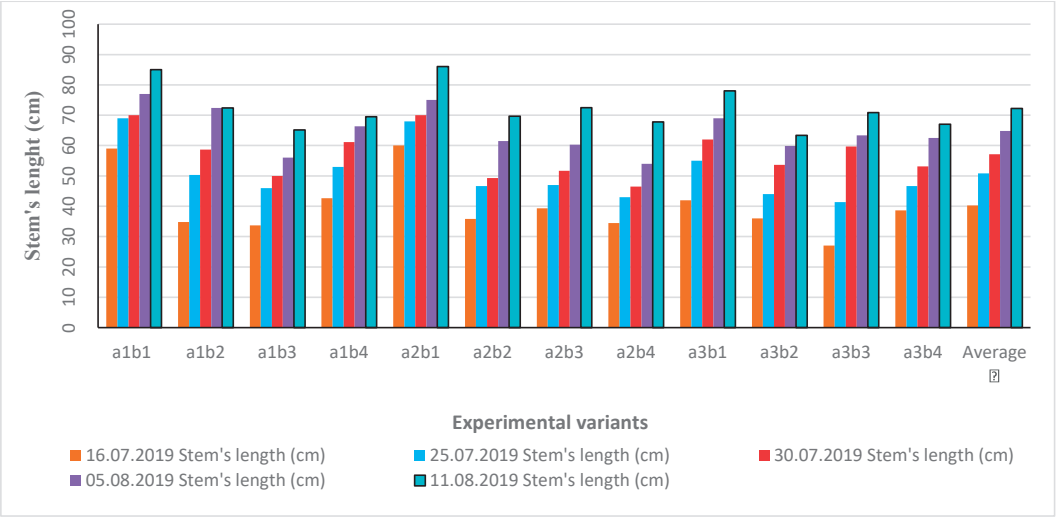

Figure 3. The values of the measured stem's length (cm), tomatoes plant- **Hera** variety

Table 2. The values of the statistically assured differences calculated for tomatoes plant- **Hera** variety

Experimental variants	Date of experimental determination									
	16.07.2019		25.07.2019		30.07.2019		05.08.2019		11.08.2019	
	Dif. (cm)	Significance level	Dif. (cm)	Significance level	Dif. (cm)	Significance level	Dif. (cm)	Significance level	Dif. (cm)	Significance level
a1b1	18.71	***	28.71	***	12.85	***	12.23	***	12.73	***
a1b2	-5.46	o	10.04	**	6.52	NS	7.66	**	0.16	NS
a1b3	-6.62	oo	5.71	*	9.71	**	-8.77	oo	-7.1	Oo
a1b4	2.38	NS	12.71	***	4.02	*	1.56	NS	-2.77	O
a2b1	19.71	***	27.71	***	12.85	***	10.23	***	13.73	***
a2b2	-4.49	o	6.38	**	-7.82	oo	-3.27	o	-2.60	O
a2b3	-0.96	NS	6.71	**	-5.48	o	-4.44	o	0.23	NS
a2b4	-5.79	o	2.71	*	-10.65	ooo	-10.77	ooo	-4.44	O
a3b1	1,71	NS	14.71	***	4.85	*	4.23	*	5.73	*
a3b2	-4.29	NS	3,71	*	-3.48	o	-4.94	o	-8.94	Oo
a3b3	-13.29	ooo	1.04	NS	2.52	*	-1.4	NS	-1.44	NS
a3b4	-1.62	NS	6.38	**	-4.05	*	-2.27	NS	-5.27	O
Average, %	**40.29**		**50.83**		**57.15**		**64.77**		**72.27**	
DL5% = 2.46; DL1% = 6.08; DL0.1% = 10.22										

***, **, *, NS indicate statistical significance at the DL5%; DL1% and DL0.1% level and nonsignificant, respectively

The experience III - Coralina variety

The differences registered with the average of the Experience III (Figure 4) in terms of their height, ranged between -13.58 cm and 21.79 cm on July 16, 2019, between -10.33 cm and 17.67 cm following the biometric determinations performed on July 25, 2019, between -14.19 cm and 16.48 cm on July 30, 2019, while the determinations made in the first decade of August highlighted differences in this biometric indicator between -20.79 cm and 13.88 cm (5.08.2019), respectively between -25.10 cm and 15.57 cm, values that were recorded on 11.08.2019.

If we do a detailed analysis on these differences it is found that they were insured during July (16.07.2019-30.07.2019), from a statistical point of view, from very significant negatives (ooo) in the case of experimental variants a2b2, a3b2, a3b3 and a3b4, up to very significant positive (***) in Control sample variants a1b1, a2b1 and a3b1. The differences in plant height at the beginning of August (5.08.2019) had very negative statistical assurance (ooo) for the experimental variants a1b3 and a3b2, distinctly significant negative (oo) to a2b2, significantly negative (o) to a3b3, insignificantly negative (NS) for variants a2b3 and a2b4, significantly

positive (*) for variants a1b2 and a1b4, distinctly significant positive (**) for a3b1 and a3b4, respectively very significantly positive (***) for Control variants a1b1 and a2b1 (Table 3).

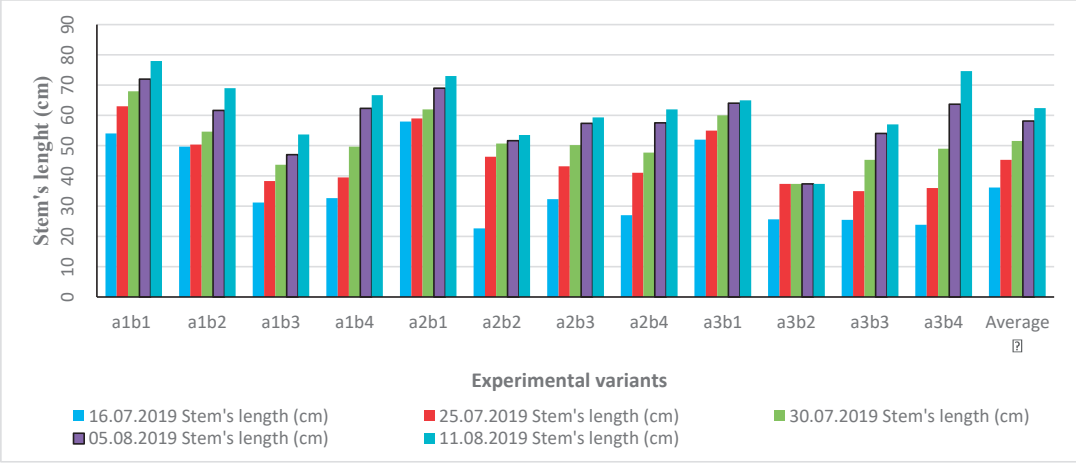

Figure 4. The values of the measured stem's length (cm), tomatoes plant- **Coralina** variety

Table 3.The values of the statistically assured differences calculated for the **Coralina** variety

Experimental variants	Date of experimental determination									
	16.07.2019		25.07.2019		30.07.2019		05.08.2019		11.08.2019	
	Dif. (cm)	Significance level	Dif. (cm)	Significance level	Dif. (cm)	Significance level	Dif. (cm)	Significance level	Dif. (cm)	Significance level
a1b1	17.79	***	17.67	***	16.48	***	13.88	***	15.57	***
a1b2	13.46	***	5.00	**	3.15	*	3.54	*	6.57	**
a1b3	-5.04	oo	-7.00	oo	-7.85	oo	-11.12	ooo	-8.76	Oo
a1b4	-3.54	o	-5.83	oo	-1.85	NS	4.21	*	4,24	*
a2b1	21.79	***	13.67	***	10.48	***	10.88	***	10.57	***
a2b2	-13.58	ooo	1.00	NS	-0.85	NS	-6.45	oo	-8.93	Oo
a2b3	-3.88	o	-2.16	o	-0.82	NS	-0.79	NS	-3.10	O
a2b4	-9.21	oo	-4.33	o	-3.85	o	-0.62	NS	-0.43	NS
a3b1	15.79	***	9,67	***	8.48	**	5.88	**	2.57	*
a3b2	-10.54	ooo	-8.00	oo	-14.19	ooo	-20.79	ooo	-25.10	Ooo
a3b3	-10.71	ooo	-10.33	ooo	-6.19	oo	-4.12	o	-5.43	Oo
a3b4	-12.38	ooo	-9.33	oo	-2.52	o	5.55	**	12.24	***
Averag, %	**36.21**		**45.33**		**51.52**		**58.12**		**62.43**	
DL5% = 2.46; DL1% = 6.08; DL0.1% = 10.22										

***, **, *, NS indicate statistical significance at the DL5%; DL1% and DL0.1% level and nonsignificant, respectively

Approximately, the same evolution is observed following the determinations made on 11.08.2019, the differences related to the size of plants being statistically ensured, from very significant negative (ooo) to experimental variant a3b2, to very significant positive (***) in the case of tomato plants tested in variants a1b1, a2b1 and a3b4., the lowest values of this biometric parameter being obtained in the case of plants belonging to the experimental variant a3b2, the plants developing under optimum lighting conditions, which led to obtaining some vigorous plants.

CONCLUSIONS

The results obtained at the biometric measurements (the size of the stems) showed that the answers were different in terms of all the factors involved: the assortment of tomatoes (3 varieties), the color spectrum of the light emitted by the LED devices (red, blue and white) but

also depending on the duration of exposure to daily treatments of additional short-term lighting (15, 30 and 45 minutes).

Thus, for tomato plants belonging to the Sonia de Buzău variety, the statistically significant was negative (ooo) response was registered for the combination of factors a2b2- (LEDs with red light x 15 minutes) as well as the combination of factors a1b4- (LEDs with blue light x 45 minutes exposure).

For the tomato plants of Hera variety, the statistically significant negative force (ooo) response was recorded at the combination of factors a2b4 (red light LEDs x - 45 minutes).

And in the case of the tomato seedlings of the Coralina variety, the statistically insured response, very negative (ooo) was registered to the experimental variant a3b2 consisting of the combination of factors a3 - LEDs white light x b2 - 15 minutes.

The analysis of the results recorded by the investigation of the biometric parameters, shows that by these additional lighting treatments, the tomato plants have developed much better compared to the plants of the control variant (without additional LED lighting treatment), which the plant stems they have gone a long way.

ACKNOWLEDGEMENTS

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THE EFFECT OF TiO_2 AND ZnO_2 NANOPARTICLES UPON SOME BIOMETRICAL CHARACTERISTICS IN SOYBEAN (*Glycine max* L. Merril) *IN VITRO* CULTURES

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Abstract

The aim of this study was to investigate the effects of two different nanoparticles TiO_2 (TiO_2 NPs) and ZnO_2 (ZnO_2 NPs) on the *in vitro* culture of soybean plants *(cv. Felix.). The TiO_2 and ZnO_2 NPs concentrations used for soybean tissue culture were 10, 100 and 1000 mg/l added to MS medium which contained no plant growth regulators. After four weeks, average height of the plants, average length of the main roots, secondary roots and fresh weight of the plantlets were measured. The results show that relatively low concentrations of TiO_2 NPs (10 and 100 mg/l) when added to the culture medium didn't have deleterious effects, but stimulated the growth and development of soybean plants. When higher concentrations (1000 mg/l) of TiO_2 NPs were added the plant growth was inhibited. The average fresh weight of a plantlet was 2200,14±51,56 mg on the medium without TiO_2 NPs and 2270,03±39,78 mg at the concentration of 100 mg/l TiO_2 NPs added. At the highest concentration of TiO_2 NPs added the average weight of the plant decreased significantly to 1616,86 ± 68,09 mg. The addition of ZnO_2 NPs to the culture media at higher concentrations than 10 mg/l showed a clear inhibitory effect on plant growth. Therefore, our results suggest that ZnO_2 NPs had a greater inhibitory effect on soybean plant growth and development than the TiO_2 NPs.*

Key words*: micropropagation, nanoparticles, titanium, zinc.*

INTRODUCTION

Nanoparticles of TiO_2 and ZnO_2 are used already on a large scale in many industries such as food, pharmaceutical, cosmetics, plastics, paper and it is expected that in the near future to become a possible source of danger of toxicity for our environment since its quantity is increasing (Coman et al., 2019; Javed et al., 2017; Zafar et al., 2016; Demir et al., 2014; Safavi, 2014; Prasad et al., 2012; Klančnik et al., 2011).

Previous reports highlight the positive but also the negative effects of ZnO_2 nanoparticles on different agricultural crops; their effects being influenced by their concentration (Javed, 2017; Zafar et al., 2016).

Other studies show that also the presence of TiO_2 nanoparticles can have both positive and negative effects on different plants' growth such as rice, peanut, black mustard, soybean, onion, sugarleaf etc. (Chutipaijit & Sutjaritvorakul, 2017; Laware & Raskar, 2014; Safavi, 2014). Among these plants, soybean (*Glycine max*), which is considered one of the most important agricultural crops plants in terms of seed protein and oil contents, has proven to be a perfect model for nanoparticle accumulation studies due to its high biomass production and ease of cultivation (Coman et al., 2019). However, to the best of our knowledge, there is no any report published about the effect of TiO_2 and ZnO_2 on *in vitro* culture of soybean plants. Therefore, the aim of this research was to investigate the effect of TiO_2 and ZnO_2 nanoparticles in different concentrations ranging from 10 to 1000 mg/l on the development of soybean cv. Felix grown in vitro. We strongly believe that the experimental

results would considerably contribute to the enrichment of knowledge regarding the interaction of TiO_2 and ZnO_2 nanoparticles with plant mechanisms.

MATERIALS AND METHODS

Characterization of TiO_2 and ZnO_2 nanoparticles

The nanoparticles used in this experiment were purchased from Sigma-Aldrich and Merck: TiO_2 (TiO_2 NPs): code 700347-25G, Lot # MCBT6314V Titanium (IV) oxide, mixture of rutile and anatase nanoparticles, < 150 nm particle size (volume distribution, DLS), dispersion, 40 wt. % in H_2O, 99.5% trace metals basis and ZnO_2 (ZnO_2 NPs) code 721077 - 100G, Lot # MKCC4480 Zinc oxide, dispersion nanoparticles, < 100 nm particle size (TEM), ≤ 40 nm avg. part. size (APS), 20 wt. % in H_2O.

Media preparation with TiO_2 and ZnO_2 nanoparticles

To investigate the toxicity of effect of Ti NPs and Z NPs plain Murashige and Skoog 1962 (MS) agar media was used (Murashige and Skoog, 1962) with no plant growth regulators added (Duchefa Biochemie B.V. Olanda, M0222, Murashige and Skoog 1962 (MS) medium including vitamins, original concentration 4405.19 mg/l). Regular sugar was added as carbon source and plant agar was used to solidify the culture media (4 g/l plant agar, Duchefa Biochemie B.V. Olanda, cod P1001**,** Plant Agar, Cas number 9002-18-0, Gel strength min. 1100 g/cm^2, Crude ash < 3%, Ash, acid insoluble < 0.5%). The pH of the media was adjusted to 5,8 before the plant agar was added as solidifying agent. The medium was dispensed (8 ml) in glass tubes of 140/25 mm. The media was autoclaved at 121ºC for 20 minutes, 1 atm. The TiO_2 NPs and ZnO_2 NPs, in concentrations of 0, 10, 100 and 1000 mg/l were added to the culture medium before autoclavation.

In vitro Culture

Felix soybean cultivar was chosen and used as plant material to carry out this experiment. This variety was crated at the Agricultural Research and Development Station Turda-Cluj as a result of the cross of Maple presto x Merit. This soybean variety develops a compact bush, erect port, and semi-definite growth. It is a high-grade variety with an average height of 94 cm. The average mass of 1000 soybean is 178 grams. The average vegetation period is 122 days (Mureşanu et al., 2010). To initiate the *in vitro* culture, the seeds of 'Felix' soybean were washed through running tap water for 10 minutes and then rinsed with double-distilled water containing one drop of Tween using a magnetic stirrer. Then, the seeds were rinsed again using double distilled water to eliminate the rest of the Tween solution. In the next step, in aseptic conditions the seeds were treated with ACE solution (20%) for 20 minutes and then rinsed with double-distilled water several times thoroughly. One seed was inoculated in each glass tube (Figure 1 a, b). The culture vessels were kept in growth chamber at 36 μmol m^{-2} s^{-1} light intensity provided by white fluorescent tubes (Philips, 36 W), at 23 ± 2°C and 50 ± 5% humidity.

Data analysis

To investigate the effects of ZnO and TiO2 NPs seed germination and plant characters, 20 glass tubes of each treatment were inoculated with 1 seed/tube in three repetitions. To analyse the explant responses, 10 plantlets/treatment were randomly selected for measurements. After four weeks of culture various biometrical measurements were made as follow: plantlets' height, length of the main and secondary roots, fresh weight of the plantlets. The results are presented as mean values with standard error. The means were further analysed using ANOVA and Tukey's HSD test ($p < 0.05$) to determine the differences among the means.

RESULTS AND DISCUSSIONS

Effect of TiO_2 NPs on soybean in vitro regeneration

The presence of TiO_2 NPs in the culture media in low concentration (10 mg/l) did not affect negatively but stimulated plantlet growth. The highest plants were developed on the culture media with 10 mg/l TiO_2 NPs concentration reaching 16.79 ± 0.40 cm. The increase of NP's concentration inhibited plant growth; at 1000 mg/l Ti NPs the average plant height recorded was 14.61 ± 0.34 cm. Regarding the maximum average length of primary and secondary roots, there were no statistically significant differences between the control and the concentration of 10 NPs, while at 1000 mg/l NPs concentration root

length decreased significantly by 47.03% (7.74 ± 0.27 cm) for both primary and secondary roots (Figure 2). Furthermore, the number of secondary roots was significantly lower at 1000 mg/l TiO_2 NPs in comparison with other concentrations (Figure 3). The average fresh weight of the plantlets on the media without NPs was 2200.14 ± 51.56 mg/plantlet and increased to 2270.03 ± 39.78 mg/plantlet at the concentration of 100 mg/l TiO_2 NPs. At 1000 mg/l TiO_2 NPs decreased by 26.52% 1616.86 ± 68.09 mg/plantlet (Figure 4).

Similar growing patterns were observed in *Oryza sativa* (Chutipaijit & Sutjaritvorakul, 2017) when different TiO_2 NPs concentrations were added to the culture media (0, 5, 10, 15, 20, 25 mg). The results show, that up to the concentration of 20 mg the nanomaterials stimulated plant regeneration. Thus, the culture medium supplemented with 20 mg l^{-1} TiO_2 nanoparticles provided the highest frequency (67%) of plant regeneration from mature seeds of indica rice cv. RD49 while higher concentrations inhibited plant growth. In addition, the results also suggest that optimum concentration of TiO_2 nanoparticles has significantly enhanced the percentages of callus induction and plant regeneration of *Oryza sativa* cultivars (Suphanburi1 and Suphanburi 90 cultivars). This research highlights also the efficiency of the culture medium supplemented with TiO_2 nanoparticles for in vitro micropropagation from mature seeds of Suphanburi1 and Suphanburi90 cultivars. Culture medium supplemented with 50 mg l^{-1} and 40 mg L^{-1} TiO_2 NPs were selected as the best media composition for callus induction and plant regeneration (Chutipaijit & Sutjaritvorakul, 2018).

Figure 1. *In vitro* culture of *Glycine max* L. 'Felix' with different TiO_2 and ZnO_2 NPs concentrations: a,b - *in vitro* culture initiation; c - culture media with 0, 10, 100 and 1000 mg/l ZnO NPs; d - *in vitro* culture of soybean with 0, 10, 100 and 1000 mg/l NPs of TiO_2.

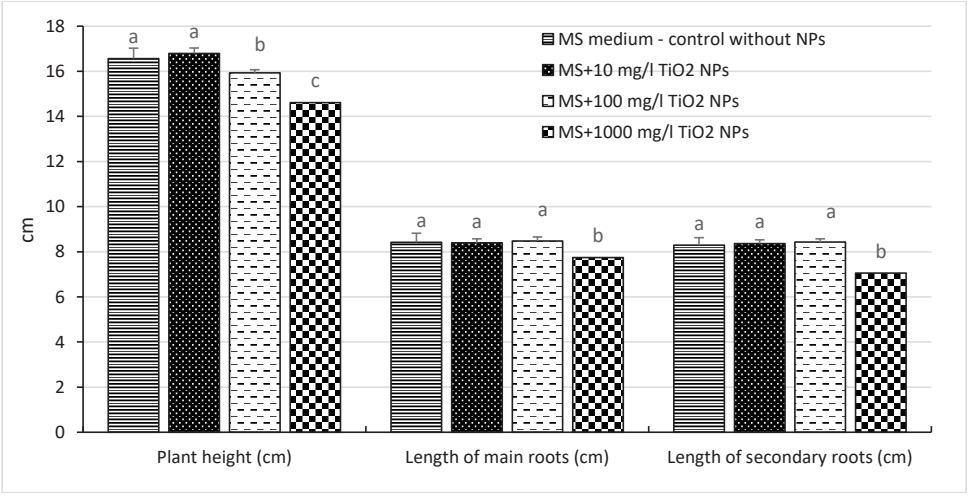

Figure 2. Effect of TiO_2 NPs on *in vitro* cultured *Glycine max* L. 'Felix' plantlet length, root length (primary and secondary) The values shown are means ± SE. Different lowercase letters above the bars indicate significant differences between the means of different treatments according to Tukey's HSD test ($p \leq 0.05$)

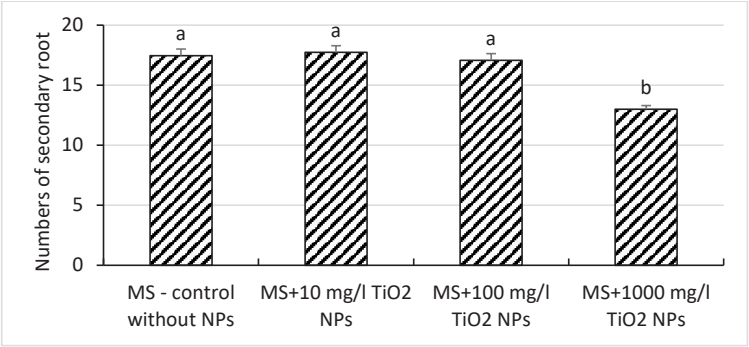

Figure 3. Effect of TiO_2 NPs on *in vitro* cultured *Glycine max* L. 'Felix' number of primary and secondary roots. The values shown are means ± SE. Different lowercase letters above the bars indicate significant differences between the means of different treatments according to Tukey's HSD test ($p \leq 0.05$)

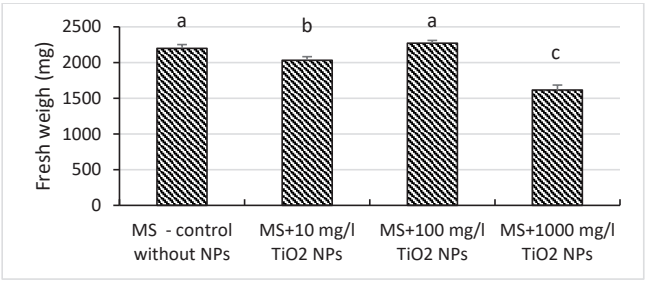

Figure 4. Effect of TiO_2 NPs on *in vitro* cultured *Glycine max* L. 'Felix' fresh weight (mg/plant). The values shown are means ± SE. Different lowercase letters above the bars indicate significant differences between the means of different treatments according to Tukey's HSD test ($p \leq 0.05$)

Effect of ZnO_2 NPs on soybean in vitro regeneration

Soybean plantlets responded differently to different concentrations of ZnO_2 NPs incorporated in the culture media. Similar to TiO_2 effects, at low concentrations the nanoparticles generated a stimulatory effect upon plant growth reaching 18.26 ± 0.57 cm as compared to control (16.56 ± 0.46 cm). Concentrations of 100 mg/l and 1000 mg/l ZnO_2 NPs inhibited plant growth and decreasing plant height by 14.32% (14.19 ± 0.29 cm) and 26.94% (12.10 ± 0.36 cm) respectively (Figure 5). Similar results have been obtained in S*tevia rebaudiana* when the presence of ZnO_2 NPs in concentrations of 0.1, 1 and 10 mg/l stimulated shoot growth, but the length of the shoots decreased as the NP concentrations increased (100 and 1000 mg/l) as compared to control (Javed et al., 2017).

At the same 10 mg/l ZnO_2 NP concentration, both average length of primary roots and secondary roots were considerably increased as compared to control. Concentrations of 100 and 1000 mg/l ZnO_2 NP reduced root development by 56.06% (3.70 ± 0.20 cm) and 75.9% (2.03 ± 0.33 cm) as compared to control (8.42 ± 0.24 cm). The same reducing pattern was observed also in the development of secondary roots (Figure 5). The average number of secondary roots was significantly lower. The increase of NPs concentrations decreased proportionally the length of secondary roots (Figure 6); thus the length of secondary roots ranged from 17.47 ± 0.54 (control) and 5.83 ± 0.36 (1000 mg/l ZnO_2 NPs). The fresh weight of the plantlets decreased in all the treatments as compared to control. It was observed that at the highest concentration of ZnO_2 NPs (1000 mg/) the average fresh weight of the plants decreased to 1061.31 ± 43.42 mg, representing a decrease of 51.77% (Figure 7). Other reports (Javed et al., 2017) show that same concentrations of ZnO_2 NPs (1000 mg/l) added to the culture media MS for *Stevia rebaudiana* led to a decrease of 56.25% from 0.16 ± 0.07 g in control to 0.07 ± 0.04 g at 1000 mg/l NPs. Concentrations of 0.1,

1, 10 and 100 ml/l ZnO_2 NPs stimulated biomass growth in Stevia.

Our results indicate that the presence of both TiO_2 and ZnO_2 NPs at the concentration of 1000 mg/l in the culture media inhibited secondary root formation and growth. Thus, it was observed that the *in vitro* plantlets emerged secondary roots only in the transition area above the level of the culture media (Figure 1c and d).

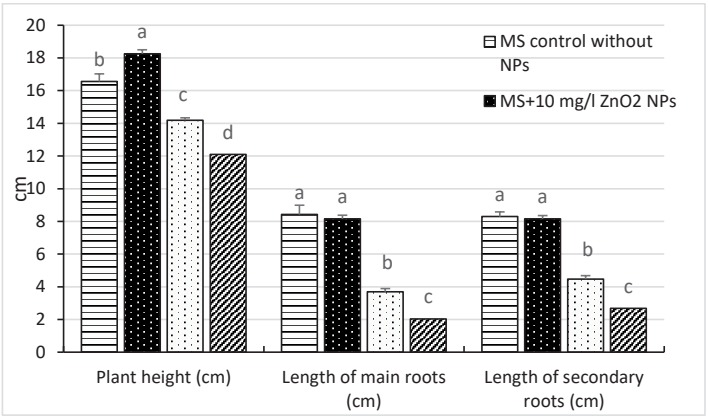

Figure 5. Effect of ZnO_2 NPs on *in vitro* cultured *Glycine max* L. 'Felix' on plant height and primary and secondary root length. The values shown are means ± SE. Different lowercase letters above the bars indicate significant differences between the means of different treatments according to Tukey's HSD test ($p \leq 0.05$)

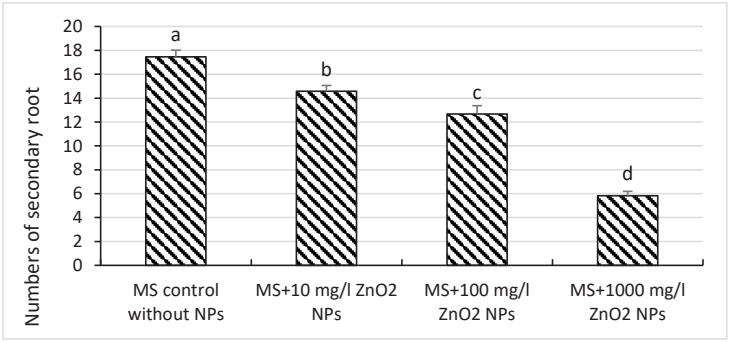

Figure 6. Effect of ZnO_2 NPs on *in vitro* cultured *Glycine max* L. 'Felix' secondary root number. The values shown are means ± SE. Different lowercase letters above the bars indicate significant differences between the means of different treatments according to Tukey's HSD test ($p \leq 0.05$)

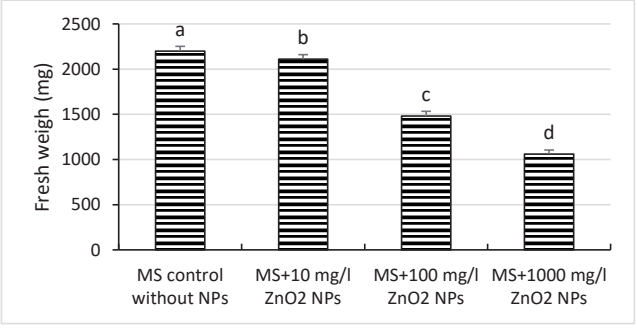

Figure 7. Effect of ZnO_2 NPs on *in vitro* cultured *Glycine max* L. 'Felix' fresh weight (mg/plant). The values shown are means ± SE. Different lowercase letters above the bars indicate significant differences between the means of different treatments according to Tukey's HSD test ($p \leq 0.05$)

CONCLUSIONS

To sum up, in this experiment we revealed the responses of *Glycine max* L. 'Felix' suggesting that TiO_2 NPs when incorporated to the culture media in concentrations of 10 mg/l or 100 mg/l did not have any negative effect on plant fresh weight or number of secondary roots. Moreover, the concentration of 10 mg/l TiO_2 NPs stimulated plant growth. Similar effects were observed also when ZnO_2 NPs were added in the same concentration simulating plant growth, but in comparison to TiO_2, ZnO_2 showed a greater inhibitory effect on the investigated plant growth parameters at concentrations of 100 and 1000 mg/l (fresh weight, number of secondary roots, length of primary and secondary roots).

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EFFECT OF VARIOUS EXTRACTION METHODS ON PEANUT PROTEIN EXTRACTION EFFICIENCY

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Abstract

Peanut allergy is one of the most common food allergies. Peanuts can cause a severe, potentially life-threatening allergic reaction (anaphylaxis). In order to improve consumer protection, our study is geared to quantification of proteins through several extraction methods. Fried peanuts samples were firstly defatted by stirring with pre-cooled acetone and 0.07% β-mercaptoethanol and then extracted with different buffers. The effect of various extraction procedures on the extraction efficiency for peanut protein was investigated by Lowry assay. Bovine serum albumin (BSA) was used as standard. Our work shows that the results depend on extraction method used.

Key words*: Lowry assay, peanut allergy, peanut extraction.*

INTRODUCTION

Food-induced allergy represents a public health problem spread worldwide affecting adults and children with a rising growth. According to Food and Agriculture Organization, the most common allergenic foods include peanuts, milk, soy, eggs, nuts, fish, shellfish, and wheat. They are responsible for 90% of the total numbers of food allergies (Ekezie, Cheng and Sun, 2018; Pele and Campeanu, 2016).

Among allergic foods, peanuts (*Arachis hypogaea*, fam. Fabaceae) represent the main causative factor of the most severe allergic reactions, including allergic and anaphylactic shocks (Xiaowen et al., 2019). Peanuts are used worldwide in food industry for oil production, peanut butter, cake decoration, roasted peanuts and snack products, extenders in meat product formulations, soups and desserts (Zhao et al., 2012). A major concern of health organizations is that even trace amounts of peanut can induce serious allergic reactions for certain people, for example threshold doses are as low as 100 μg of peanut protein (Al-Muhsen et al., 2003). Also, often peanut allergies are persistent throughout the lifetime, and only 20% sensitive individuals can overgrow it (Skolnick, 2001). Currently, some therapies have been introduced to reduce the prevalence of peanut allergy including strict avoidance and rescue medication upon accidental exposure to peanuts, oral immunotherapy, modifying or removing allergens from foods, etc. (Sitton and Temples, 2018; Bavaro et al., 2018).

Peanut kernels contain lipids, proteins and fibers along with some amount of carbohydrate, vitamins, and minerals (Pi et al., 2019). Overall, seventeen protein allergens have been identified in peanuts and are listed by the Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies (Subcommittee, 2020), named Ara h1 to Ara h17. Among these allergens, Ara h1, Ara h2, Ara h3 and Ara h6 are more abundant and associated with severe allergic reactions (Zhuang and Dreskin, 2013).

Proteins need to be extracted efficiently to ensure that an accurate representation of allergenic proteins from the source material is obtained. Therefore, the selection of an adequate method of extraction represents a major step for

further investigations. In order to obtain an optimized peanut extraction, several extraction conditions must be tested and their effects on protein characteristics need to be investigated (Ma et al., 2010). The factors that affect the extractability of proteins include: the extraction buffer (nature, pH, ionic strength), the solid-to-liquid ratio, the extraction temperature, the extraction duration, etc. (Walczyk et al., 2017; Kain et al., 2009, Kim et al., 2011; Poms et al., 2004). Usually, the main goal in optimization of protein extraction conditions is to obtain as much protein as possible. Several methods have been employed to assess the total protein content in samples like: UV detection, Lowry method, BCA assay, Bradford method, biuret method, etc. (Nishi et al., 1985; Zheng et al., 2017). The Lowry method is one of the most used procedures in order to quantify proteins, due to its sensitivity, good reproducibility, easy to apply (Upreti et al., 1988; Pires-Oliveira and Joekes, 2014).

Herein we investigated the influence of several parameters on the extractability of protein, such as: extraction buffer (pH and molarity), solid-to-liquid ratio, temperature of extraction, and duration of extraction. The protein extractability, assessed as total protein content, was quantified by Lowry assay using bovine serum albumin (BSA) as standard protein.

This knowledge will be valuable for the optimization of peanut protein extraction for medical researchers and peanut breeders.

MATERIALS AND METHODS

Materials: β-mercaptoethanol, Tris-HCl, Folin Ciocalteu reagent, sodium carbonate, sodium hydroxide, copper sulfate and trisodium citrate were purchased from Sigma-Aldrich, Germany. BSA (synthesis grade, ≥ 95%) was obtained from Merck, Germany. Acetone used was analytical grade.

Peanut sample preparation

Fried peanuts were purchased from a local supermarket (Figure 1), washed with distilled water and drained at room temperature.

Figure 1. Peanut Sample (local market)

Protein extraction and characterization

Protein extraction

Defatted peanut powder was prepared using the procedure described by Zhou et al. (Wu et al., 2016). Fried peanuts were milled with a grinder to obtain a milled powder. The peanut powder was defatted by stirring with pre-cooled acetone and 0.07% β-mercaptoethanol (1:5 w/v ratio) for 2 h at 4°C. Pellets were then filtered by a vacuum filter. The defatting process was repeating three times. The defatted powder was dried in an oven at 40°C and stored at - 20°C. The obtained defatted powder was further used at extraction of peanut protein. Protein was extracted using five different methods (Methods 1-5) varying several parameters like: extraction buffer, solid-to-liquid ratio, temperature of extraction and duration of extracti
on.

Method 1: Peanut protein extract was obtained by mixing the defatted peanut powder with 20 mM Tris-HCl, pH 7.2 with ratio of 1:10 (w/v) at 25°C for 2-6 h. The crude extract was cleared by centrifugation at 10 000 rpm for 30 min at 4°C and the supernatant was collected.

Method 2: Peanut protein extract was obtained by mixing the defatted peanut powder with 20 mM Tris-HCl, pH 7.2 with ratio of 1:10 (w/v), and stirred at 4°C for 2-6 h. The crude extract was cleared by centrifugation at 10 000 rpm for 30 min at 4°C and the supernatant was collected.

Method 3: Peanut protein extract was obtained by mixing the defatted peanut powder with 20 mM Tris-HCl, pH 8.2 with ratio of 1:10 (w/v), and stirred at 4°C for 2-6 h. The crude extract was cleared by centrifugation at 10 000 rpm for 30 min at 4°C and the supernatant was collected.
Method 4: Peanut protein extract was obtained by mixing the defatted peanut powder with 50 mM Tris-HCl, pH 7.2 with ratio of 1:10 (w/v), and stirred at 4°C for 2-6 h. The crude extract was cleared by centrifugation at 10 000 rpm for 30 min at 4°C and the supernatant was collected.
Method 5: Peanut protein extract was obtained by mixing the defatted peanut powder with 20 mM Tris-HCl, pH 8.2 with ratio of 1:20 (w/v), and stirred at 4°C for 2-6 h. The crude extract was cleared by centrifugation at 10 000 rpm for 30 min at 4°C and the supernatant was collected.

Protein quantification

Extraction efficiency for peanut protein was investigated by Lowry assay. BSA was used as standard. In short, for the Lowry assay 0.5 ml of sample extract were incubated with 0.5 ml Lowry reagent at 25°C for 10 minutes; then were added 1.5 ml Folin Ciocalteu reagent (1:10 v/v) and kept for 30 minutes at room temperature. The absorbance was measured at 760 nm as compared to blank using an UV-Vis spectrophotometer (Jasco V630, Germany).
The Lowry reagent used was prepared mixing solution A with solution B 10:1 (v/v). Solution A: 10% sodium carbonate in 0.5% sodium hydroxide. Solution B: 0.5% copper sulfate in 1% trisodium citrate.

Statistical Analysis

Extraction experiments and protein content determination assessed by Lowry method were carried out in duplicate and, respectively triplicate. All data are expressed as the mean ± standard deviation.

RESULTS AND DISCUSSIONS

As peanut is a popular food and an important source of proteins and food oil worldwide, extraction represents an important step in the acquisition of target compounds from various materials (Kain et al., 2009; Sharma et al., 2002). Hence, the knowledge of protein extractability and quality is a key factor in selecting particular proteins for possible medical research and food applications (Poms et al., 2004; Jiang et al., 2010).
A challenge to overcome when studying proteins is the selection of the most appropriate method of protein extraction. Many factors affect the extractability of proteins.
For the selection of optimal extraction conditions of peanuts the influence of several parameters on the protein extractability (extraction buffer - pH and molarity, solid-to-liquid ratio, temperature of extraction and duration of extraction) need to be investigated. The protein extractability was assessed by Lowry assay.
The Lowry method was chosen for the protein quantification due to its sensitivity and good reproducibility. Also it is a very easy method to apply in laboratory. The peanut extraction conditions are listed in Table 1.

Table 1. Peanut extraction conditions

Sample name	Extraction conditions
I.1	Tris 20 mM; pH 7.2; 1:10 (w/v); 25^0C; 2 h
I.2	Tris 20 mM; pH 7.2; 1:10 (w/v); 25^0C; 4 h
I.3	Tris 20 mM; pH 7.2; 1:10 (w/v); 25^0C; 6 h
II.1	Tris 20 mM; pH 7.2; 1:10 (w/v); 4^0C; 2 h
II.2	Tris 20 mM; pH 7.2; 1:10 (w/v); 4^0C; 4 h
II.3	Tris 20 mM; pH 7.2; 1:10 (w/v); 4^0C; 6 h
III.1	Tris 20 mM; pH 8.2; 1:10 (w/v); 4^0C; 2 h
III.2	Tris 20 mM; pH 8.2; 1:10 (w/v); 4^0C; 4 h
III.3	Tris 20 mM; pH 8.2; 1:10 (w/v); 4^0C; 6 h
IV.1	Tris 50 mM; pH 7.2; 1:10 (w/v); 4^0C; 2 h
IV.2	Tris 50 mM; pH 7.2; 1:10 (w/v); 4^0C; 4 h
IV.3	Tris 50 mM; pH 7.2; 1:10 (w/v); 4^0C; 6 h
V.1	Tris 20 mM; pH 8.2; 1:20 (w/v); 4^0C; 2 h
V.2	Tris 20 mM; pH 8.2; 1:20 (w/v); 4^0C; 4 h
V.3	Tris 20 mM; pH 8.2; 1:20 (w/v); 4^0C; 6 h

Effect of buffer on protein content (pH and molarity)

The effect of buffer on protein content is presented in Figure 2 and Figure 3.

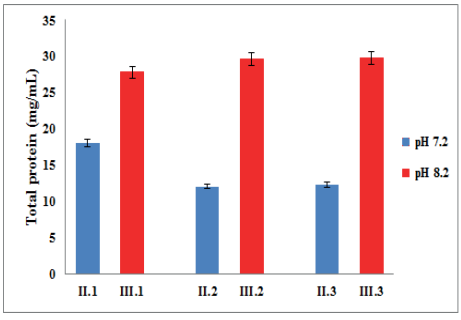

Figure 2. Effect of pH on total protein extraction

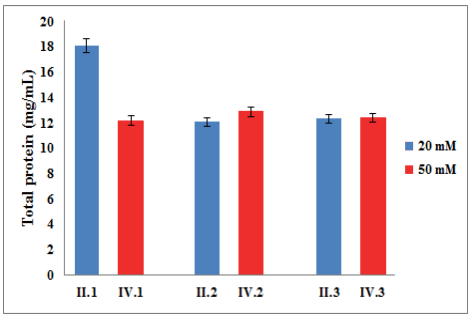

Figure 3. Effect of molarity on total protein extraction

It was observed the pH had a strong impact on the protein content in all samples. By increasing the pH value of the buffer from 7.2 to 8.2 the protein extractability increased as well. Our results were in agreement with the work of Shridhar et al. (2009) who reported that the protein extractability from edible nuts was about two times higher at pH 8.45 than at pH 7.2.

The molarity of buffer influenced the protein content in a lesser extent than the buffer pH. An exception was noticed at sample II.1 (Tris 20 mM; pH 7.2; 4^0C; 2 h) witch presented significantly higher value than sample IV.1 (Tris 50 mM; pH 7.2; 4^0C; 2 h).

Effect of solid-to-liquid ratio on total protein extraction

The effect of solid-to-liquid ratio of extraction on protein content is shown in Figure 4.

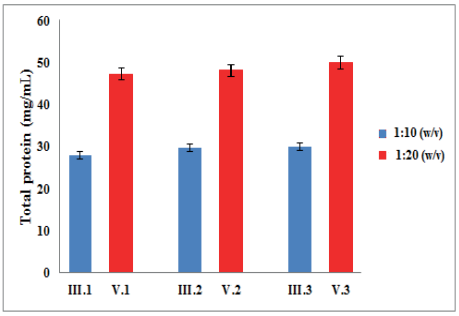

Figure 4. Effect of solid-to-liquid ratio on total protein extraction

It was observed that increasing the solid-to-liquid ratio from 1:10 (w/v) to 1:20 (w/v) the protein content increased in all samples.

These results were expected, because a higher solvent content leads to lower viscosity of the solution, thereby promoting molecular diffusion and facilitating protein extraction.

Effect of temperature of extraction on total proteins

The effect of temperature of extraction on protein content is shown in Figure 5.

In the case of the first two samples, the best results had been shown for sample II.1 (Tris 20 mM; pH 7.2; 4^0C; 2 h).

For the other samples significant differences were not observed.

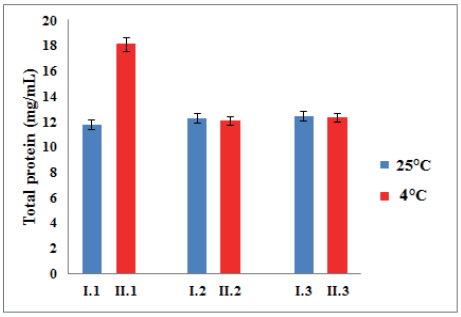

Figure 5. Effect of temperature on total protein extraction

Effect of duration of extraction on total proteins

The effect of duration of extraction of extraction on protein content is presented in Figure 6.

In the case of method I, III, IV, V slightly better results were obtained at a higher extraction time, while for method II the best extraction time was at 2 h.

Several papers reported that usually shorter extraction duration is preferred to minimize protein degradation (Kain et al., 2009).

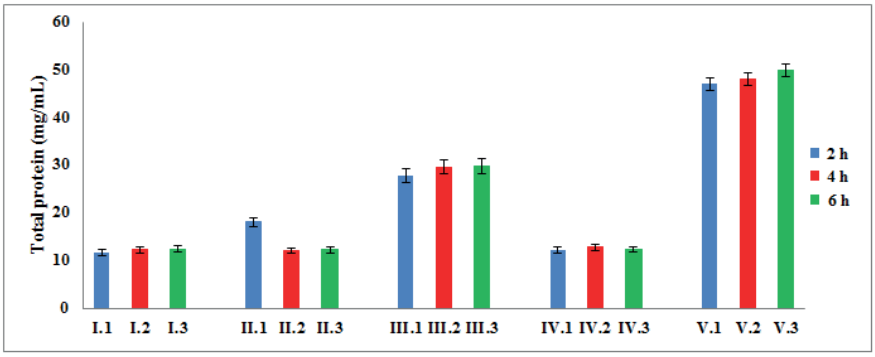

Figure 6. Effect of duration of extraction on total protein extraction

CONCLUSIONS

In this study, the influence of several extraction factors was assessed in order to identify the conditions that resulted in improved protein extractability.

Protein content increased significantly with increased pH and solid-to-liquid ratio of extraction. Buffer molarity, temperature of extraction and duration of extraction influenced the protein content in a minor extent.

These results present valuable information for the optimization of peanut protein extraction for medical researchers and peanut breeders.

Further investigations are needed to quantify the allergenic proteins from peanuts.

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EFFECT OF DRY SOURDOUGH ADDITION IN WHEAT FLOUR ON DOUGH RHEOLOGICAL PROPERTIES

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Abstract

The aim of this work was to characterise the rheological changes of wheat doughs with dry sourdough addition on mixing and pasting characteristics. Nowadays, it is well known that there is an increased demand for bakery products, obtained from natural ingredients, with good sensory characteristics. The use of sourdough in wheat flour improves bread sensory characteristics of bakery products which may present a better taste and flavour and longer shelf-life. Our study meets the demands of bakery consumers which desire products close, if it is possible to the traditional ones, by using the sourdough in the bread recipe. The advantage of this process is that the sourdough is directly introduced in the bread recipe fact that now in the "century of speed" facilities the bakery process. In order to know the way in which sourdough may change the bakery technological behaviour it is important to study dough rheological characteristics. For this purpose our study analyzes by using modern devices such as Farinograph, Extensograph and Amylograph the way in which sourdough addition changes dough rheological characteristics.

Key words*: Amylograph, bread, dry sourdough, Extensograph, Falling Number, Farinograph, wheat flour.*

INTRODUCTION

Sourdough has been used in the preparation of bread as a leavening agent for over 500 years and represents one of the oldest traditional and biotechnological products of all fermented foods (Arendt et al., 2007; Yu et al., 2018).

Sourdough is characterized as a dough composed of flour and water fermented by yeast and lactic acid bacteria and is used in the bakery industry as a leavening agent (Siepmann et al., 2018).

Poutanen et al. (2009), Manini et al. (2016) and Neysens & De Vuyst (2005) reported that sourdough due to its higher acidity, antifungal and antimicrobial activity may improve the shelf-life of the final product. Moreover, a considerable consensus has been reached on the positive effects of using sourdough in bread making, such as increased bread volume in the finished products an improved crumb structure (Clarke et al., 2004) and better sensory and aroma profile(Corsetti, 2012). Sourdough fermentation influences the rheological and functional properties of bakery products, affecting the rheology of the dough in two stages; at the first stage, that of sourdough itself and at the second stage, that of bread dough prepared with sourdough. In sourdough, fermentation decreases elasticity and viscosity of the wheat flour dough; therefore, addition of sourdough to bread dough results in less elastic and softer dough (Chavanet al., 2011; Clarke et al., 2002; Wehrle et al., 1997).

By adding sourdough in bread recipe all parameters change significantly such as dough's characteristics, nutritional value of bread, taste and flavour (Chavan et al., 2011; Katina et al., 2006; Tafti et al., 2013).

Sourdough has a major effect on dough properties and bread quality and in wheat breads, sourdough may be used to reduce phytic acid content which is a natural anti-nutrient compound found in the flour (Hansen et al.,1994; Clarke et al., 2002; Crowley et al., 2002; Lavermicocca et al., 2003; Dal Bello et al., 2007)

In recent years, the tendency of consumers towards traditional products has resulted in a huge success of traditional bread prepared with sourdough which produce a more natural taste and is healthier for the human body (Brummeret al., 1991).

Despite its long tradition and the well-documented positive effects on bread products, the use of sourdough in a dry form is not very

common in Romania however there is an increased tendency of using it The producers do not fully understand the influence of it's use on dough and finished products, which is significant (Armero et al., 1996; Gobbetti, 1998; Hammes et al., 1998; Brandt, 2001).

However, in addition to the positive aspects of adding the sourdough in the bread receipt, there are some disadvantages due the fact that it addition during mixing modify the technological process of bread making. Therefore, it is necessary that all the technological parameters of bread making to be adapted due to the dry sourdough addition in order to obtain bakery products of a good quality. Successful development of equipment for mechanized sourdough production is a positive step toward safe, competitive application of sourdough in wheat bread production. Initially, most used sourdoughs were liquid sourdoughs obtained through fermentation of wheat flour dough, that could be pumped on the fabrication process (Brümmer, 1991). The fermentation process changes the flow behaviour, viscosity and density of dough and also the chemical composition and microbial status of sourdough (Meuser et al., 1993). Many factors influence the rheological characteristics of the fermented dough. At the beginning of the mixing process, physical actions such as hydration take place, the gluten network is formed by proteins, and starch granules absorb water. Enzyme activity of amylases, proteases, and hemicelluloses causes the breakdown of several flour components (Wehrle et al., 1997). Recent researchers have led to systems that work with dry sourdough in a powder form (Meuser, 1995).

The aim of this work was to investigate the effect of the addition of dry sourdough (DS) on dough rheological properties by addition up to 5% sourdough.

MATERIALS AND METHODS

Wheat flour (harvest 2019) of a refined type was provided by S.C. MOPAN S.A. (Suceava, Romania) and dry sourdough from wheat flour by Enzymes & Derivates S.A. Company (Romania, Neamt).

The wheat flour was analyzed through the international and Romanian standard methods: falling number (ICC 107/1), wet gluten (SR90:2007) and gluten deformation index (SR 90:2007).

Dough rheological properties during mixing were analyzed by farinographic and extensographic assays.

The Farinograph device (Brabender, Duigsburg, Germany, 300 g capacity) and Extensograph device (Brabender OGH, Duisburg, Germany) was used according to ICC method 115/1.

The pasting properties of dough samples were analyzed by using the Falling Number (Perten Instruments, Sweden) device according to ICC method 107/1 and the Amylograph (Brabender OGH, Duisburg, Germany) device according to ICC method 126/1.

Statistical analysis was performed with XLSTAT (Version 2019.14.1, free trial; Addinsfot's Corporation, USA). A Tukey and ANOVA tests were performed with a 95% confidence interval.

RESULTS AND DISCUSSIONS

The wheat flour used in this study is one of a very good quality for bread making, with a low α amylase activity according to the obtained data: 0.66 g/100 g ash content, 14.0 g/100 g moisture content, 12.7 g/100 g protein content, 30 g/100 g wet gluten content, 6 mm gluten deformation index, and 442 s Falling Number value.

Effect of dry sourdough on Farinograph characteristics

The Farinograph is the standard device used for measuring water absorption capacity, and it is a very useful tool for measuring the mixing characteristics of wheat flour giving good indication of flour performance in bread making (Catterall, 1998).

According to the standard procedure (ICC 115/1) the following Farinograph indices were determined: water absorption (WA), development time of dough (DT), stability of dough (ST), and the degree of softening of dough (DS).

Table 1 shows the values obtained from the Farinograph for dough samples with different levels of dry sourdough addition. It may be seen that the addition of dry sourdough significantly increases the water absorption (WA) up to 3.2% compared to the PM3 sample and 2.8% compared to the PM4 sample.

Table 1. Farinograph parameters of wheat flour with dry different level of dry sourdough

Sample	Parameters			
	WA (%)	DT (min)	ST (min)	DS (BU)
PM1	60.0±0.01	1.7±0.01	2.5±0.01	66±2.00
PM2	60.6±0.01	1.9±0.01	2.3±0.02	77±1.00
PM3	61.8±0.01	1.5±0.01	1.3±0.02	93±1.00
PM4	63.2±0.01	1.7±0.02	1.2±0.01	127±1.00
PM5	62.8±0.02	1.5±0.01	1.1±0.01	142±1.00

Farinograph parameters: WA, water absorption (%), DT, dough development time (min), ST, dough stability (min), DS, degree of softening at 10 min (BU)

It may be seen that DT (Figure 1) do not change in a significant way and it may be noticed a slight decrease of this value to PM 5 sample. This may be due (Hoseney, 1994) to the decrease of dough pH values by the dry sourdough addition. Hoseney (1994) reported that the pH value of the dough has major influences on the mixing time so that the doughs with lower pH require shorter mixing times.

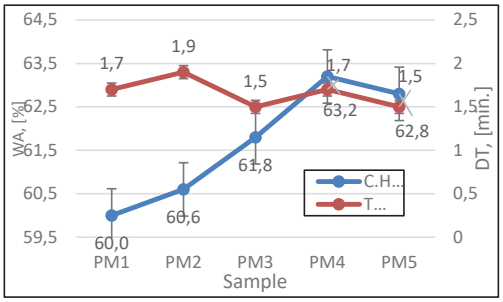

Figure 1. Representation of the correlation between water absorption and dough development time

Along DT decrease it may be seen also a decrease of ST values. Also, as it may be seen from the Figure 2 the degree of softening at 10 min (DS) increases, the maximum value being obtained for the PM5 sample which is the dough sample with the highest content of dry sourdough addition in wheat flour.

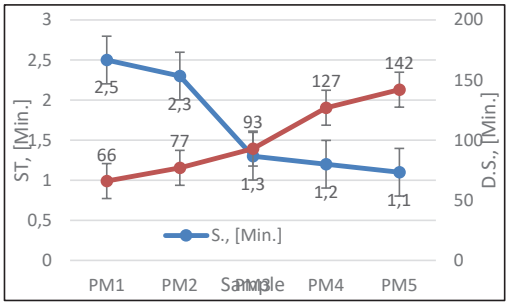

Figure 2. Representation of the correlation between dough stability and degree of softening

Effect of dry sourdough on Extensograph characteristics

The Extensograph gives information about dough extensibility and resistance to extension (Walker et al., 1996). A very desirable trait for the dough is a good combination of resistance and good extensibility.

With the increase level of dry sourdough addition from 1% to 5% the extensibility of dough gradually increased (Table 2).

Table 2. Extensograph parameters of wheat flour with different level of dry sourdough

Parameter	Sample	Time		
		45 min	90 min	135 min
Energy,E (cm^2)	PM1	52±1.00	54±1.00	58±1.00
	PM2	48±1.00	46±1.00	49±1.00
	PM3	41±1.00	40±1.00	35±1.00
	PM4	27±2.00	28±2.00	29±2.00
	PM5	39±1.00	39±1.00	39±1.00
Resistance to extension, R_{50} (BU)	PM1	230±2.00	236±1.00	235±2.00
	PM2	229±1.00	235±1.00	234±1.00
	PM3	228±1.00	237±1.00	236±1.00
	PM4	208±1.00	210±2.00	211±1.00
	PM5	207±1.00	209±1.00	210±1.00
Extensibility, E (mm)	PM1	136±1.00	137±1.00	148±2.00
	PM2	137±1.00	135±1.00	147±1.00
	PM3	135±1.00	136±1.00	149±1.00
	PM4	138±1.00	140±1.00	141±1.00
	PM5	137±1.00	139±1.00	142±1.00
Ratio number	PM1	1.7±1.00	1.7±1.00	1.6±1.00
	PM2	1.6±1.00	1.8±1.00	1.5±1.00
	PM3	1.8±1.00	1.6±1.00	1.7±1.00
	PM4	1.5±1.00	1.6±1.00	1.5±1.00
	PM5	1.4±1.00	1.7±1.00	1.4±1.00

The increase in proofing time also caused a higher value of dough extensibility. The energy or work input necessary to promote the deformation was increased with the addition of 1% to 5% dry sourdough, for all resting times. A slight decrease in deformation energy of the dough containing dry sourdough was also reported by Kulp et al. (2003), Esteve et al. (1994), Gocmen et al. (2007). They concluded that as the sourdough level increased, the resistance to extension decreased.

Effect of dry sourdough on Falling Number and Amylograph characteristics

The effects of the addition of dry sourdough on the amylograph parameters can be noted in Table 3. A significant decrease in gelatinization temperature (Tg) and peak viscosity (PV) has been noticed with the increase level of dry sourdough addition. Also a significantly decreased of the falling number values was noticed by dry sourdough addition. As it may be seen the FN value decreased up to 18.4% for the

sample with 5% dry sourdough addition in wheat flour. This fact indicates that α amylase activity in dough system increased. This may be due to the α amylase content from dry sourdough or due to the pH change from the dough up to levels to optimum α amylase activity. Also it may be noticed a decreased in PV_{max} up to 8.43% for PM5 sample. This value may be correlated with FN value. This fact is explainable since it is well known that FN is proportional to the viscosity and inversely proportional to α amylase activity (Codină et al., 2012).

Table 3.Amylograph and Falling Number parameters of wheat flour with different level of dry sourdough

Sample	Parameters		
	Tg (^{0}C)	PV_{max}(BU)	FN (s)
PM1	88.8±0.01	1195±1.00	358±2.00
PM2	88.7±0.01	1184±1.00	345±1.00
PM3	88.5±0.01	1055±2.00	330±2.00
PM4	88.4±0.01	1105±2.00	319±1.00
PM5	88.1±0.15	1102±1.00	302±1.00

Pasting parameters: Tg, gelatinization temperature, PV_{max}, peak viscosity time (min), FN, falling number.

CONCLUSIONS

By adding dry sourdough a significant impact on the dough rheological characteristics was observed. The data obtained showed that the addition of dry sourdough in the flour led to the increase of water absorption (WA) and the dough softening degree (DS) and a significantly decrease of the dough stability (ST). Also it was noticed a decrease of the resistance to extension falling number values and peak viscosity.

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COMPARATIVE STUDY OF THE QUALITY OF TRADITIONAL HONEY AND INDUSTRIAL HONEY

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Abstract

Along with the special nutritional properties, honey also has therapeutical properties, finding its place in many applications of modern medicine. The main objective of the research consisted in the qualitative testing of eight honey types, four of traditional type and four of industrial type, both organoleptic and physico-chemical and microbiological: lime honey, acacia honey, sunflower honey and polyfloral honey. The organoleptic determinations allowed us to classify all types of honey in the high quality category. In the case of microbial contamination, it was found that no honey sample developed microbial colonies. The pH determinations revealed that all commercial honey showed slightly high value than the traditional ones. In the case of viscosity, the largest differences were observed. It has been found that the traditional sunflower honey has the highest viscosity, respectively 8860cP, and the commercial sunflower honey the lowest viscosity, 200cP. The determination of reducing sugars by the Schoorl method showed that industrial sunflower honey has the highest content, 92.5%, and traditional lime honey, the lowest, 74.3%.

Key words*: honey, sugars.*

INTRODUCTION

Bee honey is one of the most biologically complex products, in which compositions, apart from directly assimilable sugars, have been found very important substances for the human body. This fact increases the importance of honey to humans, compared to any other food (Bogdanov et al., 2008).

Besides the nutritional qualities, honey is in the first place among the medicines that nature offers us through flowers and herbs (Miguel et al., 2017).

The chemical and biological composition of honey makes it, in addition to excellent food, an important pharmaceutical means (Bogdanov et al., 2008). Vitamins in honey play the role of catalysts, glucose and fructose help regulate nerve activity, dilate blood vessels, have hemostatic action (stop bleeding) (Bogdanov et al., 2004). As it decreases the acidity of the gastric juice, honey is recommended in duodenal ulcer and hyperacidity (Sultan et al., 2017).

In the therapeutic field, honey can be used both orally and by local application. The most common diseases for which the honey bee is beneficial are: digestive disorders, hepato-biliary disorders, cardiovascular disorders and nervous system disorders (Miguel et al., 2017).

Honey is very nutritious, with promising properties of antioxidant, anti-inflammatory, antibacterial, as well as cough reduction and wound healing (Jaafar et al, 2017). The main concern in the use of honey-based medicines in modern medicine is the variation of the composition and the lack of clinical studies. Therefore, honey bee is recommended as a valuable dietary supplement.

The limited availability and the high price of honey offered a greater interest for falsifying or altering its quality. Honey quality and identity parameters are considered useful for detecting these possible distortions and also for confirming the hygiene conditions for handling and storing honey (Khaliq et al., 2013).

The main objective of this research consisted in the qualitative testing of eight honey types, four of traditional type and four of industrial type, both organoleptic and physico-chemical and microbiological: lime honey, acacia honey, sunflower honey and polyfloral honey.

MATERIALS AND METHODS

The examination of honey was carried out in order to assess its quality and purity, in order to establish the state of degradation or alteration, through a series of physico-chemical and microbiological determinations (Szasz-Zima et al., 2016).

In order to carry out the experiments, 8 honey bee samples were analyzed:

1) Traditional honey (purchased from small manufacturers):

- P1 - lime honey;
- P2 - acacia honey;
- P3 - sunflower honey;
- P4 - polyfloral honey.

2) Industrial honey (purchased from commerce):

- PI - lime honey;
- PII - acacia honey;
- PIII - sunflower honey;
- PIV - polyfloral honey.

The following parameters were analyzed: organoleptic parameters (appearance, color, odor, taste, consistency), pH, total acidity, reducing sugars, refractive index, viscosity, microbiological contamination.

Organoleptic analysis consists of examining a product by evaluating the perceptible attributes of the five sense organs (organoleptic attributes), such as: color, smell, taste, touch, texture. Sensory evaluation allows us to distinguish the botanical origin of honey, to identify and quantify certain defects (fermentation, impurities and flavors).

The ***pH*** **determination** was performed by potentiometric method (Romanian Pharmacopoeia, Xth Edition, 1993). Honey contains a number of acids which include amino acids and organic acids.

The ***total acidity*** was determined by titration with 0.1N sodium hydroxide solution (Romanian Pharmacopoeia, Xth Edition, 1993). Increased acidity of honey is an indicator for a fermentation process and transformation of alcohol into organic acid

The determination of the ***reducing sugars*** was done by the Schoorl method (Jurcoane et al., 2010).

Refractometric determination of the ***water content*** The refractive index of honey varies almost linearly, depending on the water content (Cano et al., 2001). It is considered that moisture content less than 18% will prevent the fermentation.

The ***viscosity*** was determined with the Brookfield DVII+Pro viscometer. The water content greatly influences the viscosity of honey. The more water a honey contains, the more fluid it is. Also, the presence of dextrins increases the viscosity of honey and gives it a glossy, unpleasant appearance.

Determination of impurities. The impurities were determined by weighing the resulting residue after filtration and drying of 10 g honey diluted to 50 ml with water. The impurities found in the honey samples represent an important parameter that provides information on the precision of the honey processing process.

Diastatic index The determination of the *diastatic index* was performed by the Goethe method (Persano et al., 1990). The diastatic index is expressed by the amount of starch (1%) hydrolyzed in 1 hour of 1 gram of honey. *diastatic index* is a measure of freshness, correct processing and a guarantee of its authenticity.

The ***microbiological analysis*** wanted to highlight the presence of possible microorganisms (bacteria and fungi) in the tested samples (Romanian Pharmacopoeia, Xth Edition, 1993).

RESULTS AND DISCUSSIONS

Organoleptic examination performed on the honey assortments allowed us to classify them in the good quality category (Table 1).

Table 1. Organoleptic characteristics of the analyzed honey samples

Honey sample	Organoleptic characteristics
P1	Clean, homogeneous appearance; fluid; light yellow; sweet taste; strong lime aroma
P2	Clean, homogeneous appearance; fluid; light yellow; sweet taste; pleasant aroma
P3	Clean, homogeneous appearance; fluid; viscous; dark yellow; pleasant, sweet, specific taste.
P4	Clean, homogeneous appearance; fluid; light yellow; sweet taste, very aromatic
PI	Homogeneous appearance; fluid; yellow-orange, sweet taste, strong lime aroma
PII	Clean, homogeneous appearance; fluid; light yellow; sweet taste, pleasant aroma
PIII	Clean, homogeneous appearance; fluid; golden yellow; sweet taste; pleasant aroma
PIV	Clean, homogeneous appearance; fluid; light yellow; sweet taste, very aromatic

The pH of honey. Honey contains a number of acids which include amino acids and organic acids, which gives honey an acid character.
The pH values were between 3.5 for industrial sunflower honey and 4.2 for industrial lime honey. The average pH of honey is 3.9, with a typical range of 3.4 to 6.1. A lower pH value suggests a possible alteration of the honey by fermentation, The pH value tests revealed that all honey samples fall within the normal limits of the admitted pH values (Figure 1).

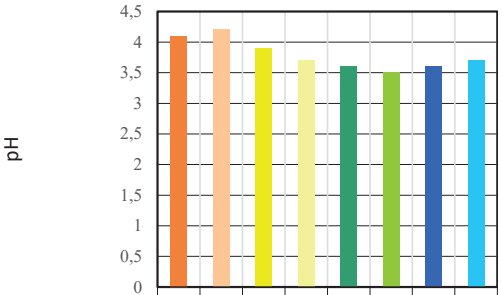

Figure 1. The pH value of of honey samples

Determination of total acidity. This parameter allows to appreciate the freshness of honey. The acidity can be exceeded in case of fermentative alteration. In the case of falsification with invert sugar syrup, the reaction can be strongly acidic, if the acid used has not been neutralized, or neutral to alkaline in case of excessive neutralization.
All the analyzed samples were within the permissible limits, according to Table 2, the highest value being found in samples P4 (traditional polyfloral honey) 2.8, and the smallest value in sample I (industrial honey of lime), 1.4.

Table 2. Total acidity of honey samples

Honey sample	Total acidity (cm^3 NaOH solution 0.1 N/100 g honey)
Limit	max. 4.0
P1	**1.4**
PI	1.6
P2	1.8
PII	2.2
P3	2.6
PIII	2.6
P4	**2.8**
PIV	2.4

Since none of the samples exceeded the maximum allowed value, we can conclude that the honey tested did not suffer any alteration or falsification and shows stability for fermentation.
Determination of reducing sugars. Sugars are the main constituent and they give honey the main characteristic, the sweet taste: glucose, fructose, and sucrose. In general, the sugar composition of honey is affected by botanical origin (types of flowers), geographical origin, climate, processing and storage time (da Silva et al., 2016)
The average content of honey in reducing sugars is around 76%. A low content indicates poor quality of honey, which makes it susceptible to fermentation, while a too high content can mean artificial intervention on its composition.
From the results presented in Figure 2 it can be observed that the values regarding the content in reducing sugars corresponds, in almost all samples, to the data mentioned in the literature. Exceptions and extremes were the samples PIII (commercial sunflower honey), 74.8% and P3 (traditional sunflower honey), 86.4%.
Comparing traditional honey samples with the industrial ones we can observe that the first ones, due to their higher sugar content, are more qualitative.
In the case of P3, due to the very high content, 86.4%, we can assume that it was counterfeit by the addition of sugar. However, if we correlate this result with those obtained for the water content and the viscosity of the sample, this increased content sugars can be justified.

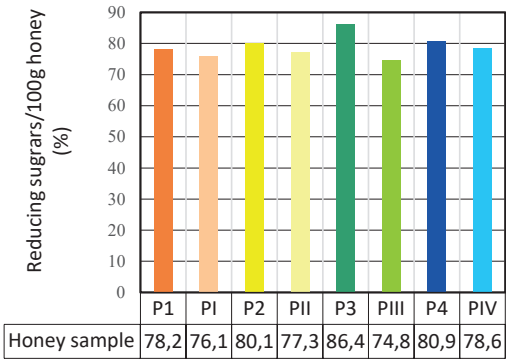

Figure 2. Content in reducing sugars of honey samples

Determination of water content. The higher moisture-in-honey content, the greater is the

possibility that the yeasts will ferment and change the flavor.The values of the refractive index varied between 1.4870-1.5044, all these data being presented in Table 3. The excess water content (max. 18%) reduces the value of honey and predisposes it to fermentation.
The low water content of P3 (traditional sunflower honey) is also supported by the following determination, of the viscosity, which could not be achieved under the same conditions as the other samples.

Table 3. Corelation between water content and refractive index of the honey samples

Honey sample	Refractive index (nD)	Water content (%)
Limit	1.4915 to 1.4993	20.8-15.0
P1	1.4937	17.1
PI	1.4915	18.0
P2	1.4987	15.2
PII	1.4920	17.8
P3	**1.5044**	**13.0**
PIII	**1.4870**	**19.8**
P4	1.4956	16.4
PIV	1.4930	17.4

Determination of viscosity. As can be seen in Table 4, the viscosity of the honey samples varied very widely. The determinations made at 22ºC, for 5 min, 8 RPM, with 06 rod, the values obtained varied between 240 cP, for PIII and 800 cP, for P2. Due to the high viscosity of sample P3, the determination was made with the 07 rod, without disk, the value obtained being 1200 cP.

Table 4. Viscosity of the honey samples

Honey sample	Rod	Viscosity (cP)
P1	06	530
PI	06	440
P2	06	**800**
PII	06	430
P3	06	8860
	07	**1200**
PIII	06	**240**
P4	06	610
PIV	06	490

Determination of impurities. Large impurities in honey are usually represented by dead bees or larvae or fragments thereof, by beeswax particles, pollen and sometimes by cellulose particles, all of which are insoluble in water. When the proportion of impurities exceeds 4% it proves negligence in extraction, purification or storage.

From the results presented in Figure 3 it can be observed that in case of 5 honey samples the content of impurities was exceeded.
The most important observation of this analysis is that all the traditional honey samples showed exceedances of the content of impurities, between 0.13-2.32%, which proves the negligence of the traditional producers regarding the processing and conditioning of honey.

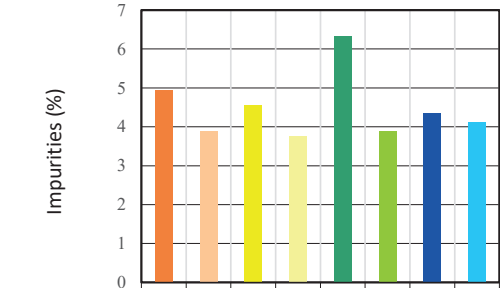

Figure 3. Content in reducing sugars of honey samples

What has caught our attention is that one of the industrial samples has slightly exceeded the content admitted for impurities.
Determination of diastatic activity. The presence of amylase in honey is a measure of freshness, correct processing (without heating exceeding 45 degrees Celsius) and a guarantee of its authenticity. The lack or small amount of diastase in honey may indicate a forgery. A diastatic index below 8 indicates a normal honey (Figure 4).

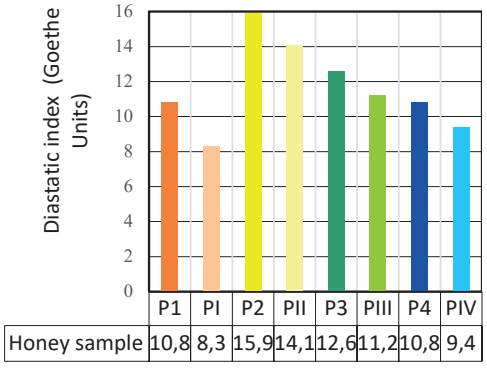

Figure 4. Diastatic index of honey samples

In honey degraded by energetic heat treatment and in the one treated with different substituents,

the activity of the diastase (amylase) will be diminished or completely null, so the value of the diastatic index will be below the normal limits, tending to zero.

The results obtained in case of determining the activity of diastasis have shown that honey, both traditional and industrial honey, are of high quality, presenting, in some cases, values much higher than the minimum allowed limit which proves the value and authenticity of the tested samples.

Microbiological analysis of the samples after 2 days, in the case of bacteria and 7 days, in the case of fungi, showed that no analyzed sample shows contamination. Therefore, all assorted honey samples are safe for human consumption.

CONCLUSIONS

Following the research conducted on honey samples it can be concluded that the tested honey samples are of good quality, observing very small differences in quality between industrial and traditional products, obtained by small producers.

The tests showed that all types of honey fall within the normal limits of the admitted values of pH, total acidity, reducing sugars, water content, viscosity, diastatic activity. Microbiological analysis demonstrated the absence of microorganisms (bacteria and fungi) from the tested samples.

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EVOLUTION OF CONTENT IN ORGANIC ACIDS OF RED WINE IN RELATIONSHIP WITH THE VARIETY AND PEDOCLIMATIC CONDITIONS FROM VALEA CĂLUGĂREASCĂ VINEYARD

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Abstract

The acidity of grapevine products has a major influence on several aspects. It is involved in the microbiological, tartan and protein stability, determines the color of wine and the speed of maturation and ageing, but in particular, determines the taste balance. This paper aimed to present the evolution of content in organic acids during the period 2017-2019 in the Valea Călugărească vineyard from three grapes cultivars: Cabernet Sauvignon, Merlot and Fetească Neagră which were analysed through Ion Chromatography (IC-MS) method. The experimental measurements established that the ripe grapes and the must contains in the beginning higher amounts of malic acid: for example in 2017, 1120 mg/l malic acid in November and only 324 mg/l in March 2018 comparing with next harvest when the malic acid content was in November 2018 - 1389 mg/l and decreased to 310 mg/l in March 2019. In terms of ecoclimatic conditions 2017 was characterized by a moderate heliothermal regime, especially in April and May, when the average annual values were overcome compared to 2018 when the heliothermic regime was high and based on relatively low water resources.

Key words*: malolactic fermentation, organic acids, red wine.*

INTRODUCTION

The agricultural sector, including grape and wine, is sensitive to climate change and the associated extreme weather. The global wine industry is already experiencing impacts, including earlier growing seasons, changes in precipitation patterns, and increased frequency and intensity of extreme weather events, all of which are affecting wine quality. To adapt to the challenges and opportunities created by climate change industry stakeholders must develop and sustain a level of adaptive capacity (Pickering et al., 2014).

Vineyards in Romania grow under temperate-continental climate, with frequent occurrences of extreme climatic conditions and with some regional variations. Prolonged high temperature during the maturation of grapes determined a high level of sugar accumulation, the loss of acids through respiration (Bucur & Dejeu, 2016).

Romania is well known and appreciated for her wines, occupying 13th in world rankings of winemakers, with approximate 5 million hl of annual wine product (Visan et al., 2017). Grapevine (*Vitis vinifera* L.*)* is one of the most important crops in Romania. According to the national statistical database published by the National Institute of Statistics, Romania has cultivated 177,000 hectares of grapevine in 2017 (NIS 2018, published in 2019) (Chireceanu et al., 2019).

The determination of organic acids in foods and beverages provides relevant information from the standpoint of monitoring the fermentation process, checking product stability, validating the authenticity of juices or concentrates and studying the organoleptic properties of fermented products (Castineira et al., 2002).

The primary varietal flavours are accumulated in the skin and grape through specific processes of the metabolism. They are determined by the genetic nature of the varieties, and by the specific pedological and climatic factors (Palade & Popa, 2015).

The character of wine is affected by range of factors in which we could include the location of vineyard, grapes varieties, agricultural engineering, the time of harvesting and the production technology. In case of lack of sun and also cold summer weather in our geographical conditions we might encounter the production of lower quality grapes, which

results in a lower content of fermentable saccharides and a higher content of organic acids, mostly of malic acid which presence is not desired. However, there is a possibility to decrease the excessive content of acids. First of all, it is related to biodegradation of acids when lactic fermentation bacteria transform malic acid into lactic acid (Kučerová & Široký, 2011).

The quality of red wine and their organoleptic characteristics are nowadays well defined when considering the effects of malo-lactic fermentation (Alonso et al., 1998).

Organic acids are important for the wine stability. Therefore, their analysis in wines is required for quality control as well as to check the evolution of acidity during the different stages of winemaking (starting from the grapes juices, continuing to the alcoholic fermentation and wine stabilization processes), since important changes in wine would be detected by alterations in the acid content (Robles et al., 2019).

Organic acids and total acidity play a pivotal role in wine sensory perception, and directly influence the overall organoleptic character of wines (Chidi et al., 2018).

Organic acids play a major role in the microbiological and physicochemical stability and sensory properties. Monitoring of multiple organic acids simultaneously is often essential and is typically conducted with ion/liquid chromatography (Ohira et al., 2014).

The main organic acids found in grapes are tartaric, malic and in a smaller extent, citric acid. Other organic acids such as, succinic, acetic, lactic, fumaric acids can also be present (Cosme et al., 2017).

In grapes, organic acids are involved in glycolytic and shikimic pathway, but can also result from the Krebs cycle or the glyoxylic pathway, where mostly they remained unchanged (compared to those from the grapes) (Niculaua et al., 2014).

Organic acids belong to the most important components that complete the overall character and taste of wine. Ripe grapes contain major amounts of tartaric and malic acids, in a minor extent, citric acid is present, too. In addition to the above-mentioned organic acids, the wines contain the products of yeast and malolactic fermentation such as acetic, lactic and succinic acids (Zeravik et al., 2015).

MATERIALS AND METHODS

Wine is an alcoholic beverage obtained exclusively from total or partial alcoholic fermentation of crushed fresh grapes or grape must and continues to surprise consumers with its incredible flavors and aromas (Raducu et al., 2019).

Experiments were conducted during the period 2017-2019 in the Valea Călugărească vineyard from three grapes cultivars: Cabernet Sauvignon, Merlot and Feteasca Neagră which were analysed through Ion Chromatography (IC-MS) method.

Ion chromatography (IC) is a standard method for wine analysis. This technique is dedicated to develop selective, sensitive, robust and as fast as possible applications for the detection of organic acids in matrix-loaded samples. Only small sample volumes in the µl range are necessary. Automatic sample preparation (e.g. Inline Ultrafiltration, Dilution or Dialysis) guarantees reproducible results and minimum time for lab work.

The advantage of ion chromatography is that chemically similar substances can be determined simultaneously in a single analysis. Acids are an important component in wine. They preserve the wine and give it expression and character. Wine contains several kinds of acids. The most important ones are tartaric acid, malic acid, and lactic acids.

Organic acids are omnipresent in winemaking. Some of them are present already in the grape while others appear during fermentation. The sum of organic acids and their composition have a direct influence on the taste of the respective wine.

Settings of the mass spectrometer were optimized for small measurements. By an adapted tuning, low concentration levels of ions can be detected. A diverter valve between IC and MS was used to only switch the flow to the MS when analytes of interest are expected. The conductivity signal is also a good tool to monitor the status of the system. During instrument calibration, automated sample preparation or the elution of matrix components, the flow is switched to the waste in order to avoid contamination of the MS detector. Metrohm IC and Waters MS are easily operated under just one software. Filtration is generally

recommended in IC to avoid blockages in the injection valve, in the capillary connection, and in the column. It is indispensable for samples containing particles. Inline Ultrafiltration combines sample injection directly with filtration. The two parts of the ultrafiltration cell are separated by a filter membrane. On one side, the sample is carried through the cell at a high flow rate. On the other side, some of the sample is drawn off through the membrane and transported to the injection valve.

The formation of filter cake is prevented by continuous flushing of particles out of the cell at a high flow rate. For organic acid analysis in wine we used: column: Metrosep A Supp 5 - 100/4.0; eluent: 5.0 mmol/l Na_2CO_3 + 5.0 mmol/l $NaHCO_3$, 1.0 ml/min; sample volume: 20 µl, inline dilution: 1:10.

RESULTS AND DISCUSSIONS

In order to find out the correlation of organic acid content with climatic conditions, a picture for the experimented years is presented below. 2017 was characterized by a moderate heliothermic regime against a background of rich water resources, especially in April and May, when it was overcome multiannual values. Pluviometric data indicate the average annual amount of precipitation being between 510-590 mm, with large variations in them from one year to another. The vegetation period (April), it started with temperatures lower than the normal (11.7ºC), and a higher water regime, confronted with multiannual values.

As shown in Table 1, the thermal regime is as follows:

Table 1. Thermal regime from April-November 2018/ April-October 2017

Month	Medium temp. ºC Apr.-Nov. 2018	Medium temp. ºC Apr.-Oct.2017	Precipitation mm Apr.-Nov. 2018	No. Precipitation days - 2018	Precipitation mm Apr.-Oct. 2017	No. Precipitation days – 2017
April	16.4	11.7	2.0	3	44.8	9
May	19.7	17.5	16.8	6	67.3	9
June	22.3	21.5	79.4	17	81.5	6
July	22.2	23.6	111.8	8	75.8	11
August	24.1	23.3	28.2	3	62.7	6
September	18.2	18.1	39.2	6	54.4	9
October	14.3	12.2	20.2	2	46.2	4
November	4.8	-	19.2	10	-	-

Compared with 2017 the year of 2018, was characterized in general by a high heliothermal regime amid relatively low water resources. Low water registered regime in April led to an uneven start to vine vegetation, there was a delay in the development of shoots in the first stages of vegetation. Low thermal registered regime in April resulted in a late start (about 10 days) and the uneven growth of the vine, which has been recovered late in May and June.

Determination of organic acids plays an important role in the evaluation of the development of wine in terms of chemistry and biochemistry.

Grapes and wine contain a variety of organic acids, in concentrations ranging depending on the variety, climate, soil, technological process applied. The organic acids from must are presented in concentrations of 3-12 g/l and the most common are: malic, tartaric, citric, ascorbic, succinic, lactic, glutaric, fumaric, pyruvic. It can be see the changes in organic acids content from Tables 2 to 5.

Tartaric and malic acid account for 70-90% of the total acids present in the grape berry, existing at roughly a 1:1 to 1:3 ratio of tartaric to malic acid (Kliewer, 1966; Lamikarna et al., 1995). For this reason, citric acid with acids like tartaric and malic are considered major products of wine-making. Tartaric acid concentration in the grapes is determined by genetic factors, but also environmental, varying between 5 and 10 g/l. In the year of 2017 the results obtained for organic acid content, before malolactic fermentation, in our experimental settings are presented in Table 2.

Table 2. Acid content in red wines before malolactic fermentation

November 2017 IC-DVV Valea Călugărească

No.	Grape variety	Citric acid (mg/l)	Tartaric acid (mg/l)	Malic acid (mg/l)	Succinic acid (mg/l)	Acetic acid (mg/l)	Lactic acid (mg/l)
0	1	2	3	4	5	6	7
1	**Cabernet Sauvignon (CS)**	150	4328	1120	623	354	245
2	**Merlot (M)**	120	4102	1015	597	395	210
3	**Fetească Neagră (FN)**	176	3975	1235	629	401	238

Table 3. Acid content in red wines after malolactic fermentation

March 2018 IC-DVV Valea Călugărească

No.	Grape variety	Citric acid (mg/l)	Tartaric acid (mg/l)	Malic acid (mg/l)	Succinic acid (mg/l)	Acetic acid (mg/l)	Lactic acid (mg/l)
0	1	2	3	4	5	6	7
1	**Cabernet Sauvignon (CS)**	130	4010	324	604	525	625
2	**Merlot (M)**	115	3980	296	585	530	710
3	**Fetească Neagră (FN)**	158	3725	308	630	525	735

Tartaric acid decreases during ripening of the grapes because of the dilution effects resulting from the growth of the grape berry (Table 2 and Table 3). Tartaric acid, naturally present in grape, must and wine is the L(+)-isomer, resulting from the biochemical conversion of glucose. In must the tartaric acid is founded in concentrations of 1-7 g/l. It is the best of the mash acid. The value of tartaric acid in Feteasca neagra was the smallest in march 2018 - 3725 mg/l and decreased from 3975 mg/l. Feteasca neagra is an authentic grape and wine specific to Valea Călugărească vineyard and reddish-brown soil.

The malic acid peak concentrations of 20 g/l in the grapes at the time they give the berry of. The amount of malic acid decreases with increasing temperature, so that the time of harvesting down malic acid concentration somewhere between 1-7 g/l.

Malic acid is a very popular substrate for yeast, especially for the malolactic bacteria. Therefore, depending on the chosen wine-making technology the concentration of malic acid in wine can greatly decrease (Table 2 and Table 3). The malic acid is the most important metab olized acid by lactic acid bacteria. For Cabernet Sauvignon we can see that from 1120 mg/l in November 2017 the concentration decreased at 324 mg/l in march 2018. Same happened with values of Fetească Neagră and Merlot.

Citric acid is found in low concentrations in grapes, 0.3-0.5 g/l, in general in the skin berry, and in the pulp, and its concentration remains low throughout the different stages of maturation. Citric acid in the wine is found in relatively low concentrations, ranging from 150-300 mg/l., rarely exceeding 700 mg/l. The Merlot variety had the smallest value of citric acid 158 mg/l in march 2018.

As we can see in the Figure 1 after malolactic fermentation is a slight decrease in acidity, because malic acid in the presence of lactic acid bacteria is transformed into lactic acid, wine becomes soft, smooth and non-invasive.

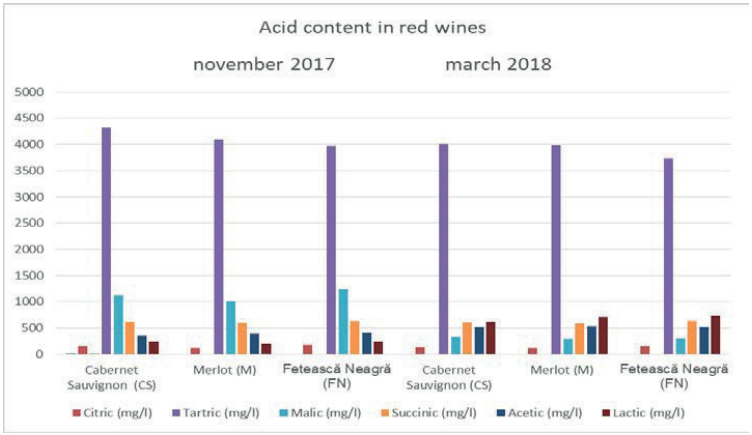

Figure 1. The evolution of acid content in red wines before and after malolactic fermentation

Malic acid is presented more in wines obtained from grapes insufficient development. Lactic acid is a secondary-product of the alcoholic fermentation and a principal-product of malolactic fermentation. As we can see the values of lactic acid rise as a sign of passing wine through malolactic fermentation. For example, the largest increase was recorded for Merlot and Feteasca Neagră, almost 500 mg/l, from 210 mg/l to 710 mg/l for Merlot. The correlation value is correlated with the strength of succinic acid alcohol, succinic acid, representing 10% of alcoholic strength in terms of volume. Acetic acid is formed during the alcoholic fermentation and has a particular importance for the organoleptic characteristics of the wine, in the wine is the main volatile acid. For Cabernet Sauvignon variety we can observed an increase from 354 mg/l in November 2017 to 525 mg/l in march 2018, under the effect of yeast strain. The year of 2018 comparative with 2017 - experimental measurements established that the ripe grapes and the must contains in the beginning higher amounts of malic acid: for example in 2017, 1120 mg/l malic acid in November and only 324 mg/l in March 2018 comparing with next harvest when the malic acid content was in November 2018 - 1389 mg/l and decreased to 310 mg/l in March 2019. In 2018 we can notice a higher value of malic acid for all the three varieties: Cabernet Sauvignon, Merlot and Feteasca Neagră, before malolactic fermentation caused by heliothermal regime high in July, August and September, against a normal rainfall and very low in September, who led to a better accumulation of sugars in grapes (Table 4 and Table 5). We can say that the pedoclimatic conditions and the soil influences the characteristics of grapes, musts and finally the acid content of wines before and after malolactic fermentation and the quality of red wines. From the Tables 4 and 5 representing the acids content before and after malolactic fermentation, it seems:

- for Cabernet Sauvignon the citric acid had a smaller value in November 2017 - 150 mg/l compered to November 2018 - 160 mg/l and it fell after malolactic fermentation in March 2019 - 157 mg/l; 3 mg/l difference in 2018 to 20 mg/l difference in 2017; for Merlot the difference remained the same, but for Feteasca Neagră the differences was from 18 mg/l in 2018 to 1 mg/l in 2019;
- the values of tartaric acid in 2017 decreases during ripening of the grapes, for all the three varieties: Cabernet Sauvignon, Merlot and Feteasca Neagră, because of the dilution effects resulting from the growth of the grape berry. Comparing with 2018 when the berries was little, but reach in sugars, because of the low thermal regime registered in April the differences between values were for example only 322 mg/l for Feteasca Neagră in 2018-2019 to 250 mg/l for Feteasca Neagră in 2017-2018;

Table 4. Acid content in red wines before malolactic fermentation

November 2018 IC-DVV Valea Călugărească

No.	Grape variety	Citric acid (mg/l)	Tartaric acid (mg/l)	Malic acid (mg/l)	Succinic acid (mg/l)	Acetic acid (mg/l)	Lactic acid (mg/l)
0	1	2	3	4	5	6	7
1	**Cabernet Sauvignon (CS)**	160	4475	1389	678	370	290
2	**Merlot (M)**	155	4120	1186	650	330	273
3	**Fetească Neagră (FN)**	148	4022	1015	608	315	249

Table 5. Acid content in red wines after malolactic fermentation

March 2019 IC-DVV Valea Călugărească

No.	Grape variety	Citric acid (mg/l)	Tartaric acid (mg/l)	Malic acid (mg/l)	Succinic acid (mg/l)	Acetic acid (mg/l)	Lactic acid (mg/l)
0	1	2	3	4	5	6	7
1	**Cabernet Sauvignon (CS)**	157	4008	310	650	548	810
2	**Merlot (M)**	150	3980	302	639	527	798
3	**Fetească Neagră (FN)**	147	3700	296	610	502	790

- the succinic acid values it remains almost unchanged during the aging step and aging of wine, for example for Merlot from 650 mg/l in November 2018 to 639 mg/l in march 2019, a difference of 11 mg/l;
- for Fetească Neagră the value of acetic acid increase from 315 mg/l in November 2018 to 502 mg/l in march 2019, a difference of 187 mg/l with 63 mg/l more than 2017-2018 values;
- the values of lactic acid rise as a sign of passing wine through malolactic fermentation. For example, the largest increase was recorded for Feteasca neagra from 249 mg/l in November 2018 to 790 mg/l in march 2019, a difference of 541 mg/l, more than 2017-2018.

In Figure 2 compared to Figure 1 we can notice difference, but in the same time similarities of the acids values from the three varieties: Cabernet Sauvignon, Merlot and Fetească Neagră, based on different factors such as temperature, light, fertilization, water supply, acidity and technological process applied that affect the malolactic fermentation who is almost finished, the ultimate goal is getting a quality red wine. The tartaric acid has the major values decreases during maturation.

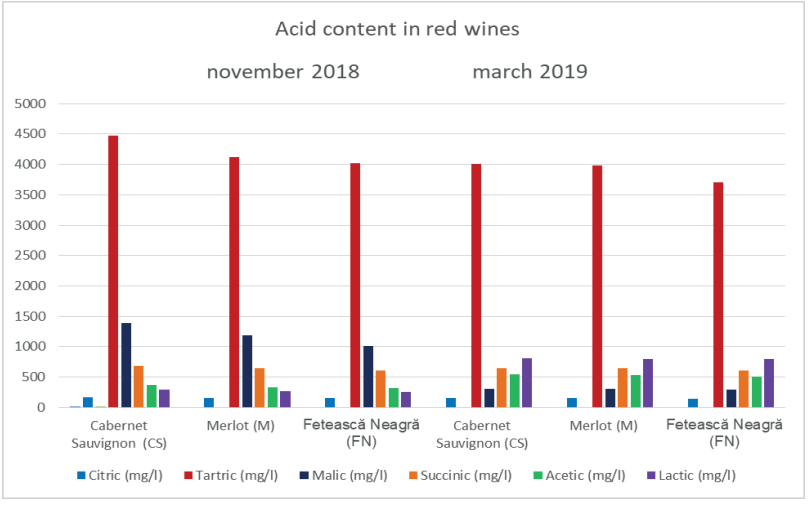

Figure 2. The evolution of acid content in red wines before and after malolactic fermentation

Organic acids in wine derive either directly from the grape, or are the result of microbiological activities that take place before, during or after alcoholic and malolactic fermentation. While the most commonly measured feature of wine acidity is the total acidity (TA) and pH, some organic acids are important markers for fermentation management and wine flavour and aroma. Malic acid is monitored to measure The progress of malolactic fermentation, acetic acid is monitored as an indicator of fermentation problems or of spoilage, and citric acid may be added to adjust acidity and chelate metal ions to prevent nutrients from precipitation resulting from the interaction of nutrients with metal ions, such as iron precipitating with phosphorus (Fowles,1992).

Tartaric acid is not metabolised by grape berry cells via respiration in the same manner as malic acid, and the level of tartaric acid in the grapes remains relatively consistent throughout the ripening process. The concentration of tartaric acid in grapes depends largely on The grape variety and soil composition of the vineyard (Ribéreau-Gayon et al., 2006).

CONCLUSIONS

The meteorological data recorded on a 2-year study, for Valea Călugărească vineyard centre, shows that the average annual temperature is around 10.8°C. Winters are relatively short and cold becomes more intense in January and the first half of February. Frost-free period is an average of 202 days. The average temperature of the hottest (July) is +22.4°C, showing variations between 20.7°C and +25.6°C. The number of days with an average temperature of over 10°C range between 175-226. The amount of active temperature is between 3300 and 4040. The total number of hours of sunshine is 2146. The phase of maturation of the grapes (the months of August-September) rainfall is

lower, favoring the ripening.

The organic acids content in wine is related to the region and it is also associated with climate during the growth and ripening of grapes. In Valea Călugărească vineyard were the climate is warmer than in the others regions and the soil helps to obtain good quality red wines, the values of tartaric acid in 2017 decreases during ripening of the grapes, because of the dilution effects resulting from the growth of the grape berry for all the three varieties, comparing with 2018 when the berries was little, but reach in sugars, because of the low thermal regime registered in April the differences between values were for example only 322 mg/l for Fetească Neagră in 2018-2019 to 250 mg/l for Fetească Neagră in 2017-2018. The excess of malic acid gives wine a distinctly more sour character, the values of malic acid were a little bigger in 2018 than in 2017, before malolactic fermentation caused by heliothermal regime high in July, August and September, against a normal rainfall and very low in September, who led to a better accumulation of sugars in grapes. The most common process is malolactic fermentation; the malic acid becomes converted to softer lactic acid, the values of lactic acid rise, in 2018 more than in 2017, as a sign of passing wine through malolactic fermentation. Succinic acid is stable and durable in time, it remains unchanged during the aging step and aging of wine, for example in Fetească Neagră from 629 mg/l in November 2017 to 630 mg/l in march 2018.

In conclusion, the content of organic acids depends by factors like: ecoclimatic conditions, region, soil, the time of harvesting and the production technology, because they determined the quality of red wine and their organoleptic characteristics, determines the taste balance.

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HEAVY METALS CONTAMINATION OF FOOD CONTACT MATERIALS IN ROMANIA

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Abstract

Exposure of consumers to heavy metals is an important issue and many researches has focused on this topic in the last decades. These metals can reach the human body through food contact materials. The aim of this study is to analyze the heavy metals content (Pb, Cd, Cr) or heavy metals migration from different food contact materials (plastic, paper, board, glass, ceramic etc.) available on the Romanian market and to establish the compliance with the imposed limit. The influence of printing inks and dyes on the metals level in food contact materials was also studied. Heavy metals analysis was performed by atomic absorption spectrometry, after wet digestion of the samples.

Key words*: food contact materials, heavy metals, packages, spectrometry.*

INTRODUCTION

Food packaging is the main way of storing, transporting and protecting food products by external factors (Ahmadkhanika & Rastkari, 2017), but these packages may have some degree of chemical contamination.

This process of contamination occurs as a result of the direct or indirect contact between the food and the packing material, resulting in the transfer of substances between the two components, a process called migration (Rather et al., 2017). Thus, packaging made from virgin materials or recycled materials can be an important source of contamination of the environment with heavy metals, through heavy metal catalysts used for polymerization processes (Whitt et al., 2012), or through additives added to improve the properties of packaging materials, such as stabilizers, antioxidants, plasticizers, slipping agents (Rather et al., 2017, Constantinescu et al., 2019). Heavy metals resulting from the migration process reach the body where they can affect the proper functioning of biological systems by accumulating them in the body where they can promote the appearance of diseases, even at low concentrations (Sood & Sharma, 2019).

The aim of this study was to analyze the content of heavy metals (lead, cadmium and total chromium) in different types of food contact materials (plastic, paper and board), but also the levels of heavy metals that can migrate from different types of packaging materials (plastic, paper and board, glass) from Romanian producers, to evaluate their compliance with the limits imposed by the legislation in force.

MATERIALS AND METHODS

Sample collection

55 samples of food contact materials (32 plastic, 19 paper/board and 4 glass) were purchased from different Romanian producers. A full description is provided in Tables 1 and 2.

Reagents

Nitric acid (HNO_3) and Hydrogen peroxide (H_2O_2) were purchased from Merck. Calibration curves were obtained using Lead standard solution 1000 mg/l Pb for AA ($Pb(NO_3)_2$ in HNO_3 2%), Cadmium standard solution 1000 mg/l Cd for AA ($Cd(NO_3)_2$ in HNO_3 2%), Chromium standard solution 1000 mg/l Cr for AA ($Cr(NO_3)_3$ in HNO_3 2%) purchased from Scharlau, and a Multielement Standard Solution 6 for ICP purchased from Sigma Aldrich. Dilutions were performed using ultrapure water (18.2 MΩ.cm). All glassware used was cleaned and decontaminated with 10% HNO_3.

Table 1. Samples description analyzed for heavy metals content

Code	Material	Description
P1	PE film	200 µ transparent and printed PE film
P2	PE bottle	Red PE bottle
P3	HDPE bag	Transparent and printed HDPE bag
P4	HDPE closure	Green HDPE closure
P5	LDPE bag	Transparent and printed LDPE bag
P6	PP film	Transparent and printed PP film
P7	PP bottle	Red PP bottle
P8	PP fabric	White PP fabric, laminated
P9	PP trays	500 CC PP trays, color black
P10	BOPP film	Transparent BOPP film
P11	BOPP bag	Transparent BOPP bag, unprinted
P12	CPP bag	Transparent CPP bag, unprinted
P13	PP cup	White PP yoghurt cup
P14	PET bottle	2500 ml green beer PET bottle
P15	PET casseroles	250 CC transparent PET casseroles
P16	PE/EVOH/PE/ PET AF wrapping film	Transparent PE/EVOH/PE/ PET AF film, unprinted
P17	PE/PA wrapping film	Transparent PE/PA film, unprinted
P18	Stretch film	Transparent stretch film
P19	Cutlery PS	Transparent PS (GPPS and HIPS) cutlery
P20	Paper bag	Brown kraft paper bag
P21	Baking paper	Natural baking paper
P22	Juice labels	Paper juice labels
P23	Beer labels	Paper beer labels
P24	Paper towels	White paper towels, printed
P25	Cardboard type III	Natural cardboard
P26	Cardboard trays	White cardboard trays
P27	Cardboard casseroles	White cardboard casseroles
P28	Corrugated board	Natural corrugated board, printed
P29	Salt packages	Printed cardboard for salt packages
P30	Cardboard inserts	Promotional cardboard inserts for chip bags
P31	Egg box	Printed egg box
P32	Fruit box	Natural fruit box, unprinted

Table 2. Samples description analyzed for heavy metals migration

Code	Material	Description
P1	PET bottle	900 ml white PET bottle for milk
P2	PET bottle	1000 ml transparent PET bottle for water
P3	PET bottle	2500 ml green PET bottle for beer
P4	PET jars	Transparent PET jars
P5	HDPE bag	Transparent and printed HDPE bag
P6	HDPE closure	HDPE closure for water bottle, color blue
P7	LDPE bag	Transparent and printed LDPE bags
P8	LDPE bottle	Red LDPE bottle for ketchup
P9	PP trays	500 ml black PP trays
P10	PP cup	200 ml PP yoghurt cup, color white
P11	BOPP bags	Transparent BOPP bags
P12	PS cup	White PS (GPPS and HIPS) yoghurt cup
P13	EPS bowl	500 CC White EPS bowl
P14	Silicone baking paper	*baking paper* coated with *silicone*
P15	Paper muffins	*White and brown paper muffins*
P16	Chocolate	White chocolate box, printed
P17	Coffee cup	White paperboard coffee cups, printed
P18	Silver cardboard trays	silver cardboard trays for cakes
P19	Pizza	Brown pizza box, upper surface printed
P20	Jar glass	Transparent jar glass
P21	Glass bottle	Uvag glass bottle for beers
P22	Glass bottle	Flint glass bottle for water
P23	Glass containers for salt	Transparent glass containers for salt

Equipment's

Lead, cadmium and chromium content were performed using an AAnalyst 600 graphite furnace atomic absorption spectrometer system (Perkin Elmer) provided with a Transversely Heated Graphite Atomizer (THGA) assembly and longitudinal Zeeman-effect background correction. Specific migration of metals was performed using a NexION 300Q Inductively Coupled Plasma Mass Spectrometer (Perkin Elmer).

Sample preparation

For heavy metals content analysis, samples were prepared by wet digestion in microwave system (MWS-2, Berghoff) using HNO_3 65% and H_2O_2 according to the program presented in Table 3.

Table 3. Microwave heating program

Step	Temperature (°C)	Duration (min)	Power (%)
1	160	5	80
2	220	40	90
3	Cooling	20	0

For the analysis of heavy metals that can migrate from the packing materials, the samples were subjected to certain test conditions. Thus, the plastics were exposed 10 days to 40 degrees (OM2), using as a food simulant 3% (v/v) acetic acid, for paper and cardboard a cold aqueous extract was obtained (24 hours at room temperature) which was stabilized with HNO_3 65%, and the glass packaging materials were tested 24 hours at room temperature, using as food simulant 4% (v/v) acetic acid.

Calibration

Calibration curve for each element was performed using reagents described above. 5 calibration points were used for each calibration curve. The correlation coefficient of each calibration curve was higher than 0.995.

RESULTS AND DISCUSSIONS

Heavy metals content

According to European Directive 94/62/EC on the management of packaging and packaging waste to prevent their impact on the environment (consolidated version 2018), transposed into Romanian legislation by Law 249/2015 on the management of packaging and packaging waste, the sum of concentrations of lead, cadmium, hexavalent chromium and mercury present in packaging or packaging components must not exceed 100 mg/kg.

From the results presented in Table 4, it can be observed that they fall within the limits imposed by the legislation in force. The values obtained for plastic materials (P1-P19) are considerably lower than for paper and cardboard packaging (P20-P32).

For plastic materials, lead levels ranged between < 0.002 and 5.997 mg/kg, < 0.0002 and 0.059 mg/kg for cadmium and between < 0.013 and 0.638 mg/kg for chromium. In the case of paper and cardboard samples higher levels were obtained for the three determined metals, so that for lead the levels ranged between < 0.002 and 14.650 mg/kg, for cadmium between < 0.0002 and 0.490 mg/kg, and for chromium between < 0.013 and 25,800 mg/kg.

Table 4. Heavy metals content in food packaging samples (mg/kg)

Sample	Pb	Cd	Cr
P1	1.507	< 0.0002	< 0.013
P2	0.024	< 0.0002	0.118
P3	1.228	< 0.0002	< 0.013
P4	0.267	< 0.0002	0.638
P5	1.066	< 0.0002	< 0.013
P6	0.937	< 0.0002	< 0.013
P7	1.577	< 0.0002	< 0.013
P8	0.423	< 0.0002	< 0.013
P9	1.618	< 0.0002	< 0.013
P10	0.813	0.059	< 0.013
P11	0.825	< 0.0002	< 0.013
P12	1.467	< 0.0002	< 0.013
P13	< 0.002	0.056	0.055
P14	< 0.002	< 0.0002	< 0.013
P15	5.977	< 0.0002	< 0.013
P16	< 0.002	< 0.0002	< 0.013
P17	< 0.002	< 0.0002	< 0.013
P18	< 0.002	< 0.0002	< 0.013
P19	0.477	< 0.0002	< 0.013
P20	5.737	< 0.0002	2.450
P21	< 0.002	< 0.0002	3.040
P22	4.301	0.005	6.722
P23	2.602	0.008	1.856
P24	< 0.002	< 0.0002	< 0.013
P25	3.674	< 0.0002	5.130
P26	14.650	0.025	25.800
P27	1.693	< 0.0002	< 0.013
P28	7.509	0.064	6.260
P29	4.703	0.490	1.402
P30	3.454	< 0.0002	4.740
P31	8.492	0.064	1.690
P32	13.177	0.301	2.000

The high levels of heavy metals in paper and board are due to the chemical additives used to process pulp and secondary fibers to obtain paper or cardboard (Mertoglu, 2017), additives such as adhesive chemicals, mineral products used to improve certain characteristics of paper (Conti, 2008; Mertoglu, 2017).

The metal levels in samples with high weight (corrugated cardboard - P25, P26, P27, P28, P31, P32) are higher compared to those with lower weight (baking paper, paper towels - P24, P21). These differences are also generated by

the presence of dyes and printing inks, which are a major source of toxic metals (Mertoglu, 2017).

Heavy metals migration

According to ResAP EC Resolution 3/2002, the maximum values of lead and cadmium that can migrate from paper and cardboard are 0.012 mg/l, respectively 0.018 mg/l.

From the results presented in Table 5, it can be observed that they fall within the limits imposed by the legislation in force and do not represent a source of contamination of food products.

Although there are no limits for the level of chromium that can migrate from this category of packaging, the obtained values were lower than the detection limit of this method.

Table 5. Heavy metals migration from paper and board materials (mg/l)

Sample	Pb	Cd	Cr
P14	< 0.002	< 0.0002	< 0.013
P15	< 0.002	< 0.0002	< 0.013
P16	0.003	< 0.0002	< 0.013
P17	0.0028	< 0.0002	< 0.013
P18	< 0.002	< 0.0002	< 0.013
P19	0.005	< 0.0002	< 0.013

For Pb and Cd migration in glass (Table 6), the results were compared with the limits presented in the standard ISO 7086-2/2000 Glass hollowware in contact with food - Release of lead and cadmium - Part 2: Permissible limits, which are 5.0 mg/l for Pb and 0.5 mg/l for Cd. The obtained values are very low, confirming the claim that glass is a very stable packaging material.

Table 6. Heavy metals migration from glass materials (mg/l)

Sample	Pb	Cd
P20	< 0.002	< 0.0002
P21	< 0.002	< 0.0002
P22	< 0.002	< 0.0002
P23	< 0.002	< 0.0002

According to the Regulation (EU) no. 10/2011 on plastic materials and articles intended to come into contact with food, plastic materials and articles shall not release substances in quantities exceeding the specific migration limits.

The values obtained are lower than maximum allowed limits for all metals (Table 7).

Table 7. Maximum allowed limits form metals according to Regulation (EU) no. 10/2011

Element	Maximum allowed limit (mg/kg)
Barium	1.0
Cobalt	0.05
Copper	5.0
Iron	48.0
Manganese	0.6
Nickel	0.02
Zinc	5.0

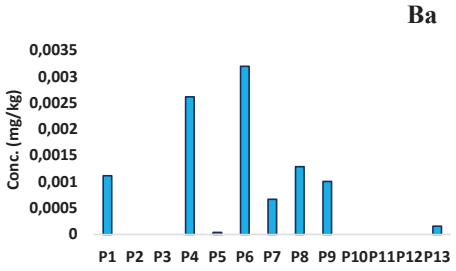

Figure 1. Barium migration from food contact materials

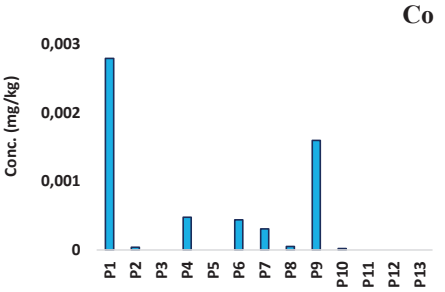

Figure 2. Cobalt migration from food contact materials

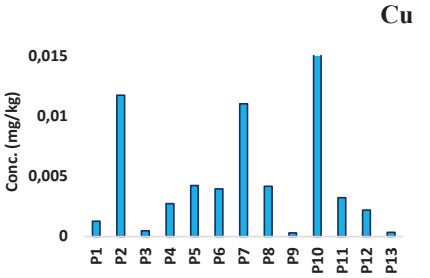

Figure 3. Copper migration from food contact materials

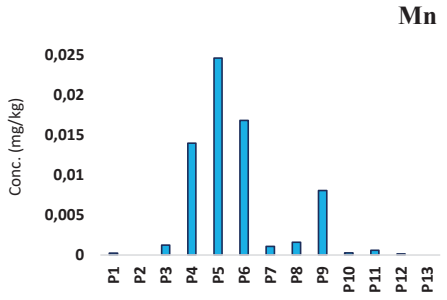

Figure 4. Manganese migration from food contact materials

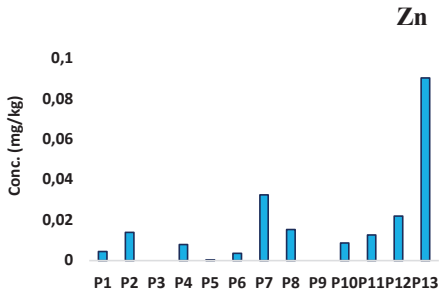

Figure 5. Zinc migration from food contact materials

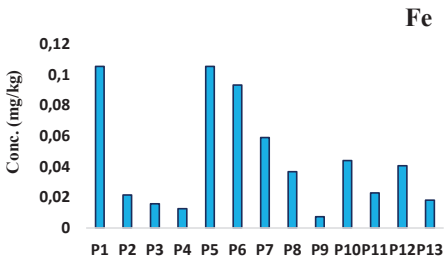

Figure 6. Iron migration from food contact materials

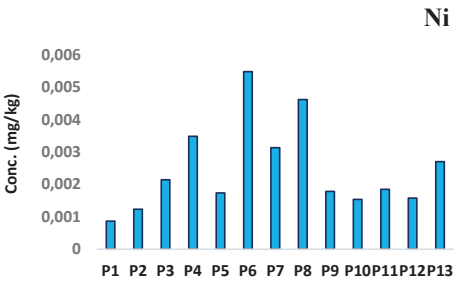

Figure 7. Nickel migration from food contact materials

Analyzing all the obtained results (Figures 1-7), it can be observed that the highest values were obtained for Fe and Zn, while the lowest values were obtained for Co.

CONCLUSIONS

Two versatile techniques, atomic absorption spectrometry with graphite furnace (GF-AAS) and inductively coupled plasma mass spectrometry (ICP-MS) were used to evaluate the heavy metals content and metals specific migration of several food contact materials (plastic, paper/board and glass).

The obtained results were compared with maximum allowed limits stated by the legislation in force: European Directive 94/62/EC on packaging and packaging waste and Romanian Law 249/2015 regarding management of food packaging and food packaging waste, Regulation (EU) no. 10/2011 on plastic materials and articles intended to come into contact with food or Resolution ResAP EC 3/2002 on paper and board materials and articles intended to come into contact with foodstuffs.

The values obtained are lower than maximum allowed limits for all metals analyzed. In terms of heavy metals content, the values obtained for paper and board are higher than for plastic.

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THE INFLUENCE OF PLANT-BASED PROTEIN INGREDIENTS ON THE QUALITY OF HIGH-PROTEIN BREAD

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Abstract

The aim of this work was to analyse the influence of plant-based protein ingredients added to bread on the overall quality of the end product. The analysis of the recent literature revealed a great interest in using plant-based protein ingredients versus animal-based ingredients. The main previous improvements consisted in the production of the bread in which about 15% wheat flour was replaced with various protein isolates from wheat, corn, potato, carob, peas, soya, lupine and beans. The impact on the properties of the dough (gluten aggregation, gluing behaviour, rheology) as well as on the quality of the bread (volume, crumb structure, crumb hardness) was investigated. The protein-rich ingredients affected gluten aggregation, gluing and determined the weakening of the gluten network in dough containing potato and pea proteins. Also, the literature indicated a high importance of the consumers' preference regarding the inclusion of functional foods with added protein in their daily diets, so that they reach the levels of intake necessary to achieve the health effects at present.

Key words*: gluten, high-protein bread, pea, plant-based proteins, soya.*

INTRODUCTION

One of the most consumed products in the world, which plays an important role in the human diet is bread (Henchion et. al., 2017). Bread is a simple food that is obtained usually from wheat flour, yeast and water. But this simple recipe can be modified or improved by using different types of flours and ingredients in order to increase its nutritional value, to obtain products for special diets and to answer to the consumers' preferences. Bread is a source of complex calories and carbohydrates (Gomez et. al., 2008), but the proteins from bread contain low levels of essential amino acids, such as lysine and threonine. In addition, the use of refined white flour reduces the nutritional density and fiber content of white bread compared to whole grain bread. It is known that the amino acid composition of legumes is complementary to that of cereals (Boye et al., 2010; Anderson et al., 2009), and they are also rich in bioactive compounds, such as fibers and phytochemicals, that is why legumes flour is often added to wheat flour in bread making process.

The nutritional properties of wheat bread can be improved by including a percentage of alternative flours from other cereals or legumes. Therefore, due to the fact that bread is a food in which other ingredients can be easily incorporated, a wide range of plant rich bakery products can be produced. The nutritional value of a food product is given by the protein digestibility value, so the quality of the proteins is very important for the characterization of the nutritional properties of a product (Bonnand-Ducasse et al., 2010; Belc et al., 2020).

Protein is a dietary essential macronutrient needed for a healthy structure and function of human's body. The quality of the proteins can be quantified according to the quantity and the profile of the essential amino acids, as well as based on the real value of the digestibility of the essential amino acids.

Protein density can be quantified based on the amount of total calories consumed to meet the daily requirement of all essential amino acids (Robert et al., 2018; Gherghina et al., 2015). The use of plant proteins provides additional nutrients and can improve the nutrient supply to consumers (Mao and Miao, 2015).

Terms of "high protein" are found in various forms, but according to Regulation (EC) no. 1924/2006, a product is considered "rich in protein" if 20% of its calories are supplied by protein. The plant ingredients sources of proteins can be classified, based on the protein content, as: flour (protein less than 65% d.m.), concentrates (protein more than 65% d.m.) and isolates (protein higher than 90% d.m.) (Boye et al., 2010). The protein content of the isolates depends on the processing of raw ingredients and this does not always reach a protein level of 90% d.m (Arntfield and Maskus, 2011).
The general objective of this paper is to identify and analyse low cost and efficient sources of plant-proteins, in order to develop new, modern, protein enriched foods.

Plant based proteins ingredients

Based on the scientific literature search, partial substitution of wheat flour with protein-rich ingredients from numerous sources was applied for bread production: legumes (Villarino et al., 2015; Turfani et al., 2017; Marchais et al., 2011), cereals (Bugusu et al., 2002), pseudo-cereals (Sanz-Penella et al., 2013) and dairy proteins (Kenny et al., 2000). The previous studied proteins sources were: wheat (gluten - *Glue*), corn (zein - *Zea mays*), potato *(Solanum tuberosum)*, carob/tomato (*Ceratonia siliqua*), peas (*Pisum sativum*), lupine (*Lupinus angustifolius*) and beans (*Vicia faba*) and they were tested regarding the impact on high-protein breads (Day, 2011; Crepon et al., 2010). Pea proteins have gained popularity in food and pharmaceutical systems due to the relatively lower content of anti-nutrients compared to soy protein (Nirali et al., 2019).
In the study conducted by Andrea et al., 2019, five commercially available high protein ingredients were used: potato protein isolate, pea protein isolate, redwood meal, gluten vital and corn protein (zein), protein isolate from blue lupine and a fine fraction high in protein from beans. In this study, 15% of wheat flour was replaced with different protein flours of different concentrations.
Therefore, the red bean meal and bean meal had the lowest levels of protein concentration (55.04% d.m.) respectively (61.25% d.m.); pea flour and gluten vital had a protein content of 80.19% d.m. and 83.11% d.m., respectively, and corn and potato flour had a protein content of over 90-91.79% d.m.
In another study the protein sources used were: flour (21.34% protein); redcurrant flour (43.17% protein); pea protein isolate (80.74% protein) and soybean meal (38.14% protein). This time the researchers chose to adjust the amount of protein in each formulation to obtain the same percentage of protein (1.5%) (Miñarro et al., 2012).
In the other studies, wheat flour was replaced approx. up to 30%, depending on the type of the used protein ingredients (flour, concentrate or protein isolate). Studies showed that the replacement of 30% from the wheat flour with bean flour with a protein content of 35.7 ± 1.2% d.m. increased the protein content from 11.6 to 16.5% d.m. In other researches it was found that the simple addition of bean flour resulted in an increase of approx. 15-30% of free peptides and amino acids, during fermentation (Rossana et al., 2017). Following the study in which 15% of the wheat flour was replaced by protein-rich ingredients, the protein content of floury mixture increased 3.5 times in comparison with wheat flour which had a protein content of 14.09% d.m. (Andrea et al., 2019).
Other plant protein sources, like quinoa and amaranth, were recently taken into consideration. Quinoa has a protein content of 12-20%, although this can be modified depending on its species. Its protein amount is at least equal to the amount of milk protein, containing also essential amino acids (Abugoch, 2009; Repo-Carrasco-Valencia & Serna, 2011; Yıldız et al., 2014).
Quinoa was compared to other cereals in terms of protein content as the quinoa flour had a protein content of 14.12%, rice flour 6.81%, barley flour 9.91%, wheat flour 13.68%, corn flour 9.42%, rye flour 10.34 and sorghum flour 10.62% (Aybuke and Nevin, 2019). In his study, Gostin (2019) tested the addition of quinoa flour to white wheat and wholemeal wheat flour the samples being obtained from (1) white wheat flour (WF, control, protein 13.2%), (2) wholemeal wheat flour substituted with 33 g/100 g (33%) white wheat flour (protein 13.7%), (3) wholemeal wheat flour (protein 14.0%), (4) wholemeal wheat flour substituted with 10 g/100 g (10%) quinoa flour (protein

13.0%), (5) white wheat flour substituted with 10 g/100 g quinoa flour (protein 13.2%), and (6) quinoa flour (protein 12.9%). Compared with the control bread (wheat flour), all breads with the addition of quinoa flour were perceived to be twice as salty and six times as bitter. Therefore, bread with quinoa flour added had very low acceptability.

According to previous studies quinoa flour can be used up to 10-13% in bread with wheat flour or corn flour for gluten-free products (Aybuke and Nevin, 2019).

Rheological analysis

Proteins have a strong influence on the rheological properties of the dough (Rafa et al., 2013; Choi and Han, 2001). Proteins, in addition to their nutritional properties, have also functional properties that play an important role in the formulation and processing of foods. The functional properties of these proteins are: the solubility capacity, the water and fat binding capacity and the foaming capacity (Boye et al., 2010; Korus et al., 2009).

In a previous study in which flour mixtures were analyzed, it was found that in the samples with raw redcurrant flour and lentil flour the dough's behavior was not different from the control but the redcurrant flour (10%) made the dough sticky (Turfani et al., 2017; Ahmed et al., 2013). During the formation of the dough with the addition of potato it was found a lack of elasticity and a higher viscosity, this could be influenced by the fact that it was applied at the same time mixing for all the recipes, and in the case of the potato exceeded the tolerance of gluten mix (Andrea H. et al., 2019). The amount of water added to the dough formation differs depending on the protein added. The amount of water varied between 523 g per sample in the case of albumin and 739 g in the case of soy protein, and the intermediate variants were lupine (568 g), collagen (617 g) and peas (639 g) (Rafa et al, 2013; Choi and Han, 2001).

When adding lupine flour (35% protein content) in a proportion of 5 and 10% respectively, it was found that the water incorporation time in the flour was higher, therefore the dough formation time increased with approximately 1.5 minutes compared to the control sample (only with flour), but not depending on the use lupin quantitative.

It was also found that samples with the addition of lupine flour have a higher dough stability, a stronger dough than the wheat flour control sample (Paraskevopoulou et al., 2010; Witczak et al., 2012).

Several tests were done to prepare, three different types of bread using a 30% level of wheat flour substitution with raw peas, sprinkled peas or fried peas. After the analysis of dough formation with the Mixolab equipment, it was found that pea flour-wheat flour mixtures had a lower water absorption than the control (wheat flour) and the dough formation time was similar with the control. But differences were found in the stability of the dough and its resistance to mechanical mixing. The resistance was reduced in all the three cases. (Millar et al., 2019).

Regarding the rheological analyses for four samples of chickpea flour, redcurrant flour, pea protein isolate and soybean meal, revealed no significant differences were found. The analyses were performed at Brabender Farinograph where 15% of wheat flour was replaced with protein flour of different percentages, respectively flour (control flour) 14.09%, gluten 83.11%, zein 91.79, potato 55.04, peas 80.19, lupine 94.51, beans faba 61.25% (Angioloni & Collar, 2012).

It was noticed that the absorption of water was higher in tomato, gluten and peas 69.8%, 70.2%, respectively 71.7%, even in potato and lupine, 65.2%, respectively 66.2%, compared to wheat flour 63.0%, when using goddess and beans was made only with the exception of the goddess (60.8%), the beans (62.2%) (Andrea H. et al., 2019).

In another study in which lupine flour and soybeans replaced 5-10% of white wheat flour, the dough stability and tolerance index increased (Kaack et al., 2002).

Loss of moisture when baking

An important parameter that influences the bread shelf-life is the loss of moisture during baking and this leads to the formation of a dry crust and therefore determines the early aging of the product. Moisture loss can be influenced by the water binding ability of the dough ingredients. Addition of lupine protein isolate has been reported to delay bread staling, and the addition of dietary fiber to bread has been

reported to delay drying (Kaack et al., 2006; Kiosseoglou et al., 2014).

As a result of the moisture determination analyses, it was found that the sample of Australian sweet lupine bread had lower moisture loss during baking (Villarino et al., 2015; Alvarez et al., 2010).

Texture

In the ordinary production of bread, wheat is used because of its properties that provide the desired texture through the formation of gluten network (Popa et al., 2014).

The effects of the Australian sweet lupine variety on the physical characteristics of the bread were evaluated by Villarino et al. (2015); Alvarez et al. (2010). Adding Australian sweet lupine flour to wheat bread resulted in a reduced bread volume and a strong texture, due to disruption of the gluten matrix by non-elastic lupine proteins and high-water absorption of Australian sweet lupine dietary fiber. The influence of the lipid and protein components of Australian sweet lupine flour on the texture properties of bread was observed, but the instrumental textural properties of Australian sweet lupine wheat bread did not differ significantly from those of wheat only bread (Villarino et al., 2015). Any difference in the size of Australian sweet lupine flour particles may in turn affect the volume of bread. The particle size reduction of refined wheat flour substitutes (bran or whole wheat) either increased or decreased the volume of bread. After increasing the percentage of protein by replacing wheat flour with bean flour, it was observed that the bread hardness increased with about 30-50% in bread with added bean flour (Andrea et al., 2019). Partial replacement of wheat flour with pea protein isolate (Marchais et al., 2011; Hogan et al., 2012) and lupine flour (Villarino et al., 2015) has been reported previously to decrease specific volume and increase hardness. Following the studies done by Bugusu et al. (2002) and Turfani et al. (2017) it was found that the volume of bread increased in samples with the addition of carob flour and zein (from corn).

Regarding hardness, one of the important characteristic of bread, the study compares the control sample (wheat flour; 11,81N) with plant protein added as follow; 11.81 N corn/corn (15.10 N), peas (16.68 N), potato (19.02 N), lupine (20.11 N) and faba beans (20.11 N) (Andrea H. et al., 2019).The addition of lupine flour (35% protein content) in a of 5 or 10% resulted in increasing the values of the hardness of the bread compared to the control bread with wheat flour. Bread hardness also increased significantly after 24 and 48 h subsequent tests, but compared to the wheat flour control sample, lupine flour samples had a softer texture, probably due to the high-water content (Paraskevopoulou et al., 2010; Sabanis et al., 2006).

Color

The addition of different ingredients rich in protein often determine changes in the color of the final baked products.

The bakery samples with beans were the darkest ($L^* = 57.07$), and the most light sample was the one with the zein (protein concentration 91.79% d.m.) ($L^* = 72.80$), being relatively close to the one with the potato protein isolate ($L^* = 70.58$), the control sample having a value of $L^* = 71.84$. The color of the crust (L∗) was measured by a Colorimeter CR-400 (Konica Minolta, Japan) using the CIE $L^*a^*b^*$ color space (Andrea et al., 2019). The values of the samples with redcurrant, lupine, vital gluten and peas had contained values between 62.33 (carob) and 66.83 (peas).

A color difference was observed between the samples with the added pea flour (in the form of raw, sprouted and fried) and the control sample (only wheat flour). The samples with added pea protein in different forms had a lower value of color parameter L^*, which results in a darker color. This may be influenced by the increase in Maillard browning reactions as a result of increased protein content (Millar et al., 2019).

Samples of bread enriched with: chickpea flour (21.34% protein); carob germ flour (43.17% protein); pea protein isolate (80.74% protein); and soy flour (38.14% protein) were subjected to color analysis and is noticed the darker color was in the sample with carob germ flour ($L^* = 73.52$) and the lightest color was at the sample with the addition of pea protein isolate ($L^* = 77.40$) (Miñarro et al., 2012).

Consumer acceptability

Acceptability regarding the appearance of a gluten-free bread with added pea protein was

slightly lower than that of the control (corn starch, potato starch, pectin, guar gum, yeast, sugar, salt, oil and water), but the samples with the addition of lupine and soy protein had a very low acceptability. The possible causes for which the latter had a low acceptability, was the specific volume and their compact structure. Bread with added protein from peas had a high degree of acceptability also in terms of color and odor evaluation, this having a particularly pleasant odor compared to the control sample (Rafa et al., 2013; Choi and Han, 2001).

When replacing wheat flour with 5-10% lupine flour and soybean flour, the parameters remained as good as in the case of wheat flour, but when increasing the substitution with more than 10% there were reported changes in the rheology of the dough, especially in the volume, weight and texture of the bread, therefore the acceptability was lower (Doxastakis et al., 2002).

In another study wheat flour (control sample with approximately 9.9% protein) was analyzed with the addition of skimmed soybean meal (approximately 48.9% protein), in the following mixtures: wheat flour with added 3% soybean meal (mixture containing about 11.8% protein), wheat flour with added 7% soybean meal (mixture containing about 14.0% protein) and another sample of wheat flour with added 7% soybean meal and 3% sugar (mixture having approximately 14.2% protein). The protein content of the flour mixture with 3%, respectively 7% soybean meal increased by 21.4%, respectively 29.1% without wheat flour. Breads were prepared from these mixtures which were subsequently subjected to sensory analysis. The samples were tested by a number of 145 panellists and the sensory characteristics sought were: breaking resistance, appearance, aroma and taste, crust texture and general properties of acceptability. From the appearance point of view, it was observed that the color of the crust has changed from white-yellow to yellow-brown with the increase of the added soybean meal percentage added. The breaking resistance increased slightly with the increase in the percentage of soybean meal added to wheat flour. The aroma and taste were more acceptable in the samples with small amount of added soy flour, being similar with the ones of control sample (only wheat flour).

The acceptability was higher in samples with the addition of 3% soybean meal, compared to samples with a higher percentage of soybean meal (Mashayekh et al., 2008).

In another study, made by Miñarro et al., (2012) the sensory analysis was carried out with the help of consumers, who tested four bread samples. The control sample was with corn starch and the other ones enriched with different proteins: chickpea flour, carob germ flour, pea protein isolate and soy flour. Bread with the addition of carob germ flour had the highest hardness values compering with the control in the after five days of storage. The sample with the addition of chickpea flour had the most volume while the bread with the addition of carob germ flour recorded the lowest volume. In terms of flavor and taste, they did not show significant differences, these being accepted by consumers. The highest score for the overall appearance was obtained by the bread with the addition of soy flour.

Leguminous flour affected the dough rheology and bread quality by altering key features such as its specific volume, structure and texture. After several experiments, Turfani et al. (2017) found that a 5% flour replacement with legume substitutes does not influence the dough formation, but if the percentage increases for example to 10%, then the volume of the bread is negatively affected. The reduced volume of bread is related to the vegetable fiber content and the legume proteins (Sivam et al., 2010).

CONCLUSIONS

Usually, the products are improved by adding proteins of animal origin but nowadays, the interest is focused on using mostly proteins from plant sources as they can bring special nutritional value due to the fiber content, they have a lower processing cost and do not have such a negative influence on the environment. Bread dough with added lupine is harder than bread dough with wheat flour. In the samples with wheat flour and pea addition, a lower water absorption was registered during the formation of the dough, which led to the differences in the stability of the dough and the increase of resistance to mechanical mixing. The acceptability of the products is influenced by the shelf life, which is related to the loss of moisture

during baking which leads in the first phase to the formation of a drier cave. The addition of Australian sweet lupine flour to wheat bread has reduced bread volume and the hardness increased. Partial replacement with bean flour, pea protein isolate and lupine flour resulted to hard hardness of bread. Pea protein isolate and lupine flour have a reducing effect on the specific volume of bread, carob flour and zein (corn) having a positive effect. Acceptability was directly influenced by the volume and texture of the bread, therefore all samples with a lower volume compared to the control sample had a lower acceptability. It is a continuous challenge to find technological procedures to facilitate the addition of plant proteins into foods in order to answer to the consumers' willingness to include functional foods with added protein in their daily diets.

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TRENDS AND CHALLENGES IN GLUTEN-FREE BAKING PRODUCTS INGREDIENTS: A REVIEW

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Abstract

Nowadays gluten-free (GF) products represent a growing sector in the food industry due to the increasing interest of gluten-intolerant and also for healthy people who are preoccupied in special diets and a healthy lifestyle. This paper reviews recent studies about different ingredients used in gluten-free baking products. The main flour sources for developing gluten-free products are cereals (rice, corn, sorghum), pseudo-cereals (buckwheat, quinoa, amaranth), minor cereals (teff, millet), and legumes (soybean, chickpea, lentil, pea). Apart from these, there are other types of flours used in baking gluten-free products from seeds (flax seeds, chia seeds, pumpkin seeds), nuts (almonds, hazelnuts, chestnuts, walnut, cashew nut), and tubers (arrowroot, tapioca, jicama, taro, potato). This research focused on the literature review regarding identification and quality parameters of various flours and ingredients used in the manufacture of gluten-free baking products.

Key words*: baking products, cereal flour, gluten-free, gluten-free flours.*

INTRODUCTION

Celiac disease (CD) also called gluten enteropathy is an inflammatory condition of the small intestine. This disease affects approximately 1% of the global population (Vici et al., 2016). The clinical symptoms of celiac disease are different from patient to patient, depending on age, duration and the extent of extra intestinal manifestations (Saturni et al., 2010).

CD develops on genetically predisposed subjects when they have an immune system abnormal reaction due to wheat gluten, barley and rye prolamins (Picascia et al., 2016). The consequences of the above reactions are the inflammation and damage to the lining of the small intestine which reduce the absorption of different nutrients such as calcium, iron, vitamins A, D, E, K and folate (Health Canada, Celiac Disease - The Gluten Connection, 2008). Based on data from a recent study, published by World Gastroenterology Organization, in Romania, the incidence of celiac disease among patients with diabetes is 3.9% (WGO, 2016).

Nowadays, more and more people even if they don't suffer from celiac disease are interested in gluten-free products because they want a healthy lifestyle. Besides satisfying hunger and providing the nutrients for humans, healthy eating should also prevent nutrition-related diseases and improve physical and mental well-being of the consumers (Jnawali et al., 2016).

There is no treatment for celiac people so far, the only solution is following a gluten-free diet which requires significant patient education, motivation and follow-up (Rubio-Tapia et al., 2013). Gluten-free products have usually lower quantities of different nutrients like fibre, magnesium and folic acid than gluten-containing ones.

There are other things that make gluten-free products hard to replace. Without gluten, doughs lack cohesiveness, elasticity and baking quality. This fact negatively influences the way of dough handling (Bendera & Schonlechnera, 2019). The risk of suffering from celiac disease is higher for people who have a first degree relative who suffers from it (Murray, 2005; Rubio-Tapia et al., 2008).

The highest risk is for monozygotic twins, next in human leukocyte antigen (HLA) - matched siblings, and then parents and children of patients with CD (Rubio-Tapia et al., 2008). The

lower probability is for 2nd degree relatives (Strong recommendation, high level of evidence). When more than one person is already diagnosed with CD in the family, it is recommended to test the whole family, including the 2nd degree relatives (HLA-DQ2/DQ8) (Rubio-Tapia et al., 2013).
Because of the increasing amount of people who suffer from wheat-related diseases and the desire for healthy eating and lifestyle, food industry began to promote new products made from cereals, other than wheat or rye on the market (Collar, 2014).

MATERIALS AND METHODS

Web of Science database was electronically searched for articles published in the last 20 years. The literature search included as document type: research article and review, on the topics: „gluten-free products", „celiac disease", „gluten-free ingredients", „gluten-free challenges" and the article title containing key words regarding gluten-free cereal and flour types.

RESULTS AND DISCUSSIONS

A gluten-free diet supposes a complete change in life style and a big challenge for celiac people due to the presence of gluten in most types of food products: breads, breakfast cereals, pasta, biscuits, cookies, cakes, bagels, soups (Jnawali et al., 2016).
The most common ingredients used for baking gluten-free products are corn and rice (Gobbettia et al., 2018).
However, a long list of pseudo-cereals, seeds, legumes and nuts (e.g. amaranth, quinoa, millet, sorghum, flax and chickpeas) could replace gluten and integrate and/or substitute the main GF ingredients, all variously improving the nutritional quality of gluten-free diet (GFD) (Kupper, 2005).
Nowadays, pseudo-cereals represent an alternative used for the development of gluten-free bakery products. Pseudo-cereals are edible seeds which belong to dicotyledonous species and they resemble to the physical appearance and high starch content with true cereals. People are interested in pseudo-cereals because these are crops which adapt to different environments from tropical to temperate climatic conditions. The most important pseudo-cereals are quinoa, amaranth and buckwheat (Martínez-Villaluenga et al., 2020).
It is a real challenge to develop gluten-free products because celiac people have various nutrient deficiencies (Hallert et al., 2002). Studies have shown that adults which are suffering from celiac disease and follow a gluten-free diet have significantly lower weight, body mass index, fat and lean body mass than control subjects (Ciacci et al., 2002). A gluten-free diet assumes an alimentation with a lower intake of dietary fibre than a diet which contains gluten (Vici et al., 2016), so using pseudo-cereals represents a good alternative to wheat flour.
These are an important source of minerals (calcium, iron and zinc), vitamins and phytochemicals such as saponins, polyphenols, phytosterols, phytosteroids, and betalains which presents a real potential health benefit (Martínez-Villaluenga et al., 2020). The major nutritional components of pseudo-cereals grain are carbohydrates which vary between 60 and 80% of the seed dry weight (Joshi et al., 2018; Joshi et al., 2019; Shukla et al., 2018). Buckwheat, quinoa, amaranth have a different content of amylose 18.3-47% of total starch, 11-12% of total starch and 7.8-34.3% of total starch, respectively (Repo-Carrasco-Valencia & Arana, 2017).
Starch can be rapidly digestible, slowly digestible or resistant, depending on how easily it is broken down in the gut (Lockyer & Nugent, 2017). Resistant starch (RS) is known for its health benefits as it cannot be digested and absorbed in the small intestine reaching the colon where it is slowly fermented by microorganisms to produce short chain fatty acids (Lehmann & Robin, 2007). According to EFSA (2011), starchy food should contain at least 14% of RS on a total basis to provide health benefits. The highest levels of resistant starch are found in common and tartary buckwheat (27-33.5%) (Skrabanja et al., 1998; Zhou et al., 2019).
Pseudo-cereals are also rich in dietary fibre. Total fibre content varies from 7.0 to 26.5% for quinoa, 2.7 to 17.3% for amaranth and 17.8% for buckwheat, which is in the same range as common cereal grains (Joshi et al., 2019; Joshi

et al., 2018; Lamothe et al., 2015). Quinoa and amaranth also contain a higher content of folic acid, approximately 78 mg/100 g and 102 mg/100 g, respectively, in comparison to wheat (approximately 40 mg/100 g).

The protein quantity and profile depend on genotype and environmental conditions. Compared to pseudo-cereals, cereals have inferior nutritional value. The protein content varies from 9.1-16.7% for quinoa, 13.1-21.5% for amaranth and 5.7-14.2% for buckwheat (Joshi et al., 2019; Joshi et al., 2018; Nowak et al., 2016; Pereira et al., 2019; Shukla et al., 2018; Thanh-Tien et al., 2018), making them key contributors to human protein intake (Martínez-Villaluenga et al., 2020). The gluten-free bread making technology is significantly different from the process of standards wheat breads.

Gluten-free doughs are much less cohesive and elastic than wheat dough. They are extremely smooth, too sticky, less pasty, and difficult to handle. According to Houben et al. (2012) most gluten-free doughs have higher water content and have a more fluid-like structure comparable to the batter of a cake. A shorter mixing, proofing, and baking times are needed, compared to wheat doughs. Gluten-free breads are characterized by smaller volumes, firmer crumbs, and softer crusts. Besides these, other disadvantages of gluten-free bread are: short shelf life, a quick staling, a dry mouthfeel, and a dissatisfactory taste. Although numerous attempts have been made to improve the quality of gluten-free bread, studies are still necessary to optimize recipes and processes.

Sourdough in GF bread

One of the main trends on the market is the use of sourdough especially for bread because it has been demonstrated the fact that this one has a positive sensory, nutritional, texture, and shelf life features influence of baked goods (De Vuyst et al., 2009; Gobbetti et al., 2014).

Overall, the sourdough fermentation has opened new ways in improving the quality and acceptability of gluten-free bread.

During sourdough fermentation, acid occurs, which enhances the swelling of polysaccharides that could partially replace gluten and improve the gluten-free bread structure (Moroni et al., 2009).

According to Coda et al. (2010), lactic acid bacteria are very important because this ferments gluten-free flours; the result is the production of functional breads enriched with bioactive compounds. *Lactobacillus plantarum* and *Lactococcus lactis* subsp. *lactis* were selected to synthesize γ-aminobutyric acid (GABA), through sourdough fermentation of wheat, rye, spelt, oat, buckwheat, rice, amaranth, millet, chickpea, soy and quinoa flour. *L. plantarum* C48 produced the highest amount of GABA with buckwheat and quinoa fermentation.

It has been revealed that using *Lactococcus lactis* subsp. *lactis* PU1 the best results were for amaranth and chickpea flour (Arendt et al., 2011). Using the same pattern, Wolter et al. (2014) incorporate *Weissella calbaria* MG1 starter sourdough to buckwheat, sorghum and teff flour; this softened the batter, which in turn influenced the rheology and baking properties.

Lynch et al. (2014) used *Lactobacillus amylovorous* DSM19280 which is known for antifungal activity to produce quinoa sourdough bread. By adding sourdough fermented by this organism, the mold free shelf life was increased for 4 days compared with gluten-free normal bread, without sourdough, which showed visible mold after only 2 days. Acid production leads to delayed mold growth.

The bread quality in terms of mechanical strength and loaf volume improved and the staling rate decreased (Axel et al., 2015). When sourdough fermented with *Lactobacillus fermentum* was added to gluten-free batter, the glycemic index decreased from 68 to 54 g/ 100 g. It was also observed that the bread texture and volume improved, and the staling was also considerably delayed (Novotni et al., 2012).

Gluten-free cereals in gluten-free biscuits

Although rice is one of the most used cereals in the production of gluten-free biscuits/cookies, researchers tried to add different ingredients for new formulation. Tavares et al. (2016) incorporate co-products generated during industrial processing; they added toasted rice bran, broken rice flour and soybean okra in the original formulation. Because of this addition, biscuits/cookies had a lighter colour, a lower water activity and a smaller specific volume compared to commercially available samples.

A mix of brown rice flour, soya flour, maize and potato starch was studied by Scober et al. (2003). The result was comparable with wheat biscuits/cookies. However, other mixtures such as brown rice flour, potatoes starch, buckwheat flour, millet flakes or brown rice flour, soya flour, maize starch, potato starch, millet flakes did not show positive results.

Oats in GF products

Oats consumption is considered safe for the majority of celiac people, but this fact is still under research by the scientific community (Comino et al., 2015).

Oats could possibly have a post-harvest contamination with gluten which can appear from the other cereals. It has been demonstrated that higher replacement levels or a total substitution of oat flour by oat bran in cookie formulations, affect negatively the acceptability by the consumers (Duță & Culețu, 2015).

Pîrvulescu et al. (2014) studied the differences between normal flours and flours obtained from germinated oats. The result shows a lower content of glucose present in the flour made from germinated oats. This demonstrates the fact that germinated flours can be used in hypoglucidic diets. It is an important thing to know because most of celiac people also suffer from diabetes.

Pseudo-cereals in GF products

Buckwheat is one of the most researched pseudo-cereals for GF biscuit formulation. During thermal treatments, buckwheat flour can maintain its antioxidant capacity (Sakac et al., 2011).

Sakač et al. (2015) replaced rice flour with buckwheat flour (10, 20 and 30% proportion) and observed the fact that the final products had high mineral availability, antioxidant potential (DPPH assay), phenolic level and raised rutin content.

Besides this, Torbica et al. (2012), demonstrated that a mixture of rice and buckwheat flour represents a successfully formulation for GF cereal-based products, like biscuits. Their products presented a good shape, appearance and a pleasant flavor. Another study revealed that buckwheat-based bakery products have a significant antioxidant content (Sedej et al., 2011). GF crackers were made with buckwheat flour and a higher antioxidant content was found, compared to the control crackers with wheat flour.

Another highly nutritious pseudo-cereal, with excellent protein quality and rich in minerals and vitamins is quinoa. Although quinoa is highly nutritious, it presents a major drawback in bakery industry because of the lack of gluten (Thejasri et al., 2017). However, there are many studies based on quinoa flour formulations. Based on an experimental design procedure, Brito et al. (2015) compared mixtures based on quinoa flour, quinoa flakes and maize starch in biscuit formulations. The result was that quinoa flour and flakes formulation have a higher content of proteins, sugars and phenolic compounds. Quinoa flakes and maize starch had a positive effect on the volume of the assessed biscuits.

Due to nutritional intake, amaranth could be very useful in developing products for celiac people. Chauhan et al. (2015) showed that the use of germinated amaranth flour produced acceptable amaranth GF biscuits with a good nutritional quality. Amaranth biscuits had a higher spread ratio than control wheat biscuits which is a desirable characteristic in biscuits. Inglett et al. (2014) also produced amaranth-oat biscuits in a 3:1 ratio, with different type of oat (whole oat flour, oat bran concentrates and steam cooked oat bran concentrate). Biscuits made from amaranth and whole oat flour had the most similar characteristics with the control.

Legumes in GF products

Nowadays 1.8% of the EU arable land is cultivated with legumes compared to 4.7% in 1961 so the Common Agricultural Policy required the production of vegetable proteins to increase (Roman et al., 2016).

Legumes flours are used to increase nutritionally the quality of GF products. All of them are an important source of nutrients such as proteins, complex carbohydrates, fibres, micronutrients and antioxidant compounds (Melini et al., 2017). However, Maghaydah et al. (2013) demonstrated the fact that it is not possible to make GF biscuits exclusively with legume flours. Biscuits with 100% lupine flour were not as good as biscuits in combination with maize flour, or maize starch, rice flour and maize flour with the addition of xanthan gum and

carrageenan. Giuberti et al. (2018) used legumes to increase nutritional value of biscuits. By adding alfalfa seed flour, biscuits showed an overall improvement of nutritional quality. Alfalfa biscuits recorded an acceptable score, but all rice biscuits resulted to have better sensory scores. According to Mancebo et al. (2016) another solution to improve the quality of GF biscuits could be the addition of legume proteins instead of adding legume flours to the formulation. The addition of proteins did not have a negative impact on sensory properties, and they increased dough consistency.

***Rosa damascena* in Gluten-free cookies (GFC)**

Rosaceae family contains different components such as terpenes, glycosides, flavonoids and anthocyanins. It has different properties such as: antioxidant, antimicrobial, analgesic, anticancer, anti-inflammatory, antimutagenic, antidiabetic and antidepressant. (Boskabady et al., 2011; Mahboubi, 2016).

The flour was obtained from dried and ground petals. The other ingredients were potatoes and corn starch, corn and rice flour, sodium bicarbonate, corn syrup, hydrogenated vegetable oil, fine granulated sugar and salt. Rosa flour (RF) GFC were made with 2.5-5-7.5-10% RF added (Gül & Tekeli, 2018).

GFC were sensory analysed. The score was higher for 10% RF concentration for taste but lower for the texture. The best score was recorded by 7.5% RF added GFC regarding odour, aroma and mouth feel (Gül & Tekeli, 2018).

CONCLUSIONS

Celiac disease affects approximately 1% of the world and this percentage is still growing. The only treatment for celiac people is adherence to gluten-free foods.

The most common ingredients used in developing GF products are corn and rice, but researchers permanently try to find new formulations.

Apart from rice and corn, there are other types of flours used in baking gluten-free products from cereals (sorghum), pseudo-cereals (buckwheat, quinoa, amaranth), minor cereals (teff, millet), and legumes (soybean, chickpea, lentil, pea), seeds (flax seeds, chia seeds, pumpkin seeds), nuts (almonds, hazelnuts, chestnuts, walnut, cashew nut), and tubers (arrowroot, tapioca, jicama, taro, potato).

A drawback, and probably the only one, of developing GF products which contain pseudo-cereals, legumes and seeds is that they have a higher price and are harder to find in comparison to corn and rice.

It is very hard to develop GF products because celiac people have various nutrient deficiencies. The production of gluten-free products assumes different challenges to face.

Gluten is an essential element which helps in structure building protein, necessary to formulate high quality cereal-based goods.

The lack of gluten affects the structure of the dough causing a liquid batter and several defects in baked products.

Although the addition of sourdough to gluten-free bread is not an approach at industrial level, studies have shown so far that the use of selected lactic acid bacteria improves the nutritional properties as well as increases the shelf life (with 4 days) of the breads. Sourdough also improves the volume, texture and sensory quality of the gluten-free breads.

For biscuits/cookies, gluten does not play a fundamental role as in bread or other soft products with biological leavening.

The texture of biscuits/cookies does not depend on protein/starch structure, but it depends especially on starch gelatinization and sugar.

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STABILITY OF *Lactobacillus plantarum, L. casei* AND *L. rhamnosus* IN TWO TYPES OF MICROCAPSULES

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Abstract

Probiotics require proper encapsulation in order to allow development of useful products with biological activity. Survival of encapsulated probiotics was monitored as a function of the carbohydrate source and of time, over a period of 45 days. The starting material was a mixed culture of Lactobacillus casei, Lactobacillus rhamnosus and Lactobacillus plantarum with a concentration of 10^{11} colony-forming unit/ml (CFU/ml). Standard culture medium with glucose allowed higher initial concentration of probiotics compared with oligofructose medium. However, reduction of viable probiotics in oligofructose supplemented microcapsules was of only 4 log CFU/g, while in glucose supplemented microcapsules was of 8 log CFU/g. Specifically, numbers of probiotics varied from 10^{10} CFU/g immediately after encapsulation to 10^{2} CFU/g, after 30 days of storage for glucose supplemented microcapsules, while, for oligofructose supplemented microcapsules, numbers were 10^{1} CFU/g initially and 10^{6} CFU/g after 30 days. Our results indicates that oligofructose is a more effective prebiotic than glucose, allowing higher survivability of probiotics.

Key words*: Lactobacillus, glucose, oligofructose, prebiotics, probiotics.*

INTRODUCTION

As has been known for more than a century, the Nobel Prize laureate, Russian researcher Elie Metchnikoff is the one who brought the new concept about the existence of "pro-life" bacteria. But what led to this concept was the hypothesis that the long and healthy life of Bulgarian peasants was due to the consumption of dairy products containing lactic bacteria. Probiotics have been defined by the FAO/WHO Expert Committee in 2001, as being "living microorganisms that, when administered in adequate quantities, provide health benefits to the host" (De Prisco et al., 2016). Taking into account all the definitions issued so far and accepted, it has been found that more than 30 species and genera of bacteria are accepted as probiotics. (Fijałkowski et al., 2016).

The probiotic agents most frequently used are those microorganisms which produce lactic-acid, including in particular *Lactobacillus* species (Serna-Cock et al., 2016). The most important benefits of these microorganisms are found especially in the intestinal level, where they are particularly concerned with the maintenance of the microbiota ecosystem (Ozyurt & Otles, 2014).

In the present study were analyzed three species from the group of lactobacilli and they are: *Lactobacillus plantarum, Lactobacillus casei* and *Lactobacillus rhamnosus*, about which the specialized literature states that they are effective in many treatments. For example, in the treatment of patients who had chronic fatigue syndrome it was used the *Lactobacillus casei* strain *Shirota* and it was observed a significant reduction on the Beck anxiety (Rao et al., 2009); Other authors have argued that the *L. plantarum* is responsible for the decrease and even treating the symptoms of colitis and irritable bowel Schultz et al. (2002); Regarding *L. rhamnosus* and other probiotics, it was found to be responsible for the anti-inflammatory induction of cytokines, interleukin-10 (Tenea et al., 2018). It is also known that there is an interaction between the innate immune system, the adaptive immune system and probiotics contributing to intestinal homeostasis. (Latvala et al., 2011).

In order for a probiotic to be used, it must follow basic principles such as: it must survive so that the concentrations of living microorganisms at the time of food consumption are above 10-10^{7} CFU g^{-1} or ml^{-1} (FAO/WHO, 2001). For more than 10 years, in order to cope with the

disruptive factors, different physical barriers to the protection of these microorganisms are analyzed. (Bernucci et al., 2017, Burgain et al., 2011). The most studied method of protection is microencapsulation, which involves retaining the culture of microorganisms in a capsule made of materials that must be generally recognized as safe (GRAS), be able to ensure the integrity of probiotic cells and be soluble in their place of action (Valero-Cases, 2015). Starch, chitosan, alginate, xanthan gum, cyclodextrins, whey proteins etc. are among the most analyzed biopolymers and used as a material for the capsule protection wall (Ashwar et al., 2018). In order to ensure a higher survival rate throughout the manufacturing process and up to the place of action of probiotics in the encapsulation matrix, a nutritious substrate such as prebiotics was incorporated. These prebiotics were defined by International Scientific Association for Probiotics and Prebiotics (ISAPP) as being "a substrate selectively used by host microorganisms that confer health benefits". From the combination of a probiotic and a prebiotic, results a symbiotic product that can increase the probiotic's survival rate during products and storage but at the same time enhances the effects on the intestine. (Raddatz et al., 2019; De Prisco et al., 2016). As far as we know, there are no studies investigating the survival of the three strains mentioned above in mixed culture. Therefore, this study aimed to analyze the viability of the three strains of *Lactobacillus* in two different capsule types for 45 days of storage at 4°C. The capsules contain different sources of carbon, one source is glucose (GLU) and the other is a prebiotic, oligofructose (OLI).

MATERIALS AND METHODS

Chemicals and reagents

De Man-Rogosa-Sharpe agar was purchased from VWR International bvba/sprl; MRS broth, manganese (II) sulfate hydrate, potassium chloride, sodium chloride sodium citrate, peptone from casein, meat extract, yeast extract, magnesium sulfate hepta-hydrate glucose, oligofructose were purchased from Sigma-Aldrich, Germania; Alginic acid Sodium salt from AppliChem GmbH. Calcium chloride hexa-hydrate from Lach - Ner Company - Czech Republic; acetic acid-ammonium salt, sodium salt tri-hydrate and sodium phosphate, acetic acid, and sodium phosphate dibasic hydrate from Across organics-Spain.

Microorganism and culture preparation

In order to determine the ability of *Lactobacillus* cultures to use oligofructose to have long-term viability, a De Man-Rogosa-Sharpe broth (MRS broth) was used as a basal medium as described by De Man et al., 1960 and a modified medium where the initial source of carbohydrate with oligofructose (OLI) was replaced. In brief, the medium containes the following components (g/l): peptone (10.0), meat extract (10.0), yeast extract (4.0), Na × $3H_2O$ acetate (5.0), K_2HPO_4 × $3H_2O$ (2.0), (NH_4) $3C_6H_5O_7$ × $2H_2O$ (2.0), $MgSO_4$× $7H_2O$ (0.2), $MnSO_4^-$ × $4H_2O$ (0.05), Tween 80 (1 ml) and glucose (20.0). For the homogenization of the two types of culture media, a homogenizing vortex (Vortexer, Heathrow Scientific ® LLC) was used for 15 min at 1400 rpm. Before being sterilized in the autoclave at 121°C for 15 min, the pH was adjusted to 6.2.

Probiotic strain and culture condition

In this study were used three strains of lactobacillus. *L. casei 431* is a registered trademark, and it was received from Christian Hansen. From this strain, pure cultures were obtained after three reactivations in MRS broth and stored at -20°C in glycerol. *Lactobacillus plantarum* and *L. rhamnosus* (BIOPROX RP 80) were purchased from Bioprox Noyant, France. Prior to use, each culture was reactivated in MRS broth at 37°C for 24 h. Then another 24 h reactivation took place at 37°C, to obtain a densitometry of 4 McFarland units for *L. casei* and 8 McFarland units for the other two strains (*L. plantarum* and *L. rhamnosus*) grown together. These values were determined on a McFarland densitometer with McFarland measuring range 0.3-15.0 at wavelength $\lambda = 565 \pm 15$ nm. After this, the cultures, also separately, were inoculated in modified MRS broth at 37°C for another 24 h. The three strains were then put together in equal proportions and incubated in modified MRS broth for 24 h at 37°C. and then for another 16 h in order to obtain the final culture for this study. The cell suspensions were subsequently subjected to micro-encapsulation as described later.

Microencapsulation procedure

For the preparation of all solutions including culture media, distilled water (Aquatron A4000D, Cole-Parmer Ltd) was used, and to avoid any contamination, sterilized reagents and glassware were used. The encapsulation technique used was extrusion, a technique described by Darjani et al. (2016), Krasaekoopt et al. (2004) and Peredo et al. (2016), and then adapted and modified to the needs of the present study. The materials used to form the encapsulation matrix were: 1.75% sodium alginate, 1% carbon source, 10% vegetable oil, 85% distilled water. All the materials were mixed with the help of a stirrer at 10,000 U/ min. The pellet was added and the mixing was continued using a magnetic stirrer (15 min/600 rpm). This pellet was obtained by centrifuging the culture for 16 h for 10 min at 2500 g at 4°C.

After this homogenization, a peristaltic pump, a 3 mm diameter hose and a 0.4 mm diameter needle were used to pass the prepared emulsion. The resulting drops reached into aliquots of 2% sterile calcium chloride at a distance of about 10-15 cm with a power of 3.2 rpm/ml/min. Once the drops arrived in the calcium chloride solution, they immediately formed micrometer-sized gel spheres. The microcapsules were allowed to stand for 30 min to harden, then harvested using vacuum pump (EZ-Stream® vacuum filtration pump). The whole process was performed by autoclaving all the solutions involved in the process (121°C, 15 min) and under sterile conditions in a horizontal laminar air-flow cabinet.

Characterization of capsules

Considering the dimensions of the micrometers, the morphological characterization and the dimensions of the microcapsules were performed using microscopy. An optical microscope and an electronic microscope were used. The morphology was performed by scanning electron microscope-SEM.

Viable cell count

For the determination of the number of living cells from the emulsion, serial dilutions were performed in distilled water. 1 ml of the final dilution was inoculated in triplicate in MRS agar plates and incubated at 37°C for 48-72 h under anaerobic conditions in anaerobic jars. The results were reported in CFU/ml.

Encapsulation yield

Regarding the determination of the number of viable cells in the fresh microcapsules, it was necessary to dissolve the capsule and release the cells. This protocol was performed by dissolving 0.1 g of fresh microcapsules in 9.9 ml of 1% sterile sodium citrate solution with pH 6 and slightly stirred at room temperature for approximately 12 min, after which serial dilutions were performed as mentioned above. From the last dilution 1 ml was inoculated into small plates with MRS agar and incubated for 48-72 h at 37°C in anaerobic jars with anaerobic generator. All experiments were performed in triplicate and the results were reported as CFU/g of microcapsules. The encapsulation yield (EY) was calculated according to the formula used by Chávarri et al., 2010; Picot & Lacroix, 2004; Rather et al., 2017:

$$\mathrm{EY} = \frac{log_{10} N_1}{log_{10} N_0} ,$$

where $log_{10}N_1$ represents the number of viable cells trapped in the capsule and $log_{10}N_0$ represents the amount of free viable cells added to the emulsion during the encapsulation process and the result of the equation is expressed as number of CFU/ml. This formula, EY, represents a criterion for measuring how the encapsulation process influences the number of viable cells (Picot & Lacroix, 2004).

Viability of encapsulated bacteria during storage

To determine the number of viable cells encapsulated during storage at 4°C for 45 days, the microcapsules were stored in sterile and sealed petri dishes. The cell viability was analyzed both from the microcapsules containing glucose in their matrix as carbon source, as well as from microcapsules with prebiotic, oligofructose.

The testing was performed on microcapsules collected from day 0, 7, 14, 28 and 45. The same protocol described above was used. After dissolution of the capsule, serial dilutions were made and then pour plated in MRS agar. Incubation was carried out under the same conditions as above. An average of three

replications were made, and was expressed as log CFU/g of microcapsules.

RESULTS AND DISCUSSIONS

Microscopic examination of alginate beads/Morphological characterization of micro-particles

One of the objectives of this study was to evaluate the effect of prebiotic on morphology and on the size of the microparticles. For this purpose, optical microscopy and electron microscopy were used. Thirty-five microcapsules were randomly selected and analyzed under the optical microscope which was equipped with a digital component (Table 1). Optical microphotographs of microcapsules showed different types of beads with a defined limit, but spherical shape was more abundant and particles were isolated without adherence to each other. This aspect leads us to the idea that this technique is improved because it produces capsules of micron size, much better than those of millimetric dimensions produced by other researchers (Hyndman et al., 1993; Arnaud et al., 1992) who have used a similar technique, and will offer a smooth texture when incorporated into products. Some researchers (Mokarram el al., 2009; Hansen et al., 2002) claim that large alginate capsules (> 1 mm), cause the coarse texture of foods that are supplemented with probiotic cultures.

Therefore, two samples of fresh microcapsules were subjected to electron microscopy (SEM) scanning: microcapsules with prebiotic and probiotic bacteria - oligofructose and microcapsules with probiotic bacteria and without prebiotic (Figure 1). It was found, according to Table 1, that there are no significant differences in morphology and size of beads. Following the SEM analysis, it was found that: microcapsules varied in size (which was also observed in optic microscopy), were compact and continuous, spherical but with irregular surface. As an explanation for the uneven surfaces, there may be a greater concentration of polymer there (Fareez et al., 2015). Also, the absence of free *Lactobacillus* cells on the surface of the capsules was noted, and as mentioned above the encapsulation process means that it is efficient from this point of view.

Evaluation of the mean diameter and distribution of microparticles size

Below, in Table 1 are given the averages of the diameters of the microcapsules that were analyzed. It is considered that the distribution and size of the capsules may be influenced by several factors such as: firstly, the stirring speed, then the ratio between water and oil (v/v) or surfactant concentration.

Table 1. Beads size expressed as mean of 35 microcapsules ± standard deviation

Parameter	Size (μm)	
	Oligofructose	Glucose
Mean	0.478 ±0.13 [a]	0.42 ±0.14 [a]
Median	0.478	0.42
Minimum	0.20	0.21
Maximum	0.65	0.62

Means in the same row with superscripts (a) not differ significantly: *P > 0.05.

Some authors believe that with the introduction of probiotic cells into the emulsion and then injected with needle into calcium chloride, the size of the microparticles (Sousa et al., 2015; Martin et al., 2013) is reduced and the capsules are more compact. This happens due to the presence of probiotics cells, but also due to the gel layer that forms which compresses the capsule matrix and thus expels some of the inner water (Sánchez et al., 2017). On the other hand, another explanation regarding the aforementioned might be that probiotic cells would replace Ca^{2+} ions which leads to a change in Ca^{2+} concentration which will lead to syneresis. At the same time, the size of the microcapsules can be influenced by other factors different from those mentioned, such as: alginate concentration, calcium chloride concentration, needle diameter, pump pressure, distance between needle and calcium chloride solution. In previous studies with microencapsulation by extrusion, Lenton et al., (2012) obtained an average size for microcapsules of 2.9 mm; Muthukumarasamy et al. (2006) obtained an average size of 2.37 mm with a similar needle (G21) with the one used in the study performed by Valero-Cases et al., 2015, for microencapsulation by extrusion, which obtained an average of 1.86 mm, which was larger than the average size obtained in this study for all the beads (0.48 mm). In other previous studies with internal emulsion microencapsulation, Cai et al. (2014) obtained

average microcapsule dimensions of 343 µm, which were similar to those of the present work obtained using the method of extrusion encapsulation.

Viable cell count and Microencapsulation efficiency and yield

The presence of prebiotic in the culture medium significantly influenced the growth of lactobacilli in final cultures of 16 h thus, the growth of lactobacilli in the culture medium with oligofructose was 11.08 ± 14.11 log CFU/ml and 12.08 ± 7.06 log CFU/ml for glucose.

This is explained by the fact that lactobacilli metabolize glucose much faster as a carbon source compared to oligofructose (Luca and Oroian, 2019).

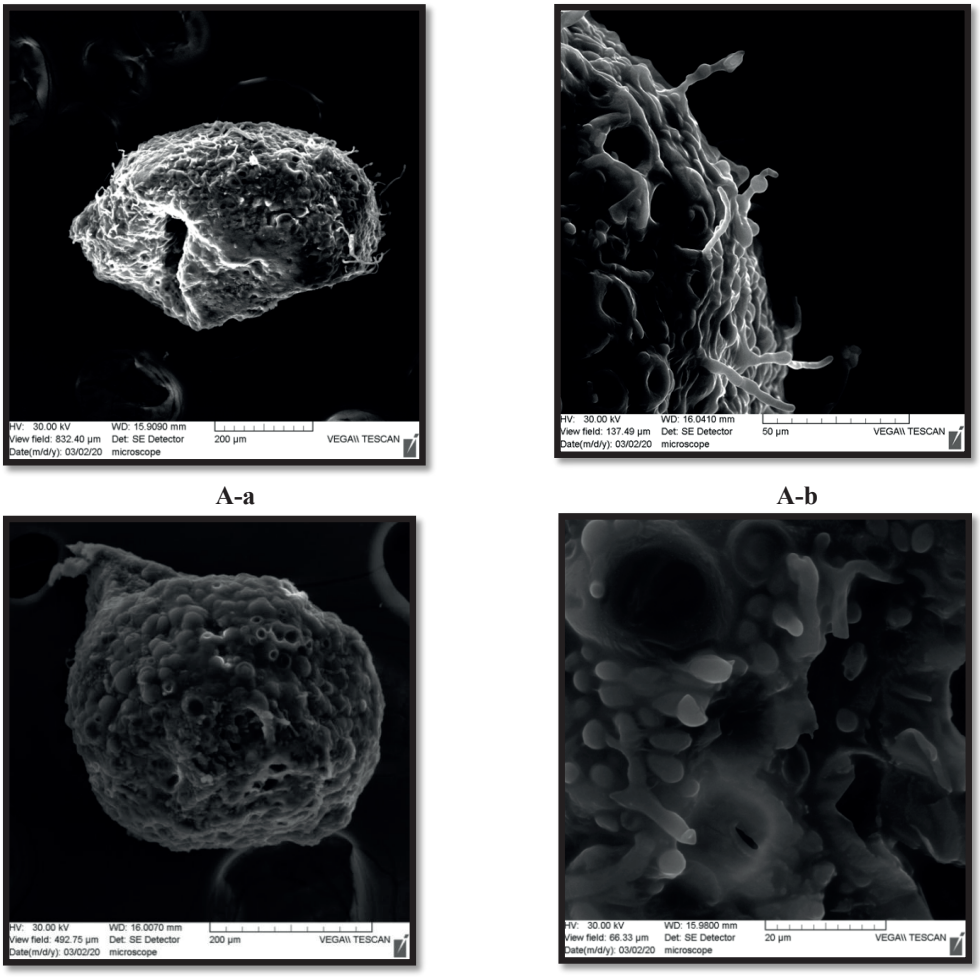

Figure 1. Scanning electron microscope photographs of fresh beads: (A-a) bead whit prebiotic; (A-b) surface of a bead with oligofructose; (B-a) bead whit glucose; (B-b) surface of a bead with glucose

Another objective of this study was to identify the most efficient encapsulation matrix of each type of microcapsule for *Lactobacillus* strains. For this it was analyzed how the *Lactobacillus* cells were protected during the encapsulation process - the of microcapsulation but also EY.

Table 2 shows the results obtained for glucose microcapsules efficiency and also for oligofructose microcapsule. It can be seen that both types have formulated an optimal encapsulation efficiency of 100% but with a significant difference between them ($p > 0.05$).

The encapsulation efficiency of the employed process was very high and is not a critical parameter that needs to be adjusted. The results showed an encapsulation efficiency of over 100%, given the fact that the number of CFU/g of capsules is almost 1 log higher than that of CFU/ml of microorganism containing emulsion. This effect appeared due to the capsules being a proper medium for encapsulated bacteria growth, as it contains prebiotics and of the fact that enumeration of encapsulated bacteria was performed approximately 6 h after the actual encapsulation. The growth of probiotics in prebiotic supplemented media was already shown to occur rather rapidly. For instance, *Lactobacillus casei* has a rate of approximately 1 log of growth over 6 h in 4 different prebiotic supplemented media (Luca and Oroian, 2019), while *Lactobacillus plantarum* and *L. rhamnosus* displayed a similar behavior with the same prebiotics (Luca et al., 2019). A probiotic encapsulation report by Sánchez et al in 2017 showed similar results but they did not use prebiotics. Other authors such as Raddatz et al., 2020, found that their capsules obtained by internal emulsification/gelation, had an EY% between 82.65% and 91.24%; capsules obtained by Jantarathin et al. (2017) using extrusion technique with *L. acidophilus*, sodium alginate and inulin had an EY % = 88.19%; Zou et al., 2011, using the internal gelling technique, produced alginate microspheres mixed or coated with other polymers containing *Bifidobacterium bifidum F-35*, and the average EY% ranged from 43% to 50%. As noted above, it is observed that maintaining cell viability is a very important factor, regardless of the type of encapsulation.

Viability of encapsulated bacteria during storage

The cellular viability of glucose and oligofructose microcapsules stored in the refrigerator for 45 days at 4°C is shown in Figure 2. After 45 days, the survival rate of *Lactobacillus* cells in glucose microcapsules indicates a decrease greater than 7.60 CFU log/g. In terms of cell survival in prebiotic microcapsules, it decreased from 2.96 x 10^{10} ± 20.3 CFU/g to 6.93 x 10^{5} ± 1.33 CFU/g, so with a loss of viability of 4, 63 CFU log/g after this period of 45 days. The survival of cells in oligofructose beads was significantly ($p < 0.05$) better than that of glucose beads.

It was statistically analyzed both the cell viability at the same time between the two types of microcapsules, but also between the obtained values of the same type of microcapsules at the same moment. It was found that there are significant differences ($p < 0.05$) in both cases at all times. These differences were maintained during the 45 days.

In the first 7 days after encapsulation, a reduction in the viability of probiotics was observed in both prebiotic and non-prebiotic microcapsules. In the first case the reduction was 1.899 log CFU/g, and in the second case the reduction was 2.90 log CFU/g. Therefore, the viability decreased during the first 7 days by 1.26% for probiotics encapsulated with oligofructose and by 1.25% for probiotics encapsulated with glucose. If in the next 7 days the decrease in cell viability of oligofructose microcapsules was insignificant of 0.073 log CFU/g, in the case of the other type of microcapsule, the cell viability decreased by approximately 2 log CFU/g. After 28 days of storage, it was observed a decrease in cell viability of 3.169 log CFU/g in prebiotic microcapsules compared to day 14 and a decrease of 5.14 log CFU/g compared to the time of encapsulation, but this decrease will not be the same after 45 days when the reduction was only 0.49 log CFU/g. A decrease in cell viability also occurs in the case of glucose microcapsules, but it was 2.53 log CFU/g less than day 14 and much higher (7.41 log CFU/g) compared to day 0. Comparing the last 2 measurement points between the cell viability of the capsules with and without prebiotic, day 28 and day 45, it was found a greater decrease of cell viability in microcapsules without prebiotic, of 1.2 log CFU/g. According to the above it can be stated that these microcapsules which have a source of carbon in their matrix the microorganisms are metabolically active in capsules at 4°C. Whether prebiotics, or glucose was used as a substrate in the capsule matrix, the encapsulation is not responsible for decreasing cell viability. In our opinion, some of the main causes would be the passage of time, the consumption of the nutritious substrate, the presence of compounds resulting from the metabolism process such as metabolic acids and

bacteriocins. Other explanations for this would be the presence of residual water in microcapsules and the fact that they were stored in petri dishes during the storage test. Each sample tested was taken from these plates, where the humidity in the atmosphere could have entered which could have led to increased water activity. (Sánchez et al., 2017). Also, it is well known that humidity has a negative effect on cell viability (Heidebach et al., 2010). Using extrusion as the encapsulation method for *Lactobacillus gasseri* and *Bifidobacterium bifidum*, Chavarri et al. (2010) reported that they observed a decrease in viability in the first 11 days of 3.34 log CFU/ml and 4.11 log CFU/ml; respectively after 14 days they did not observe any survival.

As shown in Figure 2, at the end of the storage period, the number of microorganisms in the encapsulated probiotic with oligofructose was higher than the probiotic encapsulated with glucose.

Table 2. Enumeration of probiotic cells and the encapsulation yield

	Oligofructose	Glucose
Culture of 16 h	1.39E+11±14.11	1.23E+12±7.06
Probiotic population before encapsulation (CFU/ml)	8.16E+10^{a}±23.9	1.12E+11^{a} ±8.00
Encapsulated probiotic population (cfu/g)	2.96E+11^{b} ±2.01	1.87E+11^{b} ±2.67

All values are mean ± standard deviation of three replicates

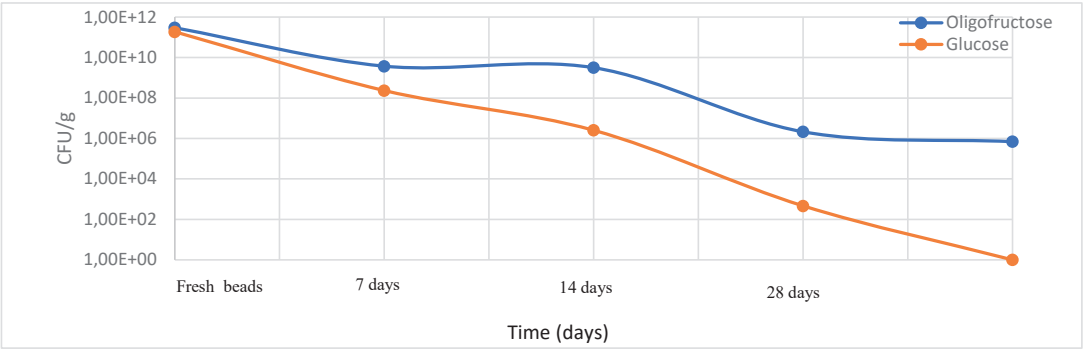

Figure 2. Stability of oligofructose and glucose microparticles of *Lactobacillus* during 45 days at 4ºC. Means (n = 3) ± SD (P < 0.05 between oligofructose and glucose)

CONCLUSIONS

In this study, two types of microcapsules obtained by the extrusion encapsulation method were analyzed. They had different carbon sources in their matrix. Checking the cell viability after encapsulation showed that the encapsulation process chosen did not reduce the cell number. The results also showed that the presence of a carbon source such as oligofructose is much more efficient than glucose in terms of cell survival, having a much greater protective role against environmental conditions. *Lactobacillus* strains had a much higher survival rate in oligofructose capsules than glucose capsules during 45 days of storage. At the same time, the results showed that regardless of the type of emulsion used for encapsulation in this case, the size and shape of the beads were similar without statistically significant differences, and this behavior could be attributed to prebiotics ability to improve capture efficiency and accumulation capacity over time of the encapsulation process.

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COMPARATIVE STUDY BETWEEN GENETICALLY MODIFIED PRODUCTS OBTAINED BY CONVENTIONAL TRANSGENESIS AND BY NEW TECHNIQUES OF TARGETED MUTAGENESIS

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Abstract

This paper presents a comparative study reguarding similarities and differences between the genetically modified products (food raw materials of vegetable origin) obtained by conventional methods of transgenesis and the product obtained by new techniques of targeted mutagenesis, like CRISPR-Cas9 method. In this article we will present briefly through explanatory drawings the genome of plants obtained by conventional random mutagenesis and targeted mutagenesis CRISPR-Cas9, mutagenesis in EU GMO legislation, the objectives of innovation and multiplication of specific plants which can contribute to a sustainable agriculture and increased food production. The products resulted from small editions, which could also have appeared spontaneously in cultures. In conclusion, CRISPR-Cas9 is the principal used technology for genome editing for simplicity and efficiency. We try to highlight the application and benefits of CRISPR-Cas9 method like a tool genome editing for agriculture and food industry.

Key words*: CRISPR-Cas9, genome editing, GMO, mutagenesis.*

INTRODUCTION

The product genetically modified organism (GMO) is an organism, in which the genetic material has been modified in a way that does not occur naturally the recombination, by mating.

The process within this definition:

1. Methods for nucleic acid recombination with final scope to obtain a new genetic material by inserting nucleic acid molecules, belonging to an organism (virus, bacterial plasmids or other vectors) and incorporating them into a host organism, in which they are capable of multiple;
2. Techniques by direct introduction of a piece by nucleic acid in an organism, of the genetic material which does not belong the organism, like microinjection, macroinjection and microencapsulation;
3. Cell fusion (protoplast), hybridization methods in which living cells have new combinations of hereditary genetic material formed by the fusion cells through naturally occurring processes.

Conjugation, transduction, transformation, polyploid induction, mutagenesis (irradiation, chemicals - alkylating agents), cell fusion (protoplast) of vegetal origin cells from plants that can change genetic material by conventional methods are not considered genetic modification in light of Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms (https://eur-lex.europa.eu/legal-content/EN/TXT/ ? qid = 1579766311266 & uri = CELEX: 32001L0018).

The product obtained by new genetic engineering techniques: oligonucleotide targeted mutagenesis, zinc finger nucleoside, transcriptional effector nucleases, repeated groups with short intermediate palindromia that were associated with the double-stranded DNA binding protein Cas9, cisgenesis, intragenesis, grafting, agro-infiltration, RNA dependent DNA methylation and reverse multiplication.

The process makes specific changes in the DNA of the plants to modify the traits, these changes can be from the modification of a single base, to the insertion or deletion of one or more genes.

The alterations of the DNA sequence produced by genome editing methods are not identified for the changes of the DNA sequence obtained naturally or by conventional mutagenesis. The genome editing could be possible used to change more than two bases into single-site DNA. This are less likely to be a natural or mutagenic process (Jones et al., 2018).

The method of genomic editing system is very used to intermediate short-acting palindromic

groups associated with the binding protein of Cas9 into the specific DNA sequences (CRISPR-Cas9).
The CRISPR-Cas9 technique allows the vegetal origin genome to be precisely modified by removing undesiderated genes or indicating the specific genes can get new functions (Wolt et al., 2016).
This new products obtained by the methods of targeted mutagenesis, like CRISPR-Cas9 system are very similar with the naturally occurring variations.

MATERIALS AND METHODS

The research methodology used into the paper has the following aspects:
• Bibliographic study by the national and international literature;
• Collecting the information within the researched specific area;
• Order, process and present of the results in a synthetic form;
• Analysis and interpretation of the results, elaboration of conclusions and recommendations.

RESULTS AND DISCUSSIONS

These new targeted mutagenesis techniques are much faster and cheaper than conventional breeding techniques. There are already several products of vegetable origin obtained by the new techniques, which are near or in the testing phase in crop or marketing.
The main differences between the food products consist in the mechanism of inducing the break on DNA sequence and their efficiency in targeting the desired sequences. Conventional mutagenesis mechanisms produce multiple local mutations of the genome, while CRISPR-Cas9 (targeted mutagenesis) method yields nucleotide point mutations.
Editing the genome through the CRISPR-Cas9 method allows the application of new genetic engineering techniques, several DNA sequences can be targeted at the same time. Are obtained the specific products much faster and at lower costs than conventional methods and is easy to apply to plants. According to the literature, CRISPRs are prokaryotic DNA segments containing short, repetitive base sequences. In a palindromic repeat, the nucleotide sequence is the same in both directions. Each repeat is followed by short segments of the distal DNA from previous exposures to foreign DNA (virus or plasmid).
Which is different from what we normally consider a GMO (Figure 1): conventional mutagenesis products obtained by natural randomisation, targeted mutagenesis products obtained by genome editing method like the CRISPR-Cas9 method and conventional transgenesis GMO obtained with techniques for nucleic acid recombination (Custers R., Flemish Institute for Biotechnology, 2019).

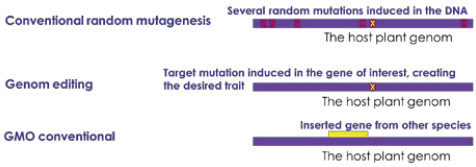

Figure 1. The host plant genome by different methods of mutagenesis and transgenesis
Source: Custers R., 2019

From the Figure 1 graphical representation of the host genome is easy to observe the important changes in the genome of plants in the case of conventional mutagenesis and the transfer of genes of interest by well-known methods of nucleic acid recombination. By comparison, the new methods of targeted mutagenesis and plant genome editing, induce minor modifications, by several nucleotides.
The predictability of phenotypic manifestations is well determined in the products obtained by targeted mutagenesis, because the modifications are minor at the genome level, compared to the conventional methods of obtaining genetically modified plants. Several random mutations are induced in the nucleic acid of the host plant genome by the conventional random mutagenesis method (Figure 2).

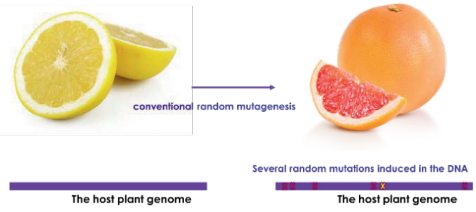

Figure 2. Representation of the conventional random mutagenesis plant genome
Source: Custers R., 2019

Target mutation induced in the gene of interest using methods of genetic engineering like CRISPR-Cas9 create the plants and vegetable food products with precise features, previously desired (Figure 3).

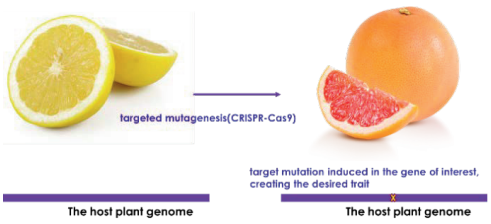

Figure 3. Representation of the targeted mutagenesis plant genome by CRISPR-Cas9
Source: Custers R., 2019

The objectives of the innovation of the products obtained by the targeted mutagenesis and the multiplication of the specific plants, according to the specialized literature are:
- increased production and poverty reduction in areas with salted or dry soils,
- high quality nutritional foods,
- reduced pressure on the soil through less work (fertilizers, pesticides),
- soil bioremediation.

The conventional random mutagenesis and targeted mutagenesis are very different at the genome level, even if phenotypically we identify the same gene expression (Figure 4).
Targeted mutagenesis, having a precise and unique genomic position, as opposed to naturally occurring and then randomized mutagenesis, where other undesirable changes occur, there will be no possibility for future undesirable characters to manifest in future generations of plants or vegetable food.

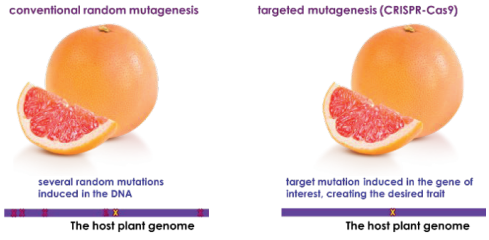

Figure 4. Representation of the comparation of conventional random and targeted mutagenesis
Source: Custers R., 2019

Changing the DNA sequence obtained by genomic editing methods cannot currently be identified by methods known by the laboratory, as compared to changing the DNA sequence obtained by natural processes or conventional mutagenesis. When the method of genome editing is used to introduce more than two base pairs into the single-stranded DNA strand, these being less probable to be natural or mutagenic, may be an exception (Jones et al., 2018).
If not held information about the changes introduced at this moment is impossible to detect these changes. Detection might be possible if there was a reference genome for comparison (Lusser et al., 2011).
On 25 July 2018, the European Court of Justice ruled that organisms obtained by mutagenesis must be considered to be GMOs, exception could be only the organisms obtained by conventional mutagenesis, which have a long safety history. The judgment of the European Court of Justice notes that the organisms obtained by the new genome editing techniques (CRISPR-Cas9 methods) are GMOs by Directive 2001/18/EC. The directive requires for this organism produced by genome editing to be developed specific detection methods within the European national reference laboratories for GMOs.
In accordance with the new legislation mentioned above, products of vegetable origin genetically modified by targeted mutagenesis methods are subject of specific market authorization legislation for genetically modified organisms, as opposed to products obtained by conventional mutagenesis by natural selection, chemical or irradiation methods (Figures 5 and 6).

Figure 5. The conventional mutations products are not subject to GMO specific legislation
Source: www.vib.be

Figure 6. The genome editing products are subject to GMO specific legislation
Source: www.vib.be

EURL - GMFF has developed a report for detection issues and the possible ways to detect these products (Jones et al., 2018).
Creating a database for genomic comparisons would be a huge economic effort for European Union. This was one of the proposals, but on the European Union market are registered 14,442 varieties of bread wheat, Durham wheat, corn, soybeans, barley, Swedish rapeseed, rapeseed and potatoes, according to the European Commission's plant variety database. According to Wikipedia, there are 7,500 varieties of apples and 10,000 varieties of tomatoes.
This would be very costly, impossible to implement and would provide relatively weak evidence. (Jones et al., 2018).

CONCLUSIONS

Plants with the same modification, obtained by targeted or natural mutagenesis, cannot be precisely identified.
Genome editing is at least as safe as conventional mutagenesis. CRISPR-Cas9 is a tool that can help achieve the objectives in a better oriented and faster way.
CRISPR-Cas9 system, known as genome editing is the most simplest and efficient technique for crop development.
The conventional random mutagenesis and targeted mutagenesis are very different at the genome level, even if phenotypically we identify the same gene expression:
- Several random mutations are induced in the host nucleic acid of the plant genome by the conventional random mutagenesis method;
- Target mutation induced in the gene of interest using methods of genetic engineering like CRISPR-Cas9 create the plants and vegetable food products with precise features, previously desired.
From the studies done so far, it appears that the CRISPR-Cas9 method for obtaining vegetable products is going to change the course of the agricultural and food industry.

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BY-PRODUCTS INGREDIENTS IN CORN BASED PASTA: EFFECTS ON THE TECHNOLOGICAL AND QUALITY CHARACTERISTICS

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Abstract

Grape peels and whey powder represent valuable by-products which contain antioxidant fibres and protein respectively that can be used to enhance gluten-free pasta nutritional and technological properties. The aim of this paper was to investigate the effect of grape peels (0, 1, 3 and 5%) or whey powder (0, 5, 10 and 15%) addition on the colour, cooking quality, microstructure, texture and sensory characteristics of gluten-free pasta based on nixtamalized corn flour. The increase of grape peels addition led to a decrease of luminosity and fracturability, higher cooking loss, cooking time and firmness, while the incorporation of whey decreased cooking loss, luminosity, fracturability and increased cooking time. The sensory profile revealed good acceptability of gluten-free pasta enriched with grape peels or whey powder, the scores depending on the addition level, the best acceptability scores being obtained for samples with the highest whey amount (15%) and with the lowest grape peels content (1%) respectively. These results revealed the possibility to develop novel corn based gluten-free pasta with higher nutritional value and acceptable technological and sensory properties by grape peels or whey incorporation.

Key words*: corn, gluten-free pasta, grape peels, valorisation, whey.*

INTRODUCTION

Nowadays the demand for gluten-free products increased among consumers suffering from celiac disease. Food industry generates significant amounts of by-products which present high biological value. Their use in food formulation has advantages from two view points: novel product nutritional value increase and environment protection though waste reduction. The main ingredients for gluten-free pasta are corn and rice flours which have relative low protein and dietary fiber contents (Bouasla et al., 2017; Giménez et al., 2013). Corn flour nutritional value can be enhanced by nixtamalization, a process which implies a thermal treatment with lime of the grains at about 90°C for 1 h (Cortés-Gómez et al., 2005). This treatment determines dough rheological properties improvement, calcium content increase and bound niacin release, but decrease fiber content by pericarp elimination (Wacher et al., 2003).

Gluten absence from pasta may cause some technological and quality issues as it has an important role on the textural characteristics of the final product (Phongthai et al., 2017). In order to minimize these negative effects, hydrocolloids and/or exogenous proteins can be used (Padalino et al., 2016). Pasta enriched with cottage cheese whey proteins presented higher nutritional value and good sensory characteristics, according to the results presented by Schoppet et al. (1976), while Marti et al. (2014) reported an improvement of pasta structure and texture after whey protein addition. Thus, whey powder which is a great source of proteins, especially albumins and globulins, vitamins and minerals (Tsakali et al., 2010) can be considered as ingredient for corn based pasta in order to enhance their nutritional and technological properties.

Gluten-free pasta fiber content can be improved by using fruits and vegetables by-products (Ciccoritti et al., 2019; Bustos et al., 2019). Grape peels are valuable by-products rich in polyphenols, fibres and minerals and were used in foods such as bread (Mironeasa et al., 2019; Deng et al., 2011), pasta (Gaita et al., 2018), pastry products (Bender et al., 2017) in order to increase the antioxidants and dietary fiber contents. Fiber-rich ingredients incorporation in pasta can negatively affect dough behaviour and final product quality (Ciccoritti et al., 2019; Mironeasa et al., 2019; Bustos et al., 2019). These effects should be minimized by finding the optimal amount of grape peels that can be added, by applying the optimization process (Mironeasa & Mironeasa, 2019).

The aim of this study was to investigate the singular effects of grape peels or whey addition in gluten-free pasta formulations based on nixtamalized corn flour. For this purpose, the raw pasta water activity, cooking quality, raw and cooked pasta colour, dough and pasta texture, microstructure, roughness and cooked pasta sensory profile were evaluated.

MATERIALS AND METHODS

Materials

Nixtamalized corn flour from white variety (Hari Masa, Mexico), whey powder (Top Ingrediente, Romania), corn starch (Sano Vita) and grape peels powder (Herbavit, Romania) were acquired from a local market of Romania and used in the experiment.

Pasta manufacturing

Proportions of 30% corn starch and 70% nixtamalized corn flour were mixed and used in pasta main recipe (C0), along with 0.50% salt and 50% water. Corn starch-flour mix was replaced by 5% (C1), 10% (C2), 15% (C3) whey powder (WP) or 1% (C4), 3% (C5), 5% (C6) grape peels powder (GP).

The ingredients were mixed for 5 min in a heavy duty mixer (Kitchen Aid, Whirlpool Corporation, USA), the dough was extruded using a short pasta accessory for the same mixer with a rigatoni mould. Pasta were dried according to the method described by Bergman et al. (1994): 30 min were kept at room temperature in open air, 60 min at 40°C, 120 min at 80°C, 120 min at 40°C in an oven, then were cooled at room temperature for 12 h and packed in polyethylene bags.

Water activity

Samples were ground using a domestic grinder and the water activity (*Aw*) was determined in triplicate with an Aqua Lab device (ICT International Amirdale, NSW 2350, Palestine) at 24.20 ± 1.28°C.

Cooking quality

Optimal cooking time

Optimal cooking time (*OCT*) was determined in triplicate according to the AACC International Approved Method 66-50.01. Five pieces of pasta were boiled in distillated water and the results were recorded when the white particles of uncooked starch disappeared after compressing the sample between two glass containers at intervals of 30 s (Espinosa-Solis et al., 2019).

Water absorption

The water absorption (*WA*) determination was achieved according to the method described by Giménez et al. (2013). Pasta sample (10 g) were boiled in 200 ml of distilled water and after *OCT* the samples were drained for 3 min and weighed, measurements being realized in triplicate.

Cooking loss

The cooking water volume resulted from the *WA* determination was measured and 30 mL were placed in glass containers and dried in an oven at 105°C until dryness (Giménez et al., 2013). After cooling, the residue was weighted. The data were collected in triplicate and the results were reported as cooking loss (*CL*) percentage of dry pasta.

Swelling index

The swelling index (*SI*) was achieved by using the method described by Piwińska et al. (2016) with some modifications. Boiled pasta used for *WA* determination were put in glass containers and dried at 105°C for 16 h, cooled and weighted. Three measurements were done and the results were expressed using the formula: $(m_1-m_2)/m_2$, where m_1 - weight of cooked pasta; m_2 - weight of pasta after drying.

Pasta colour

Colour parameters (L^* describing the lightness from black (0) to white (100), a^* expresses colour from green (−) to red (+), and b^* from blue (−) to yellow (+) nuance) of dried and cooked pasta were achieved by reflectance using a Konica Minolta CR-400 colorimeter (Tokyo, Japan). Five measurements were done for each sample.

Texture

Dough and pasta texture was evaluated by using a TVT-6700 texture analyzer (Perten Instruments, Sweden) with a 10 kg load cell.

Dough texture

Dough pieces of 50 g were tested by double compression up to 50% of the original height with a 35-mm diameter cylindrical probe, at a speed of 5.0 mm/s, a trigger force of 20 g and a recovery period between compressions of 12 s. Firmness, adhesiveness, springiness and cohesiveness were recorded in triplicate.

Dry pasta fracturability

Pasta fracturability was achieved with an aluminium break probe with a three point bend rig, set at a width of 13 mm. A single piece of sample was cut at a speed of 3 mm/s and a trigger force of 50 g. Fracturability represents the maximum force required to break the pasta, the measurements being done in triplicate.

Cooked pasta texture

Firmness, adhesiveness, stringiness and stickiness of cooked pasta were acquired by single cycle compression at 0.5 mmop (compression is specified as distance above the scale plate - mmop) with a transparent noodle probe, according to AACC 16-50 method, at a speed of 0.2 mm/s. Two pieces of sample were put on the heavy duty stand and cut with the noodle probe, three measurements being realized.

Microstructure and roughness

A Mahr CWM100 microscope (Mahr, Gottingen, Germany) from the Integrated Research, Development and Innovation Center for Advanced Materials, Nanotechnologies and Distributed Manufacturing and Control Systems (MANSiD, "Ștefan cel Mare" University of Suceava) was used in order to evaluate pasta surface microstructure and roughness, the images being registered after scanning four different areas. Mountain Map software (Digital Surf, Lavoisier, France) 8 version (trial version) was used for data processing, roughness being calculated as mean of the computed profiles of three areas.

Cooked pasta sensory evaluation

Sensory characteristics in terms of colour, taste, smell, texture, appearance and acceptability of pasta samples were evaluated in two sessions by a panel of 9 semi-trained judges. Pasta samples were boiled at *OCT* and served on white plates with 1 drop of olive oil on top of each piece. A nine-point hedonic scale was used to evaluate each characteristic.

Statistical analysis

The obtained data were processed by using the SPSS 26.0 (trial version) software for Windows (IBM, New York, USA). The differences between means were evaluated by Analysis of variance (ANOVA) and Tukey's test at 5% significance level, statistically significant differences being considered at $p < 0.05$.

RESULTS AND DISCUSSIONS

Pasta cooking quality and water activity

Pasta cooking parameters in terms of optimal time, cooking loss, water absorption and solubility index are important for final product quality. The addition of WP in pasta from nixtamalized corn flour leaded to an increase of *OCT* and decreased *WA* and *CL* compared to the control, except for C3 when *WA* was higher (Table 1). Significantly differences ($p < 0.05$) were observed for *CL*, a decreasing trend being obtained as the addition level was higher. The absence of gluten in corn pasta determines less efficient starch polymer retention in dough matrix which results in higher cooking losses (Marti et al., 2014). The addition of exogenous proteins leaded to a diminishing of cooking loss, while the *WA* was not significantly changed according to the results obtained by Marti et al. (2014). Our similar trends can be due to the protein coagulation during cooking, to their high solubility, hydration and/or emulsifying properties (Pagani et al., 1986; Marco & Rosell, 2008). Pasta containing WP presented higher A_w values compared to the control, while the swelling index presented unequal variation (Table 1).

GP addition led to an increase of *OCT*, *CL* and A_w, while *WA* and *SI* decreased as the amount was higher, statistically significant differences among samples being observed, except for *SI* (Table 1). Similar results for *WA* were reported by Bustos et al. (2019) for berry-enriched pasta, probably due to the dough network weakening by pectin (Padalino et al., 2017). The decrease of *SI* determines the same trend for *WA* as the fiber-rich ingredients compete with starch for water (Aravind et al., 2012). As compared to the control, pasta with GP presented higher cooking quality parameters values, except for *WA* and *SI* of C3 which contains the highest amount of GP. The increase of *WA* after GP addition can be related to the fiber content which has water binding capacity (Kaur et al., 2012). Cooking loss values increase can be due to the fiber interactions with dough network which may allow gelatinized starch leaching during cooking. A similar trend was reported by Aravind et al. (2012) for spaghetti enriched with insoluble dietary fiber.

Table 1. Pasta cooking quality and A_w

Sample	*OCT* (min)	*CL* (%)	*WA* (%)	*SI*	A_w
C0	5.58 ± 0.30^{ax}	10.80 ± 0.05^{bx}	234.10 ± 6.52^{ax}	0.19 ± 0.01^{abx}	0.24 ± 0.00^{ax}
C1	6.15 ± 0.12^{b}	10.72 ± 0.39^{b}	224.23 ± 1.62^{a}	0.18 ± 0.01^{a}	0.33 ± 0.01^{b}
C2	6.30 ± 0.00^{b}	9.88 ± 0.29^{ab}	231.72 ± 0.32^{a}	0.21 ± 0.00^{b}	0.44 ± 0.01^{c}
C3	6.30 ± 0.00^{b}	8.79 ± 0.49^{a}	235.50 ± 2.46^{a}	0.18 ± 0.00^{a}	0.35 ± 0.02^{b}
One way ANOVA *p* value					
	< 0.05	< 0.05	ns	< 0.05	< 0.05
C4	7.00 ± 0.00^{y}	16.76 ± 0.14^{y}	248.49 ± 4.76^{y}	0.27 ± 0.04^{x}	0.33 ± 0.02^{y}
C5	7.03 ± 0.05^{y}	16.88 ± 0.39^{y}	238.79 ± 0.79^{xy}	0.24 ± 0.06^{x}	0.33 ± 0.01^{y}
C6	7.07 ± 0.05^{y}	17.27 ± 0.05^{y}	227.82 ± 0.06^{x}	0.18 ± 0.00^{x}	0.34 ± 0.01^{y}
One way ANOVA *p* value					
	< 0.05	< 0.05	< 0.05	ns	< 0.05

Mean values with different letters in the same column are significantly different ($p < 0.05$): a-b for WP containing samples; x-y for GP containing samples C0 – control, C1 – 5% WP, C2 – 10% WP, C3 – 15% WP, C4 – 1% GP, C5 – 3% GP, C6 – 5% GP, *OCT* – optimal cooking time, *CL* – cooking loss, *WA* – water absorption, *SI* – swelling index, A_w – water activity, ns – not significant.

Colour

Pasta colour is one of the most important characteristics as it directly influences consumers purchase decision. Colour parameters of the ingredients and gluten-free pasta with WP and GP are presented in Table 2.

Table 2. Ingredients, dry and cooked pasta colour parameters

Sample	Dry pasta			Cooked pasta		
	*L**	*a**	*b**	*L**	*a**	*b**
C0	78.97 ± 0.89^{ax}	-4.25 ± 0.15^{ax}	23.20 ± 0.51^{ay}	71.16 ± 0.76^{ax}	-6.18 ± 0.07^{ax}	15.01 ± 0.58^{ax}
C1	76.78 ± 0.49^{a}	-3.96 ± 0.18^{ab}	22.69 ± 0.20^{a}	72.38 ± 0.68^{a}	-5.47 ± 0.11^{ab}	20.06 ± 0.68^{a}
C2	76.57 ± 0.20^{a}	-4.29 ± 0.28^{a}	23.97 ± 0.62a	71.15 ± 0.78^{a}	-4.56 ± 0.28^{b}	22.30 ± 0.95^{a}
C3	76.41 ± 0.15^{a}	-3.69 ± 0.25^{b}	26.73 ± 0.75^{a}	70.74 ± 0.60^{a}	-4.87 ± 0.14^{ab}	21.38 ± 0.57^{a}
One way ANOVA *p* value						
	ns	< 0.05	Ns	ns	< 0.05	Ns
C4	72.14 ± 0.40^{x}	-2.65 ± 0.13^{y}	18.13 ± 0.66^{xy}	65.78 ± 0.65^{x}	-3.43 ± 0.22^{y}	14.27 ± 0.17^{x}
C5	65.11 ± 0.86^{y}	-0.33 ± 0.11^{z}	14.46 ± 0.44^{x}	58.19 ± 0.86^{y}	-1.00 ± 0.12^{z}	9.63 ± 0.30^{y}
C6	57.47 ± 0.31^{z}	1.39 ± 0.13^{w}	12.85 ± 0.37^{x}	50.92 ± 0.94^{z}	0.77 ± 0.14^{w}	6.90 ± 0.07^{z}
One way ANOVA *p* value						
	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
WP	90.65 ± 0.11	-7.32 ± 0.08	28.04 ± 0.09	-	-	-
CSF	89.52 ± 0.06	-4.97 ± 0.01	16.48 ± 0.08	-	-	-
GP	42.61 ± 0.06	4.99 ± 0.08	6.96 ± 0.10	-	-	-

Mean values with different letters in the same column are significantly different ($p < 0.05$): a-b for WP containing samples; x-w for GP containing samples. C0 – control, C1 – 5% WP, C2 – 10% WP, C3 – 15% WP, C4 – 1% GP, C5 – 3% GP, C6 – 5% GP, L* - luminosity, a* - red-green intensity, b* - yellow-blue intensity, WP – whey protein, CSF – corn starch-flour mix, GP – grape peels, ns – not significant.

WP incorporation in nixtamalized corn pasta did not significantly affects dry and cooked pasta colour parameters ($p < 0.05$), except a^* which increased, led to a less green nuance intensity described by a^* parameter (Table 2). Phongthai et al. (2017) obtained lightness values of pasta enriched with whey protein higher than 70, which is in accordance with our findings. Control and WP containing samples presented yellow nuances, as indicated by the positive values of b^* parameter. WP addition in corn gluten-free pasta may be responsible for browning due to the Maillard reactions that can occur during drying (Manoj Kumar et al., 2019). After cooking, all colour parameters of pasta decreased, possibly due to the non enzymatic browning reactions (Marti et al., 2014).

The addition of GP led to a decrease of luminosity (L^*) and yellowness (b^*) compared to the control and with the GP increasing quantity, while the redness (a^*) increased for both dry and cooked pasta, significant differences ($p < 0.05$) being obtained among samples (Table 2). These trends could be due to the anthocyannins and tannin presence in grape peels (Fournand et al., 2006). Our results are in accordance with those reported for other fiber-rich ingredients supplementation in pasta which determined darker colors (Jayasena & Nasar-Abbas, 2012; Aravind et al., 2012; Bustos et al., 2019). Lower colour parameters values were obtained after pasta cooking, although the nuances of GP were kept after the thermal treatment, similar results being obtained by Bustos et al. (2019) for berry enriched pasta.

Texture

Dough texture

Pasta dough texture parameters are presented in Table 3. The addition of WP led to a decrease of dough firmness with the level increase, significant differences being observed ($p < 0.05$). Similar results were obtained by Asghar et al. (2009) for frozen dough with whey protein concentrates. Adhesiveness significantly ($p < 0.05$) increased as the added amount was higher. No significant changes among samples ($p > 0.05$) were obtained for springiness and cohesiveness, the most elastic dough sample being C2, while cohesiveness slightly increased with the added amount of by-products ingredients (Table 3). Compared to the control,

higher firmness and adhesiveness of C1 and lower springiness were obtained, while C2 and C3 were less firm and more adhesive, elastic and cohesive than C0. Asghar et al. (2009) also reported an increase of cohesiveness values of dough when whey protein isolates were incorporated. The textural parameters variation after WP addition can be related to elastic properties of WP when it is mixed with starch and/or to the disulphide bonds that are formed (van Riemsdijk, van der Goot & Hamer, 2011).

Table 3. Pasta dough textural parameters

Sample	Firmness (g)	Adhesiveness (g · s)	Springiness (%)	Cohesiveness (adim.)
C0	2074.00 ± 45.92^{bx}	-19.28 ± 12.66^{cy}	99.76 ± 0.00^{ax}	0.17 ± 0.00^{ax}
C1	2516.00 ± 90.83^{c}	-86.4 ± 14.76^{bc}	99.66 ± 0.00^{a}	0.15 ± 0.07^{a}
C2	1938.67 ± 95.86^{b}	-185.95 ± 93.18^{b}	99.81 ± 0.00^{a}	0.17 ± 0.03^{a}
C3	1627.67 ± 17.21^{a}	-393.05 ± 67.27^{a}	99.77 ± 0.00^{a}	0.21 ± 0.01^{a}
One way ANOVA *p* value				
	< 0.05	< 0.05	ns	Ns
C4	2240.67 ± 26.85^{y}	-77.41 ± 14.80^{xy}	99.79 ± 0.00^{x}	0.15 ± 0.06^{x}
C5	2424.67 ± 15.27^{z}	-65.82 ± 27.71^{y}	99.68 ± 0.00^{x}	0.19 ± 0.00^{x}
C6	2427.00 ± 45.07^{z}	-155.46 ± 56.89^{x}	99.59 ± 0.00^{x}	0.19 ± 0.00^{x}
One way ANOVA *p* value				
	< 0.05	< 0.05	ns	Ns

Mean values with different letters in the same column are significantly different ($p < 0.05$): a-c for WP containing samples; x-z for GP containing samples C0 – control, C1 – 5% WP, C2 – 10% WP, C3 – 15% WP, C4 – 1% GP, C5 – 3% GP, C6 – 5% GP, ns – not significant.

GP incorporation in dough leaded to significantly higher firmness and adhesiveness compared to the control, an increasing trend being observed with the level increase ($p < 0.05$). GP firmer dough can be related to the limitation of water availability due to the considerable fiber content (Eskicioglu, Kamiloglu & Nilufer, 2015; Aprodu, Șerban & Banu, 2019). Higher firmness of fiber enhanced gluten-free bread dough was also reported by Sciarini et al. (2017). Springiness and cohesiveness slightly decreased after GP addition, but no significantly differences among samples were seen ($p > 0.05$).

Pasta texture

Pasta fracturability is important to be evaluated as it can predict final product behaviour during transportation and/or manipulation. Dry pasta fracturability variation with WP or GP addition level is presented in Figure 1. The increase of WP amount led to a decrease of dry pasta fracturability, the values being higher compared to the control. Gupta (2019) also revealed an increase of fracturability when pea protein flour was incorporated in gluten-free amaranth pasta, compared to the control. Similar decreasing trend was obtained for GP enriched gluten-free pasta, C6 presenting the closest value to that of the control (Figure 1). A decrease of wheat pasta fracturability with the spirulina biomass substitution level increase was also reported by Rodríguez De Marco et al. (2014) and by Jayasena and Nasar-Abbas (2014) for pasta with lupin flour. Pasta breaking strength is related to the mould type and the extrusion conditions, lower breaking stress being related to weaker internal structure which will absorb more water (Marti et al., 2011), fact supported by the WA obtained data (Table 1).

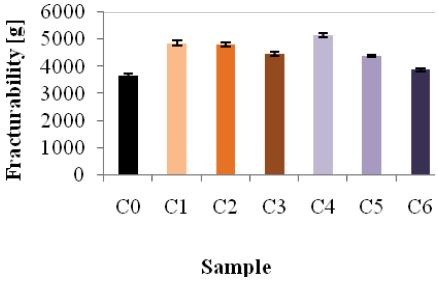

Figure 1. Gluten-free pasta fracturability: C0 - control, C1 - 5% WP, C2 - 10% WP, C3 - 15% WP, C4 - 1% GP, C5 - 3% GP, C6 - 5% GP

Textural parameters of cooked pasta play an important role in consumer's acceptability. WP addition in corn gluten-free pasta determined a decrease of cooked pasta firmness, stickiness and adhesiveness and an increase of stringiness compared to the control (Table 4). Pasta containing WP firmness did not differ significantly ($p > 0.05$) at different levels, while adhesiveness, stringiness and stickiness significantly increased ($p < 0.05$) as the addition level was higher. Stickiness values are preferred to be as lower as possible. Similar results of firmness trends were reported by Jayasena and Nasar-Abbas (2014) for pasta containing lupin flour. Gluten-free pasta dough matrix strength is given by inter- and intra molecular bonds which may disintegrate during cooking and can release extrudes during starch gelatinization which lead

to pasta stickiness increase (Jayasena and Nasar-Abbas, 2014).

Table 4. Cooked pasta textural parameters

Sample	Firmness (g)	Adhesiveness (g · s)	Stringiness (mm)	Stickiness (adim.)
C0	351.33 ± 69.26^{by}	-0.36 ± 0.05^{ax}	0.08 ± 0.06^{ax}	-0.42 ± 0.06^{cy}
C1	240.33 ± 53.08^{a}	-0.16 ± 0.02^{b}	0.43 ± 0.08^{b}	-0.64 ± 0.08^{b}
C2	245.00 ± 3.61^{a}	-0.17 ± 0.11^{b}	0.70 ± 0.10^{c}	-0.73 ± 0.02^{b}
C3	242.00 ± 21.07^{a}	-0.28 ± 0.01^{ab}	0.75 ± 0.02^{c}	-0.85 ± 0.07^{a}
One way ANOVA p value				
	< 0.05	< 0.05	< 0.05	< 0.05
C4	243.00 ± 9.85^{x}	-0.27 ± 0.12^{x}	0.33 ± 0.05^{y}	-0.23 ± 0.01^{z}
C5	249.67 ± 11.93^{xy}	-0.03 ± 0.00^{y}	0.09 ± 0.06^{x}	-0.83 ± 0.08^{x}
C6	289.67 ± 97.21^{xy}	-0.04 ± 0.05^{y}	0.42 ± 0.04^{z}	-0.46 ± 0.09^{y}
One way ANOVA p value				
	< 0.05	< 0.05	< 0.05	< 0.05

Mean values with different letters in the same column are significantly different ($p < 0.05$): a-c for WP containing samples; x-z for GP containing samples C0 – control, C1 – 5% WP, C2 – 10% WP, C3 – 15% WP, C4 – 1% GP, C5 – 3% GP, C6 – 5% GP, ns – not significant.

GP incorporation led to a decrease of firmness values compared to the control (Table 4), which is in agreement with the findings of Bustos et al. (2019) for wheat pasta enriched with berries. The incorporation of GP which is a fiber rich ingredient may affect pasta dough structure resulting in a higher components losing during cooking due to the weakening effect which may lead to textural parameters decrease (Mercier et al., 2016; Bustos et al., 2019). Adhesiveness, stringiness and stickiness values of GP containing pasta increased compared to the control. Significantly differences were obtained among samples for all the textural parameters analyzed ($p < 0.05$), an increasing trend with the addition level increase being observed for firmness and adhesiveness, while stickiness decreases (Table 4). Similar trends for firmness and adhesiveness were reported by Padalino et al. (2017) for pasta enriched with tomato peels. GP containing samples stickiness decrease with the addition level increase is in agreement with the results obtained by Ciccoritti et al. (2019) for bran containing pasta.

Microstructure and roughness

Nixtamalized corn gluten-free with WP or GP pasta microstructure is presented in Figure 2.

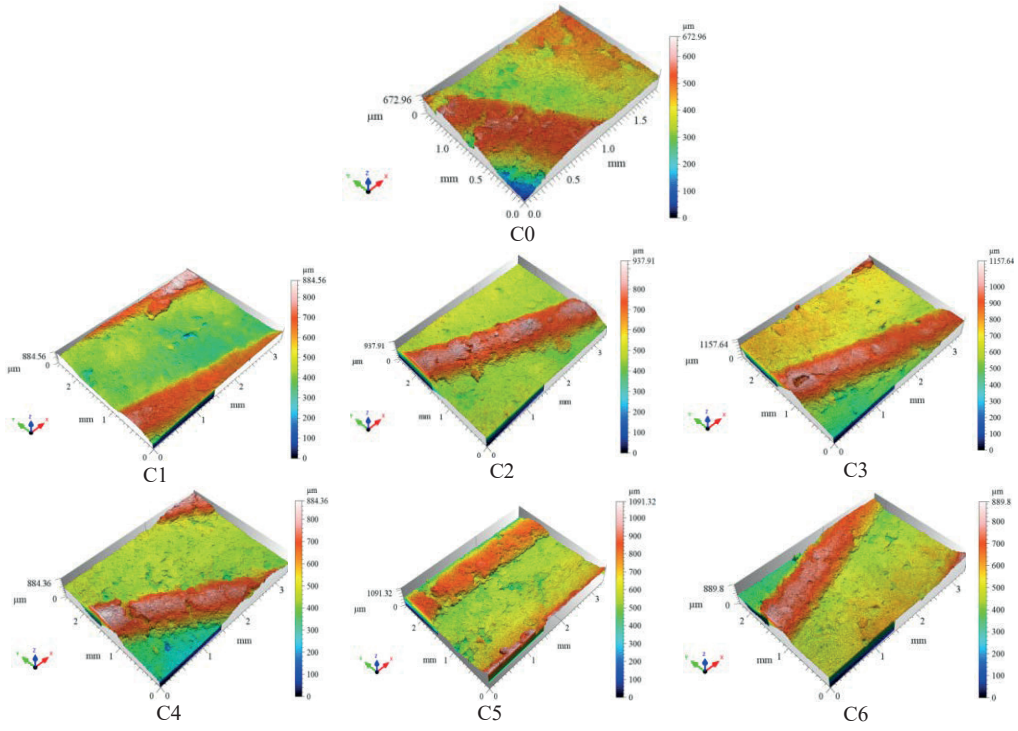

Figure 2. Gluten-free dry pasta microstructure: C0 - control, C1 - 5% WP, C2 - 10% WP, C3 - 15% WP, C4 - 1% GP, C5 - 3% GP, C6 - 5% GP

As it can be observed from Figure 2, the addition of WP led to a smoother pasta surface compared to the control. On the other hand, GP incorporation led to an increased number of holes and cracks on the surface (Figure 2) which means rougher pasta (Figure 3), this phenomena being more present in sample with highest GP amount (C6).

The presence of these cracks would allow starch, soluble dietary fiber and other solids to leach out from the matrix during boiling, leading to higher *CL* (Table 1) (Phongthai et al., 2017).

A decreasing trend of pasta roughness was obtained for all the samples containing WP (Figure 3). All WP containing samples presented lower roughness values compared to the control.

These results are in agreement with those reported by Marti et al. (2014) for gluten-free pasta enriched with whey proteins. Smoother surface can be due to the emulsifying properties of WP and to the compact structure formed with starch granules (Phongthai et al., 2017).

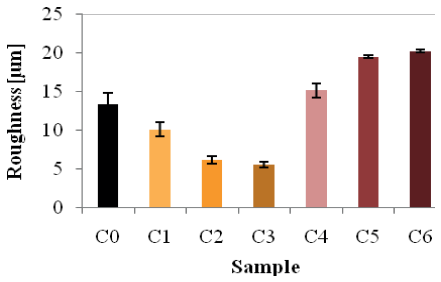

Figure 3. Gluten-free pasta roughness: C0 - control, C1 - 5% WP, C2 - 10% WP, C3 - 15% WP, C4 - 1% GP, C5 - 3% GP, C6 - 5% GP

Corn pasta with GP incorporated exhibited higher roughness values (Figure 3) which increased with the addition of by-products level increase. Surface roughness is directly influenced by the particle size as Sandberg (2015) showed that coarse bran particle size determined higher pasta roughness.

Moisture distribution within gluten-free dough matrix plays an essential role for the surface roughness as it can determine micro-cracks and uncontrolled shrinkages in the structure (D'Amico et al., 2015).

Nixtamalized corn pasta samples with WP or GP incorporation are presented in Figure 4.

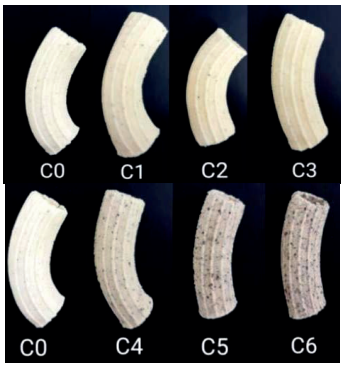

Figure 4. Gluten-free corn pasta: C0 - control, C1 - 5% WP, C2 - 10% WP, C3 - 15% WP, C4 - 1% GP, C5 - 3% GP, C6 - 5% GP

Sensory characteristics

The sensory profile of gluten-free pasta enriched with WP or GP was significantly changed compared to the control. The WP containing samples obtained higher scores for appearance, texture, taste and overall acceptability than the control samples (Figure 5). The highest scores for all the sensory characteristics studied were registered for C3, the pasta with the highest WP amount (15%). Colour and smell characteristics were appreciated with similar scores for all the samples. The yellow nuance of the pasta containing WP was preferred by consumers, as C3 sample obtained the highest scores, which is in agreement with the results presented by Jayasena et al. (2010) in the case of vegetable flours addition in pasta. Positive effects of whey powder addition on the colour, texture, flavour, taste and overall acceptability of wet noodles was also reported by Lee and Kim (2000).

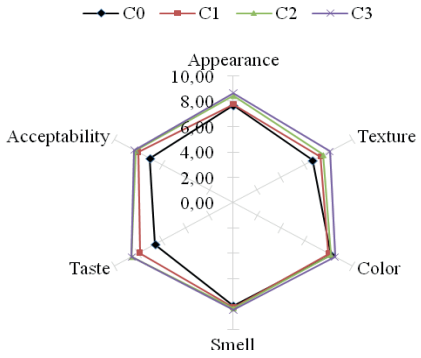

Figure 5. Sensory scores for WP pasta: C0 - control, C1 - 5% WP, C2 - 10% WP, C3 - 15% WP

GP is a fiber-rich ingredient that can negatively affect the sensory characteristics of gluten-free pasta. Sensory scores obtained for corn pasta with GP depends on the addition level used (Figure 6). The sample C4 containing the smallest amount of GP (1%) was evaluated with the highest scores for taste and overall acceptability, while in terms of colour and smell the control sample (C0) obtained the best scores.

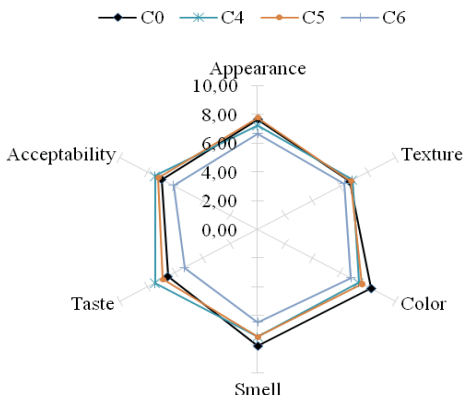

Figure 6. Sensory scores for GP pasta: C0 - control, C4 - 1% GP, C5 - 3% GP, C6 - 5% GP

The addition of 5% GP (C6) in gluten-free pasta recorder the lowest scores for all the sensory characteristics compared to the control and to the other samples (Figure 6), concluding that higher amounts than 3% of GP in corn pasta are not acceptable from sensory point of view. For high by-product addition levels the panellists noticed a gritty texture probably due to the overgrown GP particle size. The smell and the taste of the final product can be negatively influenced by the presence of polyphenols from GP (Deng et al., 2011; Gaita et al., 2018). According to the results obtained by Gaita et al. (2018), GP incorporation at levels up to 3% in wheat pasta leaded to an improvement of the sensory scores. On the other hand, cereal bran which is also a fiber rich ingredient, determined a decrease of the pasta or tortilla acceptability compared to the control (Kaur et al., 2012; Gajula et al., 2008).

CONCLUSIONS

Whey powder and grape peels are important sources of proteins and antioxidant dietary fiber respectively which make them being valuable by-products that can be used in food formulations. Whey powder addition in gluten-free pasta based on nixtamalized corn flour leaded to lower dough firmness and higher adhesiveness, roughness and fracturability of dry pasta decrease with the by-product addition level increase. In addition, the cooking quality of pasta was improved, the yellowish colour was more pronounced, cooked pasta firmness decreased while stringiness increased and better sensory characteristics were observed. Grape peels incorporation determined firmer and more adhesive dough, dry pasta fracturability decrease and roughness increase as the added amount of by-product was higher. Also a decrease of pasta cooking quality, significantly colour changes, firmer cooked pasta, and lower sensory characteristics, especially at levels higher than 3% were obtained. Thus, in order to improve the nutritional and technological characteristics of gluten-free pasta, amounts up to 10% whey powder and 1 to 3% grape peels are recommended. Our results can be useful for novel gluten-free pasta products developments in order to better satisfy consumers demand.

ACKNOWLEDGEMENTS

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ANTIOXIDANTS IN FRESH AND COOKED BROCCOLI (*Brassica oleracea* var. Avenger) AND CAULIFLOWER (*Brassica oleracea* var. Alphina F1)

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Abstract

Vegetables represent one of the most important components of the human diet. Brassica vegetables have been shown a great antioxidant capacity due to the presence of a multitude of biochemical substances such as vitamins, compounds with phenolic structure and pigments. Also it is known that some of these nutrients have a positive impact on the human health, reducing the risk of cardiovascular disease, diabetes and some types of cancer. This study evaluates the influence of two processing methods (boiling and steaming) on the stability of phenolic compounds, ascorbic acid, carotenoids and chlorophyll and on the antioxidant activity in edible part of broccoli and cauliflower. Boiling treatments caused losses of total phenolic compounds for both analysed vegetables (16.3% and 25.2%), while steaming led to comparable values with the fresh sample for broccoli and an increase by 12% for cauliflower. Thermal treatments determined a substantial loss of ascorbic acid for the analysed vegetables, ranging between 28% and 43.9%. Total carotenoid and chlorophyll content was higher in cooked samples and the antioxidant activity was lower after boiling and comparable with fresh vegetables when steaming.

Key words*: antioxidant capacity, boiling, broccoli, cauliflower, steaming*

INTRODUCTION

The breathing process and various other reactions taking place in the human body result in formation of free radicals. In recent years, specialized research has focused on these reactive forms of oxygen, as they cause cell damage, leading to cancer, inflammation or adverse changes in blood vessels. In this respect, there has been increased interest in food substances that are capable of inhibiting or diminishing the harmful effects of free radicals in the human body (Sikora et al., 2008).
Several organizations such as European Food Safety Authority (EFSA), Food and Agriculture Organization (FAO), United States Department of Agriculture (USDA), World Health Organization (WHO) recommend high consumption of fruits and vegetables (Allende et al., 2006) because they offer protection against diseases, such as cardiovascular disease, cancer and cataract (Del Caro et al., 2004).
The contribution of the *Brassica* vegetables (e.g. broccoli, cauliflower, cabbage, kale) to health may be related to the antioxidant capacity of these vegetables due to the presence of phenolic compounds, carotenoids, vitamins and minerals. It is known that vegetables with antioxidant properties are rich in phenolics, especially phenolic acids and flavonoids. Research has shown that carotenoids possess several biological functions, which are associated with reduced risk of degenerative diseases, cataract prevention, reduced incidence of macular degeneration caused by aging, and reduced incidence of coronary heart disease (Krinsky, 1994; Podsedek, 2007; Gulçin, 2012).
Broccoli (*Brassica oleracea* var. italica) is part of the Cruciferous family (Podsędek, 2007). Broccoli contains a lot of nutritional substances

as glucosinolates, flavonoids, cinnamic acid derivatives, carotenoids, ascorbic acid and minerals. Broccoli contributes to health benefits due to its anticarcinogenic, antimutagenic and antioxidant properties. (Latté et al., 2011).

Cauliflower (*Brassica oleracea* L. var. botrytis) is also a member of the Cruciferous family. The main substances of cauliflower include glucosinolates, ascorbic acid, carotenoids, phenolic compounds and vitamin E. Also, it has anticarcinogenic and antioxidant effects, like other cruciferous vegetables (Llorach et al., 2003; Costache et al., 2012).

Before eating, most of vegetables need to be cooked to enhance their palatability and taste. However, cooking brings several physical and chemical changes in the composition of vegetables (Rehman et al., 2003; Balan, 2016). These changes could be both beneficial and detrimental depending on the extent and the type of the treatment conditions. Variety of effects like destruction, release and structural transformation of the phytochemicals take place during the cooking process. Cooking treatments like boiling, microwaving (Zhang & Hamauzu, 2004), baking, frying and griddling lead to changes in texture and nutritional properties of the vegetables.

This article attempts to investigate the influence of two cooking methods (boiling and steaming) on the stability of phenolic compounds, vitamins, pigments and on the antioxidant activity. The optimum cooking method was determined.

MATERIALS AND METHODS

Materials

The freshly harvested vegetables: broccoli (*Brassica oleracea* var. Avenger) and cauliflower (*Brassica oleracea* var. Alphina F1) were obtained from Development and Research Station for Vegetables Growing from Buzau. Chemicals like Trolox, DPPH and 2,6 diclorphenol-indophenol were purchased from Sigma-Aldrich Chemical CO. Meta-phosphoric acid, ethylenediaminetetraacetic acid, sodium hydrogen carbonate and sodium carbonate were purchased from Roth. Folin Ciocalteau reagent, ascorbic acid and analytical grade organic solvents (methanol and acetone) were purchased from Merck.

Methods

Sample preparation

The heads of broccoli and cauliflower were selected considering the absence of any damages and infections. The inedible parts were removed, then the heads were washed in tap water and then cut into pieces. After homogenization, three samples were obtained from each vegetable: one was retained raw as control and two were prepared for thermal processing (one for boiling and one for steaming).

Processing methods

The two types of cooking the vegetables were selected according to the incidence in Romanian cuisine. Both treatments, boiling and steaming, were conducted to obtain similar tenderness and taste (well done but firm when bitten). All the cooking variants were conducted in triplicates for each vegetable.

Boiling: Samples of broccoli and cauliflower were immersed in boiling water (1:4 vegetables/water), in a stainless-steel vessel, at 100°C temperature and boiled for 5 minutes. After boiling, the samples were drained off and quickly cooled.

Steaming: Samples of fresh broccoli and cauliflower were placed in a steamer vessel (Food Steamer Tefal) with 250 ml water, for 20 minutes (broccoli) and 10 minutes (cauliflower), at atmospheric pressure. After steaming, the samples were quickly cooled.

Analytical methods

For evaluating the influence of different processing methods (boiling and steaming) on the antioxidant properties of broccoli and cauliflower, quantification of total phenolics, ascorbic acid, carotenoids, chlorophylls and antioxidant activity were performed as follows.

Determination of total phenolic content

The total phenolic content of fresh and cooked broccoli and cauliflower was determined using Folin-Ciocalteau reagent (Singleton & Rossi, 1965). Briefly, 1 ml of 50% (v/v) methanolic extract was treated with 5 ml Folin-Ciocalteau reagent and 4 ml sodium carbonate (7.5% w/v). After 20 minutes keeping in the dark, the blue-coloured compound was measured spectrophotometrically at 752 nm with a Specord 210 UV-VIS spectrophotometer (Analytic Jena, Germany). Phenolics content was calculated using a calibration curve obtained with gallic

acid. The results were expressed as mg GAE (gallic acid equivalents)/ 100 g dry weight (DW).

Determination of ascorbic acid

For quantification of ascorbic acid, the dye-titration method was used, according to AOAC procedure, 2000. Metaphosphoric acid extracts of uncooked and processed vegetables were subjected to titration with 2,6-dichlorophenolindophenol. In this oxidation-reduction reaction, the ascorbic acid from the extract was oxidized to dehydroascorbic acid and the indophenol dye was reduced to a colourless compound. The end point of the titration was detected when excess of the unreduced dye gave a rose pink colour in acid solution. Dehydroascorbic acid was not analysed in this study. The results were expressed in mg ascorbic acid/100 g dry weight.

Determination of pigments

Carotenoids and chlorophyll were extracted using a solvent mixture of acetone/water (80:20, v/v/). For a more efficient extraction, the vegetable sample mixed with the solvent was vortexed for 15 min, at 2000 rpm, at 20°C and then separated by centrifugation for 15 min, at 3500 rpm, at 20°C. The clear supernatant was further used for absorbance determination at 470, 646, 663 nm with Specord 210 UV-VIS spectrophotometer (Analytic Jena, Germany), as described by Lichtenthaler (1987). Using the specified equations, the results were expressed in mg/ 100 g dry weight.

Determination of antioxidant activity

The antioxidant activity was determined based on the reduction of 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical. A modified protocol was used (Culetu et al., 2016) consisting in extraction of the samples in methanol:water (1:1, v/v). After centrifugation (15 min, 3500 rpm, 20°C), 1 ml of supernatant was treated with 6 ml DPPH solution.

The reaction was carried out for 30 minutes in the dark and then the decline in absorbance was measured spectrophotometrically at 517 nm with Specord 210 UV-VIS spectrophotometer (Analytic Jena, Germany). The results were expressed in μmol Trolox/100 g dry weight.

RESULTS AND DISCUSSIONS

The processing methods used in this study had different effects on the values of antioxidant compounds (phenolics, ascorbic acid, chlorophyll and carotenoids) and on the level of antioxidant activity of the selected vegetables, as shown in Tables 1 and 2. The results obtained for all treatments were compared with those registered for fresh vegetables.

1. Total phenolic content

Phenolics represent a large group of metabolites which are present, in smaller or larger quantities, in plants. The amount of total phenolics in raw broccoli was 356.06 mg GAE/100 g DW. Researchers reported in broccoli different levels of total phenolic content ranging between 99.8 and 1204 mg GAE/100 g DW (Podsedek, 2007; Miglio et al., 2008; Ramos dos Reis et al., 2015). The big difference in the reported data may be due to the variation of the phenolic compounds according to the cultivars, agronomic conditions, harvesting stage, extraction solvent and the details of the analysis method. Thermal treatment of broccoli resulted in a loss of phenolic compounds when boiling (Table 1). The obtained value (297.97 mg GAE/100 g DW) is 16% lower than the one for fresh broccoli. The results are consistent with other works that reported the reducing of total phenolic content in broccoli up to 72%, according to the cooking time period (5-20 minutes) and also to the vegetable/water ratio (Zhang & Hamauzu, 2004; Porter, 2012; Danowska-Oziewicz et al., 2020). Steaming process didn't affect the level of total phenolics, the obtained value being comparable to the level recorded for the fresh sample (Table 1). The data reported in different studies showed a decrease of the total phenolics by up to 38% (Miglio et al., 2008) or even an increase by 18% comparing with untreated sample (Turkmen et al., 2004). When boiling, the temperature may enhance the extraction, but in the same time, the loss of biologically active compounds occurs, based on dissolution of polyphenols in water, depending on the type of vegetables and on the cooking time period.

Analysis of the fresh cauliflower revealed a total phenolics level of 381.49 mg GAE/100g DW. The reported data show different levels of phenolics, with values ranging between 27.8 and 782 mg GAE/100g DW, according to the used extraction solvent and the procedure (Podsedek, 2007; Hwang, 2019). Boiling the cauliflower sample for 5 minutes led to a 25.2% decrease in total phenolic content (Table 2). Steaming was

found to have a good effect on cauliflower, the obtained level for phenolics being 12% higher than the one of the uncooked sample.
The results are consistent with other studies that indicate an increase of the phenolic content for steamed vegetables, but there are others that reported lower levels (Girgin & Nehir, 2015; Hwang, 2019).
In their study, Vallejo et al. (2002) showed that a possible explanation for higher values of the steamed samples is the inactivation of oxidative enzymes due to the temperature and the lack of water. Another assumption is that the level of flavonoids is increased by the heating treatment due to their enhanced extractability (Stewart et al., 2000).

Table 1. Effect of cooking on antioxidant compounds and antioxidant activity of broccoli

	Fresh	Boiled	Steamed
Total phenolic content (mg GAE/100 g)	356.06±9.71	297.97±19.86	357.18±23.44
Ascorbic acid (mg/100 g)	600.32±14.34	368.94±18.25	391.45±20.11
Total chlorophyll (mg/100 g)	28.45±3.54	32.70±5.72	27.67±4.15
Chlorophyll a (mg/100 g)	22.71±3.08	27.88±5.14	22.5±3.66
Chlorophyll b (mg/100 g)	5.74±0.46	4.82±0.58	5.17±0.49
Total carotenoids (mg/100 g)	7.06±0.52	8.34±0.77	7.75±0.47
DPPH (µmol Trolox/100 g)	3420.18±94.15	2943.32±110.33	3251.74±106.28

*All values were reported at dry weight

**Values are presented as means ± SD of triplicate experiments

Table 2. Effect of cooking on antioxidant compounds and antioxidant activity of cauliflower

	Fresh	Boiled	Steamed
Total phenolic content (mg GAE/100 g)	381.49±12.50	284.94±20.12	426.43±16.59
Ascorbic acid (mg/100 g)	870.55±22.73	488.15±34.18	627.12±38.64
Total chlorophyll (mg/100 g)	nd	Nd	Nd
Chlorophyll a (mg/100 g)	nd	Nd	Nd
Chlorophyll b (mg/100 g)	nd	Nd	Nd
Total carotenoids (mg/100 g)	0.92±0.08	0.96±0.11	0.97±0.08
DPPH (µmol Trolox/100 g)	3455.04±71.43	2859.32±123.17	3401.61±99.86

*All values were reported at dry weight

**Values are presented as means ± SD of triplicate experiments

Among the two cooking methods performed in this study, boiling caused a decrease of the total phenolic content, while steaming had an increase effect.

2. Ascorbic acid

Vitamin C, in addition to being a nutrient, represents one of the most important antioxidant because it protects the body from free radicals. Because this vitamin is very sensitive at increased temperature, it is often used in research studies for evaluating the influence of different processing methods on vitamin content. The results of this study showed the effect of two cooking techniques on the level of ascorbic acid in broccoli and cauliflower. Fresh broccoli and fresh cauliflower showed the highest levels of vitamin C: 600.32 mg/100 g DW, respectively 870.55 mg/100 g DW. The performed cooking treatments (boiling and steaming) led to a significant decrease in the content of ascorbic acid (Tables 1 and 2). The highest loss was observed for both vegetables when boiling, the obtained results being by 38.55% and 44.1% lower than those recorded for the uncooked samples. This is probably mainly due to thermal degradation of ascorbic acid, but considering also water leaching (Lee & Kader, 2000). The retention of vitamin C during steaming processing was 65.2% for broccoli and 72.1% for cauliflower. These results are comparable with those reported by other researchers (Vallejo et al., 2002; Miglio et al., 2008). Although some studies reported a higher retention of ascorbic acid after the steaming

process of broccoli (up to 74%), probably based on the reduced contact with water during thermal treatment, in the present study the extended cooking time (15 minutes) led to slightly lower results. Therefore optimal retention of vitamin C may be obtained with a thermal treatment using lower quantities of water when applied for shorter time periods.

3. Chlorophyll

An important parameter for assessing the quality of the cooked vegetables, like broccoli, asparagus and other leafy vegetables, is the degree of greenness. The green colour of the vegetables is given by chlorophyll pigments. These phytochemicals (chlorophyll a and chlorophyll b) are affected by heat treatments, during which they are transformed into other compounds, like olive-coloured pheophytins. In the present study the amount of chlorophyll a and b determined in fresh broccoli are comparable with those reported in other studies (Table 1). The performed cooking methods influenced the chlorophyll content in different manner. Chlorophyll a showed a 28.8% increased value for boiled broccoli. Danowska-Oziewicz et al. (2020) explained the higher value of chlorophyll a in boiled samples by a stronger interaction of the water with the plant tissue due to a better access. Regarding steaming processing, the contents of chlorophyll a and b were not significantly influenced (Table 1). These results are consistent with other published data (Ramos dos Reis, 2015; Danowska-Oziewicz et al., 2020). Mitic et al. (2013) reported a decrease of the chlorophyll a and b content in boiled broccoli.

4. Carotenoids

Carotenoids are important secondary plant metabolites that poses biological properties. Carotenes and their oxygenated derivatives act as antioxidants, scavenging and inactivating free radicals. Due to their chemical structure, these lipophilic compounds are sensitive to extreme temperature and light exposure, but also to the enzymes, metals and other oxidants action. Because during vegetables cooking, these factors can occur, it is important to assess the recovery of carotenoids after processing.

The results presented in Tables 1 and 2 show higher amount of total carotenoids in fresh broccoli compared with fresh cauliflower (7.06 mg/100 g DW and 0.92 mg/100 g DW, respectively). Boiling of broccoli sample resulted in 18.1% increase of total carotenoids, while steaming led to an only 9.8% increase comparing with the non-processed vegetable. These results are similar to those obtained by Ramos dos Reis et al. (2015) and Miglio et al. (2008), the reported retention of carotenoids ranging between 103-131% for boiling and between 119-131% for steaming. In their study Zhang & Hamauzu (2004) found that boiling broccoli led to the decrease of total carotenoids by 13% of the value registered for fresh sample, while steaming didn't affect significantly the carotenoids retention.

When cooking cauliflower by boiling and by steaming, the amount of carotenoids was slightly increased (by 4.3% and 5.4%, respectively) comparing with the level of fresh sample.

The obtained results confirm that carotenoids are not altered to a great extent by thermal treatments with short time periods and more, their content is increased probably due to greater chemical extractability and enzymatic degradation (Podsedek, 2005). Miglio et al. (2008), accounted for the improved extractability in boiled and steamed broccoli the disruption of carotenoid-protein complexes. When analysing the main carotenoids from fresh, boiled and steamed samples, Ramos dos Reis et al. (2015) found out that lutein and cryptoxanthin content was significantly increased in both broccoli and cauliflower, while β-carotene only in broccoli. The levels of zeaxanthin and α-carotene were lower for both cooked vegetables.

5. Antioxidant activity

Antioxidant activity is considered as an indicator of the overall benefits of the antioxidant compounds of a matrix. Fresh and cooked broccoli and cauliflower were tested for their hydrophilic antioxidant activity measured by DPPH radical scavenging method and the results are presented in Tables 1 and 2. The values of the antioxidant activity recorded for fresh studied vegetables didn't differ too much (3420.18 μmol Trolox/100 g DW for broccoli and 3455.04 μmol Trolox/100 g DW for cauliflower), being similar to those reported by Li et al. (2018).

The two performed cooking methods resulted in a slightly decrease of the antioxidant activity.

Boiling broccoli led to lowering it by 14%, while steaming only by 5.1% (Table 1). When cooking cauliflower, the loss of antioxidant activity was 17.2% for boiling and 1.5% for steaming (Table 2). Antioxidant activity was significantly correlated with total phenolic content (r^2 = 0.788) and moderate correlated with ascorbic acid content (r^2 = 0.474). Other research studies reported the same behaviour when boiling or steaming, but the decreasing of the antioxidant activity varies within wide limits, depending on the oxidation of the antioxidant compounds and their leakage in water (Zhang & Hamazu, 2004; Porter, 2012). In contrast with these findings, Ramos dos Reis et al. (2015) showed an improvement of antioxidant activity after such cooking methods due to thermal destruction of cellular walls and inactivation of prooxidant enzymes. The contradictory reported data are influenced by process parameters (extent of the cooking time period, temperature, and light exposure), variety and quality parameters of raw vegetables and the analytical methods.

CONCLUSIONS

The results of this study showed modification of the content of bioactive compounds and antioxidant capacity of broccoli and cauliflower subjected to boiling and steaming. After boiling the vegetables retained lower phenolics and ascorbic acid, while total chlorophyll and carotenoid content increased. Steam treatment resulted in better retention of antioxidant compounds (except ascorbic acid), with higher results for cauliflower. Antioxidant potential was lower after boiling and comparable with fresh broccoli and cauliflower when steaming. Accordingly, it may be stated that steaming is a better processing method because it minimises the loss of antioxidant properties of the tested vegetables, mainly due to the absence of water, but maintaining the advantage of temperature on improving extractability and oxidative enzymes inactivation.

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Candida (Pichia) guilliermondii CMGB 44 WITH VERSATILE ANTIMICROBIAL ACTIVITY

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Abstract

The yeast Candida (teleomorph Pichia) guilliermondii is a biocontrol agent intensively studied as an ecological alternative for fungicides in preventing decay of plants. The strain C. (P.) guilliermondii CMGB 44, characterized using morpho-physiological tests, was accurately identified using PCR-RFLP of the ITS1-5.8S rRNA-ITS2 region. The phylogenetic genotyping studies confirmed the molecular analysis. The antifungal tests using seven phytopathogenic strains from natural isolates, showed that C. (P.) guilliermondii CMGB 44 had high inhibitory activity against Sclerotium sp., Rhizoctonia solani and Botrytis cinerea. The antimicrobial activity was also tested at 25, respectively, 28°C against four potential pathogenic Candida strains, with relative low results. The activity against Candida krusei and Candida tropicalis was augmented by adding sodium bicarbonate solution in a final concentration of 2%.
C. (P.) guilliermondii CMGB 44 presented versatile antimicrobial activity against plant and human pathogenic microbial strains. Future research will aim improvement of the antagonistic activity using an extended range of species/strains.

Key words*: antimicrobial activity, Candida, Candida (Pichia) guilliermondii, fungi, improvement.*

INTRODUCTION

The yeast species *Candida guilliermondii* is largely distributed in nature, on plants, in soil and water, comprising also human pathogenic strains involved in candidemias, especially in immunocompromised patients. The species represents the asporogenic (asexual) state of the teleomorph yeast *Pichia* (*Meyerozyma*) *guilliermondii*, of high interest for its antifungal abilities against phytopathogenic fungi and also for its ability to overproduce riboflavin (Sibirny & Boretsky, 2009; Papon et al., 2013). Although during decades *C. guilliermondii* and *P. (M.) guilliermondii* were considered as genetically heterogeneous being composed of phenotypically undistinguishable taxa, molecular biology analyses and genome sequencing techniques showed that *P. guilliermondii* represents a separate clade formed by sporogenous strains previously classified in *C. guilliermondii*. Thus, phylogenetic studies place *P. (M.) guilliermondii* in the same clade as many *Candida* species (*C. albicans*, *C. tropicalis, C. dubliniensis* and the *C. parapsilosis* branch) (Pryszcz et al., 2015; Mixão et al., 2019).
The present research focuses on the molecular identification of a new *C. (P.) guilliermondii* strain, its antagonistic interactions with fungi from natural isolates and improvement of antimicrobial activity against potential pathogenic strains of *Candida* sp.

MATERIALS AND METHODS

1. PCR-RFLP of ITS1-5.8S rDNA-ITS2 region

For the genomic DNA extraction, 100 µl of 10^8 cells/ml from an overnight culture grown in YPG medium (5 g/l yeast extract, 10 g/l peptone, 2 g/l glucose) at 28°C, 150 rpm, were centrifuged 6 min at 6500 rpm and the cell pellet was resuspended in 100 µl solution of 1% SDS supplemented with 0.8 M lithium chloride (LiCl). The suspension was incubated for 15 min at 70°C and 300 µl of 95% ethanol was added. After vortexing briefly, the suspension was centrifuged for 5 min at 13000 rpm, the pellet was washed with 500 µl of 70% ethanol, centrifuged, and the sediment was resuspended

in 40 µl TE (10 mM Tris, 1 mM EDTA, pH 8.0) supplemented with RNase A (40 µg/ml). After a new centrifugation, the debris was removed, the supernatant containing genomic DNA was preserved for 24 hours at 4ºC and used for PCR amplification. The DNA extracts were analysed by gel electrophoresis using 0.8% agarose and Tris-Borate-EDTA buffer [0.5X].

The PCR amplification of the ITS1-5.8S rDNA-ITS2 was performed in a final volume of 50 µl comprising: 2 µl genomic DNA samples, 1.2 µM of each primer ITS1 (5'TCCGTAGGTGAACCTGCGG) and ITS4 (5'TCCTCCGCTTATTGATATGC) and GoTaq Green Master Mix 2X (Promega). The amplification program was: 5 min at 94ºC, 40 cycles of 1 min at 94ºC, 30 sec at 55ºC, 2 min at 72ºC, and a final extension 5 min at 72ºC. The amplicon was digested for 90 min with 0.5 µl of *Cfo* I (5'-GCG/C-3'), *Hae* III (5'-GG/CC-3'), *Hinf* I (5'-G/ANTC-3') and *Msp* I (5'-C/CGG-3') (izoschisomere *Hpa* II) (10 U/µl, Promega). The amplicon and the restriction fragments were analysed by gel electrophoresis using 1.7% agarose and 0.5X Tris-Borate-EDTA buffer. After electrophoresis, the gel was stained with ethidium bromide, visualized under UV light (UV-VIS Spectrophotometer), digitalized and the size of the amplicon and restriction fragments was determined using the Quantity One program (Bio-Rad).

2. Phylogenetic genotyping

For the phylogenetic studies, reference strains used were: *Candida tropicalis* CMGB 165 (Collection of Microorganisms of the Department of Genetics, Faculty of Biology, University of Bucharest), *Rhodotorula glutinis* CMGB 189, *Ogatea* (*Hansenula*) *polymorpha* CMGB 85, *Pichia pastoris* CMGB 167, *Yarrowia lipolytica* CMGB 32 and *Candida albicans* ATCC 10231. The ITS1-5.8S rDNA-ITS2 amplicons were obtained and then digested with *Hinf* I according to the methods described above. The phylogenetic tree was constructed using the unweighted pair group method with arithmetic mean (UPGMA) from the Quantity One program (Bio-Rad).

3. Antifungal activity

For testing the antifungal activity of *C.* (*P.*) *guilliermondii* CMGB 44, we used seven fungal strains of *Alternaria mali, Rhizoctonia solani, Sclerotinia sclerotiorum, Sclerotium* sp., *Botrytis cinerea, Aspergillus carbonarius, Monilinia* sp. from natural isolates and preserved in the Fungal Collection of the Faculty of Biotechnology, University of Agronomic Sciences and Veterinary Medicine of Bucharest. The fungi were grown for 48 hours on PDA medium (400 g/l potato infusion, 20 g/l glucose and 15 g/l agar). Blocks of about 7 mm of fungal cultures were then spoted in the middle of Petri plates covered with PDA medium. The yeast strains were grown for 48 hours on YPGA medium (YPG medium supplemented with 20 g/l agar-agar), then streaked at 10 mm from the edges of the fungal blocks. The plates were incubated at 28ºC for seven days and checked daily. The antifungal activity of the yeasts was evaluated by measuring the inhibition zones using as reference plates without yeast cultures.

4. Screening and improving the anti-*Candida* activity

The *C.* (*P.*) *guilliermondii* CMGB 44 culture grown overnight on YPG medium at 28ºC, 150 rpm was centrifuged for 6 min at 6500 rpm. Equal aliquots of cell suspensions (10^8 cells/ml) separate (reference for screening tests) and, respectively, in mixtures with sodium bicarbonate ($NaHCO_3$) in final concentrations of 1, respectively, 2%, were plated on YMA medium (3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, 10 g/l glucose, 20 g/l agar-agar) Petri plates floated with 10^6 cells/ml of: *Candida albicans* ATCC 10231, *Candida parapsilosis* CBS 604, *Candida tropicalis* CMGB 165 and *Candida krusei* CMGB 94. The results were recorded after five days of incubation at 25ºC, respectively, 28ºC.

RESULTS AND DISCUSSIONS

1. Molecular and phylogenetic identification

Previous morpho-physiological tests allowed the classification of the yeast strain CMGB 44 as belonging to *C. (P.) guilliermondii*, forming white-yellow, smooth colonies on YPGA medium after 48 hours of incubation and pseudohyphae with small blastoconidia (Figure 1).

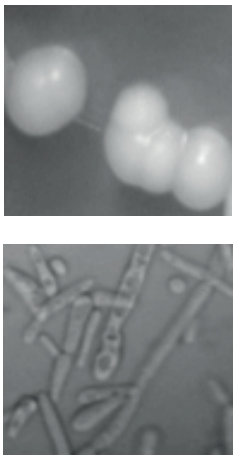

Figure 1. *C. (P.) guilliermondii* CMGB 44 (a) colonies and (b) cells and pseudohyphae

Molecular identification of the yeast strain *C.* (*P.*) *guilliermondii* CMGB 44 was based on the PCR-RFLP analysis of the ITS1-5.8S rDNA-ITS2 region, a technique intensively used in yeast taxonomy. The amplicon had 620 bp and the restriction fragments obtained using four restriction endonucleases, *Cfo* I, *Hae* III, *Hinf* I and *Msp* I, were determined and compared with those described in similar studies on *C. guilliermondii*, respectively, *Pichia (Meyerozyma) guilliermondii* strains (Table 1). The analysis of the restriction patterns revealed a high degree of similarity, confirming thus the taxonomic classification of our strain in the *C.* (*Pichia, Meyerozyma) guilliermondii* species. The variability of the size of the restriction fragments is most probable due to the different origin of the strains. Also, the *Msp* I endonuclease is not frequently used in PCR-RFLP studies and, therefore, there is limited information regarding the generated restriction profiles.

Table 1. Comparative analysis of the amplicons and restriction fragments of the ITS1-5.8S rDNA-ITS2 regions from *Candida (Pichia, Meyerozyma) guilliermondii* strains

Strain	Amplicon (bp)	Restriction fragments size (bp)				Reference
		Cfo I	*Hae* III	*Hinf* I	*Msp* I	
P. guilliermondii CMGB44	620	290, 240	370, 120, 70	320, 300	350, 140, 80	this study
P. guilliermondii	650	300, 270	400, 120, 70	310, 290	-	Bezerra-Bussoli et al., 2013
P. guilliermondii CECT 1019; 1021; 1438; 1456	625	300, 265, 60	400, 115, 90	320, 300	-	Esteve-Zarzoso et al., 1999
M. guilliermondii UAF214	607	293, 255, 49, 10	390, 116, 79, 17, 5	314, 285, 8	-	Pham et al., 2011
M. guilliermondii CECT 1019	625	300, 265, 60	400, 110, 90	320, 300	-	
M. guilliermondii NCYC 443	625	300, 265, 60	400, 115, 90	320, 300	-	
M. guilliermondii CBS 2030	605	320, 270	380, 120, 80	340, 300	-	
C. guilliermnondii	625	300, 265, 60	400, 115, 90	320, 300	-	Valles et al., 2007
P. guilliermondii	650	-	-	-	460, 90	Basílio et al., 2008
P. guilliermondii	630	260, 220, 50	450, 90	265	-	Jeyaram et al., 2008
C. guilliermondii ATCC 9058	608	-	-	-	371, 155, 82	Mirhendi et al., 2006

For a more accurate identification, we performed a phylogenetic genotyping analysis. On this purpose, the PCR-RFLP patterns obtained for *C.* (*P.*) *guilliermondii* CMGB 44 using the endonuclease *Hinf* I were integrated in a study comprising data from related *Candida* species (*C. tropicalis*, *C. albicans*) and, also, other yeast species/genera, some considered as phylogenetically distant.

According to our analysis (Figure 2), the strain *C.* (*P.*) *guilliermondii* CMGB 44 is placed near *C. tropicalis* and *C. albicans* forming a separate branch, far from *Y. lipolytica*, which conserves its place as an out-group.

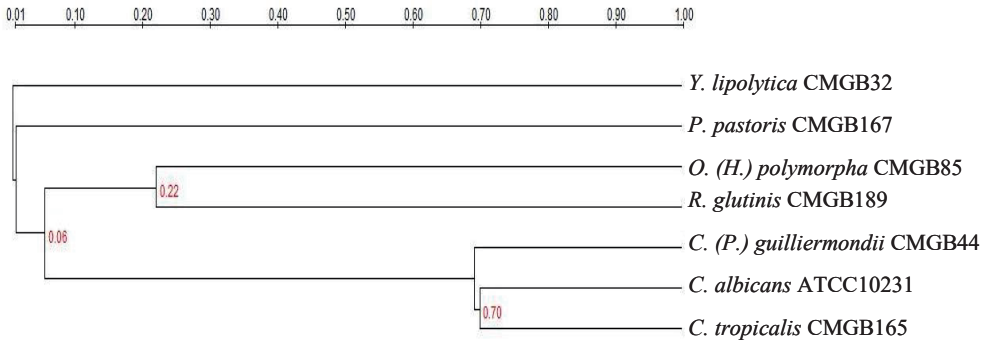

Figure 2. Dendrogram (UPGMA) representing the phylogenetic position of *C.* (*P.*) *guilliermondii* CMGB 44

Similar phylogenetic relationships were presented by Diezmann et al., (2004) during their analysis of the nuclear 18S rDNA and 26S rDNA of 73 ascomycetous taxa. Fitzpatrick et al. (2006) used the genome data for over 345.000 protein-coding genes from 42 fungal genomes, and described the close relation between the three *Candida* species. Moreover, their study explains the close position of *C. albicans* and *C. tropicalis* separately from *C.* (*P.*) *guilliermondii*, fact that might be also observed in our dendrogram. Thus, the CTG clade (organisms that translate CTG codon as serine instead of leucine) within *Saccharomycotina*, is divided in two branches in which *C.* (*P.*) *guilliermondii* represents *Candida* species with sexual reproduction. The position of the three *Candida* species on one side, and *P. pastoris* and *Y. lipolytica*, on the other side, also derived from the analysis of the variation of the GC content of all protein coding genes (Louis et al., 2012). The phylogenetic tree resulted from the analysis of 79 highly conserved sequence-specific DNA-binding proteins from 31 fungal species, also confirmed our results (Lohse et al., 2013).

2. Antifungal activity

In present, *P. guilliermondii* is one of the most promising biocontrol agents used for preventing the action of phytopathogenic fungi. The antifungal tests using seven phytopathogenic fungi from natural isolates, showed that the strain *C.* (*P.*) *guilliermondii* CMGB 44 totally inhibited the growth of *Sclerotium* sp. (Figure 3a) and had high activity against *R. solani* (Figure 3b). The growth of *B. cinerea* mycelium was also inhibited (Figure 3c), while low activity was observed in the case of *Sclerotinia* sp. (Figure 3d). On the contrary, no results were recorded against *A. mali*, *Aspergillus carbonarius* and *Monilinia* sp.

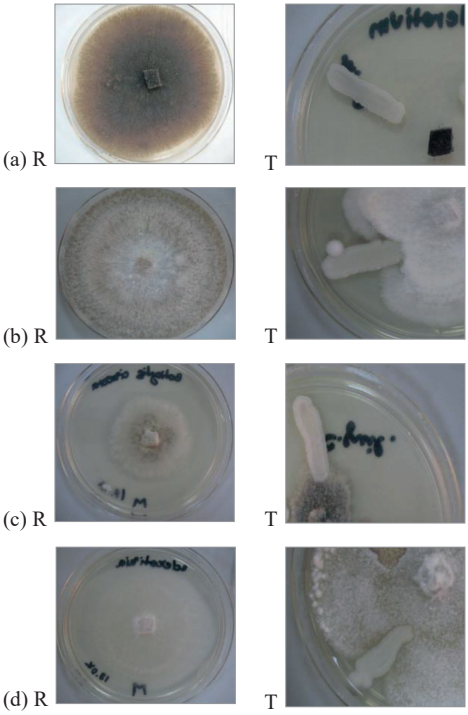

Figure 3. Antifungal activity of *C.* (*P.*) *guilliermondii* CMGB 44 against: (a) *Sclerotium* sp., (b) *R. solani*, (c) *B. cinerea* and (d) *Sclerotinia* sp. (R - reference; T - test plates)

Similar results were recorded against *B. cinerea* during other studies. Thus, *in vitro* and *in vivo* studies regarding the antifungal mechanism of action of *P. guilliermondii* showed that the yeast

was able to protect apples from *B. cinerea* by attaching to the fungal hyphae most probably due to a lectin link and by producing β-(1-3) glucanases (Wisniewski et al., 1991). The attachment of yeast cells also restricted the proliferation of *Colletotrichum capsici* causing chilli anthracnose. Moreover, the yeast competed with the fungus for the carbon (glucose, sucrose) and nitrate substrates (Chanchaichaovivat et al., 2008). Production of β-(1-3) glucanases and exochitinase was also responsible for *P. guilliermondii* activity against *Aureobasidium pullulans* from apple wounds. Furthermore, it seems that *P. guilliermondii* can trigger the production of ethylene in plant cells, which activates the phenylalaninammonium-lyase involved in the synthesis of phenols, phytoalexins and lignins wiht role in plant defense (Spadaro & Gullino, 2004). Also, a *P. guilliermondii* strain isolated from Moroccan citrus Valencia-Late oranges was effective against green mold produced by *Penicillium italicum* (Lahlali et al., 2014).

On the other hand, *C. guilliermondii* strains were proved as having antifungal activity. The biomass obtained from a *C. guilliermondii* strain isolated from heterograft tomato crop stoped the rotting of tomatoes produced by *Rhizopus stolonifer* (Zambrano et al., 2014). Other strains isolated from figs (*Ficus carica* L.) and cactus pear (*Opuntia ficus-indica*) fruits were active against *Penicillium expansum*, the mechanism being based on the competition for nitrogen sources (Scherm et al., 2003).

Several studies mentioned *Saccharomyces cerevisiae* as having growth promoting effect and biocontrol activity against *Sclerotium* or *Rhizoctonia* (Freimoser et al., 2019). The negative effect of *R. solani* was reduced by strains of *Candida saopaulonensis*, *Cryptococcus laurentii* and *Bullera sinensis* in cowpea plants (de Tenorio et al., 2019), respectively, by strains of *Candida valida*, *Rhodotorula glutinis* and *Trichosporon asahii* in suger beet (El-Tarabily, 2004). However, until present, we could not find any data on the antifungal activity of *C.* (*P.*) *guilliermondii* against *Sclerotium*, *Rhizoctonia* or *Sclerotinia* strains.

3. Anti-*Candida* activity

P. guilliermondii and its asexual form, *C. guilliermondii*, are studied mainly for their biocontrol potential for plant protection. Therefore, little is known regarding their antimicrobial activity against human pathogenic microbial strains. Moreover, although less pathogenic and invasive than other *Candida* species, such as *C. albicans*, *C. guilliermondii* strains were isolated in hospitals from immunocompromised patients with cancer and hematology diseases, or from various samples of skin, urine, blood or genital tract tissue and, therefore, might represent risk factors (Papon et al., 2013). Nevertheless, Acuña-Fontecilla et al. (2017) isolated from wine a *P. guilliermondii* strain with killer activity against bacterial strains of *Salmonella typhimurium*, *Listeria monocytogenes* and *Escherichia coli*.

The strain *C.* (*P.*) *guilliermondii* CMGB 44 was tested against four *Candida* species with pathogenic potential, at 25, respectively, 28ºC. The two different temperatures were chosen since higher temperatures are favorable to pseudohyphal / hyphal transition in *Candida* cells which expose β (1,3)-glucan easier than the yeast-form cells making them thus more succeptible to the attack of the β-(1-3) glucanases (Chen et al., 2019). The screening tests revealed no antimicrobial activity of our strain for none of the tested temperatures (Figure 4aR, 4bR; Figure 5R).

Numerous studies mentioned using sodium bicarbonate for improving the biocontrol potential of various yeast species, based on its ability to inhibit spore germination (Karabulut et al., 2003; Yao et al., 2004). Moreover, previous research (Csutak et al., 2013) showed that adding sodium bicarbonate in final concentrations of 1 or 2% to *Metschnikowia pulcherrima* cells enhanced the antimicrobial activity of the yeast against pathogenic *Candida* strains. Therefore, for further tests, we used mixtures of *C.* (*P.*) *guilliermondii* CMGB 44 cells and sodium bicarbonate.

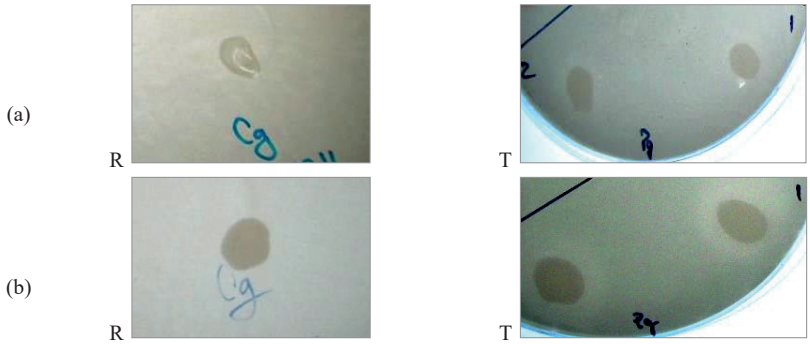

Figure 4. Antimicrobial activity of *C.* (*P.*) *guilliermondii* CMGB 44 after two days against *C. krusei* CMGB 94 at (a) 25ºC and (b) 28ºC (R - reference; T - test plates)

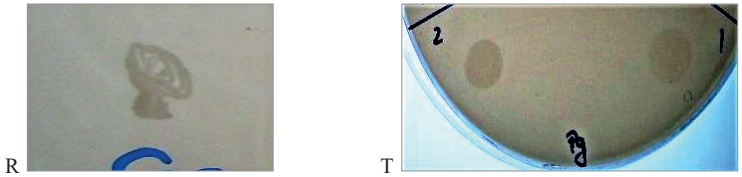

Figure 5. Antimicrobial activity of *C.* (*P.*) *guilliermondii* CMGB 44 after five days against *C. tropicalis* CMGB 165 at 28ºC (R - reference; T - test plates)

Positive results were recorded after two days of incubation against *C. krusei* CMGB 94 and *C. tropicalis* CMGB 165, even though the difference between the inhibition halos obtained using sodium bicarbonate 1%, respectively, 2%, were not significant (Figure 4T; Figure 5T). An interesting fact is that, while for *C. krusei* the temperature influenced visibly the antimicrobial activity, with a rapid response at 28ºC *vs*. 25ºC (Figure 4aT and bT) after two days of incubation, in the case of *C. tropicalis* an enhancement of the antimicrobial activity was recorded only at 28ºC after five days of incubation (Figure 5T). A possible explanation might reside in the variation of β-glucans content of the cell wall of different *Candida* species (Csutak et al., 2017).

CONCLUSIONS

Recent molecular approaches demonstrated the identity of *Candida guilliermondii*, a rare pathogenic species, and its sexual state, *Pichia guilliermondii*, intensively studied for its antimicrobial potential used in plant biocontrol. The strain *C.* (*P.*) *guilliermondii* CMGB 44 presented high antifungal activity against phytopathogenic fungi from natural isolates. Also, to our knowledge, this is the first reported case of a *C.* (*P.*) *guilliermondii* strain able to inhibit *Sclerotium*, *Rhizoctonia* or *Sclerotinia* sp. Moreover, the addition of sodium bicarbonate augmented its antimicrobial activity against *Candida* potential pathogenic strains, which represents the basis for new biomedical applications of *C.* (*P.*) *guilliermondii*. Further research will aim understanding the antagonistic mechanism of action using a wider range of plant and human pathogenic strains for developing applications both in biocontrol and biomedicine.

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COMPREHENSIVE EVALUATION OF LIPIDIC CONTENT IN DRY PET FOOD RAW MATERIALS: COMPARISON BETWEEN FRESH MEATS AND MEAT MEALS

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Abstract

In a balanced diet for companion animals, the lipid component represents an important nutrient and source of energy, moreover it increases the palatability of the food. Dogs and cats are unable to synthesize essential fatty acids (EFAs) needed for their metabolism, therefore, they must be taken with the diet. The majority of dry pet food nowadays on the market are produced starting from fresh meats and meat meals which have a different lipid composition. This study was conducted to analyse the lipid component of the raw materials used for the production of dry pet food, paying particular attention to the polyunsaturated fatty acids (PUFAs), the ω-3 and ω-6, whose presence is fundamental for pet health. The crude fats of both fresh meats and meat meals were analysed by a gravimetric method while the lipid profile was determined by LC/MS-QTOF (Liquid Chromatography/Mass Spectrometry-Quadrupole Time Of Flight) in order to evaluate the lipid component, in terms of saturated, monounsaturated and polyunsaturated fatty acids of the different raw materials used for dry pet food production. The results demonstrated that fresh meats have a better lipid profile, having a higher concentration of PUFAs compared to meat meals, thus making fresh meats the best choice as raw materials for dry pet food production from the lipid point of view.

Key words*: Dry Pet Food, Lipid Content, Saturated Fatty Acids (SFAs), Monounsaturated Fatty Acids (MUFAs), Polyunsaturated Fatty Acids (PUFAs).*

INTRODUCTION

The market of dry pet food is constantly expanding and new formulations are proposed, making it necessary to have a more accurate assessment of the raw materials used in the production process (Zicker, 2008). Pet food should ensure the right supply of nutrients so that the animal can enjoy a good state of health. In this respect, the fats present in dry pet food play a very important role, since many of these are not naturally produced by the body; therefore they must necessarily be included in the regular diet (Bauer, 2008; Lenox, 2016). Some of the main categories of fats that should be present in dry pet food are represented by monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), ω-3 and ω-6. Their intake ensures normal lipid metabolism and brings numerous beneficial effects on pet health (Ahlstrøm et al., 2004; Granato et al., 2000; Hilton, 1989; Moussa et al., 2000; Watson, 1998; Yaqoob, 2002). A few studies have also disclosed how some fatty acids can have antioxidant effects (Giordano & Visioli, 2014; Richard et al., 2008). It has been shown that the intake of PUFAs brings numerous benefits to both human and animal health (Alessandri et al., 1998; Calder & Yaqoob, 2009; Kouba & Mourot, 2011; Lara et al., 2007; Newton, 1996; Simopoulus, 2001; Sioen et al., 2008). Some of these PUFAs, such as ω-6 linoleic acid, arachidonic acid, ω-3 α-linolenic acid, eicosapentaenoic acid and

docosahexaenoic acid are essential fatty acids (EFAs) for the health of pets (Lenox, 2016; MacDonald et al., 1983; Rivers et al., 1975; Wander et al., 1997; Watson, 1998). PUFAs play a structural role in cell membranes and act as precursors to eicosanoids such as prostaglandins and leukotrienes (Lenox, 2016; Watson, 1998). It has also been shown how nutritional deficiencies of PUFAs are at the basis of pathologies affecting the skin, such as dermatitis, dry and rough coat and dry and itchy skin (Ahlstrøm et al., 2004; Bauer, 1994; Lenox, 2016; Palmquist, 2009; Watson, 1998). PUFAs are also fundamental for the reproductive efficiency of the animal, for renal function and the regulation of the immune system (Alonge et al., 2019; Brown et al., 1998; Brown et al., 2000; Filburn & Griffin, 2005; Hall et al., 2003; Lenox, 2016; Pawlosky & Salem, 1996; Wander et al., 1997). Based on all these findings, it is then clear how a healthy dry pet food should contain a suitable concentration of MUFAs and PUFAs in order to allow pets to enjoy a good state of health.

The majority of dry pet foods nowadays found on the market are produced starting from two different kinds of raw materials: fresh meats and meat meals (Thompson, 2008) (Figure 1).

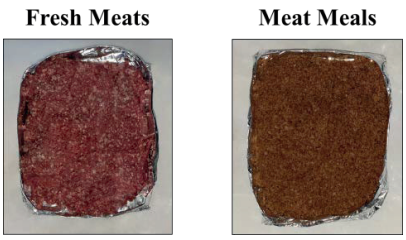

Figure 1. Representation of a type of raw material used in this study

Fresh meats are obtained from the waste of meat intended for human consumption, while meat meals derive from meat by-product processing according to the Regulation (EC) No 1069/2009 of the European Parliament and of the Council of 21/10/2009. These meals are mainly produced by pet food manufacturers to supply protein sources in order to make pet kibbles; however, the intensive industrial process they undergo may cause the onset of oxidation processes and a partial degradation of the raw materials (Camire et al., 1990; Lankhorst et al., 2007; Rokey et al., 2010; Singh et al., 2007; Tran et al., 2008; Williams et al., 2006). In fact, one of the main cause of alteration of pet food is due to the pro-oxidant action of oxygen and light (Piergiovanni & Limbo, 2010), capable of inducing the formation of radical species and Reactive Oxygen Species (ROS) that damage different molecules, lipids included (Lin et al., 1998).

An appropriate storage of raw materials, therefore, seems to be fundamental for the preservation of the nutritional qualities of the food, not least a good selection of the raw materials used for dry pet food production is an essential step for the manufacture companies to obtain better quality products.

The aim of this study is to analyse the lipidic component of the raw materials usually used for dry pet food production. The crude fats of both fresh meats and meat meals were analysed by a gravimetric method. At the same time, a lipidomic analysis was carried out using LC/MS–QTOF (Liquid Chromatography/Mass Spectrometry-Quadrupole Time Of Flight) in order to evaluate the presence and concentration of saturated, monounsaturated and polyunsaturated fatty acids in the different raw materials.

MATERIALS AND METHODS

Raw Materials

Raw materials used in this study are listed in Table 1 and they consist of: chicken fresh meat for companion animal food, 10 batches from pet food manufacturers (Italy), chicken meat meal for companion animal food, 10 batches from pet food manufacturers (Italy); pork fresh meat for companion animal food, 10 batches from pet food manufacturers (Italy), pork meat meal for companion animal food, 10 batches from pet food manufacturers (Italy); salmon fresh meat for companion animal food, 10 batches from pet food manufacturers (Italy), salmon meat meal for companion animal food, 10 batches from pet food manufacturers (Italy).

Table 1. List of raw materials used in this study.

Raw Materials		
Chicken	Fresh meat for companion animal food	10 batches from pet food manufacturers
	Meat meal for companion animal food	10 batches from pet food manufacturers
Pork	Fresh meat for companion animal food	10 batches from pet food manufacturers
	Meat meal for companion animal food	10 batches from pet food manufacturers
Salmon	Fresh meat for companion animal food	10 batches from pet food manufacturers
	Meat meal for companion animal food	10 batches from pet food manufacturers

Drying Procedure

Samples of fresh meats and meat meals were dried according to the method described by da Silva et al. (2018). Briefly, an exact amount of raw material (40 g) was dried in oven (Termaks TS 8136) at 90°C for 6 hours, then it was cooled down at room temperature in an desiccator containing silica gel. Samples were then weighed using OHAUS™ Analytical Balance (Pioneer™) until a stable weight was reached.

Determination of Crude Fat content

An amount corresponding to 1 gram of each dry sample was finely weighed through the use of OHAUS™ Analytical Balance (Pioneer™), and placed in previously weighed glass vials. Diethyl ether was then added to the vials in order to solubilize the lipid component. The samples were then shaken for 15 minutes (Multi Reax, Heidolph) to facilitate the lipid solubilization process. Subsequently, the samples were centrifuged at 6000 × g in order to precipitate the insoluble component. The supernatant was then discarded and the samples were weighed with the vials. The process was repeated until a stable weight was reached. The crude fats, corresponding to the part removed with the solvent, were calculated as the difference between the initial weight and the sample residual weight.

Sample preparation

For the lipid extraction, a quantity corresponding to 100 mg of each dry sample was carefully weighed in an Eppendorf tube and 1 mL of 10 mM Butylated Hydroxytoluene in Methanol/Methyl *tert*-butyl ether/Chloroform (1:1:1) was added. The samples were then shaken 30 minutes at 1500 rpm at room temperature in a Thermomixer (T-Shaker Thermomixers, EuroClone). Subsequently, the samples were centrifuged at 1500 × g for 10 minutes at room temperature (Eppendorf™ 5415D Centrifuge). The supernatant containing lipid fraction of the sample was then recovered (Pellegrino et al., 2014).

To release the fatty acid components of glycerolipids and phospholipids, strong basic hydrolysis was performed. An aliquot of 100 µL of the supernatant, obtained as described above, was transferred into a new 2 mL Safe-Lock Eppendorf tube with 80 µL of a freshly prepared solution of 2% NaOH in Methanol/Water 8:2. The tube was shaken and heated in a Thermomixer at 60°C for 30 minutes. Afterwards, the solution was cooled at room temperature, acidified with 20 µL of 12 M HCl and 1 mL of n-Hexane was added. The tube was vortexed for 10 seconds and centrifuged at 1500 × g for 5 minutes. Finally, 250 µL of the supernatant, containing all fatty acids, was transferred in an autosampler vial for subsequent analysis.

Determination of Fatty Acid content

LC/MS analysis was conducted using an Agilent 6530 LC/MS-QTOF system. Fatty acids were separated using a Kinetex C18 column (4.6 mm × 100 mm, 2.6 µm, Phenomenex) with a 15 minutes linear gradient from 40% to 90% of Acetonitrile/Water 60:40 (Solvent A) and Isopropyl Alcohol (Solvent B), both containing 10 mM Ammonium Acetate. The column operated at 20°C with a flow of 0.8 mL/min.

Liquid Chromatography was interfaced to Mass Spectrometer with an Agilent JetStream source. Mass Spectrometer acquired negative ions in Full-Scan mode in the mass range of 100-1700 with mass accuracy of 1.5 ppm. This was achieved by continuous infusion in the source of a reference mass solution (Agilent G1969-85001). LC/MS raw files were aligned and processed using Batch Recursive Feature Extraction algorithm of MassHunter Profinder (Agilent B.08.00). Afterwards, data with a score > 90% were imported in Mass Profiler (Agilent B.08.01). Fatty Acid Database was downloaded

from LIPID MAPS® Structure Database (LMSD) (Sud et al., 2007) and adapted to work in Agilent Mass Profiler. Only fatty acids with a score > 90% were retained. At the end of the workflow, a matrix data reporting the abundance of the peaks of 40 Fatty Acids (9 saturated, 7 monounsaturated ad 24 polyunsaturated) was created and used to determine lipid content.

Statistical analysis

Data shown in this study, regarding the analysis of the crude fat content and the lipid profile of the raw materials used for the production of dry pet food, are reported as mean values of the ten analysed batches (Table 1) ± standard error of the mean (SEM). The t-Student test was used to investigate the significance of the different lipid content in fresh meats and meat meals. The level of significance for the data was set at $p < 0.05$. All statistical tests were done using GraphPad Prism 6.00 for Windows (GraphPad Software, Inc., San Diego, CA).

RESULTS AND DISCUSSIONS

In this study the crude fat content was initially evaluated for each raw material.

The results shown in Figure 2 represent the average of the crude fat values obtained for each batch of fresh meats and meat meals analysed. The analysis reveals how fresh meats exhibit a significantly higher crude fat content compared to meat meals.

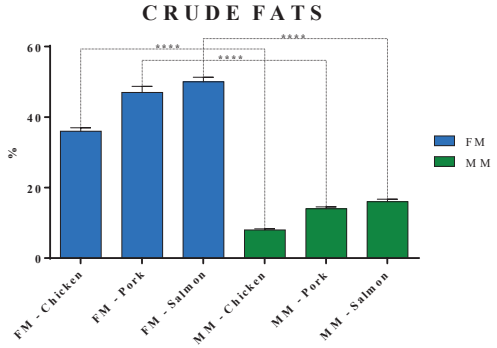

Figure 2. Crude fat content of fresh meat (FM) and meat meal (MM) for companion animal food determined by gravimetric analysis (expressed as percentage values on 100 g of dry sample). Data are reported as mean ± SEM, n = 10, ****$p < 0.0001$

The crude fat level in fresh meats, reported as weight percentage with respect to dry sample weight, ranges from about 36% in the case of chicken to 50% in the case of salmon, whereas a crude fat content lower than 20% is peculiar to all meat meals, reaching a minimum value of 8% in chicken meat meals.

As a rule, the amount of raw fats found in meat meals was always reduced by a factor of at least three compared to the amount found in the corresponding fresh meats. This feature may result from the fact that fresh meats, unlike meat meals, do not undergo treatments and manipulations that can cause loss of the crude fat content.

The crude fat content recommended for dry pet foods is no less than 15% (Case et al., 2010; Rolinec et al., 2016); thus, a deficit of fat concentration in dry pet food is dangerous, because fats are one of the main sources of energy in food and also represent the source of fatty acids (Rolinec et al., 2016). In addition, crude fats also play a key role in contributing to the palatability and the texture of food (Bauer, 2006). In this respect, the results obtained suggest that fresh meats, as well as being healthier due to their adequate lipid content, could also result more palatable for pets.

Subsequently, the fatty acid content in each raw material was evaluated through LC/MS-QTOF. The results reported in Figure 3 show the average of saturated, monounsaturated and polyunsaturated fatty acids present in the different batches of fresh meats and meat meals analysed.

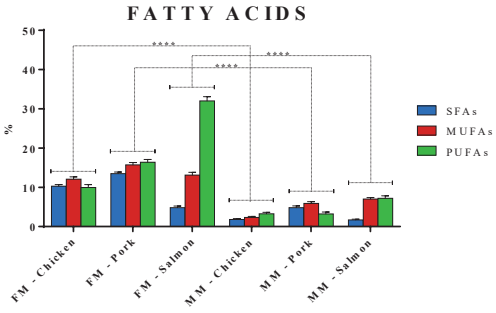

Figure 3. Fatty acid content of fresh meat (FM) and meat meal (MM) for companion animal food determined by LC/MS-QTOF (expressed as percentage values on 100 g of dry sample). Data are reported as mean ± SEM, n = 10, ****$p < 0.0001$

The results are reported as percentage values of each lipid class compared to dry samples. Broadly speaking, the content of saturated, monounsaturated and polyunsaturated fatty acids is significantly higher ($p < 0.0001$) in fresh meats for all the raw materials analysed. Saturated fatty acids (SFAs) are on average about three times more abundant in fresh meats than meat meals, with this ratio being the highest in the case of chicken samples. However, the largest concentrations are recorded for pork fresh meats, where SFAs reach the value of 13.5% relative to the dry sample.

Although fresh meats have been found to have a higher quantity of SFAs compared to meat meals, these fatty acids are mainly long-chain SFAs (LC-SFAs) (Figure 4), *i.e.* between 11 and 20 carbon atoms (C), which are less likely to increase the serum concentrations of cholesterol than very- and ultra-long-chain SFAs (VLC-SFAs and ULC-SFAs), respectively $20 < C \leq 25$ and $C \geq 26$ (Grundy, 1994; Sassa & Kihara, 2014). In fact, all the samples analysed have a concentration of LC-SFAs higher than 90% of total SFAs. In general, all the fresh meats analysed show a statistically significant higher content of LC-SFAs, VLC-SFAs and ULC-SFAs compared to meat meals.

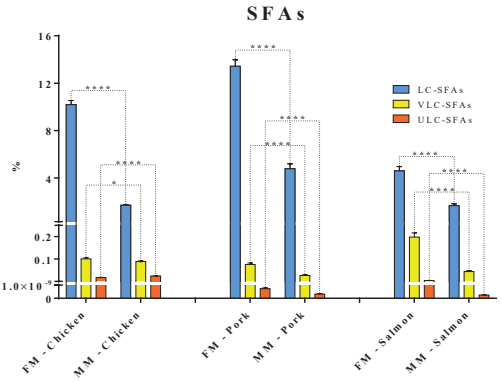

Figure 4. LC-SFA, VLC-SFA and ULC-SFA content of fresh meat (FM) and meat meal (MM) for companion animal food determined by LC/MS-QTOF (expressed as percentage values on 100 g of dry sample). Data are reported as mean ± SEM, n = 10, $*p < 0.05$, $****p < 0.0001$

Similarly, to what described above, even MUFAs are more abundant in fresh meats, with an average abundance about three times higher than the corresponding meat meals (Figure 3). As in the case of SFAs previously analysed, chicken again shows the greatest increase, about five times, compared to meat meals; while the highest MUFA concentration (15.7%) is found for pork fresh meats.

The MUFA content is mostly composed of long-chain fatty acids in all the samples analysed (Figure 5). However, fresh meats, also in this case, show a significantly higher content of LC-, VLC- and ULC-MUFAs compared to meat meals, except for ULC-MUFAs in salmon fresh meats and meat meals whose difference is not statistically significant.

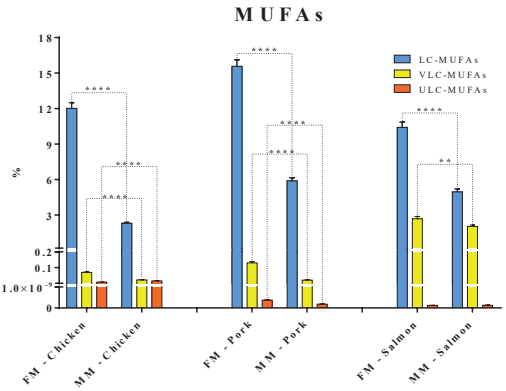

Figure 5. LC-MUFA, VLC-MUFA and ULC-MUFA content of fresh meat (FM) and meat meal (MM) for companion animal food determined by LC/MS–QTOF (expressed as percentage values on 100 g of dry sample). Data are reported as mean ± SEM, n = 10, $**p < 0.01$, $****p < 0.0001$

The LC-MUFA increase is more evident in both fresh meats and meat meals of chicken and pork, where LC-MUFAs are always significantly higher than the 98% of the total MUFA composition. This is beneficial inasmuch as some of them may play a role in lowering serum concentrations of cholesterol and modulating immune functions (Grundy, 1994; Yaqoob, 2002). Moreover, other studies showed that LC-MUFAs have positive effects on cardiovascular health, while VLC-MUFAs seem to have adverse effects (Li et al., 2014).

As far as the total fatty acid composition is concerned, particularly evident is instead the greater content of PUFAs in fresh meats, which is on average more than four times higher than the corresponding meat meals (Figure 3). The

highest concentration of PUFAs is found in salmon fresh meats, reaching 32% of the dry sample weight. This is coherent with the data found in literature according to which fish meats are richer in PUFAs than the other meats, particularly ω-3 fatty acids, which are known for their stimulatory action of anti-inflammatory responses in the case of skin diseases pet (Ahlstrøm et al., 2004; Ricci et al., 2009; Scott & Miller, 1993).

As for chicken and pork fresh meats, higher concentrations of PUFAs are again found compared to the related meat meals. In chicken fresh meats particularly rich in ω-6, the concentration of PUFAs is about 10% of the total weight, while in pork fresh meats it is 16.4%. These findings demonstrate that also for these two raw materials the fresh meat lipid profile is better than that of meat meals, in the light of the innumerable health benefits of PUFAs and for their antioxidant power as well (Alessandri et al., 1998; Giordano & Visioli, 2014; Kouba & Mourot, 2011; Richard et al., 2008; Simopoulus, 2001).

The PUFA carbon backbone length analysis shows a statistically significant higher content of LC-, VLC- and ULC-PUFAs in fresh meats than meat meals in all raw materials analysed; moreover, the LC-PUFA content results to be higher than VLC-PUFA and ULC-PUFA content in all the samples (Figure 6).

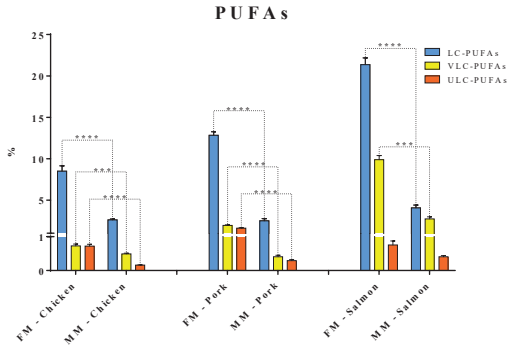

Figure 6. LC-PUFA, VLC-PUFA and ULC-PUFA content of fresh meat (FM) and meat meal (MM) for companion animal food determined by LC/MS–QTOF (expressed as percentage values on 100 g of dry sample). Data are reported as mean ± SEM, n = 10, ***$p < 0.001$, ****$p < 0.0001$

Salmon shows the highest content of LC-PUFAs and VLC-PUFAs in both fresh meats and meat meals, and the highest concentrations of these between health and promoting nutrients are found in salmon fresh meats (Calder & Yaqoob, 2009; Lara et al., 2007; Newton, 1996; Sioen et al., 2008).

These findings are consistent with the literature data that highlight how salmon is particularly rich in LC-PUFAs (Henriques et al., 2014; Sprague et al., 2016; Tocher et al., 2019). PUFAs bring numerous benefits to animal health (Lenox, 2016; MacDonald et al., 1983; Rivers et al., 1975; Wander et al., 1997; Watson, 1998), in fact, LC-PUFAs have cardioprotective, immunoprotective and anti-inflammatory effects (Palmquist, 2009).

The results obtained then show that meat meals have a significantly lower content of all the categories of fatty acids analysed. This could be justified by the treatments and processes employed for meat meal production, which could cause the degradation of the raw material with the loss of important nutrients such as EFAs (Camire et al., 1990; Lankhorst et al., 2007; Piergiovanni & Limbo, 2010; Rokey, 2010; Singh et al., 2007; Tran et al., 2008; Williams et al., 2006).

In this preliminary study, it has been shown how there are significant differences in the lipid content of the diverse raw material used for dry pet food production, which have important consequences in the quality of the final products. These results could help the manufacturing companies to shed light on which raw materials are the best choice for the production of healthier dry foods for dogs and cats.

CONCLUSIONS

Fatty acids, especially PUFAs, are essential in the diet of dogs and cats as they provide energy, modulate inflammation, act as precursors of eicosanoids and prostaglandins, play a structural role in the composition of biological membranes, affect the health of the skin and coat and more generally promote a healthy development of pets. In particular, ω-3 and ω-6 fatty acids are fundamental in animal diet, as pets are unable to synthesize them on their own. This study has shown how the different raw materials used in the production of dry pet food,

fresh meats and meat meals, have a quantitatively and qualitatively different lipid composition.

As opposed to meat meals, fresh meats appear to be the best raw materials that can be used for the production of dry food for pets, both in terms of crude fat content and in terms of MUFA and PUFA content. These results may therefore provide a new approach for the production of better-quality dry pet food, allowing manufacturer companies to better understand how to proceed in the formulation of new products with improved qualities.

In conclusion, this study has clearly shown how fresh meats, from the lipid point of view, appear to be the best choice as raw material for the production of dry food for companion animals.

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ANTIMICROBIAL POTENTIAL OF KOMBUCHA BACTERIAL BIOPOLIMER

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Abstract

During the process of the tea fermentation with Kombucha microbial consortium (SCOBY), due to the presence of acetic bacteria (Acetobacter, Gluconacetobacter) a cellulosic biopolymer is developing on the surface. On industrial level, generally, this is considered as a residue, but recent research proved that such waste can be used for different purposes in pharmaceutical and cosmetic industry as excipient or bandage on injured skin. In this context, we have conducted Kombucha fermentation on different tea sources (black tea, green tea, oolong tea and green tea added with Melissa officinalis). The polymers obtained after fermentation processes were dried under controlled conditions and tested for its antimicrobial activity on potential pathogenic microorganisms (Gram-positive and Gram-negative bacteria, as well as on different Candida species). The highest quantity of polymer was obtained on Green tea supplemented with Melissa, followed by oolong tea, green tea and black tea. All polymers showed antibacterial activity on all Gram-positive and Gram-negative bacteria, including on methicillin resistant S. aureus; highest antibacterial activity was registered in the case of oolong tea source, followed by green tea, green tea with Mellissa and black tea. No inhibitory activity was noticed in the case of Candida species.

Key words*: antimicrobial activity, bacterial cellulose, Kombucha.*

INTRODUCTION

Nowadays, Kombucha has already received the status of functional drink (Jayabalan et al. 2019), accepted by many consumers, especially in Asia, as an alternative to the sugary beverages. During the process of the tea fermentation with Kombucha microbial consortium (SCOBY) a biofilm (cellulosic polymer) is formed on the surface (Figure 1). On industrial level this is considered as a residue, but recent research proved that such waste can be used for different purposes in pharmaceutical and cosmetic industry as excipient or bandage on injured skin. The biofilm is developed by a complex microbial consortium consisting of bacteria such as *Rhodospirillales* cluster which includes *Acetobacter, Bacteria xylinum* and *Gluconobacter*) (Dutta et al., 2007; Nguyen et al., 2008). One of the specific biochemical activity of this acetic bacteria is the oxidation of glucose to gluconic acid, which further leads to the synthesis of microbial cellulose forming the biofilm that remains in the liquid surface. This form of cellulose production has the advantage that, under controlled conditions, the bacteria grow rapidly and can produce cellulose from a variety of carbon sources including glucose, ethanol, sucrose, and glycerol (Villa-Real et al., 2018). The microbial cellulose is produced extracellularly in the form of fibrils that are attached to the bacterial cell. Bacteria produce two forms of cellulose, cellulose I and cellulose II. Cellulose I is a ribbon-like polymer composed of bundles of microfibrils, while cellulose II is an amorphous polymer that is thermodynamically more stable than cellulose I (Podolich et al., 2016). During the fermentation ongoing, the membrane thickness is increased by the generation of new layers on the surface, forming a suspended structure in the culture medium.

The SCOBY contains also other various microorganisms, like yeast belonging to *Pichia* spp., *Torulopsis* spp., *Zygosaccharomyces* spp. or *Brettanomyces* spp. (Greenwalt et al., 2000; Vina et al., 2004), as well as lactic bacteria like *Lactobacillus* sp., *Lactococcus* sp. or *Lecunosctoc* sp. (Marsh et al., 2014; Chakravorty et al., 2016).

The antimicrobial activity of Kombucha was not so extensively studied, more attention going

toward its antioxidant and anti-carcinogenic effects. Data is provided only on the Kombucha soup (suspension), but no information was published, on our knowledge, in regard to the inhibitory activity of the biofilm. It is well known that the tea itself has been reported to have antimicrobial activity (Bansal et al., 2013). Many components such as organic acids (acetic acid, gluconic acid, glucuronic acid etc.), bacteriocins, proteins, enzymes, tea polyphenols and their derivatives which are produced during Kombucha fermentation have been said to contribute to its antimicrobial activity as well as other pharmacological effects (Bhattacharya et al., 2016). In early 2000, Sreeramulu and his teamproved that Kombucha exert antimicrobial activities against *S. typhimurium*, *E. coli*, *Sh. sonnei*, *C. jejuni* and *S. enteritidis* even at neutral pH and after thermal denaturation; their finding suggested the presence of antimicrobial compounds other than large proteins and acetic acid in Kombucha. Recently, the Kombucha suspension made of different tea sources was proven to have inhibitory activity on grape mould *Botrytis cinerea* (Matei B. et al., 2017). Also, Ansari et al. (2017) reported that the Kombucha green tea has an antibacterial activity against *Staphylococcus aureus* and *Salmonella typhimurium*.

One report on bacterial cellulose antimicrobial activity was provided by Kukharenko et al. (2014). After combining bacterial cellulose from *Gluconacetobacter xylinus* isolated from Kombucha with polymeric biocide polyhexamethylene guanidine hydrochloride (PHMG-Cl), the polymer showed excellent efficacy against multidrug resistant strains *Staphylococcus aureus* and *Klebsiella pneumonia,* as well as on yeast.

The aim of this work was to test the antimicrobial activity of Kombucha cellulosic polymers from four different tea sources, respectively black tea, green tea, oolong tea and green tea added with *Melissa officinalis*.

MATERIALS AND METHODS

Production of Kombucha biofilm

Kombucha samples were prepared adding in boiled water 6 g/l of different tea (*Camellia sinensis*) sources: black tea (KN), green tea (KV), oolong tea (KO) and green tea added with *Melissa officinalis* (KVM) from the market; the infusion time was 15 minutes. The sweetened tea (90 g sucrose/l), after cooling, was inoculated 5% Kombucha SCOBY suspension from former cultures maintained in the laboratory at room temperature, aside the direct action of sun light. The fermentation was conducted during 17 days at 28ºC. In the end of the fermentation, the upper polymer was harvested and dried under ventilation at 35ºC for 18 days; its weight was measured by an analytical balance.

Culture media

To test the antimicrobial activity specific media for each microbial group were used, as follows: Nutrient Agar (Merck, Germany) and Nutrient Broth (Merck, Germany) for bacterial use and YPG (Oxoid) and Malt Extract Agar (Roth, UK) for the fungi. All media were autoclaved at 121ºC for 15 minutes.

Tested microorganisms

The inhibitory tests were performed on thirteen potential pathogenic bacteria and on four *Candida* species (Table 1). For the inhibitory activity tests the bacterial inoculum was prepared in nutrient broth for 48 hours at 37ºC, while the fungi, in liquid YPG for 24 hours at 28ºC.

Table 1. Tested microorganisms for their susceptibility to be inhibited by Kombucha polymers

No.	Tested microorganisms	Origin
1.	*Bacillus cereus* CP1	Faculty of Biotechnology collection
2.	*Bacillus pumilus* MI 26	Faculty of Biotechnology collection
3.	*Bacillus licheniformis* MI 27	Faculty of Biotechnology collection
4.	*Staphylococcus aureus* ATCC 25923	American Type Culture Collection
5.	*S. aureus* ATCC 6538	American Type Culture Collection
6.	*S. aureus* ATCC 43300 MRSA	American Type Culture Collection
7.	*S. epidermidis* ATCC 51625 MRSE	American Type Culture Collection
8.	*S. epidermidis* ATCC 12228 MSSE	American Type Culture Collection
9.	*Enterococcus faecalis* MI 28	Faculty of Biotechnology collection
10.	*Salmonella typhimurium* ATCC 14028	American Type Culture Collection
11.	*Escherichia coli* ATCC 8739	American Type Culture Collection
12.	*Pseudomonas aeruginosa* ATCC 9027	American Type Culture Collection
13.	*Candida albicans* ATCC 10231	American Type Culture Collection
14.	*Candida parapsilosis* ATCC 20019	American Type Culture Collection
15.	*Candida guilermondii* MI 40	Faculty of Biotechnology collection
16.	*Candida krusei* MI 41	Faculty of Biotechnology collection

Inhibitory activity test

For the inhibitory activity was employed an adapted method of the disk diffusion test (Kirby Bauer test) in which the antibiotic discs were replaced with discs made of the Kombucha cellulosic polymer (5 mm diameter). As positive controls were used ampicillin disk of 10 µg (BioAnalyse, Turkey) in the case of bacteria and fluconazole of 25 µg (BioAnalyse, Turkey) for the fungi. The formed halo was measured; the plates were performed in duplicate.

RESULTS AND DISCUSSIONS

Production of Kombucha polymers

After 17 days of fermentation, the membrane developed on the upper phase of the suspension (Figure 1) was harvested and dried at 35ºC.

Figure 1. Aspects of Kombucha biofilm (polymer) developed after 17 fermentation days in black tea

Aspects of the dried biomass can be observed in Figure 2, while the microfiber developed by the acetic bacteria activity in black tea is presented in Figure 3 (under optical microscopy, 10x). In the end, the dried biomass was weight.

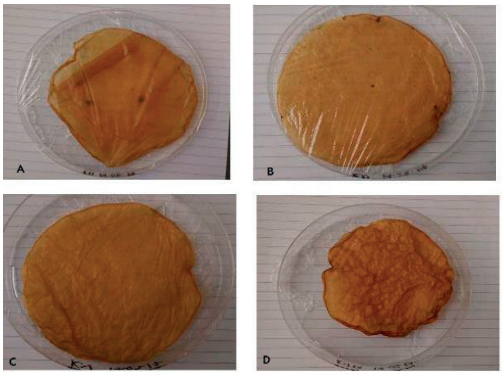

Figure 2. Aspects of Kombucha biofilms dried during 18 days at 35ºC (A: black tea; B: oolong tea; C: green tea; D: green tea + *Melissa officinalis*)

Under the same fermentation conditions, adding the same size inoculum in different tea types, the highest quantity of polymer biomass was obtained in the case of the green tea added with *Melissa officinalis* (KVM: 6.6 g/l Kombucha suspension), while the lowest quantity was registered for the black tea (KN: 3.5 g/l Kombucha suspension), being almost half of the KVM (Figure 4).

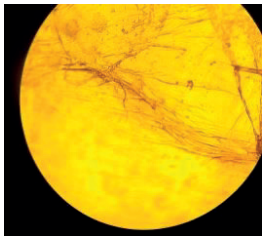

Figure 3. Optic microscopy image of Kombucha polymer microfibre made of black tea (KN) (10x)

In the case of oolong (KO) and green tea (KV) was obtained close quantities of biomass (5.3-5.5 g/l); oolong tea, also named blue-green tea, is a semi-fermented tea, with closer biochemical composition to the green tea, than the black tea, which is a completed fermented product (more oxidized); all are made of *Camellia sinensis* leafs under different processing conditions.

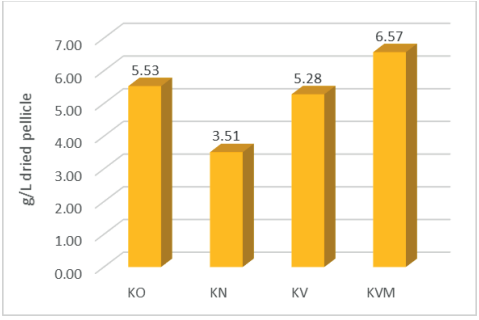

Figure 4. Dried Kombucha biofilm biomass obtained from different tea sources: black tea (KN); green tea (KV); oolong tea (KO); green tea + *Melissa officinalis* (KVM)

Antimicrobial activity of Kombucha polymers

Regarding the inhibitory activity on the Gram-positive bacteria (Table 2 and Figure 5), all Kombucha biofilms showed high inhibitory activity against the *E. faecalis*, higher than the antibiotic control, the ampicillin of 10 µg. Also, the addition of *Melissa officinalis* in the green

tea led to a product with high inhibitory activity on *B. licheniformis*. Except the staphylococci, all biofilms exhibited moderate inhibitory activity on all Gram-positive bacteria. However, should be emphasized that all the biofilms still had some inhibitory activity on all methicilin resistant staphylococci, while the ampicillin, hasn't showed any inhibitory activity.

Table 2. Inhibitory activity of Kombucha biofilm on potential pathogenic Gram-positive and Gram-negative bacteria

Bacteria species	Amp. (10 µg)	KO	KN	KVM	KV
Gram-positive bacteria					
B. cereus CP1	-	++	++	++	++
B. licheniformis MI 27	++	++	++	+++	++
B. pumilus MI 26	+++	++	+	++	++
E. faecalis MI 28	++	+++	+++	+++	+++
S. aureus ATCC 25923	++	+	+	+	+
S. aureus ATCC 6538	+++	+	+	+	+
S. aureus ATCC 43300 MRSA	-	+	+	+	+
S. epidermidis ATCC 51625 MRSE	-	+	+	+	+
S. epidermidis ATCC12228 MSSE	-	+	+	+	+
Gram-negative bacteria					
S. typhimurium ATCC 14028	++	++	+	++	++
E. coli ATCC 8739	-	++	++	++	++
P. aeruginosa ATCC 9027	-	++	+	++	+

Legend: Amp (Ampicillin); (-): no inhibitory activity; (+): low inhibitory activity; (++): moderate inhibitory activity: (+++): high inhibitory activity

Also, the inhibitory activity was tested on Gram-negative bacteria with pathogenic potential (Table 2 and Figure 6). No high inhibitory activity (+++) was noticed against such potential pathogenic bacteria. However, most of the Kombucha biofilm inhibited moderately the development of Gram-negative bacteria, especially the *E. coli* strain.

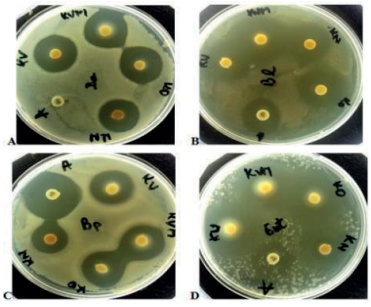

Figure 5. Inhibitory activity of Kombucha biofilm on different Gram-positive bacteria:
A - *B. cereus* CP1; B - *B. licheniformis* MI 27; C - *B. pumilus* MI 26; D - *E. faecalis* MI 28

As a general remark, the biofilm made of black tea showed less remarkable antibacterial activity, while the most promising is the green tea added with *Mellisa officinalis*. *Mellisa officinalis* it was previously reported having inhibitory activity in aqueous extract against *Listeria monocytogenes* and *S. aureus* (Purcaru et al., 2018). Some other plants were reported to increase the antimicrobial effect of Kombucha suspension. Battikh et al. (2013) reported that the antimicrobial activity observed in the fermented infusions with *Lippia citriodora*, *Rosmarinus officinalis*, *Foeniculum vulgare* and *Mentha piperita* was not only significant against the tested Gram-positive and Gram-negative pathogenic bacteria, but also against all *Candida* strains tested except *C. krusei*.

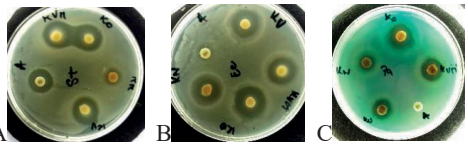

Figure 6. Inhibitory activity of Kombucha biofilm on different Gram-negative bacteria:
A - *S. typhimurium* ATCC 14028 (St); B - *E. coli* ATCC 8739; C - *P. aeruginosa* ATCC 9027

Another step was to test the action of Kombucha biofilms on some *Candida* spp. with potential pathogenic characteristics. None of the tested biomass show any inhibitory activity (Table 3), despite former results reported by Bathik et al (2013) on Kombucha suspension.

Table 3. Inhibitory activity of Kombucha biofilm on potential pathogenic *Candida* spp.

Candida spp.	Fluc. (25µg)	KO	KN	KVM	KV
C. albicans ATCC 10231	+++	-	-	-	-
C. parapsilosis ATCC20019	+++	-	-	-	-
C. guilliermondii MI 40	+++	-	-	-	-
C. krusei MI 41	+++	-	-	-	-

Legend: Fluc (Fluconazole); (-): no inhibitory activity; (+): low inhibitory activity; (++): moderate inhibitory activity: (+++): high inhibitory activity

In our study we have produced on lab scale bacterial biofilm starting from different tea infusion sources; we have obtained the lowest biofilm quantity in the case of the black tea, which is not in total accordance with data reported by El-Salam (2012). It may be presumed that some of the biochemical compounds of the black tea may inhibit the

development of the bacteria group responsible for the biofilm development.

CONCLUSIONS

The use of different tea sources for Kombucha fermentation have an important influence on the quantity of the produced polymeric biofilm. In our trials, the highest dried biomass was obtained in the case of green tea added with *Melissa officinalis* (6.6 g/l Kombucha suspension), while the lowest quantity, almost half, was registered for the black tea (3.5 g/l Kombucha suspension.

Among the Gram-positive bacteria, the most inhibited specie was *Enterococcus faecalis* in the case of all the biofilms. An important finding is that, even the control, ampicillin, had not inhibitory activity on methicillin resistant staphylococci, still Kombucha biofilms have antagonistic effects, even on a low degree. In addition, most of the Kombucha biofilms inhibited moderately the development of Gram-negative bacteria, especially the *E.coli* specie. No inhibition was noticed against any *Candida* species taken into account in this work.

Our findings are coming to support the potential use of Kombucha bacterial biopolymer for the production of different pharmaceutical/medical devices, like bandages or cosmetic products, like masks, with antibacterial activity.

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COMPARATIVE ANALYSIS OF *Artemisia annua* EXTRACTS OBTAINED BY MODERN AND CLASSIC EXTRACTION TECHNIQUES

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Abstract

The aim of our study was to assess the artemisinin and phenolic compounds content of extracts obtained from Artemisia annua by classic solvent extraction comparatively with the extract obtained by microwave. For the classic technique, the solvents used were water, methanol, ethanol, and acetone. For microwave extraction, the solvent was ethanol 90% (v/v). The phenolic compounds were identified by HPTLC and artemisinin was also quantified by HPLC. In all samples rutin, hyperoside, chlorogenic and cafeic acids were identified. The highest quantity of artemisinin was found in extracts obtained by microwave extraction. The highest DPPH radical scavenging was obtained for aqueous extract, whereas methanolic extract exhibited almost a constant activity in different concentrations. The results obtained for total antioxidant capacity by the phosphomolibdenum method were similar to the DPPH method.

Key words*: Artemisia annua, artemisinin, microwave extraction.*

INTRODUCTION

Artemisia annua in a perennial plant from *Asteraceae* family, indigenous from temperate Asia. It is used in Chinese traditional medicine for a long time for anti-malarial, immune-suppressive, anti-inflammatory and anti-cancer properties (Alesaeidi & Miraj, 2016). Now it's naturalized in countries from temperate climates, including Romania. The chemical composition includes sesquiterpenoids, flavonoids, coumarins, proteins, and steroids. The main chemical compound of the plant is artemisinin (sesquiterpenoid) (Ikram & Simonsen, 2017). Based on scientific research, for the treatment of uncomplicated malaria due to *Plasmodium falciparum*, the World Health Organization (WHO) recommends artemisinin-based combination therapy (ACT) Also, the WHO statement shows that further studies are necessary, in the field of fundamental and clinical research (WHO, 2012). In 2015 the estimated number of people infected with malaria parasites in sub-Saharan Africa was 114 million (WHO, 2016).

Based on research studies WHO does not recommend the utilisation of *Artemisia annua* in different forms (as tea or compacted plant material), because of the low content of artemisinin in the plant (WHO, 2012).

Recent studies have shown that flavones and artemisinin have synergic anti-malarial activity (Czechowski, 2019).

Because of the previous scientific results, the extraction techniques of artemisinin are very important. The efficiency of extraction depends on the chosen technique, respectively on the raw material, solvent - solid ratio, the temperature of extraction and duration of the extraction (Zhang et al., 2018).

Safety risks such as solvent toxicity and the presence of solvent residues in extracts, together with low yield, have stimulated the development of other extraction technologies, clean or environmentally friendly technologies, which can minimize or eliminate the use of organic solvents.

These techniques are also known as cold extraction techniques, in which the stability of the extracted compounds is not affected and the energy required for extraction is reduced (Tiwari, 2015).

Our goal in this study is to comparatively evaluate the chemical composition in artemisinin and phenolic compounds and antioxidant capacity of extracts of *Artemisia*

annua obtained by classic solvent extraction and microwave extraction techniques.

Microwaves are electromagnetic fields in the range of 300 MHz to 300 GHz, with two perpendicular oscillating fields, the frequencies of the electric field and the frequencies of the magnetic field. The solvent enters the solid matrix by diffusion, and the solute is dissolved to reach a concentration limited only by the characteristics of the solid (Angiolillo et al., 2015).

Microwaves are a non-contact heat source that can generate more efficient heating, accelerate energy transfer and reduce the thermal gradient. Several classes of compounds such as essential oils, antioxidants, pigments, flavors and other organic compounds can be effectively separated using this method (Li et al., 2013).

According to Leadbeater (2014), the use of microwave equipment is a flourishing technology, because it is possible to generate higher temperatures easily, safely and in a reproducible manner; reaction time can be reduced; the yield can be increased; and the purity can be improved, compared to conventional heating methods. This technique can be performed either with or without the addition of any solvent (Oroian & Escriche, 2015).

Li et al. (2013) provide an overview of the techniques that are available for extracting bioactive compounds using microwaves, without solvents. They have shown that this can be an alternative to other techniques, with the benefits of time reduction, energy consumption, solvent use, and no CO_2 emissions.

Grigoras et al. (2012) conducted a comparative study of conventional methods, maceration and green extraction using pressurized liquid, ultrasound, and microwave. The microwave assisted methodology provided the highest concentration of the bioactive compounds in the extract obtained.

MATERIALS AND METHODS

Raw material - Artemisia annua was purchased from Dorel Plant SRL. A voucher specimen of *Artemisia annua* is deposited in INCDCF-ICCF Herbarium.

Sample preparation: Classic extraction - the samples were prepared by extraction with ethanol (T1), water (T2), methanol (T3), and acetone (T4) - vegetal material/solvent rate - 1/10 m/v, for 1 h at the boiling temperature of the solvent. The process was followed by filtration of the extracts, solvent evaporation at reduced pressure until the precipitates appeared. Each precipitate were dissolved in 50 ml 50% ethylic alcohol (v/v) and frozen until analysis.

Microwave-Assisted Extraction: raw material was extracted under the influence of microwave energy using 90% ethanol (v/v) with the variation of extraction parameters as follows:

Codification of extracts	Raw material (G	Pressure (W)	Temperature (°C)	Time (min)
M1	30	180	30	8
M2	30	330	60	8
M3	30	330	40	6
M4	30	330	40	12

The extract thus obtained was evaporated in vacuum and redissolved in 15 ml 50% ethylic alcohol (v/v) and frozen until analysis.

HPTLC Analysis - Fingerprint chromatography of the samples for the identification of the chemical compounds was made according to the method described by Wagner and Bladt, 1996. The samples (3-7 µl) and references substances of phenolic compounds and artemisinin (1-5 µl) (10^{-3}M T5 - artemisinin, T6 - hyperoside, T7 - chlorogenic acid, T8 - chologenic acid, T9 - caffeic acid, T10 - rutin Sigma-Aldrich) were spotted on 20 x 10 Silica gel 60F254 TLC plate in 10 mm band length. The spots were made with a Hamilton- Bonaduz, Schweiz syringe, and CAMAG LINOMAT 5 instrument. For artemisinin identification the mobile phase was n-hexane: ethyl acetate 75:25 (v/v) (system A) and for polyphenolic compounds was ethyl acetate-acetic acid-formic acid-water 100:11:11:27 (v/v/v/v) (system B). For pre-saturation, the mobile phase was put in the TLC twin-chamber for 30 min at ~20°C, before the analysis. After development (up to 90 mm), plates were dried and visualized, for B system in Natural Product followed by PEG4000 reagent and for A in sulphuric anisaldehyde. The fingerprints were evaluated at UV with WinCats and VideoScan software for A system and in visible light for B the system.

HPLC Analysis - Chromatographic separation was achieved on a LaChrom CLASIC MERCK-HITACHI system, with DAD detector; a Kromasil 100-5C18 150*4.6 mm column at 30

± 1°C, using gradient elution. Quantification of artemisinin was performed using a mobile phase consisting of two solutions - phosphate buffer solution, pH = 3.0 as A solution and acetonitrile as B solution at an initial flow rate of 1 ml/min; with an injection of 20 ìl.

*Free radical scavenging w*as made for different concentrations (1%, 0.1%, 0.01% in methanol) of the extracts and measured against 1, 1 diphenyl-2-picryl hydrazyl radical (Sigma-Aldrich) (Sanchez-Moreno et al. 1998). Briefly, 2950 ml of the DPPH methanolic solution (0.0025 g/l) were mixed with 50 µl aliquots of the extract. For blank solution, the sample was replaced with methanol. After standing for 30 minutes of the room temperature for the solution was measured UV absorbance at 517 nm. The DPPH% scavenging effect was calculated using the following equation:

% inhibition = $\{(D_0 - D_1)/D_0\}$ x 100.

Where D_0 is the absorption of blank solution and D_1 is the absorption of the extract.

Total antioxidant capacity (TAC) assay - was made for ethanolic solution of the extracts (concentration 1%, 0.1%, 0.01%) according to Prieto et al., 1999, phosphomolybdenum method. To 2.7 ml of phosphomolybdenum solution (0.6 M sulphuric acid, 28 mM sodium molybdate, and 4 mM ammonium phosphate) was added 0.3 ml of extract solution. The absorbance of the samples, incubated for 90 minutes at 95^0, was measured after cooling them to room temperature at 695 nm with UV-VIS spectrophotometer. The blank solution was ethanol. The antioxidant capacity was expressed as ascorbic acid equivalent (AA) to 1 mg of the active substance. The calibration curve is linear for ascorbic acid in the range of 0.001 to 1 mg/ml, n = 6, $r^2 = 0.999$.

RESULTS AND DISCUSSIONS

The HPTLC fingerprint profile is utilized as an identification method of compounds, compounds classes, of plant species respectively.

The fingerprint of *Artemisia annua* extracts - A system - (Figure 1) is characterized by the presence of artemisinin (T5) compound in all four samples as a pink spot at Rf~0.4.

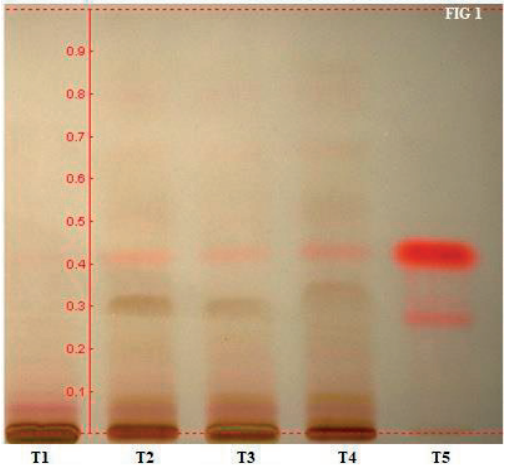

Figure 1. HPTLC fingerprint of *Artemisia annua* extracts - identification of artemisinin (reference substance)

In Figure 2, *the polyphenolic fingerprints of Artemisia annua extracts - B system - are* characterized *by the presents of all four reference substances:*

Reference substance	*Rf*	*Description*
T6 - hyperoside	Rf~0.68	orange fluorescent spot
T7 - chlorogenic acid	Rf~0.53	blue fluorescent spot
T8 - caffeic acid	Rf~0.95	blue fluorescent spot
T9 - rutin	Rf~0.45	orange fluorescent spot

In all four samples was also identified two green spots with Rf between 0.2 and 0.3 and an orange sport at Rf~0.18, mostly due to flavonoid glycosides and two blue florescent spots at Rf~0.8 and Rf~0.9, as caffeoyl and dicaffeoyl quinic acids (chlorogenic acids derivates).

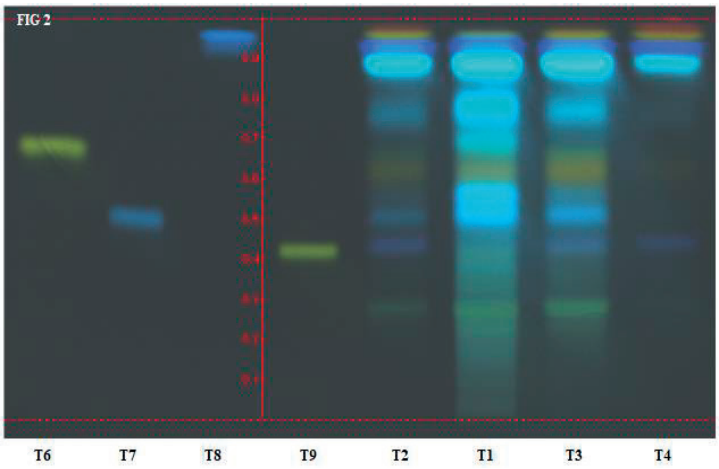

Figure 2. HPTLC fingerprint of *Artemisia annua* extracts - identification of phenolic compound

HPLC was performed for the quantification of artemisinin in all samples. The results are presented in Table 1.

Table 1. Artemisinin content in extracts (mg/100 ml)

Extract	Artemisinin (mg/100 ml)
T1	34.12
T2	13.39
T3	12.65
T4	22.23
M1	27.39
M2	60.37
M3	21.45
M4	92.45

Antioxidant activity was performed by DPPH (1,1 diphenyl-2-picryl hydrazyl) radical scavenging method for all the extracts obtained. The DPPH assay method is based on the reduction of the stable free radical DPPH (Shekhar & Anju, 2014). Changing the solution color from purple to yellow reflects the ability to scavenge DPPH free radical. (Brighente et al., 2007; Ionita, 2005).

Total antioxidant capacity assay (TAC) is based on the reduction of Mo (VI) to Mo (V) creating a green colored phosphomolybdenum at V complex, acidic pH, in the presence of extracts that contain antioxidants compounds. (Wan et al., 2011).

The next two figures present the antioxidant activity of the extracts obtained by the two methods DPPH - Figure 3 and TAC- Figure 4.

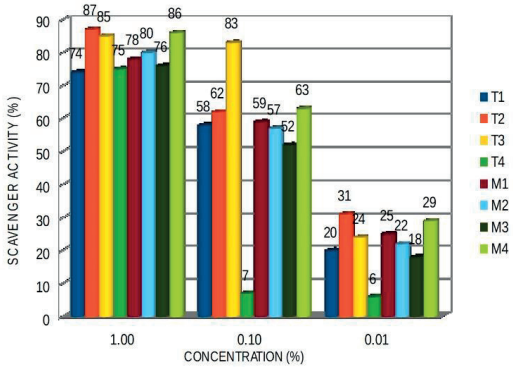

Figure 3. Scavenger activity of the DPPH

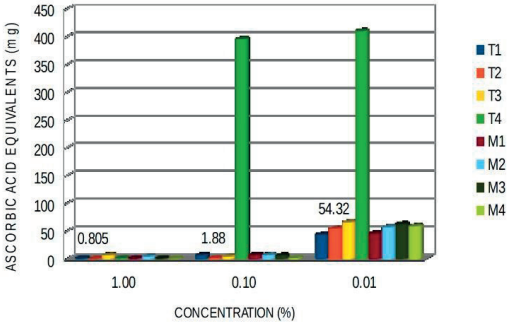

Figure 4. Total antioxidant capacity

The results obtained for both methods show that the antioxidant activity the T1 - water extract and M4 ethanol microwave extract have the highest effect, T1 being slightly superior.

The antioxidant activity is mainly attributed to polyphenolic compounds by research results (Cai et al., 2004; Kırca & Arslan, 2008; Surveswaran et al., 2007). There are also recent studies about water extract obtained from *Artemisia annua* that contain artemisinin and have DPPH radical scavenging activities if 91%, respectively higher than extracts obtained in other solvents (Kim et al., 2015). A review made by Alesaeidi & Miraj in 2016, shows that artemisinin and its flavonoids (a class of polyphenols) have a synergistic effect not only for anti-malarial activity, but also for immunosuppressive, anti-inflammatory, and anti-cancer properties of the plant activity.

CONCLUSIONS

The present study indicates that the water extract obtained by the solvent classical extraction technique and the last one of the extracts obtained by microwave technique are the most valuable for antioxidant properties, and also that the modern technique has superior results for artemisinin extraction.

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METHODS FOR SCREENING OF NOVEL L-ASPARAGINASE FROM MICROORGANISMS

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Abstract

L-asparaginase is a hydrolase able to mediate the cleavage reaction of the amide bond from asparagine to ammonia and aspartate. This hydrolysis reaction plays a very important role in the medicine, bringing the solution to diseases considered incurable such as acute lymphoblastic leukemia (ALL), malignant diseases of the lymphoid system, different lymphomas, being a treatment used in chemotherapy schemes. Despite the adverse effects that it can trigger in the body, L-asparaginase remains the main treatment for such diseases. It is well known that microorganisms, mainly bacteria, are able to produce important quantities of L-asparaginase. However, the level of this enzyme need to be improved in order to reduce the costs, and this could be realized both by identification of new microbial producers or optimization of the production technologies. Moreover, the new sources of L-asparaginase must be free of glutaminase and urease activity, these enzymes being involved in the side effects of enzymatic treatment of ALL. For this reason, the identification of new sources of L-asparaginase, free of glutaminase as well as urease, remains an important goal for researches in domain. The present review aims to discuss the microbial sources of L-asparaginase, the methods used for screening new microbial strains isolated from different sources able to produce large quantities of L-asparaginase, the molecular aspects of the main enzyme producers, as well as the characteristics of enzymes and their applications.

Key words*: different sources, L-asparaginase, molecular aspects, new microbial, screening.*

INTRODUCTION

There are more than 50 years from the first demonstration of the anti-tumour activity of *Escherichia coli* L-asparaginase II (Broome, 1961) and during the time, several applications (including commercial) in food and therapeutics have been described. The importance of this enzyme is proved by the high global demand for it, 380 million USD being used in 2017 for its commercialization. It is estimated that the total request of L-asparaginase to increase to up 420 million USD by 2025 (Alam et al., 2019, Muneer et al., 2020).

The L-asparaginases are widespread in nature, being found in plants, microorganisms (Gram-positive and Gram-negative bacteria, filamentous fungi, yeasts, algae) or animal tissues (pancreas, liver, brain, ovaries or testes, kidneys, spleen and lungs from fish, mammals or birds).

The applications in medicine, in the treatment of Acute Lymphoblastic Leukemia (ALL) are based on the effect of L-asparaginase that diminishes the supply of asparagine to cancer cells, leading to apoptosis. However, the clinical data proved that the depletion of glutamine due to the dual glutaminase and L-asparaginase activity of some of the commercial enzymes may lead to secondary effects like pancreatitis, central nervous system dysfunction, haemostasis abnormalities and immunological reactions due to antibody production (Ashok et al., 2019). Moreover, in some commercial preparations was detected the presence of urease activity, which can reduce the efficacy of enzymatic treatment of ALL. For this reason, as well as for industrial production of enzymes, new sources of L-asparaginase, that is free of glutaminase and urease activity, both prokaryotic and eukaryotic origin, remains the main objective of many scientists and are essential so to overcome the disadvantages associated with actual commercial products (*Escherichia coli* and *Erwinia chrysanthemi* asparaginases, and their derivative.) However, based on the main characteristics as producers (easy cultivation in non-expensive media, high biomass accumulation or increased secretion of the extracellular enzymes) microorganisms remain the main sources of the L-asparaginases.

CHARACTERISTICS OF L-ASPARAGINASE

L-asparaginase is an amidohydrolase characterized by the ability to catalyse L-asparagine to L-aspartate and ammonia (Shi et al., 2017).

The mechanism of action of L-asparaginases involves two steps with an intermediate - beta-acyl-enzyme (Figure 1). The first step of hydrolysis produced by L-asparaginase involves the activation of the nucleophilic residue of the enzyme by NH_2 and the production of the intermediate product beta-acyl-enzyme from L-asparagine. The second step of the process presume the hydrolysis of this intermediate to L-aspartic acid, with liberation of NH_3 (Shakambari et al., 2019).

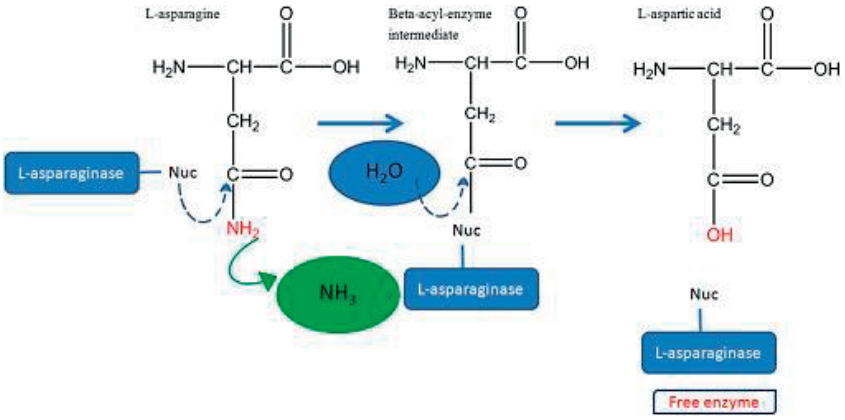

Figure 1. Mechanism of action of L-asparaginase (according Shakambari et al., 2019)

Normal cells as well as leukemic cells require L-asparagine for protein synthesis, but unlike malignant cells, normal cells are capable of synthesizing asparagine by using metabolic pathways that involve transaminase enzyme and asparagine synthetase. The transaminase converts oxaloacetate into an intermediate aspartate, which later on transfers an amino group from glutamate to oxaloacetate producing α-ketoglutarate and aspartate; then, aspartate is converted to asparagine by asparagine synthetase (Batool et al., 2016). In neoplastic cells, asparagine synthetase in not produced and exogenous asparagine is essential for protein synthesis. The presence of L-asparaginase in tumour tissues determines the hydrolysis of all exogenous asparagine, leading to the death of the cancer cells.

Based on the studies performed with bacterial L-asparaginases it was shown that the enzyme exists as a tetramer but hexameric, dimeric, and monomeric forms were detected in enzymes isolated from various sources (Batool et al., 2016). It was shown that the L-asparaginase is active as a homotetramer, with monomers of about 330 amino acid residues (Qeshmi et al., 2018). Bacterial L-asparaginases have many common biochemical properties (optimum pH, optimum temperature etc) with similar tertiary and quaternary structures. The results obtained demonstrated that most of the enzymes work optimally at a temperature of 40°C with a maximum range of pH 6.0-10, but thermostable L-asparaginases which work at 80-85°C were identified (Muneer et al., 2020). The properties of the L-asparaginases during biochemical processes as well as the kinetic parameters are vital in order to use enzymes efficiently in different industrial processes and, for these reasons, intensive studies are performed not only in identification of novel sources of enzymes but in characterization them.

SOURCES OF L-ASPARAGINASE

Occurrence of L-asparaginases in fungi, yeasts, bacteria, algae, plant cells, and animal cells, and their antitumor effects were reviewed by various scientists (Zuo et al., 2015; Shrivastava et al., 2016; Queshni et al., 2018; Shakambari et al., 2019; Muneer et al., 2020). The most intense studies were performed with microorganisms, both prokaryotic and eukaryotic. The microbial L-asparaginase studied until now can differ in their cellular location and properties, namely periplasmic asparaginase, extracellular asparaginase, intracellular asparaginase, and

glutaminase-asparaginase, and plays a role in basic metabolism (Zuo et al., 2015). Based on the studies performed with *E.coli* L-asparaginases, two types of enzymes that differ in their affinity for L-asparagine were described: type I of L-asparaginases are cytoplasmic enzymes that have a lower affinity to asparagine; the type II is found in the periplasmic space and has a high affinity to the substrate (L-asparagine), explaining their anti-leukemic activity (Souza et al., 2017; Cornea et al., 2002).

BACTERIAL L-ASPARAGINASE

Commercial asparaginases are produced using *Escherichia coli* and *Erwinia chrysanthemi* but a large number of Gram-positive and Gram-negative bacteria were identified as producers of L-asparaginase (Table 1). Bacterial L-asparaginases are differentiated by different affinity, different stability and different pH, the production rate that may be affected by the concentration of C and N sources, temperature, aeration rate, pH, time and inoculation (Ali et al., 2017).

The kinetic properties and biochemistry of the L-asparaginase vary depending on the microbial source. For example, L-asparaginase produced by *Erwinia* spp. is considered less toxic compared to the enzyme produced by *E.coli* that could cause allergic reactions. But the enzyme produced by *Erwinia* has a shorter half-life comparing with the *E. coli* enzyme (Prakasham et al., 2007).

Gram-negative bacteria (like *E. coli*) produce two types of L-asparaginase: L-asparaginase I (cytosolic enzyme that show relatively low affinity to L-asparagine and relatively high specific activity towards L-glutamine) and L-asparaginase II (periplasmic enzyme that displays low to negligible activity against L-glutamine in addition to high specific activity against L-asparagine) (Qeshmi et al., 2018).

In other Gram-negative bacteria, like *Rhizobium etli* were identified two L-asparaginases: a thermostable and constitutive L-asparaginase I, and a thermolabile, inducible (by asparagine) L-asparaginase II, repressible by the carbon source (Moreno-Enriquez et al., 2012). It was shown, according to the nucleotide sequence of the gene and the amino acid sequence of the protein, that *R. etli* asparaginase II are different from *E. coli* and *E. chrysanthemi* L-asparaginases. The potential application in chemotherapy of the L-asparaginase II from *R. etli* is related to the glutaminase-free property (Moreno-Enriquez et al., 2012).

In the genus *Bacillus* two L-asparaginases were also identified; they are encoded by specific genes: *ansZ* gene (encodes a functional L-asparaginase with 59% similarity to *E. chrysanthemi* L-asparaginase and 53% identity to the L-asparaginase II from *E. coli*) and *ansA* gene (encodes type I L-asparaginase) (Yano et al., 2008).

The production of L-asparaginase has been reported in different Actinobacteria, mainly in *Streptomyces* genus, isolated from various sources (soil, plant material, rivers, marine ecosystems etc) (Arévalo-Tristancho et al., 2019). These enzymes belong to the L-asparaginase II family, as it was demonstrated by phylogenetic tree analysis of *ansA* gene sequence, and the amino acid sequences of the enzymes are conserved among the similar proteins from different *Streptomyces* species (Mena et al., 2015). The advantages of L-asparaginase from streptomycetes are: production in extracellular manner, minimal or no L-glutaminase activity, and high activity at physiological conditions.

Relative recent studies demonstrated that several extremophiles (thermophiles and extreme halophilic bacteria) (*Pyrococcus, Thermus, Salinococcus, Thermococcus* etc) (Table 1) are able to produce thermostable L-asparaginase, efficient in a large range of pH (6.0-10).

Bacterial endophytes are now considered by many scientists as potential sources of L-asparaginase (Chow and Ting, 2015). According to Nongkhlaw and Joshi (2015), a number of endophytic bacteria isolated from ethnomedicinal plants identified as *Serratia marcescens* cenA, *Bacillus subtilis* cenB, *B. methylotrophicus* PotA and *B. siamensis* C53 are able to produce important quantities of L-asparaginase. Other authors (Krishnapura and Belur, 2015), reported high level of enzyme in 31 endophytic bacterial strains isolated from medicinal plants of the Family Zingiberaceae.

The marine environment is considered a good source of new bacterial strains capable of producing various bioactive compounds, including L-asparaginase. Recent studies

reported increased level of L-asparaginase produced by marine *Bacillus velezensis* (Mostafa et al., 2019), strains of *B. licehniformis* isolated from Red Sea (Alrumman et al., 2019), *Pseudonocardia endophytic* isolated from mangrove ecosystem (Muneer et al., 2020), actinomycetes (Dhevagi and Poorani, 2005) etc.

Table 1. L-asparaginase producing bacteria (adapted from Shakambari et al., 2019 and Muneer et al., 2020)

Bacteria	Species	References
Gram-negative	*E. coli*	Netrval, 1977, Cachumba et al., 2016
	Acinetobacter calcoaceticus	Joner et al., 1973 (cited by Shakambari et al., 2019)
	Erwinia cartovora	Maladkar et al., 1993 (cited by Shakambari et al., 2019)
	Acinetobacter soli	Jiao et al., 2020
	Pseudomonas stutzeri	Hosamani and Kaliwal, 2011 (cited by Muneer et al., 2020)
	Serratia marcescens	Agarwal et al., 2011(cited by Muneer et al., 2020)
	Rhizobium etli	Angélica et al., 2012 (cited by Muneer et al., 2020)
	Salmonella typhimurium	Kullas et al., 2012 (cited by Muneer et al., 2020)
	Thermus thermophilus	Pritsa and Kyriakidis, 2001 (cited by Muneer et al., 2020)
	Aeromonas sp.	Muslim, 2014
	Pseudomonas proteolytica	Shukla et al., 2014 (cited by Muneer et al., 2020)
	Pseudomonas aeruginosa	Kamble et al., 2012 (cited by Muneer et al., 2020)
	Pseudomonas fluorescens,	Sindhu and Manonmani, 2018
	Pectobacterium carotovorum	Jetti et al*.,* 2018
	Enterobacter aerogenes	Baskar et al., 2013 (cited by Muneer et al., 2020)
	Acinetobacter baumannii	Muslim, 2014
	Erwinia aroideae *Hydrogenomonas eutropha*	Ali et. al., 2017
	Aquabacterium sp.	Sun et al., 2016
Gram-positive	*Streptomyces gulbargensis,* *Streptomyces venezuelae*	Naveena et al., 2012 (cited by Muneer et al., 2020)
	Bacillus pseudomycoides *Paenibacillus denitriformis*	Joshi and Kulkarni (2016) Muneer
	Bacillus licheniformis, B. circulans	Gulati et al. (1997), Alrumman et al. (2019)
	Bacillus firmus (AVP 18)	Rudrapati and Audipudi (2017) (cited by Muneer et al., 2020)
	Streptomyces alkaliphilus, *Staphylococcus roseus*	Jha et al. (2012) (cited by Muneer et al., 2020)
	Staphylococcus capitis	Paglla et al. (2013) (cited by Muneer et al., 2020)
	Bacillus subtilis (hsw x 88)	Feng et al. (2017)
	Bacillus amyloliquefaciens	Yim and Kim (2019)
	Paenibacillus barengoltzii	Shi et al. (2017)
	Corynebacterium glutamicum	Ahmad et al. (2012)(cited by Muneer et al., 2020)
	Anoxybacillus flavithermus	Maqsood et al., 2020
	Lactobacillus reuteri (DSM 20016)	Aishwarya et al. (2017)
	Salinicoccus sp.	Jha et al. (2012) (cited by Muneer et al., 2020)
	Bacillus spp.	Safary ey al (2019)
Archaea	*Pyrococcus furiosus*	Jayam and Kannan (2014) (cited by Muneer et al., 2020)
	Pyrococcus yayanosii (CH1)	Li et al. (2018) (cited by Muneer et al., 2020)
	Thermococcus kodakaraensis	Chohan and Rashid (2013) (cited by Muneer et al., 2020)
	Thermococcus gammatolerans	Xue et al. (2014) (cited by Muneer et al., 2020)

FUNGAL ASPARAGINASES

There are many studies that demonstrated that eukaryotic microorganisms such as yeasts and filamentous fungi have potential for the production of L-asparaginase.

The advantages of the fungal enzymes are related to several characteristics: are stable, are excreted in the extracellular environment, are easy to extract and process downstream, and could be obtained in high quantities in relative cheap culture conditions (Dias et al., 2017; da Cunha et al., 2019). More than 85% of fungal L-asparaginases are produced by ascomycetes, and only 11% by species of basidiomycetes.

The most studied fungal L-asparaginase are originated from strains of *Aspergillus, Penicillium, Fusarium, Cladosporium* are studied for their ability to produce L-asparaginase (Table 2).

Table 2. Fungal producers of L-asparaginases

Fungal genus/group	Species	References
Aspergillus	*Aspergillus alliaceus,*	Gulati et al. (1997)
	Aspergillus tubingensis,	Gonçalves et al. (2017)
	Aspergillus oryzae (CCT 3940)	Dias et al. (2016)
	Aspergillus tamari, Aspergillus terreus	Sarquis et al. (2004)
	Aspergillus niger, Aspergillus nidulans,	Baskar and Renganathan (2011) (cited by Muneer et al., 2020)
	Aspergillus fumigatus	Benchamin et al. (2019) (cited by Muneer et al., 2020)
	Aspergillus sydowii	Ali et al. (2017)
Trichoderma	*Trichoderma viride*	Chow and Ting (2015)
	Trichoderma sp.	Muneer et al., 2020
Fusarium	*Fusarium culmorum* (ASP-87), *Fusarium equiseti*	Meghavarnam and Janakiraman (2018)
	Fusarium solani	Gulati et al. (1997)
	Fusarium oxysporum	Patro and Gupta (2012) (cited by Muneer et al., 2020)
	Fusarium roseum	Shakambari et al., 2019
	Fusarium incarnatum	Ali et al., 2017
	Fusarium tricinctum	Doriya and Kumar, 2016
	Fusarium verticillioides, Fusarium proliferatum	Chow and Ting, 2015
Penicillium	*Penicillium notatum*	Eisele et al. (2011)
	Penicillium sp.	Gulati et al. (1997)
	Penicillium simplicissimum	Chow and Ting, 2015
	Penicillium digitatum	Shrivastava et al., 2016
	Penicillium brevicompactum	Cachumba et al., 2016
Colletotrichum	*Colletotrichum gloeosporioides, Colletotrichum siamense*	Chow and Ting, 2015
Alternaria	*Alternaria* sp.	Doriya and Kumar, 2016
	Alternaria alternata	Shakambari et al., 2019
Cladosporium	*Cladosporium* sp.	Chow and Ting, 2015, Mohan et al., 2013
	Cladosporium cladosporioides	Ali et al., 2017
Beauveria	*Beauveria bassiana*	Nageshwara et al. (2014) (cited by Muneer et al., 2020)
Trichosporon	*Trichosporon asahii*	Ashok et al. (2019)
Yeasts	*Pichia polymorpha, Saccharomyces cerevisiae*	Jha et al. (2012) (cited by Muneer et al., 2020)
	Candida utilis	Shakambari et al., 2019
	Yarrowia lipolytica	Darvishi et al., 2019
Basidiomycetes	*Flammulina velutipes*	Eisele et al. (2011)

Generally fungal L-asparaginases are obtained by submerged fermentation (SmF) but the method has several disadvantages: it is too expensive, low concentration of the enzyme, large amount of effluents.

For these reasons, solid state fermentation (SSF) is preferred, as the yield of the product is higher (Mohan et al., 2013; Meghavarnam and Janakiraman, 2018).

Studies performed with strains of *Fusarium culmorum* cultivated on SSF media containing soybean meal and wheat bran in SSF medium reported 8.2 fold higher L-asparaginase production than in SmF (Meghavarnam and Janakiraman, 2018). Similar results were obtained by Souza et al., (2017) with strains of the genus *Aspergillus* in both solid and submerged fermentation.

Due to their properties, yeasts are considered as important producers of proteins useful for many applications. Relative recent data demonstrated the ability of strains of *Saccharomyces cerevisiae, Pichia* sp., *Yarrowia lipolytica* or *Candida utilis* to produce important quantities of L-asparaginase in presence of organic sources of nitrogen (2% proline, for example) (Sarquis et al., 2004, Darvishi et al., 2019; Shakambari et al., 2019, Muneer et al., 2020).

Despite the increased interest for compounds produced by macromycetes, the synthesis of L–asparaginase was detected only in strains of *Flammulina velutipes* (Eisele et al., 2011).

L-ASPARAGINASE FROM PLANTS

It was demonstrated that in higher plants, asparagine is catabolized by two metabolic routes: by transamination of the amino nitrogen by the asparagine-oxo-acid aminotransferase (as reported for soybean, pea, and lupine) or by L-asparaginase, releasing aspartate and ammonia (as in temperate legumes) (Qeshmi et al., 2018). The highest levels of L-asparaginase activity were detected mainly in leaves or roots of various plant species (Michalska et al., 2006). Two forms of L-asparaginase are described in higher plants: they are immunologically distinct, based on their dependence/independence of potassium and on the affinity for asparagine (Michalska and Jaskolski, 2006).

In seeds of *Lupinus* two types of enzymes have been also identified: a potassium independent form found in *L. arboreus* and *L. polyphyllus* and a potassium dependent form found in other *Lupinus* species and in *Pisum sativum* (Mohamed et al., 2015).

Significant amount of L-asparaginase was detected in green chillies (*Capsicum annum* L.) and tamarind (*Tamarindus indica* but the potential applicability in chemotherapy is reduced due to the low affinity to asparagine the simultaneous activity on glutamine (glutaminase activity) and urea (urease activity) (Bano and Sivaramakrishnan, 1980).

METHODS FOR SCREENING AND QUANTIFICATION L-ASPARAGINASE

The screening of microbial producers of L-asparaginase is based on the increase of pH as a consequence of ammonia liberation by enzymatic activity on a specific substrate (asparagine, glutamine, urea or $NaNO_3$). This property was used for development of rapid plate detection techniques using a pH indicator dyes incorporated in culture media that contain asparagine as sole nitrogen source (Gulati et al., 1997). Generally, two types of dyes are used for detection of pH modification: phenol red and bromothymol blue. Phenol red (PR) indicator is yellow under acidic condition and turns pink under alkaline condition due to increase in pH (Figure 2).

Bromothymol blue (BTB) is another pH indicator, coloured yellow at acidic pH, with a transient green color at neutral pH, 7.0 and dark blue at higher pH 8.0-9.0, useful for screening L-asparaginase activity, with an increased sensitivity and precision than the previous method (Mahajan et al., 2013) (Figure 3).

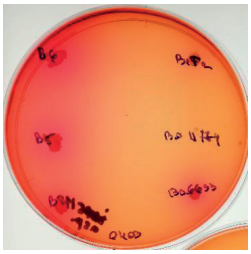

Figure 2. Screening of L-asparaginase producing bacteria in media containing asparagine and phenol red (original)

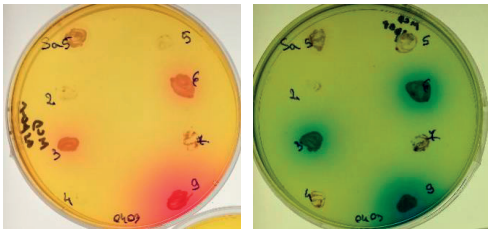

Figure 3. Screening of L-asparaginase producing yeasts in media containing asparagine and PR (left) or BTB (right) (original)

The level of enzymatic activity of L-asparaginase is very important in order to evaluate the efficiency and the practical approaches of the enzyme. The majority of the methods determine the amount of ammonia or aspartic acid released during the reaction or after the degradation of the asparagine (Batool et al., 2015). The method involves the enzymatic source (the supernatant, crude microbial lysate or purified enzyme) and Nessler reagent which leads to the appearance of a colour due to the liberation of ammonia during the enzymatic reaction (Shakambari et. al., 2019). Such methods allow the study of the influence of environmental factors on the enzymatic, and to optimize the pH and temperature values of the process (Goswami et al., 2019).

APPLICATIONS OF L-ASPARAGINASE

It is well documented that microbial L-asparaginase could be used in medicine to treat lymphoblastic leukemia and lymphosarcoma. Bacteria like *E. coli, E. carotovora, Bacillus* sp., *Enterobacter aerogenes, Corynebacterium glutamicum, P. stutzeri* and actinomycetes are described as good sources for asparaginase

(Narayana et al., 2008). More recent, fungal asparaginase has been shown to have acrylamide reduction effects in bakery products, chips, products that have a high starch component and which lead to high temperatures turn into carcinogenic products (Meghavarnam et al., 2018) (Figure 4).

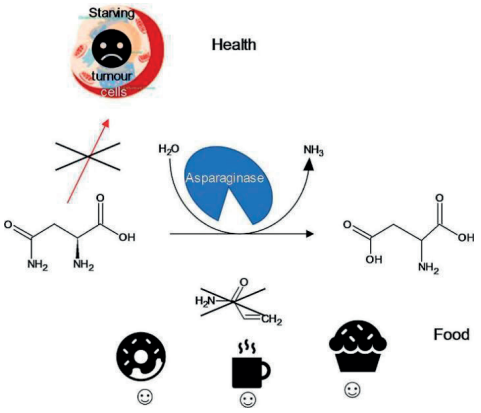

Figure 4. L-asparaginase hydrolyzes asparagine to aspartic acid, thus preventing feeding of asparagine to tumor cells and formation of acrylamide in heat-processed foods (Qeshmi et al., 2018)

The optimization of culture condition of the potential microbial producers allows the synthesis and the recovery of large amounts of the enzyme (Table 3).

ROLE OF L-ASPARAGINASE IN MEDICINE

For more than 30 years, L-asparaginase has been widely used in the treatment of acute lymphoblastic leukemia (ALL), the therapy being based on the intravenous injection of the enzyme resulting from *E. coli* and *E. chrysanthemi,* in combination with other drugs or radiotherapy (Qeshmi et al., 2018).

Table 3. Microbial strains producing L-asparaginase and their yield (adapted from Cachumba et al., 2016)

Name of the L-asparaginase producer	L-asparaginase activity
Pectobacterium carotovorum MTCC	35.24 U mg^{-1}
Bacillus licheniformis RAM-8	697.1 U mg^{-1}
Bacillus subtilis hswx88	23.8 U ml^{-1}
Bacillus aryabhattai ITBHU02	680.5 U mg^{-1}
Bacillus licheniformis MTCC 429	597.8 U mg^{-1}
Penicillium brevicompactum 829	574.24 U mg^{-1}
Fusarium solani	121 U/ml
Cladosporium sp.	83.3 U mg^{-1}

The amino acid L-asparagine is essential for the growth of tumor cells. This amino acid is not required for normal cell growth because they can synthesize the amount necessary for normal protein synthesis. Therefore, the L-asparaginase deprives the exogenous L-asparagine tumor cells so their growth is stopped (Han et al., 2014).

Bacterial L-asparaginase (from *E. coli* an *Erwinia chrysanthemy*) successfully treated acute lymphoblastic leukemia and other lymphoid malignancies. Several factors are involved in the mechanism of anti-leukemic action of L-asparaginase, for example: the hydrolysis capacity of L-asparaginase, pharmacological factors of serum clearance of the enzyme, growth of tumor cells resistant to asparaginase, activation of the immune system by producing anti-asparaginase antibodies (Shrivastava et al., 2016).

Three form of L-asparaginase are currently available for clinical applications: native L-asparaginase from *E. coli*, a PEG ylated (PEG: polyethylene glycol) form of L-asparaginase (PEG-asparaginase), and L-asparaginase from *Erwinia chrysanthemi* (*Erwinia* asparaginase). In treatment protocols, *E. coli* asparaginase or PEG asparaginase is used as a first-line treatment of childhood ALL, while *Erwinia* asparaginase has been adopted in European and US protocols for second - or third-line treatments (Shakambari et al., 2019).

Besides the advantages, bacterial L-asparaginases presents a number of problems: they can trigger allergic reactions, cause high toxicity including hepatotoxicity, acute pancreatitis, thrombosis and hyperglycemia, leading in some situations to the resistance of the disease to asparaginase (Shrivastava et al., 2016).

Hypersensitivity reactions resulting from the production of anti-asparaginase antibodies have been observed in 60% of patients at one time during *E. coli* asparaginase therapy. Symptoms include: anaphylaxis, pain, edema, Quincke edema, urticaria, erythema, rash, pruritis and major skin lesions (Pieters et al., 2010).

APPLICATIONS IN FOOD INDUSTRY

Foods containing starch, when subjected to high temperatures, may undergo changes, forming harmful compounds for the health of the consumer. One of these harmful compounds is

represented by acrylamide, formed in foods cooked at high temperatures. In fried potatoes, acrylamide is made up of reducing sugars and asparagine, being compounds of the Maillard reaction (Bethke and Bussan, 2013).

Acrylamide is generated as a by-product and usually occurs at temperatures above 100ºC and is noted for changing color and flavor in fried or baked foods containing starch. A significant amount was detected in fries, radishes, crispy chips, toast, cakes, biscuits, cereals and coffee (Alam et al., 2018). Recent studies demonstrated that the application of fungal L-asparaginase (isolated from *Aspergillus oryzae*) during potato frying significantly reduces the level of acrylamide (Dias el al., 2017). Similar aspects were observed with L-asparaginase isolated from *Fusarium culmorum*: the application of different concentration of this enzyme during the cooking of potatoes and bread reduce with 90% the formation of acrylamide (Meghavarnam and Janakiraman, 2018).

Many other L-asparaginases from diverse sources, such as *A. niger, B. licheniformis*, *B. subtilis, Cladosporium* sp., *Paenibacillus barengoltzii* or *Thermococcus zilligii* have been tested: the use of the enzyme allowed for a decrease from 34 to 97% of the acrylamide level in diverse food goods, namely biscuits, crisp bread, French fries, and sliced potato chips (Qeshmi et al., 2018).

The reduction of acrylamide level is based on the consumption of the asparagine precursor (Figure 4), without negative impact on organoleptic properties of the processed foods.

To mitigate the acrylamide formed in food, there are currently two commercial fungal asparaginases recognized by the FAO as a safe food additive: Prevent ASeTM 202 from a strains of *A. oryzae,* and Acrylaway® from a genetically modified *A. oryzae* (Xu et al., 2016).

CONCLUSIONS

L-asparaginase enzyme is a very important enzyme both for chemotherapy and for food industry. Bacterial L-asparaginase are currently used in the treatment of ALL, as first-line drug. Fungal asparaginases are useful in food industry, as food additive, for reduction the level of acrylamide produced during cooking at high temperatures.

The identification of new sources of L-asparaginase, free of glutaminase and urease activities represents the main objective of studies and are essential to overcome the disadvantages associated with actual commercial products.

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PRELIMINARY RESULTS ON BACTERIAL CROSS-RESISTANCE ASSESSMENT ASSOCIATED WITH POVIDONE IODINE USAGE

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Abstract

Povidone iodine (PVP-I) is a valuable, widespread antiseptic that could trigger bacterial cross-resistance to antibiotics when used at sub-lethal concentrations, although no acquired bacterial cross-resistance has been reported for PVP-I. In this study are assessed the changes in the antimicrobial susceptibility profile of Staphylococcus aureus and Staphylococcus epidermidis strains after five exposures to 0.3125 % PVP-I. For all Staphylococcus epidermidis tested strains the mean of the minimum inhibitory concentrations (MICs) and of the minimum bactericidal concentrations (MBCs) significantly decreased after repeated exposures to sub-lethal concentrations of PVP-I compared to the initial values. There was no significant change from baseline in mean results of the disk diffusion test of any antibiotic for the tests performed on the analysed strain.

Key words*: cross-resistance, Staphylococcus aureus, Staphylococcus epidermidis, povidone iodine, resistance.*

INTRODUCTION

Povidone iodine (PVP-I) is a valuable, widely used antiseptic in the healthcare industry.
Iodine compounds have immediate antimicrobial activity over a wide range of viruses, spores, bacteria, fungi and protozoa (Paulson, 1999). The antimicrobial effect of PVP-I is driven by cell wall penetration and binding capabilities with amino acids and unsaturated fatty acids leading to cell membrane disruption.
An extensive use of biocides at sub-lethal concentrations could trigger bacterial cross-resistance to antibiotics (Molina-González, 2014; Dopcea et al., 2019), thus still no acquired bacterial cross-resistance has been reported for PVP-I (Dopcea & Matei, 2018). Microorganisms of the genus *Staphylococcus* are one of the most numerous bacteria found on the human skin and they are an important source of healthcare-associated skin infections (Merezeanu, 2016).
In this study we aimed to assess the changes in the antimicrobial susceptibility profile of *Staphylococcus aureus* and *Staphylococcus epidermidis* when exposed to PVP-I.

MATERIALS AND METHODS

Four strains from ATCC were used: *Staphylococcus epidermidis* ATCC 12228 (MSSE), *Staphylococcus epidermidis* ATCC 51625 (MRSE), *Staphylococcus aureus* ATCC 43300 (MRSA) and *Staphylococcus aureus* ATCC 25923 (MSSA).
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of PVP-I (Alfa Aesar) for each tested strain were determined before and after exposure using a modified broth macrodilution method (CLSI, 2012, NCCLS 1999). Briefly, PVP-I was diluted into concentrations between 10% and 0.0195%. The inoculum suspension for each strain was adjusted to 1 x 10^6 CFU/ml and 10 µl of inoculum was added into the test tubes containing 2 ml of PVP-I diluted at different concentrations in Mueller-Hinton broth (MHB) (Tulip Diagnostics (P) Ltd.), also two control test tubes, a positive control tube containing

only PVP-I and a negative control tube containing only MHB, were inoculated. All the tubes were then incubated at 30°C for 24 h. The MIC was defined as the lowest concentration of PVP-I that completely inhibits growth of the tested strain in the tubes. For determination of MBC, 0.5 ml aliquots were plated on tryptic soy agar containing lecithin and polysorbate 80 (TSA+N) (Scharlau, Spain) from all tubes having no visible growth, followed by incubation at 37°C for 24 h. MBC was defined as the lowest PVP-I concentration corresponding to the TSA+N plate with no bacterial growth observed after incubation.

Antimicrobial susceptibility was determined for all strains before and after PVP-I exposure using the disk diffusion test (CLSI, 2006). The following antibiotics were used: penicillin (10 µg), gentamicin (10 µg), tetracycline (5 µg), ofloxacin (5 µg) and doxycycline (30 µg) (all from Becton Dickinson).

Five exposures variants of the strains to 0.3125% PVP-I were performed. Inoculum was produced from overnight culture and adjusted to 1 x 10^6 CFU/ml. Each exposure consisted of 1 ml Mueller Hinton Broth (MHB) (Accumix - Tulip Diagnostics), 1 ml PVP-I and 0.1 ml inoculum. The samples were kept for 24 h at 30ºC. All procedures were repeated two times for a total of three replicates.

Paired sample t test was used to compare MICs, MBCs, and the sizes of antibiotic zones of inhibition before and after exposure to PVP-I.

RESULTS AND DISCUSSIONS

The mean MICs and MBCs results are summarized in Table 1 and Table 2, respectively. Following all five exposure variants to PVP-I, the mean MICs and MBCs had the same values as before exposure for both *Staphylococcus aureus* strains, but for both *Staphylococcus epidermidis* strains a significant reduction in MICs and MBCs of PVP-I after exposure was recorded.

Table 1. Mean MICs results for all strains

	Baseline	After exposure to PVP-I
MSSE	0.3125%	0.1563%*
MRSE	0.3125%	0.1563%*
MRSA	0.3125%	0.3125%
MSSA	0.3125%	0.3125%

* Significantly different from baseline if p≤0.05

Table 2. Mean MBCs results for all strains

	Baseline	After exposure to PVP-I
MSSE	0.6250%	0.3125%*
MRSE	0.6250%	0.3125%*
MRSA	0.6250%	0.6250%
MSSA	0.6250%	0.6250%

* Significantly different from baseline if p≤0.05

The disk diffusion method results are summarized in Table 3 and their evolution between baseline and after PVP-I exposure for all tested strains are shown in Figure 1 for penicillin, Figure 2 for gentamicin, Figure 3 for tetracycline, Figure 4 for ofloxacin and Figure 5 for doxycycline.

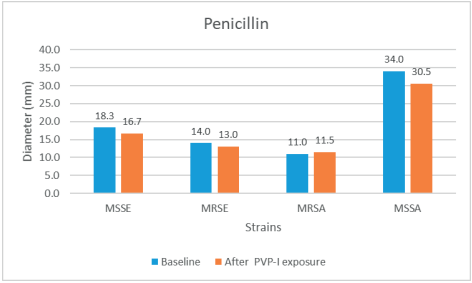

Figure 1. Disk diffusion method results on penicillin for all strains before and after exposure to PVP-I

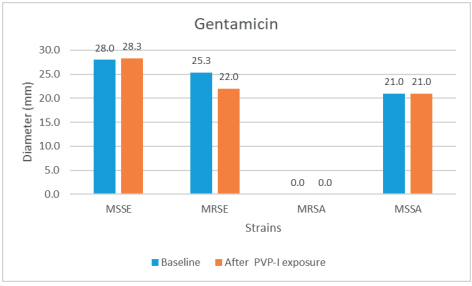

Figure 2. Disk diffusion method results on gentamicin for all strains before and after exposure to PVP-I

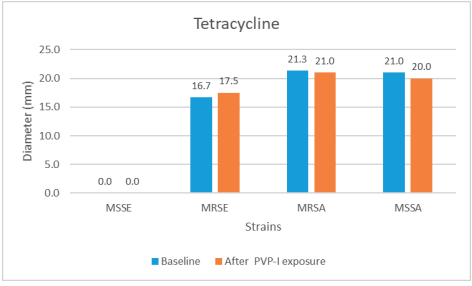

Figure 3. Disk diffusion method results on tetracycline for all strains before and after exposure to PVP-I

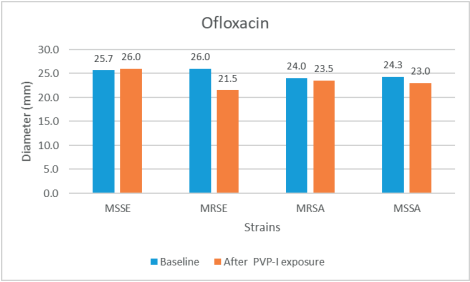

Figure 4. Disk diffusion method results on ofloxacin for all strains before and after exposure to PVP-I

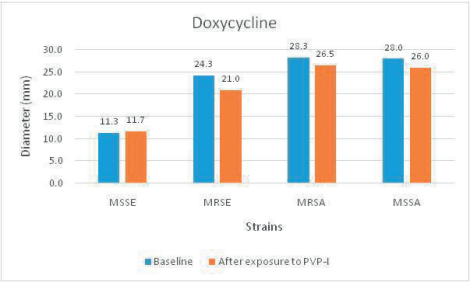

Figure 5. Disk diffusion method results on doxycycline for all strains before and after exposure to PVP-I

After the exposure to PVP-I a decrease in mean results of the disk diffusion tests compared to baseline ranging between 0.3 mm and 4.5 mm was shown. This decrease was observed on both *S. aureus* strains (MRSA and MSSA) for tetracycline, ofloxacin and doxycycline, and moreover for penicillin only on MSSA, also on *S. epidermidis* MRSE strain for penicillin, gentamicin, ofloxacin and doxycycline, however *S. epidermidis* MSSE strain showed a decrease only for penicillin.

There is no significant change from baseline in mean results of the disk diffusion test of any antibiotic for any strain (Table 3). For all four strains there was no clinical change in antimicrobial susceptibility to any of the antibiotics tested following exposure to PVP-I (Table 3). Dopcea et al. (2019) reported no clinical change in antimicrobial susceptibility to any of the antibiotics tested following exposure of the same strains to octenidine dihydrochloride, but when these strains were exposed to chlorhexidine digluconate a clinically decreased susceptibility was observed to penicillin and tetracycline for MSSA and gentamicin for MRSA.

Table 3. Disk diffusion method results for all strains before and after exposure to PVP-I

Penicillin
[Mean diameter (mm) ± SD]

	Baseline		After exposure to PVP-I	
MSSE	18.33 ± 0.15	R	16.67 ± 0.25	R
MRSE	14.00 ± 0.10	R	13.00 ± 0.28	R
MRSA	11.00 ± 0.00	R	11.50 ± 0.07	R
MSSA	34.00 ± 0.26	S	30.50 ± 0.21	S

Gentamicin
[Mean diameter (mm) ± SD]

	Baseline		After exposure to PVP-I	
MSSE	28.00 ± 0.20	S	28.33 ± 0.23	S
MRSE	25.33 ± 0.25	S	22.00 ± 0.71	S
MRSA	0.00 ± 0.00	R	0.00 ± 0.00	R
MSSA	21.00 ± 0.17	S	21.00 ± 0.00	S

Tetracycline
[Mean diameter (mm) ± SD]

	Baseline		After exposure to PVP-I	
MSSE	0.00 ± 0.00	R	0.00 ± 0.00	R
MRSE	16.67 ± 0.15	I	17.50 ± 0.07	I
MRSA	21.33 ± 0.15	S	21.00 ± 0.14	S
MSSA	21.00 ± 0.10	S	20.00 ± 0.14	S

Ofloxacin
[Mean diameter (mm) ± SD]

	Baseline		After exposure to PVP-I	
MSSE	25.67 ± 0.15	S	26.00 ± 0.10	S
MRSE	26.00 ± 0.20	S	21.50 ± 0.35	S
MRSA	24.00 ± 0.10	S	23.50 ± 0.21	S
MSSA	24.33 ± 0.06	S	23.00 ± 0.00	S

Doxycycline
[Mean diameter (mm) ± SD]

	Baseline		After exposure to PVP-I	
MSSE	11.33 ± 0.06	R	11.67 ± 0.12	R
MRSE	24.33 ± 0.12	S	21.00 ± 0.28	S
MRSA	28.33 ± 0.15	S	26.50 ± 0.21	S
MSSA	28.00 ± 0.17	S	26.00 ± 0.00	S

[S]Susceptible
[I]Intermediate
[R]Resistant
*Significantly different from baseline if p≤0.05

The usage of PVP-I as an antiseptic, even at sub-lethal concentration, does not lead to significant variations in antimicrobial susceptibility profile of the strains used in this study.

Although *Staphylococcus aureus* strains, and *Staphylococcus epidermidis* strains did not showed increased MIC and MBC values to PVP-I following exposure and no resistance to these strains was demonstrated, still PVP-I should be tested for resistance against other isolated strains from healthcare facilities.

Among other antiseptics PVP-I remains one of the best options for human skin disinfection in the healthcare facilities, due to its' effectiveness,

safety for use in humans and lack of induced resistance.

CONCLUSIONS

Both *S. epidermidis* strains became more susceptible to PVP-I following exposure to PVP-I.

There was no clinical change in antimicrobial susceptibility profile of any strain used in our study following exposure.

In the conditions of this study the antimicrobial susceptibility profile of all tested strains to penicillin, gentamicin, tetracycline, ofloxacin and doxycycline suffered variations, either to increase the susceptibility or to decrease the susceptibility, which were not statistically significant, compared to the initial values before PVP-I exposure.

Staphylococcal exposure to PVP-I, under the conditions of this study, did not change significantly the antimicrobial susceptibility profile.

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PURIFICATION FLOW OF COSMETIC CAMELINA OIL

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Abstract

Camelina oil is obtained by pressing the seeds of Camelina sativa. Their pressing is done by a cold process. The oil samples used were obtained from the Camelina sativa - Mădălina variety, which was cultivated at the Belciugatele farm, with a working point Moara Domneasca (Găneasa commune, Ilfov county) between 2017-2018. The present project aims at the use of camelina oil in cosmetic preparations, using 4 types of oil grown in an ecological system, but their storage was different namely: some of the samples were kept at ambient temperature, in plastic dark blue cans, their capacity being 10l, but they were filled with 5l oil, in parallel being kept experiments at the refrigerator in brown glass containers, fully filled, in order to obtain the purest oil, but also the most complete one in terms of preservation of the biochemical qualities. The determinations made consisted of: appearance, odor, color, determination of iodine and saponification indices, as well as composition in fatty acids. All these determinations have led to the most efficient flow of camelina oil purification used in the cosmetic industry.

Key words*: bentonite, Camelina sativa, purification, volcanic tuff, zeolite.*

INTRODUCTION

Camelina is an oilseed plant belonging to the *Brassicaceae* family, which has low agronomic requirements (Putnam et al., 1993). *Camelina sativa* (L.) Crantz (2n = 40) is part of the same families with mustard, rapeseed, shepherd's purse, but also the plant that was used as a model for research in molecular genetics: *Arabidopsis thaliana* (Vollmann and Eynck, 2015).

Camelina sativa has a high adaptability to climate conditions, being able to grow in temperate climate, semi-arid environments, and short season regions (Ehrensing and Guy, 2018). Due to its increased adaptability, this plant can be found and developed in various countries, so the plant is distributed almost throughout the North American continent and in Europe.

From the fatty acid profile point of view, this is unusual with a high alpha-linoleic acid content and relatively low erucic acid concentrations (Zubr and Matthaus, 2002).

The applications for camelina oil include paint industry, biodiesel fuels as well as cosmetics. (Bonjean and Goffic, 1999; Bernardo et al., 2003).

The presence of polyunsaturated fatty acids gives sensitivity to camelina oil to lipid oxidation, but remains sufficiently stable during storage due to antioxidants present in the seed (Ni Eidhin et al., 2003; Abramovič and Abram, 2005). Camelina oil is the main compound obtained from Camelina seeds, and its yield is between 30-40% DM (Budin et al., 1995; Zubr, 2003). The oil obtained from Camelina seeds has a varied content in fatty acids, having a supply of 50-60% unsaturated fatty acids, 35-40% content of omega 3 as well as a content of 15-20% omega 6. It has a content high in omega 3, being one of the richest plant sources related to this fatty acid (Ratusz et al., 2016). Some of the most important attributes of camelina oil refer to its high productivity, which does not require a special and complicated cultivation technology, as well as its multiple uses in various industries (Toncea et al., 2013).

In one research made by Popa et al. (2019) about physico-chemical characterization of oil from *Camelina sativa* seeds grown in Romania, they had analyzed three types of oil (romanian and spanish oil).

The analysis of the oil extracted from the three Camelina varieties lead to the conclusion that the samples are very similar. The both are similar from the point of view of the type of extraction and the fatty acid profile. Another result is about the fertilization and the use of fertilizers, which do not conduct to significant quantitative or qualitative changes in the oils. They all are in accordance with the physico-chemical parameters specified by the European Pharmacopoeia for virgin linseed oil (Popa et al., 2019). Other studies on chemical composition have referred to the amino acids content. Camelina cracks were analyzed and the results indicated a high content of essential amino acids with values between 3.89% and 16.12% (Bătrâna et al., 2019). Another very important aspect regarding the composition of amino acids, refers to the essential amino acids that were found in all three varieties analyzed, namely: 38% for Madalina 37.9% in Calena variety, and for the local variety of BUASVMT 38.58%, along with 9 other non-essential amino acids (Bătrâna et al., 2019). It is well known that only plants can synthesize essential amino acids, so animals must obtain them from food. Their lack can significantly affect the quality of life and the appearance of certain diseases, therefore their importance should not be neglected.

Crăciun et al. (2018) made a research about the benefits and effect of the *Camelina sativa* oil for the skin. They had a revolutionary discover namely: Camelina oil, through its rich composition in fatty acids, accelerates the dermo-epidermal "de novo" synthesis, stimulating the keratinocytes differentiation and turn-over and also fibroblasts cellular division and collagen synthesis (Crăciun et al., 2018).

The linoleic and linolenic acids found in camelina oil have some interesting effects following topical application, as: tissue regeneration, involvement in membrane lipid transport and protective effect against chemical and enzymatic agents (Crăciun et al., 2018).

MATERIALS AND METHODS

For this project, we used the oil that was obtained by pressing the seeds of *Camelina sativa* from the variety Mădălina. This variety was cultivated in an ecological system at Belciugatele farm, having a working point Moara Domnească from Găneasa commune, Ilfov county during 2017-2018. The oil filtration was performed by a cold process, followed by the retention of the 4 samples being performed differently. Two of these (samples 1 and 2) were stored at room temperature in cans with a capacity of 10 l, but partially filled.

Contrary to this, the other two samples (samples 3 and 4) were kept in the refrigerator at temperatures of 4 degrees Celsius in brown glass containers, fully filled. A cold mechanical press was used in order to obtain the oil.

The extraction equipment used is called IEU-00 from the National Institute of Research and Development for Machinery and Installations designed for Agriculture and Food Industry-INMA Bucharest. The seeds were conditioned before pressing to eliminate impurities, using an ICS equipment from INMA-Bucharest. The oil was left at room temperature for 48 hours in order to decant, after that the oil being separated from the sediment.

Camelina oil purification method

The filtration was performed with the help of 3 adjuvants, namely: zeolite, bentonite and volcanic tuff. The samples were filtered differently, namely:

- Sample 1 - stored at ambient temperature - pretreatment with 0.05% bentonite - treatment with 0.05% bentonite - stored at 4°C;
- Sample 2 - stored at ambient temperature - pretreatment with 0.05% zeolite - treatment with 0.05% bentonite - stored at 4°C;
- Sample 3 - stored at 4 °C - 0.05% bentonite treatment - stored at 4°C and
- Sample 4 - stored at 4°C - 0.05% zeolite treatment - stored at 4°C.

Two of the 4 samples were filtered through a single stage and the other 2 samples were pre-treated. For one-step purification, the samples were treated with bentonite respectively zeolite at a concentration of 0.05%.

Purification performed by two steps introduced a pretreatment with 0.05% zeolite, respectively, to subsequently perform a filtration with 0.05% bentonite (Figure 1).

The working protocol used was aimed at removing as much of the impurities as possible to obtain an oil as pure as possible.

Figure 1. Camelina oil filtration

The filtering method (Table 1) was the same for all samples as follows:
Seeds => Seed pressing (V1) => Adding hot water (V1 + 10 mL) => Centrifuging oil (V2) => Adding bentonite or zeolite => 30 minute rest => Oil filtering (V3)
The pH is determined both before and after filtering the oil.
The addition of hot water over the oil sample aims to "wash" the oil of impurities. Once the hot water is added, rotating movements are performed for a few minutes in order to carry out the oil washing process as well as possible. After this process, the oil together with the water is distributed in vacuums and centrifuged at 5000 rpm for 5 minutes. After centrifugation, when the water has separated from the oil, a rapid movement to remove the water from the oil is performed. When all the vacuums have been emptied of oil as well as the removed water, over the oil collected from the centrifugation, the 0.05% bentonite and zeolites will be added, leaving to stand for 30 minutes. When the 30 minutes of rest had elapsed, the oil was filtered resulting in the amount of oil remaining after filtration, oil that will be analyzed and used in cosmetic preparations (Figure 2).

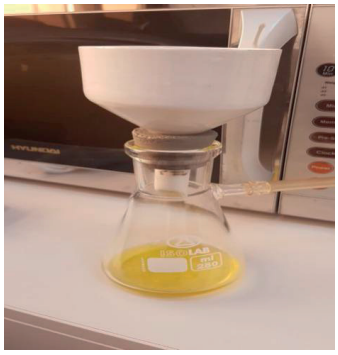

Figure 2. Camelina oil after filtration

The storage of samples before filtration was different, so that samples 1 and 2 were stored in plastic cans at room temperature, and samples 3 and 4 were stored in a dark color container at a temperature of 4°C. After filtration, storage was done in dark colored bottles in the refrigerator, at a constant temperature of 4°C (see Table 1).

Table 1. Filtering protocol (Copaci et al., 2019)

No.	Seeds (g)	V1 (ml)	V1+ 10 ml	V2 (ml)	Bentonite (g)	Zeolite (g)	Rest (min)	pH1	pH2	V3 (ml)	The color of the oil
1	400	90	100	79	0.4	0	30	5	5.5	70	light yellow, no deposits, clear
2	400	90	100	83	0.4	0	30	4.5	5.5	65	light yellow, no deposits, clear
3	200	50	60	48	0.22	0	30	5	5.5	40	dark yellow, no deposits, opaque
4	200	53	63	48	0	0.402	30	5	5	31	dark yellow to green, no deposits, opaque, water bubbles

RESULTS AND DISCUSSIONS

Following these purification processes, the main purpose is to find and optimize the best filtration method as well as the oil purification. The pretreatments applied were with bentonite or zeolite in a concentration of 0.05%, having the same methodology as the subsequent filtration. In the case of oil samples 1 and 2 after the pretreatment with bentonite and zeolite respectively, a treatment with 0.05% concentration bentonite was applied. For sample 3, only 0.05%

bentonite treatment was applied, and for sample 4 zeolite 0.05% treatment.

For the analyzed parameters, the values can be found in Table 2. In the literature, until now, the camelina oil has not been well individualized for use in cosmetic products. Due to the fact that it is not individualized, there is no standard in the European Pharmacopoeia, but also in the American Pharmacopoeia (USP), being analyzed as a newly introduced ingredient.

In order to be able to characterize each parameter, it will be analyzed in comparison with the oils already used and standardized for the cosmetic industry. The macroscopic purpose of these filtrations was to remove the existing physical impurities following the pressure, and at the physio-chemical level not to affect their chemical properties, namely: relative density d2020, refractive index nD20, acidity index (mg KOH/g), the iodine index (g I/100 g), the saponification index (mg KOH/g) and the peroxide index (meq O_2/kg).

Based on the analyzes performed at the physio-chemical analysis center S.C. BIOTEHNOS SA, the obtained results could be comparable with those already existing in the literature, determined by Abramovič and Abram (2005) for *Camelina sativa* oil from Slovenia: density at 20°C being 0.9207 ± 0.0001 g/cm^3, index of refraction at 25°C having a value of 1.4756 ± 0.0001, the peroxide determination being 2.38 ± 0.01 meq O_2/kg, and the acid value -6.2 ± 0.1.

Of the 4 samples, with regard to color, only the oil in sample 4 (without pretreatment + treatment with 0.05% zeolite) had small changes compared to the rest of the samples, namely "weak opalescent liquid", but it complied with the accessibility conditions. imposed. The yellow color and the specific odor of the plant were fully satisfied by all the samples regardless of the filtration method applied. Ullmann's encyclopedia did not report color changes or odor respectively, so that the oil was not degraded by filtration with zeolite or bentonite.

For the sample without pretreatment + treatment with 0.05% zeolite the peroxide index was within the allowed limits, having a value of 9.44 meq O_2/kg. The storage time of vegetable oils depends mainly on the phospholipid profile and the presence of antioxidants. External factors such as temperature, air and light are also decisive factors (Zubr and Matthaus, 2002). In the specialized literature, a peroxide limit of maximum 10 meq O_2/kg was set by SON (Standard Organization of Nigeria) (2000) and NIS (Nigerian Industrial Standard) (1992) (Zahir et al., 2017). Its high α-linoleic acid (ALA) composition is well known, and this high content indicates the susceptibility of camelina oil to oxidation.

Camelina sativa has a much higher content of fatty acids compared to other oilseeds, having a high content (over 50%) in polyunsaturated fatty acids especially OMEGA-3 type where we find α-Linolenic acid (18: 3n - 3) in a percentage of 38% and 15% linoleic acid (18: 2n - 6), followed by other important acids such as: oleic acid and eicosenoic/gadoleic acid.

Table 2. Chemical and physical characteristics of Camelina oil
(Certificate of analysis no. 77 / FC1 / 05.05.2019, S.C. BIOTEHNOS S.A.)

Physical chemical characteristics	Conditions of admissibility	Results			
		Sample 1	Sample 2	Sample 3	Sample 4
Peroxide index, meq O_2/kg	Max 10,0	**188,59**	**188,56**	**37,78**	9,44
Color	yellow	yellow	yellow	yellow	yellow
Acidity index, mg KOH/g	Max 10	1,462	1,409	1,730	1,997
Relative density, d2020	0,9100-0,9300	0,9278	0,9270	0,9233	0,9232
Refraction index, nD20	1,4700-1,4800	1,47751	1,47753	1,47747	1,47748
Saponification index, mg KOH/g	160-200	183,08	183,95	181,63	180,21
Iodine index, g I/100 g	130-170	145,02	141,82	146,70	147,28
Aspect	Clear or slightly opalescent liquid	Clear liquid	Clear liquid	Clear liquid	slightly opalescent liquid
Odor	plant specific	plant specific	plant specific	plant specific	plant specific

Another parameter analyzed was the pH of the four samples before and after filtering (Figure 3). In the case of sample 1 (stored at ambient temperature - pretreatment with 0.05% bentonite - treatment with 0.05% bentonite - stored at 4°C)

the initial pH was 5, and after treatment, the pH increased to 5.5.
For sample 2 (stored at ambient temperature - pretreatment with 0.05% zeolite - treatment with 0.05% bentonite - stored at 4°C) the initial pH was 4.5, identifying a pH of 5.5 after filtering. As with the other 2 samples, at sample 3 (stored at 4°C - 0.05% bentonite treatment - stored at 4°C) the initial pH of 5, increased to 5.5.
Last but not least, sample 4 (stored at 4°C - 0.05% zeolite treatment - stored at 4°C), the sample that did not show pH changes after the filtration. As can be seen from the above data, the filtration with zeolites without an effective pretreatment did not change the pH of the oil, a parameter that has utility for choosing the best filtration method (Table 3).
pH is very important no matter in which industry the oil is used, but for the cosmetics, food and pharmaceutical industry, this parameter is vital, and can have toxic and harmful implications for the human body. Fortunately, no filtration method has significantly altered the quality of the oil and thus the pH.

Figure 3. pH analysis of samples

Table 3. pH determination

Sample	Treatment	pH before filtration	pH after filtration
1	stored at ambient temperature - pretreatment with 0.05% bentonite - treatment with 0.05% bentonite - stored at 4°C	5	5.5
2	stored at ambient temperature - pretreatment with 0.05% zeolite - treatment with 0.05% bentonite - stored at 4°C	4.5	5.5
3	stored at 4°C - 0.05% bentonite treatment - stored at 4°C	5	5.5
4	stored at 4°C - 0.05% zeolite treatment - stored at 4°C	5	5

CONCLUSIONS

From our results we can see that the best method of purification was illustrated by sample 4 (without pretreatment + 0.05% zeolite treatment), samples 1, 2 and 3 do not correspond to a very important parameter, respectively, the "Peroxide index". As it is not possible to speak of a complete description of the camelina oil for its use in cosmetic products, the filtration technology applied to sample 4 (without pretreatment + treatment with 0.05% zeolite) had the best results in terms of maintaining the qualities nutrients, lipid profile, resistance to oxidation.
Oxidative stability is a significant distinguishing feature for camelina oil, which makes the emphasis not only on the filtration method but also on the pre- and post-filtration conditions.

Although the storage conditions after filtering were the same, a defining parameter was temperature prior to this process. For samples 3 and 4 even though they were kept at the same storage conditions, the zeolite filtration was much better able to meet all the required parameters.
Although the other samples were not significantly affected by the pH adjuvant filtering, it is still considered the best method that had no influence. As we said, the best results for maintaining the pH were recorded for sample 4, which further strengthens the selection of this method as the most suitable for preserving the oil properties after filtering.

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BIOTECHNOLOGICAL APPROACHES TO *Paulownia IN VITRO* PROPAGATION AND *IN VIVO* ADAPTATION

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Abstract

Different species and hybrids of Paulownia are valuable technical, bioenergetic, medicinal and decorative crops cultivated all over the world. We investigated in vitro microclonal propagation of hybrid Paulownia elongata × Paulownia fortunei via auxiliary buds activation. The phytohormone regulation of the growth and development of cuttings was studied on the base of modified MS medium with different concentrations of 6-benzylaminopurine with and without 0.2 mg/l 3-indole-acetic acid. The addition of 2.0 mg/l alone appeared to be optimal for new shoot formation. The best for rooting was ½MS supplemented with 1.0 mg/l indole-3-butyric acid. The mass loss of wood at the stage of cellulose decomposition was ranged from 57.3 to 63.9% in two versions of soil profiles containing water retention layer. The initial results allow estimating P. elongata × P. fortunei as a plant which demonstrated stable adaption potential as bioenergy tree.

Key words*: adaptation, biochar, microclones, phytohormones, rhizogenesis.*

INTRODUCTION

The biomass of woody plants, which is predominantly wood, can provide alternative energy raw materials and simultaneously reduce the emissions of toxic gases that accompany the combustion of hydrocarbons (Lemus and Lal, 2005). *Paulownia* is an important energy, decorative, landscaping plant that is becoming more widespread in the world as well as in Ukraine. It is a fast-growing woody energy plant, which makes it possible to create high-productive plantations with a long service life. Last decade *Paulownia* tree has been considered as "magic" because of its fast growth rate and the high amount of the wood generated in a short time period. Each *Paulownia* tree aged 5-7 years old can generate 1 m^3 timber in the plantation with density of 2000 plants/ha, offering a total production of 330 t/ha. In the areas planted with a smaller number of plants *per* area unit can reach a production of 150 t/ha (Icka et al., 2016). However, the investigations of the possibility of *Paulownia* cultivation on different types of soil and in specific climatic conditions to preserve its properties as a bioenergy crop remains relevant. *Paulownia* is actively investigated as a medicinal plant for humans (Zhang et al., 2019; Wang et al., 2019) and animals (Yang et al., 2019). Molecular and genetic characteristics of this genus are being intensively studied in connection with problems of taxonomy, phylogeny and systematics (Li et al., 2020), the nature of gene expression and its regulation by biotic and abiotic factors (Fan et al., 2015; Wang et al., 2019). At the present stage of production this woody, vegetative propagating crop needs a sufficient amount of high quality planting materials. The technology of the plant microclonal propagation via the activation of axillary buds *in vitro* allows obtaining quickly the required number of cuttings of a particular genotype. In general, this technology consists of three main steps. The first one is the sterilization of plant material and its introduction into culture *in vitro*. The second one is the sterile multiplication, most often via activation of axillary buds with the formation of newly formed shoots and their following 5-7-fold cutting. The third step is the rooting of the obtained microclones *in vitro*. The next technological operation is the transplantation of rooted microclones into the soil, their adaptation and *in vivo* cultivation according to the

requirements of a crop (Cherevchenko et al., 2008). For many species of *Paulownia*, the choice of the type of microclonal propagation and the optimization of all its stages is an urgent task. The solution of this task is in the plane of the use of phytohormones and other biologically active substances both in the nutrient media at the stages of cutting and rooting *in vitro* as well as at the stage of regenerated plantlets adaptation in the soil.

For species *P. tomentosa*, *P. elongata*, a hybrid *P. tomentosa* × *P. fortunei*, there is contradictory information regarding the efficiency of representatives of main classes of phytohormones such as auxins, cytokinins and their combinations in microclonal propagation *in vitro* (Bahri and Bettaieb, 2013; Rahman et al., 2013; Pozoga et al., 2019). The study of other biologically active substances which mechanism differs from classical phytohormonal regulation is especially interesting at the stage of rooting and plantlets adaptation in the soil. Different techniques and methods were used for this purpose. Thus, positive effect of bacteria *Bacillus meganterium* ONU 500 on adaptation of *P. tomentosa* microclones in soil after *in vitro* conditions was shown (Tesliuk and Avramovich, 2019). For intensification of *in vitro* rooting *P. tomentosa* × *P. elongata*, vermiculite instead of agar was used and the addition of 2.0-2.5 g/l activated charcoal was effective (Filipova et al., 2019). In this regard the use of biochar as an admixture to a nutrient medium or substrate for *in vivo* plant growth and development is of significant interest and was not previously been studied for microclonal propagation of *Paulownia*. Biochar (biocoal) is a substance derived from biomass carbonation. It can be used to bind carbon from the atmosphere (Mulabagala et al., 2015). Biochar can be added to soils to improve substrate function. There is growing interest in the use of biochar to mitigate the effects of global warming and increase plant bioproductivity (Wang et al., 2012). It has been established that biochar can accelerate plant growth by improving the chemical, physical and biological properties of soil (Glaser et al., 2002; Lehmann and Rondon, 2006). The positive effect of biochar is related to neutralization of pH of acidic soils and improving their physical properties - the ability to retain water, preserve nutrients, and reduce nutrient loss under leaching (Lehmann et al., 2003; Lehmann, 2007). The negative impact of biochar on plant bioproductivity was recorded at a deficiency of microelements distorted by high pH values of biochar (Mikan and Abrams, 1995). Differences in the plant response on biochar depend on the properties of a sample, soil type, plant species (Chan et. al., 2007; Rondon et. al., 2007; Majeed et al., 2018).

Thus, the study of the peculiarities of the influence of biologically active substances on the efficiency of microclonal propagation by activation of auxiliary buds for industrial cultivars of *Paulownia* is actual. In this regard, the purposes of this work were to investigate the effect of phytohormones of the class of auxins and cytokinins as well as their combinations at the cutting stage and the effect of biochar at the rooting stage for optimization of *Paulownia* microclonal propagation *in vitro*. We also aimed to investigate the special bioenergy properties of *Paulownia* microclones after cultivation *in vivo* on different types of soil substrate.

MATERIALS AND METHODS

Hybrid *Paulownia elongate* × *Paulownia fortunei*, the industrial cultivar, was used as the material of the investigations. The donor plants were grown in the vegetative vessels of 20 liters in volume in the substrate consisted of 9 black soil: 1 sand, moistened twice a week. The donor annual plant represented itself a tree of 1 m height with one erect main shoot, which housed non-lignified branches with opposite leaves. In the sinus of each leaf one auxiliary bud was situated. Cuttings of 1 cm length were isolated from donor plants, directly from young, non-lignified branches of 2-3 months old for further sterilization and explantation onto the nutrient medium. Leaves were removed from the selected cuttings before explantation to eliminate superfluous, undesirable contamination. Thus, the cuttings included a region of a stem and two buds located into the nodes. These cuttings were sterilized in a saturated solution of calcium hypochlorite for 10 minutes and washed five times with sterile distilled water. Sterile cuttings were implanted on a nutrient medium for induction of shoot development via activation of axillary buds.

This medium contained macro-, microcomponents MS (Murashige and Skoog, 1962), 2.5 mg/l lysine, 30 g/l sucrose, 7 g/l agar but did not contained phytohormones. Cuttings were cultivated for 20 days at a temperature of 25°C, 16 h photoperiod and the light intensity of nearly 1500 luxes. After 20 days, as soon as the newly formed shoots had been developed from buds the first sterile cutting was made on the medium of the same composition - MS with 30 g/l sucrose, 7 g/l agar, without phytohormones. After 20 days in culture the newly formed shoots had grown, and these ones were cut ones more. Received cuttings consisted of a stem region of 1.0 cm in length and two opposite auxiliary buds with the residues of leaf scapes (Figure 1).

Figure 1. Cuttings of *P. elongate* × *P. fortunei* explanted on the nutrient media for microclonal propagation

They were used as the material in the first experiment on the effect of phytohormones on the effectiveness of the next (the second) cycle of sterile cutting. This experiment was carried out in the base of the control medium for cutting contained macro-, microcomponents and vitamins MS, 2.5 mg/l lysine, 30 g/l sucrose and 7 g/l agar. To study the potential of the multiplication, cytokinin 6-benzylaminopurine (BAP) in concentrations of 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l was added to the control medium as well as the combinations of these concentrations of BAP with 0.2 mg/l auxin 3-indole-acetic acid (IAA) - Table 1.

Table 1. The content of phytohormones in the medium for cutting of *P. elongate* × *P. fortunei* as the scheme of experiment

Trial	IAA (mg/l)	BAP (mg/l)	Trial	IAA (mg/l)	BAP (mg/l)
1 (Control)	0.0	0.0	7	0.2	0.0
2		0.5	8		0.5
3		1.0	9		1.0
4		1.5	10		1.5
5		2.0	11		2.0
6		2.5	12		2.5

Note: IAA = 3-indole-acetic acid, BAP = 6-benzylaminopurine

Cuttings in the second sterile cycle were cultivated at a temperature of 25°C, 16-hour photoperiod and the light intensity of nearly 1500 luxes. In the experiment on the effect of phytohormones on the multiplication of *P. elongate* × *P. fortunei* shoots 50 cuttings were explanted *per* a variant. The analysis of the results was carried out on the 30th day of cultivation counting from the explantation of cuttings in the second sterile cutting cycle.

The effects of phytohormones on the growth and development of cuttings were estimated on the following traits:

- the shoot formation frequency, % - percentage ratio of the number of cuttings with at least 1 newly formed shoot to the total number of cuttings explanted;
- a number of newly formed shoots *per* 1 explanted cutting, pcs. - ratio of total number of newly formed shoots to total number of cuttings explanted;
- a number of internodes *per* 1 newly formed shoot, pcs. - the ratio of the total number of internodes on newly formed shoots to the total number of newly formed shoots;
- a number of internodes of newly formed shoots *per* 1 explanted cutting, pcs. - the ratio of the total number of internodes on the newly formed shoots to the total number of cuttings explanted. This trait is integral and conbines both the number of newly formed shoots *per* a cutting and the number of internodes *per* a newly formed shoot.
- the length of newly formed shoots, cm;
- the frequency of rooting,% - the percentage ratio of the number of rooted cuttings to the total number of cuttings explanted.

In the second experiment, the effect of biochar on root formation of *P. elongate* × *P. fortunei* cuttings *in vitro* was studied in the next, third cycle of sterile cutting. The control medium for

rhisogenesis contained reduced twice concentration of macro-, microcomponents and vitamins MS, 2.5 mg/l lysine, 20 g/l sucrose, 1.0 mg/l indole-3-butyric acid (IBA) and 7 g/l agar. The experimental medium included all the components of the control medium added with 5 g/l biochar. Based on sunflower husk biochar was obtained from the private company in Odessa city.

Cuttings in the third cycle were also cultivated at a temperature of 25°C, 16 hours photoperiod and the light intensity of nearly 1500 luxes. In the experiment on rhizogenesis of *P. elongate* × *P. fortunei* 100 cuttings were explanted *per* a variant. The analysis of the results was carried out on the 30th day of cultivation counting from the explantation on rooting media.

To evaluate the results of the second experiment the following traits were estimated:

- the frequency of rooting, % - the percentage ratio of the number of rooted cuttings to the total number of cuttings explanted;
- the length of the root system, cm;
-the density of the root system, points. Density assessment was performed on a point system in the range from 1 to 10 points: the most developed root system was evaluated at 10 points and the least developed - at 1 point;
-the length of newly formed shoots, cm.

The testing of *P. elongate* × *P. fortunei* adaptation to six artificial soil profiles was carried out at Pokrov land reclamation station of Dnipro State Agrarian and Economic University, which is situated in Dnipropetrovsk region, south-east of Ukraine. The following models of technogenic edaphotops were used to test the growth of *P. elongate* × *P. fortunei* plants as following:

1. 100 cm of loamy like loam and red brown clay mix (LLL+RBC);
2. 40 cm black soil + 60 cm sand (BS+Sand);
3. 40 cm black soil + 60 cm gray-green clay (BS+GGC);
4. 100 cm red-brown clay (RBC);
5. 100 cm black soil (BS);
6. 40 cm black soil +10 cm sand + 50 cm red-brown clay (BS+Sand+RBC).

A comparative thermogravimetric analysis of branches of one year age trees of *Paulownia elongate* × *Paulownia fortunei* grown on different artificial soil profiles was carried out to obtain the wood thermal stability information. The analysis was performed using the derivatograph Q-1500D of the "F. Paulik-J. Paulik-L.Erdey" system (Kharytonov et al., 2017). Differential mass loss and heating effects were recorded. The results of the measurements were processed with the software package supplied with the device. Samples of annual wood were analyzed dynamically at a heating rate of 10°C/min in air atmosphere. The mass of each sample was 100 mg. Aluminum oxide was used as a reference substance.

The reliability of all the results was evaluated at a significance level of 0.05 using the Student's test.

RESULTS AND DISCUSSIONS

The stage of sterile cutting needs the optimization of the processes that ensure the activation of auxiliary buds of *P. elongate* × *P. fortunei*, the formation of as many as possible shoots from axillary buds of an explant and internodes on newly formed shoots to increase the rate of multiplication, rapid growth of new shoots in length and reduction or absence of untimely, spontaneous rooting. The shoot formation frequency of *P. elongate* × *P. fortunei* on all investigated nutrient media for cutting was 100%, so for all cuttings explanted the activation of at least one axillary bud of each cutting had taken place (Figure 2).

Figure 2. *P. elongate* × *P. fortunei* newly formed shoots after sterile cutting and 30 days cultivation *in vitro*

The number of newly formed shoots *per* 1 explanted cutting for IAA-free media averaged 1.71 pc., but under 0.2 mg/l IAA it tended to decrease and amounted to 1.57 pcs. (Table 2).

Table 2.The influence of phytohormone composition of the nutrient medium for cutting *P. elongate* × *P. fortunei* on the development of newly formed shoots

Phytohormones in medium for cutting	Number of newly formed shoots *per* 1 explanted cutting, pcs.	Number of internodes *per* 1 newly formed shoot, pcs.
Control (hormone free)	1.3 ± 0.2	3.3 ± 0.3
0.5 mg/l BAP	1.6 ± 0.2	3.2 ± 0.4
1.0 mg/l BAP	1.6 ± 0.2	3.6 ± 0.4
1.5 mg/l BAP	1.9 ± 0.2	3.5 ± 0.4
2.0 mg/l BAP	2.0 ± 0.2	3.5 ± 0.3
2.5 mg/l BAP	1.9 ± 0.2	3.1 ± 0.3
0.2 mg/l IAA	1.02 ± 0.04	3.5 ± 0.3
0.2 mg/l IAA + 0.5 mg/l BAP	1.7 ± 0.3	2.4 ± 0.3
0.2 mg/l IAA + 1.0 mg/l BAP	1.7±0.2	2.7 ± 0.3
0.2 mg/l IAA + 1.5 mg/l BAP	1.6±0.2	2.9 ± 0.3
0.2 mg/l IAA + 2.0 mg/l BAP	1.8±0.2	3.2 ± 0.2
0.2 mg/l IAA + 2.5 mg/l BAP	1.6±0.2	3.1±0.2

The highest quantitative value of the trait in this experiment was observed under the influence of 2.0 mg/l BAP, which significantly differed from the control and the medium with 0.2 mg/l IAA and tended to exceed the values of the other variants. The number of internodes *per* 1 newly formed shoot of *P. elongate* × *P. fortunei* varied without auxin treatment from 3.1 to 3.6 pcs., on average 3.4 pcs., but under IAA it varied from 2.4 to 3.5 pcs., in average 3.0 pcs., that is, tended to decrease under the influence of auxin load. No significant differences for this trait were observed between media of on the non-auxin background. Otherwise, under 0.2 mg/l IAA the addition of low BAP amounts significantly reduced the number of internodes *per* 1 newly formed shoot. At the same time increased BAP contents ensured the development of the trait at a level that did not significantly differ from that on the BAP-free medium with 0.2 mg/l IAA. Therefore, the variant of the phytohormone composition of the medium for sterile cutting with 2.0 mg/l BAP was the most favourable both for a number of newly formed shoots *per* 1 explanted cutting as well as for a number of internodes *per* 1 newly formed shoot of *P. elongate* × *P. fortunei*.

The number of internodes of newly formed shoots *per* 1 explanted cutting (Figure 3) on the average on a non-auxin background was 5.73 units, and on the same variants of concentrations of BAP, but under 0.2 mg/l IAA showed a tendency to decrease and amounted to 4.53 pcs.

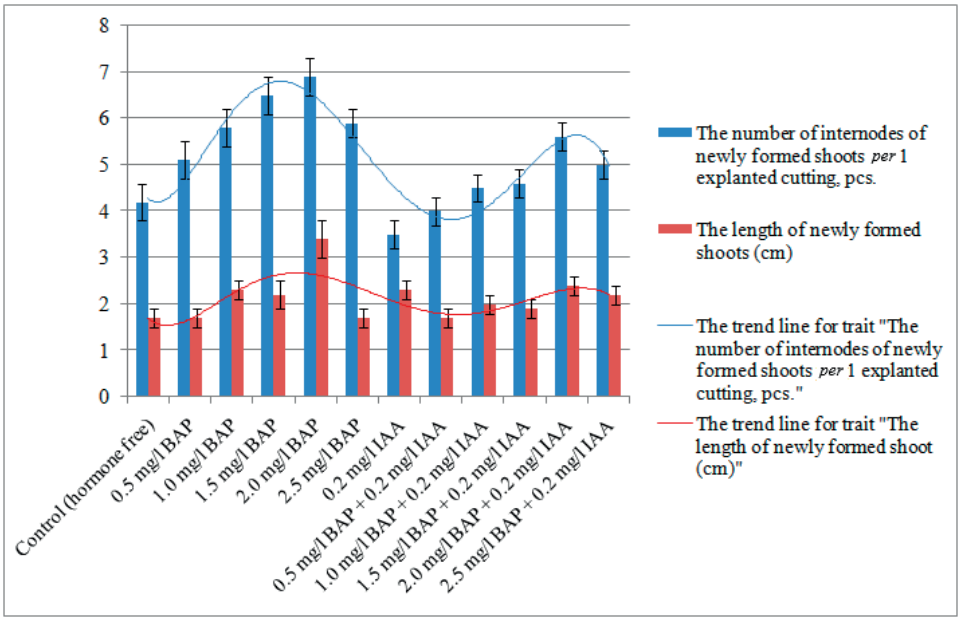

Figure 3. Effect of phytohormone composition of the medium for cutting on the number of internodes of newly formed shoots *per* 1 explanted cutting and the length of newly formed shoot of *P. elongate* × *P. fortunei in vitro*

On the non-auxin background, the highest value (6.9 pcs.) was provided with the addition of 2.0 mg/l BAP. However, comparing the values on MS + 2.0 mg/l BAP and MS + 0.2 mg/l IAA + 2.0 mg/l BAP shows that the addition of IAA reduces the positive effect of BAP on the number of internodes of newly formed shoots *per* explant. The best phytohormonal composition for sterile *P. elongate* × *P. fortunei* cutting when estimating the integral value of internodes number of newly formed shoots *per* 1 explanted cutting was MS + 2.0 mg/l BAP. Figure 3 clearly shows the trend line for this integral trait, which shows the tendency of the enhancement of this integral indicator according to the growth of BAP contents. There is a similar trend line for this trait under auxin background, which was formed with 2.0 mg/l IAA, but at a slightly lower level of values. Likewise, the trend line is positioned to indicate the length of newly formed shoots.

The length of new shoots of *P. elongate* × *P. fortunei* which were formed due to the activation of auxiliary buds *in vitro* varied on different media from 1.7 cm in control up to 3.4 cm for medium with 2.0 mg/l BAP.

There were significant differences in comparison with control for media with 1.0, 1.5 and 2.0 mg/l BAP (Figure 3). Variants with 0.5 or 2.5 mg/l BAP did not differ from control significally. The addition of 0.2 mg/l IAA in the medium for cutting contributed to a significant increase in the length of newly formed shoots compared to the control. The simultaneous application of 0.2 mg/l IAA and BAP did not significantly affect the shoot length compared to the control, but reflected a certain downward trend. The best option for the phytohormonal composition to increase the length of newly formed shoots of *P. elongate* × *P. fortunei* was 2.0 mg/l BAP in a medium for cutting. At the stage of sterile cutting of *P. elongate* × *P. fortunei in vitro* as well as in microclonal propagation of other plant species, it is important to avoid spontaneous rooting, as this can redistribute nutrients in a plantlet and reduce the yield of cuttings *per* an explant. As rooting at the stage of cutting is a rare event, the size of the samples used in our study was some insufficient and the differences between media were not very noticeable. However the observations show that the highest frequency of spontaneous root formation was observed for media without BAP - the control medium without phytohormones (66%) and the medium with 0.2 mg/l IAA (69%). Addition of BAP at the studied concentrations inhibited spontaneous root formation compared to control media to 12-30% under non-auxin background and to 6-53% under 0.2 mg/l IAA. Phytohormone composition in medium for cutting, which was the best in the analysis of the previous traits, 2.0 mg/l BAP, provided a significant reduction in spontaneous rooting to control and to all variants on auxin background, while did not differ significantly from the rest concentrations of BAP under non-auxin background.

Therefore, the given study revealed a clear pattern of the influence of BAP in the concentration range from 0.5 mg/l to 2.5 mg/l on the growth and development of *P. elongate* × *P. fortunei* cuttings *in vitro* both on non-auxin background and under the influence of auxin IAA at a concentration of 0.2 mg/l. This pattern means an increase of the positive effect of BAP in the range of concentrations from 0.5 mg/l to 2.0 mg/l and a tendency to inhibit the development of cuttings while increasing the concentration of BAP up to 2.5 mg/l. The positive effect of BAP on growth and development of *P. elongate* × *P. fortunei* cuttings is somewhat offset with the inclusion into the nutrient medium of auxin IAA, but nevertheless prevents a significant inhibitory effect of IAA. BAP also restrains spontaneous rooting of cuttings. Thus, it should be recommended to use 2.0 mg/l 6-benzylaminopurine at the stage of sterile cutting of *P. elongate* × *P. fortunei in vitro* to ensure maximum number of cuttings *per* an explant for the next cycle of sterile cutting and prevent unwanted spontaneous rooting.

It is somewhat difficult to compare the results of the effect on the microcutting of *Paulownia*, since the information available relates to different species, their hybrids and cultivars. Thus, in the investigation of Bahri and Bettaieb (2013) for *P. tomentosa* on 6-benzylaminopurine, indole-3-butyric acid and their combinations it was found that on MS medium with 30 g/l sucrose the best shoot multiplication was taken place at 1.0 mg/l IBA while rooting - at 0.5 mg/l IBA. Rahman et al. (2013) for the same species *P. tomentosa*

observed the most effective shoot multiplication on MS added with 30 g/l sucrose, 2.5 mg/l BAP, 0.5 mg/l naphthylacetic acid, but root formation - on ½ MS with 0.5 mg/l IBA. Ipekci and Gozukimizi (2003) in studies of somatic embryogenesis of *P. elongata* for the development of artificial seeds production technology have found that for MS + 30 g/l sucrose + 500 mg/l casein hydrolyzate thidiazuron (10 mg/l) was better than BAP, IAA, naphthylacetic acid, kinetin. For cuttings of hybrid *P. tomentosa* × *P. fortunei*. M. Pozoga et al. (2019) considered the most effective for microclonal propagation the supplementation of ½MS with 20 g/l sucrose, 0.5 mg/l BAP, whereas under 0.2 mg/l BAP the growth of plants became slow, and at 1.0 mg/l BAP callusogenesis was observed. For root formation on cuttings these authors recommended to add 1.0 mg/l IBA to ½MS + 20 g/l sucrose. In our study, the influence of phytohormones at the stage of sterile cutting of *P. elongata*×*P. fortunei* showed the most significant positive effect with the 2.0 mg/l BAP while the concentrations of BAP 0.0, 0.5, 1.0, 1.5, 2.5 mg/l both on the non-auxin background and under 0.2 mg/l IAA provided lower cutting efficiency and shorter length of shoots.

Such differences in the response of plant material to the same phytohormone concentrations can be explained by investigations of different species of *Paulownia* as well as basic nutrient media varied in the content of macro-, microelements, vitamins and sucrose. The certain contribution to the variation of results was made by different conditions of plant growth *in vitro*, in particular temperature and light regimes, especially in the investigation of Pozoga et al. (2019). This approach proves once again the need to test nutrient media for specific species or industrial cultivars of *P. elongate* × *P. fortunei* as well as for specific cultivation conditions.

In the following experiment, the effectiveness of biochar (5 g/l) in the medium for rhizogenesis of *P. elongate* × *P. fortunei* was studied in order to obtain the most developed root system of cuttings at the rooting stage *in vitro* for further transplantation into soil (Figures 4, Figure 5a and Figure 5b).

As it was mentioned in "materials and methods", the control medium for rhizogenesis contained reduced twice contents of macro-, microsalts and vitamins MS, 2.5 mg/l lysine, 20 g/l sucrose, 1.0 mg/l IBA and 7 g/l agar. The rooting frequency on both media was 100%. That is the development and rooting of all explanted cuttings occurred. As it was shown by the experiment, the addition of biochar at some rate inhibited the development of the root system of cuttings at the stage of rooting *in vitro*. Thus,under the influence of biochar the length of the root system decreased by 36.5% compared to control, its density decreased by 31.4%, while the length of the newly formed shoots remained at the level of control.

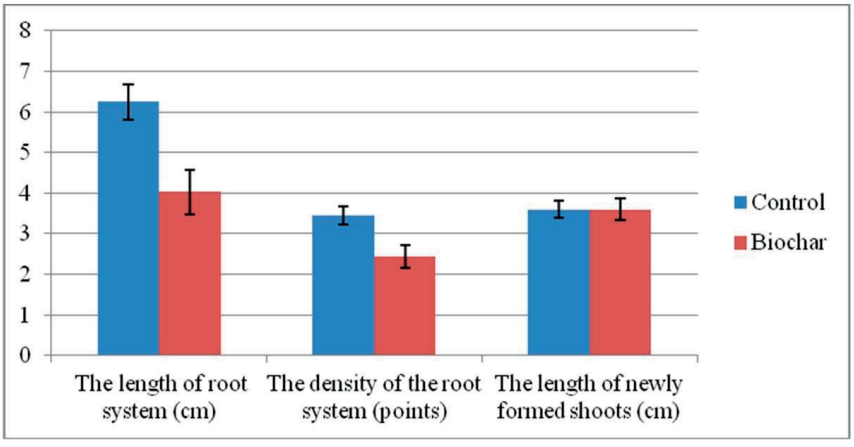

Figure 4. Effect of biochar (5 g/l) in the medium for rhizogenesis at the rooting of cuttings of *P. elongate* × *P. fortunei in vitro*

Figure 5a. *In vitro* formed plantlets of *P. elongate* × *P. fortunei* on control

Figure 5b. *In vitro* formed plantlets of *P. elongate* × *P. fortunei* on control + 5 g/l biochar media for rhizogenesis

In general, we performed the five-fold cutting of sterile *P. elongate* × *P. fortunei* plants in isolated culture. However, there was a progressive decrease in auxiliary buds activation, shoot growth and rooting frequency in generations, so the fourth and fifth cycles of cutting were conducted on MS medium supplemented with 30 g/l sucrose, 2.5 mg/l BAP, 4.0 mg/l GA_3, 0.5 mg/l IAA, 2.5 mg/l adenine and 0.05 mg/l kinetin. On this medium in the late cycles of microclonal propagation the shoot formation on *P. elongate* × *P. fortunei* cuttings occurred at a frequency of about 97%. The use of a nutrient medium with half the content of macro-, microelements and vitamins MS, 15 g/l sucrose, 1.0 mg/l BAP and 0.05 mg/l IAA allowed to root 100% of cuttings obtained *in vitro*.

After transferring to the soil regenerated plants were adapted well enough and grown first in 100 cm^3 soil cells and then in 1000 cm^3 soil vegetation vessels.

The cultivation of regenerated plants in the soil at models of technogenic edaphotops made it possible to evaluate the wood characteristics of there regenerant plants after cultivation. The thermolysis process of wood biomass of *P. elongate* × *P. fortunei* consisted of two phases (Figure 6 and Figure 7).

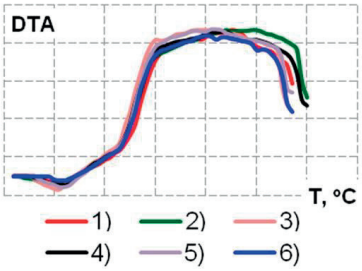

Figure 6. Difference Thermo Analysis of curves of the thermal decomposition of wood samples of *P. elongate* × *P. fortune:*
1 - LLL+RBC; 2 - BS+Sand; 3 - BS+GGC; 4 - RBC; 5 - BS; 6 - BS+Sand+RBC

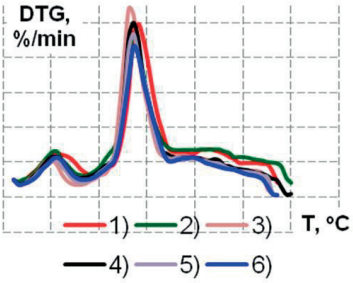

Figure 7. Differential termogravimetry graphic the total mass change % of *P. elongate* × *P. fortunei* wood samples total mass change, %:
1 - LLL+RBC; 2 - BS+Sand; 3 - BS+GGC; 4 - RBC; 5 - BS; 6 - BS+Sand+RBC

The first period included the processes of evaporation of water, volatile compounds and decomposition of the main components of the wood. The second phase, in turn, included the destruction of hemicelluloses and cellulose (stage 1), the decomposition of lignin and the formation of a non-combustible residue (stage 2). The first phase began in the temperature range of 50-60ºC and ended in the range of 160-190ºC. This process was characterized mainly by endothermic reactions. *P. elongate* × *P. fortunei* wood contained a small amount of volatile components. The process speed was also low. One peak was observed at a temperature of 100-110ºC. The highest rate of decomposition was observed at a temperature of

90ºC in the samples of the profile no. 3 (BS+GGC). The loss of mass was also the greatest - 6.1%. Decomposition of hemicelluloses and cellulose in profiles no. 1 (LLL+RBC) and no. 2 (BS+Sand) occurred in the temperature range 170-180°C ÷ 380-400°C. In other variants, this process took place in the region of lower temperatures 160ºC ÷ 350-370ºC. Exothermic reactions took place at this stage. Their destruction was delayed due to the specific composition of hemicelluloses in *Paulownia* wood. Therefore, the peak of hemicellulose decomposition is overlapped by the peak of cellulose decomposition (Figure 6). The mass loss at this stage was the greatest and ranged from 57.3% (profile no. 6, BS+Sand+RBC) to 63.9% (profile no. 2, BS+Sand) comparative to other experiment profiles. The lowest temperature (260°C) of the maximum mass loss rate was recorded for the profile no. 3 (BS+GGC). The last stage of thermolysis was characterized by the greatest exothermic effect in the range of 410-450°C. It was observed less pronounced in the region of lower temperatures (380-390°C) in the profile no. 3 (BS+GGC). The most pronounced and prolonged exothermic effect was in profile no. 2 (BS+Sand) (Figure 7). The rate of lignin decomposition was low, with no pronounced peaks. The mass loss ranged from 25.4% (profile no. 2, BS+Sand) to 31.3% (profile no. 6, BS+Sand+RBC). It should be noted that *Paulownia* wood has low ash content. The proportion of non-combustible residue did not exceed 5-7%. Thus, the results allow estimating *P. elongate* × *P. fortunei* as a plant which demonstrated stable adaption potential as bioenergy tree.

CONCLUSIONS

Microclonal propagation of *Paulownia elongate* × *Paulownia fortunei* at the stage of *in vitro* auxiliary buds activation and sterile cutting has been investigated to choose the optimal phytohormones combination among 6-benzylaminopurine concentrations in the range of 0-2.5 mg/l with or without 0.2 mg/l 3-indol-acetyc acid. The most effective one occurred the addition of 2.0 mg/l 6-benzylaminopurine into the medium MS with 2.5 mg/l lysine, 30 g/l sucrose and 7 g/l agar. This approach at the 30th day of cultivation *in vitro* allowed reaching 100% shoot formation frequency, in average 6.9 internodes of newly formed shoots *per* 1 explanted cutting and new shoots of 3.4 cm in length. At the stage of *in vitro* root formation of *Paulownia elongate* × *Paulownia fortunei* microclones the most suitable medium was ½MS supplemented with 2.5 mg/l lysine, 20 g/l sucrose, 1.0 mg/l indole-3-butyric acid and 7 g/l agar while the addition of 5 g/l biochar inhibited the root system development.

The thermal stability of the wood of plants grown on profile (40 cm black soil + 60 cm green gray clay) was slightly lower than in other versions of the experiment. The mass loss at the stage of cellulose decomposition was the greatest and ranged from 57.3 to 63.9% in two profiles with water retention layer comparative to other experiment trials.

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THE GIANT BAMBOO AND THE EVOLUTION OF THE EUROPEAN INDUSTRY TOWARDS THE SUSTAINABILITY OF MATERIALS

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Abstract

The text is the result of four years of study and experimentation on giant bamboo, of which over 2000 hectares have been planted in Italy from 2015 to today. A small experimental cultivation has been present since 2016 at the UASVM Bucharest. More than 30 million hectares of bamboo are grown worldwide. For the characteristics of its wood and for the abundance and continuity of production, it is to be considered one of the fundamental resources that Europe can draw on for an industrial turn towards the sustainability of materials. Bamboo finds application in many supply chains and makes it possible to create products with a negative $C0_2$ rate. Having overcome the initial diffidence for something that does not belong to European culture and tradition, with our pioneering activity we have found that, with the appropriate care and the right techniques, the plant can thrive in the temperate areas of our continent exactly as it thrives on them latitudes of China. Planted once, it produces oxygen, food and sustainable raw materials for about 100 consecutive years. The researches that were the basis of the preparation of the present paper aimed, on the one hand, the increase of the aerial biomass production realized by the giant bamboo plants with the age of 4 years at the surface unit, the dynamics of the sequestration of the organic carbon from the atmosphere, but also the improvement of the physical characteristics (moisture content) and resistance to mechanical actions of fibers, all against the background of practicing technological links as gentle with the environment. The results of the research have shown that this species is a viable alternative for reducing greenhouse gas emissions due to the ability to sequester large quantities of CO_2 from the atmosphere, while also representing an excellent building material, due to the superior physical and mechanical properties of other woody species. Exactly what the world of the future needs.

Key words*: bamboo, farming, industry, sustainability, prosperity.*

INTRODUCTION

If there are things that you will always need, they are: oxygen, food and sustainable raw materials. Bamboo, the fastest growing plant in the world, produces all this with abundance and quality. The sustainability of raw materials is today the central theme at world level, governments are moving accordingly. It is therefore clear that the great business of the coming years is precisely represented by sustainability. The prosperity economy model is the natural evolution of the profit economy. Prosperity Economy identifies profit as the natural result of widespread well-being, a shift paradigm that improves the quality of the environment and our life. When a big change is coming, there are two choices: ride it or suffer it, so industry can see sustainability as a problem or an opportunity.

The program stems from the experience gained from years in the world of the giant bamboo cultivation in Italy, a new type of crop for the European market, with a huge development potential.

The program is based on the observation that our civilization is inevitably developing on the use of natural raw material and on the consumption of food of non-animal origin with a high nutritional content.

The pioneering experiences of the recent years have served to understand the correct agronomic needs of the plant, to acknowledge the rules of planting and cultivation, the suitability and preparation of the land, the procedures for the correct management of the plant in order to obtain abundance of production.

The cultivation of bamboo enhances the agricultural land through a rich production, abundant and suitable for many uses (Ashby M.,

2009). A bamboo plant produces wood, food and oxygen, for 80 to 100 consecutive years. Bamboo is a giant grass, a grass that exists in over 1500 different species, partly with a tropical climate, partly with a temperate climate (Bezze G. et al., 2017). With over 30 million hectares spread throughout the world (FAO, 2010b) and annual sales of over 60 million dollars, bamboo offers a natural and abundant tool to alleviate poverty, protect the environment and mitigate climate change. The plant is intertwined with the tradition and culture of many rural and tribal populations and has been an integral part of their cultural, social and economic conditions since time immemorial (Pauli G.A., 2010). It was nicknamed "friend of the people", "green gas", "the plant with a thousand faces", "green steel", "green gold".

From 2015 to today in Italy crops have been started for a total of 2000 hectares. Reaches 25 meters in height and 15 cm in diameter it is considered one of the most nutritious foods in the world and has high CO_2 absorption values (Morris D., 2006). It's the fastest growing plant in the world with peaks of 1 m/day Due to its characteristics wood is called "green steel". Each hectare produces hundreds of tons of material annually. It is cultivated without the use of chemicals, preserving the organic life of soils and aquifers.

During its life, one hectare of bamboo absorbs 1000 tons of CO_2 and produces 20 m^3 of material every year. Bamboo production therefore has a negative CO_2 rate. We can reverse the CO_2 process. Due to the organoleptic characteristics of the sprout and the mechanical characteristics of its wood, the plant finds application in many sectors such as: cellulose, textiles, construction, objects, cosmetics, pharmaceuticals, biomass, animal feed.

Great strides are being made in the search for new materials based on vegetable fiber. Almost everything that is currently produced with plastic is replaceable, and in the future many products currently made of minerals and metals will also be replaceable.

Europe has always purchased raw materials from other countries. On this new industrial chain, we have the climatic conditions and the know-how to be producers of raw materials. Who produces the raw material is at the top of an industrial pyramid.

No chemical treatments are used, bamboo is antifungal and natural pesticide, the organic quality of the soil and the purity of the underlying aquifers are preserved.

This underground biomass makes bamboo capable of surviving and regenerating when surface biomass is destroyed by fire. Bamboo, once developed, is also tolerant to flooding and drought.

Several aspects of bamboo biology make it excellent for stabilizing unstable soil and preventing soil erosion, thanks to its extensive root systems, that can measure up to 100 kilometers per hectare and live for about a century (Liese W., 1998). Bamboo is included among the phytoremediation plants for the ability to grow in polluted sites, absorb the heavy metals present in the soil and thus carry out a real remediation action.

MATERIALS AND METHODS

The research objectives

The research was carried out during the year 2019 within a giant bamboo plantation that was established in 2016 in the Ripapersico area, Italy, on a type of clay-alluvial soil.

The fundamental purpose of the experiment was to verify the influence of the different technological links (planting density and fertilization scheme) on increasing the weight biomass production and the technological characteristics of the wood obtained at harvest in same time.

The objectives of the research were:

a) to identify the optimal technological links in order to increase biomass accumulation;

b) to identify the optimal technological links in order to increase carbon biomass accumulation;

c) determination of the physic-mechanical properties of the fibers belonging to giant bamboo plants aged 4 years.

The laboratory analyzes and determinations aimed at:

- biometric determinations regarding the production of fresh and dry biomass/plant - by summing the biomass of the biomass components of the plants, respectively stems, branches and leaves (kg/plant);
- biometric determinations regarding the production of total fresh and dried biomass (tons/ha) - by reference to the density of plants at the surface unit, according to the sowing scheme (1200 plants/ha);

- researches regarding sequestered carbon biomass in bamboo plants aged 4 years (kg/plant, respectively tons/ha);
- tests regarding the moisture content of bamboo fibers (%), within a certain time interval;
- tests regarding the compressive strength of bamboo fibers (N/mm^2), within a certain time interval.

The experimental method used in research

The experience with the giant bamboo was one of a bifactorial type, placed in the field according to the method of the subdivided plots, in three repetitions, the experimental factors considered in the study being the following:

FACTOR A - fertilization scheme, with 3 graduations:

a1 - non fertilized (Control);

a2 - fertilized with 250 kg/ha Zeolite;

a3 - fertilized with 500 kg/ha Zeolite.

FACTOR B: irrigation regime, with 3 graduations:

- b1 - non irrigated (Control);
- b2 - irrigated with a norm of 1000 l water/ha;
- b3 - irrigated with a norm of 1500 l water/ha.

By combining the two experimental factors resulted 9 experimental variants, the processing and interpretation of the experimental results being done by analyzing the variance, according to the method of field placement of the experiment.

Observations and determinations made during the research

To determine the components of the aerial biomass on the giant bamboo, destructive strains samples were collected separately for each experimental variant.

The samples were brought to the laboratory where the aerial biomass components were separated from the plants, respectively the culms, branches and leaves and their weighing, thus resulting in the production of fresh biomass reported on the plant, the results of the weighing being used subsequently to determine the total biomass production obtained at the surface unit.

After the determination of the fresh biomass the analysis samples were dried in the oven, at a constant temperature of 60ºC, for 24 hours, for the culms and their branches, respectively 20ºC, for the leaves.

The biomass carbon sequestrated into bamboo plants been obtain by multiplying total biomass production resulted after the biometric tests with default carbon fraction value 0.47 (Eggleston H.S. et al., 2006).

In order to evaluate the physic-mechanical characteristics of the giant bamboo fibers, 5 culms from each experimental variant were selected, culms that were harvested by shaving at ground level and were then allowed to air dry 6 months, until the color of the stems became yellowish, specific to the bamboo wood for industrial processing.

The bamboo culms were cut into small samples being taken from the ridge internode in accordance with the provisions of ISO 22157: 2019, 1st Part. The samples were taken along the ridge length, from three different points, respectively at the base of the culm, at the middle and at the apical part of the culm, the selection internodes being free of knots.

To perform the compression strength test we used a universal testing machine, applying a constant load of 0.01 mm/s on the test pieces, the maximum load obtained for each sample being recorded. After the compression test was completed, 25 mm x 25 mm specimens were taken to determine the moisture content of the fiber. For this purpose, the specimens were initially weighed and then dried in a hot air drying oven at 103 ± 2ºC for 24 hours. In order to quantify the amount of moisture remaining after being dried in the air-drying oven the samples were retested.

RESULTS AND DISCUSSIONS

Research results regarding fresh the aerial biomass production

The experimental results obtained from the determination of the aerial biomass of the bamboo plants belonging to each experimental variant (Table 1) showed a great variability due to the influence of the irrigation regime and the biological fertilization scheme practiced within the experiment, the values of this biometric parameter increasing directly in proportion to the quantity of water and the dose of fertilizer administered to the bamboo plants, with values that were between 4.73 kg fresh matter/plant, the lowest production of fresh biomass recorded in the case of the control variant (a1b1), in conditions of non-fertilization and non-irrigation of the plants and 64.33 kg fresh matter/plant, in the experimental variant a3b3,

this variant realizing the highest production of fresh biomass, against the background of the basic fertilization of the plants with 500 kg/ha Zeolit and of the administration of a norm of irrigation of 1500 liters water/ha.

Performing a detailed analysis on the dynamics of biomass accumulation within the 9 experimental variants studied, it is found that, compared to the control variant a1b1, the rest of the experimental variants recorded production increases ranging between 1.76 kg fresh matter/plant and 59.60 kg fresh matter/plant, spores that had statistically insignificant positive assurance (-) or significantly positive (x), in the experimental variants in which the plants were irrigated with a norm of 1000 liters water/ha, respectively 1500 liters water/ha, under conditions of non-fertilisation a1b2 and a1b3), distinctly significantly positive (xx), in the variants in which the bamboo plants benefited from the nutritional contribution brought by the administration of 250 kg/ha Zeolit (a2b1, a2b2 and a2b3) and very significantly positive (xxx), under the conditions of fertilization with 500 kg/ha Zeolit, regardless of the irrigation norm administered to the plants (a3b1, a3b2 and a3b3).

Table 1. Fresh aerial biomass production as result of the experimental factors influence

Experimental variants	Aerial biomass (kg f.m./pl.)	Difference (kg f.m./pl.)	Total biomass (t f.m./ha)	Difference (t f.m./ha)	**Signification**	
a1b1 (Control)	**4.73**	**Control**	**5.67**	**Control**	**-**	**-**
a1b2	6.49	1.76	7.79	2.12	-	-
a1b3	8.99	4.26	10.78	5.11	x	x
a2b1	10.83	6.10	12.99	7.32	xx	xx
a2b2	14.63	9.90	15.55	9.88	xx	xx
a2b3	16.81	12.08	20.18	14.51	xx	xx
a3b1	52.98	48.25	63.58	57.91	xxx	xxx
a3b2	60,64	55.91	72.77	67.10	xxx	xxx
a3b3	64.33	59.60	77.19	71.52	xxx	xxx
$DL_{5\%} = 1.96$; $DL_{1\%} = 4.64$; $DL_{0,1\%} = 15.42$ $DL_{5\%} = 2.32$; $DL_{1\%} = 5.36$; $DL_{0,1\%} = 18.19$						

The total biomass production from the giant bamboo plants at the surface unit ranged from 5.67 tons fresh matter/ha to 77.19 tons fresh matter/ha, the highest yields being obtained when administering a dose of 500 kg/ha Zeolit, the plants belonging to these experimental variants producing over 57 tons fresh matter/ha, far superior to those obtained by practicing the other fertilization schemes, irrespective of the irrigation regime practiced within the experiment.

Compared to the control variant (a1b1), the production increases ranged from 2.12 tons fresh matter/ha and 71.52 tons fresh matter/ha, statistically insignificant positive (-), in the case of the experimental variant a1b2, significantly positive of 5.11 tons fresh matter/ha, variant a1b3, distinctly significantly positive (xx), between 7.32 tons fresh matter/ha and 14.51 tons fresh matter/ha in the variants a2b1, a2b2 and a3b3 and very significantly positive (xxx), between 57.91 tons fresh matter/ha and 71.52 tons fresh matter/ha in the experimental variants a3b1, a3b2 and a3b3, variants where the most efficient technological links were practiced, links that had a direct impact in increase the accumulation rate of biomass at the surface unit.

Research results regarding the dry aerial biomass

Following the determination of the aerial biomass production as a dry substance (Table 2) the same dynamics of growth production was observed with the increase of the Zeolite dose and the irrigation norm, the biomass productions that were recorded between 3.31 kg dry matter/plant and 25.83 kg dry matter/plant, minimum production of dry aerial biomass obtained in the case of control variant a1b1, higher quantities of dry matter being stored in the bamboo plants tested in the experimental variants where for the filling of the nutrients needed of the plants administered 500 kg/ha Zeolit, against the background of administering an irrigation norm of 1000 liters water/ha or 1500 liters water/ha (a3b2 and a3b3).

Compared with the control of the experience (a1b1), the production differences were between 0.99 kg dry matter/plant and 22.52 kg dry

matter/plant, differences that were provided statistically insignificant positive (-), in the experimental variant a1b2, significantly positive (x), for variants a1b3, a2b1, a2b2 and a2b3 and very significantly positive (xxx), for experimental variants a3b1, a3b2 and a3b3.

Table 2. Dry aerial biomass production as result of the experimental factors influence

Experimental variants	Aerial biomass (kg d.m./pl.)	Difference (kg d.m./pl.)	Total biomass (t d.m./ha)	Difference (t d.m./ha)	**Signification**	
a1b1 (Control)	**3.31**	**Control**	**3.97**	**Control**	**-**	**-**
a1b2	4.30	0.99	5.16	1.19	-	-
a1b3	5.78	2.47	6.93	2.96	x	X
a2b1	5.57	2.26	6.68	2.71	x	X
a2b2	7.13	3.82	8.55	4.58	x	Xx
a2b3	8.36	5.05	10.03	6.06	x	Xx
a3b1	20.56	17.25	24.67	20.70	xxx	Xxx
a3b2	24.09	20.78	28.90	24.93	xxx	Xxx
a3b3	25.83	22.52	30.99	27.02	xxx	Xxx
$DL_{5\%} = 1.44$; $DL_{1\%} = 7.13$; $DL_{0,1\%} = 11.90$ $DL_{5\%} = 1.32$; $DL_{1\%} = 3.99$; $DL_{0,1\%} = 10.12$						

The experimental results regarding the total biomass production, demonstrated a linear increase of the values of this biometric parameter, directly correlated with the increase of the water quantity and of the fertilizer dose applied to the plants, the best answer to the practice of these technological links obtained on the bamboo plants that were fertilized with 500 kg/ha Zeolit, the total biomass production of dry matter for these experimental variants ranging from 24.67 tons dry matter/plant and 30.99 tons dry matter/ha (Table 2.). These results can be attributed both to the contribution of microelements present in this natural fertilizer and to the ability of Zeolite to favor water conservation in the soil for a long time and to favor cationic exchange in soil solution, thus increasing the accessibility for plants of the nutrients present in the soil.

The differences regarding the production of dry biomass realized at the surface unit within the 8 experimental variants, compared with the control variant, non-fertilized and non-irrigated (a1b1) ranged between 1.19 tons dry matter/plant and 27.02 tons dry matter/ha, differences statistically insignificant positive (-) of 1.19 tons dry matter/plant (a1b2), significantly positive with production increases between 2.96 tons dry matter/plant and 2.71 tons dry matter/plant in variants a1b3, respectively a2b1, distinctly significantly positive (xx) between 4.58 tons dry matter/plant (a2b2) and 6.06 tons dry matter/plant (a2b3) and very significantly positive (xxx) with production increases of over 20.70 tons dry matter/plant in experimental variants a3b1, a3b2 and a3b3.

Research results regarding carbon biomass sequestrated

The results regarding the carbon biomass sequestered by the giant bamboo plants of 4 years old are centralized in Table 3. Analyzing the data entered in this table it is observed that the capacity of the plants to sequester the organic carbons from the atmosphere has registered very large variations between the experimental variants taken in the study, there is a direct correlation between the irrigation regime, the fertilization scheme and the values recorded by this parameter, the rate of growth and vegetative development of the plants and implicitly of carbon assimilation being maximum (12.14 kg carbon biomass/plant) under the conditions of the administration of 500 kg/ha Zeolit, against the background of the administration during the vegetation period of the plants of an irrigation norm of 1500 liters water/ha (a3b2).

In the experimental variants in which the fertilization of the plants was done with a dose of 250 kg/ha Zeolit the biomass of carbon sequestered in the plants ranged between 2.62 kg carbon biomass/plant and 3.93 kg carbon biomass/plant while, in the case of the non-fertilized bamboo plants due to the deficient growth and vegetative development the rate of carbon sequestration has been greatly diminished the values of this indicator being

between 1.56 kg carbon biomass/plant and 2.72 kg carbon biomass/plant.

Compared with the control variant a1b1 for which the carbon biomass was only 1.56 kg carbon biomass/plant, the rest of the experimental variants tested in the research registered differences from a statistical point of view from insignificant positive (-) in variants a1b2 and a2b1, significantly positive (x) of 1.16 kg carbon biomass/plant in the case of variants a1b3, distinctly significantly positive (xx) between 1.78 kg carbon biomass/plant and 2.37 kg carbon biomass/plant in the case of experimental variants a2b2 and a2b3, differences that have become very significantly positive (xxx) with more than 8.11 kg carbon biomass/plant in the experimental variants in which the plants benefited from the optimal supply with the nutrients essential for the good development of physiological processes responsible for the growth and harmonious development of plants.

Table 3. Carbon biomass sequestrated as result of the experimental factors influence

Experimental variants	Carbon biomass (kg/pl.)	Difference (kg/pl.)	Total carbon biomass (t/ha)	Difference (t/ha)	Signification	
a1b1 (Control)	**1.56**	**Control**	**1.87**	**Control**	**-**	**-**
a1b2	2.02	0.46	2.43	0.56	-	-
a1b3	2.72	1.16	3.26	1.39	x	X
a2b1	2.62	1.06	3.14	1.28	-	X
a2b2	3.35	1.78	4.02	2.15	xx	Xx
a2b3	3.93	2.37	4.71	2.85	xx	Xx
a3b1	9.66	8.11	11.60	9.73	xxx	Xxx
a3b2	11.32	9.76	13.58	11.72	xxx	Xxx
a3b3	12.14	10.59	14.57	12.70	xxx	Xxx
$DL_{5\%} = 1.12$; $DL_{1\%} = 1.59$; $DL_{0,1\%} = 5.13$ $DL_{5\%} = 0.83$; $DL_{1\%} = 1.68$; $DL_{0,1\%} = 6.34$						

The same evolution is also observed when analyzing the total biomass of carbon sequestered by giant bamboo plants at the surface unit (Table 3.) the assimilation of organic carbon from the atmosphere intensifying with the increase of the amount of water and natural fertilizer administered to the plants.

The values of this indicator were between 1.87 tons carbon biomass/ha and 14.57 tons carbon biomass/ha, the most valuable in terms of the capacity of assimilation and sequestration of the carbon from the atmosphere being the bamboo plants with the most intense vegetative growths, the contribution Zeolite administration as a natural fertilizer against the background of optimum supply of water plants finding in the increase in height and diameter of the culms and also in increasing the value of the leaf surface index of the leaves, vegetative organs in which was sequestered large amount of organic carbon.

Compared to the control of the experience (a1b1) in the rest of the experimental variants studied the capacity of the bamboo plants to assimilate the organic carbon from the atmosphere increased with differences recorded being between 0.56 tons carbon biomass/ha and 12.70 tons carbon biomass/ha with statistically assurance insignificant positive (-) to variant a1b2, significantly positive (x) to variants a1b3 and a2b1, distinctly significantly positive (xx) in the case of experimental variants fertilized with 250 kg/ha Zeolite and irrigated with a norm of 1000 liters of water/ha (a2b2) or 1500 liters water/ha (a2b3) and very significantly positive (xxx) under the conditions of administration of 500 kg/ha Zeolit regardless of the irrigation rule administered to plants.

Research results regarding the bamboo fibers moisture content

The tests on the water content of the bamboo fibers determined at 1 month, respectively 6 months after the air drying time, showed that the values of this physical parameter were influenced by both experimental factors (the fertilizer dose and the amount of water administered to the bamboo plants) and as well from the sampling place of the wood samples, respectively from the base, the middle or the top of the culm, the experimental results obtained from the determinations being centralized in Table 4.

Overall, the average water content of giant bamboo fibers at 1 month after the air drying time of the culms was between 19.15%, the minimum value obtained in the case of the control variant (a1b1) and 21.05%, the content the highest in water of fibers recorded in the

samples from the variant in which the plants were optimally supplied with both water and nutrients (a3b3).

In a detailed analysis of the results recorded after determining the water content of the bamboo fibers taken from the 9 experimental variants studied we find that in the samples collected from the base of the culm the humidity of the fibers varied between 18.23% and 19.62%, in the samples taken from the middle of the culm the values of the recorded humidity were between 19.11% and 21.73% whereas, the samples collected from the apical internode of the culm had a moisture content that oscillated between the limits of 20.13 % and 21.81%, the variation of this parameter increasing by approximately one percentage unit from the base to the tip of the stem.

Table 4. Bamboo fibers moisture content as result of the experimental factors influence

Experimental variants		Moisture content after 1 month (%)	Average after 1 month (%)	Difference after 1 month (%)	Moisture content after 6 months (%)	Average after 6 months (%)	Difference after 6 months (%)	**Signification**	
a1b1 (Control)	Bottom	**18.23**	**19.15**	**Control**	**13.25**	**14.20**	**Control**	-	-
	Middle	**19.11**			**14.22**				
	Top	**20.13**			**15.15**				
a1b2	Bottom	18.34	19.49	0.34	13.32	14.50	0.30	x	-
	Middle	19.53			14.55				
	Top	20.61			15.63				
a1b3	Bottom	18.46	19.58	0.43	13.48	14.60	0.40	x	x
	Middle	19.59			14.61				
	Top	20.71			15.73				
a2b1	Bottom	18.63	19.74	0.59	13.65	14.76	0.56	xx	x
	Middle	19.72			14.74				
	Top	20.87			15.89				
a2b2	Bottom	18.92	19.92	0.77	13.94	14.94	0.74	xx	xx
	Middle	19.88			14.90				
	Top	20.97			15,99				
a2b3	Bottom	19.03	20.51	1.36	14.05	15.53	1.33	xxx	xxx
	Middle	21.09			16.11				
	Top	21.42			16.44				
a3b1	Bottom	19.27	20.64	1.49	14.29	15.66	1.46	xxx	xxx
	Middle	21.16			16.18				
	Top	21.51			16.53				
a3b2	Bottom	19.48	20.81	1.66	14.50	15.83	1.63	xxx	xxx
	Middle	21.33			16.35				
	Top	21.64			16.66				
a3b3	Bottom	19.62	21.05	1.90	14.64	15.86	1.66	xxx	xxx
	Middle	21.73			16.20				
	Top	21.81			16.75				
$DL_{5\%} = 0.33$; $DL_{1\%} = 0.48$; $DL_{0,1\%} = 0.92$ $DL_{5\%} = 0.38$; $DL_{1\%} = 0.61$; $DL_{0,1\%} = 0.87$									

The water content of the samples collected from the 8 experimental variants registered compared with the control variant differences between 0.34 and 1.90 percentage points being from a statistically significant positive point (x) to the samples collected from the unfertilized experimental variants (a1b2 and a1b3), distinctly significantly positive in the samples collected from the variants in which the plants were fertilized with 250 kg/ha Zeolite, under non-irrigation or irrigation conditions with a irrigation norm of 1000 liters water/ha (a2b1 and a2b2) and very significantly positive (xxx) in the case of the experimental variant where on the background of the fertilization with 250 kg/ha Zeolit was administered during the vegetation period of the plants an irrigation norm of 1500 liters water/ha (a2b3), as well as in the samples that were taken from the culms of the plants that benefited from supplementing the necessary nutrients by administering 500 kg/ha Zeolite (a3b1, a3b 2 and a3b3).

As the drying time of the bamboo culms collected from the 9 experimental variants increased the moisture content of the fibers decreased by about 5 percentage points compared to the initial moment of the determinations (after 1 month) so that the values of this physical indicator approached the considered value reference STAS regarding the moisture content of the

fibers, respectively 12%. Thus, we observe the same dynamics of moisture loss inside the fibers as we move from the tip of the culms to their base, the lowest humidity being determined in the case of samples taken from the internodes from the base of the culm with values between 13.25% and 14.64%, followed closely by the samples collected from the internodes from the middle of the culm with values between 14.22% and 16.20% and those collected from their apical internodes with values that ranged between 15.15% and 16.75%.

Following the free air drying of the bamboo culms for 6 months the average moisture content of the specimens varied between 14.20% and 15.86%, the bamboo fibers being considered to be of good quality as a result of the combined action of the experimental factors studied (fertilization scheme x irrigation scheme), from the point of view of this physical indicator the differences from the control variant a1b1 being very close between 0.30 and 1.66 percentage points with statistical assurance of from insignificant positive (-), to very significantly positive (xxx).

Research results regarding the bamboo fibers compressive strength

The samples taken from the giant bamboo stalks air-dried for 1 month had a different resistance to compression, resistance that increased as the determinations advanced from the base to the top of the culms regardless of the experimental variant from which the culms were collected. Thus, the fibers present in the samples taken from the base of the culms have the least resistance to the compression test being recorded values of this mechanical indicator between 48.17 N/mm^2 and 77.43 N/mm^2, those collected from the middle the culms have an increased compressive strength, respectively between 53.12 N/mm^2 and 78.54 N/mm^2, the samples taken from the apical internode of the culms being the most resistant to the mechanical action on the fibers, the compression resistance in these fibers varying between 57.34 N/mm^2 and 79.41 N/mm^2 (Table 5).

Table 5. Bamboo fibers compressive strength as result of the experimental factors influence

Experimental variants		Compressive strength after 1 month (N/mm^2)	Average after 1 month (N/mm^2)	Difference after 1 month (N/mm^2)	Compressive strength after 6 months (N/mm^2)	Average after 6 months (N/mm^2)	Difference after 6 months (N/mm^2)	**Signification**	
a1b1 (Control)	Bottom	**48.17**	**52.87**	**Control**	**53.77**	**58.49**	**Control**	-	-
	Middle	**53.12**			**58.75**				
	Top	**57.34**			**62.97**				
a1b2	Bottom	51.43	58.28	5.41	57.06	63.91	5.42	x	x
	Middle	58.96			64.59				
	Top	64.47			70.10				
a1b3	Bottom	58.12	64.13	11.26	63.75	69.79	11.30	xx	xx
	Middle	66.13			71.76				
	Top	68.24			73.87				
a2b1	Bottom	60.03	66.33	13.46	65.66	71.96	13.47	xxx	xxx
	Middle	67.92			73.55				
	Top	71.05			76.68				
a2b2	Bottom	62.13	68.18	15.31	67.76	73.81	15.32	xxx	xxx
	Middle	68.33			73.96				
	Top	74.08			79.71				
a2b3	Bottom	66.23	70.31	17.44	71.86	72.61	14.12	xxx	xxx
	Middle	69.56			75.19				
	Top	75.16			80.79				
a3b1	Bottom	69.06	73.51	20.64	74.69	79.14	20.65	xxx	xxx
	Middle	74.67			80.30				
	Top	76.81			82.44				
a3b2	Bottom	72.18	75.45	22.58	77.81	81.08	22.59	xxx	xxx
	Middle	76.13			81.76				
	Top	78.06			83.69				
a3b3	Bottom	77.43	78.46	25.59	83.06	84.09	25.60	xxx	xxx
	Middle	78.54			84.17				
	Top	79.41			85.04				
$DL_{5\%} = 4.02$; $DL_{1\%} = 9.97$; $DL_{0,1\%} = 11.33$ $DL_{5\%} = 4.36$; $DL_{1\%} = 10.03$; $DL_{0,1\%} = 12.71$									

The average values of the compressive strength of the bamboo fibers were also influenced by the interaction of the experimental factors studied in this research so that, under conditions of non-fertilization of the plants the compressive strength of the fibers as a mean of the irrigation regime ranged from 52,87 N/mm^2 and 64.13 N/mm^2. Following the administration of a dose of 250 kg/ha Zeolite the fibers were more resistant to mechanical action with average values between 66.33 N/mm^2 and 70.31 N/mm^2 by supplying 500 kg/ha Zeolite the opposite resistance of fibers to the mechanical action of compression increasing significantly, their compressive strength exceeding 73.00 N/mm^2 (77.43 N/mm^2 - 78.46 N/mm^2).

The differences recorded after the determination of the resistance of the giant bamboo fibers to compression, in comparison with the variant taken as an experimental control had a statistically significantly positive assurance in the case of the a1b2 variant (5.42 N/mm^2), distinctly significantly positive (xx), at variant α1b3 (11.26 N/mm^2) and very significantly positive (xxx) at the rest of the experimental variants with differences ranging from 13.46 N/mm^2 to 25.59 N/mm^2.

After 6 months of natural drying of the stems, the resistance of the fibers to compression has increased significantly especially due to the reduction of their moisture content, the average values recorded being between 58.49 N/mm^2, the lowest compression resistance manifested by the fibers belonging to of the culms harvested from the control variant (a1b1) and 84.09 N/mm^2 in the case of fibers belonging to the strains harvested from the experimental variant a3b3.

Throughout the strain the resistance of the fibers to the mechanical action followed the same trend as in the first stage of the compression tests (1 month after the drying of the culms), increasing from the base to the tip of the culm with the average value between 53.77 N/mm^2 and 83.06 N/mm^2 in the case of samples taken from the basal internode of the culm, between 58.75 N/mm^2 and 84.17 N/mm^2 in the samples collected from the internode from the middle of the culm, respectively between 62.97 N/mm^2 and 85.04 N/mm^2 for the fibers present in the specimens collected from the apical internode of the culm.

Compared with the compressive strength opposed by the samples belonging to the control variant (a1b1), in the case of the other experimental variants tested during the research the compressive strength of the fibers increased after 6 months by over 5.42 N/mm^2 being insured from the point of view statistically significant positive (x) in variant a1b2, distinctly significantly positive (xx) at variant a1b3 and very significantly positive (xxx) for the rest of experimental variants studied.

The evolution of the mechanical characteristics of the giant bamboo fibers with the age of 4 years can be taken into account the specific anatomical and biological particularities of this species, with the dynamics of fiber formation inside the strain depending on the intensity of the vascularization of the strain, with the physiological process that takes place with the highest intensity in the apical part of the culm which is why the higher number of fibers are formed in the upper internodes of the stem, thus increasing their resistance to mechanical actions.

CONCLUSIONS

Due to its many and varied uses giant bamboo is the species with the fastest growth rate, the extraordinary capacity of regeneration of this plant being the reason why the culture has started to gain more and more land not only in the countries with tradition in the cultivation of bamboo, but also in the area of temperate climate, including in Romania.

The production of biomass (fresh and dried matter) was directly influenced by the mineral fertilization scheme and irrigation regime practiced during the research, the highest biomass production being realized in the case of the bamboo plants that were optimally supplied with water as well as with the nutrients necessary for the growth and harmonious development of the plants, thus obtaining yields of over 77 tons fresh biomass, respectively productions of over 30 tons dry matter at the surface unit the giant bamboo thus representing a sustainable source of biomass that can be used as a raw material in the pulp and paper industry or as a renewable source of green energy.

Although at present the giant bamboo culture in Europe is regarded as a challenge, in the near

future this culture will certainly be regarded as an alternative in reducing greenhouse gases, due both to the rapid growth rate, the extraordinary capacity of regeneration, the longevity of the species, but also the extraordinary ability of this species to fix CO_2 from the atmosphere, the plants tested in the present research sequestering over 14.5 tons carbon biomass/ha by using friendly practices with the environment.

The moisture content of the fibers was influenced by both the experimental factors studied in the study and the sampling point of the culms, the values of this physical parameter increasing from the base of the stalk to its apical part and directly proportional to the dose of fertilizer and the amount of water administered to the plants during the vegetation period, the humidity of the fibers after 6 months of air drying being about 16%.

The resistance of the fibers to compression was influenced by their moisture content at the time of the mechanical tests but also by the anatomical-morphological properties of the species, the highest resistance to the mechanical action being recorded in the samples taken from the apical internodes of the culm which are highly vascularized and in which the greatest number of fibers are formed.

The compressive strength of giant bamboo fibers after the natural drying of the stems for 6 months increased directly in proportion to the increase in the number of fibers formed in the culm internodes and inversely proportional to the moisture content of the fibers, the values recorded by this parameter exceeding 79 N/mm^2 (higher than steel) under the conditions of practicing efficient technological links which makes the bamboo stems to be considered very resistant to mechanical actions, thus being successfully used as a resistance material in the field of construction.

In these new industrial chains, Europe no longer needs them: we have the know-how and we have the climate to produce the raw material for the next years on our own. And we know that whoever produces the raw material is placed at the top of the pyramid of an industrial chain and therefore has control over it.

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BIOTECHNOLOGICAL ASPECTS OF FERULOYL ESTERASE

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Abstract

Industrial processes lead to high amounts of lignocellulosic wastes, that are not valorised properly. Feruloyl esterase are a group of accesory enzymes used in the biodegradation of lignocellulose, capable of releasing ferulic acid that is located either between lignin and hemicellulose or between hemicelluloses. The importance of feruloyl esterase is given mainly by the fact that its action allows other enzymes to hydrolyse xylan or lignin. The released ferulic acid has several applicatios in various industries such as food, feed, medical, biofuel, pulp and paper etc. Feruloyl esterases are produced by several microorganisms (bacteria or fungi), but research studies are still focused on finding more sources for these enzymes. There are different methods for detecting microbial strains able to produce feruloyl esterase, the most used being plate screening methods.

Key words*: ferulic acid, feruloyl esterase, lignocellulose, xylan.*

INTRODUCTION

Massive agro-industrial and food processing lead to an accumulation of lignocellulosic biomass, one of the most abundant biomasses, with almost 200 billion tons (Gunjal et al., 2020). Lignocellulosic wastes, are either discarded or used as animal feed (Sarangi & Sahoo, 2010), without being properly valorised (Gropoșilă-Constantinescu et al., 2017; Trulea et al., 2016). The microbial degradation of these lignocellulosic structures is considered to be the best alternative to obtain valuable compounds, without affecting the environment and using low-cost processes. One major impediment in lignocellulose valorization is linked to its recalcitrance and structural complexity, making it difficult to depolymerize.

The structural complexity of lignocellulose is given mainly by its components: cellulose, hemicellulose and lignin (Dumitru et al., 2018). Therefore, for a complete depolymerisation several main enzymatic systems are required: cellulases, xylanases and ligninases.

Xylanases are a group of enzymes with the ability to completely hydrolyse xylan (hemicellulose's main component) into smaller fragments, being divided into two categories: primary enzymes (endo β-1,4 xylanase and β-xylosidase) and accessory enzymes (α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, ferulic esterase and p-coumaric acid esterase (Xue et al., 2012). Although most of them are labelled as secondary enzymes, their activity is very important due to their capacity to remove side chains from the main xylan structure and improve the accessibility of other enzymes such as cellulases, xylanases or pectinases (de Oliveira et al., 2014).

Feruloyl esterases or ferulic acid esterases (FAE, E.C. 3.1.1.73) are a group of enzymes involved in ester bonds hydrolyzation and release of ferulic acid (4-hydroxy-3-methoxy-cinnamic acid) from lignocellulosic structures (Figure 1).

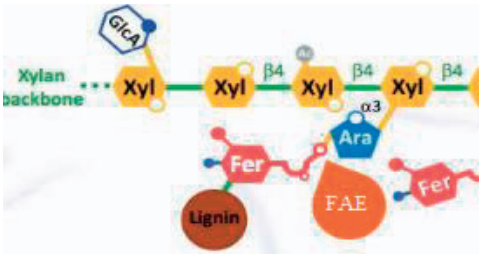

Figure 1. Feruloyl esterase mode of action (Dilokpimol et al., 2017)

Ferulic acid is either linked to arabinose or galactose residues (Figure 1) and is considered to be the most abundant and ubiquitous

hydroxycinnamic acid found in plants (Sarangi and Sahoo, 2010). As seen in Figure 1, ferulic acid is located either between hemicellulose and lignin, either between hemicelluloses (Wong, 2006). Therefore, its release could also beneficially enhance the activity of several lignin modifying enzymes (phenol oxidases or hem containing peroxidases) (Kumar and Chandra, 2020). During lignin biodegradation, FAE acts as a mediator for laccase and can also help in depolymerising polysaccharide complexes (Ozer et al., 2020).

There are many sources known as having high ferulic acid content such as: wheat, rice, oat, grains, fruits (pineapple, banana), coffee, vegetables (beetroot, spinach, artichoke), bamboo, beans, nuts etc. (Kumar & Pruthi, 2014; Topakas et al., 2007). The amount of ferulic acid from these sources varies between 0.5-2% (Kumar & Pruthi, 2014) and most of the times ferulic acid is extracted in trans isomeric form (Figure 2).

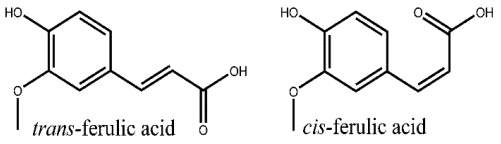

Figure 2. Ferulic acid isomeric forms (Horbury et al., 2016)

Amongst these substrates, agricultural by products are considered to be most desirable, due to economic and environmental reasons.

FERULOYL ESTERASE IMPORTANCE

The importance of feruloyl esterase derives mainly from its participation in releasing ferulic acid from lignocellulose, especially in combination with endoxylanases (Nagar et al., 2010).

Ferulic acid is used in various industrial applications such as: medical, pharmaceutical, cosmetics, food, feed, environmental etc. (Huang et al., 2011; Chen et al., 2020; Liu et al., 2020; Dilokpimol et al., 2017; Topakas et al., 2007). Its biotechnological value is related to some of its properties, such as: antimicrobial, antiallergic, anti-diabetic, antioxidant, anti-ageing, anti-inflammatory, antitumoral, neuroprotective, antithrombosis, antibiotic or even emulsifying (Sarangi & Sahoo, 2010; Liu et al., 2020; Pellerito et al., 2020; Shi et al., 2016; Sakai et al., 1999; Hong et al., 2016; Eom et al., 2016; Kumar & Pruthi, 2014; Grigore et al., 2019; Nichita et al., 2016).

Although initially ferulic acid was produced via chemical synthesis, in the last years it was considered necessary a more environmental approach via microbial fermentation.

Thus, scientists are still focused on isolating new and improved strains that display FAE activity.

Feruloyl esterases are capable of hydrolysing phenolic compounds esterified from lignocellulosic structures in a less aggressive manner, as opposed to chemical treatments that affect the environment (Jiao et al., 2014).

Other biotechnological applications of feruloyl esterase are: animal feed additives (improve nutrient assimilation), pulp and paper industry (enhance endoxylanases activity in bio-bleaching processes), bio-polymers, food industry (juice clarification, bread quality improvement, flavours etc.), bio-fuel, pharmacology etc. (Fazary & Ju, 2007; Bhathena et al., 2008; Topakas et al., 2007; Ozer et al., 2020; Sharma et al., 2020).

SCREENING PROTOCOLS OF FERULOYL ESTERASE

There are several ways to identify feruloyl esterase activity, one of the most used method being plate screening protocols.

A simple, low-cost and efficient method is described by Donaghy et al. (1998), where the microbial strains were cultivated in a minimal agar medium with the following composition (g/l): 1.3 $(NH_4)_2SO_4$, 0.25 $MgSO_4$ x 7 H_2O, 0.37 KH_2PO_4, 0.07 $CaCl_2$ x 2 H_2O, 0.03 $FeCl_3$, 1.0 yeast extract and 20 agar (for microbiological purposes). The screening media was supplemented with 0.3 ml ethyl ferulate solution (prepared in dimethylformamide), after the sterilization and partial cooling of the medium. Ethyl ferulate acted as the only carbon source, so only microorganisms that will produce FAE will be able to grow and hydrolyse the medium. After incubation at 30°C for 24 hours, the plates were analyzed based upon their hydrolysis zone around the colony, which indicated feruloyl esterase activity, as seen in Figure 3.

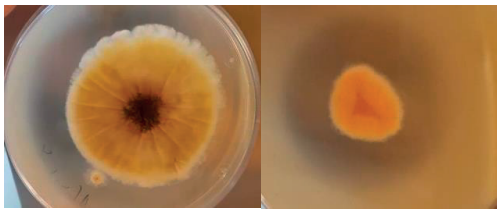

Figure 3. Hydrolysis zones around microbial colonies indicating FAE activity (original)

Although the most used and precise methods for assaying FAE activity are based on HPLC techniques (Fazary and Ju, 2007), a simple and economic protocol was described in several studies (Qi et al., 2011; Dilokpimol et al., 2017; Fazary & Ju, 2007; Mastihuba et al., 2002).
The method is based on quantifying the released ferulic acid from the hydrolysed substrate. The assay mixture was comprised of: 0.8 ml phosphate buffer 100 mM (pH = 6.00), 0.2 ml sample (from the microbial culture) and 15 µl ethyl ferulate solution (prepared in dimethylformamide).
After the incubation at 37°C for 2 h, the samples were placed in a water bath at 99°C for 3 minutes and subjected to spectrophotometric analysis at λ = 338 nm. With a ferulic acid solution (prepared in dimethylformamide) was constructed a calibration curve, that allowed to determine FAE activity. One unit of FAE was defined as the amount of enzyme that released 1 µmol of ferulic acid per minute under the assay conditions.

SOURCES OF FERULIC ACID ESTERASE

Several studies demonstrated that both fungi and bacteria display feruloyl esterase activity, in their way to hydrolyse the ester bond that connects ferulic acid to polysaccharides (de Oliveira et al., 2014; Wong, 2006).
According to Brenda Enzymes Database, the main producers of feruloyl esterases are belonging to various genera: *Aspergillus, Penicillium, Lactobacillus, Fusarium, Streptomyces, Ruminococcus* etc. (Tables 1 and 2).
The isolated enzymes have various characteristics that differs in accordance with the microbial source: substrate specificity, enzymatic type (A, B, C or D), preferable inductors, hydrolysis of methyl esters, release of free diferulates etc. (Fazary & Ju, 2007; Topakas et al., 2007).
Feruloyl esterases display their activity in a broad range of pH (3-10) and temperature (20-75°C) (Dilokpimol et al., 2016).
The bacterial strains that have displayed high feruloyl esterase activity are included in Table 1.

Table 1. Bacterial strains registered as FAE producers

Bacteria	Reference
Bacillus subtilis	Topakas et al., 2007
B. amyloliquefaciens	Topakas et al., 2007; Wang et al., 2017; Topakas et al., 2007; Fazary & Ju, 2007
Butyrivibrio fibrisolvens	Topakas et al., 2007
B. proteoclasticus	Goldstone et al., 2010; Topakas et al., 2007
Sporotrichum thermophile	Mukherjee et al., 2007; Fazary & Ju, 2007
Streptomyces olivochromogenes	Faulds et al., 1997; Wong, 2006; Donaghy et al., 2000
S. avermitilis	Ferreira et al., 1999; Garcia et al., 1998
S. cinnamoneus	Uraji et al., 2018
Cellvibrio japonicus	Topakas et al., 2007
Lactobacillus acidophilus	Topakas et al., 2007; Xu et al., 2017
L. fermentum	Russo et al., 2016; Topakas et al., 2007; Xu et al., 2017
L. plantarum	Esteban-Torres et al., 2013
Fibrobacter succinogenes	Donaghy et al., 2000; Topakas et al., 2007
Clostridium thermocellum	Blum et al., 2000; Fazary & Ju, 2007; Wong, 2006; Topakas et al., 2007
Pseudomonas fluorescens	Blum et al., 2000; Fazary & Ju, 2007; Ferreira et al., 1999; Wong, 2006; Donaghy et al., 2000;

In order to secrete FAE into the culture medium, scientists have used solid state or submerged fermentations (Fazary and Ju, 2007). Solid state fermentation (SSF) was considered to be the best option for fungal sources, listed in Table 2.
An important step obtaining ferulic acid from fermentations was selecting the best substrates, that will both provide energy and necessary compounds, required for inducing FAE production (Fazary & Ju, 2007).
Some of the best substrates from food and agro-industrial wastes are the ones with high amount of esterified ferulic acid, such as: wheat bran, maize bran, maize fibre, sugar beet pulp, de-starched wheat bran, sugar cane bagasse, corn bran, oat hulls (Fazary & Ju, 2007; Mathew & Abraham, 2005; Camacho-Ruiz et al., 2016; Topakas et al., 2007).

Table 2. Fungal strains registered as FAE producers

Fungi	Reference
Aspergillus awamori	Donaghy et al., 2000; Wong, 2006; Topakas et al., 2007; Fazary & Ju, 2007
A. nidulans	Fazary & Ju, 2007; Topakas et al., 2007
A. flavus	Zhang et al., 2013; Li et al., 2015
A. niger	Ferreira et al., 1999; Wong, 2006; Fazary & Ju, 2007; Topakas et al., 2007; Li et al., 2015
A. oryzae	Wong, 2006; Topakas et al., 2007; Garcia et al., 1998
A. terreus	Li et al., 2015; Topakas et al., 2007
A. nidulans	Fazary & Ju, 2007; Topakas et al., 2007
Penicillium brasilianum	Panagiotou et al., 2007; Topakas et al., 2007
P. chrysogenum	Li et al., 2015
Schizophyllum commune	Faulds et al., 1997; Donaghy et al., 2000
Fusarium oxysporum	Fazary & Ju, 2007; Wong, 2006; Topakas et al., 2007
F. proliferatum	Topakas et al., 2007; Fazary & Ju, 2007
Trichoderma reesei	Topakas et al., 2007

CONCLUSIONS

Lignocellulose is an abundant biomass that still needs more research for its valorization. An economic and environmental approach is its degradation with microorganisms that possess specific enzymatic systems (cellulases, xylanases, ligninases).

Feruloyl esterases are a group of enzymes involved in degradation of lignocellulose by hydrolysing ester bonds and releasing ferulic acid.

Since ferulic acid connects lignin to hemicellulose or hemicelluloses between each other, its displacement is linked to lower recalcitrance of the biomass and a more approachable structure for other enzymes.

In this study, there were presented some of the most used methods for detecting microbial feruloyl esterase activity.

FAE are produced both by bacteria (*Lactobacillus, Ruminococcus, Streptomyces*) and fungi (*Aspergillus, Penicillium, Fusarium).*

Feruloyl esterases have many biotechnological applications such as: food, feed, medical, cosmetic, pharmaceutical, environmental, fuel pulp and paper etc.

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EFFECT OF SOME STRESSORS ON BIOLOGICAL AND BIOCHEMICAL PARAMETERS IN THE *Rd* GREEN MICROALGA

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Abstract

Photosynthetic microorganisms, in addition to the major planetary role in solar energy conversion and biogeochemical cycles, have a major contribution to a wide range of biotechnological applications. In this paper, we present our original results concerning the use of different concentrations of nitrate as stressor for enhancing the lipid content in our isolate, a green microalga named Rd. The effects on cellular growth, lipid, total protein, chlorophyll a and b, and carotenes content are also presented. The results are discussed with respect to applicative and basic research approaches for a deeper understanding of the mechanisms involved in the response of microorganisms to stress.

Key words*: green microalgae, lipids, nitrate, total proteins.*

INTRODUCTION

The ability of photosynthetic microorganisms to synthetize intracellular lipid droplets is an important topic both for fundamental research as well as for practical applications (Xin et al., 2010; Sharma et al., 2012; Borowitzka, 2013; Benemann, 2013; Ardelean, 2015; Ardelean et al., 2017; 2018; Lari et al., 2016; Moisescu et al., 2018; Velea et al., 2017; Sibi et al., 2016; Tale et al., 2017).

One strategy to increase the synthesis of lipids concerns the use of so-called stressors, such as the limitation in nitrogen or phosphorus availability, the presence of heavy metals, gamma irradiation at low doses which are not inhibitory for cellular growth, or treatment with exogenous hydrogen peroxide (Li et al., 2008; Choi et al., 2015; Ermavitalini et al., 2017a; 2017b; Sibi et al., 2016; Tale et al., 2017; Gomes et al., 2017).

In this context, the aim of this paper is to show the effect that different nitrate ($NaNO_3$) concentrations have on lipid, chlorophyll *a* and *b*, carotenes, and total protein content in *Rd* strain, a new isolated green microalga.

MATERIALS AND METHODS

The photosynthetic microorganisms. The *Rd* green microalga strain was previously selected by random mutagenesis (Ardelean et al., 2018), due to increased lipid content as well as a practical important characteristic, namely growth in liquid medium as microcolonies which significantly promote harvest by rapid gravitational sedimentation. The *Rd* strain was cultivated on four variations of BG_{11} medium, with different concentrations of nitrate ($NaNO_3$): classic BG_{11} (with 1.5 g/l $NaNO_3$), BG_0 (without $NaNO_3$), and the modified BG_{11} (with 0.03 or 0.375 g/l $NaNO_3$).

Lipid content was estimated using the phospho-vanillin method (Park et al., 2016). Microalgal paste was resuspended in 2:1 parts of chloroform: methanol (v/v) by manually shaking the tube vigorously for a few seconds or until the biomass was dispersed in the solvent system. Finally, a 0.73% NaCl water solution was added to produce a 2:1:0.8 system of chloroform: methanol: water (v/v/v). The phospho-vanillin reagent was prepared by dissolving 0.75 g vanillin in 125 ml distilled water and mixed with 500 ml of 85% phosphoric

acid solution. The final concentration of reagent was 1.2 mg/ml vanillin in 68% phosphoric acid. Algal oil was dissolved in chloroform (10 mg in 10 ml for a final concentration of 1 mg/ml), and different concentrations (10 to 150 µg) of standard lipid samples were prepared in clean glass vials. The vials were incubated at 90°C for 10 min to evaporate the chloroform. 0.1 ml of concentrated sulphuric acid was added to each vial and then heated at 90°C for 10-20 min. After cooling on ice for about 5 min, 2.4 ml of phospho-vanillin reagent was added and allowed to develop for 10 min, until the colour of the sample turned pink (Park et al., 2016).

The equation for etalon curve is the following y = 0.0034 x -0.006 (R^2 = 0.9826), using oil extracted from mixture of microalgae populations.

Nile red labelling of lipids has been done as previously described (Ardelean et al., 2018).

Chlorophyll *a* and *b* was extracted in 90% methanol and the concentration calculated as previously described (Ardelean et al., 2018).

Carotenoids were measured spectrophotometrically using the modified method of Mackinney (1941) as presented by Boyer (2006). Briefly, a known volume of culture was centrifuged at 4000×g for 10 min. The supernatant was decanted and the same volume of methanol was added to the pellet. The mixture was incubated in a water bath at 55°C for 15 min and then centrifuged at 4000×g for 10 min. The absorbance (A) of the extract was measured against a blank of free methanol at 650, 665, and 452 nm. Carotenoids were estimated as µg/ml of culture suspension using the following equation: Carotenoids (µg/ ml) = 4.2 A452 – [0.0246 (10.3 A665 – 0.918 A650)].

Total soluble proteins were estimated using the biuret method. After carotenoids extraction, residual cells were extracted using 1 N NaOH in a boiling water bath for 2 h. Serum bovine albumin was used to construct the calibration curve, the equation being the following: y = 0.0346 x -0.0004 (R^2 = 0.9998). This equation was further used to calculate the concentrations of total proteins in our experimental samples.

RESULTS AND DISCUSSIONS

In Figure 1 there is presented the quantity of lipids in the green microalga *Rd*, grown in classic BG_{11} medium (with 1.5 g/l $NaNO_3$) or in different variants of this medium: BG_0 (without $NaNO_3$) or modified BG_{11} (with either 0.03or 0.375 g/l $NaNO_3$).

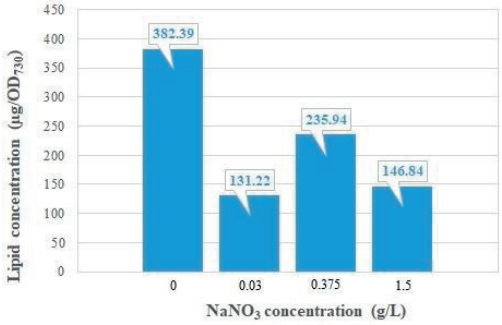

Figure 1. Lipid content of *Rd* green microalga grown in various $NaNO_3$ concentrations

The lipid content varies significantly when the green microalga *Rd* is grown in the same mineral medium, but with different $NaNO_3$ concentrations. The highest value was obtained for cells previously grown in BG_{11} and then transferred in BG_0, in order to induce nitrogen starvation.

These results are in general agreement with other reports concerning the increase in lipid content following nitrogen starvation (Li et al., 2008). However, we have no explanation for why at very low $NaNO_3$ concentration in the growing medium (0.03 g/l $NaNO_3$) the lipid content is lower (131.22 µg lipids/unit OD) than in the case of 0.375 g/l $NaNO_3$ (382.39 µg lipids/unit OD). It can be speculated that even at that very low $NaNO_3$ concentration, the metabolism is not sufficiently stimulated to change.

These empirical observations could be helpful not only for practical oriented research but also for fundamental research.

In Figure 2, there are presented cells of *Rd* strain grown in BG_{11} with 0.03 g/l $NaNO_3$ and labelled with Nile red for lipid inclusions, in both green and red fluorescence.

In Figure 3, in the *Rd* cells grown in BG_0 and labelled with Nile red, the lipids droplets are seen much clearer, their concentration being higher (i.e., 382.39 µg lipids/unit OD) as compared to the cells grown in BG_{11} (Figure 2) where the concentration of lipids is lower (i.e., 131.22 µg lipids/unit OD).

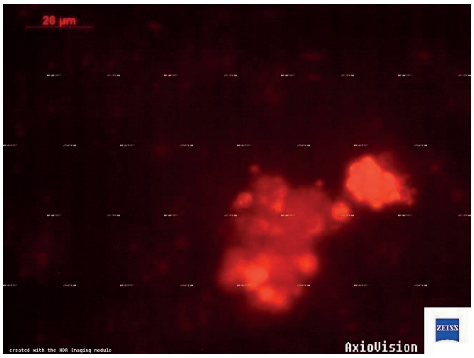

Figure 2. Microscope images of the same microscopic field showing *Rd* cells grown in BG_{11}, and labelled with Nile red for lipid inclusions

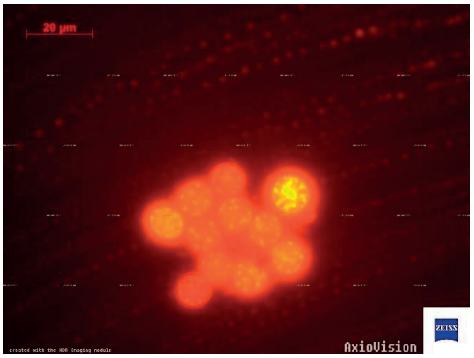

Figure 3. Microscope images of the same microscopic field showing *Rd* cells grown in BG_0, and labelled with Nile red for lipid inclusions.

The results concerning the content in chlorophyll *a* and *b*, total proteins and total carotenes in *Rd* grown in either BG_{11} or BG_0 are presented in Table 1.

Table 1. The content in chlorophyll *a* and *b*, total proteins and carotenes in *Rd* strain grown in BG_{11}or BG_0

Culture	Biomass (mg)	Lipids (µg/DO_{730})	Proteins	Chl *a*	Chl *b*	Carotenes
			(µg/ml)			
BG_0	0.20	382	5.1	4.8	1.0	2.6
BG_{11}	0.14	147	3.3	2.8	1.3	1.9

The high decrease in total protein content in *Rd* cells grown in BG_0, where the lipid content is highly increased suggests that, in the absence of exogenous $NaNO_3$, some of the proteins are mobilised and converted to lipids, which are deposited inside the cells as droplets.
However, low dose gamma irradiation seems to be less efficient in promoting lipid accumulation compared with $NaNO_3$ starvation (results not shown).

CONCLUSIONS

Different nitrogen concentration is able to induce changes in lipid composition of the green microalga *Rd* as compared with cells grown in classic BG_{11} medium. The highest lipid concentration was obtained under complete nitrogen starvation, when no nitrate was present in the extracellular medium (BG_0).
In these conditions (BG_0) other cellular components also exhibit different variations in their concentrations.

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SCREENING OF HALOTOLERANT BACTERIA PRODUCING HYDROLYTIC ENZYMES WITH BIOTECHNOLOGICAL APPLICATIONS

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Abstract

Hydrolytic enzymes produced by halotolerant/halophilic microorganisms display polyextremophilic properties, which determines particular industrial interest. The goal of this work was to detect at least one halotolerant bacterial strain for the ability to produce multiple enzymatic activities (amylases, proteases, lipases, cellulases) with biotechnological importance. Eighteen halotolerant bacterial strains, isolated from hypersaline water located in Lopătari, Romania, have been used in this study. Hydrolytic enzymes production was assayed by plate screening on basal media supplemented with following sources: 1% soluble starch (for amylase activity); 1% carboxymethylcellulose (for cellulase activity); skimmed milk (for protease activity); 1% Tween 80 and $CaCl_2$ (for lipase activity). Positive reactions have been shown by the presence of a clear halo or precipitates around the bacterial isolates. All strains had at least one enzymatic activity. The lipases and amylases activities were the predominant hydrolytic activities detected. Our results revealed the ability of different halotolerant bacterial strains to present a combination of two or three hydrolytic enzymes: 10 isolates have produced amylases and proteases enzymes; 2 isolates - lipases and proteases enzymes; 1 isolate - amylases, proteases and cellulases enzymes and 3 isolates - proteases, lipases and cellulases enzymes, respectively. Potential enzymes production by isolate 11.5 also was evaluated in submerged cultivation on different substrates. Among the substrates tested, the production of hydrolytic enzymes by isolate 11.5 was enhanced when grounded cereals mixture 1% was used as sole substrate as carbon and nitrogen source: 312 U/ml (amylases), 0.22 U/ml (cellulases) and 0.09 U/ml (proteases).

Key words*: halotolerant bacterial isolates, hydrolytic enzymes, plates screening.*

INTRODUCTION

Microbial communities of hypersaline environments represent valuable sources with industrial applications such as food/feed, chemical, pharmaceutical, detergents, environmental, bioremediation and biosynthetic processes, being one of the main reasons for studying them (Oreon, 2010; Kamekura and Enache, 2010; Patel and Saraf, 2015; Yin et al., 2015; Flores-Gallegos et al., 2019; Liu et al., 2019). Ventosa et al. (1998) classified halophiles into slight halophiles growing optimally at 1-5% sodium chloride (NaCl); moderate halophiles growing optimally at 5-20% NaCl; and extreme halophiles growing optimally at 20-30% NaCl. Halophiles (especially bacteria and Archaea) have been isolated from few Romanian saline areas: Lake Telega (Enache et al., 2007), Salina Unirea (Cojoc et al., 2009), Balta Alba (Neagu et al., 2014) Techirghiol (Enache et al., 2009) and Lopătari (Proca et al., 2017). In a recent study, Proca et al. (2017) reported that of a total of 44 bacterial strains isolated from a hypersaline water in Lopătari, România, 4.5% demonstrated a salt large tolerance ranging from 0% to 12.5%; 7% ranging from 2.5% to 30%; 41% ranging from 5 to 25%, respectively.

In relatively recent years, an interesting research topic was considered the selection of the best halophiles microorganisms producing a wide range of extracellular hydrolytic enzymes (Sánchez-Porro et al., 2003; Cojoc et al., 2009; De Lourdes Moreno et al., 2013; Suganthi et al., 2013; Babavalian et al., 2014; Enache et al. 2014; Neagu et al., 2014; Karray et al., 2018). These enzymes are good candidates for the use in industrial processes, because they are active under extreme conditions such as high salt concentration, low water activity (aw), at extreme pH and temperature (Kamekura and

Enache, 2010; Oreon, 2010; De Lourdes Moreno et al., 2013; Patel and Saraf, 2015; Flores-Gallegos et al., 2019).

Bacterial strains isolated from different saline environments have been investigated as hydrolytic enzymes producers, such as proteases (Sánchez-Porro et al., 2003; De Lourdes Moreno et al., 2013; Suganthi et al., 2013; Babavalian et al., 2014; Karray et al., 2018), amylases (Amoozegar et al., 2003, Enache et al., 2009; De Lourdes Moreno et al., 2013; Babavalian et al., 2014; Karray et al., 2018), cellulases (Babavalian et al., 2014; Karray et al., 2018), lipases (De Lourdes Moreno et al., 2013; Babavalian et al., 2014; Moreno et al., 2016; Karray et al., 2018) or xylanases (Babavalian et al., 2014; Karray et al., 2018).

There are few Romanian researches reporting the screening of the extremely halophilic microorganisms producing hydrolytic enzymes (Enache et al., 2009; Cojoc et al., 2009; Enache et al. 2014; Neagu et al., 2014). Enache et al. (2014) reported the ability of halophilic bacteria isolated from salted lakes and salt crystals from the salt mine, to produce a wide range of extracellular hydrolytic enzymes able to degrade several macromolecular substrates, such as sugar based polymers or proteins. In another study, Neagu et al. (2014) reported the capacity of halophilic bacterial strains isolated from Balta Albă salty lake to synthesize two or three different enzymes.

The aim of this work was to select the halotolerant bacteria isolated from a hypersaline water in Lopătari, România as producers of hydrolytic enzymes (amylases, cellulases, lipases and proteases). After the initial screening, a halotolerant strain exhibiting relevant and divers hydrolitic activities (isolate 11.5) has been selected for in depth characterisation of its enzymatic activity.

MATERIALS AND METHODS

Microorganisms and growth conditions

Eighteen bacterial isolates originating from a hypersaline water in Lopătari, România (Proca et al. 2017) have been used in this study. The bacterial strains were grown and maintained on Nutrient Agar (Merck, Germany) containing 5% NaCl.

Plate screening of enzymes producing bacteria

All halotolerant bacterial isolates were streaked in line on the surface of culture media distributed in Petri dish.

Amylase activity was evaluated on nutrient agar medium with 5% NaCl supplemented with 1% of soluble starch. After incubation at 37°C for 2 days, the cultures were flooded with 0.3% I_2-0.6% KI solution. Positive reaction was indicated by a clear zone surrounding a halotolerant bacterial isolate.

Cellulase activity was tested on nutrient agar medium with 5% NaCl supplemented with 1% carboxymethylcellulose (CMC). After incubation at 37°C for 2 days, the hydrolysis zone was visualized after staining with 0.1% Congo red solution and washed the plate with 1 M NaCl.

Protease activity was detected on milk agar medium containing skim milk (0.1% fat): water (v/v) (1:2) and 2% agar. After incubation at 37°C for 2 days, the halotolerant bacterial isolates showing clear zone of the degradation of casein were read as positive for protease production.

Lipase activity was determined on nutrient agar medium containing 1% Tween 80 as a lipid substrate and 0.01% $CaCl_2xH_2O$. Positive reactions were directly observed by a precipitation zone around the inoculated halotolerant isolates.

Production of hydrolytic enzymes by halotolerant isolate 11.5 in submerged cultivation

The bacterial isolate 11.5 was cultured in nutrient broth medium with 5% NaCl supplemented with soluble starch (0.5% and 1%), carboxymethylcellulose (0.5% and 1%) and respectively skim milk (diluted 1: 2) as substrates. Also, we used grounded cereals mixture 1% (maize, wheat, sunflower, soybean, in unknown quantities) as sole substrate for carbon and nitrogen source. Fermentation carried out in orbital incubator shaker operating at 120 rpm, at 37°C for 72 hours. At every 24 h, the cultures were harvested by centrifugation at 4000 g for 10 min at 4°C. The cell-free culture supernatant was used as preparation of crude enzyme to measure the activities of amylase, cellulase, and protease.

Enzymatic activity

Amylase activity. 0.5 ml sample was added with 0.5 ml of 0.2 M phosphate buffer and 1 ml of 1% (w/v) starch disolved in 0.2 M phosphate buffer and 20% (w/v) NaCl. The reaction mixture was incubated for 10 min at 30°C. The reaction was stopped by 2 ml of 3,5-dinitrosalicylic acid and the mixture was heated at 100°C for 5 min in boiling water bath. 8 ml of water was added and the absorbance was determined at 546 nm. One unit of enzyme activity (U) was defined as the amount of enzyme liberating 1 μg of maltose per minute under the assay conditions. The amount of enzyme was determined by dinitrosalicylic acid (DNS) method of reducing sugar described by Miller (1959) and maltose was used as a standard.

Cellulase activity. The reaction mixture consisting of 2 ml of a 1% (w/v) CMC in 50 mM citrate buffer pH 6.4 as a substrate and 0.2 ml of crude enzyme solution was incubated for 10 min at 50°C. The reaction was stopped by 3 ml of 3,5-dinitrosalicylic acid and the mixture was heated at 100°C for 15 min in boiling water bath. One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 mg of reducing sugar per minute under the assay conditions, according to the standard method (Miller, 1959), and glucose was used as a standard.

Protease activity. Protease activity was determined using casein as a substrate. The reaction mixture containing 5 ml of 0.65% (w/v) casein solution, prepared in 50 mM potassium phosphate buffer was mixed with 1 ml of protein as crude cell-free enzyme source and incubated at 37°C for 10 minutes. The reaction was stopped by adding 5 ml of 110 mM trichloroacetic acid (TCA), followed by and incubation at 37°C for 10 minutes. After filtration using a 0.45 µm polyethersulfone syringe filter, 2 ml samples filtrate were mixed with 5 ml of sodium carbonate and 1 ml of Folin's reagent and incubated at 37ºC for 30 minutes. The absorbance was determined at 660 nm and tyrosine was used as a standard. One unit of protease activity (U) was defined the amount in micromoles of tyrosine equivalents released from casein per minute under the assay conditions (Folin and Ciocalteu, 1927; Anson, 1938).

RESULTS AND DISCUSSIONS

Screening of halotolerant bacterial isolates for extracellular enzymes

On-plate screening has been used to evaluate eighteen halotolerant bacteria for their ability to produce extracellular enzymes.

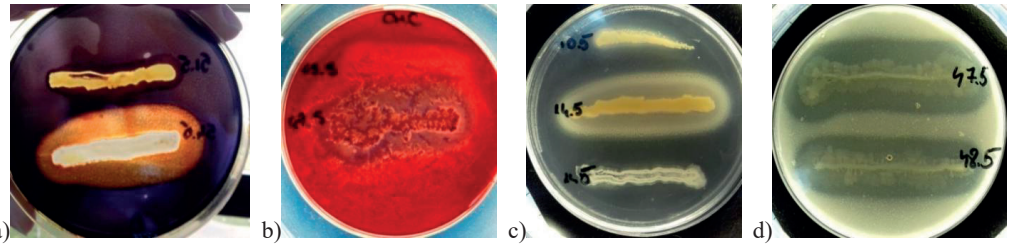

Figure 1. Plate screening aspects of enzyme assays for halotolerant bacterial isolates: amylase (a), cellulase (b), lipases (c), proteases (d)

Aspects of the of positive results for the hydrolytic enzymes screening have been visualised by the ability to produce halo or precipitate zones around the microorganisms, as seen in Figure 1; as procedure, the diameters of clear zones were measured in millimeters (mm). Lipase and amylase were the most abundant activities shown by 16 and 12 strains, respectively (Table 1). The minimum amylase activity was recorded on strain coded 51.5 and lipase activity on strain coded 57.5. Six strains were able to produce proteases. The isolates 11.5, 47.5, 48.5 presented the highest clear zone formed around the inoculation wells on milk agar plates and followed by the isolates 46.5 and 63.5, and the 46.5 showed the lowest. Cellulase activity was detected only in the case of four strains coded 11.5, 47.5, 48.5, and, respectively 63.5 (Table 1). In another study, Enache et al. (2014) reported the ability of halophilic bacteria

isolated from salted lakes and salt crystals from the salt mine, to produce a wide range of extracellular hydrolytic enzymes able to degrade several macromolecular substrates, such as starch, casein, Tween 80, and carboxymethylcellulose.

Our results revealed the ability of several halotolerant bacterial strains to produce a combination of two or three hydrolytic enzymes: 10 isolates have produced amylases and lipases enzymes; 2 isolates - lipases and proteases enzymes; 1 isolate - amylases, proteases and cellulases enzymes; 3 isolates - cellulases, lipases and proteases enzymes, respectively (Table 1).

Table 1. Plate screening of enzymatic activity from the halotolerant bacterial isolates

No.	Isolate code	Amylases	Cellulases	Lipases	Proteases
1.	10.5	+		+	-
2.	11.5	+++	+++	-	+++
3.	13.5	++		+++	-
4.	14.5	++		++	-
5.	45.5	-	-	++	++
6.	46.5	-	-	+	+
7.	47.5	-	+++	++	+++
8.	48.5	-	+++	++	+++
9.	50.5	++	-	+++	-
10.	51.5	+	-	-	-
11.	54.5	+++	-	+++	-
12.	55.5	++	-	++	-
13.	57.5	-	-	+	-
14.	58.5	+++	-	++	-
15.	59.5	++	-	+++	-
16.	60.5	++	-	++	-
17.	62.5	+	-	++	-
18.	63.5	-	+++	++	++

+: 0.1-0.9 mm; ++1.0-1.9 mm;: +++ : > 2 mm; −: no enzymatic activity

Neagu et al. (2014) also reported the capacity of halophilic bacterial strain isolated from Balta Albă salty lake to synthesize two or three enzymes: one halophilic bacterial strain combined cellulase, esterase and gelatinase activities and other seven strains combined two hydrolytic activities, either esterase and gelatinase or cellulase and gelatinase.

Karray et al. (2016) isolated halophilic strains from Chott El Jerid, a hypersaline lake in the south of Tunisia and identified as members of the genera: *Salicola, Bacillus, Halorubrum, Natrinema* and *Haloterrigena*. Most of these isolates were able to produce hydrolytic enzymes such as amylase, protease, lipase, cellulase, xylanase, pectinase and some of them showed combined activities.

In our study, the isolate 11.5 registered high enzymatic activity in the case of three hydrolytic enzymes, respectively amylases, cellulases, proteases. Data not published (personal data) indicate that the isolate 11.5 exerted also a broad-spectrum antimicrobial activity. Due to these facts, the isolate was kept for further investigations as a halotolerant bacteria of biotechnological potential.

Preliminary studies on extracellular hydrolytic enzymes of halotolerant isolate 11.5

Initially, the hydrolytic enzymes production of the isolate 11.5 was evaluated by submerged cultivation during 72 hours using different substrates: 1% starch, CMC 1% and skim milk: water (1:2). The enzymatic activity was determined at different time intervals: 24, 48 and 72 hours.

The amylase and cellulase activities increased 10-fold (Figure 2a and b) and 2-fold for the protease activity (Figure 2c) after 48 h.

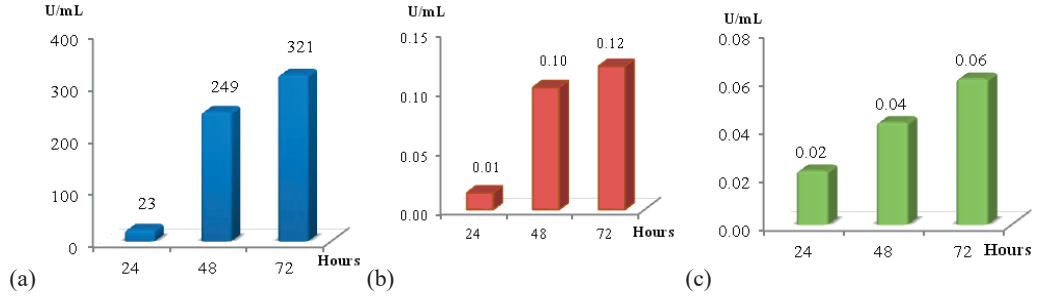

Figure 2. Enzymes production of 11.5 isolate on different substrates during 72 hours: amylases (a), cellulases (b), proteases (c)

Also, the 11.5 potential for enzymes production was evaluated in submerged cultivation on different substrates and in different concentrations (Figure 3).

The amylase activity increased 2.39-fold after increasing the starch concentration from 0.5% to 1%. A similar result of the amylase activity was also obtained when were used grounded cereals mixture as sole carbon and nitrogen source under submerged cultivation (312 U/ml) (Figure 3a).

The cellulase activity of isolate 11.5 increased 1.33-fold when increasing the concentration of CMC from 0.5% to 1%. A similar result of the cellulase activity was also obtained when grounded cereals mixture was used as sole carbon and nitrogen source under submerged cultivation (0.22 U/ml) (Figure 3b).

Regarding the protease activity, it increased 1.29-fold under submerged cultivation on grounded cereals mixture comparing to skim milk medium (Figure 3c).

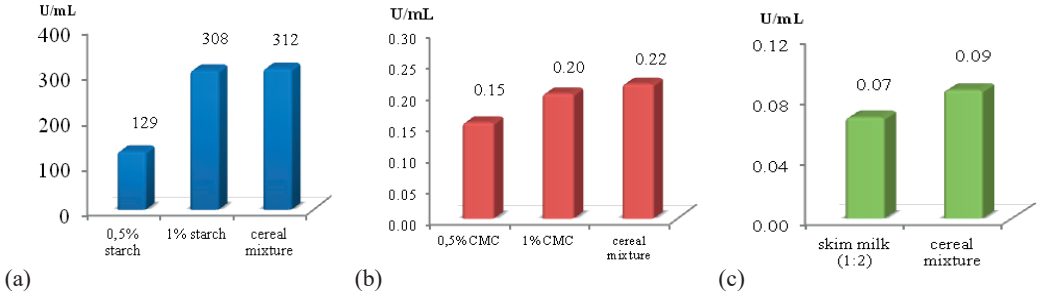

Figure 3. Effect of different concentrations of substrates on enzymes production of the isolate 11.5: amylase (a), cellulase (b), protease (c)

CONCLUSIONS

The results of the present study show that the halotolerant bacterial strains isolated from a hypersaline water in Lopătari, România could be a potential source of hydrolytic enzymes production. From the total halotolerant bacterial isolates (eighteen) 66.7% produce two enzymes types, 22.2% produce a combination of three different hydrolytic enzymes and only 11.1% produced one hydrolytic enzyme.

The present study showed that the production of hydrolytic enzymes by the isolate 11.5 was enhanced when grounded cereals mixture was used as sole carbon and nitrogen source under submerged cultivation reaching the following values: 312 U/ml (amylases), 0.22 U/ml (cellulases) and 0.09 U/ml (proteases).

As a consequence, the eventually minimization of the use of nutrients, and chemicals sources could reduce considerable the production costs on an eventual industrial scale-up.

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DIMINISHING FOOD LOSS AND WASTE: A CHALLENGE TO MAINTAIN SUSTAINABLE FOOD CHAIN AND COMBAT THE GLOBAL PROBLEM OF HUNGER AND MALNUTRITION

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Abstract

Malnutrition is a global issue that no country can afford to avoid. According to recent reports, the number of hungry people rises from 785 to 822 million from the year 2015 to 2018. Though food production is increased from the past few decades to make up the increasing demand for food, the loss of food and the wastage of food that occurs from the agricultural field to the consumer end are highly alarming. About one- third of agricultural production gets wasted every year. The judicious use of these food wastes can attenuate the crisis of hunger and malnutrition. Food loss and wastage of food not only signify wastage of wasting food particles but also inevitably address the wastage of most important available resources like land capable of agriculture, water footprints, energy, manure, chemical compounds, money and manpower. Climate change is another factor that disrupts food production. These two factors together challenge food security which is a basic human right. To a certain extent, food loss can be reduced by the use of proper food handling, use of technology etc., and edible wasted biomass can be transformed to harmless, alimental and simpatico products that can alleviate the nutrient deficiencies as well as undernutrition and enhance the sustainability of the total food system. Though loss and waste of food are not only the principal reason behind undernutrition through the reduction of the loss and wastage of food, the global problem of hunger and malnutrition can be eradicated to a certain extent. Thus, this research paper tries to discuss the cause, revival of the loss of food and wastage of food along with how the conversion of these wastes can produce nutritious food products that can not only maintain a sustainable food chain but also can alleviate the global problem of malnutrition.

Key words*: biomass, food commodities, food loss, food security, malnutrition.*

INTRODUCTION

The problem of scarcity of food increases with the growing population of the world and 98% of people from developing countries are deprived of adequate food to sustain a healthy life.

Studies by Engstorm and Carlsson-Kanyama (2004) and Kader (2005) revealed that about 30-40% of food losses occur in the nations where the agricultural infrastructure is poor and if these losses can be prevented there will be an enormous effect on food safety and security.

FAO has distinguished the terms of food "losses" and "waste" (FAO, 2013b). Namely, "Food loss" can be defined as the food which lost its edible biomass during its deportment throughout the food supply chain. The foods get damaged and spoiled and of reduced quality, before it reaches the market for the consumers. Food loss is an unlimited process or the institutional/legal framework.

"Food waste" refers to the food that maintains its quality throughout the food supply chain but thrown away or discarded which is related to the behaviour of the consumer and the retailers. The lack of awareness and a mindfull choice to throw food away lead to the wastage of large amount of food.

"Waste" of food mostly takes place in developed countries and is related to the behaviour of consumers. It occurs because they have high per capita earnings and as a result, the opportunity cost of time becomes lower in numerous countries with medium/high-income rate (Hall et al., 2009). Furthermore, food "losses" mainly takes place in developing countries. Because food losses primarily associated with technical limitations such as inappropriate harvesting practice, lack of storage and cooling facilities and financial limitations and managerial boundaries. Per capita "food wasted" by people in Europe and North America was stated to be 95-115 kg per year. But, in sub-Saharan Africa and South and South-East Asia, per capita wastage is only 6-11 kg/year (Gustavsson et al., 2011) - Figure 1.

In the US, yearly 40 million tons of food discarded by households, foodservice and retailers and this amount are adequate to give food to all the malnourished people around the world. Famine would disappear from the world if the quarter amount of food is saved which is wasted in the US, UK, and Europe (Stuart, 2009). An estimation of global hunger in 2013 reported that "the world produces enough food to feed everyone". But still, coincidentally, it was estimated that one in eight people or some 870 m people are undernourished.

The loss and wastage of food are symbolising the gap between the production and consumption of food at the consumer level, which is commonly seen in the whole world via the food value chain.

MATERIALS AND METHODS

Relevant research papers and reviews were searched on several scientific databases such as Google Scholar, PubMed, Scopus and Science Direct. The search was mainly focused on food losses and waste, their prevention, climate change and food loss, and malnutrition. The following terms were searched for selection of relevant papers such as "food loss", "food waste", "climate change", "malnutrition", "hunger", "famine", "global data on the loss and wastage of food", "reduction of the loss and wastage of food".

The selected research papers were reports, observations, basic studies, relevant reviews preferably with global data. Importance was given to more recent reports, reviews and original articles mainly published in the last ten years. The number of reports and articles has increased each year as the importance given to the reduction of the loss and wastage of food to reduce the incidence of chronic hunger and malnutrition.

RESULTS AND DISCUSSIONS

1. Food loss and wastage toward the food supply chain

The wastage of food occurs in all stages of the food supply chain. Agricultural products are travelled a long way and gone through various stages up to packaging before they reach the market, specifically, to the buyers or consumers. Finally, not only the product changes its originality but also lost some of its major parts in this process (Hoering, 2012).

There are two different scenarios in developed and developing countries that should be considered while considering the reasons for food waste. The difference is mainly based on economic capacity, manpower, technical knowledge, educations and opportunities to go into the chain of food supply system.

Lipinsky et al. (2013) stated industrialized Asian nations and developed nations are accountable for fifty-six percentage of total loss and wastage of food. Simultaneously, total loss and wastage of food in nations which are developing account for forty-four percentage. As the studies conducted in 2011, global food loss is 36.17% that was primarily at the consumer level. In developed countries, the major loss of food materials occurs at the consumer level. At the same time developing countries witness the loss mainly occurs during the production level (Pachón, 2013).

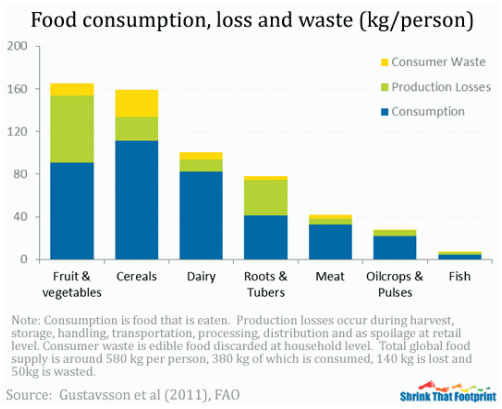

Figure 1. Food consumption, loss and waste (kg/person)

2. Food loss, waste and global climate change

Food production and wastage of food are responsible for greenhouse gas production which was estimated to be 3.3 million tons. This accelerates climate change. Studies also revealed that food waste is responsible for the third biggest emission of greenhouse gases. The main reason behind this is the energy used during food production as well as the use of a large amount of fossil fuel during production and processing of food, cooking and transport of food materials to the market worldwide. The food which is thrown away or discarded

produces methane gas produced at landfills is another reason for global warming (Figure 2).

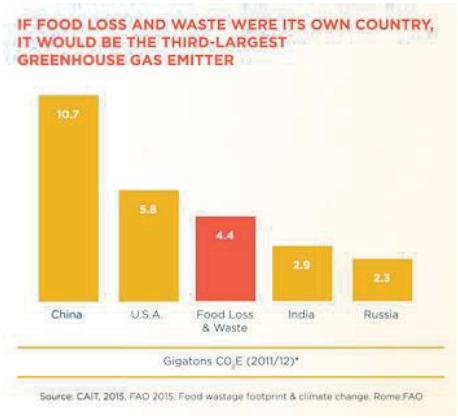

Figure 2. Impacts of food waste on climate change

3. Estimation of the loss and wastage of food

Though some primary materials on the loss and wastage of food were evaluated the international works of literature do not supply adequate data on this matter. Most studies were taken by Tristram Stuart (2009) and the FAO, and particularly on the investigation carried out by Gustavsson on behalf of the FAO. Depending on various stages of the supply chain of food, it was estimated that, at the various stages in the time of production, the global food loss is 24%, 24% during the post-harvest stage and at the consumer level it is 35%. And altogether, these multifarious stages are responsible for more than eighty percentage of the loss and wastage of food globally (Lipinsky et al., 2013). Research conducted by The World Bank Group (2014) stated that there is a role of socioeconomic status in case of the loss and wastage of food. It was found that lower-income group families waste less food compared to high-income families. A study by the Institution of Mechanical Engineers (2013) showed that wheat consist of 21 million tons is wasted in India every year due to a lack of proper storage facility and distribution system. According to Pachón (2013), about 230 million people in India went hungry per day. FAO statistics (2013) highlighted that 557 million Asian people are suffering from starvation. At the same time, food loss in developing countries like Africa, South and Southeast Asia and Latin America were attributed to the loss of crops in the field and post-harvest losses. According to Stuart (2009), the US, Europe and the UK manage to produce double amount of food which is dietary required for their inhabitants. But half of food also is wasted in the supply chain of food system.

4. Food loss, waste and malnutrition

The amount of food that is lost and concurrently wastage of food remains edible for human consumption. Pascoe (2011) stated that fifteen percentage of the food that is wasted can supply enough food for the malnourished habitants of Mexico. Food security does not only depend on the food supply problem, but it also involves accessibility i.e. purchasing capacity and food prices. Improving the proficiency of the food supply chain can help to reduce the price of the food and therefore increase access for the consumers (FAO, 2011). There should be collaboration between governments and other agencies to establish a sustainable food environment for people. Major criteria should include food supply, food system, food environment and change behaviour of the consumers (World Cancer Research Fund/American Institute for Cancer Research, 2018). Food loss at the production level should be checked by the developing countries as the number of malnourished people is high in those regions. Public health initiatives for improving the health of the population must take into account the food system within-country but also from a global point of view (Waterlander et al., 2018) and include priorities to target a decline of risk factors and disease (International Council for Science, 2017).

5. Prevention of the loss and wastage of food

There are a variety of reasons for the loss and wastage of food in developed and developing countries. In poor countries, food loss mainly occurs at the production and post-harvest level while in rich countries food gets wasted at the buyers' level. Developing countries should organize their farmers for diversifying and up scaling their production and marketing. Food and packaging industries should work together and invest to improve the infrastructure and transportation facilities. Involvement of the public and private sector is also necessary for achieving these goals. While in developed countries, different strategies are needed for

reducing food loss. Increasing awareness among consumers, retailers and food industries is also needed. There is a need to discover good and valuable utilization for the food which is wasted (FAO, 2011).

In addition to this, the wasted food or the edible biomass can be converted to nutrient-rich, safe and appealing foods that help to improve the nutrient intake of the vulnerable groups. The recovery of wasted biomass into the food supply chain also promotes a sustainable food system (Augustin et al., 2019).

CONCLUSIONS

This work highlighted the loss and wastage of food, some probable reasons for wastage of food in the total value chain. Research findings indicated various anomalies in each step in developed and developing counties though the data is difficult to find especially in the case of developing countries. This discussion related to the loss and wastage of food pointed out that food loss is common in countries which are developed while the incidence of food waste is high in developed countries. Both are vastly responsible for economic loss and related to the food security and public health. In addition to that, the discarded food is not only wastage of wealth but also a loss of natural resources. The data also showed that if the world's food loss and food waste can be reduced to a minimum, there will be no incidence of famine and malnutrition. Therefore, awareness is necessary both at the production and consumer level to minimize the loss and wastage of food for a better future of our world

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RESEARCH ON THE POTENTIAL COSMETIC APPLICATION OF A POLY-HERBAL PREPARATION

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Abstract

Plants are widely used for the development of new products for cosmetics and pharmaceutical applications. In recent years, it is increasingly recognized that the active properties of plant extracts, which are usually complex mixtures of many components, are not due to the activity of a single component, but is the result of the synergistic effects of the elements of the mixture. The aim of this study was to demonstrate the feasibility of a poly-herbal formulation based on Daucus carota L. and Prunus armeniaca L. to be included in cosmetics as an active ingredient. Three types of extracts were taken into consideration: hydroalcoholic, hydroglycerin and macerate in grapeseed oil. HPLC analysis revealed that the hydroalcoholic extract contains polyphenolcarboxylic acids rather than flavonoids. As regards fatty acids quantified by GC, slight differences between sample and control were recorded. The potential effect of the three extracts on murine fibroblasts was evaluated and the proliferation rate was assesed by MTS test. It was showed that none of the extracts are not exhibiting cytotoxic effects. Except for the maximum concentration tested of the hydroglycerin extract, at which the cell viability was around 50%, most of the samples exceeded the 80% threshold. In the case of the hydroglicerin extract, it was observed a strong stimulating effect on cell proliferation compared to control group. It can be concluded that this poly-herbal combination could be used successfully as cosmetic ingredient for its wound healing and regenerative properties.

Key words*: Daucus carota L., flavonoids, polyphenolcarboxylic acids, Prunus armeniaca L.*

INTRODUCTION

Global market for skin-care products is characterized by constant growing in the period 2015 to 2021 with the highest share for the anti-aging segment (Research and Markets, 2015). Herbal preparations are a modern trend in the field of beauty but also in the pharmaceutical one. These agents are gaining popularity because these products provide the body with nutrients and enhance health, while also providing satisfaction, as they do not contain synthetic chemicals and have fewer side effects, compared to synthetic cosmetics. In recent years, it is increasingly recognized that the active properties of plant extracts, which are usually complex mixtures of many components, are not due to the activity of a single component, but is the result of the synergistic effects of the elements of the mixture. Currently, an important factor that determines the growth of the cosmetics market is innovation along the chain, which results in new products with diverse and innovative formulations and added functionality, produced in a sustainable way and in accordance with consumer expectations (Boeriu, 2015).

The plant metabolites with potential cosmetic applications include phenolics, polyphenols, flavonoids, terpenoids, steroids, steroidal saponins, sterols, sugars, polysaccharides, lignans, carotenoids, organic acids, anthocyanins and coumarins. A number of plant sources have been explored by the cosmetics industry to create innovative combination of ingredients with specific pharmacological actions such as antiallergy, antiinflammatory, moisturizing, procollagen, anticarcinogenic, antiageing, antihyperpigmentation and UV protective (Dorni et al., 2017).

The genus *Daucus*, belonging to the family Apiaceae (Umbelliferae), comprises about 60 annual and biennial species mostly distributed in north temperate regions of the world (Fu et al., 2010) and widely used în cosmetics for its astringent, vulnerary, antiinflammatory, maturative, analgesic, depurative, antiaging, tissue regenerative, beautifying properties

(Gilca et al., 2018). Wound-healing property of ethanolic extract of *Daucus carota* L. (carrot) root may be attributed to the various phytoconstituents like flavonoids and phenolic derivatives present in the root and the quicker process of wound healing could be a function of either its antioxidant or antimicrobial potential (Patil et al., 2012). High quality pectin also contributes to its soothing properties (Toma et al., 2019). In a recent study, among the species identified to have the highest dermatologic importance, *Daucus carota* L. had an index of 72.28 on a scale of 100, after *Brassica oleracea* L. (100), *Matricaria chamomilla* L. (79.17), *Arctium lappa* L. (74.82) (Gilca et al., 2018).

Apricot kernel oil (*Prunus armeniaca* Blanco), has been included in cosmetic preparations as moisturiser or emollient agent (Lube and Verpoorte, 2011). In traditional oriental medicine, apricot (*Prunus armeniaca* L.) seed has been used to treat skin diseases such as furuncle, acne vulgaris and dandruff due to its antimicrobial, antioxidant and antiinflammatory properties (Lee et al., 2014).

Fatty acid composition of the oils from the kernel samples of 42 wild apricot (*P. armeniaca*) genotypes collected from Kullu, Keylong, and Udaipur region of Himachal Pradesh and Nubra, Leh and Kargil region of Jammu and Kashmir (India) was determined and the principal fatty acid emerged as oleic acid (52.41-80.76%) and linoleic acid (12.19-39.79%) (Mandal et al., 2007).

The present study aimed to demonstrate the feasibility of a poly-herbal formulation based on *Daucus carota* L. and *Prunus armeniaca* L. to be included in cosmetics as an active ingredient. In this respect, three types of extracts were taken into consideration: hydroalcoholic, hydroglycerin and macerate in grapeseed oil and their rejuvenating potential was evaluated.

MATERIALS AND METHODS

Plant material

Sliced dried carrots and apricot kernel powder were purchased from a local market.

Chemicals

All reference compounds were purchased from Sigma Aldrich-Fluka. All other chemicals were analytical grade reagents.

Preparation of extracts

Hydroalcoholic extract was prepared by mixing 1 part vegetal material (equal parts of both herbs) with 10 parts of 50% hydroalcoholic solution (vegetal material/solvent ratio = 1/10 m/v) and further ultrasonicated for 30 minutes at 70°C. The resulted solution was filtrated and concentrated under reduced pressure to dryness. The residue was dissolved in ethylic alcohol 50% (v/v) to 0.25 g/ ml concentration.

Hydroglycerin extract was prepared by soaking 1 part vegetal material (equal parts of both herbs) with 10 parts of 70% glycerin (vegetal material/solvent ratio = 1/10 m/v) for 10 days at room temperature, in darkness, with occasional stirring, followed by filtration.

For the third type of extract, 1 part vegetal material (equal parts of both herbs) was soaked in 10 parts grapeseed oil and macerated for 10 days at room temperature, in darkness, with occasional stirring, followed by filtration.

Determination pf total phenolics

Total phenolics content of all three extracts was determined according to the Folin - Ciocalteu method adapted for microtitration format (Tatzber et al., 2020). The total phenolic content was expressed as gallic acid by reference to the gallic acid standard calibration curve in mM. Samples were determined in duplicate.

GC analysis

GC analysis was performed for identification of fatty acids in the oily extract was carried out by using an Agilent 6890N gas chromatograph equipped with a FID detector, 7683B autosampler and a capillary column HP INNOWax (60 m x 0.32 mm; film thickness 0.25 μm). The injector and detector temperatures were kept at 250°C. Nitrogen was used as carrier gas, a flow rate of 1.5 ml/min. Total analysis time was 31 min. Identification of the main components was carried out by the comparison of the GC retention times against those of the reference standards. The fatty acids contents were expressed as weight percentages, % *w*/*w* (g fatty acids/100 g of sample).

HPLC analysis

A method for quantification of polyphenols was developed for the hydroalcoholic extract. A Merck - Lachrom system with DAD L2455

detector was used. Phytochemicals were separated on a Intersil ODS3 analytical column (4.6 mm x 250 mm). The chromatographic separation was carried out using a mobile phase with phosphoric acid: water 0.05% as solvent A (pH = 2.8) and methanol as solvent B at a flow rate of 1-1.5 ml/ min. The injection volume for all samples and standard solutions was 10 µl.
A gradient flow of mobile phase and a gradient of mobile phase composition were applied: A:B (70:30) for 50 min, changing to A:B (30:70) for 10 min, then A:B (70:30) for another 10 min. Before use, all mobile phases were filtered through a 0.20 µm membrane (CHROMAFIL® O-20/25) and dis-aerated in an ultrasonic bath.

Cell viability assay

Cell viability was examined using an MTS assay kit (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, WI, USA). Murine fibroblasts (L929, ATCC) were cultivated in complete media (EMEM, 10% horse serum, 1% antibiotic, ATCC) and seeded into 96-well plates (1×10^4 cells/well). After 24 hours, different doses of each type of extract were applied in triplicate. Cell viability was measured 24 h after incubation. The results of MTS assay were obtained by measuring absorbance using a microplate reader (LKB Chamaeleon) at 492 nm. All experiments were repeated three times.

Scratch assay with fibroblast cell line

The fibroblasts L929 (1×10^4 cells) were seeded in 24-well cell culture plate. Linear scratch was made in confluent cell monolayer using 200 ml pipette tip. Cell debris were washed out with plain medium. The sample tested was hydroglycerin extract 5% and it was compared to a non-treated group. All experiments were made in duplicate. Images of cellular gap were captured periodically (0, 8 and 24 h) on Nikon inverted microscope.

RESULTS AND DISCUSSIONS

Three types of extracts suitable for inclusion in cosmetic formulations - oily, hydroglycerin, hydroalcoholic extracts - were analyzed.

Determination of total phenolics

In the three samples tested, the polyphenol content ranged between 0 in the oily extract and 2.2 mM in the hydroalcoholic extract. Hydroglycerin extract showed 1.34 mM polyphenols content.

GC analysis

The results are showed in Table 1 represent the percentage composition in fatty acids of the oily extract - determined by the ratio of the separate components on the chromatogram. No changes were recorded for myristic and linolenic acids. The variation in the fatty acid profile of the oily extract was insignificant comparing to control (grapeseed oil). Compared to the extraction solvent (grape seed oil), we noticed a slight decrease in the linoleic and palmitic acids contents while stearic and oleic acids contents increase. Several studies showed that apricot seeds oil have high content of monounsaturated oleic acid which is favourable in human nutrition and also have significant beneficial effect on the appearance and function of the skin (Stryjecka et al., 2019).

Table 1. Fatty acids composition of the oily extract

Fatty acids	Composition (%)	
	Grape-seed oil	Oily extract of *D.carota* and *P. armeniaca*
C14:0 (myristic acid)	0.06	0.06
C16:0 (palmitic acid)	8.16	8.13
C18:0 (stearic acid)	3.74	3.78
C18:1c (oleic acid)	26.52	27.03
C18:2c (linoleic acid)	60.10	59.65
C18:3α (α-linolenic acid)	0.68	0.68

HPLC analysis

As reference substances we selected ubiquitous phenolic compounds (polyphenolcarboxylic acids - caffeic, chlorogenic, rosmarinic, ferulic and flavonoids - rutin, quercetin, kaempferol, luteolin). The analysis was conducted only for the hydroalcoholic extract due to high viscosity of the other two extracts.
The results (Table 2) showed the absence of flavonoids (both aglycones and glycosides) and a high concentration of chlorogenic acid in the hydroalcoholic extract. Previously, Sharma reported that chlorogenic acid was the main hydroxycinnamic acid identified in different carrot tissues, accounting for 42.2% to 61.8% of total phenolics (Sharma et al., 2012).

Table 2. Chemical composition of alcoholic extract 50% (flavonoids and polyphenolcarboxylic acids) determined by high performance liquid chromatography

(HPLC-DAD)

Compound	Concentration mg/ 100 ml
Chlorogenic acid	3.970
Caffeic acid	0.210
Rutin	-
Quercetin	-
Kaempferol	-
Rosmarinic acid	0.072
Ferulic acid	0.163
Luteolin	-

MTS assay

Fibroblasts are critical in supporting normal wound healing, involved in key processes such as breaking down the fibrin clot, creating new extra cellular matrix (ECM) and collagen structures to support the other cells associated with effective wound healing, as well as contracting the wound (Bainbridge, 2013).

All extracts (hydroglycerin, hydroalcoholic, oily) based on *Daucus carota* - *Prunus armeniaca* combination had a strong stimulating effect on cell proliferation, comparing to controls. Except for the maximum concentration of the hydroglycerin extract, at which the cell viability was around 50%, most of the extracts exceeded the 80% threshold (Figures 1-3).

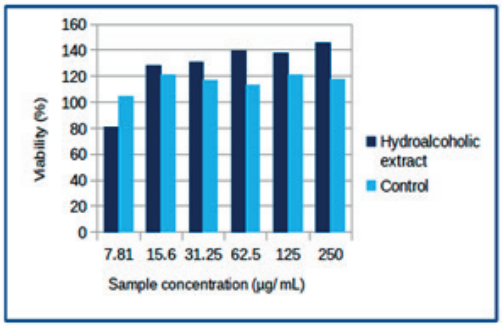

Figure 1. Viability of fibroblast cells after exposure to several concentrations of hydroalcoholic extract

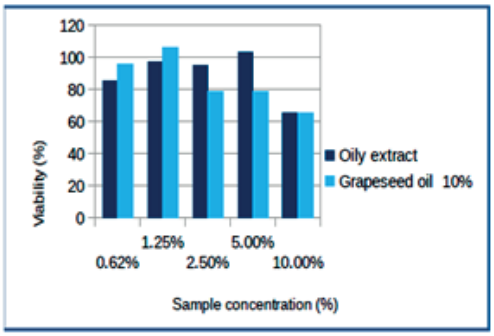

Figure 2. Viability of fibroblast cells after exposure to several concentrations of oily extract

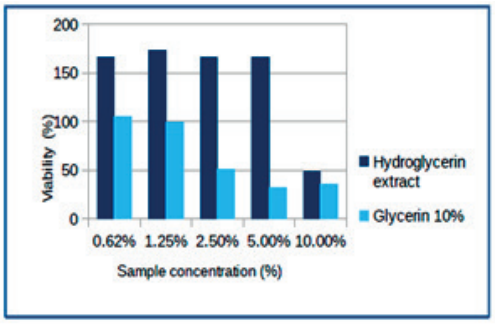

Figure 3. Viability of fibroblast cells after exposure to several concentrations of hydroglycerin extract

Scratch assay

In the present study, L929 (fibroblasts) cells were used in scratch assay. Using image analyzer, the time required to close the gap in the confluent cell monolayer in presence of 5% hydroglycerin extract was studied. The time taken to close the gap was compared with untreated cell culture.

The experimental results showed that formulation at 5% concentration closed the gap in the scratch of fibroblasts more efficiently as compared to control group.

Comparative cell migration at 0, 8 and 24 h in non-treated and formulation-treated is showed in Figure 4.

Figure 4. Scratch assay on L929 cell line

The hydroglycerin extract tested in this assay contains a high amount of polyhenols and it is widely known that plant phenolics play important roles in tissue repair mechanisms. As it was showed by Patil et al., wound-healing property of *Daucus carota* L. root extract may be attributed to the phenolic derivatives present in the root which accelerate the process of wound healing by antioxidant or antimicrobial effects (Patil et al., 2012).

CONCLUSIONS

The study proposed a poly-herbal formulation for cosmetic use based on *Daucus carota* L. and *Prunus armeniaca* L., two plants with long tradition of use in skin ailments.

All of the three extracts tested (hydroglycerin, oily and hydroalcoholic) had a strong stimulating effect on fibroblast cells proliferation, comparing to controls. Spectrofotometric and HPLC analysis showed high content of phenolic compounds in hydroglycerin and hydroalcoholic extracts that can contribute to pharmacological effects.

It can be concluded that preparations based on *Daucus carota* and *Prunus armeniaca* ingredients are suitable for inclusion in cosmetic preparations due to their antioxidant, anti-aging and wound healing properties.

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IDENTIFICATION OF OUTDOOR POLLEN - RESULTS FROM A POLLEN TRAPPING EXPERIMENT

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Abstract

Many allergenic plants are wind-pollinated and produce large amount of pollen that has the ability to float in the air. An efficient preventive treatment for pollen allergy is the avoidance of exposure. This can be done if there is a detection of the regional atmospheric concentrations for various pollen types. The present study shows the results of an outdoor pollen trapping experiment in the vicinity of an area rich in vegetation, the Carol Park in Bucharest, during a week in May 2018. Pollen analysis was done using stained microscope slides at a magnification of 400×. Microscopic images of all the pollen grains on the slides were photographed with a Sony Cyber-shot® digital camera (Carl Zeiss Vario-Tessar 5× zoom lens). Eight pollen types were identified, these were: inaperturate; monoporate; triporate; pollen with 4 pores; periporate; tricolpate; tricolporate and saccate pollen.

Key words*: Carol Park (Bucharest), light microscope (LM), microphotographs, outdoor pollen.*

INTRODUCTION

Pollen cytoplasm contains some allergenic proteins that normally have a role in fertilization in plants, however, since pollen size is small (microscopical) and because these allergenic proteins are water-soluble and can be released, they can reach other targets too (humans, animals) causing allergy. Pollen allergy (pollinosis) is one of the most common respiratory alergy in Romania. The incidence of this type of allergy has been increasing in Europe in the past decades (D'Amato, 2007).
Many allergenic plants are wind-pollinated and produce large amount of pollen that has the ability to float in the air (Ianovici, 2007). An efficient preventive treatment for pollen allergy is the avoidance of exposure. This can be done if there is a detection of the regional atmospheric concentrations for various pollen types. Several European scale projects aiming to develop such a system of forecasting hourly pollen concentrations all over Europe are being carried out (European Aerobiology Society, 2020), also there are specific training courses and meetings for aerobiologists (Berger, 2020a). A standard methodology is applied for the measurements of the pollen content in the air - pollen traps are used and a determination and counting of pollen grains using light microscopy (Berger, 2020b; Galan et al., 2014). Identification of pollen types is based on the knowledge of the pollen morphology and description for the pollen types usually found in air samples and is carried out at magnification 400× (Bucher & Kofler, 2020).
At present, in Romania, there is a need to develop a network of aerobiological monitoring laboratories. Therefore, following previous studies (Enache et al., 2019), an attempt to identify different pollen types from the air in late spring, using a pollen trapping experiment carried out in the vicinity of an area rich in vegetation, was considered relevant.

MATERIALS AND METHODS

Carol Park is one of the oldest parks in Bucharest (dates from 1906) and is well known for several monuments of historical value as well as the beautiful panoramic views and vegetation, which includes numerous trees (Figure 1). To analyze pollen types found in the air, 10 microscope slides with a sticky surface were kept outdoors on a 1st floor balcony of a house situated next to the park for a week in May 2018 (6-11 May), then they were brought to the Laboratory of Biology of the Faculty of Biotechnologies of the University of Agronomic Sciences and Veterinary Medicine of Bucharest.

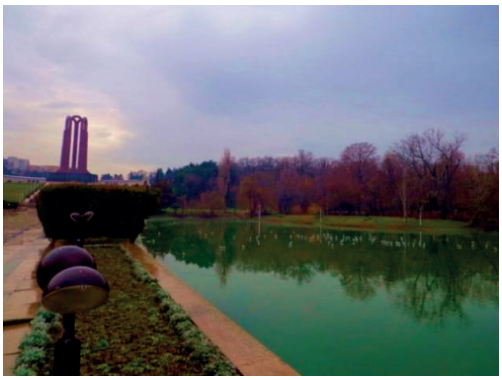

Figure 1. View from Carol Park in winter (the Mausoleum monument can be seen on the back left)

Slides were stained with toluidine blue for better contrast and a coverslip was added (Figure 2). An optical microscope Micros Austria was used (ob. 40×) and the microscopic images of all the pollen grains on the slides were photographed with a Sony Cyber-shot® digital camera (Carl Zeiss Vario-Tessar 5× zoom lens). The measurements were done with an ocular micrometer and the calibration ratio was 2.5 µm.

Figure 2. Slides used in the present study

Comparisons of the images were made with pollen descriptions found in the literature (Tarnavschi et al., 1981; 1987; 1990; Șerbănescu-Jitariu et al., 1994) or on various Internet sites (Pollen-Wiki-Der digitale Pollenatlas, Stebler, 2020a; the PalDat-Palynological Database or the Pollen Atlas of Bucher and Kofler, 2020), as well as with our collection of allergenic pollen images (Enache et al., 2019). The classification of pollen according to size is from Stebler (2019b).

RESULTS AND DISCUSSIONS

The list of main plant genera/species of relevance for aerobiological studies found in Romania (Berghi, 2012) includes species that have pollen that can be classified in one of the following 8 pollen types:

- inaperturate: Cupresaceae - *Juniperus* sp. (junipers); Salicaceae - *Populus* sp. (poplar);
- monoporate: Poaceae - wild grasses and cultivated grasses (including ornamental grasses);
- diporate: Moraceae - *Morus* sp. (mulberry);
- triporate: Urticaceae - *Urtica* sp. (nettle), *Parietaria officinalis* (common pellitory); Betulaceae - *Corylus* sp. (hazel), *Betula* sp. (birch);
- 4 to 6 pores: Betulaceae - *Carpinus* sp. (hornbeam), *Alnus* sp. (alder); *Ulmaceae* - *Ulmus* sp. (elm);
- periporate: Plantaginaceae - *Plantago lanceolata* (plantain); Amaranthacee - *Amaranthus* sp.; Juglandaceae - *Juglans* sp. (walnut);
- tricolpate: Aceraceae - *Acer* sp. (maple); Fagaceae - *Quercus* sp. (oak); Platanaceae - *Platanus* sp. (plane trees); Oleaceae - *Fraxinus* sp. (ash); Asteraceae - *Ambrosia elatior*, *A. trifida* (ragweeds), *Xanthium strumarium, X. commune* (cockleburs);
- tricolporate: Fagaceae - *Castanea* sp. (chestnut), *Fagus* sp. (beech); Polygonaceae - *Rumex* sp. (dock, sorrels); Asteraceae - *Artemisia vulgaris* (mugwort); Tiliaceae - *Tilia* sp. (lime tree/linden); Salicaceae - *Salix* sp. (willow); Oleaceae - *Ligustrum* sp. (privet).

Differences between plant genera/species in one category can be determined based on pollen descriptions in each case (Bucher and Kofler, 2020).

For the microscope slides that were analysed a total of 456 microphotographs were obtained and 8 pollen types were identified.

Only one granule was inaperturate; while several pollen grains were present in each of the other 7 categories. Bisaccate medium to very large pollen grains (Pinaceae) were over-represented on these slides (Figure 3).

Small or medium size monoporate pollen grains were also seen, due to their characteristic shape and annulus present around the pore, they can be identified as Poaceae (Figure 4).

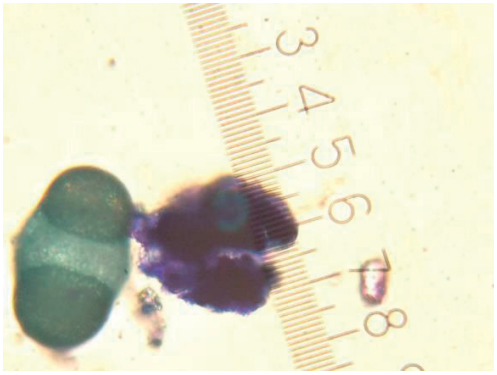

Figure 3. Large bissacate pollen grain (~ 80 µm)

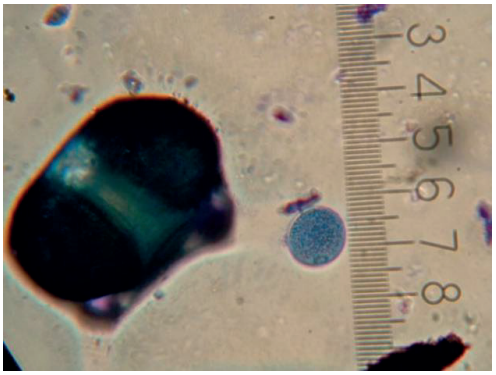

Figure 4. Medium size monoporate pollen grain (~ 30 µm) with annulus next to very large bissacate pollen grain (~ 115 µm)

Hazel and birch have similar shapes and have both 3 protruding pores (Figure 5), however these are larger in hazel therefore in polar view birch looks more circular (Figure 6).

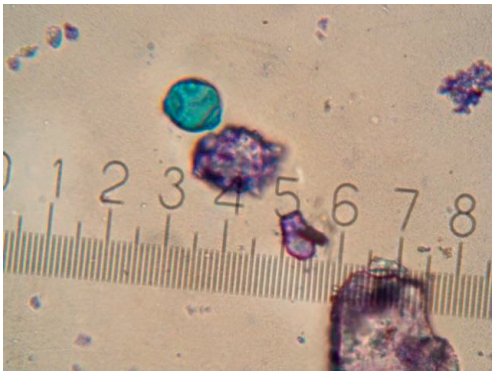

Figure 5. Triporate pollen grain with protruding pores (~ 22,5 µm) - semi-lateral view

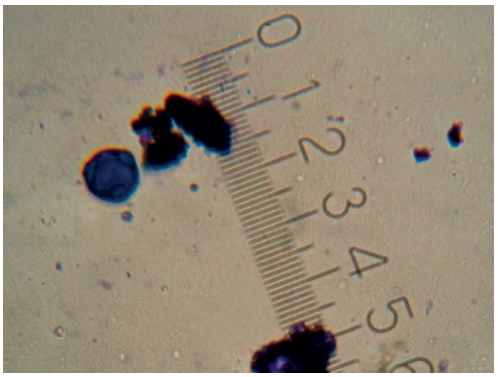

Figure 6. Triporate pollen grain (~ 25 µm) - apical view

Four or five pores have hornbeam and alder, both are circular in polar view (Figure 7), but alder has distinctive protruding pores. Also there is a size difference between them: alder pollen is small, while hornbeam is medium size.

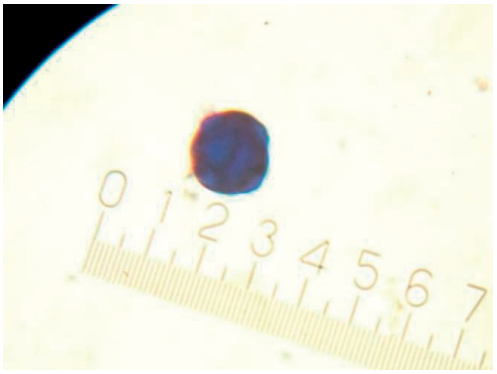

Figure 7. Pollen grain with 4 pores (~ 37.5 µm) - apical view

Two types of round, medium size periporate pollen grains were also seen on the slides in the present study (Figures 8-9), however there are details that differentiate them - one is operculate (possible *Plantago lanceolata*), while the second type has pores with a lenticular thickening around them (*Jugans* pollen).

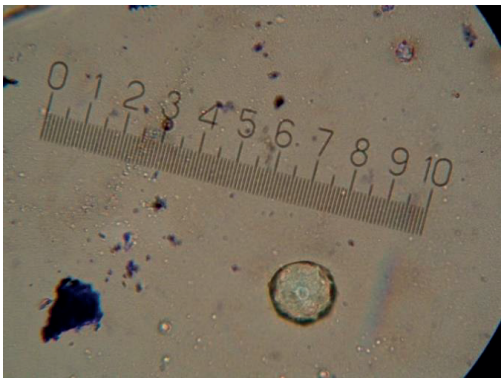

Figure 8. Periporate operculate pollen grains (~ 37.5 µm)

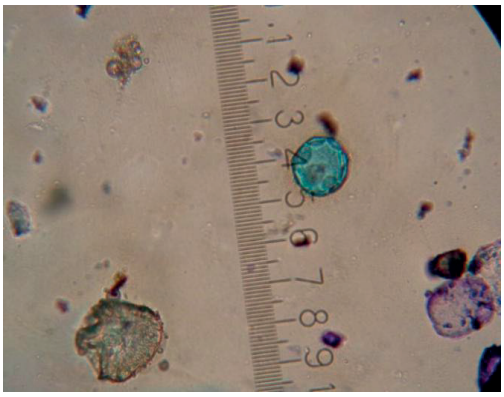

Figure 9. Periporate pollen grains (~ 37.5 µm), lenticular thickening around the pores can be seen

Small size tricolpate pollen grains were also noted (Figure 10), but not sufficient details were present on the pictures to be able to analyze them further.

Medium size tricolporate pollen grains possible *Fagus* (beech) and *Tilia* (lime tree) could also be found (Figures 11-12).

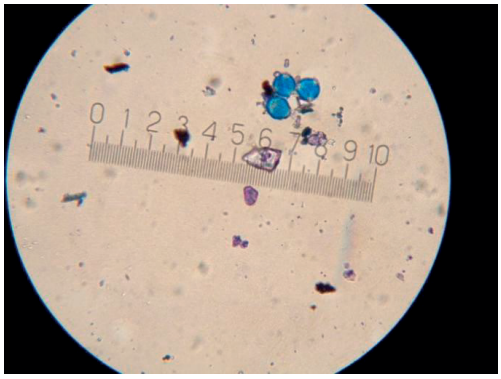

Figure 10. Tricolpate pollen grains (~ 20 µm)

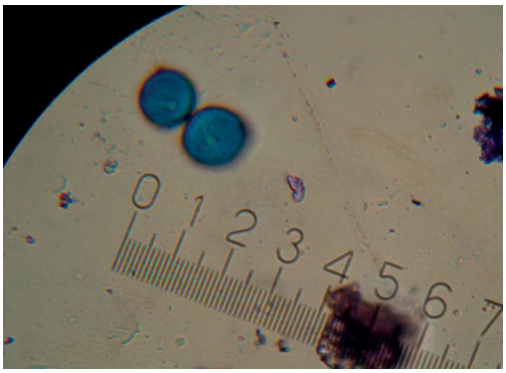

Figure 11. Tricolporate pollen grains with colpus and pore visible in lateral view (~ 30 µm)

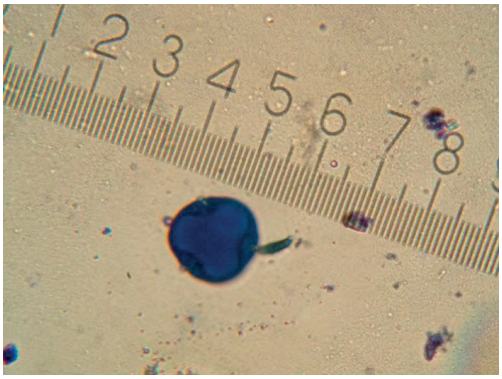

Figure 12. Tricolporate pollen grains with triangular convex polar shape with mid-wall apertures that have a thickening around them (~ 35 µm)

CONCLUSIONS

A simple pollen trapping experiment was used in the present study to analyze the morphology of some pollen grains from the air. Pinaceae pollen was over-represented on the slides, but several other pollen types were determined, reflecting the vegetation found in the immediate vicinity.

The inventory of plant species with allergenic potential in certain areas of the city and the knowledge of their flowering periods - the establishment of the pollen calendar, brings important data for pollen allergy sufferers, children, but also for the general population.

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