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SUMMARY

SUBSECTION I: AGRICULTURAL BIOTECHNOLOGY

SUBSECTION II: BIOTECHNOLOGY IN VETERINARY MEDICINE

SUBSECTION III: FOOD BIOTECHNOLOGY

SUBSECTION IV: INDUSTRIAL AND ENVIRONMENTAL BIOTECHNOLOGY

SUBSECTION V: FOOD SAFETY

SUBSECTION VI: MISCELLANEOUS

AGRICULTURAL BIOTECHNOLOGY

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A REVIEW OF *IN VITRO* STUDIES ON *Allium tuncelianum* (Kollman) Ozhatay, Matthew, Siraneci

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Abstract

Allium tuncelianum (Kollman) Ozhatay, Matthew, Siraneci is an endemic plant species only grown in Turkey. Unlike common garlic, it has only one clove bulb and it can also produce fertile flowers and seeds. Due to the similarity of its flavor and taste to Allium sativum, it is called 'tunceli garlic' and 'ovacik garlic' in the region. In recent years, the amount of consumption has increased due to revealing the benefits of biochemical content to human health. For this reason, Allium tuncelianum has been collected from nature for domestic and medical purpose by herbalists and local people. So, it is in danger of extinction due to unconscious and over-exploitation from the nature. In recent years, different strategies have been developed to protect Allium tuncelianum from destruction. Germination problems of its seeds have led researchers to use in vitro techniques. These studies focus to develop an efficient protocol for propagation and conservation of this endemic species. In this review, in vitro studies on Allium tuncelianum were evaluated.

Key words*: Allium tuncelianum, Tunceli garlic, in vitro propagation.*

INTRODUCTION

Allium tuncelianum (Kollman) Ozhatay, Matthew, Siraneci has a limited distribution in eastern Anatolia in the Tunceli and Erzincan areas. It is locally called as 'Tunceli garlic' or 'Ovacik garlic'. *Allium tuncelianum* is originally named as *Allium macrochaetum* Boiss and Haussk subsp. *tuncelianum* Kollmann. It is an important endemic species for Turkey. It was discovered in 1980s. The bulbs and young leaves of *A. tuncelianum* are used as vegetable and spice locally, being very similar in flavor to *Allium sativum* (Ozhatay & Mathew, 1995; Etoh & Simon, 2002; Yanmaz & Ermis, 2005; Kosar et al., 2006; Ipek et al., 2008; Yanmaz et al., 2010; Baktir et al., 2013; Kiralan et al., 2013; Aasim, 2015; Yarali & Yanmaz, 2016).

Consumption of *Allium tuncelianum* has several benefits such as stimulates the body's immune system, lowers the level of sugar and cholesterol in the blood, improve blood circulation thus reduces the risk of heart attack (Agbas et al., 2013; Aasim, 2015; Atila et al., 2017). In addition, *Allium tuncelianum* has a strong antioxidant and antiradical activity than *Allium sativum* L. Because of high amount of p-Coumaric acid content of *Allium tuncelianum*, it has much higher antioxidant activity compared with the *Allium sativum*. And also in terms of fatty acid compositions *Allium tuncelianum* is observed having more effective level of essential omega acids compared to the common garlic (Sehitoglu et al., 2014; 2018).

Picking of endangered geophytes for trade is banned in Turkey for conservation purpose in agreement with the "Convention on the International Trade in Endangered Species (CITES)". However, *Allium tuncelianum* has been collected from nature for domestic and medical purpose by herbalists and local people in Turkey. So, it is in danger of extinction due to unconscious and over-exploitation from the nature (Yanmaz et al., 2010; Aasim, 2015). Conservation efforts can be complemented by development of *in vitro* conservation protocols along with improved agronomic techniques suitable for cultivation of the plant in other areas of Turkey. The application of *in vitro* culture techniques for the protection of

endemic *Allium tuncelianum* is less common than for *Allium sativum*. Therefore, extensive studies are needed to develop tissue culture techniques for Tunceli garlic with significant advantages (Kosar et al., 2006; Yazar, 2006; Kizil et al., 2014; Aasim, 2015). In recent years, different strategies have been developed to protect *Allium tuncelianum* from destruction. However, there are a limited number of studies on *in vitro* conservation of endemic 'Tunceli garlic'. In this review, *in vitro* studies on *Allium tuncelianum* were evaluated.

IN VITRO STUDIES

Unlike *Allium sativum*, *Allium tuncelianum* has only one clove bulb and it can also produce fertile flowers and seeds (Yazar, 2006; Yanmaz et al., 2010; Agbas et al., 2013; Baktir et al., 2013; Kiralan et al., 2013; Aasim, 2015; Kizil & Khawar, 2015; Takim, 2015; Yarali & Yanmaz, 2016; Babacan et al., 2017) (Figures 1, 2 and 3).

Figure 1. Bulbs of *Allium tuncelianum* (Photographed by: Faika YARALI KARAKAN)

Figure 2. Inflorescence of *Allium tuncelianum* Photographed by: Faika YARALI KARAKAN)

Figure 3. Seeds of *Allium tuncelianum* (Photographed by: Faika YARALI KARAKAN)

Allium tuncelianum multiplies naturally by seed or vegetative by newly regenerated bulb attached to mother bulbs. However, the percentage of new regenerated bulbs and the number of plantlets per bulb is too low for practical regeneration purposes. In addition, seeds have germination problem (Yanmaz et al., 2010; Kizil et al., 2014). *In vitro* techniques *are* useful tool to develop propagation methods for *Allium tuncelianum* (Yanmaz et al., 2010). For this reason, researchers aimed to develop *in vitro* protocols by using *in vitro* techniques, such as shoot and root culture, leaf culture, bulb culture, *in vitro* seed germination and gynogenesis.

Root and shoot culture

In vitro root and shoot tip culture methods used to propagation of *Allium tuncelianum* by Yazar (2006). Murashige and Skoog (MS) basal medium supplemented with BA (0.0, 0.1 and 1.0 mg/l), 2,4-D (0.0, 1.0 and 2.0 mg/l), NAA (0.0, 1.0 and 2.0 mg/l) were used in root tip culture. Root tip explants prepared from root tissues are planted in petri dishes then cultured at 25 ± 10°C under dark conditions and taken 1 month later under fluorescent light to 16/8 h. In shoot tip culture, bulbs were opened under a binocular microscope then explants having 0.5-1.0 cm long leaflets were prepared after bulbs were disinfected. They were cultured in MS basal medium supplemented with BA (0.0, 0.05 and 0.1 mg/l); 2,4-D (0.0, 0.1 and 0.5 mg/l); NAA (0.0, 0.1 and 0.5 mg/l). As a result of the research, it was stated that callus formation couldn't be provided in root tip culture

experiments. But in the shoot tip culture experiment, shoot formation started approximately 1 month after shoot tip planting. After 4 sub-cultures, it was determined that MS medium containing IAA gave out better results than NAA with regard to the number of shoots. The highest shoot rate was obtained from the medium supplemented with 0.05 mg/l BA+0.1 mg/l IAA with 76%. This was followed by medium supplemented with 0.1 mg/l BA, 0.1 mg/l BA + 0.1 mg/l NAA with 67% and 0.05 mg/l BA + 0.5 mg/l IAA with 65%, 0.05 mg/l BA with 56%. In a similar study, Yanmaz et al. (2010) aimed to develop a novel micropropagation method for *in vitro* propagation of *Allium tuncelianum* by root tip and shoot culture techniques. Root tips were obtained from 18 days old *in vitro* plantlets. To determine the best combinations of the growth regulators; 2,4-D and NAA (0, 1.0, 2.0 mg/l) and BA (0, 0.1, 1.0 mg/l) were used in MS medium. According to results, the root tip culture was not found as a proper method for shoot proliferation. On the other hand, shoot culture was found effective on shoot formation. As an average, 1 or 2 shoots were obtained per explant. The researchers stated that *Allium tuncelianum* could be propagated at lower doses of plant growth regulators such as IAA and BA (0.1 mg/l, 0.1 mg/l) via *in vitro* shoot culture. Contrary to these findings Kizil et al. (2014) suggested that root tip explants were most suitable for bulblet regeneration of *Allium tuncelianum*. They used MS medium supplemented with 1.0, 2.0, 3.0, 4.0, 5.0 mg/l 2,4- D and 1.0, 2.0, 3.0, 4.0, 5.0 mg/l BAP and 0.5 mg/l NAA. The results indicated that root tip explants were most suitable for bulblet regeneration on MS medium containing 5.0 mg/l BAP and 0.5 mg/l NAA. Similarly, Icgil (2012) stated that root explants showed bulblet regeneration on root tips on MS medium containing various concentrations of BAP and NAA. The bulblet regeneration rate from root explants ranged from 13.33% to 100%. When the effect of MS medium containing different concentration of BAP and 0,5 mg/l NAA on the bulblet regeneration rate was examined, it was seen that the bulblet regeneration rate and the number of bulblets per explant decreased as the concentration of BAP increased in the media containing 1, 2 and 3 mg/l BAP + 0.5 mg/l NAA. The bulblet regeneration rate increased to 60% at a concentration of 4 mg/l BAP + 0.5 mg/l NAA and reached 100% at a concentration of 5 mg/l BAP + 0.5 mg/l NAA. For obtaining virus-free plant, Taskin et al. (2013) aimed to combine meristem culture technique by shoot tip culture technique. They used two different culture media (Medium 1; MS + 0.5 mg/l 2-IP + 0.2 mg/l NAA + 30 g/l sucrose and Medium 2: MS + 2 mg/l BA + 0.5 mg/l IBA + 30 g/l sucrose) and two garlic species, *Allium sativum* and *Allium tuncelianum*. They stated that Medium 2 was found more effective in term of number of shoots than Medium 1. In the first propagation, 14.10 shoots/plant and 4.63 shoots/plant were obtained from Medium 2 and Medium1, respectively. And also Medium 2 has been successful at subculture. It was obtained 13.27 shoots per plant from Medium 2. Similar results were obtained in shoot tip culture. 11.37 and 2.41 shoots per plant were obtained from Medium 2 and Medium 1 in the first propagation, respectively. Considering the explant types, meristem explants were found to be more successful compared to shoot tip explants for both *Allium sativum* and *Allium tuncelianum*. Real-time PCR analysis revealed that *in vitro* plants obtained from meristem culture do not have any onion yellow dwarf virus (OYDV) and leek yellow stripe virus (LYSV). Contrary to these findings OYDV and LYSV viruses were detected in plants obtained via shoot tip culture.

Leaf culture

Kizil et al. (2014) used leaf tips, the middle portions of leaves and leaf bases explants and MS medium supplemented with different concentrations of 2,4- D, BAP and NAA. They stated that regeneration or callusing was not observed on day 28 of culture on leaf tips or on the middle portion of leaves on MS medium containing 0.5-1.0 mg/l BAP + 0.5 mg/l NAA (five combinations). In addition, no bulblet regeneration was induced from leaf bases on MS medium supplemented with 1.0 mg/l BAP plus 0.5 mg/l NAA, or 5.0 mg/l BAP plus 0.5 mg/l NAA. A maximum of 13.3% regeneration was recorded on MS medium supplemented with 1.0 mg/l BAP plus 0.5 mg/l NAA. All

other culture media showed low regeneration percentages (6.7% each). Mean values of 1.0, 1.0 and 0.7 bulblets per leaf base were recorded on MS medium containing 2.0 mg/l BAP plus 0.5 mg/l NAA, 3.0 mg/l BAP plus 0.5 mg/l NAA, or 4.0 mg/l BAP plus 0.5 mg/l NAA, respectively. Similarly, İcgil (2012) stated that no regeneration was recorded on leaf tip explant from MS medium with different concentrations of BAP-NAA, 2,4-D. Contrary to these findings, the highest regeneration rate (13.3%) on petiole explants was obtained from MS medium containing 2.0 mg/l BAP and 0.5 mg/l NAA.

Bulb culture

Icgil (2012) investigated the effects of different concentrations of BAP, NAA, 2,4-D on different bulb explants such as; longitudinally sectioned ½ and ¼ bulb explants. It was determined that plant growth regulators substances and explant types were effective on the shoot formation. While the highest shoot number per explant (83.33%) was obtained from MS medium containing 2 mg/l 2,4-D and no shoot formation was observed from MS medium containing 1 mg/l 2,4-D from longitudinally sectioned 1/2 bulb explants. While the highest shoot number per explant (3) was obtained from MS medium supplemented with 1 mg/l BAP+ 0.5 mg/l NAA, the lowest shoot number per explant (0.67) was obtained from MS medium containing 2 and 3 mg/l BAP+ 0.5 mg/l NAA from longitudinally sectioned 1/4 bulb explants. Contrary to these positive findings, Kizil et al. (2014) stated that vertically-sectioned half or quartered bulb or both horizontally-sectioned upper and lower half-bulb explants were unsuitable for the regeneration of new bulblets. They cultured all these explants on MS medium supplemented with different concentrations of 2,4-D, BAP and NAA. Their results showed that no bulblet regeneration was obtained from any vertically-sectioned half or quartered bulb and both horizontally-sectioned upper and lower half-bulb explant.

Over wintered ‘Tunceli garlic’ bulbs had a potential to produce bigger bulbs than unwintered materials. These bulbs had a significant effect on uniform plant formation. This is very important for cultivation of *Allium tuncelianum* (Yanmaz et al., 2010). Aasim (2015) used wintered and unwintered half cloves. Bulb explants were cultured at MS medium supplemented with 0.25, 0.50 and 1.0 mg/l BA and 0.25, 0.50 and 1.0 mg/l of KNAA for regeneration. As a result, the study determined that unwintered and wintered upper and lower half clove explants failed to regenerate new bulblets. However, proximal half clove of the wintered bulbs was evaluated as the best explant for regeneration on MS medium supplemented with 0.50 mg/l BA with 0.50 mg/l KNAA. The rooted bulbs were acclimatized and transferred to pots and fields. It was concluded that the protocol could be safely used to conserve this plant.

Seed germination

Allium tuncelianum has fertile black seed that can easily be used for propagation. But they undergo deep seed dormancy soon after maturity. For this reason, the aim of *in vitro* studies is to eliminate seed dormancy. Dormancy can be broken with the cold treatment given to garlic before planting. Kizil et al., (2017), aimed to break seed dormancy of *Allium tuncelianum* and determine the conditions for induction of bulblets on these seeds. They collected ‘Tunceli garlic’ seeds from field grown plants. After being surface sterilized, seeds were germinated on MS medium with or without 20 g/l sucrose followed by their culture on 1 × 1900 mg/l, 2 × 1900 mg/l, 4 ×1900 mg/l and 6 × 1900 mg/l KNO_3 to increase bulb diameter. At the end of the study, it was reported that bulb formation rate on each of the germinated seeds was not parallel to seed germination rate. A total of 34% seeds (with 138 seeds that converted to bulbs) and 28.5% (with 94 seeds that converted to bulbs) on MS medium with and without 20 g/l sucrose, respectively. The results showed that MS medium containing sucrose had significantly positive effect on seed germination and bulb induction and vegetative growth. The best increase in bulb diameter was noted on MS medium containing 1×1900 mg/l KNO_3 after 178 days with bulblet diameter and bulblet weight of 0.54 cm and 0.048 g, respectively.

Gynogenesis

There have been conducted out several studies for *in vitro* propagation of *Allium tuncelianum* but breeding studies were not carried out. Today, biotechnological breeding methods offer great benefits in breeding. Using dihaploidization techniques provides great advantages in obtaining the inbreed lines used in hybrid breeding in a short time. Different methods have been improved for *in vitro* haploid production, but only gynogenesis has been reported to be successful in *Alliums*. Yarali and Yanmaz (2016) aimed to ensure optimization technique for *Allium tuncelianum* which were used successfully for other *Allium* species. It is the first research about determining of gynogenic induction frequency of *Allium tuncelianum* via flower bud culture. They used BDS medium supplemented with 0, 1 and 2 mg/l of 2,4-D and BAP and their combinations to determine the effect of plant growth regulators on gynogenic embryo induction. They stated that BDS medium supplemented with different combinations of auxin (2,4-D) and cytokinin (BAP) were effective on callus development on explants. The highest callus formation rate was obtained from BDS medium supplemented with 2+1 mg/l 2,4-D+BAP and 2+2 mg/l 2,4-D+BAP. In this research, callus development was provided on flower buds at 55.28% but plantlets could not be achieved from callus. This study is important due to the guidance for future studies about haploid plant production on *Allium tuncelianum*.

CONCLUSIONS

In vitro studies on *Allium tuncelianum* were carried out by several researchers. This subject is important, considering that it is an endemic species and has biochemical content, important for human health. Researches have been aimed to reveal the well-established protocols for *in vitro* propagation and conservation, and in part, some successful results have been achieved. Efforts have been done to regenerate plants under *in vitro* conditions. But, there is need for doing extensive work for development of comparatively more efficient and reproducible *in vitro* protocols for propagation, breeding and conservation purposes of *Allium tuncelianum*.

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BIOTECHNOLOGICAL RECYCLING OF FRUIT TREE WASTES THROUGH ORGANIC CULTIVATION OF MUSHROOM SPECIES

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Abstract

The excessive and long-term accumulation of large amounts of redundant lignocellulose materials, as outcome wastes from the specific activities of all fruit tree farms across the whole country in Romania, has become a huge problem which needs to be solved by using biological means for their conversion into beneficial products. Thus, the main aim of this work was to solve this problem by recycling the fruit tree wastes through organic cultivation of two mushroom species, Ganoderma lucidum and Pleurotus ostreatus. The fruit body productions of each one of these mushroom species registered the highest levels as 1,830 g for G. lucidum and 2,750 g for P. ostreatus, relative to 5 kg of substrates made of fruit tree wastes. According to these results, the suitable biotechnological procedures for recycling of apple, plum and cherry tree wastes through organic cultivation of mentioned mushroom species is presented in this paper.

Key words*: biotechnology, Ganoderma lucidum, Pleurotus ostreatus, lignocellulose wastes.*

INTRODUCTION

It is well known that every year huge amounts of redundant lignocellulose materials outcome from any orchard as fruit tree wastes which cause serious environmental troubles if they accumulate in the local fruit tree farms or they are burned on the soil of other areas (Chahal, 1993; Carlile & Watkinson, 1996).

All these natural but redundant materials, mainly composed of dried trunks and branches of fruit trees, could be recycled as main substrates for solid-state cultivation of mushroom species belonging to the group of Basidiomycetes (Stamets, 1993; Moser, 1994).

In this respect, the experiments were set up on testing and optimizing the biotechnological processes of fruit tree wastes recycling through controlled cultivation of edible and medicinal mushroom species *Ganoderma lucidum* and *Pleurotus ostreatus,* in order to get their carpophores to be used as food and nutraceuticals (Smith, 1998).

The main aim of this research work was focused to find out the best biotechnological procedure for recycling the fruit tree wastes from orchards through the organic cultivation of certain mushroom species on these wastes made of lignocellulose materials and finally get the carpophores of edible and medicinal mushrooms.

MATERIALS AND METHODS

Mushroom species used in experiments

As a mushroom species belonging to the group of white rot fungi, *Ganoderma lucidum* (Curt. Fr.) P. Karst is a wood degrading fungus, belonging to lignin decomposers. Until now, *G. lucidum* species has been cultivated mainly on wood substrates or as fungal mycelium in synthetic liquid media in small scale production processes (Stamets, 1993; Cohen et al., 2002). On the other side, *P. ostreatus* (Jacquin ex Fries) Kummer is a mushroom species with a high potential to grow on lignocellulose wastes and form mushroom fruiting bodies during their biological cycles (Sanchez, 2010).

In order to achieve the experiments related to biotechnological recycling of fruit tree wastes through organic cultivation of mushroom species, selected pure cultures of mushrooms from the culture collection belonging to the University of Pitesti were used. The stock cultures were maintained on malt-extract agar (MEA) slants at 25°C for 5-7 days and after that, they were stored at 4°C. To achieve the experiments, the mushroom pure cultures were transferred in 250-mL flasks containing 100 mL of MEB medium (20% malt extract, 2% yeast extract and 20% peptone solution in pure water up to 100%) and let to grow at 23°C

on rotary shaker incubators at 110 rev min^{-1} for 5-7 days (Petre et al., 2014)

Substrate variants for mushroom cultivation

For the optimal cultivation of mushroom species *G. lucidum* and *P. ostreatus*, there were set up three variants of mushroom cultivation substrates, mainly consisting of natural compounds like woody wastes made of sawdust resulted from milled branches of apple, plum and cherry, which were chopped, mixed and hydrated (24-30 h) with a solution made of following ingredients: wheat bran, yeast extract, calcium carbonate and tap water, as it is shown in Table 1.

Table 1. The composition of substrate variants for the controlled cultivation of mushroom species

Substrate ingredients	The composition of each substrate variant (w/w)		
	S1	S2	S3
Sawdust from milled apple branches	70	-	-
Sawdust from plum milled branches	-	70	-
Sawdust from cherry milled branches	-	-	70
Wheat bran	10	10	10
Yeast extract	3.5	3.5	3.5
Calcium carbonate	1.5	1.5	1,5
Tap Water	15	15	15

Then, all three variants of substrates, S1, S2, S3 were soaked in a nutritive aqueous solution made of natural ingredients, having the composition presented in Table 1, and then were placed in thermorezistant polypropylene bags with 5 kg weight, subsequently being sterilized in an autoclave at the temperature of 121^{o}C, for 50 min. After cooling, the contents of sterilized polypropylene bags containing the substrate variants were aseptically inoculated with the pure cultures of mushroom species *G. lucidum* and *P. ostreatus*. Next, all bags with the variants of substrates, previously disinfected by sterilization and inoculated with pure cultures of mentioned mushroom species, were placed into automatic growing chambers and kept at the constant temperature of 23^{o}C, for 15-30 days depending on the mushroom species used in experiments. Then, during the incubation, the whole mycelial biomass, developed inside the substrates, placed in polypropylene bags, formed the fruit bodies belonging to both mushroom species.

During the process of fruit body formation the culture parameters were set up and maintained at the following levels depending on each mushroom species used in experiments: the air temperature, 18-20^{o}C, the air flow volume, 5-7 m^3/h, air flow speed, 0.2-0.3 m/s, the relative moisture content, 95-97%, the light intensity, 500-1,000 luces for 8-10 h/day. The whole period of mushroom growing from the inoculation up to the fruit body formation lasted between 30-35 days for *P. ostreatus* and 60-70 days for *G. lucidum*.

RESULTS AND DISCUSSIONS

After the first stage of primordial formation, the carpophores belonging to both mushroom species have developed continuously in a fast growing process. Thus, during the five crop stages of such biological process, the carpophores of both species were studied regarding their body development and increasing in significant weight. In this respect, during a period of time lasting between 30 and 70 days, the mature carpophores belonging to both mushroom species were collected and after that, they were weighted.

The results regarding the harvest of carpophores for each one of the mushroom species were registered and assessed during a period of time lasting from 35 up to 70 days, depending on the mushroom species used in experiments, as it is shown in Tables 2 and 3.

Table 2. The mushroom harvest variation, depending on each crop stage and substrate variant, during the cultivation of *G. lucidum*

Crop stage	Mushroom harvest on substrate S1* (w/w)	Mushroom harvest on substrate S2* (w/w)	Mushroom harvest on substrate S3* (w/w)
I	610	490	530
II	475	350	450
III	350	270	320
IV	240	210	250
V	155	140	120
Total weight (g)	1,830	1,460	1,670

*The average of harvest which were registered during three repeated cultivation cycles

Table 3. The mushroom harvest variation, depending on each crop stage and substrate variant, during the cultivation of *P. ostreatus*

Crop stage	Mushroom weights on substrate S1* (w/w)	Mushroom weights on substrate S2* (w/w)	Mushroom weights on substrate S3* (w/w)
I	730	770	590
II	650	630	430
III	510	530	310
IV	370	470	250
V	310	350	210
Total weight (g)	2,570	2,750	1,790

*The average of weights which were registered during three repeated cultivation cycles

Regarding the registered results, it is of great importance to take into consideration that each value of weight, presented both in Table 2 and Table 3 means the average of weights which were registered during three repeated cultivation cycles, by keeping constant the environmental factors (the air temperature at 18-20^{o}C, the air flow volume, 5-7 m^{3}/h, the air flow speed, 0.2-0.3 m/s, the relative moisture content, 95-97%, the light intensity, 500-1,000 luces for 8-10 h/day).

Comparing the values of registered amounts of mushroom carpophores belonging to *G. lucidum* species, it has to be mentioned that the highest total weights were noticed when it was used the substrate variant S1, followed by the substrate variant S3, and finally, by the substrate variant S2.

Significant differences of mushroom weights between the crop stages were noticed in the case of *G. lucidum*, respectively between the first three crop stages corresponding to the mushroom harvest on substrate variants S1 and S2, as they are shown in Table 2.

At the same time, there were determined significant differences between the crop stages of *P. ostreatus*, mainly between the first three crop stages related to the mushroom harvest on substrate variants S2 and S3, like they were presented in Table 3.

In Figures 1, 2 and 3, the carpophores belonging to *G. lucidum* mushroom species, which have developed on substrate variants S1, S2 and S3, are displayed.

Figure 1. *G. lucidum* carpophores, developed on the substrate S1

Figure 2. *G. lucidum* carpophores, grown on the substrate variant S2

Figure 3. *G. lucidum* carpophores, grown on the substrate variant S3

The collected carpophores belonging to the mushroom species *P. ostreatus* are illustrated in the Figures 4, 5 and 6.

Figure 4. Bunches of *P. ostreatus* carpophores, developed on the substrate variant S1

Figure 5. A bunch of *P. ostreatus* carpophores, developed on the substrate variant S1

Figure 6. *P. ostreatus* carpophores, grown on the substrate variant S3

Regarding the results of *P. ostreatus* harvesting, almost the same significant differences between the crop stages were noticed, but the highest total weights of mushroom carpophores were registered for the substrate S2, followed by S1 and the last one being the substrate S3.

According to the registered results, the optimal biotechnology for recycling fruit tree wastes by using *G. lucidum* and *P. ostreatus* mushroom species was established and it is shown in Figure 7:

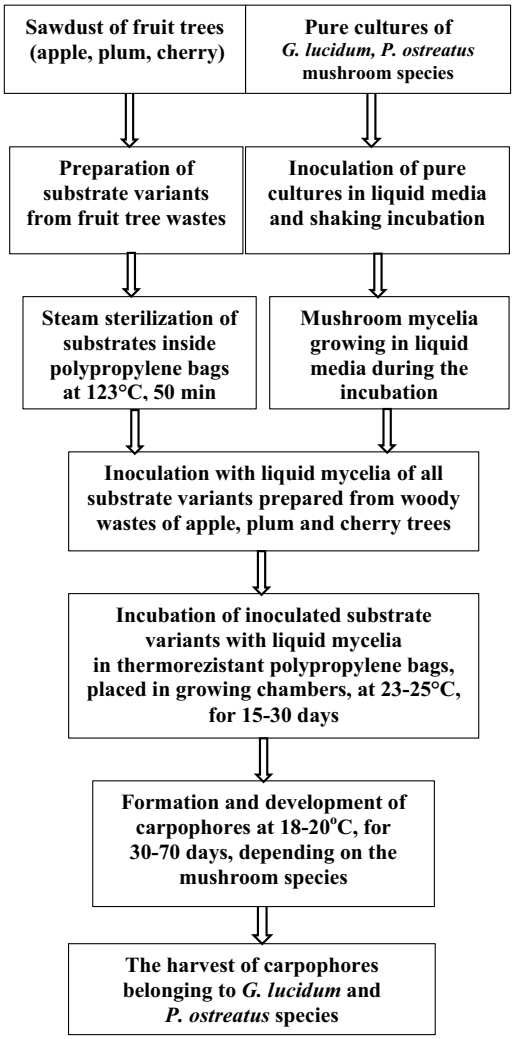

Figure 7. Biotechnology for recycling the fruit tree wastes by using *G. lucidum* and *P. ostreatus* mushroom species, through organic cultivation

The most important advantage of using such biotechnology for recycling the fruit tree wastes by using *G. lucidum* and *P. ostreatus* is that the carpophores which were collected from the substrate variants are 100% natural food products, being obtained through the organic cultivation of these mushroom species.

It is expected to implement this biotechnology for recycling the fruit tree wastes, by following the controlled process previously presented, by decomposing such lignocellulose materials and getting significant amounts of carpophores, as well as providing the environment protection in orchards designed for growing apple, plum and cherry trees.

CONCLUSIONS

According to the registered results, the best substrate variant for *G. lucidum* cultivation was determined as being S1 and for *P. ostreatus* mushroom species it was proven to be the substrate variant S2.

By comparing the amounts of carpophores belonging to *G. lucidum* mushroom species harvested on the substrate variants which have been used in experiments, it has to be mentioned that the highest total weights were noticed when it was used the substrate variant S1, followed by the substrate variant S3, and finally, by the substrate variant S2.

Speaking about the mushroom species *P. ostreatus*, there were registered the highest total weights of mushroom carpophores when the substrate variant S2 was used, followed by the substrate variant S1 and the last one being the substrate S3.

Significant differences of mushroom weights between the crop stages were noticed in the case of *G. lucidum*, respectively between the first three crop stages corresponding to the mushroom harvest on substrate variants S1 and S2. At the same time, there were determined significant differences between the crop stages of *P. ostreatus*, mainly between the first three crop stages related to the mushroom harvest on substrate variants S2 and S3.

The fruit body productions of each one of these mushroom species have registered the highest levels as 1,830 g for *G. lucidum* and as 2,750 g in the case of *P. ostreatus*, relative to 5 kg of substrate variants S1 and respectively S2, mainly made of fruit tree wastes.

However, in-depth experiments regarding the optimal valorizing of different types of lignocellulose wastes coming every year from fruit tree growing works through controlled cultivation of mushroom species like *G. lucidum* as well as *P. ostreatus* are going to be carried out in the next period of time.

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PRELIMINARY RESULTS REGARDING THE TESTING OF TREATMENTS WITH LED ON THE SEED GERMINATION OF *Lycopersicum esculentum* L.

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Abstract

The present study discusses the preliminary results obtained from testing the influence of LED treatments over seed germination of three different Lycopersicum esculentum L. *varieties. The respective varieties have been chosen for their determined growth pattern, which would make them suitable for implementation within a vertical farming system. Speeding up the germination process and ensuring that most, if not all the seeds germinate is relevant in obtaining healthy and productive L. esculentum* L. *crops suitable for indoor farming, which will provide a sustainable solution for a round-the-year constant production which can meet the market request, no matter the climatic conditions specific to the area. The experimental results obtained show that treatments with light emitted of LED (white, blue, red and natural light), on the seed germination of Lycopersicum esculentum* L. *varieties (Buzău 4C, Rio Grande ST and Saint Pierre ST), may influences: the sprouts rate, the fresh weight of sprouts, the fresh weight of cotyledons, the fresh weight of hypocotyls, the fresh weight of roots, the length of hypocotyls and length of roots.*

Key words*: Lycopersicum esculentum* L., LED, seed germination.

INTRODUCTION

The present article discusses preliminary results regarding the testing of LED treatments on seed germination of *Lycopersicum esculentum* L.

Lycopersicum esculentum L. is an annual herbaceous plant species from the *Solanaceae* family, originating from South America, Peru, Bolivia and Ecuador (Popescu, 2008). The *Solanaceae* family is, from either an economical, industrial or nutritional point of view, extremely important, as it includes very valuable species, *Lycopersicum esculentum* L. being one of the most representative (Kimura & Sinha, 2008). Aside from being one of the most commonly consumed fresh vegetable around the world, the tomato (*Lycopersicum esculentum* L.) bears a fruit that has many nutritional qualities (Suarez et al., 2007). Therefore, it is only natural for scientists to be experimenting in improving the quality and the production rate of *L. esculentum* L., thus obtaining a remarkable number of mutants, which express a wide variety of selected traits (Kimura & Sinha, 2008).

Previous research conducted on *L. esculentum* L. has shown a very strong connection between productivity and the interaction between temperature and light, from seed germination to harvesting (Verkerk, 1955).

The intensive exploitation of arable land, the need for more and more space to satisfy the needs of the planet's population, call for sustainable solutions, like the concept of vertical farming (Despommier, 2009). Applying this modern agricultural concept requires the development of technologies and selection of species that will enable it.

Also, smart management of resources is important (resources such as energy), thus making LEDs extremely efficient (Yeh & Chung, 2009).

However, there are many inconsistencies when it comes to the physiological effects of LEDs over the course of the plant's development (Berkovich et al., 2017).

The industrial importance of *L. esculentum* L. crops is undeniable therefore it is desirable that the production be increased without affecting the quality of the final product. There are many aspects that press on food

industry and urge the development of modern agricultural concepts. The discussions surrounding the concept of Vertical Farming are increasing, as global population and food demand increase (Kalantari et al., 2017).
As far as lighting solutions go, previous studies have shown increased efficiency in the use of LEDs for growing sprouts and plants, such as pomegranate (*Punica granatum*). The obtained sprouts have presented an ideal development state that resulted in extremely efficient transplantations (Bantis et al., 2018).
Other studies conducted on *Cucumis sativus* have shown that the production of qualitative sprouts has increased while using LED supplementary illumination, while harvesting was conducted eight days earlier in comparison to a conventional, natural light based protocol (AGROBIZNES.MD, 2017).
Another study on the effect of LEDs on *Lycopersicum esculentum* L. seed germination was concluded with a recommendation of a certain balance between blue light and red light for a faster development (Yingchao Xu et al., 2017). Moreover, studies conducted on *Lactuca sativa* L. have demonstrated LED efficiency, not only from the yield point of view, but also when it comes to energy costs and quality of harvest (Poulet et al., 2014; Lin et al., 2013).

MATERIALS AND METHODS

The seeds used in order to obtain the preliminary results regarding the testing of germination under LED treatments have been selected as the adult plants have a determined growth, meaning they will not need any supplementary support during their vegetation period and should be suitable for a vertical farming system as well (Popescu & Zăvoianu, 2013).
The biological material used in the experiment consisted of seeds which have proceeded from three *Lycopersicum esculentum* L. varieties: Buzău 4C, Rio Grande ST, Saint Pierre ST - respectively 60 seeds from each variety, thus enabling the performance of 3 repetitions by 15 experimental variants (one variant contains 15 seeds from one of the tested varieties) as follows:

- V_1, V_2, V_3, V_4 – experimental variants for Buzău 4C variety;
- V_5, V_6, V_7, V_8 – experimental variants for Rio Grande ST variety;
- V_9, V_{10}, V_{11}, V_{12} – experimental variants for Saint Pierre ST variety.

The working method, in terms of the *Lycopersicum esculentum* L. biological material is compliant with the conditions of the *in vitro* method, thus meaning the seeds have been subjected to aseptization by means of Domestos solution (2.5 ml of Domestos, 97.5 ml of aseptized distilled water) for 30 seconds, followed by three washing sessions with aseptized distilled water (10 minutes for each wash). The seeds have been inoculated on aseptized gauze and placed in transparent containers. The gauze has been moistened with a share of 17 ml of aseptized distilled water upon inoculation, followed by another share, of 10 ml on the third day post inoculation.
The experimental device was made up of three sets of LEDs (Light Emitting Diodes), which have emitted light out of the white, blue and red light spectrum. The technical specifications of LEDs are: power 18 W, voltage 220 V, light flux 435 lm and dominant wavelength (Livadariu & Maximilian, 2017).
The light variants used in the experiment are as follows: W- white LEDs, R- red LEDs, B- blue LEDs, N- natural light.
The experimental variants have been exposed to the different types of lighting as follows:

- V_1, V_5, V_9 – white LEDs (W);
- V_2, V_6, V_{10} – blue LEDs (B);
- V_3, V_7, V_{11} – red LEDs (R);
- V_4, V_8, V_{12} – natural light (N).

The incubation of the seeds was conducted under the following conditions: temperature of 22°C ± 2°C with enforcement of light treatment for 16 h within a 24 h period.
For each inoculation, quantitative determinations (sprouts rate, fresh weight of sprouts, fresh weight of cotyledons, fresh weight of hypocotyls and fresh weight of roots) as well as morphometric determinations (length of hypocotyls and length of roots) have been conducted.

RESULTS AND DISCUSSIONS

The preliminary results regarding seed germination of *L. esculentum* L. under the influence

of LEDs show clearly noticeable differences in different aspects of sprout development.
The first aspect that should be discussed is the average value of seed germination (Figures 1, 2 and 3).
Significant differences can be noticed for the Rio Grande ST variety (V1, V2, V3, V4), as results show that blue LED lights (B) have had quite an impact in the encouragement of seed germination, in comparison with the red LEDs (R). The results for the natural light (N) are slightly under the ones for the blue LEDs, while for the seeds treated with white LEDs, even though in day 7 (D7) the number of germinated seeds was similar to the results in the red LED variant (R), in day 18 (D18), the gap is noticeably greater. Therefore, these results show that in this variety's case, blue LEDs (B) were the most efficient in stimulating germination, while the red LEDs (R) were the most inefficient.
For the Buzău 4C variety (V5, V6, V7, V8) tested in this experiment, the results for the germination rate (Figure 2) are not very different from the ones for Rio Grande ST variety (Figure 1).

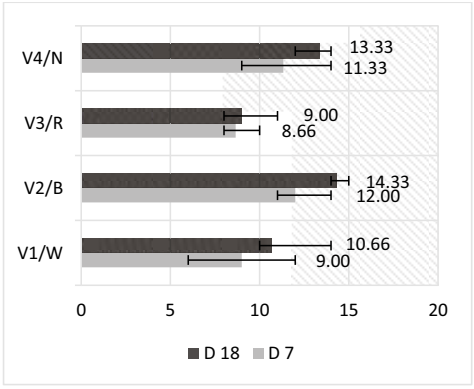

Figure 1. The average value of the rate of seed germination (no.) for the Rio Grande ST variety, in day 7 (D7) and day 18 (D18) post incubation

The results show that in this case as well, the blue LEDs (B) has represented one of the most efficient lighting method. An interesting observation is that the results for day 18 (D18) post inoculation, for the white LEDs (W) and the natural light (N), are the same. The only difference seems to be an increase in the germination speed, as on day 7 (D7) there were more germinated seeds in the variant exposed to white LEDs, in comparison to the one exposed to natural light. In a more general approach, the discrepancy between the blue LED lighting variant (B) and the rest of the lighting variants (W, R, N) is the most obvious, while there seems to be little to no difference in their influence on germination. However, the red LEDs (R) prove themselves to be, again, most inefficient.

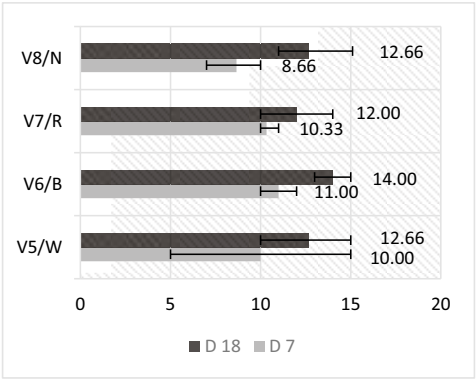

Figure 2. The average values of the rate of seed germination for the Buzău 4C variety, in day 7 (D7) and day 18 (D18) post incubation

As for the Saint Pierre ST variety (V9, V10, V11 and V12) tested (Figure 3), the germination rates for the natural light (N), red LED (R), blue LED (B) and white LED (W) lighting variants are very similar. However, in terms of numbers and end results (D 18), the white LED (W) light has been apparently most effective followed closely by the results of blue LED lighting.

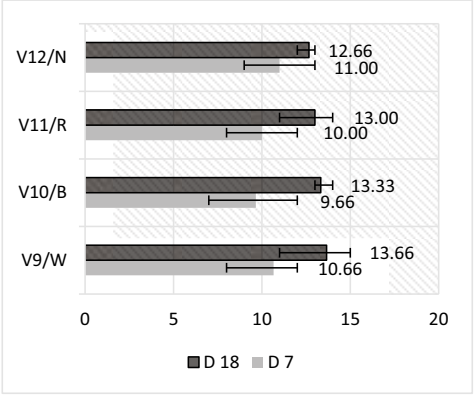

Figure 3. The average values of the rate of seed germination for the Saint Pierre ST variety, in day 7 (D7) and day 18 (D18) post incubation

Also, it seems that the germination speed was higher in the white LED variant - as suggested by the observations for day 7 (D7).
After germination, the results regarding the development of the sprouts show differences depending on the lighting variants (Figure 4). Therefore, as noticeable in Figure 4, for the experimental variants that represent the Rio Grande ST variety (V1, V2, V3 and V4), as well as for the experimental variants that represent the Buzău 4C variety (V5, V6, V7 and V8) the value of the fresh weight of the sprouts is highest under the blue LED lighting (B). However, for the Saint Pierre ST variety, blue LEDs (B) seem to have been the most inefficient, followed shortly by the natural light (N).

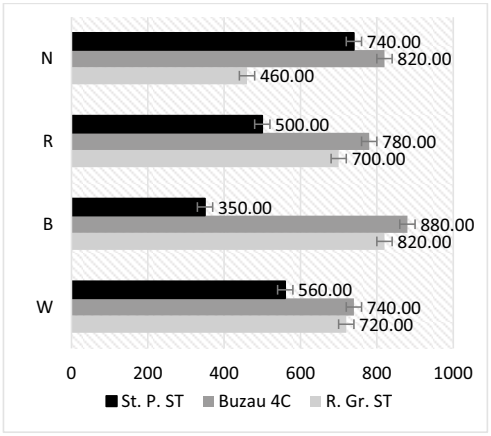

Figure 4. The average values of the fresh weight of sprouts (mg), varieties tested reported to light variants (W, B, R, N)

Moreover, the discrepancies seem to repeat themselves when it comes to the value of the fresh weight of cotyledons (Figure 5), blue LEDs (B) being most efficient for the first two varieties tested, while for the Saint Pierre variety, it proves itself most inefficient, in this case, natural light (N) taking the leading place.
There is an obvious similarity when it comes to the results regarding the fresh weight of the hypocotyls (Figure 6), between all tested varieties, as they all seem to prefer natural lighting (N). There is a small discrepancy between the results for red LEDs (R) and natural light (N) variant results, thus suggesting the two lighting options tend to have similar effects on the fresh weight of the hypocotyls.

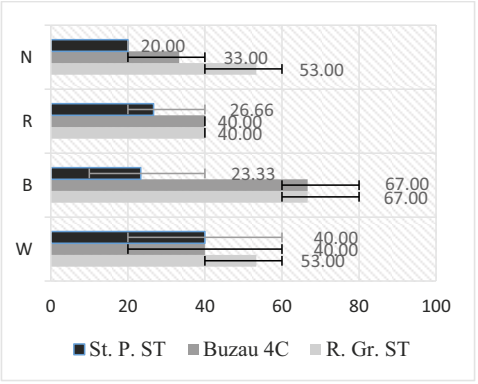

Figure 5. The average values of the fresh weight of cotyledons (mg), varieties tested reported to light variants (W, B, R, N)

Also, during the experimental observations, one has noticed that in the case of white LED lighting (W) and blue LED lighting (B), all the hypocotyls from all the varieties tested, in all their repetitions, have developed a purple coloration. There has been no sign of such coloration in any of the other experimental light variants.
Another interesting observation concerns the development of the roots, more precisely, adventive roots, in the case of plants treated under the blue LED (B) variant. Adventive roots have grown up to the middle of the hypocotyl length in some cases. This kind of root growth is not abnormal for *L. esculentum* L. However, the other experimental variants, tested under different types of lighting have not shown such an obvious particularity.

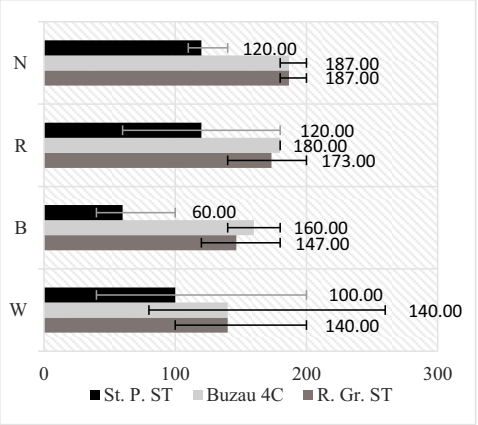

Figure 6. The average values of the fresh weight of hypocotyls (mg), varieties tested, reported to light variants (W, B, R, N)

Regarding the development of the roots (Figure 7), the outcome of the experiment shows that blue LED light (B) seems to encourage it, at least for the first two varieties tested, Rio Grande ST (V1 to V5) and Buzău 4C (V6 to V10).

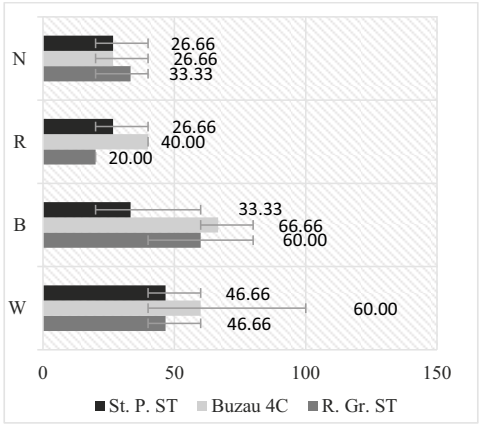

Figure 7. The average values of the fresh weight of roots (mg), varieties tested, reported to light variants (W, B, R, N)

The discrepancy is most obvious with the Buzău 4C variety, the fresh weight of roots reaching the highest value on day 18 post inoculation. Natural light (N) does a poor job in stimulating root growth for all tested varieties, with a slight discrepancy for the Rio Grande ST, which seems to have done a bit better than the other two. Quite equally inefficient when it comes to root growth stimulation is the red LED light (R) variant. White LEDs (W) and blue LEDs (B) show most potential in this aspect of the experiment.

The average values of the root length, presented in Figure 8, show that each variety has a certain preference when it comes to the development in length of the radicular system. These preferences are strangely different and further studies should be conducted over this aspect.

However, when it comes to the length of the hypocotyl (Figure 9), results show that the preference for the red LED (R) lighting variant is obvious for all tested varieties, especially in the case of the Rio Grande ST variety.

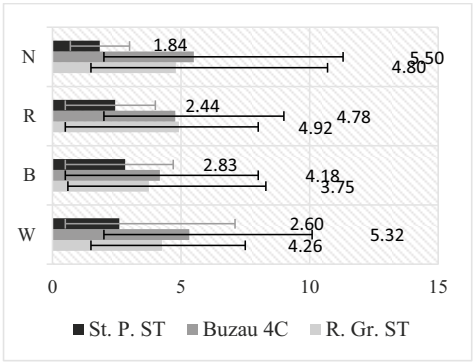

Figure 8. The average values of root length (cm), varieties tested, reported to light variants (W, B, R, N)

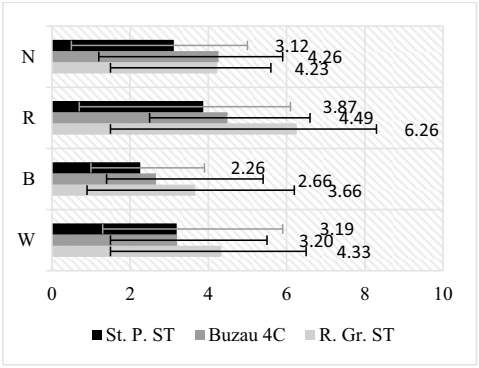

Figure 9. The average values of the hypocotyl length (cm), varieties tested, reported to light variants (W, B, R, N)

Therefore, the right combination of the different colours of the spectrum may provide most efficient lighting, thus enabling farmers to apply modern farming methods, for a fast, round-the-year, constant production of qualitative food.

However, further studies should be conducted in order to be able to draw clear conclusions, while other parameters should be taken into consideration, such as temperature variations or light intensity.

CONCLUSIONS

The experiment conducted on the three *Lycopersicum esculentum* L. varieties shows that some LED lighting variants may prove themselves very useful in different development stages (seed germination, sprout development).

The results indicate certain lighting preferences that each variety may have, depending on their state of development.

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COMPARATIVE ANALYSES OF PLANT RESPONSES TO SALINITY IN RELATED TAXA: A USEFUL APPROACH TO STUDY SALT STRESS TOLERANCE MECHANISMS

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Abstract

The progressive salinisation of irrigated cropland is causing substantial losses in agricultural production, a problem that will worsen due to climate change effects. Enhancing crop salt tolerance is a sensible strategy to achieve significant increases in crop yields, but requires a deep understanding of the underlying mechanisms. When challenged by salinity, all plants, regardless of their degree of tolerance, activate a series of basic responses, including the control of ion transport, the synthesis of compatible solutes for osmotic adjustment, or the activation of antioxidant systems. Yet, for a given species, the biological relevance and the relative contribution of different responses to the mechanisms of salt tolerance remain largely unknown. Over the last years, we have performed comparative analyses on the responses to salinity in different taxa, genetically related but with varying levels of tolerance. Correlating salt-induced changes in the concentrations of suitable biochemical stress markers with the relative tolerance of the investigated species, we are obtaining novel and interesting information on those mechanisms. Some examples with taxa of several genera are discussed, to show the usefulness of our approach.

Key words*: climate change, ion transport, osmolytes, salt stress, salt tolerance.*

INTRODUCTION

Modern agriculture is largely dependent on a limited number of cultivars of a few major crops, derived, directly or indirectly, from the 'Green Revolution' of the 1960s and 1970s (Borlaug & Dowswell, 2005). These cultivars were developed for a high-input, industrialised agriculture and can provide high yields under optimal – artificial – growing conditions, in either greenhouses or open fields, although generally require large amounts of agrochemicals (chemical fertilisers, herbicides and pesticides) and irrigation water; they are, however, relatively sensitive to stressful conditions, such as cold, high temperatures, waterlogging, drought or salinity. In fact, for all major crops there is a large difference between average yields and the record yields obtained under the most favourable growing conditions; these losses, which can vary from 50% to more than 80%, depending on the species, are mostly due to abiotic stress conditions affecting the plants in the fields; among them, drought and soil salinity are the environmental factors most relevant for this reduction of crop productivity (Buchanan et al., 2000).

In the current climate change scenario – with increasing average temperatures, reduced rainfall and alteration of the normal seasonal weather patterns – crops in arid and semi-arid regions are being affected by drought periods which are longer, more frequent and more intense than in the near past.

The progressive 'secondary' salinisation of irrigated land – by the accumulation of toxic ions dissolved in irrigation water – is also contributing to the extension of desertification of former fertile cropland.

Therefore, increasing, or even maintaining the present production levels is becoming a severe challenge for agriculture.

Additional factors, not directly dependent of climate change effects – such as the massive movement of rural population to the cities, change of land use (for urban development, tourism or industrialisation) leading to a further reduction of the area of available farmland, or

the growing demand of cereals and oilseed crops for biofuel production, competing with food – are also limiting the potential productivity of agriculture at the global level.

Crop production is still growing, both in absolute terms and *per capita*; however, the rate of growth has been decreasing since the mid-1980s. Nowadays, there is enough food to feed everybody on earth – although that food is not evenly distributed – but if this trend continues, soon this will not be true anymore.

STRATEGIES FOR INCREASING AGRICULTURAL PRODUCTION

In the present circumstances, it is clear that an increase in crop production cannot be based on a significant extension of the total agricultural land area, which is actually decreasing. As water for irrigation is becoming an increasingly scarce resource, it is also not possible to enlarge the area of land cultivated under irrigation, which is much more productive than rain-fed farmland. Also, there is an urgent need to switch the present agricultural practices to a more sustainable agriculture, stopping or reducing depletion of natural resources and the destruction of areas of high ecological value. Therefore, we should also exclude growing our present crops in low-fertility, marginal land, which would require the use of large amounts of chemical fertilisers and would not be sustainable.

An extension of the global area of biotech (transgenic) crops, including the development of varieties with new traits, will also contribute to increasing food production, as they provide higher yields than the corresponding conventional crops (ISAAA, 2017). Nevertheless, these transgenic plants are derived from 'Green Revolution' varieties and pose the same problems than non-transgenic cultivars regarding high inputs requirements and sustainability issues.

Many other strategies are being tried to increase, or at least maintain crop yields, but in the frame of a more sustainable agriculture. They include, for example, organic agriculture, which is obtaining good results in terms of productivity as well as a business, due to the interest of consumers on food products obtained without the use of agrochemicals. There is also a 'new generation' of fertilisers, namely, slow-release and controlled-released fertilisers; they can (modestly) increase crop yields when used at the same doses than traditional fertilisers – or maintain the same production at lower doses – but their main advantage is that they could help protect the environment, being less contaminating for soil and water and having a smaller 'carbon footprint'. The application to crops of 'biostimulants', a disparate group of different substances (humic substances, protein hydrolysates, seaweed extracts, chitin-derived biopolymers, or some chemical elements) or microorganisms (beneficial fungi and bacteria) should also be mentioned as a means to enhance the efficiency of plant nutrition, facilitate growth under stress conditions and/or improve the quality of the harvested product (Boscaiu et al., 2018; Xu & Geelen, 2018).

Developing drought and salt-tolerant crops

All approaches mentioned above will no doubt contribute to improving crop yields, but the expected increase in food production will not be sufficient to cope with population growth. Since the most substantial reduction in productivity is due to environmental abiotic stress conditions, especially drought and salinity, the most effective alternative would be to develop crop varieties more resistant to salt and water stress. For this, all available strategies should be used including, obviously, traditional breeding techniques. This approach has not been very successful in the past, due to the complexity of the stress-tolerance traits, but now the breeder can use an array of modern molecular tools – marker-assisted selection (MAS), next-generation sequencing (NGS) technologies, high-throughput genotyping platforms, among others – which significantly increase the efficiency and reduce the time required to carry out breeding programmes; in fact some successful examples of crops with enhanced salt or water stress tolerance obtained by 'classical' breeding, have been reported in recent years, for instance several specific cultivars mentioned by Fita et al. (2015). Genetic engineering can also be used to express 'stress-tolerance' genes in transgenic crops; here again, many laboratory experiments point to the feasibility of this strategy. However, up

to now no biotech crop with enhanced abiotic stress tolerance is commercially cultivated in our fields, except for a drought-resistant maize variety, developed by Monsanto and BASF, expressing a bacterial RNA chaperonin gene (Castiglioni et al., 2008). Progress is also being made in the recovery and improvement of local or neglected crop varieties, and in the domestication of wild plants with relatively higher stress tolerance than our standard crops. Some of those wild species are extremely resistant in nature to salinity (halophytes) or drought (xerophytes), and could be the basis of a sustainable, 'saline' or 'arid' agriculture (Fita et al., 2015; Boscaiu et al., 2018).

We can conclude that the biotechnological improvement of crop abiotic stress tolerance, especially to drought and soil salinity, is the most promising strategy to quickly increase crop yields and food production, needed to feed a growing human population in the next few decades. To reach this goal, applying the different strategies mentioned in the previous paragraph, a deep understanding of the mechanisms underlying abiotic stress tolerance in plants is required.

ELUCIDATION OF THE MECHANISMS OF STRESS TOLERANCE IN PLANTS

Paradoxically, most studies on drought and salt tolerance in plants have been carried out using species that are not tolerant, mostly the model *Arabidopsis thaliana* or some crops, such as tobacco or rice. It is doubtful that the results obtained can be generalised to all species, but this is generally assumed, partly due to the confusion in the literature between two related, but distinct concepts: *responses* to stress and stress *tolerance*.

Stress responses *vs*. tolerance to stress

All plants, independently of their degree of tolerance, use the same general responses to water and salt stress (and also to other abiotic stresses), based on the activation of a series of conserved mechanisms, including: **i)** control of ion transport and ion homeostasis, at the cell and whole plant levels; **ii)** biosynthesis and accumulation of compatible solutes or osmolytes for osmotic adjustment; **iii)** activation of antioxidant enzymes and synthesis of antioxidant compounds – since drought and high soil salinity generate oxidative stress as a secondary effect; **iv)** changes in gene expression leading to the synthesis of 'protective' proteins, such as heat-shock proteins, LEA proteins, osmotin, and many others.

Stress tolerance is obviously based on the activation of these conserved responses, justifying to some extent the use of non-tolerant models for its study. However, it is clear that the *efficiency* of the response must largely vary in different species, as plants show a very wide range of tolerance. Furthermore, the relative contribution of specific responses to the mechanisms of tolerance may also vary and, for a given species, is generally unknown.

Our experimental approach for the study of abiotic stress tolerance in plants

We are investigating the mechanisms of salt and drought tolerance in different plants, including some wild species, vegetable and ornamental crops and forest trees, such as taxa of the genera *Plantago*, *Juncus*, *Limonium*, *Phaseolus*, *Tagetes*, *Portulaca* or *Picea*, among others. Our work is based on the hypothesis that performing comparative analyses of the responses to stress of taxonomically – and, therefore, genetically – closely related taxa that show different degrees of stress resistance should help to distinguish those responses that are relevant for tolerance from those which are not, and thus contribute to elucidate general mechanisms of tolerance in plants. In our studies, as 'closely related taxa' we have included: **i)** wild species of the same genus, adapted to natural habitats affected by varying types and levels of environmental stress; **ii)** different ecotypes/varieties/cultivars of the same species, which often also show differences in stress resistance; **iii)** a crop and some of its wild relatives; or **iv)** different populations/provenances of the same species.

Our experimental strategy consists on applying salt stress (different concentrations of NaCl) and water deficit (withholding irrigation) treatments to the plants under controlled greenhouse conditions and determining the stress-induced changes in the levels of different biochemical stress markers associated to specific response pathways – ions, osmolytes,

antioxidant compounds, antioxidant enzyme activities. Correlation of the contents of these markers with the relative tolerance of the investigated taxa – estimated from their distribution in nature and/or the relative growth inhibition caused by the stress treatment – allows establishing which specific responses are involved in each case in tolerance mechanisms.

In the following sections, we describe a brief selection of published results to show the usefulness of the strategy outlined above. These examples are limited to salt tolerance mechanisms based on the control of ion transport and osmotic adjustments, in taxa of three genera: *Phaseolus*, *Plantago* and *Juncus*. Salt tolerance based on other plant responses, such as the activation of antioxidant systems, or the mechanisms of tolerance to drought, will not be mentioned here.

SALT TOLERANCE IN *Phaseolus*

Experimental material and salt treatments

Plants of three cultivars of common beans (*Phaseolus vulgaris*) – 'The Prince', 'Judía de Franco' and 'Maxidor' – and one cultivar of the runner bean (*P. coccineus*) – 'Moonlight' – were grown for three weeks in the presence of 0 (control), 50, 100 or 150 mM NaCl. After the treatments, the plants were harvested, and several growth parameters were determined: stem length, number of leaves, leaf fresh and dry weight, and leaf water content.

Based on the salt-induced inhibition of growth, the following ranking of salt tolerance was established: 'Maxidor' > *P. coccineus* ('Moonlight') > 'Judía de Franco' > 'The Prince'

Control of ion transport

Generally, Na^+ and Cl^- ions accumulate in plant leaves, to a greater or lesser extent, in response to NaCl treatments. In *Phaseolus*, Na^+ leaf levels increased with increasing external salinity, in a concentration-dependent manner, only in the most salt-sensitive cultivar, 'The Prince'. In 'Maxidor', the most tolerant, Na^+ content was maintained at the same level than in the non-stressed control at all external salt concentrations tested. In the second-most tolerant cultivar, 'Moonlight' (of *P. coccineus*) and in 'Judía de Franco', Na^+ increased significantly (but only slightly in 'Moonlight') in the presence of the highest NaCl concentration tested (150 mM), but not at lower salinities. Cl^- ions accumulated in leaves in parallel with increasing external salinities, in all tested cultivars, and reaching in all cases absolute levels much higher than those of sodium. However, the qualitative patterns of accumulation were similar to those of Na^+; that is, the highest contents were measured in the least salt tolerant cultivar ('The Prince'), and the lowest in the most tolerant *cv*. 'Maxidor'. There is, therefore, a *negative* correlation between tolerance and the efficiency of ion transport to the aerial part of the plants, indicating that, in *Phaseolus*, salt tolerance is based, at least partly, on the inhibition of Na^+ (and, to a lesser extent, Cl^-) transport to the leaves.

Osmotic adjustment

According to the 'ion compartmentalisation hypothesis' (Wyn Jones et al., 1977), Na^+ and Cl^- ions must be sequestered in the cell vacuoles to avoid reaching toxic levels in the cytoplasm; this requires the accumulation of compatible solutes in the cytosol, to maintain cellular osmotic balance. Proline (Pro) is one of the commonest plant osmolytes. Leaf Pro contents increased in response to the salt treatment in the four *Phaseolus* cultivars, but its accumulation showed a negative correlation with their relative salt tolerance: Pro reached the highest levels in *cv*. 'The Prince' and the lowest in 'Maxidor'. This means that Pro cannot be directly involved in the mechanisms of salinity tolerance in *Phaseolus*, although it appears to be a reliable biochemical salt stress marker, accumulating in those cultivars that are relatively more sensitive and therefore more stressed in the presence of salt.

Regarding other putative osmolytes, glycine betaine did not accumulate in response to the salt stress treatment in any of the four *Phaseolus* cultivars. Sucrose and fructose contents clearly increased in *cv*. 'The Prince' in parallel with increasing external salinity, but not in the other three cultivars. Of those tested, only *myo*-inositol appears to be a functional osmolyte in this genus as its accumulation in

leaves correlates positively with the relative salt tolerance of the bean cultivars.

SALT TOLERANCE IN *Plantago*

Experimental material and salt treatments

Three species of the genus *Plantago*, *P. crassifolia*, *P. coronopus* and *P. major*, were selected for this study. The two first species are halophytes, growing in natural saline ecosystems, whereas *P. major* is considered a glycophyte and is only found in the field in habitats of low salinity. According to their distribution in nature, the relative salt tolerance of these species is: *P. crassifolia* ≥ *P. coronopus* > *P. major*. Plants of the three species were grown in the greenhouse for four weeks, watered with NaCl solutions of increasing concentration, from 0 (control) to 800 mM. Quantification of the degree of salt-induced growth inhibition confirmed the relative tolerance to salinity indicated above, for the three analysed *Plantago* species.

Control of ion transport

Leaf ion (Na^+, Cl^-) contents increased in the three *Plantago* species in parallel with increasing external salinity, both ions reaching similar absolute levels. Contrary to *Phaseolus*, in this case, the highest ion contents were measured in the two halophytes, slightly higher in the most tolerant *P. crassifolia*; the most sensitive *P. major* showed substantially lower concentrations of Na^+ and Cl^- in the leaves. This accumulation pattern indicates that, in *Plantago*, salt tolerance is based on the active transport of ions to the leaves. Interestingly, both halophytes showed relatively high leaf concentrations of Na^+ (in the most tolerant *P. crassifolia*, also of Cl^-) in control, non-stressed plants, whereas the two ions were present at much lower levels in the glycophyte *P. major*. This observation suggests that salt-tolerant plants of this genus can use inorganic ions as osmotica, even under conditions of low soil salinity, which supports the existence of active mechanisms of ion transport from roots to the aerial part of the plants.

Osmotic adjustment

It is well established that sorbitol is the functional osmolyte in the genus *Plantago*. Accordingly, we measured relatively high leaf concentrations of this polyalcohol in the three selected *Plantago* species. The highest value, ~ 2 mmol g^{-1} DW, was observed in the presence of 800 mM NaCl in *P. crassifolia*, the most tolerant species. However, both the absolute levels and the patterns of accumulation of sorbitol in response to salt stress were similar in the three species. Therefore, even though sorbitol could be necessary for cellular osmotic balance, it cannot be responsible for the differences in salt tolerance, since there is no differential accumulation of the osmolyte in the three analysed taxa. Pro contents, on the other hand, were very low in the controls and largely increased in response to the salt treatment, but only at high external salt concentrations (600-800 mM NaCl) and, most important, only in the halophytes, not in the salt-sensitive *P. major*. Therefore, salt tolerance in *Plantago* seems to be partly dependent on the activation in tolerant species (but not in salt-sensitive ones) of the synthesis of a secondary osmolyte, Pro, in response to high salinity stress.

SALT TOLERANCE IN *Juncus*

Experimental material and salt treatments

In this case, we also selected three species, two halophytes (*Juncus maritimus* and *J. acutus*) and one glycophyte (*J. articulatus*). According to the salinity of the natural habitats of these species, and the degree of salt-induced growth inhibition in controlled salt treatments, the relative tolerance to salinity of these species is *J. maritimus* > *J. acutus* >> *J. articulatus*. Salt treatments were carried out by watering the plants with increasing NaCl concentrations, from 0 (controls) to 400 mM, during eight weeks.

Control of ion transport

Plants of the three selected *Juncus* species showed a progressive increase in the *root* levels of Na^+ and Cl^-, correlated to the increase of NaCl concentration in the watering solution. The absolute ion concentrations reached and the patterns of accumulation were similar for the two ions and the three species, regardless of their relative tolerance to salt. We also observed a concentration-dependent increase of Na^+ and Cl^- contents in the *shoots*, in response

to increasing salinity; although the concentration of the two ions was similar, as in roots, there were significant differences between species. We observed a clear *negative* correlation between ion concentrations and salt tolerance: the highest levels were reached in the less tolerant *J. articulatus*, followed by *J. acutus*, whereas *J. maritimus*, the most tolerant, showed the lowest accumulation of Na^+ and Cl^-. Therefore, salt tolerance in *Juncus* is associated with the *inhibition* of ion transport from the roots to the aerial part of the plants.

Osmotic adjustment

Glycine betaine (GB) and total soluble sugars (TSS) both increase with increasing salinity in the shoots of salt-treated *Juncus* plants. Although these osmolytes may contribute to osmotic balance in this genus, their patterns of accumulation and the concentrations reached were similar in the three selected species, so that salt-induced GB and TSS biosynthesis cannot be responsible for the observed differences in tolerance. Pro contents, on the other hand, were very low in the controls and increased > 20-fold in the presence of 400 mM NaCl, but only in the salt-tolerant *J. maritimus* and *J. acutus*; in the glycophyte *J. articulatus*, the increase of Pro concentration with respect to the control, although statistically significant, was less than twofold. Therefore, Pro accumulation is most likely involved in the mechanisms of salt tolerance in *Juncus*, as it correlates *positively* with the relative degree of tolerance of the investigated species.

SALT TOLERANCE AND K^+ HOMEOSTASIS

An increase in cellular Na^+ concentration is generally accompanied by a decrease in K^+ levels, as the two cations compete for the same transport proteins (Rodríguez-Navarro, 2000). Mechanisms leading to the maintenance of relatively low Na^+/K^+ ratios under high salinity conditions are considered relevant for tolerance. A reduction in leaf K^+ contents in response to salt treatments has been observed in different species, in agreement with the general behaviour mentioned above, whereas in other species K^+ is maintained at the same level than in the non-stressed controls. Interestingly, some tolerant plants show a peculiar pattern of salt-induced changes in leaf K^+ contents, first decreasing at low or moderate salinities, and then increasing again in the presence of higher external salt concentrations. This indicates the specific activation of K^+ transport from roots to leaves in response to strong salt stress conditions. We have observed this phenomenon, for example, in the tested halophytes of the genera *Plantago* (*P. crassifolia* and *P. coronopus*) and *Juncus* (*J. maritimus* and *J. acutus*), but not in the more salt-sensitive species *P. major* and *J. articulatus*, respectively. These data support the relevance of the activation of K^+ transport to the aerial part of the plants in different tolerant taxa.

SUMMARY OF SALT STRESS TOLERANCE MECHANISMS IN THE SELECTED GENERA

In *Phaseolus*, salt tolerance is dependent, at least in part, on specific mechanisms blocking transport of Na^+ cations (and, to a lesser extent, of Cl^- anions) from roots to leaves, and on the accumulation of *myo*-inositol for osmotic balance and as an osmoprotectant. Pro is a reliable marker of stress, accumulating to higher levels on those taxa which are more sensitive to stress (and, therefore, more stressed at the same salinity levels), as compared to related more tolerant taxa. However, Pro does not seem to be directly involved in salt tolerance mechanisms (Al Hassan et al., 2016c).

In *Plantago*, salt tolerance depends on the efficient transport of toxic ions (Na^+, Cl^-) to the leaves – where they are predominantly stored in vacuoles – and on the activation of K^+ transport under high external salinity. Plants of this genus use sorbitol as the main osmolyte for osmotic adjustment, but tolerant species also accumulate a secondary osmolyte (Pro) at high external salinity. Salt-tolerant *Plantago* taxa can accumulate Na^+ (or both, Na^+ and Cl^-) in the leaves, to be used as osmotica, also under low soil salinity conditions (Al Hassan et al., 2016d).

Finally, in *Juncus*, salt tolerance is associated with the inhibition of Na^+ and Cl^- transport from roots to shoots, the activation of K^+

transport to the leaves in the presence of high external salt concentrations, and the salt-induced accumulation of Pro for osmotic adjustment and osmoprotection (Al Hassan et al., 2016a).

A GLIMPSE TO OTHER SPECIES

Additional studies have revealed that some of the responses to salt stress mentioned above for *Phaseolus*, *Plantago* or *Juncus* are relevant for tolerance also in other plants species, unrelated taxonomically. For example, in oleander (*Nerium oleander*) salt tolerance is dependent on mechanisms blocking transport of toxic ions to the leaves (as in *Juncus*), and on the accumulation of GB and TSS as functional osmolytes (Kumar et al., 2017). In Norway spruce (*Picea abies*), salt stress activates transport of Na^+ and K^+ from the roots to the needles and accumulation of Pro, whereas soluble sugars do not seem to be involved in tolerance mechanisms (Schiop et al., 2015). In *Inula crithmoides*, an extremely tolerant succulent halophyte, salt tolerance is based on the efficient transport of toxic ions to the aerial part of the plants (as in *Plantago*), the activation of K^+ transport from the roots at high external salinity, and the use of GB as the main physiological osmolyte, with contribution of some sugars (arabinose, fructose and glucose) to osmotic adjustment under stress (Al Hassan et al., 2016b); similar mechanisms seem to operate in the genus *Limonium* – which includes a large number of halophytes – except that Pro, not GB, is the major functional osmolyte (Al Hassan et al., 2017). Inhibition of Na^+ transport to the leaves and maintenance of high K^+ concentrations even at high salinity levels are also important for salt tolerance in the genus *Silene* (Kozminska et al., 2018).

CONCLUSIONS

The general conclusion of the work carried out in our laboratory over the last years, and partly summarised here, is that the mechanisms of salt stress tolerance – as well as tolerance to drought and other abiotic stresses – vary widely in different plant species, even though they are based on the same conserved responses. Our strategy of performing comparative studies of the responses to stress in closely related taxa, but showing varying degrees of tolerance, has been proved to be very useful for the elucidation of those mechanisms. However, the studies must be carried out in different genera, species, cultivars or populations, as no single model, not even *Arabidopsis thaliana*, can provide a general and accurate view of the subject.

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PHYLOGENETIC ANALYSES OF SUGARCANE CULTIVARS USING SIMPLE SEQUENCE REPEAT MARKERS

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Abstract

Sugarcane (Saccharum sp.) is one of the world's most commercial and extensively grown crops. The breeding of sugarcane is keystone of all advanced sugarcane industries and several research institutes. The success of sugarcane breeding program lies in the appropriate selection of genetically rich and diverse genotypes. Research rationale of present study was to analyze genetic diversity among 24 promising flowering and non-flowering sugarcane cultivars. Molecular marker based screening was done with PCR analysis using 10 SSR primers. SCM-32 primer showed highest polymorphic bands. The genetic similarity and UPGMA clustering were analyzed for all 24 sugarcane cultivars. The similarity index values for S19, S21, S22 and S23 suggested them as closest ones and S17 as the most distant one. UPGMA clustering based dendrogram showed that the correlation between Jaccard coefficient and similarity index is high and significant. All 5 clusters showed a mixture of flowering and non-flowering cultivars, indicating that molecular marker can play a potential role in sugarcane breeding programs than morphology based analysis.

Key words*: cluster analysis, genetic similarity coefficients, simple sequence repeats, sugarcane.*

INTRODUCTION

Sugarcane (*Saccharum* sp.) is the most prominent source of sugar and bio-fuel across the globe. Thus, it is one of the most commercially important crops. More than 75% of world sugar production is achieved from sugarcane which is grown across 100 countries. The sugarcane breeding is the mainstay of all advancements in sugarcane based industries and technologies in most of the research institutes. Hence, the application of modern molecular techniques will strengthen varities to achieve high levels of yield (Silva & Bressiani, 2005).

The success of sugarcane breeding program lies in the appropriate selection of genetically rich and diverse genotypes. However, understanding the genetic diversity among the cultivars may provide platform for improved traits.

Studies have shown that genetic markers greatly aid in early identification of genotypes as they are rapid, reliable and reproducible. Thus, allowing early identification of genotypes when compared with conventional techniques, viz., morphological and biochemical markers (Sindhu et al., 2011).

Generally, molecular markers are found to be useful in genetic diversity, systematic and phylogenetic analysis. Nevertheless they have also proved to be advantageous in construction of genetic maps and genetic linkage studies in combination with other markers (Anderson, 2007; Bosland et al., 2012).

Microsatellites (Simple Sequence Repeats) are ubiquitous on eukaryotic genomes. Apparently, their sequence patterns are capable to induce hyper-variability in multiple repeats at particular locus, during DNA replication and recombination. This kind of variation among microsatellite markers has proved to be advantageous for genetic marking.

Further, these markers exhibit high level of polymorphisms and extensively exploited for evaluating genetic diversity, in construction of genetic map and cultivar identification. Microsatellite markers are likely to have many applications in sugarcane genetics and breeding including germplasm analysis, cultivar

identification, parent evaluation and marker assisted breeding (Cordeiroa et al., 2000).

Several types of molecular markers have been successfully employed for better understanding genetic relatedness among the commercial crops such as Wheat (Mohapatra et al., 2003) and Phaseolae (Vir et al., 2009). Among sugarcane crops different molecular markers have been used. Recently, genetic markers such as RAPD, ISSR and ITS, have been used to study genetic diversity among *Erianthus* and *S. spontaneum* species (Zhang et al., 2004). Singh et al. (2010) analyzed the genetic diversity among eighty four genotypes of *Saccharum barberi*, *S. spontaneum* and *S. officinarum* origin which included Indian and non-Indian commercial cultivars. Thirty two microsatellite markers consisting of sugarcane cDNA derived microsatellite markers (SCM), genomic microsatellites and unigene sugarcane microsatellite markers (UGSM) were used. More recently, Sindhu et al. (2011) reported the use of sequence tagged microsatellite sites (STMS) in genetic diversity analysis and concluded that 12 unique markers may aid in varietal identification. In the present study, SSR markers were employed to assess genetic diversity of 24 commercial cultivars. Similarly, Singh et al. (2017) investigated genetic relatedness among 24 sugarcane cultivars using 12 RAPD markers. The objective of the present study was to analyze genetic diversity among 24 promising flowering and non-flowering sugarcane cultivars.

MATERIALS AND METHODS

Plant Material

In the present study, 24 sugarcane commercial cultivars were used for the analysis of genetic diversity which is widely grown across the Northern Karnataka, India (Table 1). All cultivars were maintained at S. Nijalingappa Sugarcane Research Institute, Belgavi, Karnataka. Young leaf samples were collected and kept at -20°C until further analysis.

Genomic DNA Extraction

Genomic DNA was extracted following the protocol of Doyle and Doyle (1987). The DNA quality was confirmed by agarose gel electrophoresis (0.8%) and quantified with the aid of Nano-Drop spectrophotometer (ND-1000, version 3.1.1, USA). The DNA samples were diluted to 20 ng μl^{-1} for polymerase chain reaction (PCR) amplification.

Table 1. List of Sugarcane Cultivars – Flowering and Non Flowering

Sample Code	Name of the Cultivars
Flowering	
S1	Co 2012-109
S2	Co06027
S3	Co 11024
S4	Co 10023
S5	Co 10024
S6	Co 2001-15
S7	CoC 671
S8	Co SNK 0632
S9	Com 0265
S10	Co SNK 09268
S11	Co 13006
S12	Co 10027
Non-flowering	
S13	Co 2012-23
S14	Co 2012-24
S15	Co 11023
S16	Co SNK 7658
S17	Co SNK 07337
S18	Co SNK 07680
S19	Co SNK 09227
S20	Co SNK 09293
S21	Co SNK 09232
S22	CO SNK 0811324
S23	Co SNK 83495
S24	Co 86032

Simple Sequence Repeat Marker Analysis

Ten microsatellite markers were selected based on the previous studies of Singh et al. (2010, 2017). PCR reactions were carried out in a 25µl reaction volume comprising genomic DNA (20 ng), forward primer (0.5 µl), reverse primer (0.5 µl), dNTP (1.5 µl), *Taq* buffer (1×), *Taq* DNA polymerase (1.5U) and finally making the volume to 25 µl using nanopure water. The PCR amplification was performed using Mastercycler gradient (Eppendorf) with the following conditions; the initial denaturation at 95°C for 5 min, following 40 cycles of denaturation at 94°C for 1 min; annealing condition was set depending on the standardized annealing temperature (Table 2) of each SSR primer with common time duration of 1 min extension at 72°C for 2 min and ended with final extension step for 10 min. The PCR reactions were repeated thrice for each primer for better reproducibility. Only highly reproducible and polymorphic primers were chosen for the data analysis.

Scoring of DNA Bands

Fragments that were clearly readable were considered for data analysis. Each amplified product was considered to be a unit character and the populations were scored for their presence (1) or absence (0) of a band on the gel (Botstein et al., 1980; Anderson et al., 1993) and the cluster analysis was performed. Dendrogram was plotted with the aid of DendroUPGMA online server and similarity matrix was calculated using Jaccard's coefficient.

RESULTS AND DISCUSSIONS

The present investigation was undertaken to evaluate genetic relatedness within 24 cultivars of *Saccharum* sp. These 24 accessions were maintained by S. Nijalingappa sugar institute, Belgaum, Karnataka, India. Among 24 cultivars, 12 were flowering (S1-S12) and 12 were non flowering (S13-S24) (Table 1).

Sugarcane is a heterozygous and considered to be genetically complex aneu-polyploid species. Studies revealed that sugarcane readily undergoes inbreeding depression upon selfing (Stevenson, 1965). Hence, it is highly essential to understand the genetic diversity of *Saccharum* sp. in order to work on genetic improvement of the germplasm for commercial purpose. Thus, in this study, we have made an effort to decipher the genetic diversity of this species using microsatellite markers especially SSR. A total of 10 SSR primers were used which are specific for *Saccharum* sp. Among ten primer sets, eight primers showed reproducible bands (Figure 1). The detailed information about the primers used in this study is tabulated in Table 2.

We noted that primer SCM-32 amplified highest polymorphic bands as compared with other selected primers. The above mentioned primers amplified a total of 56 alleles out of 15 loci with a range of 1 to 6 alleles and an average of 5.3 alleles per locus; proving the efficiency of these SSR markers as a potential tool for detecting genetic variations in cultivars of studied *Saccharum* sp.

Based on generated SSR profiles, cluster analysis was performed using Jaccard similarity index and unweighted pair group method with arithmetic mean (UPGMA) to plot a dendrogram representing genetic diversity among 24 accessions (Figure 2). The genetic similarity indices among cultivars ranged from 0.083 to 1 (Table 3). The cultivar S17 showed least genetic similarity; the cultivar S18 with similarity index 0.083 and S19 was 100% similar to S21, S22 and S23 with the similarity index of 1. Further, cluster analysis categorized the cultivars into five clusters (Figure 2).

Table 2. List of SSR Primers with their Annealing Temperatures used in the present study

Oligo (Name)	Primer (Sequence)	AT
SCM4-F	CATTGTTCTGTGCCTGCT(18)	52
SCM4-R	CCGTTTCCCTTCCTTCCC(18)	
SCM21-F	CCCTCCCATAACACACAC(18)	55
SCM21-R	TTGACAGCCCAAAGAGTT(18)	
SCM27-F	TTCTCTGACTTCCAATCCAA(20)	56
SCM27-R	ATCAAGCACGCCCGCCTC(18)	
SCM32-F	GATGAAGCCGACACCGAC(18)	55
SCM32-R	AGTTGCCTGTTCCCATTT(18)	
SOMS58-F	CCGCTTTCAACCTCTACAC(19)	52
SOMS58-R	GGCTTGGTGATTCTTCTCT(19)	
SOMS118-F	GAGGAAGCCAAGAAGGTG(18)	57
SOMS118-R	TAGAGCGAGGAGCGAAGG(18)	
SOMS135-F	TCTTCAACTTCCTCTGCCT(19)	55
SOMS135-R	GTTCCTGACTGTTCCCTTG(19)	
SOMS148-F	GATGACTCCTTGTGGTGG(18)	52
SOMS148-R	CTTGACGACCCTGCTGCT(18)	
UGSM60-F	CGACTCCACACTCCACTC(18)	55
UGSM60-R	CCGAACACCACCTTCTTG(18)	
UGSM542-F	ACCTCCACCTCCACCTCAGTTC(22)	55
UGSM542-R	CGTTCAGCTTCAGGGTGTCGAT(22)	

AT: Annealing Temperature (ºC)

Cluster 1 consists of one cultivar, S9. Cluster 2 consists of two groups; first group consists of S7, S12, S18, S20 cultivars. Second group consists of S4, S6, S8, S11 and S15. S17 with lowest genetic similarity is situated in the cluster 3. Cluster 4 consists of three groups. Cultivars S13, S16, S24 are situated in the first group, cultivars S19, S21, S22, S23 with 100% similarity are situated in the second group and cultivars S2, S3 are situated in the third group. Cluster 5 consists of S1, S5, S10 and S14. All clusters showed the mixture of flowering and non-flowering cultivars which suggests the application of large number of SSR markers for precise differentiation of cultivars. However, the present SSR markers used in the study is revealing the wide genetic diversity in studied cultivars suggesting that SSR markers are important tool to assess the genetic diversity and relatedness in *Saccharum* sp. commercial cultivars (Singh et al., 2010).

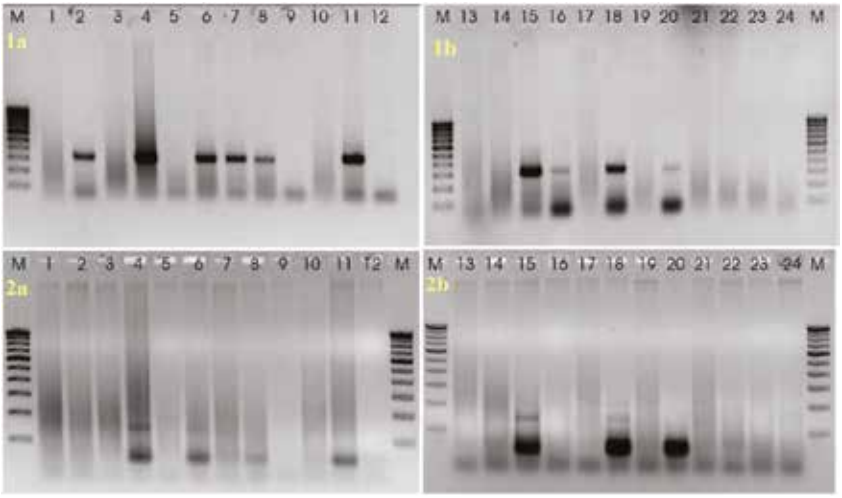

Figure 1. SSR Marker Analyses of Sugarcane Cultivars (1a: SOMS118-F, 1b: SOMS118-R, 2a: UGSM542-F, 2b: UGSM542-R)

Table 3. Similarity Index Computed with Jaccard Coefficient

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
S1	1	0.625	0.625	0.263	0.667	0.250	0.231	0.385	0.125	0.700	0.211	0.143
	0.429	0.545	0.429	0.250	0.222	0.231	0.571	0.154	0.571	0.571	0.571	0.429
S2		1	0.714	0.211	0.556	0.267	0.364	0.417	0.143	0.455	0.222	0.250
	0.286	0.333	0.462	0.500	0.250	0.250	0.667	0.273	0.667	0.667	0.667	0.286
S3			1	0.211	0.556	0.267	0.364	0.417	0.143	0.600	0.222	0.250
	0.286	0.333	0.462	0.286	0.250	0.250	0.667	0.273	0.667	0.667	0.667	0.286
S4				1	0.316	0.765	0.444	0.556	0.118	0.421	0.941	0.529
	0.176	0.421	0.579	0.111	0.167	0.529	0.105	0.471	0.105	0.105	0.105	0.111
S5					1	0.400	0.417	0.462	0.250	0.636	0.263	0.308
	0.222	0.500	0.500	0.222	0.200	0.417	0.500	0.333	0.500	0.500	0.500	0.222
S6						1	0.571	0.600	0.154	0.438	0.706	0.692
	0.143	0.438	0.625	0.143	0.214	0.692	0.133	0.615	0.133	0.133	0.133	0.067
S7							1	0.667	0.222	0.357	0.471	0.800
	0.091	0.357	0.467	0.333	0.182	0.636	0.182	0.700	0.182	0.182	0.182	0.200
S8								1	0.182	0.615	0.588	0.538
	0.273	0.400	0.600	0.273	0.071	0.538	0.250	0.583	0.250	0.250	0.250	0.273
S9									1	0.200	0.125	0.222
	0.250	0.200	0.154	0.250	0.200	0.222	0.200	0.250	0.200	0.200	0.200	0.250
S10										1	0.368	0.267
	0.300	0.538	0.643	0.182	0.167	0.357	0.400	0.286	0.400	0.400	0.400	0.300
S11											1	0.562
	0.188	0.368	0.526	0.118	0.176	0.471	0.111	0.500	0.111	0.111	0.111	0.110
S12												1
	0.091	0.267	0.467	0.200	0.182	0.800	0.083	0.889	0.083	0.083	0.083	0.091
S13	1	0.300	0.143	0.200	0.167	0.091	0.400	0.100	0.400	0.400	0.400	0.500
S14		1	0.438	0.182	0.400	0.267	0.400	0.200	0.400	0.400	0.400	0.300
S15			1	0.231	0.133	0.571	0.308	0.500	0.308	0.308	0.308	0.143
S16				1	0.167	0.200	0.400	0.222	0.400	0.400	0.400	0.500
S17					1	0.083	0.333	0.091	0.333	0.333	0.333	0.167
S18						1	0.083	0.889	0.083	0.083	0.083	0.091
S19							1	0.091	1.000	1.000	1.000	0.400
S20								1	0.091	0.091	0.091	0.100
S21									1	1.000	1.000	0.400
S22										1	1.000	0.400
S23											1	0.400
S24												1

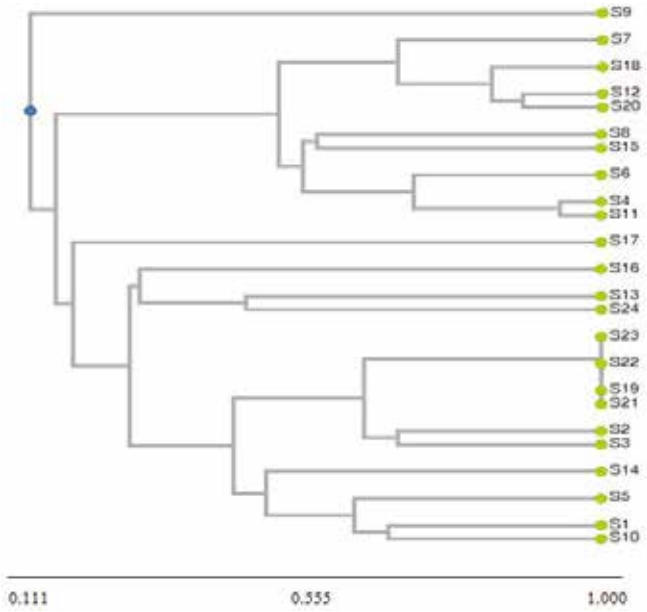

Figure 2. Phylogenetic Tree Analyses Based on Jaccard Coefficient

CONCLUSIONS

In sugarcane, cultivars morphological tools have limited implication in progeny identification as all sugarcane cross often consist of hybrids, selfs, and off-types. Hence molecular markers can play vital role in sugarcane breeding program. Among the various molecular markers, SSR markers may efficiently be used to evaluate the genetic polymorphism to establish the breeding and conservation strategies for cultivated plants like *Saccharum* sp.

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RAPID DNA ISOLATION AND ISSR-PCR OPTIMIZATION FOR FIBROUS LEAF TISSUES OF WILD PALMS OF SOUTHERN PENINSULAR INDIA

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Abstract

Isolation of pure DNA is a significant component for PCR amplification and DNA fingerprinting analysis of plant species. Leaves of wild palms are generally hard, enormously fibrous, and are difficult to grind. The 25 species belong to 9 genera such as Areca (one species), Arenga (one species), Bentickia (one species), Borassus (one species), Calamus (fifteen species), Caryota (one species), Corypha (one species), Phoenix (three species) and Pinanga (one species) of the family Arecaceae and were collected from various parts of Southern India. An experiment was done to isolate the high molecular weight DNA from fibrous young leaves of twenty five wild palm species followed by several modifications in the novel CTAB DNA isolation method. DNA was isolated with slight modification in the CTAB method with liquid nitrogen and the quantification of obtained DNA was measured using a spectrophotometer and 0.8% agarose gel electrophoresis. DNA was further diluted with $T_{10}E_1$ buffer and optimized for ISSR-PCR amplification. Hence, the described protocol has proven to be advantageous due to its simple, efficient, affordable reagents resulting in a high molecular weight DNA of good quality from leaf fibrous tissues.

Key words: *Arecaceae, CTAB, genomic DNA isolation, ISSR, Western Ghats.*

INTRODUCTION

Palms are woody monocotyledons belongs to family Arecaceae or Palmae. They are one of the prominent biotic components of the forest ecosystem. Oftenly they fascinate with graceful architecture; they usually dominate the landscape of tropical habitats by providing wide range of utility for human life. Palms have been extensively exploited by the local communities for food, fodder, handicrafts and construction purpose. Food and allied products have gained importance due to its nutritional quality and also the fibrous tissue present in palm species is very flexible, strong and has a significant role in furniture industry. Thus, a large number of inhabitants depend upon the palms and palm byproducts for their livelihood, which ultimately leads to the consumption of land areas for supplying physical space and expanding population and industry. Globally, there are 211 genera consists of 3000 palm species, whereas in India there are about 22 genera and 105 species present in three major geographical regions such as Peninsular India, Northeastern India and Andaman and Nicobar Islands (Takhtajan, 1987). Palms are less distributed in few regions of India, especially in Gangetic plains and in the lower hill valleys of North India.

Various molecular biology tools were utilized for isolation of genomic DNA to attain appropriate purity. Several conventional methods have been established for isolation of pure and integral DNA from plant tissues (Saghai Maroof et al., 1984; Doyle & Doyle, 1990; Scott & Playford, 1996; Sharma et al., 2000; Pirttilä et al., 2001; Drábková et al., 2002; Shepherd et al., 2002; Mogg & Bond, 2003; Haymes, 1996). Thus, plant species comprises of same or related genera may exhibit tremendous variability in the complex pathways of expendable functions. Due to variability among species or genera the same DNA extraction procedure cannot be utilized for all the plant species (Porebski et al., 1997; Ribeiro & Lovato, 2007; Fatemeh et al., 2018). Whereas, cetyltrimethylammonium bromide

(CTAB) process and its modifications have proved as one of the prominent methods for good quality DNA for amplification based on polymerase chain reaction (PCR) downstream applications. Numerous commercial extraction kits are available such as DNeasy Plant Mini Kits but due to high cost price, sensitivity for certain temperature, the constituent of the buffers is unknown to the user, and the lysis step is not always sufficient for some types of plant material (Ahmed et al., 2009). So using such commercially available kits hence proved to be laborious, time consuming and expensive. Certain limitations necessitate the advancement of universally accepted protocol for isolating DNA from diverse plant species. Hence, in the present study genomic DNA isolation protocol was optimized for 25 species that belong to 9 genera such as *Areca* (one species), *Arenga* (one species), *Bentickia* (one species), *Borassus* (one species), *Calamus* (fifteen species), *Caryota* (one species), *Corypha* (one species), *Phoenix* (three species) and *Pinanga* (one species) of the family Arecaceae. This method does not require any hazardous chemicals. Based on the quantity and quality of the DNA obtained by this modified protocol is well enough to perform thousands of PCR-based reactions and also used for DNA manipulation techniques, such as restriction digestion, AFLP, Southern blotting and cloning. Due to its low cost price, consumption of short time period makes this method one of the phenomenal technique for isolating genomic DNA in wild palms. The main objective to develop this protocol was to make this technique readily available for isolating highly pure genomic DNA from fibrous leaves of wild palm and to optimize the DNA concentration. The isolated high quality genomic DNA is amenable to ISSR (Inter-Simple Sequence Repeats).

MATERIALS AND METHODS

Collection of Samples

Young leaves of twenty-five wild palm belong to 9 genera which are basically an endemic, endangered and rare species which possess limited distribution range and only available in selected regions with the elevation of 400 m to 2600 m were collected from KFRI (Palmetum), Peechi, Kerala and some of them were also collected from wild habitats of Western Ghats region of Chikmagalur, Shivamogga, Dakshina Kannada and Uttara Kannada districts of Karnataka. Soon after collection, leaf samples were transferred to the laboratory and washed in 70% alcohol, blotted with tissue paper, quickly frozen by dipping in liquid nitrogen and kept at -80°C until further use.

Reagents, Chemicals and Laboratory Materials

CTAB extraction buffer consisted of 2% CTAB, 5 M NaCl, 0.5 M EDTA and 1 M Tris-HCl. Stock solutions for the different CTAB buffer components were prepared to homogenize the following protocol. Other reagents and materials such as 3% β-mercaptoethanol, 15% PVP-4.0, propanol, ethanol (absolute), Tris-EDTA ($T_{10}E_1$) buffer solutions were prepared. Laboratory pestle and mortar, 1.5 and 2 mL microcentrifuge tubes, micropipettes, microtips, pair of scissors, and a 40-well holding racks were used for the experiments. Centrifugation was done by Centrifuge 5415-R at maximum speed of 14000 rpm.

DNA Extraction and Quantification

CTAB method was followed for the extraction and isolation of DNA as prescribed by Doyle and Doyle (1987). Several modifications were made to the original protocol in order to fit with the current experimental conditions. The CTAB buffer was prepared using 2% cetyltrimethylammonium bromide, 1 M Tris-HCl pH 8.0, 0.5 M EDTA pH 8.0, 5 M NaCl, 3% β-mercaptoethanol and 2% PVP. About 0.5-1 g leaf sample was ground into fine powder using liquid nitrogen in a mortar and pestle and 4 ml of preheated 2× CTAB lysis buffer at 65 to make the paste. The mixture is incubated at 65°C for 90 minutes for lyses and lysate was extracted with phenol: chloroform: isoamyl alcohol (25: 24: 1) upon cooling to room temperature. The DNA is precipitated by adding an equal quantity of prechilled isopropanol. The DNA pellet is washed twice in 70% alcohol, air dried, dissolved in $T_{10}E_1$

and stored at -20°C until further use. Gel electrophoresis of genomic DNA was done with 0.8% agarose gel in 1× TAE buffer (Mini Sub System, BioRad, India). The image of the gel obtained is shown in Figure 1.

ISSR-PCR based Amplification using Extracted DNA Samples

An experiment was conducted to optimize DNA concentration by Polymerase Chain Reactions (PCR). A total volume of 10 µl PCR reaction mixture consisted of Taq buffer, $MgCl_2$, primers (UBC 834 AGAGAGAGAGAGAGAGYT), dNTPs, Milli Q water and Taq polymerase (3 U/µl), along with DNA sample. PCR amplification was carried out in a 10 µl reaction volume containing 1 µl genomic DNA, Taq Buffer 1.5 µl, $MgCl_2$ 1 µl, UBC 834 1 µl, dNTPs 1 µl, Milli Q water 3.75 µl and Taq Polymerase 0.25 µl. Amplification was carried out in initial denaturation step at 95°C for 3 min, followed by 32 cycles denaturation 95°C for 30 sec. UBC 834 Primer annealing temperature was 59.1°C, for 45 sec, 72°C for 2 min (primer extension) and a final extension at 72°C for 10 min. Electrophoresis was carried out to perform ISSR-PCR products on 1.8% agarose gel with 1× TAE and a standard 1 kb ladder.

Agarose Gel Electrophoresis

The PCR products were visualized on a 1.8% agarose gel with a standard 50 bp ladder. Agarose gels were prepared using 1× TAE buffer. A concentration of 0.6 µl of ethidium bromide was added to the gel for visualization of the bands. The PCR product mixture was loaded along with loading dye 0.0042 g bromophenol blue in 0.0607 g of sucrose solution. The gel profile of each ISSR primer amplicons was visualized in a UV Trans-illuminator Gel Documentation System (Vilber Lourmat Infinity-1000/26 M).

RESULTS AND DISCUSSIONS

High yield pure DNA was obtained for twenty-five different species of Arecaceae members followed by few modifications in the novel CTAB DNA isolation protocol (Doyle and Doyle, 1987) (Table 1). Fresh leaf samples are recommended for genomic DNA isolation. If any tissues are immersed in liquid nitrogen it will be brittle hard to ease crushing into powder, the main purpose is to maintain low temperature of plant material. This step can be skipped for spongy tissues and easy to grind material like flower petals. Cellular DNA extraction from hard, fibrous leaves of palm is very complex when compared to flower petals and soft leaves. Most of the developing countries still facing problem of unavailability of liquid nitrogen as its storage and maintenance also quite difficult. Cetyltrimethylammonium bromide (CTAB) is one of the phenomenal methods for DNA extraction from a variety of plant materials (Sambrok et al., 1989). Generally, there will be certain contaminants associated with plant DNA interfere PCR reactions such as polyphenolic compounds and polysaccharides (Krishna et al., 2012).

Table 1. Qualitative Analysis of Genomic DNA Isolated from Wild Palms of Southern Peninsular India

Palm Species	OD Ratio	DNA ng/µL
Areca triandra Roxb.	1.85	763.7
Arenga wightii Griff.	1.85	470.6
Bentinckia condapanna Berry ex Roxb.	1.75	297.6
Borassus flabellifer Linn.	1.96	797.7
Calamus brandisii Becc.	1.98	532.8
Calamus delessertianus Becc.	1.97	381.1
Calamus dransfieldii Renuka	1.99	225.2
Calamus hookerianus Becc.	1.99	512.4
Calamus karnatakensis Renuka & Lakshmana	1.78	209.3
Calamus lacciferus Lakshmana & Renuka	2.02	437.5
Calamus lakshmanae Renuka	1.89	398.0
Calamus metzianus Schltdl.	1.89	215.9
Calamus nagbettai R.R. Fernald & Dey	1.98	195.8
Calamus prasinus Lakshmana & Renuka	1.97	235.7
Calamus stoloniferus Renuka	1.91	352.3
Calamus thwaitesii Becc.	1.83	228.8
Calamus travancoricus Bedd.	1.88	202.6
Calamus vattayila Renuka	1.78	672.4
Calamus viminalis Willd.	1.97	816.3
Caryota urens L.	2.01	254.8
Corypha umbraculifera L.	1.69	748.3
Phoenix loureiroi Kunth	1.88	280.4
Phoenix pusilla Gaertn.	1.78	249.8
Phoenix sylvestris (L.) Roxb.	1.96	286.5
Pinanga dicksonii (Roxb.) Blume.	1.81	507.1

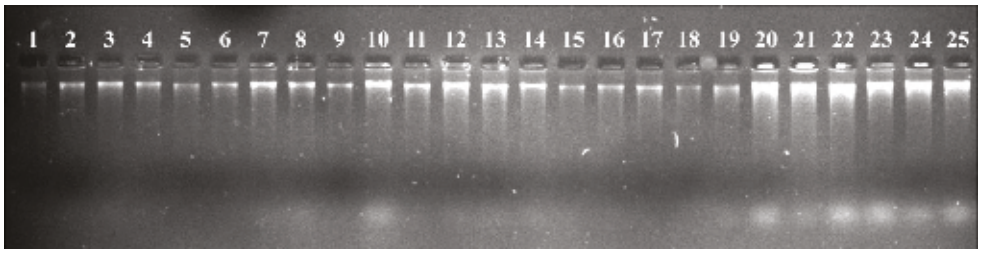

Figure 1. Genomic DNA Isolated from Twenty Five Wild Palms: 1 – *Areca triandra*; 2 – *Arenga wightii*; 3 – *Bentinckia condapanna*; 4 – *Borassus flabellifer*; 5 – *Calamus brandisii*; 6 – *C. delessertianus*; 7 – *C. dransfieldii*; 8 – *C. hookerianus*; 9 – *C. karnatakensis*; 10 – *C. lacciferus*; 11 – *C. lakshmanae*; 12 – *C. metzianus*; 13 – *C. nagbettai*; 14 – *C. prasinus*; 15 – *C. stoloniferus*; 16 – *C. thwaitesii*; 17 – *C. travancoricus*; 18 – *C. vattayila*; 19 – *C. viminalis*; 20 – *Caryota urens*; 21 – *Corypha umbraculifera*; 22 – *Phoenix loureiroi*; 23 – *P. pusilla*; 24 – *P. sylvestris*; 25 – *Pinanga dicksonii*

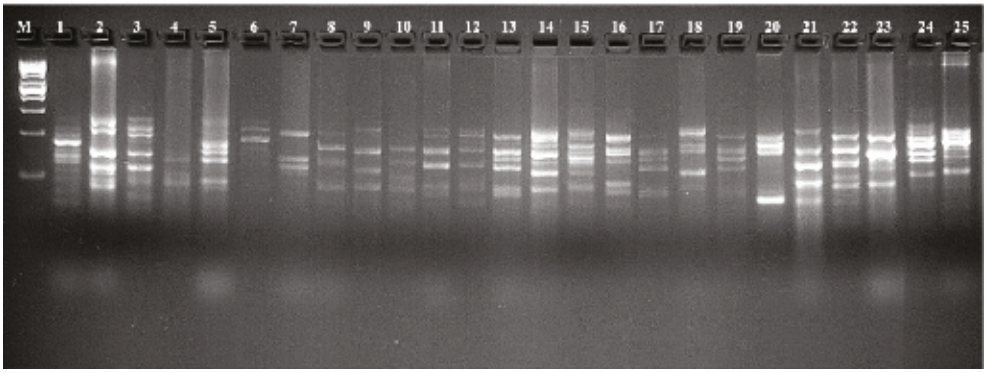

Figure 2. Optimization of DNA Concentration for ISSR-PCR: M-1kb DNA Ladder: 1 – *Areca triandra*; 2 – *Arenga wightii*; 3 – *Bentinckia condapanna*; 4 – *Borassus flabellifer*; 5 – *Calamus brandisii*; 6 – *C. delessertianus*; 7 – *C. dransfieldii*; 8 – *C. hookerianus*; 9 – *C. karnatakensis*; 10 – *C. lacciferus*; 11 – *C. lakshmanae*; 12 – *C. metzianus*; 13 – *C. nagbettai*; 14 – *C. prasinus*; 15 – *C. stoloniferus*; 16 – *C. thwaitesii*; 17 – *C. travancoricus*; 18 – *C. vattayila*; 19 – *C. viminalis*; 20 – *Caryota urens*; 21 – *Corypha umbraculifera*; 22 – *Phoenix loureiroi*; 23 – *P. pusilla*; 24 – *P. sylvestris*; 25 – *Pinanga dicksonii*

Polyphenols are potent oxidizing agent present in various plant species which are capable of decrease in the yield and purity of the DNA (Katterman & Shattuck, 1983; Peterson & Boehm, 1997; Porebski et al., 1997). Avoidance of contamination is made by adding PVP along with CTAB which promotes to bind with polyphenolic compounds by forming a compound with hydrogen bonds (Maliyakal, 1992).

Secondary metabolites such as phenolics, terpenes and alkaloids are very difficult to separate from DNA (Ziegenhagen & Scholz, 1998). For removal of polysaccharides and to avoid damage in leaf tissue, NaCl is used in DNA extraction buffer (Fang et al., 1992). Phenol: chloroform: isoamyl alcohol is used for exclusion of chlorophyll and various coloring agents such as pigments and dyes. To remove detergents and proteins precipitation steps were increased by increasing the speed and time of centrifugation. The modification necessitates amplifying high quality and quantity of genomic DNA. The purity and quantity may differ among applications (Hussein et al., 2005; Zidani et al., 2005).

The minimum OD 1.69 and maximum of 2.02 was observed (Table 1). High quality DNA was obtained from all the wild palm leaf samples and amount of DNA isolated between 195.8 µg/ml and maximum 816.3 µg/ml. Reading was taken using spectrophotometer. Both the nucleic acids (DNA and RNA) absorb at 260 nm, so evaluation of concentration of DNA

sample is done with same spectrophotometric conditions.
A protein also absorbs light at this wavelength so it is possible to obtain contamination of protein in high concentration which gives a false result in sample. Though, protein, also absorb light at 280 nm by evaluating both A260 and A280 it is easy to measure the ratio of nucleic acid to protein in the solution and thus estimate the accuracy of DNA concentration. The absorbance ratio obtained was (A260/A280) 1.8-2.0 is considered acceptable (Sambrook & Russell, 2001; Iruela et al., 2002; Wang et al., 2011; Wang et al., 2012). Lower values comprise of high level of protein contamination and estimation of the DNA concentration will not be accurate.

It is necessary to obtain high quality DNA that is proportionately free from the various contaminants found in plant cells (Shiv et al., 2017). Naturally large amount of proteins are found in most of the plant species and several other components are rectified which can bind firmly to nucleic acids during isolation of DNA and can interfere in DNA amplification (Angeles et al., 2005; Ribeiro & Lovato, 2007). Storing of isolated DNA is a significant factor, generally at -20°C gave positive percentage. This is an expected result because room temperature is certainly not a suitable condition for storing isolated DNA (Supriya et al., 2019).
The DNA obtained was used for PCR analysis and possess high intensity amplification. PCR amplification also showed that the DNA was of high quality, free from several interfering compounds and that it would be capable for various other DNA analyses such as ISSR (Lavanya et al., 2008; Sunil Kumar et al., 2012; Shafiei-Astani et al., 2015). The present study was carried out to optimize the concentration of total genomic DNA for the PCR using ISSR primers. The amplification of DNA from PCR analysis with ISSR primer was clear as shown in Figure 2.
The absence of RNA, polysaccharides and the amplification of molecular bands are evident of a good DNA quality with modified Doyle and Doyle protocol. The satisfactory quality and DNA yield was obtained from this protocol and hence considered as a simple, cost effective and time saving protocol can further be used for extraction of DNA from large populations of wild palm.

CONCLUSIONS

Here we have illustrated a very efficient, simple, cost-effective and less time consumable modified CTAB DNA extraction method that provides high-quality DNA from wild palms containing an elevated concentration of polyphenolic compounds and polysaccharides. For removal of polysaccharides and to avoid damage in leaf tissue, 5 M NaCl is used in DNA extraction buffer. This method eliminates the need to use environmentally hazardous chemicals to obtain high-quality genomic DNA. The resulting optimized CTAB protocol facilitates the isolation of high quality genomic DNA amenable to ISSR and various other processes such as enzyme digestion and cloning techniques. A fibrous leaf of palm is very complex when compared to flower petals and soft leaves, this method proved to be phenomenal especially the plant with high fibrous tissue. The CTAB percentage was increased though there will be certain contaminants associated with plant DNA interfere PCR reactions such as polyphenolic compounds and polysaccharides. Prevention of contamination is made by adding PVP along with CTAB which promotes to bind with polyphenolic compounds by forming a compound with hydrogen bonds. Therefore this method can be recommended for low-technology laboratories for high-throughput sample preparation suitable for various molecular analytical techniques.

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MOLECULAR DIVERSITY IN WILD POPULATIONS OF *Pinanga dicksonii* (Roxb.) Blume *(Arecaceae)* FROM WESTERN GHATS OF KARNATAKA USING MICROSATELLITE MARKERS

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Abstract

A precise understanding of genetic diversity and relatedness of Pinanga dicksonii (Roxb.) is an important component in its genetic improvement and germplasm conservation. P. dicksonii is an understory endemic palm of Western Ghats of Karnataka. The genetic diversity analysis among nine genotypes of P. dicksonii has been carried out using SSR markers. Among the 10 tested SSR markers, 9 successfully revealed polymorphism, SSRs demonstrating 123 alleles in total, with a range of 20 to 22 alleles at each primer. Allele frequency at each locus ranged from 22.22% to 100% with a mean of 71.92%. The PIC of each primer varied from 0.25 to 0.87 with an average of 0.38. The UPGMA-based clustering analysis performed by NTSYS pc program (version 2.0) revealed that among the 9 studied genotypes, there was a high level of polymorphism among some genotypes, as well as genetic similarities, with index values ranging from 0.473 to 0.928.

Key words*: genetic diversity, Pinanga dicksonii, SSR markers, UPGMA, dendrogram.*

INTRODUCTION

Palms are one of the significant economically essential classes of tropical and subtropical plants which play a vital role in food and raw material. Although they are under-explored species, they certainly amplify the chances of survival for people in developing countries. Throughout the world, they are represented by 212 genera encompassing 3000 species. Whereas, India it is very well represented by 22 genera with 105 species. Small quantities of palm species occur elsewhere in India, predominantly in Gangetic plains and in the lower hill valleys of Northern India (Bhat, 2011; Renuka & Sreekumar, 2012).

P. dicksonii (Roxb.) Blume is an endemic palm of the Western Ghats of Karnataka and Kerala, commonly recognized as Ivory crown shaft palm (Bhat, 2011). It is commonly grown across the moist area, as an understory-solitary palm. Usually, stem emerges beneath stolon. It is found as colonizer under favorable conditions in the evergreen forests of Western Ghats. The plant grows up to 5-7 meters height. It is widely distributed in Puttur Ghat, Kerekatte, Gerusoppa, Kuduremukha and Agumbe forests at an elevation of 250-1000 m. Some local tribes consume the fruits of this plant as a substitute for betel nuts. The stem is used to make walking sticks and as building material for huts (Renuka, 1998).

Molecular markers are relevant for genetic diversity assessments being able to define population relationships. They are not harmful to the environment and can be detected in all stages of plant growth and development (Mondini et al., 2002). But isozymes markers are not adequately variable due to their low polymorphism (Ghesquiere, 1985; Purba et al., 2000). In genetic diversity analysis, Random Amplified Polymorphism DNA (RAPD) has certain limitations, like poor reproducibility of amplification (Rafalski, 1997). Restriction fragment length polymorphic DNA (RFLP) is another vigorous molecular marker which requires a relatively large amount of purified DNA with high molecular weight, which is a

time-consuming and overlong protocol (Maizura et al., 2006). Ultimately, Amplified fragment length polymorphism (AFLP) is scored either as a presence/absence polymorphism. Simple sequence repeats (SSRs) are most commonly used molecular markers in studying interspecies and intraspecies genetic diversity of the microsatellite loci. Microsatellites can be implemented as mono locus co-dominant markers by amplifying single microsatellite loci into molecular markers by designing primers with the aid of specific sequence flanking (Kumar et al., 2009). Microsatellites offer a high level of polymorphism (Ellegren, 2004; Moges et al., 2016). As a result, they act as unique markers for studying population genetics, and gene mapping (Hearne et al., 1992; Jarne & Lagoda, 1996). Among all types of PCR based molecular markers, the SSRs are more informative, efficient and also cost-effective. The factor of abundance, co-dominance, highly reproducible and interspersed nature of microsatellite loci throughout the genome make the SSR marker the most potential and highly applicable in genetic diversity studies (Matin et al., 2012).

Several previous reports suggest that SSR markers are suitable for studying genetic variation and relationship among different genotypes, thus finds its application as a potent molecular marker in studying genetic variation (Eujayl et al., 2001; Russell et al., 2004). SSRs markers are widely used for studying the genetic diversities of different plant species, viz., maize (Garcia et al., 2004; Afaf et al., 2009), *Medicago sativa* L. (Wang et al., 2014), *Coffea canephora* Pierre ex A. Froehner (Hendre et al., 2014); raspberry and blackberry (Eric et al., 2005), *Myrica rubra* (Yun et al., 2012), cucumber (Hu et al., 2011), *Cynodon transvaalensis* (Tan et al., 2014), garlic (Meryem et al., 2015) and rice (Matin et al., 2012; Shahriar et al., 2014; Nivedita et al., 2016). Many investigators have also demonstrated the use of SSR markers (Powell et al., 1996; Afaf et al., 2009).

In the present study, we used SSR markers to analyze the intraspecies genetic diversity and relationship among the 9 wild genotypes of *P. dicksonii* from different locations of Karnataka region of Western Ghats.

MATERIALS AND METHODS

Plant Material

Nine genotypes of *P. dicksonii* were considered for this study. This plant is an endemic species for Karnataka and Kerala. It has a limited distribution range and is available only in the specified zones (Figure 1). The leaf samples were collected from forest areas of Uttara-Kannada, Shivamogga, Dakshina Kannada, Chikmagalur and Udupi districts of Karnataka. Collected plants are listed in Table 1. Young leaves were collected from the wild habitat and stored at -80°C prior to DNA extraction.

DNA Isolation and Purification

The DNA extraction followed the CTAB method described by Doyle and Doyle (1987) with certain modifications. CTAB lysis buffer (2×) was prepared by mixing 2% cetyltrimethylammonium bromide, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 2% β-Mercaptoethanol and 2% PVP-40. An amount of 7.5 ml 2× CTAB lysis buffer was preheated at 65ºC and mixed with 2 g leaf powder, prepared by grinding the leaf samples in liquid nitrogen. The mixture was then incubated for lysis at 65ºC for 60 min. The lysate was extracted with Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1) and DNA were precipitated by adding an equal quantity of pre-chilled isopropanol. The DNA pellet was washed in 70% alcohol, air dried and dissolved in $T_{10}E_{1.}$ The DNA samples were stored at -20ºC until further use.

DNA Quantification

The genomic DNA was profiled using 0.8% gel electrophoresis in 1× TAE buffer (Mini Sub System, BioRad, India). Gel imaging was performed using UV trans-illuminator (VilberLourmat Infinity-1000/26 M). Genomic DNA was quantified using NanoDrop Spectrophotometer (Quawell 3000-UV Spectrophotometer). The genomic DNA samples having absorbance ratios of 260/280 between 1.8-1.9 were used for SSR amplification.

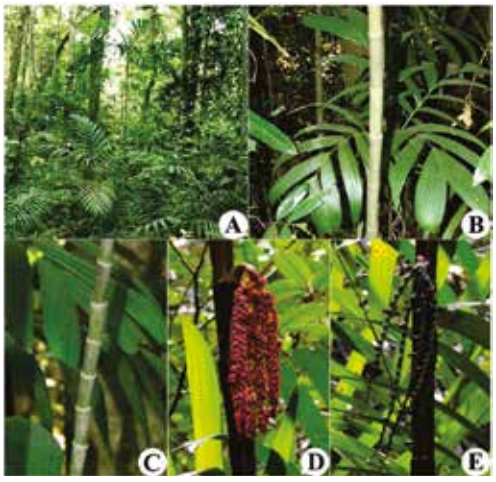

Figure 1. *P. dicksonii.* A – habit; B – leaves; C – trunk; D – inflorescence; E – fruit

SSR Primers

A total of 10 palm specific microsatellite markers (Table 3) were used to estimate the genetic diversity among 9 genotypes of *P. dicksonii* considered in this study. SSR primers were purchased from Sigma Aldrich, Bangalore.

SSR-PCR Amplification

The SSR primers were chosen selectively based on those which yielded amplification of microsatellite loci in the genome of the studied plant species. PCR amplification was carried out in a 20 µl reaction volume containing Taq buffer 1×, dNTP mix 2 mM, Forward Primer 5 pM, Reverse Primer 5 pM, Taq Polymerase 1 U, genomic DNA 100 ng. Amplification was carried out using The Master Cycler Gradient thermal cycler (Eppendorf AG 22331, Hamburg, Germany) under the following conditions: initial denaturation at 95ºC for 30 sec, and 40 cycles of denaturation at 95ºC for 15 sec, specific primer annealing temperature for 15 sec, primer extension at 68ºC for 1 min and a final extension at 68ºC for 5 min.

Agarose Gel Electrophoresis

The PCR products were visualized on a 2% agarose gel prepared in 1× TAE buffer, with a standard 50 bp ladder. A concentration of 0.05 µg/ml of ethidium bromide was added to the gel for visualization of the bands. The PCR products were mixed with loading dye 0.25% bromophenol blue in 40% of sucrose solution. The gel profile of each SSR primer amplicons was visualized in a UV trans-illuminator Gel documentation system (Infinity-1000/26M, VilberLourmat, France).

Scoring of Amplified Fragments

The best gel profile showing the perfectly resolved bands of each allele was considered for further analysis. Clear PCR products were transcripted in binary code, where each amplicon was recorded as one (1) for presence, and as zero (0) for the absence of DNA band in the gel. The number of alleles per loci was documented; accordingly average allele frequency was estimated. Polymorphism information content value was calculated by following a standardized method for each primer in order to evaluate the primer efficiency (Botstein et al., 1980; Anderson et al., 1993). Cluster analysis and dendrogram were drawn using NTSYS-pc software version 2.0 by UPGMA method, and the similarity matrix was deduced using Dice coefficient (Garcia-Vallvé et al., 1999).

RESULTS AND DISCUSSIONS

In the present investigation, 9 genotypes of *P. dicksonii* were studied for their intra-species genetic diversity and clustering. The OD values ranged between 1.82 and 1.97 (Table 2). High-quality DNA was obtained from all the *Pinanga* leaf samples and high amount of DNA was isolated, between 110.0 µg/ml and 498.5 µg/ml. A total of 10 SSR markers reported in earlier studies were selected for generation of polymorphic SSR in the present study (Table 3) (Singh et al., 2008; Ngoot-Chin et al., 2010; Noorhariza et al., 2012). Among the SSR markers used, 9 primer pairs showed amplification of DNA fragments. These markers were coded as sEg00090, sEg00113, sEg00036, sMo00020, sMo00130, sEg00067, sMo00154, sMo00138 and sEg00038. Primer pair sEg00090 revealed the most relevant results among all tested primers, as this primer pair amplified the highest number of polymorphic bands (Figure 2).

Table 1. Plant sampling locations and their accession names

Collection area	Accession name	Latitude (°N)	Longitude (°L)
PutturGhat, Puttur, Dakshina Kannada District	Putghat	12° 50′	75° 31′
Bhagavathi, Kuduremukha, Chikmagalur District	Bgvti	13° 11′	75° 11′
Jamble, Kuduremukha, Chikmagalur District	Jmble	13° 13′	75° 15′
Gerusoppa, Uttara Kannada District	Grspa	14° 13′	74° 39′
Agumbe, Thirthahalli, Shivamogga District	Agmbe	13° 30′	75° 05′
SK Border, Sringeri, Chikmagalur District	Skbrdr	13° 17′	75° 08′
GanapathiKatte, Sringeri, Chikmagalur District	Gptkte	13° 25′	75° 19′
Jamble, Koppa, Chikmagalur District	Jmbko	13° 31′	75° 21′
Gulaganjimane, Sringeri, Chikmagalur District	Glgjmne	13° 20′	75° 10′

Table 2. Nano-drop values for genomic DNA quantification of *P. dicksonii*

Samples	260/280	ng/ml
Putghat	1.88	110.0
Bgvti	1.83	159.5
Jmble	1.83	120.1
Grspa	1.85	169.1
Agmbe	1.97	199.4
Skbrdr	1.82	271.9
Gptkte	1.86	183.5
Jmbko	1.92	498.5
Glgjmne	1.91	240.7

Nine tested markers efficiently produced 123 amplicons with a range of 20 to 22 alleles. The gel profile of each primer pair was analyzed and the allele frequency at each locus was estimated ranging from 22.22% to 100% with a mean of 71.92%, demonstrating the efficiency of each primer based on allele frequency. Genetic diversity or similarities among the 9 genotypes were calculated at each locus for allelic Polymorphism Information Content (PIC). The calculation for each SSR primer pair used in the study was made following the standardized method (Botstein et al., 1980; Anderson et al., 1993), by analyzing the allele frequencies of 9 primers among all 9 genotypes. According to the prescribed standards, the PIC value must be between zero (0) and one (1) depending upon primer efficiency in order to demonstrate the allelic variation. In this study, the PIC of the loci ranged from 0.25 to 0.87 with a mean of 0.38. PIC value for primer pairs sEg00090, sEg00113, sEg00036, sMo00020, sEg00038, sMo00130, sEg00067, sMo00138 and sMo00154 was 0.277778, 0.623457, 0.876543, 0.277778, 0.0, 0.255144, 0.592593, 0.0 and 0.598765, respectively, where primer sEg00036 showed highest PIC and sMo00020 showed lowest PIC value among all primers used. This indicates the efficiency of the selected markers in presenting the polymorphism among related genotypes of *P. dicksonii.* Afaf et al. (2009) have reported that SSR markers are twice more informative than AFLP and RAPD in terms of revealing alleles per locus. The alleles were scored individually based on the comparison with molecular weight ladder. The similarity matrix was derived based on Jaccard-coefficient and the dendrogram was constructed by Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The dendrogram showed two distinct groups (Figure 3) in which the genotypes Puttur Ghat (Putghat), Bhagavathi (Bgvti) and Gerusoppa (Grspa) formed a cluster A whereas genotypes Agumbe (Agmbe), Jamble (Jmble), SK Border (Skbrdr), Ganapathi Katte (Gptkte), Jamble-Koppa (Jmbko), and Gulaganjimane (Glgjmne) formed a distinctive cluster B. The cluster A was further divided into 2 subgroups, with Puttur Ghat (Putghat) and Bhagavathi (Bgvti) as one subgroup with the highest similarity index of 0.928, and Gerusoppa (Grspa) formed an individual subgroup with a similarity index

with its neighboring subgroup of 0.733. Cluster B possessed two closest subgroups, with one subgroup formed between SK Border (Skbrdr) and Ganapathi Katte (Gptkte) genotypes with a similarity index of 0.875, indicating their genetic relationship and the other subgroup formed between genetically closely related Jambli-Koppa (Jmbko) and Gulaganjimane (Glgjmne) genotypes as revealed by their similarity index of 0.882. Whereas Agumbe (Agmbe) and Jamble (Jmble) genotypes appeared to form separate individual lineages in cluster B, showing their genetic divergence from the other genotypes under study.

Table 3. List of SSR primer pairs sequences and annealing temperature used in the present study

SSR Markers	Sequence 5′→3′	Repeated motif	Amplicon (bp)	Annealing temperature (°C)
sEg00090	F- TCAGAAATGCCTACATCAAAC R- AGGGACACGAGAATACATACA	(AT)9	230-250	62.3
sEg00113	F- GTCACCGAACCCTAATAAAAT R- ATGCAGTTGAGGACAAAAAG	(CT)15	100-110	62.3
sEg00036	F- GGACCCTTTTTGTTACTGTTT R- AGCCTACCACAACTTCCTTT	(AG)9	115-125	62.3
sMo00020	F- CCTTTCTCTCCCTCTCCTTTTG R- CCTCCCTCCCTCTCACCATA	(AG)15	100-110	60.7
sEg00038	F- ATCAAGCGGCAGTTATGAGAT R- ATACATTATTCCCACCACCA	(AAT)9	140-150	60.7
sMo00130	F- TAAGCAAAAGATCAGGGCACTC R- GGCTGGTGAAAATAGGTTTACAAAG	(AAG)11	170-178	60.7
sEg00067	F- GATTAAGTCCCAACCGTCTC R- TAAGAGAGCACGCAGTTCAG	(TGTA)6	145-155	60.7
sMo00138	F- AGGGTTGTCGCTCCAATTTAT R- GGCATCTTTTTGACCTGTAGAAG	(TTTTC)6	70-78	60.7
sMo00154	F- CAAAAGGGTTGTTTGTATACGTG R- TGCATGAATATCCTCTCAAAGTTAC	(TG)7cgcgcgtgtgcgcgtg(TA)8	168-175	60.7
sEg00066	F- ACTGATGCAGGAAAGAGGAA R- GAAGTACACAAGGTAAGTTCATAG	(AT)8	-	-

F - Forward; R - Reverse

Table 4. Similarity matrix index of 9 wild genotypes of *P. dicksonii*

	Putghat	Bgvti	Jmble	Grspa	Agmbe	Skbrdr	Gptkte	Jmbko	Glgjmne
Putghat	1								
Bgvti	0.928	1							
Jmble	0.533	0.571	1						
Grspa	0.733	0.666	0.500	1					
Agmbe	0.687	0.625	0.692	0.562	1				
Skbrdr	0.611	0.555	0.600	0.500	0.750	1			
Gptkte	0.705	0.647	0.600	0.500	0.866	0.875	1		
Jmbko	0.578	0.526	0.562	0.555	0.705	0.823	0.823	1	
Glgjmne	0.578	0.611	0.562	0.473	0.705	0.823	0.823	0.882	1

The similarity index values ranged from 0.473 to 0.928. The highest similarity index value observed was 0.928 which was shown by several samples.

The *P. dicksonii* genotypes Putghat and Bgvti showed highest similarity index value of 0.928, the same was revealed in the dendrogram, indicating they are the closely related among all the genotypes, possessing the fewest divergence between each other.

On the contrary, the lowest similarity index value observed was 0.473 between Glgjmne and Grspa genotypes, indicating the highest genetic divergence between these genotypes. A high genetic similarity was also seen between Glgjmne and Jmbko (0.882).

The similarity matrix index is given in (Table 4). These values suggest that the genetically related plants subjected to this study have some variability at DNA level.

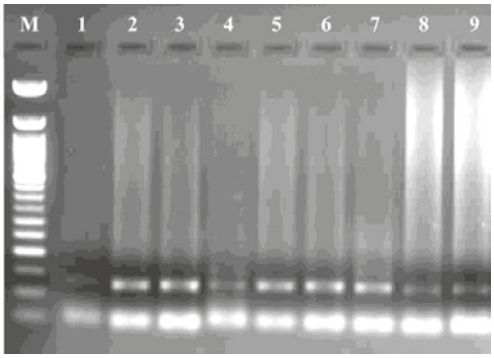

Figure 2. Gel profile showing the amplicons produced by primer pair sEg00090. 1 – Putghat; 2 – Bgvti; 3 – Jmble; 4 – Grspa; 5 – Agmbe; 6 – Skbrdr; 7 – Gptkte; 8 – Jmbko; 9 – Glgjmne

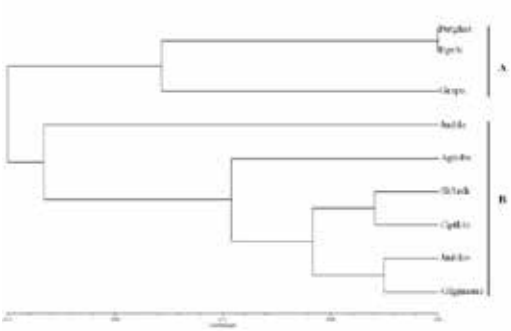

Figure 3. Dendrogram indicating phylogenetic relationships among 9 *P. dicksonii* genotypes constructed using UPGMA cluster analysis based on Jaccard similarity index obtained from 9 SSR markers

Reports indicate the immense application of SSR markers in various studies on plant interspecies or intraspecies genetic variation. Zhao et al. (2012) identified and categorized gene based SSR markers in date palm (*Phoenix dactylifera* L.). The genetic structure and diversity of *Acrocomia emensis* (Arecaceae) using SSR molecular markers showed low level of genetic variability among the populations and high, positive and significant inbreeding quality, which determines the level of homozygotes (Neiva et al., 2016). Such studies lay a strong platform for improvement of plant quality for better of human welfare and also in the conservation of respective germplasm. SSR markers are most widely and commonly used tool in studying the genetic diversity among plants (Eric et al., 2005; Afaf et al., 2009; Hu et al., 2011; Matin et al., 2012; Yun et al., 2012; Hendre et al., 2014; Shahriar et al., 2014; Tan et al., 2014; Wang et al., 2014; Meryem et al., 2015; Nivedita et al., 2016). Various studies have demonstrated the importance of SSR markers (Powell et al., 1996; Afaf et al., 2009). The molecular evaluation of genetic diversity within the 9 studied genotypes of *P. dicksonii* showed high genetic similarity in plants from nearby locations, while plants from different geographic location exhibit a low level of genetic similarity.

CONCLUSIONS

This study throws the light on genetic diversity of the endemic plant species, *P. dicksonii* found to grow in the wild. This species is not very well known and needs to be studied and conserved to maintain its' germplasm. The present study provides evidence on the genetic variation observed in the collected plant samples from the wild condition of Karnataka and Kerala. The present study also reveals the ability of SSR markers to generate polymorphic bands within *P. dicksonii* palm species. Among the 10 SSR markers used in this study, 9 have successfully amplified the plant genomic DNA fragments. This study reveals the existence of genetic differentiation among the collected plant species. Among the analysed plant samples, there was a high level of polymorphism as well as genetic similarity. This variation is indicative of the evolution of the species in their natural habitat. The plants from different geographical locations exhibited less genetic similarity while species from the same localities showed more genetic similarity. Thus more studies of this nature need to be conducted to assess the phylogeny of the plant species at the molecular level and hence aid in the conservation of their germplasm.

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IDENTIFICATION OF A SMALL HEAT SHOCK PROTEIN GENE FROM PISTACHIO

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Abstract

High temperature and water scarcity drastically influence adversely growth and production of pistachio (Pistacia vera). In this case, breeders have been faced this challenge between resistant to abiotic stress factors and high yield of varieties development. Understanding the responses of plants to abiotic stress factors will make significant contributions to solve this problem. Pistachio is among the important agricultural products for Turkey, which is grown in hot and dry regions during summer months. High temperature stress caused protein dysfunctions in plants. Small Heat Shock Proteins (sHSP) play an important role due to its function to protect the structures of denatured proteins against these stresses. In this study, we determined partial gene sequence of Small Heat Shock Protein from P. vera via degenerate primers prepared from National Center for Biotechnology Information. Herewith, it can be used for gene expression in abiotic stress studies in pistachio plants.

Key words*: abiotic stress, chaperone, phylogeny, Pistacia vera, sHSP.*

INTRODUCTION

Pistacia genus (Anacardiaceae family) has been reported to consist of at least eleven species. Of those species, *P. mexicana* and *P. texana* are of USA and Mexico originated. The other species are mainly distributed within the Mediterranean region, Western and Central Asia and the Middle East (Esmail-Pour, 2001). Turkey is of the important pistachio producer and suppliers in the world and possesses many wild species of pistachio nut. Of those species, *P. vera, P. terebinthus, P. khinjuk, P. atlantica, P. mutica, P. palaestina* and *P. lentiscus* exhibit distribution in different regions of Turkey. The main pistachio rootstock used in Turkey is *P. vera*, and followed by *P. khinjuk, P. terebinthus* and *P. atlantica* (Acar et al., 2017). Of those species, pistachio (*Pistacia vera*) is the most important commercial agricultural products. *Pistacia* is the only commercially grown *Pistacia* species compared to other *Pistacia* species. Other *Pistacia* species are commonly evaluated as rootstock in addition to different purposes as soap, coffee and medical purposes in Turkey (Ertürk et al., 2015).

Production is concentrated especially in the south-eastern Anatolia region of Turkey. This region is classified as a dry and semi-dry area. For this reason, cultivation is done almost without irrigation.

Pistachio is an economically long-lasting fruit species. Therefore, selecting rootstock is a very important factor in new pistachio orchard layout. Although pistachios are relatively tolerant to abiotic stress factors such as drought and salt, drought stress adversely affects growth, dry matter, and yield (Esmaeilpour et al., 2015).

In addition to heat stress, plant sHSPs are also produced under other stress conditions such as drought and salinity. Small heat shock proteins (sHSPs) play an important role against abiotic stress factors. sHSPs are known to protect cells in response to stress from the detrimental effect of stress. However, the mechanisms of cell protection by sHSPs are largely unknown (Sun

et al., 2002). In recent years, molecular studies related to abiotic stresses such as drought, heat stress have increased with rapid and sharp changes in environmental conditions. However, the molecular studies of pistachio have remained extremely limited.

There are only a few genome sequences in The National Center for Biotechnology Information (NCBI) about *Pistacia* species (Jazi et al., 2016).

This study was designed to identify a partial sHSPs gene sequence for used gene expression study in *Pistacia* species.

For this reason, along with the study, the present results and identifications are considered to contribute to the forthcoming studies regarding gene expression profiles in response to the abiotic stress conditions.

MATERIALS AND METHODS

Plant Materials

Leaf samples were collected from the collection orchard of the Kilis 7 Aralik University Agricultural Research and Practice Center (TUAM). Leaves of plants were collected in triplicate from pistachio trees and immediately frozen in liquid nitrogen for stored at –80^{o}C.

DNA Extraction

DNA was extracted from young leaf tissue using CTAB DNA isolation method (Untergasser, 2008). Subsequently, an RNase treatment was performed on the eluted DNA samples. Purity and concentration of the DNA were checked both on 1% (w/v) agarose gels and by µDrop Plate spectrophotometer. (Thermo Scientific Multiskan GO).

PCR analysis

Two degenerated primers Forward 5'- CGCYTCYTCAACACCAACGC – 3' and Reverse 5'- GGCGGAGATGAAGAACGG – 3'were designed based on the conserved sequence of known sHSPs in NCBİ. PCR reaction was performed in a 20 ml reaction volume. The PCR temperature profile was 94°C for 5 min followed by 35 cycles of 94°C for 45 sec., 55°C for 45 sec., 72°C for 1 min and a final extension step at 72°C for 10 min. The PCR products were separated on 1.5% agarose gel and purified DNA bands from agarose gel by the PCR Gel purification kit (The Vivantis Gel DNA Recovery Kit). Positive bands were sequenced on an ABI377 Automated Sequencer (Applied Biosystem), and the resulting sequences were verified and subjected to cluster analysis.

Sequence analysis of sHSPs: The searches for nucleotide and amino acid sequence similarities were conducted with BLAST programs at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

Phylogenetic analysis of sHSPs gene

A phylogenetic tree was constructed by using the MEGA 7.0 software by the maximum-likelihood (ML) and neighbor-joining (NJ). For statistical reliability, the nodes of the tree were evaluated by boot- strap analysis with 1000 replicates.

RESULTS AND DISCUSSIONS

Plant sHSPs are all encoded by nuclear genes and are divided into six classes. sHSPs are localized in cell of cytosol, nucleus, plastids, endoplasmic. In eukaryotes, sHSPs are abundant and different in high plants compared to other eukaryotes (Waters et al., 1996; Lee & Vierling, 2000). Heat shock proteins as a molecular chaperone are not only triggered by heat stress but they are also expressed in response to the effect of osmotic stress. In this context, there are some studies reporting the sHSP-encoding genes induced by water stress (Coca et al., 1996), cold stress (Pla et al., 1998), heavy metal (Györgyey et al., 1991), UV radiation (Murakami et al., 2002).

In this study, the partial sequence of HSP gene from *Pistacia vera* was reported for the first time. Based on sequences, a phylogenetic tree was constructed by using MEGA7.0.

Plants were separated and formed two distinct branches in the phylogenetic tree.

The relationship in the tree was generally displayed a good agreement by being taxonomy (Figure 1). Pistachio, a member of commonly known as the cashew family has shown a 100% similarity with mango from the same family in comparison to other species. Moreover, sHSPs gene sequence of *Pistacia vera* exhibited 66 % similarity with *Citrus sinensis*.

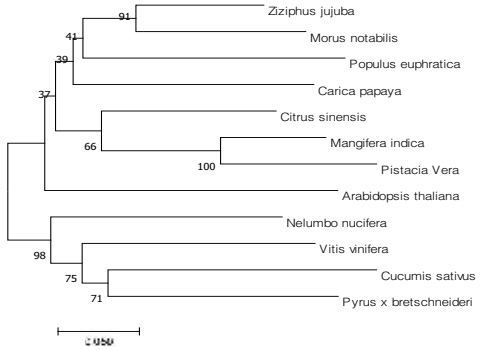

Figure 1. A phylogenetic tree of HSP family members constructed with the neighbor-joining method

The common names, species names and the GenBank accession numbers are the same as those in Table 1. Numbers at each branch indicate the percentage of times a node is supported in 1000 bootstraps pseudo-replication by neighbor-joining.

Table 1 Sequences used in the phylogenetic tree

Sequence ID	Plant Species	Localization
EU513278.1	*Mangifera indica*	----------
XM_006490055.3	*Citrus sinensis*	mitochondrial
XM_016022420.2	*Ziziphus jujuba*	mitochondrial
XM_010265959.2	*Nelumbo nucifera*	chloroplastic
XM_022053869.1	*Carica papaya*	mitochondrial
XM_010091379.2	*Morus notabilis*	mitochondrial
XM_011012544.1	*Populus euphratica*	mitochondrial
XR_002031999.1	*Vitis vinifera*	mitochondrial
EU289284.1	*Arabidopsis thaliana*	mitochondrial
XM_004147155.2	*Cucumis sativus*	chloroplastic
XM_009350311.2	*Pyrus x bretschneideri*	chloroplastic

CONCLUSIONS

Along with the present study, we-for the first time-isolated and identified a small heat shock protein gene from pistachio. Moreover, we can underline and note that there a few molecular studies for *Pistacia* species hitherto, highlight-ting the present first report to be fundamental for the ahead studies related to possible gene expression profiles against abiotic stress factors.

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Additional Information

For access partial gene sequence of Small Heat Shock Protein of *P. vera* by being FASTA format at www.orobanche.net/P.vera.shsp.fasta.

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RECENT DISCOVERIES IN *Varroa destructor* TREATMENT, PREVENTION AND PARASITE - HOST INTERACTION

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Abstract

The European honey bee, Apis mellifera, as most insects of the world, is currently facing major difficulties and, particularly for honey bees, this results in significant colony losses. One of the most stressful factors for A. mellifera is the ectoparasitic mite, Varroa destructor. V. destructor invasions are largely treatable and preventable, however they bring forth great challenges to A. mellifera populations and breeders, making apiculture increasingly time and resource consuming. The global research in apiculture pathology is mostly focused on Varroa sp. This review will be focusing on the recent literature in Varroa treatment, prevention and parasite - host interaction.

Key words*: Apis mellifera, Varroa destructor, pathology, host-parasite interaction.*

INTRODUCTION

Varroa destructor's original host was the Asian honeybee, *Apis cerana* which through coevolution was able to develop tolerance toward the mite. This trait was not carried over to the western honeybee, as the host-parasite relationship between *Apis mellifera* and *V. destructor* is relatively recent (Le Conte et al., 2007). A *Varroa* infestation can, therefore, eradicate a colony of *A. mellifera* within 1-3 years, if left untreated. The lack of a balanced host-parasite relationship between the European honeybee and the mite has facilitated a world-wide spread of *Varroa*, within a relatively short period of time.

A codependent relationship with humans means they always receive adequate treatment against infestations, in order to keep the colonies healthy and productive. At first glance, this relationship may seem advantageous for the bees, because they don't have to suffer major losses or be weakened by *Varroa* infestations, however at a second glance, such grooming hides a darker side. Namely, it breaks the cycle of natural selection which is required to become tolerant to pathogens. Thus, under constant treatment, any individual who can reproduce, regardless of its genetic sensitivity, is able to pass on its genes, which hinders the possibility of host adaptability. Examples of *V. destructor* resistant *A. mellifera* populations can be found in most parts of the world (DeJong et al., 1997; Fries et al., 2006; Le Conte, 2007). These honey bee populations prove that through long-term exposure to the *Varroa* mite, resistance can be developed.

This review will focus on recent discoveries in the host-parasite relationship of *Varroa destructor* and *Apis mellifera*, new treatment methods and the underlying mechanisms of resistance towards the mite.

Varroa destructor AND VIRAL INFECTIONS

In addition to the numerous negative effects *Varroa* directly inflicts upon *A. mellifera*, mite infestations are usually also associated with viral infections (reviewed by Tantillo et al., 2015). Recent research has helped shed light on *Varroa*'s role as a viral vector and how infections can become a contributing factor in colony losses.

Deformed wing virus (DWV) copy number in honeybee pupae is directly associated with the copy number in infesting *V. destructor* (Wu et al., 2017). The presence of large DWV copies induces immunosuppession in the honeybee in order to more easily replicate (Di Prisco et al.,

2016), which acts as an additional stressor and adds to the likelihood of a colony to perish.
Studies suggest that a longer phoretic stage does not necessarily mean a more successful reproductive cycle but that the longer the phoretic stage lasts, there is a higher chance of deformities to appear on the young honeybee. Additionally, DWV load increases with the time spent in the phoretic stage, thus leading to more frequent and severe infections (Piou et al, 2016).
DWV severity, transmitted by *V. destructor* can be dependent on the climate. Overt infections are much more common in temperate climates than they are in tropical climates, without any differences in infestation rates (Anguiano-Baez et al, 2016). This could happen in part because *Varroa* is a better vector for viruses in temperate climates. This theory is supported by Giacobino et al. (2016), who showed that colonies in temperate climates had a much higher viral load compared to colonies in subtropical climates.
This study, however, also reports that *Varroa* infestation levels were higher in temperate climate compared to tropical climate, as was the case for viral load. Currently there is no knowledge of DWV in honeybees in Australia (Roberts et al., 2017). This could be due in part because *Varroa destructor* has only recently been able to spread to this continent and because Australia's climate is partly tropical and mostly arid, which, as established above, are poor conditions for the DWV. The fact that *V. destructor* infestations are milder in Australia and usually doesn't lead to colony losses supports the idea that honeybee mortality is a result of multiple stress factors working together against the bees.

FRESH INSIGHTS IN METHODS OF *Varroa* CONTROL

As far as *Varroa* control goes, the most efficient and widely used methods consist of either synthetic 'hard' chemicals or plant based 'soft' chemicals (Rosenkranz et al., 2010). These treatments function as miticides against *Varroa* and, although effective, they also bring numerous negative side effects for the honeybees, including mortality (Gregorc et al., 2018). Severity of these effects is dependent on the age of the bees and on the level of social interaction (Van Buren et al., 1992). An additional disadvantage to chemical treatments is that *Varroa* can become resistant, which is why efficient management practices are equally as important in *Varroa* control (Thoms et al., 2018). Environmental conditions seem to be the predominant factor in mite infestation levels, followed closely by beekeeper management (Giacobino et al., 2017).
No new active compounds against *Varroa* were discovered in the past 25 years (Mutinelli, 2016), although some recent studies present promising results. Lithium salts were shown to completely eliminate *Varroa* mites in caged environments, without affecting worker bee mortality as compared to untreated controls (Ziegelmann et al., 2018).
Plant extracts offer a great alternative to conventional chemical treatments. These "soft" chemicals offer a similar antiacaricidal effect and are potentially less toxic.
Fumigation with oregano essential oil can rid a colony of *Varroa* within the first two weeks of treatment, while not showing toxic effects towards the honey bees. The constant output of essential oil through fumigation results in a more efficient treatment (Sabahi et al., 2017). Plant based extracts such as *Thymus algeriensis* essential oil also offer a great solution against *Varroa*.
This extract contains large quantities of thymol, which is a known antivarroa agent (Noureddine et al., 2016) and has been shown to reduce mite populations by 32.6%, without harming the honeybees (Kouache et al., 2017). Mild acaricide effect was shown in sage - *Salvia officinalis* L. (Lamiaceae) - essential oil (Bendifallah et al., 2018) and costic acid extracted from *Dittrichia viscosa* proved to be 80% as effective as commercial acaricides (Sofou, 2017).
In addition to good management practices and chemical treatments, the use of technological methods, like the removal of drone brood (Wantuch and Tarpy, 2009) offers an efficient and cost effective solution against *Varroa*. Irradiation of honeybee colonies did not seem to influence *Varroa* infestation levels and overall effectiveness in pest control could be described as mild, at best (de Guzman et al., 2019).

The use of *Stratiolaelaps scimitus*, a mite that feeds on small insects, showed promising effects against varroa infestations. This method isn't 100% safe though, since the mite also consumed honeybee eggs in lab conditions, but not in the hive (Rondeau et al., 2018) and treatment applied in late or early fall was not efficient in controlling varroa (Rondeau et al., 2019).

*Bacillus thuringiensis*is is virulent and pathogenic in small insects and acarids, including varroa (reviewed by Chandler et al., 2001), however, it does not affect the honeybee and can be used alongside conventional treatments for *Varroa* control (Alquisira-Ramírez et al., 2017). Overall, bacteria, especially from the *Bacillus* and *Lactobacillus* genus, act as probiotics and bring important benefits for the honeybees by increasing the immune response, stimulating queen egg laying and significantly increasing honey yield (reviewed by Audisio, 2017)

Entomopathogenic fungi could also reduce varroa damage to honeybee brood by both infecting the parasite and preventing varroa-associated suppression of honeybee immunity. Three immune genes of the honeybee, hymenoptaecin, pUf68 and BlCh, are usually suppressed by varroa. When inoculated with *Metarhizium anisopliae* and *Beauveria bassiana*, varroa cannot suppress the expression of these genes (Hamiduzzaman et al., 2012).

The sensory limitations of the varroa mite can be used against it. Given the lack of sight, the varroa mite is dependent on chemoreceptors to find its next host (Dillier et al., 2006). By inhibiting the chemoreceptors, varroa will have difficulties in choosing the right host. One way in which olfactory detection can be inhibited is through the use of racemic compounds (Govardhana et al., 2016)

In addition to grooming and hygienic behaviors, honeybees were also found to change normal behavior in order to alleviate pathogenic pressures. *A. mellifera* colonies have been found to change foraging patterns as a response to pressure from varoosis. Colonies infested with *V. destructor* increased the number resin foragers, thus increasing the quantity of collected resin as a means of self-medication (Pusceddu et al., 2019).

TREATMENT RESISTANT MITES

Chemical treatments offer the most effective solution for treating varoosis but they also bring forth multiple downsides amongst which toxicity for the honeybees, pollution of bee products and development of treatment resistance in *Varroa* (Rosenkranz et al., 2010). While product pollution and toxicity are negligible in terms of severity and economic impact, the spreading of treatment resistant *Varroa* mites could be disastrous for honeybee populations. The following part of the review will be focusing on recent scientific discoveries in resistance to treatment.

Evidence for resistant *Varroa* populations has started to emerge at the end of the 20th century (Lodesani et al., 1995; Hillesheim et al., 1996; Milani, 1999) and continue to emerge to this day. Recent studies have helped shed light on resistance mechanisms. A link was found between two novel mutations at Leucine 925 of the Voltage-Gated Sodium Channel gene (L925M, L925I) and resistance to pyrethroids, tau-fluvalinate and flumethrin, in USA (Gonzales-Cabrera et al., 2016). Mutations at this residue were also found in Pyrethroid resistant mites from Southern England (Gonzales-Cabrera et al., 2013) and in the Czech Republic (Stara et al., 2018; Hubert et al., 2014). This mutation was found in 98% of mites that went through miticide treatment and in only 45% of non-treated individuals which means that when selective pressure is applied, mite populations can develop resistance to the treatment. A connection between point mutations at position 925 in the sodium channel gene and treatment resistance has been confirmed in a biological assay (Stara et al., 2019).

Varroa destructor is a highly inbred species, due to its reproductive mechanism. Genetic diversification only occurs once the *Varroa* population grows, in the middle of the honeybee productive period, when brood cells are populated by more than one foundress. Applying antivarroa treatments before this stage, when the *Varroa* population is low and goes through a population "bottleneck" could help with fixating variants responsible for miticide resistance (Beaurepaire et al., 2017).

These findings are alarming considering the slow development of new control methods and

the fast spreading of the mite. Hierarchical genetic variation can be found at a colony level, which indicates that *Varroa* transmission doesn't only happen vertically from one generation to the next but also horizontally, between hives and apiaries (Dynes et al., 2016). Horizontal transmission is facilitated by *Varroa*'s capacity to quickly climb on its host (Peck et al., 2016). *V. destructor* has also been found in drone congregation areas, which increases the mite's transmission capabilities even further (Mortensen et al., 2018).
Luckily, though, bee populations have a few aces up their sleeves.

WESTERN HONEYBEE RESISTANCE AGAINST *Varroa*

The oldest Western honeybee population resistant to *Varroa* was recorded in 1997 by De Jong and coworkers. Twenty Italian honeybee colonies infested with *Varroa* were brought in 1984 to the Island of Fernando de Noronha, off the coast of Brazil. They were genetically isolated, as to prevent genetic contamination and were left to face *Varroa* without any treatment. This population survived the infestation and is still alive to this day (De Mattos et al., 2016).
The first experimental insight on *A. mellifera* resistance to *V. destructor* was brought forth in 2006 by Fries et al. After three years a *V. destructor* infested, untreated *A. mellifera* population of 150 colonies had an 80% mortality rate during winter. This rate decreased to 13% and 19% in the 5th and 6th year respectively, while infestation levels in the fall also significantly decreased. This is a great example of adaptability by *A. mellifera* and *V. destructor*, and proves that coevolution is possible when selective pressure is applied.
Varroa surviving colonies also show a similar mortality rate when compared to treated colonies, at the expense of lower honey productivity (Le Conte et al., 2007).
When compared to control populations, *V. destructor* resistant colonies have a similar hygienic and grooming behavior but the reproductive success of *Varroa* is significantly reduced (Locke and Fries, 2011). When compared to *A. mellifera,* mites infesting *Apis cerana* had similar reproductive initiation success, because infested individuals would be removed. Consequently, affected brood in *A. cerana*, was not able to reach maturity, supporting the idea that resistance is based on behavioral traits (Lin et al., 2018).
A *V. destructor* surviving *A. mellifera* population from Norway was analyzed in order to find traits which helped reduce the reproductive success of *Varroa*. A 10% shorter than normal post capping period was found to differentiate resistant colonies from susceptible ones (Oddie et al., 2018). Spermatozoa capacitation in inseminated mites takes 5 days. This could explain, as the phoretic phase is not vital (Ruijter, 1987), why a shortened post capping period would be problematic for *Varroa* (Häußermann et al., 2016).

CONCLUSIONS

Although behavior traits seem to offer a complete explanation of defense mechanisms for *Varroa* resistant honey bees (i.e. *Apis mellifera scutellata)*, most comparative studies link resistance to physiological traits. While it is currently unknown what the molecular basis for resistance against *V. destructor* is, studies suggest that interferences in the moulting hormone biosynthesis are a likely cause. Further research is needed to fully understand these mechanisms.
Additionally, in order for the two species, *A. mellifera* and *V. destructor,* to coevolve and create a balanced host-parasite relationship, selective pressure needs to be applied. The success of breeders in obtaining resistant *A. mellifera* populations should inspire global programs of resistance-based selection.

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COMPARATIVE STUDY ON EXTRACTION METHODS OF PECTIN FROM BY-PRODUCTS OF JUICED CARROTS

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Abstract

Carrot juice is a product of interest in food industry. Pomace, a by-product of the carrot juice industry, contains a significant amount of pectin. This natural polymer is rich in galacturonic acid and is used as food additive due to its gelling, thickening and stabilizing properties. The objective of this study was to compare different extraction methods of carrot pectin. Sodium citrate buffer, pH 5.0 and carrot pulp were used for pectin extraction. Methods used to obtain pectin required temperature, microwaves, ultrasounds and Celluclast 1.5L enzyme treatment. The extraction yield of each method was determined. The obtained pectin was characterized by the content of galacturonic acid, the degree of esterification, the degree of emulsification and cytotoxicity in a fibroblast cell line. The enzymatically extracted carrot pectin contained at least 65% galacturonic acid and had a high degree of esterification and emulsification. In vitro cytotoxicity tests have demonstrated the biocompatibility of pectin extracts. The results of this study have showed that valuable carrot pectin with high content of galacturonic acid and good biocompatibility can be obtained from carrot pomace. The enzymatic extraction method could be further studied for various industrial applications, and the obtained carrot pectin could be useful especially in food supplements.

Key words*: cytotoxicity, emulsion, esterification, galacturonic acid, pectin.*

INTRODUCTION

Food industry produces several by-products during fruits and vegetables processing, especially for natural juices manufacture, which can be valorised. The generated pomace is a valuable by-product that contains pectin, besides other components of interest. Pectin is a group of natural polysaccharides, rich in D-galacturonic acid units linked by α-1,4 glycosidic bonds, present in the cell walls of the superior plants. The galacturonic acid from pectin chain is largely esterified with methoxy and acetyl groups (Harholt et al., 2010). Due to its gelling, thickening and stabilizing properties, pectin is intensively used in the food industry, especially sweets, as an additive. It is a very safe food additive with no consumption limit. It is also used in the medical, cosmetic and pharmaceutical industries (May, 1990). There are no indications on its genotoxicity and it has no side effects or allergenic potential (Mortensen et al., 2017). At the beginning of the 20th century, the German apple juice producers used the remaining residue to obtain pectin, which was sold as a gelling agent. At present, apple pomace and citrus peels are the main sources for obtaining commercial pectin (Dranca & Oroian, 2018). 85.5% of the market pectin is from citrus peels, 14% from apple pomace and 0.5% from sugar beet pulp (Cirimina et al., 2016). New sources of pectin have been studied, such as tomatoes (Grassino et al., 2016), carrots (Jafaria et al., 2017), watermelon (Petkowicz et al., 2017), banana peels (Happi Emaga et al., 2008), passion fruit peels (Liew et al., 2014; Vasco-Correa et al., 2017), dragon fruit peels (Tongkham et al., 2017), grape pomace (Minjares-Fuentes et al., 2014) etc.

Both the extraction method and the source influence the quantity and the properties of the obtained pectin. The temperature and the buffer pH are parameters of great importance, which can vary during pectin extraction process. Pasandide et al. (2017) extracted a high esterified pectin from an aqueous solution of *Citrus medica* peel, at different temperatures and periods of extraction, with a maximum yield of 21.85%. Liew et al. (2014) obtained a high esterified pectin from passion fruit peels, varying the pH of citrate buffer and the maximum yield

corresponded to pH 2. Commercial pectin was also extracted from citrus peels or apple pomace using hot dilute acid at pH 2 (May, 1990).

Currently, environment-friendly methods based on microwaves, ultrasounds and enzymes are used in pectin extraction. Tongkham et al. (2017) obtained pectin from an aqueous solution of dragon fruit peels using microwaves at different powers and the maximum yield was 23.11%. Minjares-Fuentes et al. (2014) prepared a high esterified pectin with a yield of 29.4% from grape pomace in citric acid using ultrasounds. A maximum output of 19% pectin was obtained from apple pomace using a treatment with Celluclast 1.5L enzyme (Wikiera, 2015).

The use of pectin on a larger scale requires new methods of extraction and valorisation of different sources, in order to obtain products with convenient properties. Carrot (*Daucus carota*) is a root plant, consumed worldwide in both fresh and cooked state for its high content in minerals and carbohydrates. During carrot juice preparation, an important amount of pomace is obtained, with a high content of valuable compounds including carotenoids, dietary fibres and pectin (Sharma et al., 2012). Jafaria et al. (2017) extracted the carrot pomace pectin in citric acid.

The purpose of this study was to compare the enzymatic methods of pectin extraction with the chemical methods using carrot pulp in citrate buffer as a source. The physical and chemical characterization of pectin was performed in order to obtain a product with improved properties for applications in the food and cosmetics industry.

MATERIALS AND METHODS

Materials

Carrots originating from Romania were bought from the supermarket, washed, cut and dried at 50°C, for 24 h. Then, the material was powdered using a grinder and stored in food bags, at -20°C until analysis.

Enzymatic extraction method

The powdered carrot was incubated in citrate buffer, pH 5 containing 88 U Celluclast 1.5L, in two different weight ratios of 1: 60 (variant a) and 1: 15 (variant b), 50°C, for 20 h (Sabater et al., 2018; Wikiera et al., 2015). The enzyme activity was stopped by boiling the mixture at 100°C, for 10 min. Controls (variants a1, b1) were prepared in the same conditions without adding the enzyme. The extracts were filtered through Whatman paper to remove the vegetal residues.

Thermal extraction method

Carrot powder (0.5 g) was incubated in 15 ml citrate buffer, pH 5, at 120°C, for 2 h (Pasandide et al., 2017). After cooling at room temperature, the solution was filtered through Whatman paper (variant c) and further processed for analysis.

Microwaves-assisted extraction method

In the case of microwaves-assisted extraction, a weight ratio of 1: 100 between the carrot powder and the citrate buffer was used. The homogenized solution was exposed to microwaves, at a power of 560 W, for 160 s (Tongkham et al., 2017). After cooling, the solution was filtered through Whatman paper and further analysed (variant d).

Ultrasonic extraction method

A mixture of carrot powder and citrate buffer pH 5, in a weight ratio of 1: 30 was exposed to ultrasounds treatment, at 10 kHz frequency, at 60°C, for 40 min (Minjares-Fuentes et al., 2014). The vegetal residues were removed by filtration and further processed (variant e).

Purification method

Pectin was purified by precipitation of filtrate with ethanol. In the first step, 2 volumes of ethanol were added over the obtained filtrate and incubated at 4°C, overnight. Then, it was centrifuged at 7000 g, for 30 min. The supernatant was discarded and the precipitate was washed with 10 ml absolute ethanol and separated by centrifugation at 7000 g, for 30 min. Pectin extract was dried at 50°C until constant mass was reached.

All pectin extracts were prepared in two separate experiments, in triplicate.

Yield determination

For each extraction method, the yield was calculated using the following equation:

% yield = (final weight/initial weight) x 100 where the final weight was the amount of dried pectin and the initial weight was the dried amount of raw material.

Determination of galacturonic acid

Determination of galacturonic acid content was performed by orcinol method (Moldovan et al., 2008). Briefly, over 1 ml of pectin solution, 3 ml orcinol reagent (orcinol mixed with FeCl3 and concentrated HCl) were added. The mixture was incubated at 100°C, for 40 min with gentle shaking. After cooling, the absorbance of each processed sample was read at 665 nm using a UV-VIS spectrophotometer. A standard curve was built using dilutions of 0.15 mM galacturonic acid solution. Determinations were performed in triplicate.

Determination of esterification degree

Determination of the esterification degree (DE) was performed by titrimetry (Liew et al., 2014). A pectin solution of 1 mg/ml concentration was titrated with NaOH, in the presence of 2 drops of phenolphthalein, until the colour turned pink (initial NaOH volume). The mixture was left at room temperature, for 2 h to de-esterize galacturonic acid. HCl was used to neutralize excess NaOH until the solution became colour-less. After that, 2-3 drops of phenolphthalein was added again and the obtained mixture was titrated with NaOH until pink (final NaOH volume).

$$\%DE = \left[\frac{\text{Final NaOH(ml)}}{\text{Final NaOH (ml)} + \text{Initial NaOH(ml)}}\right] \times 100$$

Determination of emulsification degree

Determination of the emulsification degree was performed by vortexing a mixture of 0.5 mg/ml pectin solution and sunflower oil containing 0.02% sodium azide, in equal parts, at maximum speed (Jafaria et al., 2017). Then, the emulsion was centrifuged at 4000 g, for 5 min. The emulsification degree was calculated using the following equation:

$$\%\text{Emulsification} = \left(\frac{\text{Emulsion volume}}{\text{System volume}}\right) \times 100$$

where emulsion volume was the volume of emulsion phase and the system volume was the volume of total system.

Cell cytotoxicity tests

Cells from mouse fibroblast NCTC clone L929 cell line were seeded in 96-well culture plates and incubated in 5% CO_2 humid atmosphere, at 37°C, for 24 h. For experiments, the culture medium was replaced with fresh medium containing pectin extracts in the concentration range of 50-1000 µg/ml and the plates were incubated in standard conditions for 24 h and 48 h. Cell viability was determined at the end of incubation period by MTT assay, as previously described (Scudiero et al., 1988; Quentin-Leclercq et al., 1992). MTT assay consists in the reduction of tetrazolium bromide salt by mitochondrial dehydrogenases present in metabolically active cells. Briefly, the culture medium was replaced with MTT solution, followed by incubation at 37ºC, for 3 h. The formed formazan crystals were dissolved in isopropanol by gentle shaking and the absorbance of the coloured solution was read at 570 nm using a microplate reader. Untreated cells served as negative control, considered as 100% viable cells, while cells treated with 0.03% H_2O_2 represented the positive control. Determinations were performed in triplicate.

Cell morphology observations of the cultures treated with pectin extracts for 48 h were performed using an optic microscope equipped with a digital camera.

RESULTS AND DISCUSSIONS

In this study, pectin was extracted from carrot pulp in sodium citrate buffer, pH 5.0 using the enzymatic, thermal, microwave- and ultrasound-assisted methods.

Extraction of pectin and yield

The purified pectin extract was dried to obtain a yellowish or white powder (Figure 1).

The yield of pectin extraction from carrot in citrate buffer, pH 5, varied between 7-20%, according to each treatment procedure (Figure 2). Thus, the highest value was 20%, in the case of microwave extraction of pectin from carrot pulp. In the case of ultrasonic extraction, the yield was almost 3 times lower than that of microwave-assisted extraction of pectin. Enzymatic extraction of pectin in variant b had a yield of 17%, which was with 23.52% higher than the yield obtained in enzymatic treatment variant a.

Figure 1. Pectin extracts obtained from carrot pulp by enzymatic (a), thermal (c) and microwave-assisted (d) methods

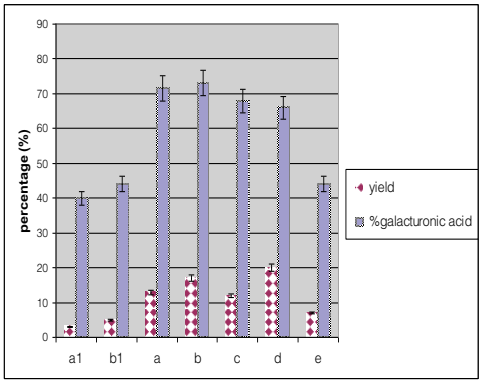

Figure 2. Extraction yield and galacturonic acid content of carrot pectin obtained by enzymatic (1: 60) (a) with its control (a1), enzymatic (1: 15) (b) with its control (b1), thermal (c), microwave-assisted (d) and ultrasonic (e) extraction methods

Pectin extracts obtained in the same conditions, but enzyme-free (a1 and b1 controls) had a very small yield of 3% and 5%, respectively, indicating that Celluclast was efficient in pectin extraction.

Celluclast 1.5L is a cocktail of enzymes with xylanolytic and cellulolytic activity. This enzymatic cocktail helps to release the pectin by destroying the cellulosic wall of the plant cell (Wikiera et al., 2015). Still, the yield of enzymatic extraction variant b was with 15% lower than the yield of microwave-assisted extraction. Thermal extraction of pectin had a yield of 12%. Similar studies indicated a yield of 23.1% in the case of microwave-assisted extraction of pectin from dragon fruit peels, in citrate buffer pH 2, for 10 min, at a power of 600 W (Tongkham et al., 2017).

Liew et al. (2016) extracted the pectin from the passion fruit by the enzymatic method using Celluclast and obtained a maximum yield of 9.17%. Wikiera et al. (2015) obtained apple pectin with a maximum yield of 19%. Jahari et al. (2017) obtained pectin from carrot pomace using a temperature of 50- 90°C for 30-150 min, at a pH ranging between 0.5-2.5. By varying temperature, time and pH parameters, yields ranged between 5-15.2%. The maximum yield was obtained in the case of pH 1.3, 90°C for 79.8 minutes.

Galacturonic acid content

The method of pectin extraction from carrot pulp influences its galacturonic acid content. According to FAO, pectin used in the food industry have at least 65% galacturonic acid content, as an indicator of its purity (May, 1990). In this study, pectin obtained by enzymatic extraction with Celluclast had a high content of galacturonic acid (Figure 2), ranging from 71.5% (variant a) and 73% (variant b). The percentage of galacturonic acid in thermal extracted pectin was 68% and in microwaves extracted pectin was 66%. The pectin extracted by ultrasound had a lower percentage of galacturonic acid (44%).

By enzymatic extraction, using Celluclast, we obtained a pectin with a 73% galacturonic acid content. Jafaria et al. (2012) showed that pectin obtained from carrot pomace, under acidic conditions, at high temperature contained 75.5% galacturonic acid. In turn, pectin obtained from passion fruit by enzymatic extraction using an

extract from *G. klebahii* had 85.4% galacturonic acid content (Vasco-Correa et al., 2017).

Esterification degree

The esterification degree represents the percentage of galacturonic acid carboxyl groups esterified with methoxy (in most cases) or acetyl groups.

The pectin extracted by different extraction methods in this study had a high degree of esterification. It was higher than 70% for all treatment procedures, as presented in Figure 3. The highest esterification degree of pectin extracted from carrot was 87%. A product with an esterification degree of 81% was obtained by thermal extraction. The microwave-assisted extraction resulted in a product with 77% esterification degree, while ultrasound-extracted pectin had a degree of esterification of 75%.

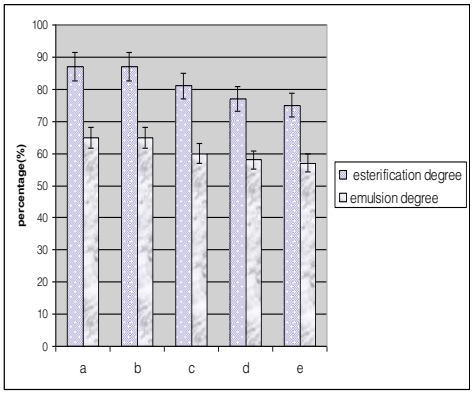

Figure 3. The esterification and emulsion degree of carrot pulp pectin obtained by enzymatic (1: 60) (a), enzymatic (1: 15) (b), thermal (c), microwave-assisted (d) and ultrasonic (e) extraction methods

A similar result was obtained by Liew et al. (2016) for enzymatically extracted passion fruit pectin, presenting 86.96% esterification degree.

Pectin esterification degree is >50% for high methylated pectin or <50% for low methylated pectin, respectively. The esterification degree affects pectin properties (Liew et al., 2014). The high methylated pectin can form gels at low pH. Low esterified pectin forms gels in the presence of Ca^{2+} (Venzon et al., 2015).

Venzon et al. (2015) attempted to decrease the pectin esterification degree by treatment with NaOH for 1 h, at 55°C. Lowering the esterification degree with a few percent, the pectin solution viscosity was reduced.

Emulsion degree

Pectin can be used as an emulsifier in the food industry and its quality depends on the degree of emulsification. In this study, enzymatically obtained carrot pectin had the highest degree of emulsification (65%) (Figure 3). The pectin obtained by ultrasounds treatment had a lower emulsification degree (57%). Thermally obtained pectin had the emulsification degree of 60%, while that of microwaves-extracted pectin was 58%. A similar degree of emulsification of 60.3% was reported for pectin obtained from carrot pomace (Jafaria et al., 2017).

Cytotoxicity tests

Cytotoxicity of pectin extracts was tested in NCTC fibroblast culture after 24 h and 48 h of cultivation, by MTT assay and cell morphology observations. The results are presented in Figure 4.

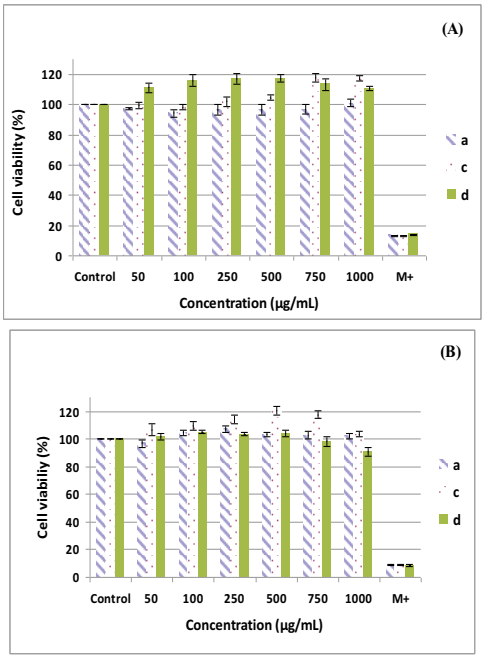

Figure 4. Cytotoxicity of carrot pectin extracted enzymatically (a), thermal (c) and microwave-assisted (d) and cultivated in NCTC fibroblast cell culture for 24 h (A) and 48 h (B)

The viability was high after 24 h of NCTC cells cultivation in the presence of pectin, surpassing 94% (Figure 4. A).
It was observed that cells cultivated with microwaves-extracted pectin with concentrations of 50-1000 μg/ml presented high values of viability, between 110.94-117.61%.
The viability of cells cultivated with thermally-extracted pectin increased in a concentration-dependent manner, with the highest value at 1000 μg/ml concentration. After 48 h of cultivation in the presence of pectin extracts, the cell viability was similar or higher than that of control cells, considered 100%, excepting microwaves-extracted pectin at 1000 μg/ml (Figure 4. B).
Thermally-extracted pectin maintained a high value of cell viability, ranging between 103.72% and 124.44%, for concentrations of 50-750 μg/ml.
Cell morphology of NCTC fibroblasts cultivated in the presence of pectin extracts was observed after 48 h of cultivation (Figure 5). No change was observed in the morphology of pectin-treated cells, compared to untreated cells. Fibroblast cells presented the characteristic spindle-shape phenotype. These observations confirmed the quantitative data obtained by MTT assay.

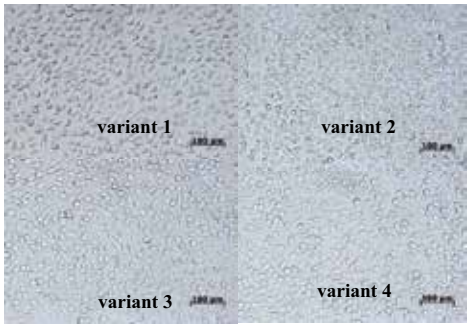

Figure 5. Morphology of NCTC cells cultivated with 250 μg/ml pectin extracted by enzymatic method (1), microwaves method (2) and thermal method (3), for 48 h. Control cells were cultivated in plastic culture plates (4)

The cytotoxicity tests demonstrated that all three types of carrot pectin were not cytotoxic. Moreover, thermally-extracted pectin stimulated the growth of NCTC cells.

CONCLUSIONS

All results demonstrated that high quality pectin extract could be obtained from carrot pulp by environmentally friendly methods based on enzymatic or microwaves treatment. They avoid strong acids or high temperature treatment for long periods of time. Each method of extraction influenced pectin extract characteristics. Thus, pectin extracts were highly esterified (>70%), had a high degree of emulsification (>50%) and over 65% galacturonic acid content. The best yield was obtained in the case of microwaves extraction, while the highest percentage of galacturonic acid was found in enzymatic-extracted pectin. The MTT assay results showed that thermally-, enzymatically- and microwaves-extracted pectin did not have cytotoxic effects in a fibroblast cell culture. In conclusion, the high quality pectin extracted from carrot pulp could be further studied for various industrial applications, especially in food supplements.

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EFFECT OF CLIMATE CONDITIONS ON THE RIPENESS QUALITY INDICATORS OF SOME RED GRAPES FROM VALEA CĂLUGĂREASCĂ VINEYARD

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Abstract

Grape maturation is a very complex biochemical process which influences wine quality. During maturation, grapes accumulates the sugars content, polyphenols, flavors, nitrogenous compounds, mineral substances, enzymes, vitamins, and other chemical compounds that participate in the formation of the wine quality. Grape maturation is strongly influenced by climatic conditions, so the quality of raw materials for wine varies widely from one year to another, from one vineyard to another. The aim of the present work is o evaluate the effects of pedo climatic influence of the year 2017 on grape maturation and quality parameters for 3 red cultivars in Valea Călugărească vineyard. For this purpose, three grapes cultivars: Cabernet Sauvignon, Merlot and Fetească neagră were analised from characteristics such us : sugar content, total acidity, 1000 grain weight , glucoacidimetric index. The experimental measurements were conducted during august-september of 2017 year. The research established the relation between the heliothermal regime of those year and quality indicators of the raw material for obtaining a good technological, phenolic and aromatic maturation for a superior quality of red wine.

Key words*: glucoacidimetric index, grapes characteristics, grape maturation, sugar content, total acidity.*

INTRODUCTION

Wine is a complex matrix which contains many classes of compounds like sugars, alcohols, acids, tannins, and other components like minerals and proteins. Its composition is influenced by many factors related to the specific production area, like grape variety, soil and climate, ripening of the grapes, yeasts, and winemaking technique (Gonzálvez et al., 2009; La Torre et al., 2006).

There are various reasons for which the concentrations of some major and trace elements in wines are further monitored (Geana et al., 2014). Some of them are related to the effects these elements may have on the organoleptic properties of the wines (Lara et al., 2005) and to their ability to discriminate wines according to the geographical region in which grapes were grown (Geana et al., 2013) as well as to detect wine adulteration (Geana et al., 2014).

The presence of phenolic substances in wine and the pedoclimatic conditions are fundamental, these being a major contributor to the formation of specific characteristics such as color, aroma, and flavor, making the difference between different assortments of wine (Mitic et al., 2010).

For these reasons, there is an increased tendency to study the wine composition in its minor constituents with the aim to achieve better characterization, contributing thus to the commercial enhancement of the product (Geana et al., 2014).

The type and concentration of the phenolic compounds in wine depends on grape variety, ripening, atmospheric conditions, viticulture and winemaking techniques. Generally, the major determinant factor for the variation in the polyphenolic content of different red wines throughout the world is probably the amount of sunlight to which the grapes are exposed during cultivation (Geana et al., 2011).

To determine the optimum moment to harvest red wine grapes, as well as the sugar/acidity ratio (technological ripeness), it is also important that the grape has a high concentration of easily extractable phenolic compounds in the skin, such as anthocyanins and tannins, and that the seeds have a hard-outer coating (Cuzmar et al., 2018).

The optimum moment to harvest red wine grapes is therefore defined by technological and phenolic ripeness (Ribereau-Gayon et al., 2006).

Maturity could be described as the time when the analytical parameters such as sugar content, acidity, and other compounds reach the proper balance, and the varietal characteristics, including aroma, flavor and color are fully developed for the style of wine to be produced. The determination of harvest time is a complex compromise between availability of harvesting labor or a mechanical harvester, the weather, the likelihood of pest and disease damage, and the stage of ripeness of the grapes. Timing is most critical when all the fruit is harvested concurrently since only rarely is it economically feasible to selectively and repeatedly harvest for fruit of a particular quality for winemaking. The best harvest decisions are made by grape growers and winemakers who work closely together using a practical, integrated approach toward maturity assessment[1].

The objectives and goals of the work is to study the effects of pedoclimatic influence of the year 2017 on grape maturation and quality parameters for 3 red cultivars in Dealu Mare region. The aim of this study was to quantify the different components during the maturation process of Cabernet Sauvignon, Merlot and Fetească Neagră grapes grown in Valea Călugărească vineyard center, from the beginning of maturation until harvest to verify whether they meet the requirements to produce high-quality wines.

MATERIALS AND METHODS

Most varieties for wine grapes fall into maturation in seasons like IV and V. The period of grapes ripening is 45-50 days (second half of August-September month) that harvesting grapes period begins after the 15th to the 20th of September.

Technological ripeness

The maturation process is followed from the entrance of the first fruits in grape (early ripeness) by taking periodic samples of the grape who are analysed in the laboratory.

At the beginning of ripening, grape samples are taken from 5 to 5 days, and as the aging process progresses, samples are taken more often from 3 to 3 days. The sample of grapes come from at least 10-20 stocks of plot, located in various points. There are taken small portions of bunches from the grapes who are in the middle of the hub, grapes on the sunny side and the ones inside the hub.

Sampling is taken in plastic bags where a label is insert, with the following characteristics: variety from which the sample is taken, in our case from Cabernet Sauvignon, Merlot and Fetească Neagră; date of harvest; the name of the place where the plantation is; any data on plantation. Samples of grapes are brought on the same day in the laboratory to be analysed.

Sample analysis consists of the following determinations: weight of 100 grains, total sugar content and acidity of the grapes and glucoacidimetric index. First is proceeded to detaching bunches grain from residues by cutting with laboratory scissors above grape grain burelet so the berries remain intact and not lose the juice.

Healthy grapes berries are counted, separating the healthy from the damaged ones. Grapes berries are weighted at technically, laboratory balance setting the weight of 100 berries.

After weighting the berries are crushed in gauze bag and by hand squeezing the must is divided. Must collection is made in conical flasks Erlenmeyer glasses which then are kept in the fridge for 2-3 hours for clarifying the wine pressing. From the clear must, unfermented, are determined the total acidity and total sugar content. The sugars content is determined out by refractometer method. The total acidity is determined by the titrimetric method, neutralizing the acidity of the must with a 0,1 N NaOH solution in the presence of bromothymol blue indicator.

The tests are listed in the register of laboratory and the graphs are drawn based on their maturing varieties.

Starting from the fact that the sugar content of grapes during maturation moves backwards with the total acidity, for determining the ripeness degree was considered the report sugars content/total acidity (S/A):

Glucoacidimetric index (S/A) = Sugars (%)/ Total Acidity (g H_2SO_4/l)

[1] Vineyard and Vintage view, July/August (1995), *10*(4), 325.

Glucoacidimetric index values in the full maturation of the grapes are between 35-45, depending on the variety. Between these limits the grapes values have reached optimum degree of maturity that insures good quality wines.

Phenolic ageing

The rich grape phenolic compounds (anthocyanins, tannins) is a technological condition for insuring the quality of red wines.

When colored grapes (black) have reached ripeness, peeled grains make up cell membranes to disrupt through the action of enzymes and let to distribute in must (juice grapes) the colorants materials, so the grapes are makeing phenolic maturation.

Seed maturity is a controversial concept because oenologists often wait too long for the grapes to reach this hypothetical maturity stage and excessively delay the harvest without achieving any advantages (Casassa et al., 2013) suggested that delaying harvest to achieve seed browning may be a relatively lesser factor affecting tannin extraction during maceration. This controversy springs from a confusion between seed browning and seed maturity; the former is related to a chemical composition as well as to representative colour, and the latter to acoustic and mechanical properties (Torchio et al., 2012), seed texture (Le Moigne et al., 2008), chemical properties (Kennedy et al., 2000), as well as seed colour properties (Ristic & Iland, 2005).

RESULTS AND DISCUSSIONS

Valea Călugărească vineyard center shows viticulture potential for the culture of vine varieties to obtain high-quality red wines. For the elaboration of the study concerning the climate, vineyard center have used data recorded at the meteorological station Valea Călugărească (latitude 44º59', longitude 26º13', altitude 210 m).

The global radiation, considered as the most important factor of climate, in its viticultural center Valea Călugărească has some annual values ranging around 125 kcal/cm^2.

The highest values of global radiation records during the warm year (April-September), varies around 92.5 kcal/cm^2 horizontal surface.

The number of insolate hours totalling the average amount in 2071 hours by year providing a normal ripening grapes and wood chords.

Pluviometric data indicate the average annual amount of precipitation being between 510-590 mm, with large variations in them from one year to another.

Distribution of rains during the vegetation period is uneven, in May-June a single maximum rainfall. Hail falls fairly infrequently, but can produce significant damage.

From the ecoclimatic point of view, the year of 2017 was characterized by a moderate regime heliothermic, against a background of rich water resources, especially in April and May, when it was overcome multiannual values.

The vegetation period (April), it started with temperatures lower than the normal (10.9ºC to 11.7ºC), and a higher water regime (107 l/m^2 to 44.8 l/m^2), confronted with multiannual values.

Air wettability in 2017 year was higher by 1.3% in April, with 0.6% in June, by 7.7% in July and 1.6% less in May, and by 4.3% in August (Table 1) compared to the previous year.

Tabel 1. Rainfall, wettability during the period April - October 2017

Year	Month	Precipitation (mm)		No days with rain	Hygroscopicity %	
		Normal	IV-X 2017	>10	Normal	IV-X 2017
2017	IV	44.8	107.0	9	67.7	68.3
	V	67.3	56.4	9	68.4	65.9
	VI	81.5	84.7	6	70.1	69.5
	VII	75.8	86.6	11	67.5	71.0
	VIII	62.7	36.4	6	66.5	61.1
	IX	54.4	40.2	9	70.7	65.3
	X	46.2	86.8	4	77.0	76.4

The optimal time for harvesting the grapes was established both by the weight of the berries and grapes glucoacidimetric index (sugar levels to acidity). At first these indices were followed up by 5 to 5 days, then with 5-6 days before harvesting, the grapes were pursued every day (maturation stroke).

Vintage achieved optimal time when the weight of 100 grains reached the maximum value, the acidity of the grapes won't change, and the

sugar level from the berries has not increased for 2-3 days.

All these factors can be found in experimental records below (Tables 2, 3, 4, 5):

Table 2. Ripening grapes on 24.08.2017

No.	Variety	Parcel	Characteristics of grapes			
			Sugar (g/l)	Total Acidity (g/l H_2SO_4)	W 100 berries (g)	Glucoacidimetric Index
1	Cabernet Sauvignon (CS)	4406	140.80	7.09	119.19	20
2	Cabernet Sauvignon (CS)	4403	155.60	6.90	109.27	23
3	Cabernet Sauvignon (CS)	4409	153.50	6.52	103.42	24
4	Merlot (M)		187.50	4.10	122.04	46
5	Fetească Neagră (FN)		155.60	4.92	110.45	32

Table 3. Ripening grapes on 30.08.2017

No.	Variety	Parcel	Characteristics of grapes			
			Sugar (g/l)	Total Acidity (g/l H_2SO_4)	W 100 berries (g)	Glucoacidimetric Index
1	Cabernet Sauvignon (CS)	4406	150.00	6.04	120.00	25
2	Cabernet Sauvignon (CS)	4403	172.60	5.65	107.86	31
3	Cabernet Sauvignon (CS)	4409	160.00	6.15	105.00	26
4	Merlot (M)		174.80	4.44	97.70	39
5	Fetească Neagră (FN)		179.00	4.34	135.69	41

Table 4. Ripening grapes on 07.09.2017

No.	Variety	Parcel	Characteristics of grapes			
			Sugar (g/l)	Total Acidity (g/l H_2SO_4)	W 100 berries (g)	Glucoacidimetric Index
1	Cabernet Sauvignon (CS)	4406	168.00	5.30	120.00	32
2	Cabernet Sauvignon (CS)	4403	172.60	5.07	113.43	34
3	Cabernet Sauvignon (CS)	4409	183.30	4.35	114.09	42
4	Merlot (M)		225.80	3.04	132.92	74
5	Fetească Neagră (FN)		189.60	3.72	151.30	51

Table 5. Ripening grapes on 14.09.2017

No.	Variety	Parcel	Characteristics of grapes			
			Sugar (g/l)	Total Acidity (g/l H_2SO_4)	W 100 berries (g)	Glucoacidimetric Index
1	Cabernet Sauvignon (CS)	4406	182.3	4.40	119.24	41
2	Cabernet Sauvignon (CS)	4403	202.40	4.63	118.47	44
3	Cabernet Sauvignon (CS)	4409	183.30	4.35	114.09	42
4	Merlot (M)		225.80	3.04	120.98	74
5	Fetească Neagră (FN)		189.60	3.72	157.71	51

From the point of view of grape maturation the following results have been registered:

- Following the dynamics of grape ripeness by setting specific parameters we concluded that the highest accumulation of sugar was recorded in Merlot, which achieved the maximum on the 14.09.2017, 225.8 g/l (Table 5). Over a period of 13 days, Merlot has accumulated 38 g of sugar, a daily average of about 3 g/l. Regarding Cabernet Sauvignon grapes the accumulation are slower, fact that made the grape harvest to start later compared to other black varieties.

- The total acidity expressed in g/l H_2SO_4 with sugar content pursue the ripening evolution of grapes. Acidity reduction is quantitatively the most important biochemical process that occurs in grapes during maturation. In the variety Cabernet Sauvignon we may notice a decrease in acidity from 7.09-6.52 g/l H_2SO_4 (Table 2) up to the target of 4.63-4.35 g/l H_2SO_4 (Table 5). For Merlot variety a significant decrease can be observed up to 3.04 g/l H_2SO_4 (Table 4), which was maintained constant for 7 days - 3.04 g/l H_2SO_4 (Table 5). For Fetească neagră total acidity decrease became constant, from values of 4.92 g/l H_2SO_4 (Table 2) up to 3.72 g/l H_2SO_4 (Table 4), and the value remains the same after a week. The lowest total acidity was registered to Merlot variety.
- Weight of 100 berries is an important parameter in determining the optimum date for harvesting grapes and it must reach the maximum. Both Cabernet Sauvignon and Fetească neagră are reaching increase values from 114.09 to 118.47 g Cabernet Sauvignon (Tables 4 and 5) and 157.71 g Fetească neagră (Table 5). Merlot increased from 97.70 g (Table 3) to 120.98 g (Table 5).
- Values of glucoacidimetric index are between 35-45 which indicates a full maturity of the grapes, as demonstrated by the experimental measurements carried out, so: for the Cabernet Sauvignon and Fetească Neagră the values are starting from 20.32 (Table 2) to 31.41 (Table 3), reaching a maximum at 42-44 for Cabernet Sauvignon (Table 5) and 51 for Fetească neagră (Table 5). Merlot maximum values are recorded to 74 (Table 4 and Table 5).

To highlight the degree maturation of grape varieties under study I prepared the schedule of ripening-indicating the weight evolution of berries, accumulation of sugars and the reduce of the acidity from grape Figure 1 to Figure 4.

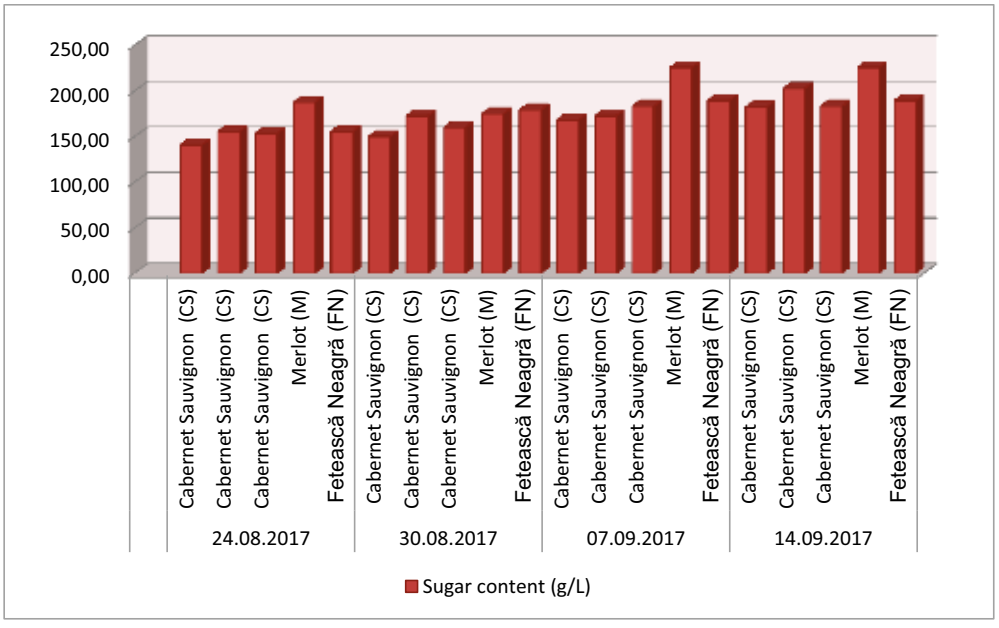

Figure 1. Graphical representation of grapes sugar content (g/l) from august-september 2017

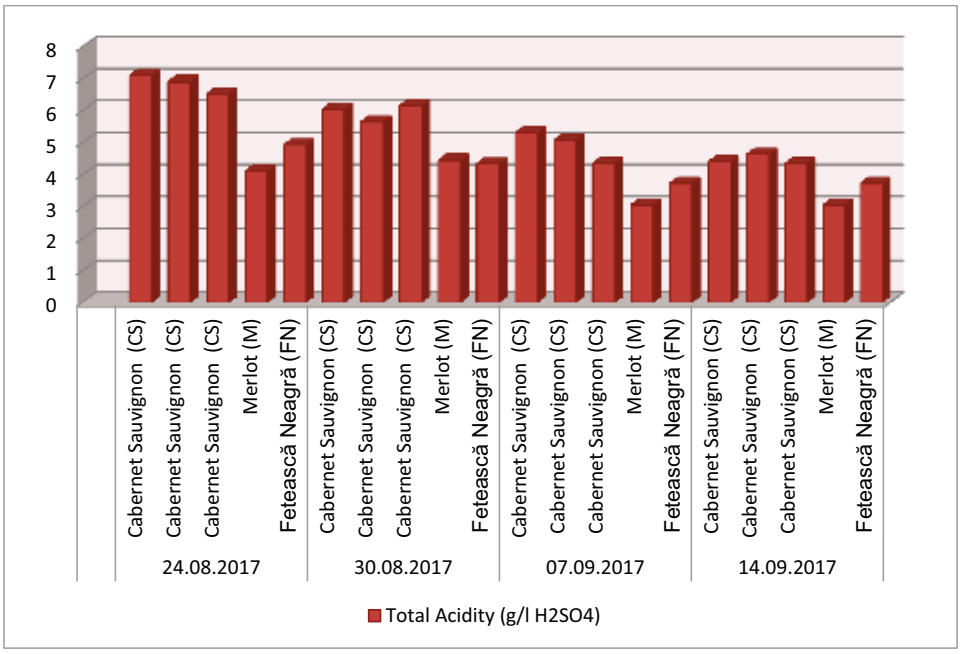

Figure 2. Graphical representation of grapes total acidity (g/l H_2SO_4) from august-september 2017

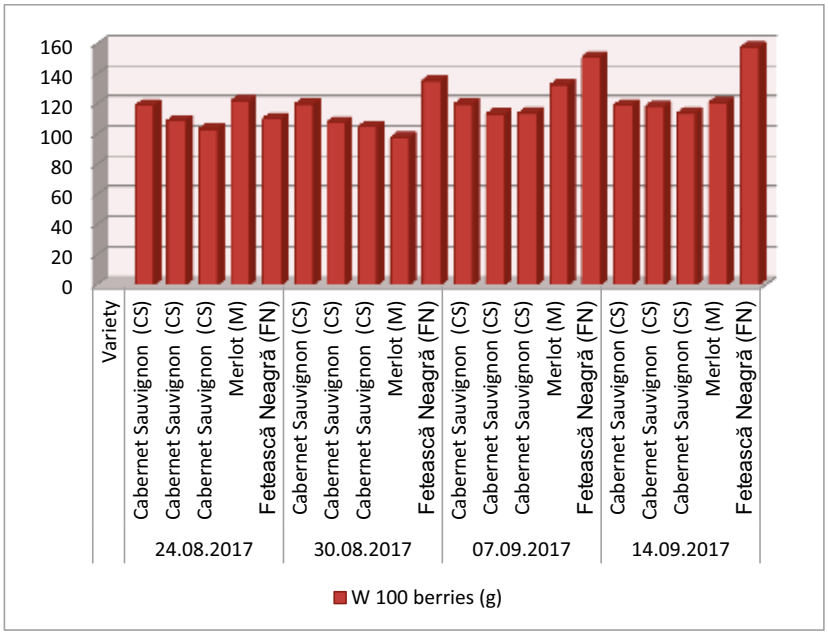

Figure 3. Graphical representation of grapes W 100 berries (g) from august-september 2017

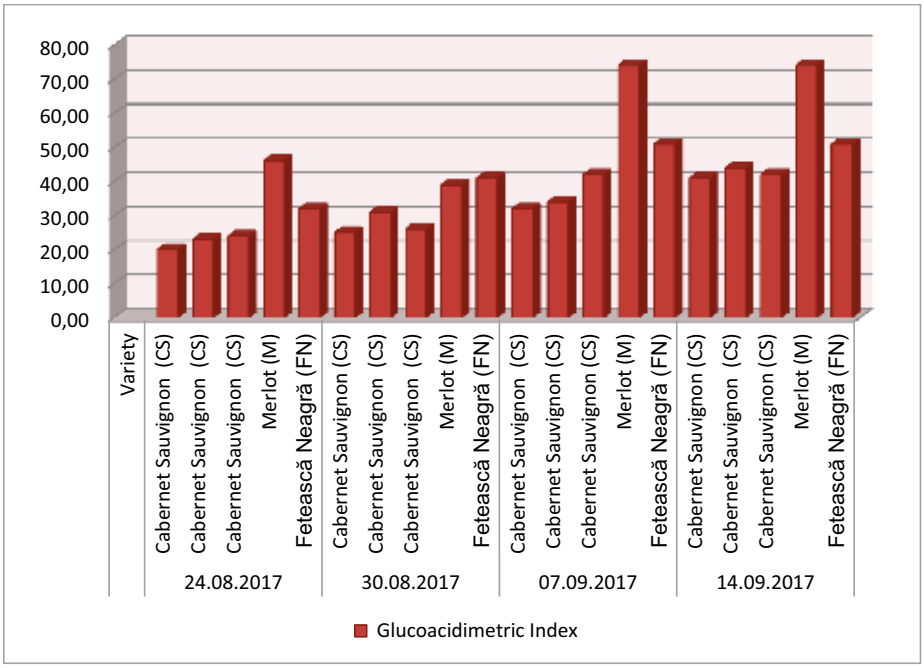

Figure 4. Graphical representation of grapes glucoacidimetric index from august-september 2017

Full maturation of the grapes (MF) is when the grapes reached their maximum weight and curve of evolution begins to descend; grape sugar content is also the maximum (Figure 1) and the curve of evolution of sugar remains stationary for a few days; total acidity decreesed substantially (Figure 2) and evolutionary curve indicating a slow decrease acidity.

In the full ripeness of the grapes the largest as the maximum weight of the grapes is reached (Figure 3). Any delay harvest thereafter translates into loss of production (harvest).

A combination of sugar and acid concentrations are generally used to determine whether the grapes have reached optimum ripeness (Falcao et al., 2008). Environmental factors are important to obtain high quality *V. vinifera* grapes for winemaking. The wine style that a region produces is the result of the specific local climate and soil characteristics. Climatic changes therefore have the potential to bring about changes in wine styles (Falcao et al., 2008).

Grape berries, like other berry fruits, undergo a complex series of physical and biochemical changes during development (Deluc et al., 2007) as we can see from the presented measurements.

The grape berries mass during maturation is correlated with sugar accumulation, availability of water in the soil and atmosphere, and the number of seeds. Smaller fruits ultimately release larger quantities of minerals including potassium, calcium, and magnesium, which greatly influence pH and total titratable acidity. Smaller grapes berry also affect the organoleptic characteristics of the wine due to the release of high quantities of tannins, which are present in large concentrations in the seeds and may turn the wine astringent (Conde et al., 2007).

CONCLUSIONS

Valea Călugărească vineyard centre belongs to a temperate climate, with the most warm month temperature (July) with more than 22ºC and a maximum rainfall in early summer.

Thermal regime is characterized by annual average temperatures of 10.4-10.6ºC and a sum of active temperatures ranging between 3300-4040ºC.

Autumns are generally warm, dry and quite lengthy, which allows carrying out in good conditions of the process of maturation of the grapes and coloring substances accumulation in red wine varieties.

Winters are relatively short, the average temperature of the coldest month of stage (January) being -2.1ºC. Number of days that records average temperatures more than 10ºC varies between 175-226 days/year.

Grapes varieties reach maturation stages according to their biological nature and the evolution of climate conditions of that year.

Grape maturation has three main aspects: technological maturation (refer to the accumulation of sugars in the grapes and to reduce acidity); phenolic ripeness refers to the accumulation of anthocyanins and tannins in grapes; aromatic maturation refers to the accumulation of the primary grape flavors.

Winemaker technologist who is interested in obtaining good quality wines follows the evolution of ripening grapes, in order to determine the best time to harvest.

In red grape varieties, the polyphenol composition can be perceived as the key element that significantly differentiates the final products, considering their specific agro-biological characteristics, in conjunction with the biosynthetic pathways of phenolics. Therefore, the harvest stage, the temperature, the duration of the maceration-fermentation process, and the winemaking technique are among the factors involved in the quality of the resulting product (Palade & Popa, 2018).

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IN VITRO RESEARCH ON THE INHIBITORY EFFECTS OF FENNEL, SAGE AND SEABUCKTHORN ESSENTIAL OILS ON SOME FOOD SPOILAGE FUNGI

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Abstract

Essential oils from natural plants are antimicrobial agents that can be used to control food spoilage and pathogenic food; they have long been used as flavoring agents in beverages and food. The antimicrobial activity of essential oils is attributed to a number of small terpenoids and phenolic compounds that provide antifungal or antibacterial activity. The experimental research carried out in this work focus on the in vitro study of the antimicrobial effect of fennel and sage essential oils on two food spoilage molds with the evaluation of the minimal lethal concentration (MLC). The fungi used in experimental work were Aspergillus niger and Penicillum expansum from Faculty of Biotechnologies collection. Minimum lethal concentrations were determined using a modified disc diffusion method in agar after puncture the fungus in the center of the Petri dishes. Sage and fennel essential oils proved to be the strongest antifungic agents, the minimum volumes that inhibited the growth and development of two fungi ranging between 14 to 19 µL. Seabuckthorn essential oil, even in higher doses of 250-300 µL, did not show antifungal activity.

Key words*: sage oil, fennel oil, seabuckthorn oil, in vitro, modified microdiffusion method, in vitro antimicrobial activity.*

INTRODUCTION

Different chemical and synthetic chemicals have been used in the food industry as antimicrobials to prevent the development of food microorganisms, but the current trend in using preservatives has led to the use of essential oils. Essential oils (EO) have the potential to prevent the development of micro-organisms, which contribute to the degradation of food. Due to the composition of essential plant oils and their high antimicrobial extracts and spectrum, associated with their low toxicity, they are potential natural food preservatives (Conner, 1993).

The essential oil of *S. officinalis* comprises α- and β-t-butone monomers, camphor, 1,8-cineol and borneol, and sometimes higher amounts of sesquiterpene, α-humulin and β-cariopilin. Di- and triterpenes have been found in leaves (Máthé et al., 2007), i.e. manol. There is a high chemical variability among *S. officinalis* essential oils, however, it can be generally argued that the predominant α- and β-thujone constituents (Newall et al., 1996). *S. officinale* seed oil has antimicrobial properties, mainly attributed to the presence of tijons (Bradley, 2006; Newall et al., 1996).

Fennel (*Foeniculum vulgare* Mill*., Apiaceae*) is a well known aromatic plant species. Mature fruits and essential oil of fennel are used as flavoring agents in food products suchas liqueurs, bread, pickle, pastries and cheeses.

The essential oil of fennel are mainly concentrated in the fruits and provide the unique aroma and taste, they are composed of aroma of several monoterpenes and phenylpropanoids. Trans-anethole, often is the most prevalent constituent, counts for the anise taste,fenechone provides the bitterness and estragole (methyl-chavicol) the sweetness. According to some studies esssential oil of fennel and its seed extracts have been reported to have antimicrobial and anticonidic activity (Abed, 2007).

Seabuckthorn is used as a functional food ingredient - the beans are particularly rich in vitamin C and flavonoids. Both soft parts (pulp

and bark) and seeds contain oil and high levels of tocopherols and vegetal sterols (Kallio et al., 2002). Sea buckthorn oil is characterized by a unique fatty acid content compared to other vegetable oils.
This oil contains rare palmitooleic acid (omega-7) which is a component of skin lipids and stimulates regenerative processes in the epidermis and healing of wounds. Seabuckthorn oil contains saturated fatty acids in the form of palmitic acid (30-33%), stearic acid and has a wide range of essential unsaturated fatty acids (UFA), especially so-called PUFAs (polyunsaturated fatty acids); these include alpha-linolenic acid (omega-3), gamma-linolenic acid (omega-6), linoleic acid (omega-6), oleic acid (omega-9) and eicosanoic acid (omega-9).
The research have focused on the *in vitro* study of the antimicrobial effect of fennel, sage and seabuckthorn essential oils on two food spoilage molds (*Aspergillus niger, Penicillium expansum*) with the evaluation of the minimal lethal concentration (MLC).

MATERIALS AND METHODS

Antimicrobial agents and strains

Three types of essential oils from *Foeniculum vulgare*, *Salvia officinalis* and *Hippophae rhamnoides*, purchased from the company Hofigal (Bucharest, Romania), were used to carry out the experiments. Fungi *Penicillum expansum and Aspergillusniger* were provided from collection of Faculty of Biotechnologies, University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania. The fungus cultures were prepared on PDA plate and incubated at 25°C for 7 days.

Antifungal assay

The antifungal activity of essential oils was determined by modified disc diffusion method on PDA and the medium was prepared according to the instructions on the package.
Subsequently, filter paper disks (6 mm Ø; Whatman) were placed on the surface of Petri dishes and impregnated with different quantities of essential oils from 3 µl to 20 µl (Figure 1).
In case of seabuckthorn essential oil was used higher doses of 250-300 µl.

Fig. 1. The distribution of Whatman filter paper disks (Φ = 6 mm) impregnated with EO in the Petri plates

Finally, 2 µl of spore suspension of each fungus culture (the initial concentration is 10^6 ufc/ml) was inoculated on culture medium in the center of the plate. All determinations were performed in three replicates and the results were statistical analysed. The negative control were performed with paper disks with ethanol, used for EO extraction. Also, the positive control were tested. The dishes were sealed with parafilm to prevent the evaporation of essential oils and incubated for 7 days at 25°C. The efficacy of the treatment was evaluated after 7 days of incubation by measuring the diameter of the inhibition zone.

Minimum lethal concentration (MLC) determination

Medium was inoculated with 2 µl spore suspension of strains *Aspergillus niger* or *Penicillum expansum* activated in PDA, to achieve a concentration of 10^6 spores/ml. Filter paper disks with different quantities of essential oils were placed on the surface of the medium. The control sample consisted of a plate with culture media without essential oils. All experiments were conducted in 3 replicates and results were statistical analysed. The plates were incubated for 7 days at 25°C. The colony diameters of fungal species used in treatment and control samples were measured.
The minimum lethal concentration (MLC) was defined as lowest concentration inhibiting visible growth of the tested fungi as the samples were measured.

RESULTS AND DISCUSSIONS

Antifungal activity of fennel, sage and seabuckthorn essential oils was determined against two antifungal strains *Aspergillus niger* and *Penicillium expansum*.

The obtained results showed that the diameter of the colony growth is significantly depended by the dose of EO and the fungal species tested (Figures 2 and 3).

The rate of fungal inhibition is directly proportional to the concentration of tested EO. Result showed that sage and fennel oils have antifungal activity at 19 µl (MLC), after 7 days at a temperature of 25°C, against *Apergillus niger*. In the same conditions, against the development of *Penicillum expansum* the minimum lethal concentration (MLC) was 14 µL for both fennel and sage EO. Following the experimental results, seabuckthorn oil has no antifungal activity, at the same condition of incubation, microorganisms growing normally even at rates of 250-300 µl.

For *Aspergillus niger* at lower concentration (3 µl) fennel EO is stronger than sage EO, as the colony diameter (cm) decreased from 7,3 cm for control group to 5,4 cm, while for sage EO decreased to 6,4 cm.

Also, we noticed that the 50% decrease of colony diameter compared to the control group was generated by 9 µl fennel EO for *P. expansum* and 6-7 µl for *A. niger*.

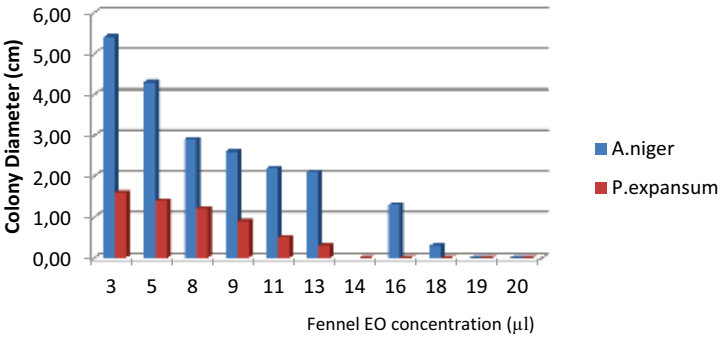

Figure 2. *Aspergillus niger* and *Penicillum expansum* average colony diameter (cm) after 7 days of incubation in PDA medium exposed fennel essential oils (EO)

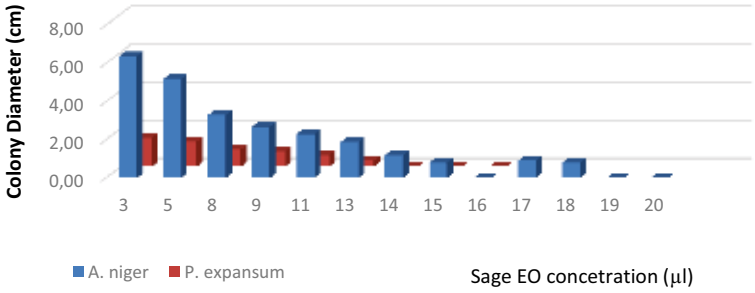

Figure 3. *Aspergillus niger* and *Penicillum expansum* average colony diameter (cm) after 7 days of incubation in PDA medium exposed sage essential oils (EO)

The investigation of the antimicrobial activity of the tested natural essential oils showed that only sage and fennel EO have antifungal effects while seabuckthorn EO even in higher doses do not has antifungal activity (Table 1).

Table 1. *Aspergillus niger* and *Penicillum expansum* colony diameter (cm) after 7 days of incubation in PDA medium exposed seabuckthorn essential oil

Essential oil	Fungal strains	Control group Colony diameter cm/day	Concentration essential oils (µl)	Colony Diameter (cm)/ day 7	Standard deviation (SD)
Seabuckthorn	*Aspergillus niger*	7.5	250	7.2	± 0.51
			300	6.7	± 0.15
	Penicillum expansum	3.5	250	3.4	± 0.12
			300	3.1	± 0.06

CONCLUSIONS

The research showed that two types of essential oils (fennel and sage EO) inhibited the growth of the tested fungi, while in the presence of seabuckthorn oil fungi developed normally and even in high doses 250-300 µl seabuckthorn EO has not antifungal effects. Also, the results of this study showed that the tested oils, sage and respectively fennel EO, have a different pattern of action in their antifungal activity. Both, sage and fennel essential oils, can be used as natural antifungal agents.

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HEAVY METALS AND PHYSICO-CHEMICAL COMPOSITION OF MATERNAL BREAST MILK AND COLOSTRUM

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Abstract

Mother's milk is a very valuable food for newborns, providing all the nutrients necessary for children's health. Colostrum is an important source of biologically active natural components and due to antimicrobial agents, can reduce gastrointestinal infections in newborns. Physical and chemical parameters were analyzed from maternal breast milk and colostrum for five days. The fat content of the colostrum shows the lowest values in the first postpartum days, after which it increases, reaching the highest values on day 5. Heavy metals in colostrum and mother's milk were evaluated considering their area of origin and all the samples were positive regarding Al, Pb, Rb, Sr, Cr. The variation in the metal concentration in maternal breast milk and colostrum could be due to their geographical origin and furtherly can affect the quality of milk.

Key words*: Breast milk, colostrum, heavy metals, physico-chemical composition.*

INTRODUCTION

Breast milk is an important nutritional source for the growth and development of newborns, an extremely complex and highly variable biofluid that has evolved over millennia to nourish infants and protect them from disease whilst, their own immune system matures (Fujita et al., 2012).

Colostrum contains a wide range of biologically active substances, including immunoglobulins, hormones, growth factors and at least 60 enzymes, ensuring immunological and anti-inflammatory properties against many diseases.In this century toxic heavy metals represent a major source of the ever-increasing problem of environmental pollution. Of these, lead (Pb), mercury (Hg), cadmium (Cd), zinc (Zn), copper (Cu) and iron (Fe) are the most common toxic heavy metals that can be present in the human milk, exhibiting harmful impact for the human body.When these elements arrive in the main feed sources, they can easily spread to all food chains, thus affecting the entire food chain.

Besides this aspect, another risk is bioaccumulation of heavy metals in living organisms which are considered primary elements of the food chain. It is observed that the human body, the last key point of the trophic chain retains the highest concentrations of heavy metals due to the bioaccumulation effect (Vinodhini & Narayanan 2008). Lead is a heavy metal naturally occurring in the earth crust and has nobeneficial impact on living organisms. Bioaccumulation of Pb arise in maternal bones, lately being released along with calcium intoher blood,and subsequently providingits pathway into breast milk.

Mercury is a toxic metal, ubiquitous in nature, is secreted in the breast milk from exposed mothers and may affect infant neurodevelopment (Johnson et al., 2009; Evers et al., 2005; Wu et al., 1991).

The development of cadmium (Cd) toxicity is known to be related to zinc (Zn) and copper (Cu) accumulation in the liver, kidney of animals (Sato & Nagai, 1989; Liu et al., 1992, 1994) and humans (Nordberg, 2004; Godt, 2006). During the lactation period, Cd is transported from maternal plasma to mammary gland and secreted into breast milk together with Cu and Zn (Sower et al., 1993; Kalkwarf et al., 1999).

Zinc is an essential trace element that has a complex biological role, playing the main

function in the activation of numerous enzymatic systems that can regulate and degrade pituitary hormones of appetite (Shay & Mangian, 2000).

Copper is a chemical element, soft, malleable, and ductile metal with high thermal and electrical conductivity. Deficiency of Cu, as well as toxicity, may present a concern for children, although precise copper concentrations have not been established yet for this age group (Lönnerdal, 1998).

Iron is the most common element on Earth, mainly forming Earth's outer and inner core. It is the fourth most common element estimated in the Earth's crust. Being a key factor, some of the bacteriostatic properties of human milk are associated with levels and/or bioavailability of this element in breast milk (Rowland et al., 1980).

Breast milk influences the intestinal microflora ensures the structural and functional maturity of mucous membranes, reduces the risk of allergies and autoimmune disorders, and contributes to the proper development of the gastrointestinal, central nervous, endocrine and immune systems (Leon-Cava et al., 2002). Due to the fact that heavy metals and trace elements are found in low concentrations in milk samples, ICP-MS is a suitable and precise analytical technique. In addition, the acid digestion assay has been considered to show the best results in sample preparation (Huynh et al., 2015; Dico et al., 2015). Therefore, the aims of this study were to evaluate the heavy metal profile and physicochemical composition of maternal breast milk and colostrum depending on the reference region. In Cluj, Sălaj, Bistrița-Năsăud, Turda, Baia-Mare, Satu-Mare, Târgu-Mureș, Cehu Silvaniei and Carei no published study has evaluated the levels of heavy metals in breast milk and colostrum. Considering the toxic potential of these compounds and the importance of milk for children development, as a source of protein, fats and other essential nutrients we analyzed the physicochemical parameters to determine the influence of postpartum on protein; fat; lactose content of milk from the mothers used in that study. Moreover, the aim was to observe the differences between these parameters in both milk and colostrum samples and if high-fat content influences heavy metals assimilation.

MATERIALS AND METHODS

Sampling

Twenty milliliters of breast milk and colostrum samples were collected aseptically from each lactating mother using sterile breast pumps, in sterile containers and stored at 4ºC until analyzes were performed. The samples were collected from Romanian localities such as: Cluj, Sălaj, Bistrița-Năsăud, Turda, Baia-Mare, Satu-Mare, Târgu-Mureș, Cehu Silvaniei and Carei.

Characteristics of the Lactating Mothers

In the present study analyzed colostrum and milk samples were collected from a total of 45 women (N = 5) per locality. Colostrum samples were obtained from day 1 to day 5 after postpartum. After three months, milk samples were harvested from the same mothers. The average age of the mothers was ranged between 25-30 years. Healthy mothers were included in the study, mothers suffering from any illnesses or infections were excluded from the present study. They did not smoke or drink alcohol and coffee during pregnancy and breastfeeding period.

Physico-chemical analysis

Lactoscan (Milk analyzer Lactoscan) device was used for physico-chemical analysis. A method previously reported by (Marchis et al., 2018). The following % of parameters were determined: fat, protein and lactose. A number of 5 samples were used for each locality.

Chemicals

The chemicals from the present work were of analytical reagent grade. Hydrochloric acid fuming HCl 37% (Merk, Germany), nitric acid HNO_3 65% (Merk, Germany), ultrapure water, Milli-Q (Millipore, Bedford, MA, USA), Hydrogen peroxide H_2O_2 30% (Merk, Germany), and ICP multi-element standard solution 1000 mg/l (Merk, Darmstadt, Germany).

Quality Assurance

For the quantitative determination of the desired elements, the external calibration method was performed. With the help of interpolation, the concentration of the analyte in the unknown sample can be easily deter-

mined. Therefore, calibrations were performed with multi-element standard solutions (Merk, Darmstadt, Germany) at different concentration levels and then the calibration curves were drawn.

Mineralization of samples

Heavy metals determination was based on an assay previously reported by Coroian et al., (2017). Milk and colostral samples were subjected to microwave digestion with 8 ml Nitric acid (65% HNO_3) and 2 ml Hydrogen peroxide (30% H_2O_2). The milk samples were digested according to the following program: (i) temperature 145°C, pressure 30 (bar), ramp time 5 min, hold time 15 min; (ii) temperature 180°C, pressure 30 (bar), ramp time 1 min, hold time 10 min; (iii) temperature 120°C, pressure 30 (bar), ramp time 1 min, hold time 15 min; (iv) temperature 100°C, pressure 0 (bar), ramp time 1 min, hold time 10 min. Digested samples were allowed to cool to ambient temperature, transferred to polypropylene tubes and diluted to 25 ml of ultrapure water. The samples were filtered through a 0.45 µm cellulose membrane filter. Simultaneously, blank samples were prepared.

Determination of minerals and heavy metals

For determination of heavy metals from colostrum and milk samples a number of 5 samples were used for each locality. Determination of minerals and heavy metals from milk and colostrum samples was evaluated by inductively coupled plasma mass spectrometry or ICP-MS used for the identification and quantification of Cr, Fe, Cu, Na, Mg, Cu, Al, Pb, Rb, Sr, and Zn elements at a concentration level of mg/L or higher concentrations by the appropriate dilution of the sample. Determination of Pb, Zn, Cu, Fe and Cr traces was determined according to SR EN 14082: 2003; LOD - 0.05 mg/l; LOQ -0.1 mg/L. As equipment was used in that study Digestor Berghoff MWS-3+ Microwave (Eningen, Germany) was used followed by ICP-MS ELAN DRC II Perkin-Elmer.

RESULTS AND DISCUSSIONS

Maternal breast milk samples were analyzed for seven heavy metals, Pb, Rb, Cr, Zn, Sr, Cu, Al and four trace elements, Na, Mg, Ca and Fe obtained from different regions of Romania. Heavy metals and essential mineral concentrations from different regions of Romania are described in Table1 and Table 2. Values represent the average of concentrations ± standard deviation.

Table1. Essential mineral concentrations from breast milk

Area/metal	Average ±s.d. (mg/l)			
	Na	Mg	Ca	Fe
Cluj	178.79±2.39	36.57±0.74	640.98±2.47	1.76±0.05
Sălaj	147.18±3.20	29.08±0.71	614.63±2.50	0.71±0.02
Bistrița-Năsăud	71.22±2.83	30.33±1.07	590.88±2.55	0.85±0.04
Turda	158.86±0.93	32.09±0.74	608.94±2.63	0.91±0.01
Baia-Mare	178.07±2.27	25.53±0.51	578.00±4.71	1.20±0.02
Satu-Mare	188.26±2.41	37.71±0.82	555.66±3.85	1.09±0.02
Târgu-Mureș	181.03±3.05	40.36±0.57	380.98±5.98	0.91±0.02
Cehu Silvaniei	157.54±3.75	34.25±0.90	479.90±2.12	1.07±0.03
Carei	166.79±3.29	35.37±0.87	636.78±3.39	1.07±0.01

The highest Na counts from breast milk where observed in the samples from Satu-Mare (188.26±2.41) following in descending order by > Târgu-Mureș (181.03±3.05) and Cluj (178.79±2.39) > Baia-Mare (178.07±2.27) > Carei (166.79±3.29) > Turda (158.86±0.93) > Cehu Silvaniei (157.54±3.75) > Sălaj (147.18±3.20) and Bistrița-Năsăud (71.22±2.83) mg/l, respectively. Magnesium levels from samples acquired from Târgu-Mureș presented elevated values compared with another regions. Altun et al. (2018) using ICP-MS noticed higher values of sodium compared with our results and the values ranged between (44.7-1703) mg/l in breast milk samples from Turkey. According to Björklund et al. 2012 Na presented increased concentration 217±77 mg/l compared to our results. Similar concentrations of Mg was found in the milk from Satu-Mare (37.71±0.82), Cluj (36.57±0.74), Carei (35.37±0.87) and Cehu Silvaniei (34.25±0.90). Our results are in line with those reported by Altun et al. (2018) and Björklund et al. (2012) with the Mg mean values of 32.6±15.5 and 28±4.8 mg/l. The most decreased values of magnesium parameter resulted in Baia-Mare (25.53±0.51) and Sălaj (29.08±0.71) mg/l samples.

One of the most important mineral for skeletal system development and namely calcium presented high values in the area of Cluj (640.98±2.47) followed by Carei (636.78±3.39) > Sălaj (614.63±2.50) > Turda (608.94±2.63) >

Bistrița-Năsăud (590.88±2.55) > Baia-Mare (578.00±4.71) > Satu-Mare (555.66±3.85) > Cehu Silvaniei (479.90±2.12) and Târgu-Mureș (380.98±5.98), mg/l, respectively. Ca levels from Swedish and Turkish mother milk demonstrated lesser concentrations compared to our results, between 305±45 and 193±53.2 mg/l, respectively (Altun et al., 2018; Björklund et al., 2012). Iron is a transition metal, playing an important key-role in human metabolism and is responsible for oxygen transport and oxygen storage in the muscular system. Fe showed increased concentrations in maternal breast milk from Cluj (1.76±0.05) and similar values among the samples were found in samples from Baia-Mare (1.20±0.02), Satu-Mare (1.09±0.02), Cehu Silvaniei (1.07±0.03) and Carei (1.07±0.01). In addition, iron content from Târgu-Mureș (0.91±0.02) and Turda (0.91±0.01) specimens demonstrated a similarity between values. Moreover, minimal concentration of Fe was observed in samples from Sălaj (0.71±0.02) and Bistrița-Năsăud (0.85±0.04), mg/l. Futhermore, resulted Fe values in breast milk are higher than concentrations reported in studies from Australia (Mohd-Taufek et al., 2016), Sweeden (Björklund et al., 2012), and Turkey (Altun et al., 2018), 47±99, 339±134 µg/l and 1.65±1.43 mg/l, respectively.

Table 2. Heavy metals concentrations from maternal milk samples

Area	Average ±s.d. (mg/l)						
	Pb	Rb	Cr	Sr	Cu	Zn	Al
Cluj	0.04±0.01	1.03±0.04	0.30±0.01	0.22±0.03	0.97±0.08	1.56±0.03	1.87±0.12
Sălaj	0.08±0.00	0.90±0.02	0.41±0.02	0.27±0.02	1.87±0.06	1.33±0.03	2.20±0.15
Bistrița-Năsăud	0.01±0.00	1.09±0.01	0.20±0.01	0.28±0.03	0.05±0.01	0.18±0.03	1.61±0.18
Turda	0.03±0.01	1.12±0.01	0.57±0.02	0.45±0.03	0.64±0.16	0.86±0.03	2.93±0.28
Baia-Mare	0.05±0.01	1.13±0.02	0.76±0.03	0.56±0.03	2.87±0.08	1.13±0.03	4.97±0.05
Satu-Mare	0.06±0.01	1.16±0.02	0.80±0.01	0.50±0.02	1.24±0.09	1.33±0.06	5.79±0.20
Târgu-Mureș	0.03±0.14	1.15±0.03	0.88±0.03	0.38±0.01	2.25±0.11	1.53±0.03	4.03±0.06
CehuSilvaniei	0.02±0.01	1.00±0.08	0.36±0.01	0.39±0.01	0.59±0.08	0.95±0.03	3.97±0.23
Carei	0.09±0.02	1.20±0.04	0.60±0.01	0.40±0.01	1.08±0.10	1.05±0.03	4.02±0.06

From the detected concentrations of toxic heavy metals, lead was found in relative reduced amounts in breast maternal milk samples compared among other metals. The highest concentration of Pb mg/l was identified in maternal milk from Carei (0.09±0.02) followed by reduced amounts in the areas of > Sălaj (0.08±0.00) and > Satu-Mare (0.06±0.01) > Baia-Mare (0.05±0.01) > Cluj (0.04±0.01) > Târgu-Mureș (0.03±0.14) > Turda (0.03±0.01) > Cehu Silvaniei (0.02±0.01) > Bistrița Năsăud (0.01±0.00), respectively. According to World Health Organisation, the acceptable Pb concentrations in breast milk are reported to reach values between 2 and 5 ng/g (Choi et al., 2008). Swedish maternal breast milk samples demonstrated reduced levels of Pb (1.5±90 µg/l) compared to ours. Chao et al. (2014) findings showed that Pb levels varied between different lactation stages indicating values ranged between (0.45 and 22.36) ng/ml. Rb did not present significant variations among the samples and comprise the values in ranged between 0.90-1.20 mg/l. Values of Rb were reported in a study of Björklund et al. (2012) showed decreased concentrations (714±108 µg/l) of Rb compared to those obtained by us. Cr levels of breast milk varied between 0.88 and 0.20 mg/l. Recommended Cr intakes in maternal breast milk for infants aged from < 6 months should comprise doses ranged from 10-40 µg (Anderson, 1998). Elevated amounts of chromium were find areas such as Târgu-Mureș (0.88±0.03) and Satu-Mare (0.80±0.01) and the most reduced in samples from Bistrița-Năsăud (0.20±0.01) and Cluj (0.30±0.01) (mg/l). In contrast to our result, Cr was find in lower concentrations in the samples analyzed by Björklund et al. (2012) with a mean of 0.30±0.27 µg/l. Maternal breast milk strontium

concentrations values resulted between 0.22 and 0.56 mg/l. Lowest Sr content was detected in Cluj (0.22±0.03) followed by Sălaj (0.27±0.02) and Bistrița-Năsăud (0.28±0.03). Lower levels of Sr from human milk samples are reported in Swedish mother milk specimens comprising means of 33±12 µg/l.

However, increased Sr numbers were detected in Baia-Mare (0.56±0.03) followed by a slight decrease in Satu-Mare (0.50±0.02)>Turda (0.45±0.03) > Cehu Silvaniei (0.39±0.01) > Târgu-Mureș (0.38±0.01) > Bistrița-Năsăud (0.28±0.03) > Sălaj (0.27±0.02) > and Cluj (0.22±0.03) (mg/l), respectively. Copper plays an important role in the processes of heme and hemoglobin biosynthesis. Therefore, its deficiency as well as iron can cause anemia. Zinc stimulates the hormonal activity of the pituitary gland contributing to the normal development of the body increasing its weight.

However, deficiency of this trace element leads to growth retardation and weight loss. Greatly increased copper values were noticed in the samples from Baia-Mare (2.87±0.08) and Târgu-Mureș (2.25±0.11). Intermediate values were indicated for areas such as Sălaj (1.87±0.06) and Satu-Mare (1.24±0.09) followed by decreased concentrations in Carei (1.08±0.10) > Cluj (0.97±0.08) > Turda (0.64±0.16) > Cehu Silvaniei (0.59±0.08) and Bistrița-Năsăud (0.05±0.01) (mg/l), with the minimal amount of Cu. Copper concentrations from the present study are higher compared to results reported by others (Mohd-Taufek et al., 2016; Björklund et al., 2012; Altun et al., 2018). Specimens from the region Cehu Silvaniei exhibit similar concentrations of Cu (0.59±0.08) compared to the region of Şanlıurfa from Turkey (0.54±0.46), mg/l. According to European Union Scientific Food Committee, the recommended doses of Cu and Zn for 6-11 month children are 0.3 mg/d and 4.0 mg/d, respectively (Mandic et al., 1996).

Another important mineral indicator, zinc, was detected in predominant concentrations in samples from Cluj (1.56±0.03) and Târgu-Mureș (1.53±0.03) (mg/l). Sălaj and Satu-Mare Zn values revealed a similarity among concentrations (1.33±0.03), (1.33±0.06), respectively. Decreased Zn values were found in Bistrița-Năsăud (0.18±0.03) milk samples. Cu and Zn concentrations from human milk samples are reported by various authors (Mohd-Taufek et al., 2016; Björklund et al., 2012; Altun et al., 2018).

The results regarding Zn concentrations are in concordance with Taufek et al. (2016) that obtained similar concentrations of this element in milks samples (1390±211 µg/l). Slightly elevations of Zn are presented in the study of Björklund et al. (2012) and Altun et al. (2018) with the means of 3471±979 µg/l and 2.89±3.23 mg/l. According to the study of Levi et al. (2018) milk obtained from Argentina mothers was subjected to acid digestion through ICP-MS showing median means for elements such as Na, Mg, Ca, Zn as follows 139/101 (28-1360), 31/30 (15-52), 247/246 (151-370) and 1.7/1.4 (0.17-7.9) (ng/g), respectively.

Predominantly high concentrations of Al were detected in samples from Satu-Mare (5.79±0.20), Baia-Mare (4.97±0.05), Târgu-Mureș (4.03±0.06) and Carei (4.02±0.06). Aluminum is exerting nephrotoxic and hepatotoxic potential at low concentrations leading to poisoning in children and adults Chao et al. (2014). Average concentrations of aluminum were noticed in breast milk from Cehu Silvaniei (3.97±0.23), Turda (2.93±0.28) and Sălaj (2.20±0.15) (mg/l), respectively. The lowest values for that parameter was registred in Bistrița-Năsăud (1.61±0.18) and Cluj samples (1.87±0.12) (mg/l), respectively. Aluminum and lead are heavy metals with neurotoxic potential, and are responsible to induce nervous system disorders in children Rebelo and Caldas (2016). Further, lesser concentrations are reported in maternal breast milk specimens from Swedish healthy mothers with a mean of 185±584 µg/l.

Cadmium, lead, aluminum, and copper are heavy metals presenting mainly negative impact on the development of biological organisms, and according to governmental regulations, the limits of these elements in food should be restricted. Contamination of breast milk with heavy metals from Lebanon showed different loads of arsenic (2.36±1.95), cadmium (0.87±1.18) and lead (18.17±13.31) (µg/l) (Bassil et al., 2018).

Concentrations of Na, Mg and Ca from colostrum are represented in Figure 1. Sodium concentration from maternal colostrum varied

between 142.7 and 189.2 mg/l. Bistrița-Năsăud concentrations demonstrated the lowest Na content (88.56 mg/l) compare to samples from other regions. Additionally, a similar fact was observed in our breast milk samples from the same region (71.22 mg/l). Calcium values from maternal colostrum were in the range of 521.3-632.1 mg/l. Baia-Mare and Târgu-Mureș samples revealed the highest content of Ca.

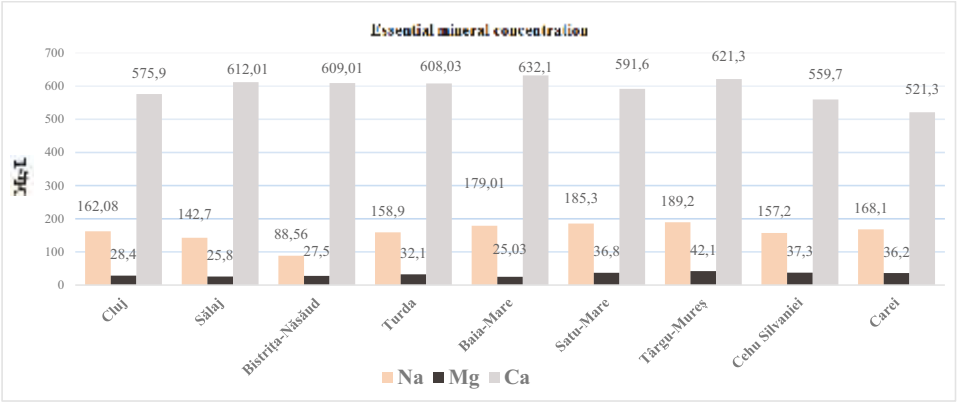

Figure 1. Values for essetial minerals obtained from colostrum depending on the reference region

Interestingly, by using ICP-MS, our study revealed elevated calcium contents from maternal milk samples and colostrum and that may be attributed due to Romanian cultural gastronomy consisted of different traditional types of cheeses and other dairy products. Magnesium from colostral specimens presented values in the range between 25.03 and 42.1 mg/l. Moreover, increased numbers were registered in the colostrum samples obtained from Târgu-Mureș and the lowest in the region of Baia-Mare. These results can be correlated with Mg concentrations that were acquired from maternal breast milk samples with the similar values in a range 25.53 and 40.36 mg/l, respectively.

Figure 2 indicates the content of heavy metals, Pb, Rb, Cr, Sr, Cu, Zn Al, and Fe from maternal colostrum depending on the reference region. From the analyzed heavy metals from colostrum, lead being the most toxic was in the range of 0.01-0.09 mg/l. Pb values in colostrum from Cluj indicated lowest concentration. However, samples from other areas exhibit elevated concentrations of Pb. Hence, Pb concentration from the present study was higher in colostrum compared to breast milk samples, a similar characteristic remarked in a study of Chao et al. (2014). Lead concentrations in Croatian (non-smoker) transitional milk was 3.4 µg/kg dry matter; mature milk showed lower values of 2.6 and colostrum Pb concentrations were predominant, 5.0 µg/kg dry matter, respectively (Letinić et al., 2016).

Suciu et al. (2008) results denoted that Câmpia Turzii (Turda) region is considered least contaminated area showing Cu, Cr, Pb, values varying from 15.70 to 63.20 ppm, 20.70 to 62.40 ppm and 27.00 to 868.60 ppm, respectively. Rb and Cr in mother colostrum samples collected from different regions of Romania presented levels ranged between 0.83-1.36 and 0.26-1.02 mg/l, respectively. Highest rubidium concentrations detected in colostrum were noticed in Turda samples, and chromium increased concentrations are attributed to Sălaj samples. The excessive level of Sr among all samples was 0.66 mg/l, identified in colostrum from Baia-Mare. Instead, the lowest and the highest concentrations of Cu from the studied localities were observed in Bistrița-Năsăud 0.03 mg/kg and Baia-Mare 2.83 mg/kg

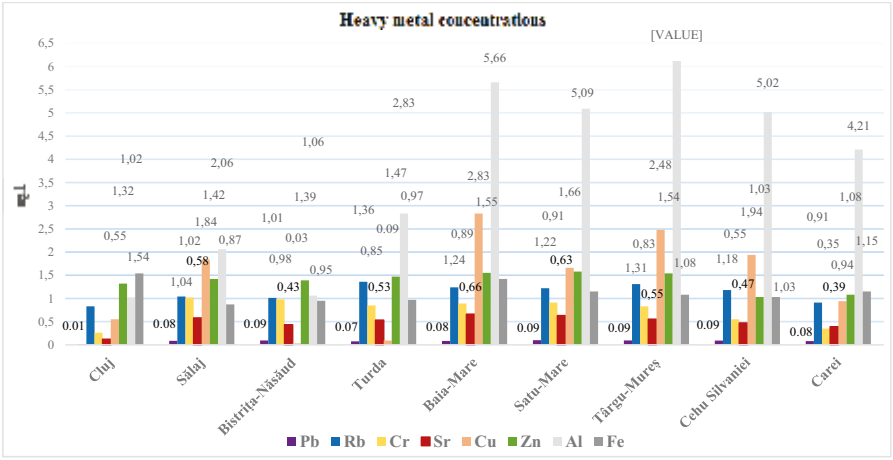

Figure 2. Heavy metal concentrations obtained from colostrum depending on the reference region

Rodna (Bistrița-Năsăud) samples from mining area soil presented Cu variations among the means from 8.5 to 108 mg/kg and Pb 3.5-4712 mg/kg (Nimirceag, 2012). Interestingly, >90% of samples had increased lead concentrations above the normal values. Concentrations of Zn in the samples with the highest concentration was 1.58 (Satu-Mare), followed in order by reduced concentrations in Baia-Mare (1.55) >Târgu-Mureș (1.54) > Turda (1.47) > Sălaj (1.42) > Bistrița-Năsăud (1.39) > Cluj (1.32) >Carei (1.08) and Cehu Silvaniei (1.03) mg/l, respectively. In a Ph.D. thesis reported soil samples from Zlatna (central Transilvania) locality showed numerical variations for Pb (160.5-563), Cd (0.94-3.28), Cu (111-446.5) and Zn (84-576.5) (mg/kg) (Buzgău, 2013). According to Geana et al. (2011) study, Cr concentrations from soil samples of Sălaj showed means of 16.36 and for Cluj-Tarnita area 83.41 mg/kg. Zinc amounts for Sălaj samples presented values of 22.2 and 150.44 mg/kg for Cluj-Tarnita locality. Moreover, for either locality Pb varied between 1.44 and 9.88 mg/kg. We found that aluminum concentrations were the highest in colostrum from Târgu-Mureș (6.12) followed by Baia-Mare (5.66). Senila et al. (2011) remarked that Baia-Mare area is highly polluted by heavy metals. Baia-Mare is considered polluted due to processing wastes from non-metallic ores and anthropogenic activities such as Pb, Zn and Cu refineries Damian et al. (2008). Lowest values of Al mg/l are represented in Cluj (1.02) and Bistrița-Năsăud (1.06) colostral specimens. Iron concentration in colostrum was not much different among the samples and was between the level of 0.87 and 1.57 mg/l. In both types of samples, predominant Fe values were detected in Cluj. On another hand, decreased amounts in either sample were observed in Sălaj region. Heavy metal content measurements obtained from Rovinari (South-West of Romania) soil samples between 2009 and 2010 presented values for copper depending on sampling depths varied from 11.4 to 157.4 mg/kg. Most elevated Cu concentration was 157.4 mg/kg, being over the alert threshold. Soil zinc concentrations presented differences in 2009 between 38.6 and 118.4 and for 2010 values from 25.8 to 91.8 mg/kg. Regarding Pb, in 2009 the values ranged from 3.2 to 20.8 and for 2010 the means were 4.4 to 11.0 mg/kg.

Figure 3 indicates means ± standard deviation, for fat content analyzed from mother colostrum during the days 1-5.

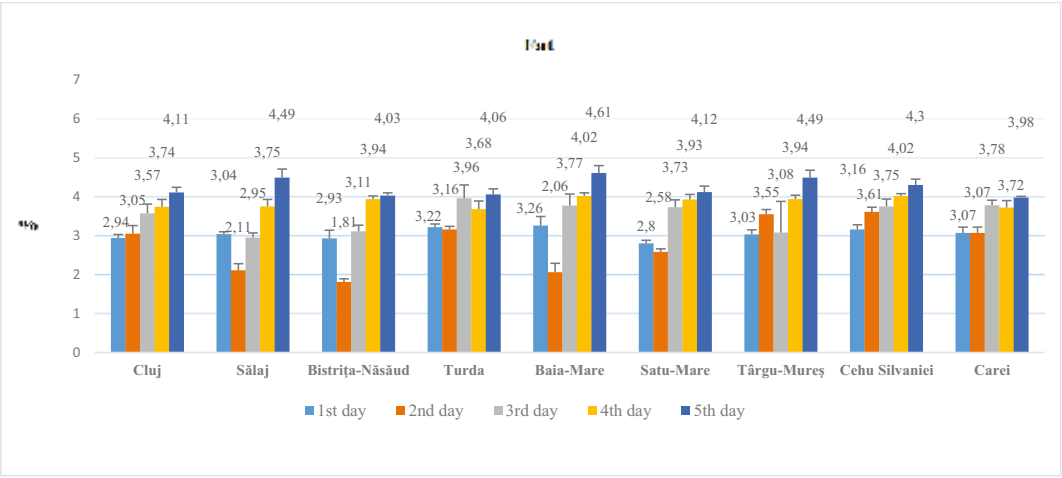

Figure 3. Fat content from maternal colostrum depending on the region

The fat content varied between 3.046±0.06 for the 1st day (Sălaj) and 4.49±0.22 g/100 ml for 5th day, postpartum period.

It can be noticed that regardless of the sampling area, the fat content shows the lowest mean values on day 1 (2.80-3.26), after which this parameter gradually increases reaching the highest values on day 4 (3.68-4.02) and day 5 postpartum (3.98-4.61) g/100 ml.

The fat content for the colostrum period is within the characteristic values for the postpartum period.

Lactose content evaluated from mother colostrum during the days 1-5 is shown in Figure 4. The lactose content is influenced by the colostral period, a physiological phenomenon reported by Shi et al. (2011) that observes the highest lactose levels during the colostral period and decreases in transitional milk and reaching the lowest values in mature milk. Proteins and lipids are behaving in a similar way.

Protein content obtained from maternal colostrum during the days 1-5 is depicted in Figure 5.

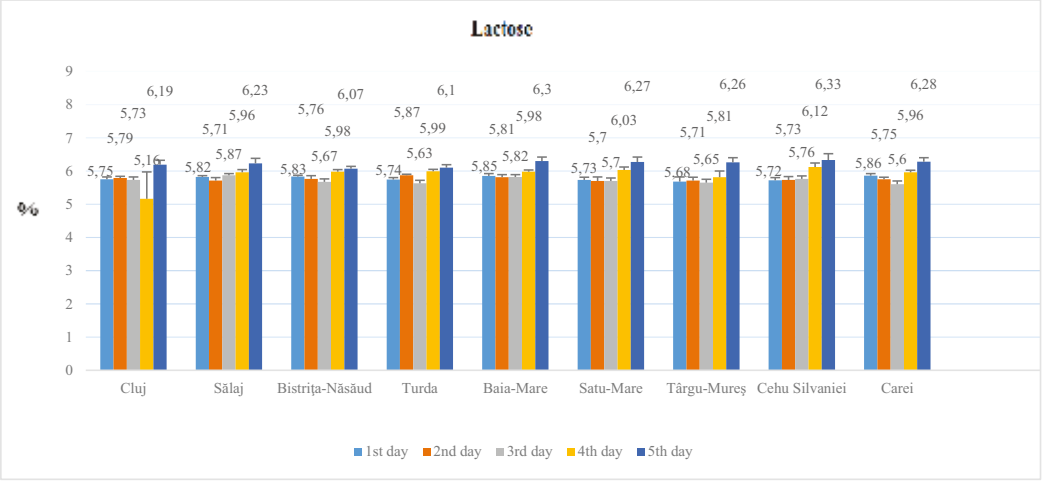

Figure 4. Lactose content from maternal colostrum

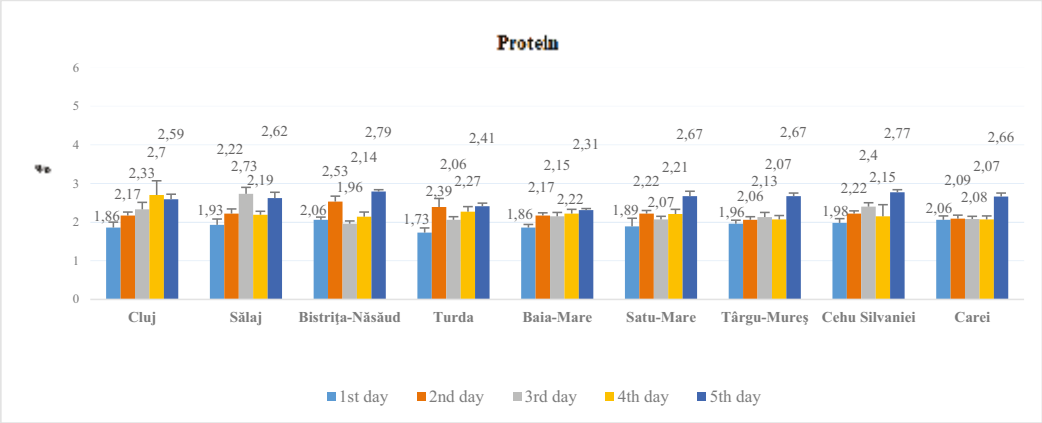

Figure 4. Protein content from maternal colostrum

Protein composition resulted in maternal colostrum, behaves as lactose and fat, previously described parameters. During the 1st-day the protein profile showed lesser values of means (1.73 and 2.06%), compared with the other days. Notably, regardless of regions between the 2nd (2.06-2.53), 3rd (1.96-2.73) and 4th-day (2.07-2.70), the protein profile moderately elevates reaching the highest concentrations on day 5 (2.31-2.79%), respectively. Moreover, samples from Cehu Silvaniei (2.77±0.07) and Bistrița-Năsăud (2.79±0.05) exhibited the highest protein contents compared to other regions during postpartum, while Turda (2.41±0.08) and Baia-Mare (2.31±0.04) %, presented decreased mean values.

Figure 6 indicates the total average of values ± standard deviation of physico-chemical parameters from all the localities described in the present study.

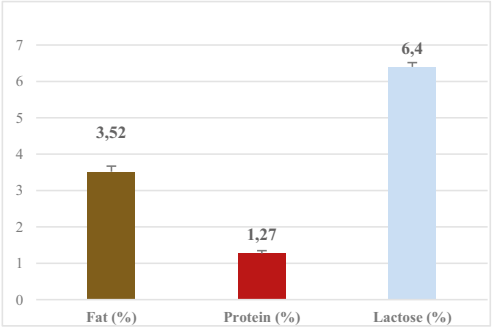

Figure 6. Basic physico-chemical composition of maternal milk

From the present study, it can be observed that maternal breast milk exhibits lower concentrations of protein (1.27±0.07) and fat (3.52±0.15), compared to colostrum nutritional parameters.

Moreover, lactose from milk samples showed an increase compared to maternal colostrum samples.

Maternal breast milk samples from collected from Indian women presented a median fat composition of 3.02% slightly decreased compared to our average value of 3.52±0.15, Bedi et al. (2013).

CONCLUSIONS

The physico-chemical parameters of colostrum are influenced by postpartum, as can be observed on day 5 of postpartum when the values are reaching the highest increases. Maternal milk and colostrum have a balanced compositional and nutritive matrix for the development of young children. Lactose was the component with the highest level in the mother's milk. Hence, it may be concluded that the variation in the metal concentration in maternal breast milk and colostrum could be due to their geographical origin. The high level of heavy metals could potentially affect the breast milk and therefore the infant health.

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APPLICATION OF HURDLE TECHNOLOGY AS A NOVEL APPROACH TO NEW DIETARY FIG-BASED PRODUCTS DEVELOPMENT IN RURAL AREAS OF ALBANIA

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Abstract

Application of hurdle technology even more is getting a special attention from food scientist and producers, due to its economical convenience, simplicity and flexibility in use. In Albania fig fruit is widespread, usually sold in summer in local markets as fresh fruit, and during the year could be found as dried figs, which is traditionally sun-dried, and a small amount is preserved with addition of sugar. A big challenge still facing rural areas in Albania is the lack of preservation methods and capacities, the high perishable nature of fig and the supply of local markets with imported fig products, which could lead in a stock creation of country produce. So the application of hurdle technology was the aim of this work, as a novel approach to new dietary dried fig-based products development, with the attempt to minimize stock creation of dried figs produce, and market diversification with a range of products competing imported fig products. This study may serve as suggestions for further development of dried fig-based products, also may have an impact for rural areas development in Albania.

Key words*: dried fig-based product development, hurdle technology, rural development.*

INTRODUCTION

Nutrition is strongly linked with people health and longevity, besides the need for consuming to meet minimal requirements for energy, and a good health is an asset for everyone.

One of the fruit that is known from antiquity as symbol of longevity is fig, being an excellent source of minerals, vitamins, dietary fibres and amino acids, and it's free of fat and cholesterol (Solomon et al., 2006; Veberic et al., 2008). Have potential health-promoting constituents as phytosterols (Jeong & Lachance, 2001), carotenoid (Su et al., 2002), anthocyanins (Solomon et al., 2008; Del Caro & Piga, 2008; Duenas et al., 2008) and polyphenols (Del Caro & Piga, 2008; Veberic et al., 2008).

The tree is deciduous in nature, it is earliest cultivated and ranked third among fruit trees cultivated in Albania, and is well-grown in regions of Berat, Tirana, Elbasan, Shkodra, Himara etc. (Hoxha & Kongoli, 2018).

Fruit is utilized by the rural people of the fig growing regions in Albania, where most of the local production is consumed fresh while the remainder goes for drying figs with traditional technique of sun-drying, also preservation with addition of sugar to jam and marmalade processing, also another a typical product produced is "*gliko*".

Hurdle technology is even more in the focus of food scientist and industries for application, due to its economical convenience, simplicity and flexibility in use, other than providing nutritious, tasty and stable products, and that is why is considered here as a novel approach for development of new products.

As promoters to our work for converting dried figs into new value added products with enhanced shelf-life, were the challenges faced yearly by farmers of rural fig growing areas, the lack of fig preservation techniques and methods, waste of fig due to high perishable nature, stock creation of dried fig, need for its utilization during winter months, in the meantime helping in development of rural areas, needs for diversified products and competing imported products in the market etc.

So the actual work may represent a preliminary contribution, filling the gap so far as no scientific work or documented data are available for development of new dietary dried fig-based products and suggesting their recipes for further implementation.

The actual study may serve as a true reflection of the importance and potential of this crop,

and would provide sustainable means to the development of rural fig growing areas in Albania, also would be useful to farmers and industries for utilization, adoption and development of new minimally processes based on hurdle technology application as novel approach to preserve figs during the winter months.

The actual conducted work, beside the aim to develop tasty, healthy, and nutritious new dietary dried fig-based products with added value, served as preliminary work for testing the product samples in-house and to a set of customers, prior launching in the market and commercial production.

MATERIALS AND METHODS

New product development

In this study was used 'Roshnik' sundried fig variety, which is an autochthonous variety mostly grown in Berat region, and well known for its suitability for drying.

The combination of preserving factors were based on the nature of dried fig for processing and converting into new value added products with minimal processes (Figure 1).

Collected and sorted dried figs, after were cleaned and washed, were blanched by dipping the fruits into hot water (temperature 88 ± 2°C) (1: 1 ratio) for 1 min and then cutting and grinding.

The dried fig grinded was thoroughly mixed in an open pan with continuous stirring with other ingredients. For recipes development were used different proportions of dried figs, including other locally available dried fruits and additives selected with intend to enhance nutritional, quality parameters, to prevent microorganism growth, and having low cost in the same time. Ingredient used were as follow: dried fig (59.5-96.5%), dried cranberry (till 35%), dried apricot (21-25%), walnut (7.3-22%), hazelnut (till 2%), unsweetened cocoa powder (till 1.5%), coconut flour (till 1.5%), oats (till 3.7%), finely grated orange zest (0.3-3.3%), grounded cinnamon (till 0.17%), grounded clove (till 0.017%), citric acid (lemon or orange juice) (till 3%), and vanilla extract (0.04-0.85%). In some of the products were used coats of: sesame seeds, coconut flour, pumpkin seeds and walnut.

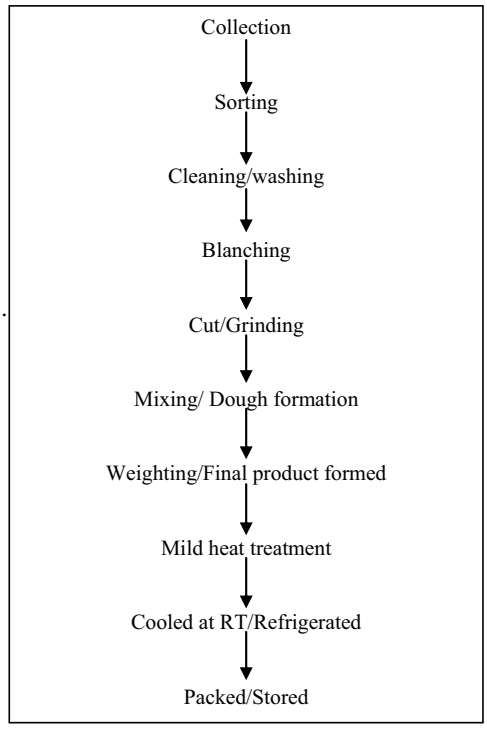

Figure 1. Flow chart for the development of new dietary dried fig-based products

Preparation of 11 recipes for each product from FP_0 to FP_{10} (are coded FP: fig-based product, and numbered in subscript to distinguish each product recipe from another).

Mixing process lasted 1 ± 0.5 h, till formation of dough, and immediately the final products were differently shaped, after equally weighted in portions of 25 ± 0.5 g and 100 ± 0.5 g.

Mild heat treatment (for 7 ± 1 h at 55 ± 1°C) was applied for further moisture content reduction. The final products after cooled to room temperature were kept in refrigerator until packaging and/or further quality parameters evaluation.

The final products packed with plastic wrap, were stored at ambient conditions.

Quality parameters evaluation

Dried fig fruits were evaluated for quality parameters, besides which was content of total ash (AOAC, ref. 942.05), total protein (AOAC, ref. 976.05), total fat (AOAC, ref. 963.15), total carbohydrates (Hedge & Hofreiter, 1962), reducing sugars (AOAC, ref. 925.36), crude fibre (AOAC, ref. 962.09), and the energy

expressed in kcal, was calculated using Atwater factors.

The methods used for evaluation of quality parameters of new developed dried fig-based products, were for moisture AOAC (ref. 934.06), for titratable acidity AOAC (ref. 942.15), and for pH AOAC (ref. 981.12).

Sensory evaluation

The sensory characteristics of the new products, including: appearance, aroma, color, texture, flavor, and overall acceptability, were evaluated by 10 semi trained panel members, in order to get the most acceptable level from the new developed recipes. To rate the products, to each panel member was given a sensory evaluation form of composite scoring (20 point for each characteristic), also was used fresh water for rinsing the mouth prior tasting the next sample.

Data processing

The analysis of Mean, Standard Deviation and bar diagram was used for the result obtained for each determined parameter, at least in three independent replicates.

RESULTS AND DISCUSSIONS

Data obtained from evaluation of quality attributes of dried fig fruit (Table 1), showed that dried fig variety 'Roshnik' possess nutrient content in such amounts that its consumption may provide a good source of energy till 298.57 kcal per 100 g product. Based on obtained results, dried fig evaluated could serves as a good source of nutrients which might play a beneficial role for a good health, especially for mineral content and fiber content respectively for total ash till 2.60 g/100 g and fiber till 8.85 g/100 g, which have resulted in greater amounts compared to similar study of Vora et al. (2017).

From the results for evaluated quality parameters, is noted that dried fig 'Roshnik' variety has attributes that make it suitable to be transformed into new products with added value, offering thus products with high nutritive value. Furthermore development of new products, other as an effective way for utilization of dried figs with added value during winter months, have further strengths, as provides gluten free, with no added sugar, and healthy products.

Table 1. Quality parameters of dried fig, as the main ingredient of new products developed

Quality parameters	Mean	SD
Carbohydrate (g/100 g)	69.34	0.107
Protein (g/100 g)	2.66	0.011
Fat (g/100 g)	1.17	0.001
Fiber (g/100 g)	8.85	0.023
Ash (g/100 g)	2.60	0.015
Reducing sugar (g/100 g)	59.58	0.081
Energy (kcal)	298.57	0.435

The results of quality attributes evaluated for new dried fig-based products developed, showed that for moisture content of new products ranged 14.85-20.98% (Figure 2), based on these values could be considered as safe interval for preserved product, but other supporting microbiological determinations are foreseen to be performed, as this is an ongoing study. Between different recipes, additions of other ingredients had an impact in lowering moisture content compared to FP_0, which is the product with the highest proportion of fig (almost 100%), and here we have considered as control for other recipes. From result it is noted that different ingredient had different impact in moisture content, especially in the case of addition of walnuts product FP_7, also for coated products with sesame seeds, pumpkin seeds, walnuts resulted to have lower moisture content.

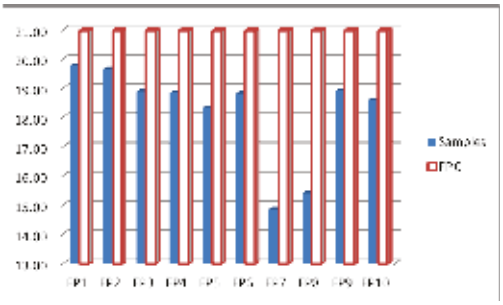

Figure 2. Moisture content of new products

Referring to Figure 2 products FP_7 and FP_8 results with lowest moisture content, maybe due to the ingredient used in their recipes, which can have more affinity to bind water,

and as result is expected a reduced water activity, which is one of the most important hurdle factors.

Titratable acidity parameter was found to be in the range 1.53-2.80% citric acid (Figure 3). Between products FP_9 resulted to have the highest total acidity values, maybe due to the presence in product of dried cranberry. Also, with an impact in total acidity value rising showed orange zest used in two recipes for product FP_4 and FP_{10}. Dried apricot used in product FP_8 and FP_{10}, showed to have similar effect in total acidity content, whereas there were not noted significant differences between other products.

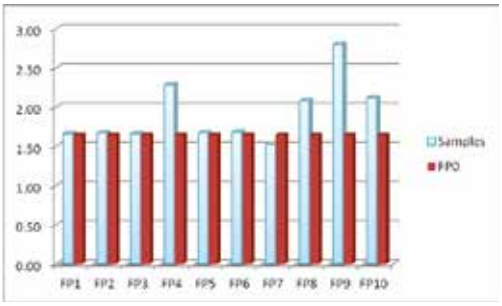

Figure 3. Titratable acidity content of new products

The pH of products ranged from 3.47 to 4.44 (Figure 4), which could considered as safe interval for microorganism growing, and is one of the most important hurdles for food preservation. The low pH value might be influenced by the presence of added lemon and/or orange juice, besides other ingredient used. Among different products, the highest pH content had product FP_7 (fig plus walnut), while with the lowest pH content had FP_9 (fig plus dried cranberry).

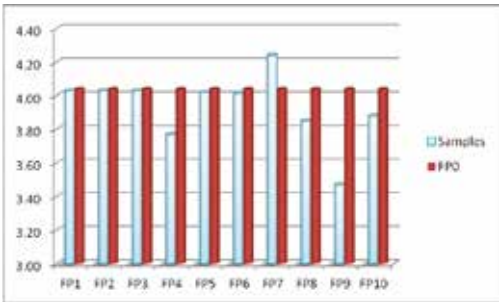

Figure 4. pH values of new dried fig-based products

An effect in lowering pH had presence of orange zest in products FP_4 and FP_{10}, and dried apricot in products FP_8 and FP_{10}. Usage of other ingredients had no effect in pH values, as between other products were not noted significant differences compared to FP_0.

The average results for appearance, aroma, color, texture, and flavor for different new products evaluated by panel members are listed in Table 2.

The highest score for appearance has product FP_6, for aroma has the product FP_4, for color has the product FP_5 and FP_9, which had the darker color by the presence of cocoa and cranberry respectively, for the texture there was no significant differences between products, where for the flavor was highly scored product FP_4.

Table 2. Sensory properties of new dried fig-based products

Quality attributes	Score	Products					
		FP_0	FP_1	FP_2	FP_3	FP_4	FP_5
Appearance	20	16	18.7	18.7	17.7	16.3	17.7
Aroma	20	16.4	16.7	16.4	17.7	19.4	17.7
Color	20	17.5	17.5	17.5	17.5	17.5	17.8
Texture	20	17.5	17.5	17.4	17.4	17.9	17.4
Flavor	20	16.4	16.9	16.7	17.8	19.5	18.1
Total score	100	83.8	87.3	86.7	88.1	90.6	88.7

Quality attributes	Score	Products				
		FP_6	FP_7	FP_8	FP_9	FP_{10}
Appearance	20	18.7	17.8	16	18.4	18.5
Aroma	20	16.7	16.7	16.4	17.8	18.2
Color	20	17.5	17.5	17.5	17.8	17.5
Texture	20	17.5	17.5	17.5	17.5	17.5
Flavor	20	16.7	16.7	16.4	17.8	18.7
Total score	100	87.1	86.2	83.8	89.3	90.4

According to the results of overall acceptability (Figure 5), product FP_4 resulted to be mostly accepted by panel members, as this product had the highest scores 90.6 out of 100, followed by products FP_{10} and FP_9. In general products with added value were highly scored compared to FP_0. Between product FP_8 and FP_0 was no differences in total score, noting that presence of dried apricot was not very distinguishable compared with that of fig, for the used proportions in that recipe. Whereas the coats

used were noted to have an impact in appearance of products, as those products FP_1 (sesame seeds), FP_2 (pumpkin seeds), FP_3 and FP_5 (coconut), FP_6 (walnut plus oats) have the highest score compared to FP_0.

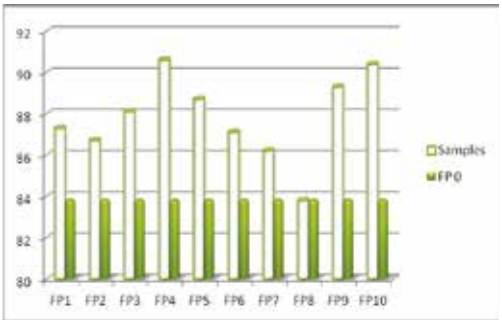

Figure 5. Overall acceptability scores of new dietary dried fig-based

Below are presented some of new dried fig-based products developed for this study, which resulted mostly evaluated and sensorially accepted by panel members (Figure 6), which might be attractive for consumers too, and for their success in the market further work would be suggested.

Figure 6. Some from the new dried fig-based products developed: a) fig+apricot (FP_8); b) fig+craneberry (FP_9); c) fig+orange zest (FP_4); d) fig+walnut (FP_7); e) fig+coconut (FP_6)

CONCLUSIONS

From this study can be concluded that Albanian dried figs possess considerable amount of nutrients, especially fiber and minerals, and may serve as good source energy. Due to its attributes is suitable to be effectively utilized for new dietary dried fig-based products, as one alternative way for adding value to the crop, in the meantime may serve as an income source to people that cultivate fig in rural areas of Albania.

Application of hurdle technology and development of new recipes provided tasty, natural, nutritive and healthy products, with low water content and pH, which are important hurdles for food preservation.

With regards to food stability of these new products, and since this is an ongoing study, further microbiological analyses are going to be performed in order to support our first results. Furthermore inclusion of cranberry, orange zest, also other additives in recipes added value to products, based on evaluation quality parameters and overall acceptability results, new products may be attractive to consumers as a potential for marketing as innovative products, which may compete other imported fig-based products in market.

The knowledge of new preservation method based on application of hurdle technology, and the quality parameters of these new products, may encourage the farmers to develop the products in a larger scale.

Further work is needed to be developed for new dried fig-based products success in the future.

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RESEARCHES REGARDING THE ANTIOXIDANT POTENTIAL OF SELECTED *Brassicaceae* VEGETABLES REPRESENTATIVE FOR HUMAN NUTRITION

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Abstract

Brassica vegetables belong to the Cruciferous family and include different kinds of cabbage (white, red), cauliflower and broccoli. These vegetables are recognised for their contribution to human health and nutrition. Brassica vegetables are extensively studied, analysed and characterised lately due to their antioxidant character and antioxidant capacity. The aim of the study was to investigate the antioxidant capacity of four white cabbage varieties ('De Buzău', 'Buzoiana', 'Măgura', 'De Işalniţa'). The content of total polyphenolics of fresh vegetables (Folin Ciocalteu procedure) was assessed as well as carotenoids and chlorophyll pigments (spectrophotometric methods) and vitamin C content (titrimetric assay using 2,6-dichlorphenol indophenol). The antioxidant activity of the vegetables was determined according to DPPH (2,2-diphenyl-1-picrylhydrazyl) protocol. The results showed the highest concentration of antioxidant compounds for Magura variety, while the lowest one was for De Isalnita variety. A study regarding stability of antioxidant capacity during storage for three months was performed too.

Key words*: Brassicaceae, cabbage, antioxidant capacity, polyphenolics, carotenoids, chlorophyll.*

INTRODUCTION

Recent research associated the reduced risk of cardiovascular disease with a rich diet in fruit and vegetables (Kaur & Kapoor, 2001). They are also an excellent source of antioxidants as polyphenolic compounds, carotenoid and chlorophyll pigments and vitamins (ascorbic acid, vitamin E, vitamin K) (Krinsky, 2001). The nutritional quality of vegetables has been extensively studied focusing on the role of diet in human health (Tiwari & Cummnis, 2013).

Brassica vegetables represent a group of horticulture species that are very important in human nutrition (Cartea & Velasco, 2008). Vegetables from the Brassicaceae family are known for their antioxidant properties that are scientifically correlated with lower risk of developing prostate cancer by 40%. Cruciferous are an excellent source of antioxidant and glucosinolate vitamins, being the precursors of a group of isocyanates that have been shown to be compounds with anticarcinogenic activity (Kris-Etherton et al., 2002).

White cabbage (*Brassica oleracea* var. *capitata* f. *alba*) is among the world's most commonly cultivated vegetables. Due to its affordable price and availability at local markets, white cabbage stands out as an important source of phytonutrients in the human diet. It may be stored raw for long periods of time and hence could be available throughout the year. Throughout history it has also been known as 'medicine for the poor' and has been used for the general improvement of health and the treatment of various inflammation and gastrointestinal (Hatfield, 2004; Cavender, 2006; Passalacqua et al., 2007). White cabbage is an inexpensive, very nutritive source of food, providing nutrients and health-promoting phytochemicals.

Phytochemicals have attracted much recent scientific attention and it is well known that white cabbage is a significant source of

glucosinolates, phenolic compounds, carotenoids and various vitamins. Several reviews have been published on the phytochemistry and health benefits of *Brassica* vegetables (Podsędek et al., 2006; Cartea et al., 2011; Jahangir et al. 2009; Björkman et al., 2011; Kapusta-Duch et al., 2012; Avato & Argentieri, 2015), but to the best of our knowledge nobody has focused specifically on white cabbage (*Brassica oleracea* var. *capitata* f. *alba*).

Polyphenolic compounds or "phenolics" are a complex group of compounds of plant origin. (Blasco Antonio J. et al., 2005). The interest of phenolic acids is continuously increasing because of their antioxidant properties among others (Cadenas E. & Packer, 1996). The "total phenolics" determination is very difficult because of their chemical complexity, difficult extraction from plant matrix, and the presence of complex interferences in food samples. Total phenolics in white cabbage has been reported by different authors (Kaulmann et al., 2014; Vicas et al., 2013; Deng et al., 2013) and the range of concentrations was between 9.3-1043.6 mg galic acid/100 g fresh weight.

Vitamins and carotenoids are essential compounds which promote human health and are responsible for accurate functioning of human metabolism and immune response. Considerable attention was focused on ascorbic acid (AA), known for its reductive properties and for its use on a wide scale as an antioxidant agent in foods; it is also important for therapeutic purposes and biological metabolism (Raoof, Ojani & Beitollahi, 2007). Due to its properties, vitamin C represents an important quality indicator of foodstuffs (Wawrzyniak, Ryniecki & Zembrzuski, 2005) and contributes to the antioxidant capacity of food (Glevitzky et al., 2008; Popa et al., 2010; Pisoschi et al., 2008; Pisoschi et al., 2010; Pisoschi et al., 2011). Vitamin C concentration in white cabbage ranges between 23.0-55.8 mg ascorbic acid/100 g fresh weight (Podsęsdek et al., 2006; Tiwuri & Cummins, 2013).

The determination of leaf pigment content is another important analytical tool in the field of plant physiology (Pompelli et al., 2012). Therefore, the chlorophyll (Chl) level is an accurate indicator of plant vigour and is routinely measured in physiological research. Carotenoids are synthesized by all plants and many microorganisms (bacteria and fungi), but not by animals, including humans, who therefore rely on dietary uptake. Due to the correlation of carotenoid intake and chronic diseases, methods allowing the rapid, accurate determination of carotenoids in these matrices are highly desired. Without prior separation carotenoids may be determined in plants together with chlorophylls, absorbing at similar wavelengths (Bieler et al., 2010). In white cabbage, the level of chlorophyll varies between 1.5 and 3.2 mg/100 g fresh weight and the level of carotenoids ranges between 0.01and 0.12 mg/100 g fresh weight.

According to the USDA National Nutrient Database for Standard Reference and dietary intake recommendations for adults (USDA), the white cabbage contains approximately 72 % of the recommended daily value (DV) for vitamin K, and 44 % of DV, 11 % of DV and 10 % of DV for vitamin C, folate and vitamin B6, respectively.

The variation in antioxidant content is caused by many factors such as geographical region, climate, variety, harvest maturity, growth conditions, soil condition and post-harvest conservation and processing method (Goncçalves et al., 2004).

The aim of the present work was the assessing of antioxidant capacity of white cabbage varieties (*Brassica oleracea* var. *capitata* f. *alba*) correlated with total phenolics, caretonoids and chlorophyll content and vitamin C level. The stability of these biochemical parameters was monitored during three months storage period.

MATERIALS AND METHODS

Materials

Four white cabbage varieties were analysed: 'De Buzău', 'Buzoiana', 'Măgura' and 'De Işalniţa'. All varieties were cultivated (Research and Development Station for Vegetables Buzău, Romania) in the same conditions, the same location, with the same agro-technical practices and harvested when reached the optimal maturity.

After harvesting the samples were transported and analysed at the Food Chemistry Laboratory. Then were selected the

inflorescences without infection or mechanical damage weighting about 2 kg each one.
Chemical substances 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,6 diclorphenol-indophenol were purchased from SIGMA-ALDRICH CHEMICAL CO. Meta-phosphoric acid, ethylendiaminetetraacetic acid, sodium hydrogen carbonate and sodium carbonate were purchased from ROTH. Folin-Ciocalteau reagent and ascorbic acid were purchased from MERCK. The organic solvents (methanol and acetone) were of analytical grade (MERCK).

Methods
Fresh samples were cleaned, cut and homogenized for optimum results. Methanol: water (1: 1, v/v) and acetone: water (80: 20, v/v) were used for extraction. Triplicates were prepared for each one.

Determination of total phenolics
The phenolics content was measured with Folin-Ciocalteau reagent (Singleton & Rossi, 1965) using gallic acid as standard. The samples were prepared by mixing 1 ml with 5 ml Folin-Ciocalteau reagent and 4 ml sodium bicarbonate (7.5% w/v). The solution was kept in the dark, at room temperature, for 20 min; the absorbance was measured at 752 nm with a Specord 210 spectrophotometer (Analytic Jena, Germany). Total phenolics content was expressed as mg gallic acid equivalents per 100 g fresh weight (mg GAE/100 g FW), calculated based on a calibration curve obtained with 1 mg/ml gallic acid solution.

Determination of ascorbic acid
The dye-titration method was used, according to AOAC procedure, 2006. Metaphosphoric acid extracts of vegetables were analysed by titration with 2,6-dichlorophenolindophenol reagent (DCIP). In this oxidation-reduction reaction, ascorbic acid in the extract was oxidized to dehydroascorbic acid and the indophenol dye reduced to a colourless compound. End point of the titration was detected when excess of the unreduced dye gave a rose pink colour in acid solution. The tests were carried out on white cabbage. Dehydroascorbic acid was not analysed in this study. The results were expressed in mg ascorbic acid/100 g fresh weight.

Determination of pigments
Carotenoid and chlorophyll pigments were extracted from 3 g fresh white cabbage using a mixture of acetone/water (80: 20, v/v/). The final mixture was vortexed (15 min., 2000 rpm, 20°C) and centrifuged (15 min., 3500 rpm. 20°C). The obtained extract was filtrated and the absorbance was recorded at 470, 646, 663 nm with Specord 210 spectrophotometer (Analytic Jena, Germany) as described by Lichtenthaler (1987). The results were expressed in µg /1 g fresh weight.

Determination of antioxidant capacity using DPPH protocol
The method is based on the color modification (from purple to yellow) of DPPH (2,2-difenyl-1-picrylhydrazyl) radical.
A modified protocol was used (A. Culetu et al., 2016) and consisted in extraction of the samples in methanol:water (1: 1, v/v). One ml of extract was treated with 6 ml DPPH. Following a 30 minutes rest in the dark, the absorbance at 517 nm was measured with a Specord 210 spectrophotometer (Analytic Jena, Germany).
The results were expressed in µmol Trolox/g fresh weight.

Stability of cabbage varieties
In order to determine the stability over time, cabbage samples were stored in the Vegetables-Fruits Section at a temperature of 20°C, for 3 months period. After two months and three months, the same parameters was re-analysed for the selected cabbage samples.

RESULTS AND DISCUSSIONS

Samples of the white cabbage varieties were assayed for antioxidant phytonutrients: phenolics, vitamin C, chlorophyll and carotenoid pigments. Total phenolics content (TP) ranged between 5.78 and 7.28 mg GAE/g FW, with highest value for ‘De Buzău’ cabbage variety (Figure 1). The obtained results showed rather similar level of TP for ‘De Buzău’, ‘Buzoiana’ and ‘Măgura’ varieties, while for ‘De Işalniţa’ variety this was about 20% lower.

It is difficult to compare these results with those of other authors because the reported values are spread over a wide range (9.3-1043.6 mg GAE/100 g FW). This high variability suggests that TP content may be influenced by a lot of factors, not only by the tested variety, geographic origin, agro-technical practices, harvest time, but also by extraction method and analytical determination parameters.

Regarding the ascorbic acid (AA) content, the highest value was registered for 'Măgura' cabbage variety with 42.85 mg/100 g FW (Figure 1). The obtained results are similar to those reported by other authors (Podsędsek et al., 2006; Tiwuri & Cummins, 2013).

The tested white cabbage varieties showed close values, the results obtained for the chlorophyll pigments being in the order: 'Buzoiana' variety > 'Măgura' variety > 'De Buzău' variety > De Işalniţa variety (Figure 1).

In terms of carotenoids, the highest value was recorded for 'Măgura' variety (2.73 µg/g) and the lowest for 'De Buzău' variety (1.7 µg/g) (Figure 1). The obtained results are close to previously published works of Fernandez-Leon et al. (2014).

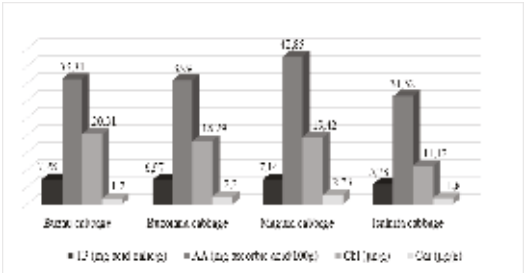

Figure 1. Variation of antioxidant parameters for different varieties of cabbage (TP - total phenolics; AA - ascorbic acid; Chl - chlorophyll; Car – carotenoid)

The antioxidant capacity, based on DPPH radical scavenging activity, assayed for cabbage varieties showed comparable results for 'De Buzău', 'Buzoiana' and 'Măgura' varieties, and about 25% lower for 'De Işalniţa' variety (Figure 2). Best correlation (r^2 0.899) was found between antioxidant capacity and the total phenolic content of the selected cabbage varieties. This suggests that total phenolic content may be used to predict the antioxidant activity of cabbage.

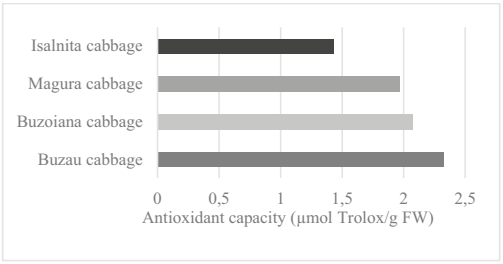

Figure 2. Antioxidant capacity of cabbage varieties using DPPH radical

The stability of antioxidants during cabbage storage was assayed for three months. After two months of storage an insignificant decrease of the amount of phenolic compounds was registered (Figure 3).

But the determinations made after three months showed 30% lower level of the total phenolics for all the tested cabbage varieties ($p<0.05$).

These results suggest that phenolic compounds were affected by abiotic factors (light, temperature, relative humidity) especially after 60 days storage.

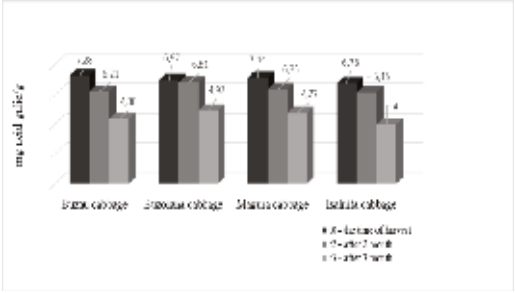

Figure 3. Stability of phenolics during storage

Analysing the stability of vitamin C during storage, the results presented in Figure 4 showed a significant decrease of the obtained values after two months ($p<0.05$).

The results confirm ascorbic acid sensitivity to degradation during handling and storage of fruits and vegetables (Balan et. al., 2016; Tiwuri & Cummins, 2013).

The loss of ascorbic acid is attributed to the conversion to dehydroascorbic acid and further on to 2,3-diketogulonic acid, favoured by exposure to oxygen, heavy metals, alkaline pH and high temperature.

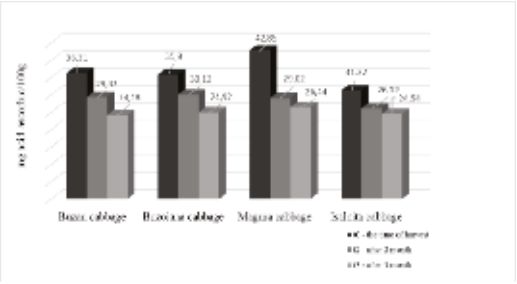

Figure 4. Stability of ascorbic acid during storage

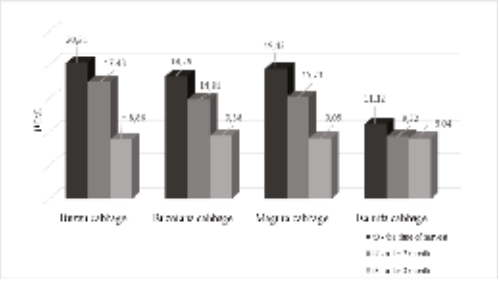

Figure 5. Stability of chlorophyll during storage

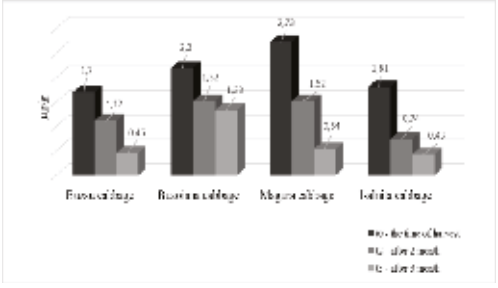

Figure 6. Stability of carotenoids during storage

With regard to chlorophyll and carotene pigments, it can be seen that they decreased over time, along with storage. Their degradation is due to the temperature and lack of natural light (Figures 5 and 6).

CONCLUSIONS

The antioxidants content in cabbage depends on the tested varieties. The highest concentration of antioxidant compounds was found in 'Măgura' variety, while the lowest one in 'Işalniţa' variety. Antioxidant capacity assayed with DPPH method was best correlated with total phenolic content. Cabbage storage for three months period at 20°C resulted in significant decrease of antioxidant compounds, especially vitamin C and chlorophyll and carotenoid pigments.

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ISOLATION AND IDENTIFICATION OF EFFECTIVE MICROBIAL STRAIN FOR ACCELERATED BIODEGRADATION OF LEATHER INDUSTRY WASTE

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Abstract:

Ecological biotechnology reduces the negative impact of industrial activities to the environment, by different bioremediation processes. Such biotechnological measures are non-polluting and environmentally safe. The use of specific microorganisms for the degradation of leather residues is an ecological alternative and an ecological tool for bioremediation. The aim of this study was to isolate some microbial strain capable to accelerate the degradation of leather waste, reducing the pollution that causes environmental damage. Three isolated bacterial strains, DA7, DA10 and DA13, were selected for their ability to produce extracellular proteases. These strains were identified with the Biolog-Microbial Identification System as Brevundimonas diminuta, Bacillus cereus/thuringiensis and Bacillus cereus, respectively. Studies revealed that the best enzymatic activity is higher after 120 hours cultivation, at 35°C temperature, 135 rpm and pH 7.0 units.

Key words*: bacterial, isolation, identification, protease, leather, degradation, waste, pollution.*

INTRODUCTION

Accelerated and disorganized industrial development has generated several environmental hazards. Therefore, it is demanding to promote sustainable development of rationally used natural resources to maintain existing ecosystems or to restore contaminated environments. This could be achieved by reducing, degrading, collecting or recycling the polluting materials as well as by improving industrialization processes, diminishing the side effects on humans, animals and on the environment.

Proteolytic enzymes catalyze the hydrolysis of the peptide bonds between amino-acids residues of protein (Dhillon et al., 2016). Such enzymes are produced by a wide range of microorganisms, like bacteria, including actinomycetes, molds, yeast etc. (Akcana & Uyar, 2011).

Selected microorganisms have the capacity to produce proteolytic enzymes using leather as unique source of carbon and nitrogen. In bacteria, this enzyme is produced mainly by strains belonging to *Bacillus* genus, especially, *B. licheniformis*, *B. horikoshii, B. sphaericus, B. furmis, B. alcalophilus, B. subtilis* (Ellaiah et al., 2011). *Bacillus* species are the main producers of extracellular proteases and industrial sectors frequently use *B. subtilis* for the production of various enzymes (Dubal et al., 2008).

The aim of the present study was to evaluate the proteases producing potential of three selected bacterial strains isolated from leather waste.

MATERIALS AND METHODS

Bacterial isolation

The microorganisms were obtained from leather waste decomposition for 90 days in the soil.

Samples of this substrate was introduced into sterile medium with pH 7.0 containing 0.5 g/l NaCl, 0.5 g/l $CaCO_3$, 0.35g/l K_2HPO_4.

After 48 h of incubation at 35°C and 135 rpm, the culture was diluted ten times and plated on agar Petri dishes.

Bacterial characterization

Newly isolated bacteria (90 colonies) were tested on three specific media: PCA containing 0.5% tryptone, 0.1% glucose, 0.25% yeast

extract and 2% agar, pH 7; Starch-Agar, containing 0.5% tryptone, 1% starch, 0.25% yeast extract, 2% agar, pH 7.2 and CMC-Agar containing 0.5% tryptone, 0.1% carboxymethyl cellulose, 0.25% yeast extract, 2% agar, pH 7. Cultures were incubated for 48h at 35°C in order to select the highest enzyme producing strains (Habib et al., 2012).
Protease production was evaluated on Slim Milk Agar.

Biomass production of leather degrading bacteria
The isolates were grown in minimal media containing 1.0 g/l NaCl, 0.05 g/l $CaCl_2$, 0.7 g/l KH_2PO_4, 0.9g/l $MgSO_4$, 2.38 g/l K_2HPO_4, 3.0 g/l sucrose, 0.6 g/l leather and incubated 120 h at 135 rpm, 35°C.
Biomass growth was evaluated by measuring the optical density (O.D.) at 600 nm using Biomate 3 spectrophotometer.
Proteolytic activity was spectrophotometric measured at 578 nm, according to Anson method (1938). The reaction mixture contained 0.5 mL of enzymatic solution and 1 mL of 1% casein in 0.2M phosphate buffer (pH 7). Samples were incubated at 37°C for 10 min. The enzymatic reaction was stopped with 2 mL of 5% tricloracetic acid. The reaction mix was kept 30 min at room's temperature and then it was filtrated through 0.22 µm membrane. For every 0.5 mL filtrate was added 0.5 mL HCl 0.2N, 2 mL NaOH 0.5N and 0.6 mL Folin-Ciocâlteu 1:2. After 30 min. at room's temperature the extinction was measured.

Identification of the selected strains using Biolog-Microbial Identification System - GEN III Bacterial identification
The selected bacterial isolated were biochemically identified using the GEN III Biolog-Microbial Identification System.
Selected microbial strains were grown over night, in isolated colonies, on BUG (Biolog Universal Growth) media at 33°C. Fresh biomass was resuspended in Biolog specific inoculation fluid, type A or B (depending on bacterial multiplication rate), at 84 to 88% turbidity (at 590nm according to the standard protocol). Specific 96 well GEN III microplates were inoculated with freshly prepared bacterial suspension, 100 µl/well. Plates were then incubated at 33°C for 22-24 hours. After 22 to 24 hours of incubations, the optical densities were measured at 590nm and 750nm using the semi-automated Biolog Spectrophotometer. The phenotypic pattern was analyzed with MicroLog3 software compared with 2650 different microbial species included in the Biolog database.

RESULTS AND DISCUSSIONS

Isolation of leather degradation bacteria
A total number of 90 isolates was harvested from the leather wastes after 90 days of decomposition in soil. These bacterial strains were tested for different enzymes production like cellulase and amylase (Figure 1).

Figure 1. Amylase producing bacterial stains

In order to reveal the CMC-ase activity, the culture plated were treated with 0.1% Congo red solution and washed with 1% acetic acid. After these two step treatment the bacterial strains having cellulose activity presented a clear halo around their colonies. To detect amylase producing bacteria, the microbial cultures grown on starch containing medium were flooded with iodine solution. The enzyme producing strains revealed a clear halo around their colony. However, the remaining non-degraded starch formed an indigo-colored compound in the presence of iodine.

Protease-producing bacteria
Around the proteases-producing bacteria it was observed an opalescent halo, having 0.2-1.1 cm diameter, after first 24 hours of incubation at 35°C. Most of the tested isolates were milky white colored, dotted, with a diameter between 0.1-1 cm, with regular shape. Other colonies were yellow colored punctiform with irregular edges, having a diameter between 0.1-0.5cm (Table 1).
Oliveira et al. (2016) found, on Slim Milk Agar (SMA), hydrolysis zone ranging from 2.5 to 1

mm, while Barros et al. (2013) observed clearance zones ranging from 2.5 to 10.0 mm for *B. subtilis* stains grow on SMA plates at 30°C for 24 h. Singh et al. (2010) reported that among 70 proteolytic bacteria isolated from soil, among which 40% were considered good protease producers, exhibit clearing zones higher than 3 mm on milk agar plates after their incubation for 20 to 30 h at 37°C.

Those that presented the best activity following tests were selected for further experiments.

Bacterial biomass productivity

The maximum biomass production (0.590 U/ml) was obtained in the minimal media supplemented with 3 g of sucrose and leather substrate, after incubation at 35°C (Table 2).

Table 1. Results of bacterial- and halo-diameter measurements after 24 h of incubation at 35°C on Slim Milk Agar for protease detection

Sample	Bact. diam. (cm)	Halo diam. (cm)	Sample	Bact. diam. (cm)	Halo diam. (cm)	Sample	Bact. diam. (cm)	Halo diam. (cm)	Sample	Bact. diam. (cm)	Halo diam. (cm)
DA1	0.3	0.3	DA25	0.9	0.9	DA49	0.2	0.4	DA73	0.3	0.7
DA2	0.9	0.8	DA26	0.3	0.6	DA50	0.3	0.4	DA74	0.2	0.3
DA3	0.2	0.3	DA27	0.7	0.9	DA51	0.1	0.5	DA75	0.2	0.5
DA4	0.7	0.7	DA28	0.2	0.3	DA52	0.5	0.9	DA76	0.7	0.9
DA5	0.7	0.8	DA29	0.4	0.4	DA53	0.1	0.3	DA77	0.9	0.7
DA6	0.8	0.8	DA30	0.1	0.3	DA54	0.2	0.6	DA78	0.1	0.5
DA7	**0.8**	**1.0**	DA31	0.8	0.7	DA55	0.7	0.7	DA79	0.1	0.3
DA8	0.2	0.3	DA32	0.9	0.9	DA56	0.8	0.8	DA80	0.3	0.5
DA9	0.5	0.7	DA33	0.7	0.8	DA57	0.3	0.7	DA81	0.1	0.5
DA10	**1.0**	**1.1**	DA34	0.1	0.6	DA58	0.6	0.9	DA82	0.4	0.8
DA11	0.1	0.2	DA35	0.1	0.3	DA59	0.7	0.7	DA83	0.7	0.7
DA12	0.3	0.5	DA36	0.2	0.3	DA60	0.7	0.8	DA84	0.8	0.7
DA13	**0.9**	**0.9**	DA37	0.2	0.5	DA61	0.9	0.6	DA85	0.8	0.5
DA14	0.7	0.9	DA38	0.2	0.3	DA62	0.8	0.9	DA86	0.5	0.9
DA15	0.8	0.9	DA39	0.3	0.4	DA63	0.7	0.9	DA87	0.2	0.5
DA16	0.3	0.7	DA40	0.6	0.8	DA64	0.5	0.9	DA88	0.2	0.6
DA17	0.4	0.7	DA41	0.9	0.9	DA65	0.3	0.6	DA89	0.7	0.8
DA18	0.1	0.4	DA42	0.7	0.9	DA66	0.2	0.5	DA90	0.1	0.4
DA19	0.5	0.6	DA43	0.7	0.8	DA67	0.8	0.8			
DA20	1.0	0.9	DA44	0.5	0.7	DA68	0.9	1.0			
DA21	0.9	0.9	DA45	0.9	0.9	DA69	1.0	0.9			
DA22	1.0	0.8	DA46	0.9	0.9	DA70	0.5	0.7			
DA23	0.1	0.4	DA47	0.3	0.7	DA71	0.5	0.8			
DA24	0.7	0.8	DA48	0.7	0.8	DA72	0.9	0.8			

Table 2. Bacterial biomass production after 48 h

Sample	D.O 600 nm	Sample	D.O 600 nm	Sample	D.O 600 nm	Sample	D.O 600 nm
DA1	0.187	DA24	0.197	DA47	0.210	DA70	0.187
DA2	0.198	DA25	0.191	DA48	0.282	DA71	0.266
DA3	0.183	DA26	0.207	DA49	0.297	DA72	0.278
DA4	0.180	DA27	0.189	DA50	0.202	DA73	0.295
DA5	0.188	DA28	0.203	DA51	0.271	DA74	0.266
DA6	0.191	DA29	0.297	DA52	0.189	DA75	0.263
DA7	**0.299**	DA30	0.239	DA53	0.186	DA76	0.251
DA8	0.189	DA31	0.215	DA54	0.205	DA77	0.280
DA9	0.197	DA32	0.199	DA55	0.201	DA78	0.222
DA10	**0.590**	DA33	0.219	DA56	0.180	DA79	0.278
DA11	0.201	DA34	0.281	DA57	0.191	DA80	0.235
DA12	0.203	DA35	0.263	DA58	0.207	DA81	0.290
DA13	**0.347**	DA36	0.254	DA59	0.219	DA82	0.244
DA14	0.219	DA37	0.233	DA60	0.189	DA83	0.188
DA15	0.193	DA38	0.279	DA61	0.231	DA84	0.207
DA16	0.204	DA39	0.289	DA62	0.276	DA85	0.183
DA17	0.289	DA40	0.255	DA63	0.203	DA86	0.189
DA18	0.277	DA41	0.199	DA64	0.218	DA87	0.199
DA19	0.180	DA42	0.277	DA65	0.297	DA88	0.272
DA20	0.287	DA43	0.264	DA66	0.246	DA89	0.263
DA21	0.288	DA44	0.200	DA67	0.239	DA90	0.209
DA22	0.182	DA45	0.185	DA68	0.222		
DA23	0.181	DA46	0.191	DA69	0.214		

Spectrophotometric quantification of proteolytic activity

Bacterial growth and protein digestion were evaluated for the three selected bacterial strains (DA7, DA10 and DA13). Results showed that DA10 was the most efficient strain, reaching the highest OD values at 578nm. Enzyme activity was evaluated as 0.556 U/ml after 24 h, and 0.987 U/ml after 120h of incubation (Table 3).

Identification of selected microorganisms

Based on the Biolog GEN III fingerprints of each strain, the selected bacteria were identified as *Brevundimonas diminua* (strain DA7) – Figure 2, *Bacillus cereus/thuringiensis* (strain DA10), and *Bacillus cereus* (strain DA13).

The Biolog identification revealed a similarity index higher than 0.5 for each analysed strain, and distance value of at least 2 points between the most similar microbial profiles, which reveals a clear species-specific identification for DA7 and DA13.

However, this identification system cannot reveal all de differences between *B. cereus* and *B. thuringiensis*.

Table 3. Proteolytic activity evaluated by spectrophotometric quantification at 578 nm

	Incubation time					
Strain	24 h	48 h	72 h	96 h	120 h	Average
DA 13	0.231	0.415	0.586	0.938	0.993	0.633
DA 10	0.556	0.630	0.815	0.906	0.987	0.779
DA 7	0.183	0.139	0.426	0.506	0.616	0.374

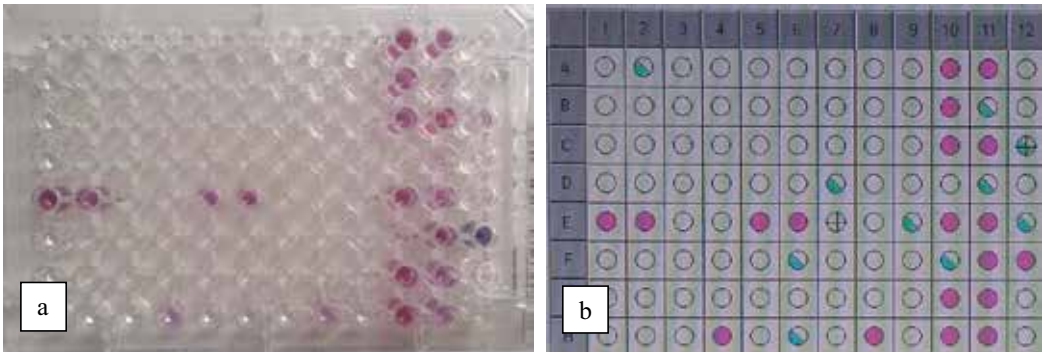

Figure 2. *Brevundimonas diminua* strain DA7:
a. Inoculated GEN III plate after 22 h of incubation at 33°C; b. MicroLog3 scanned fingerprint of the microplate

CONCLUSIONS

Three bacterial strains were isolated from leather degrading wastes.

The selected bacterial strains exhibit the highest proteolytic activity of 0.987 U/ml after 120 hours of cultivation, at 35°C.

Upon identification, it was revealed that the microbial strains were: *Brevundimonas diminuta* (DA7), *Bacillus cereus/thuringiensis* (DA10) and *Bacillus cereus* (DA13).

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EFFECT OF ACUTE GAMMA IRRADIATION ON GENERATION TIME, LIPID, CHLROPHYLL *A* AND CAROTENS, IN *Chlorella sorokiniana* UTEX 2130 AND *Synechocystis* PCC 6803

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Abstract

Microalgae are microorganisms very important for fluxes of matter, energy and information on planet Earth as well as for a plethora of biotechnological applications. In this paper, we present our original results concerning the use of acute gamma irradiation (0.9 Gy/s) to challenge the cells of Chlorella sorokiniana UTEX 2130 and Synechocystis PCC 6803.The main results obtained on Chlorella sorokiniana UTEX 2130 are: a) a great decrease in the generation time to 56% after 10 Gy irradiation, to 60% after 50 Gy irradiation, and to77% after 100 Gy irradiation, whereas b)the relative lipid content increased by 20% and 50% after 10 Gy and 100 Gy (as compared with the non-irradiated control). The main results obtained on Synechocystis PCC 6803 are: a) the generation time decreased to 90% after 10 Gy irradiation, to 85% after 50 Gy irradiation, whereas b) there is an increase in the chlorophyll a content (by 33%) and carotene (by 22%) after an irradiation of 50 Gy.

Key words*: acute gamma irradiation, lipids, carotenoids, Chlorella sorokiniana, Synechocystis PCC 6803.*

INTRODUCTION

Photosynthetic microorganisms, both prokaryotes and eukaryotes, have the ability to use water, carbon dioxide and some minerals to perform complex endergonic biochemical reactions enabling them to synthesize a plethora of cellular constituents thus sustaining their multiplication. Furthermore, some of them are capable of mixotrophic growth, using both inorganic and some organic substances as primary carbon sources. This is why 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 (Whitton, 2012 and references herein). One such direction concerns the ability of the majority of microalgae to accumulate lipids inside the cell (Sheehan et al., 1998; Chisti, 2007; Li et al., 2008; Liang et al., 2009; Demirbas, 2010; Huang, 2010; Mata et al., 2010; Amaro et al., 2011; Schuhmann et al., 2012; Borowitzka, 2013; Rawat et al., 2013; Velea et al., 2014; Ardelean & Manea, 2016; Ardelean et al., 2017; 2018).

One important task is to increase the content of lipid inclusions of the strains in order to develop an economically viable biotechnology. In this respect, the main strategies are: i) the selection from naturally occurring strains of those with high lipid content; ii) the mutagenesis of this naturally occurring populations in order to further select the most productive cells; iii) the use of genetic engineering tools (including site directed mutagenesis and metabolic engineering) to change the metabolic flux toward lipid deposition, and iv) the control of growing conditions (the so called stressors) such as nutrient depletion, nitrogen starvation, pH, and temperature shock. All these strategies have the potential to increase the lipid content (% mass/mass) in microalgae (Sheehan et al.,

1998; Sharma et al., 2012; Sibi et al., 2016; Yong et al., 2019). Recently, a new stressor, namely Co60 γ-irradiation at low, non-lethal doses, received attention with respect to the induction of increased lipid content in irradiated microalgae (Tale et al., 2018; Ermavitalini et al., 2017).

The aim of this contribution is to challenge the cyanobacterium *Synechocystis* PCC 6803 and the green alga *Chlorella sorokiniana* UTEX 2130 by acute, non-lethal gamma irradiation in order to check if any changes in their phenotypic traits, with special emphasis on the generation time (in both strains) and lipid inclusions (only in the green alga) will occur.

RESULTS AND DISCUSSIONS

Doubling time

In Table 1 are presented the results concerning the calculated generation time (GT) for the cyanobacterium *Synechocystis* PCC 6803 irradiated at 10Gy, 50Gy, and 100Gy, as compared with the non-irradiated control.

Table 1. Generation time for *Synechocystis* PCC 6803 at different irradiation doses

Irradiation dose (Gy)	Generation time (h)	%
Control	77.09	100.00
10	68.93	89.41
50	65.26	84.65
100	81.29	105.45

The GT in control is taken as 100% and all other results are reported to the control. One can see that the GT is shorter in populations irradiated at 10Gy and 50Gy, as compared with the control, whereas the irradiation at 100Gy showed a longer GT, within the range of standard deviation (5%). The irradiation at 500Gy and 1000Gy indicated the cell death (results non shown).

In Table 2 there are presented the GT for the green alga *Chlorella sorokiniana* UTEX 2130 irradiated at 10Gy, 50Gy, and 100Gy, as compared with the non-irradiated control. The results showed that the GT is much shorter in populations irradiated at 10Gy, 50Gy, and 100Gy as compared with the control. The irradiation at 500Gy and 1000Gy induced the cell death, as in the case of cyanobacterium *Synechocystis* PCC 6803.

These results obtained on both prokaryotic and eukaryotic photosynthetic microorganisms are promising with respect to the aim of our goals, especially in the case of *Chlorella sorokiniana* UTEX 2130 where differences in GT reached values up to 30-40% in irradiated populations as compared with the control. However, at this time, there is no causal explanation for these differences and further studies are necessary in order to elaborate a true working hypothesis.

Table 2. Generation time for *Chlorella sorokiniana* UTEX 2130 at different irradiation doses

Irradiation dose (Gy)	Generation time (h)	%
Control	188.59	100.00
10	105.34	55.86
50	118.89	63.04
100	145.01	76.89

Lipid inclusions content

In Table 3, is presented the estimated lipid inclusions content of *Chlorella sorokiniana* UTEX 2130 population irradiated at 10 Gy and 100 Gy, as compared with non-irradiated control. The estimation of lipid inclusions content by Nile red fluorescence method showed a much stronger increase in the fluorescence signal after Nile red addition in irradiated populations (10 Gy and 100 Gy) as compared with the control.

Table 3. The intensity of the fluorescence signal without (-) and with (+) Nile red and the calculated ratio (+/-) and % difference

Irradiation dose (Gy)	(-) Nile Red	(+)Nile Red	Numerical ratio (+/-)	%
Control	2222	9402	4.23	100.00
10	1594	8152	5.11	120.80
100	1823	11655	6.39	151.06

These results are very promising because the increased lipid droplet content (20% and 50% in the 10Gy and 100Gy irradiated cells, occurs in populations with shorter GT than in the control (55.86% and 76.89%, respectively). Interestingly, the increase in lipid droplet content occurs when cells are grown in normal conditions, without any attempt (so far) to manipulate the growing conditions in order to facilitate intracellular deposition of lipid droplet, such as the decrease of nitrogen concentration (Yong et al., 2019).

In Figure 1 there are presented individual cells of *Chlorella sorokiniana UTEX* 2130 after

labelling with Nile red, irradiated populations as compared with the control. There are presented only images obtained in green fluorescence,

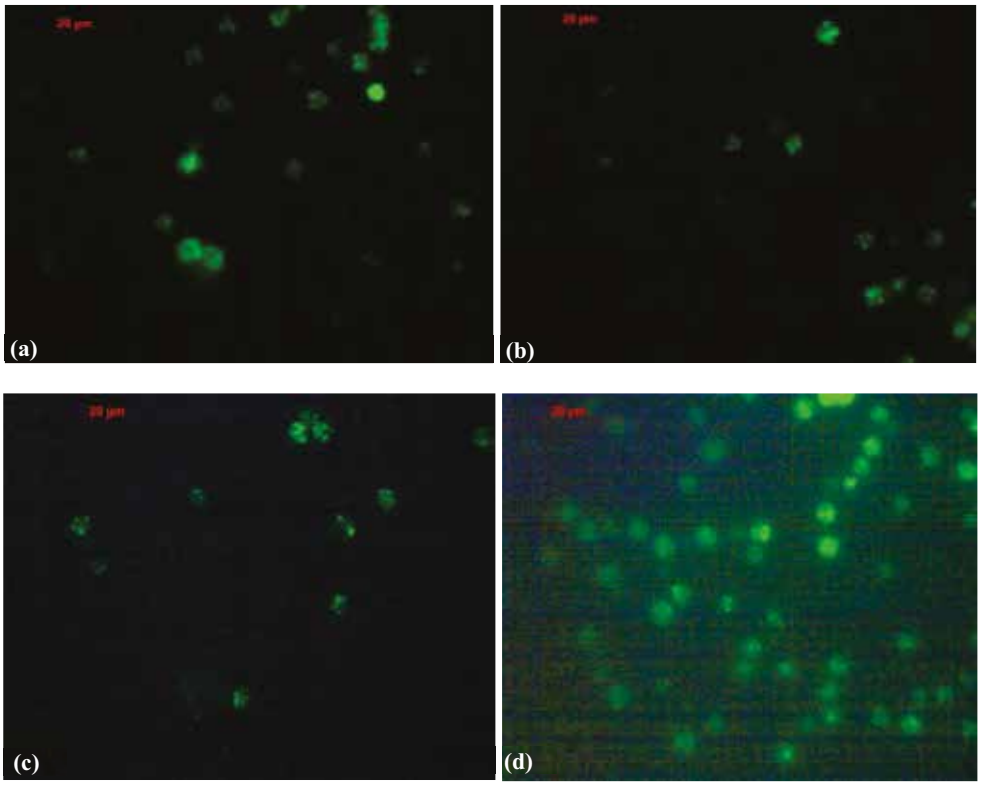

Figure 1. Individual cells of *Chlorella sorokiniana* UTEX 2130 after labelling with Nile red: (a) control, non-irradiated cells; (b) 10Gy irradiated cells; (c) 50Gy irradiated cells; (d) 100Gy irradiated cells

where only the emission of Nile red in the hydrophobic ambient of lipid droplets is evident (Greenspan, 1985) and one can see an increased surface (corresponding to labelled lipid droplets) emitting green fluorescence. The images in red fluorescence are not shown as the red fluorescence of Nile red lipid droplets is mixed together with the red fluorescence of chlorophylls in the alga.

Microscopic investigations show larger areas inside the cells and also a larger number of cells emitting green fluorescence, which indicates that these cells contain a higher lipid inclusions content. These microscopic images only document the presence of lipid droplets without any numerical quantification, as no image analysis has yet been performed on them. The lipid inclusions content was not estimated in the cyanobacterium *Synechocystis* PCC 6803 as wild cyanobacterial strains do not have true lipid inclusions, besides polyhydroxybutyrate which is a common inclusion in cyanobacteria.

Chlorophyll *a* and carotenoids content in *Synechocystis* PCC 6803

In the next table one can see the concentration of chlorophyll *a* and carotenoid pigments in *Synechocystis* PCC 6803.

Table 4. The concentration of chlorophyll *a* and carotenoid pigments in *Synechocystis* PCC 6803

Irradiation dose (Gy)	Chlorophyll *a* (µg/mL)	Carotenoid pigments (µg/mL)
Control	12.65	8.20
10	14.48	9.37
50	16.77	10.05
100	14.71	9.76

The results in the above table show that there are some changes in the chlorophyll *a* and carotenes content. Taking the control as 100% the increase in chlorophyll *a* concentration went up to 114, 132, and 116%, respectively at 10, 50, and 100Gy. The same increases were registered for the total carotenes (i.e. 114, 122, and 119%).

The cyanobacterium *Synechocystis* PCC 6803 and the green alga *Chlorella sorokiniana* UTEX 2130 had been chosen for our experiments as they are versatile photosynthetic microorganisms intensively used in both fundamental and applicative research (Whitton, 2012; Lizzul et al., 2018 and references herein) able to grow either strictly photosynthetically but also mixotrophic or even heterotrophic, not only on pure organic substances but also on waste feedstock, with short doubling times. The scientific community strongly agree that these strains have a strong industrial potential (Lizzul et al., 2018; Whitton, 2012 and references herein; Hu et al., 1990) studied the effect of gamma radiation on the growth and morphology of *A. platensis*. They reported that low doses of gamma rays, less than 1 kGy, could stimulate its growth. Small changes in the morphology of the filament were found at doses less than 0.5 kGy. The LD_{50} was 1.0 kGy, while 2.5 kGy caused 100% lethality. Wang et al. (1998) studied the effect of gamma radiation (up to 6 kGy) on the growth and morphology of four different strains of *Arthrospira* sp. and concluded that it showed resistance to gamma irradiation with stimulation of growth at low doses, while the filaments would break up or even disintegrate at high doses. Although many studies have evaluated the biological response of microalgae to high doses of gamma radiation, few studies have focused on stimulation of bioactive compounds production in *A. platensis.*

Abomohra et al. (2016) studied the influence of gamma radiation on the growth and production of some active substances of the cyanobacterium *Arthrospira platensis*. In their important paper they found the following: i) biomass production was significantly inhibited by 21 and 34%, with respect to the control at 2.0 and 2.5 kGy, respectively and chlorophyll *a* content showed 11% reduction at 2.5 kGy compared to the control. In addition, phycobiliproteins productivity showed a significant decrease by 10 and 36% below the control at 2 and 2.5 kGy of gamma radiation, respectively whereas protein production was decreased significantly with the concomitant increased of carbohydrate production by 106, 246 and 146%, respectively and lipid content increased significantly over the control at 0.5 kGy. Interestingly, carotenoid productivity showed significant increase at all used gamma doses up to 155% over the control.

In a recent paper (Ermavitalini et al., 2017), the work done on *Botryococcus* sp. irradiated at low doses (2, 4, 6, 8 and 10 Gy) showed that irradiation using gamma rays changed the characteristics of their growth, biomass, percentage of total lipids cell and fatty acid profile. More precisely, the highest biomass and lipid content found in 10 Gy irradiated microalgae are 0.833 g biomass and 41 % lipid content. Furthermore, fatty acid profile of *Botryococcus* sp. control has 6 fatty acids while 10 Gy irradiated microalgae has 12 fatty acids, with the long-chain fatty acids increased, whereas short-chain fatty acids decreased. Tale et al. (2018) used gamma irradiation as a stressor to trigger lipid accumulation in two strains of *Chlorella sorokiniana* (i.e. *C. sorokiniana* KMN2 and *C. sorokiniana* KMN3). These strains were treated by Co60 γ-irradiation, in the range of 100 to 1100 Gy, much higher than the dosses used by us in the algal strain *Chlorella sorokiniana* UTEX 2130. The authors (Tale et al., 2018) reported the enhancement of the lipid content of more than 40% of biomass as well as the level of intracellular reactive oxygen species. They also showed that the expression patterns of regulatory genes involved in lipid biosynthesis, namely acetyl-CoA carboxylase and diacylglycerolacyl transferase, were up regulated immediately after irradiation and were highest 3 days post irradiation (Tale et al., 2017). The authors also performed the analysis of the fatty acids composition in irradiated and control populations, showing that γ-irradiation induced lipid enhancement is accompanied by higher accumulation of shorter carbon chain fatty acid (C-16) compared to longer chain fatty acids. They claimed that the novel strategy of using gamma radiation as a faster andextremely potent stressor for triggering lipid biosynthesis

in microalgae has immense potential in industrial biodiesel production. They put forward the hypothesis that γ-irradiation in microalgae causes oxidative stress and up-regulationof lipid biosynthetic pathway which in turn leads to lipid accumulation.

Another study (Badri et al., 2015) showed that *Arthrospira* sp. PCC 8005 is highly tolerant to gamma raysand can survive to at least 6400 Gy (dose rate of 527 Gy/h). Their detailed proteomic and transcriptomic analyses performed after irradiation with 3200 or 5000Gy showed a decline in photosystem II quantum yield, reduced carbon fixation, and reduced pigment, lipid, and secondary metabolite synthesis. On the other hand, transcription of photo-sensing and signalling pathways, and thiol-based antioxidant systems was induced. Furthermore, transcriptomics did show significant activation of ssDNA repair systems and mobile genetic elements (MGEs) at the RNA level. Interestingly, the cells did not induce the classical antioxidant or DNA repair systems, such as superoxide dismutase (SOD) enzyme and the RecA protein. *Arthrospira* sp. cells lack the catalase gene and the LexA repressor. Based on the observation that irradiated *Arthrospira* cells did induced strongly a group of conserved proteins, the authors (Badri et al., 2015) put forward the hypothesis that these proteins could be involved in the response of cyanobacterial cells to irradiation, which remains to be checked.

In our opinion, the interactions between photosynthetic microorganisms (both prokaryotes and eukaryotes) and gamma irradiation, at low, non-mutagenic doses, is still in its infancy.

CONCLUSIONS

Acute gamma irradiation in *Chlorella sorokiniana* UTEX 2130 showed the followings: a) a great decrease in the GT to 56% after 10Gy irradiation, to 60% after 50Gy irradiation, and to7 7% after 100 Gy irradiation, whereas b)the relative lipid content increased by 20% and 50% after 10Gy and 100Gy as compared with the non-irradiated control.

Acute gamma irradiation in *Synechocystis* PCC 6803 show that: a) the GT decreased to 90% after 10Gy irradiation and to 85% after 50Gy irradiation, whereas b) there is an increase in the chlorophyll *a* content (by 33%) and carotene (by 22%) after an irradiation of 50 Gy.

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LACCASE: MACRO AND MICROBIAL SOURCES, PRODUCTION, PURIFICATION AND BIOTECHNOLOGICAL APPLICATIONS

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Abstract

Laccase belongs to the blue multicopper oxidases and participates in degradation of polymers, ring cleavage of aromatic compounds and cross-linking of monomers. It is distributed in higher plants and fungi. It is present in Ascomycetes, Deuteromycete, and Basidiomycetes and abundant in lignin-degrading white-rot fungi. Lacasse has been reported to be produced by different mushrooms (Trametes, Ganoderma, Pleurotus) and by filamentous bacteria (Streptomyces) or fungi (Aspergillus). The article proposes a comparative analysis of the optimal conditions for laccase production in the case of macromycetes and some micromycetes, like fungi or filamentous bacteria, meanwhile describing the isolation, purification and characterization of the laccase produced by such organisms. All these issues will be approached through the biotechnological application of these enzymes (dye decoloration, bioremediation etc).

Key words*: laccase, macromycetes, micromycetes, bacteria, fungi.*

INTRODUCTION

Laccase was first detected in the Japanese lac tree *Toxicodendron verniciflua*. Later, it was found in other plants, in many insects and in fungi, including yeasts (e.g., *Cryptococcus*), molds (e.g., *Penicillium*), mushrooms (e.g., *Agaricus*), and white-rot fungi (e.g., *Pleurotus*) (Mikolasch et al., 2009).

Laccases play an important role in the food proccessing industry, dye decolorization, bioremediation and biodegradation, pulp and paper industry (Couto et al., 2006) and some medical applications. Laccase is an enzyme used for degradation of pesticide and insecticide, organic synthesis and pulp delignification (Couto et al., 2008), waste detoxification, biosensor and analytical applications, textile dye transformation and food technology. Recently the activity of laccases have been efficiently applied to nanobiotechnology due to their ability to catalyze electron transfer reactions without additional cofactor. The technique for the immobilization of biomolecule such as layer-by-layer, self-assembled monolayer technique and micropatterning can be used for preserving the enzymatic activity of laccases (Agematu et al., 1993).

LACCASE – BIOCHEMISTRY

In recent years, enzymes, like laccase, have gained great importance in industries. Laccases are the oldest and most studied enzymatic systems present in nature. These enzymes contain 15-30 % carbohydrate and have a molecular mass of 60-90 kDa.

Laccase is a copper-containing molecule, 1,4-benzenediol: oxygen oxidoreductases (EC 1.10.3.2) found in higher plants and microorganisms. These oxidoreductases are glycosylated polyphenol oxidases that contain 4 copper ions per molecule that carry out 1 electron oxidation of phenolic and its related compound, and reduce oxygen to water (Couto et al., 2006; Gianfreda et al., 1999). When the substrate is oxidized by laccase, it loses a single electron and usually forms a free radical which may undergo further oxidation or non-enzymatic reactions including hydratation, disproportionation, and polymerization (Faccelo et al.,1993). These enzymes are polymeric and generally contain one of each type 1, type 2 and type 3 copper

center/subunit where the type 2 and type 3 are closer together forming a trinuclear copper cluster.

Laccases are divided into "low-redox potential" and "high-redox potential", depending on the structure and properties of the copper center.

The high-redox potential laccases occur mainly in basidiomycetes, especially white-rot fungi, the low-redox potential laccases seem to be widely distributed in molds, bacteria, insects, and plants.

Unlike most enzymes, laccases have the ability to display their activity on a wide range of substrates like monophenols, diphenols, methoxyphenols, polyphenols, aromatic amines, benzenethiols and even some inorganic compounds such as iodine (Burlacu et al., 2018).

Given their versatility and broad substrate specificity, laccases, as a family of copper-containing oxidases catalyzing a variety of oxidations, could become one of the most important biocatalysts in fungal biotechnology. Because of this, their biochemical properties and molecular evolution are of considerable interest and have been summarized in several reviews (Mikolasch et al., 2009).

Laccase activity was determined using guaiacol as substrate. The reaction mixture contained 1 ml enzyme sample, 3 ml sodium acetate buffer (10 mM, pH 5.0) and 1 ml guaiacol (2 mM). The mixture was incubated at 30^{o}C for 15 min. The changes in absorbance due the oxidation of guaiacol in the reaction mixture were recorded by spectrophotometer at 450 nm, with a molar extinction coefficient for guaiacol (Ɛ450 = 6740 M-1 cm^{-1}). One unit of enzyme activity (U/ml) is defined as the amount of enzyme that oxidized 1 µmol of guaiacol per minute (Nicolcioiu et al., 2018).

MACROBIAL SOURCES OF LACCASE

Research studies in recent decades have been focused on the different mushroom species, which belong particularly to Macrofungi (Macromycetes). Ascomycetes and Basidiomycetes are two of the most important group of Macrofungi that have been intensively investigated regarding various aspects. Enzymatic activity research of these mushrooms was, is and will be one of the important topics for understanding their physiological and biochemical features in order to emphasize the considerable potential for biotechnological and industrial applications.

Laccase is one of the most studied enzymes, because, according with Krupodorova et al. (2014), its positive reaction was detected in 21 species, frequently in 2-4 days after inoculation. It formed a reddish-brown zone around the mycelium. The most active producers of laccase were *Lentinus edodes*, *L. luscina* and *Coprinus comatus* (the latter two mentioned species are referred to soil saprotrophic). Krupodorova et al. (2014) mentioned laccase presence in two out of four brown-rot species (*Agrocybe aegerita* and *Fomitopsis pinicola*). Souza et al. (2008) found silencing genes of laccase in the genome of brown-rot microorganisms. Investigations showed laccase activity for the species *L. luscina, Crinipellus schevczenkovi, A. aurea, Hypsizygus marmoreus, L. schimeji, Oxyporus obducens* and *S. litschaueri* for the first time.

The existence of laccase has been detected in earlier studies in similar species: *A. agerita*, *C. comatus*, *Fomes fomentarius*, *Fomitopsis pinicola, Flamulina velutipes, Ganoderma lucidum*, *G. applanatum, Grifola frondosa, L. edodes*, *P. eryngii* and *P. ostreatus*. This enzyme has been found in *P. djamor, H. myxotricha, L. sulphureus* and *T. versicolor* (Krupodorova et al., 2014).

Nicolcioiu et al. (2018) research regarding laccase activity has lead to the conclusion that among all tested isolates *Pleurotus ostreatus* var. 'Florida', *Trametes versicolor* and *Ganodrema applanatum,* the fungal strains were more efficient in laccase production than *Laetiporus sulphureus* and *Flammulina velutipes*.

MICROBIAL SOURCES OF LACCASE

Considering microbial sources, laccase is produced by a large variety of bacteria (Table 1) and filamentous fungi (Table 2).

In lower fungi, as *Zygomycetes* or *Chytridiomycetes*, the production of laccase was not demonstrated (Baldrian et al., 2006).

There are also laccase-producing yeasts, like *Kluyveromyces dobzhanskii, Pichia manshurica* (Wakil et al., 2017) and *Cryptococcus neoformans*, but they have far fewer known representants than other classes of microorganisms.

Table 1. Bacterial laccase sources (after Desai and Nityanand, 2011)

Sources	References
Bacteria	
Azospirillium lipoferum	Givaudan et al., 1993; Faure et al., 1994
Bacillus subtilis	Martins et al., 2002
S. maltophilia AAP56	Galai et al., 2009
Streptomyces coelicolor	Dube et al., 2008

Table 2. Fungal laccase sources (after Baldrian, 2006)

Sources	References
Fungi	
Aspergillus nidulans	Scherer and Fischer, 1998
Botrytis cinerea	Slomczynski et al., 1995
Chalara paradoxa	Robles et al., 2002
Chetomium thermophilum	Chefetz et al., 1998
Magnaporthe grisea	Iyer and Chattoo, 2003
Mauginiella sp.	Palonen et al., 2003
Melanocarpus albomyces	Kiiskinen et al., 2002
Monocillium indicum	Thakker et al., 1992
Myrothecium verrucaria	Sulistyaningodyah et al., 2004
Neurospora crassa	Froehner and Eriksson, 1974
Ophiostoma novo-ulmi	Binz and Canerascini, 1997
Podospora anserina	Durrens, 1981
Rhizoctonia solani	Wasaki et al., 1967
Trichoderma giganteum	Wang and Ng, 2004

The properties of laccase includes: extracellular localization, molecular weight of approximatively 60 to 70 kDa (typical in fungi), isoelectric pH point between 3.0 and 7.0, optimal pH in fungi between 3.6 and 5.2 (highly dependent on the substrate), usually monomeric structure (homodimeric laccases were found too in *Gaeumannomyces graminis, Monocillium indicum, Podospora anserina*), optimal temperature around 55-60°C (for *B.cinerea*). In spectrophotometry, purified laccase has a blue appearance around 600 nm.

Bacterial laccases have a higher thermostatbility and halotolerance than fungal laccases and are thought to be more valuable in dye decolorization, biofuel production and biobleaching.

There are some actinomycetes with lignolytic-activity, like *Trichoderma* sp. or *Botryosphaeria* sp., that displayed laccase production.

The laccase produced by *Monocillium indicum* was the first *Ascomycetae* laccase characterized, that had similar immunological features with *Coriolus versicolor* and *Agaricus bisporus* laccases and with lignin peroxidase activity similar to *Phanerochaete chrysosporium* laccase (Thakker et al., 1992; Shraddha et al., 2011).

Pseudomonas putida was revealed to be a laccase-producing bacteria, with the attribute of decolorizing synthetic dyes and industrial effluents.

Streptomyces cyaneus and *Streptomyces ipomoea* exhibit laccase activity, the former having an activity tens of times higher (Margot et al., 2013). Although it is known that *Streptomyces griseus* is also a producer of laccase, in the cited assessment it did not show this kind of enzymatic activity - the localization of the laccase could be responsible for the absence of activity detected.

A fungal laccase, produced by and isolated from the Ascomycete *Chaetomium* sp., was characterized with the ability to decolorize different dyes, even in the presence of high concentrations of sodium chloride (Mtibaa et al., 2017). The purified laccase from this fungus is able to degrade or transform various synthetic dyes, such as Acid Orange, Direct Red, Direct Blue and RBBR (Remazol Brilliant Blue R), with and without mediators.

Another laccase, able to decolorize synthetic dyes, is the one produced by *Spirulina platensis*, a cyanobacterium. Afreen et al. (2017) purified and characterized the laccase from this *Spirulina* genus and evaluated its decolorization property on Reactive Blue 4, the results indicating an almost complete decolorization, without any mediators.

A psychrotolerant bacterial strain of *Serratia marescens* was studied for its laccase production (Kaira et al., 2015). It was proved to synthesize laccase even under extreme

conditions, which is likely to be beneficial for biotechnological applications.

The laccase obtained from Ascomycetae *Myceliophtora thermophila* is suitable for industrial pulp bleaching and delignification (Babot et al., 2011).

ISOLATION, PURIFICATION AND CHARACTERIZATION OF LACCASE

Isolation of laccase

When the enzyme is immobilized, it becomes more resistant to alteration in the environment, allowing easy recovery and reuse of enzyme for multiple purposes. That is why researchers are moving towards the efficient methods of immobilization that influence the properties of the biocatalyst. Laccase has been studied with a wide range of different immobilization methods and substrates (Shraddha et al., 2011).

A variety of methods include immobilization on polyamide matrices, on glass supports, on epoxy-activated carriers, on magnetically separable silica spheres, on magnetic chitosan microspheres, on nanoparticles and on kaolinite. Laccase may be immobilized by entrapment in alginate - chitosan microcapsules or in Cu-Al and Cu-alginate beads. In most cases, immobilization lead to increased enzyme stability and improved resistance to changes in temperature and pH (Mikolasch et al., 2009).

Purification of laccase

Ammonium sulfate is being used for the enzyme purification. Researchers have found much more methods such as protein precipitation by ammonium sulfate, anion exchange chromatography, gel filtration chromategraphy and desalt/buffer exchange of protein. Single-step laccase purification from *Neurospora crassa* takes place by using celite chromatography and 54 fold purifycation was obtained with a specific activity of 333 Umg^{-1} (Grotewold et al., 1998). Laccase from LLP13 was first purified with column chromategraphy and then purified with gel filtration (Kiiskinen et al., 2004a; Kiiskinen et al., 2004b). Laccase from *T. versicolor* is purified by using ethanol precipitation, DEAE-Sepharose, Phenyl-Sepharose and Sephadex G-100 chromatography which is a single monomeric laccase with a specific activity of 91.443 Umg^{-1} (Hess et al., 2002). Laccase from *T. versicolor* is purified with ion exchange chromatography followed by gel filtration with a specific activity of 101 UmL^{-1} and 34.8-fold purification (Cordi et al., 2007). Laccase from *Stereum ostrea* is purified with ammonium sulfate followed by Sephadex G-100 column chromatography with 70-fold purification (Viswanath et al., 2008). Laccase from fruiting bodies is purified with ammonium sulfate precipitation with 40-70% saturation and DEAE cellulose chromategraphy then 1.34 and 3.07 fold purification is obtained, respectively (Shraddha et al., 2011).

Laccase can be immobilized on different pyrolytic graphite (best support), ceramics supports and on a carbon fiber electrode where it acts as a biosensor. At the 12th day, maximum laccase activity of 40.774 UL^{-1} was achieved (Minussi et al., 2007). An optical biosensor is fabricated by using stacked films for the detection of phenolic compounds; 3-methyl-2-benzothiazolinone hydrazone (MBTH) was immobilized on a silicate film and laccase on a chitosan film (Alimin Abdul et al., 2009).

Characterization of laccase

Laccase was first characterized when it was extracted from the Japanese lacquer tree *Rhus vernicifera* in 1883. Later, in 1896, it was demonstrated that laccases were also present in fungi (Desai and Nityanand, 2011).

In higher plants, laccases can be found in *Rhus vernicifera, Rhus succedanea, Lactarius piperatus, Prunus persica*, *Acer pseudoplatanus, Chaetomiaceae* sp.

According to Takao Saito et al. (2003), the purified laccase produced one band on an SDS-PAGE gel at the apparent molecular mass of approximately 73 kDa (Figure 1A).

This laccase was used in gel filtration chromatography on a HiLoad 16/60 Superdex 200 pg column, the molecular mass of the native enzyme being estimated at 80 kDa. The isoelectric point (pI), determined by analytic isoelectric focusing, was 3.5 (Figure 1B). This pI is similar to that of the laccase from the basidiomycete PM1 (Coll PM et al., 1993), which has an acidic isoelectric point.

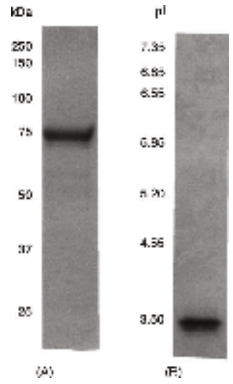

Figure 1. SDS-PAGE (A) and IEF (B) of the purified strain I-4 laccase (Takao Saito et al., 2003)

To determine the state of its catalytic center, the laccase was characterized spectroscopically. The purified laccase had a blue color, typical of copper-containing proteins. The UV-VIS spectrum of laccase showed a peak absorption at about 611 nm, typical for the type I Cu(II), that is responsible for the deep blue color of the enzyme. A shoulder at about 333 nm suggests the presence of type III binuclear Cu(II) pair (Eggert et al., 1996). The EPR spectrum of the laccase showed the superimposed signals from type I and type II Cu(II), each in a different coordination environment. The parameters of the type I Cu(II) signal were gII = 2.21 and AII = 8.3×10^{-3} cm^{-1}, and those of the type II Cu(II) signal were gII = 2.25 and AII = 1.93×10^{-2} cm^{-1}. These spectral characteristics are similar to those of other blue copper proteins that have four copper atoms (Dedeyan et al., 2000; Shin KS et al., 2000).

APPLICATIONS OF LACCASE IN BIOTECHNOLOGY

Food Processing Industry

In the food industry, laccase is used for the elimination of undesirable phenolic compounds in wine and beer stabilization, in baking, in juice processing and in the bioremediation of wastewater (Shraddha et. al., 2011).

Wine browning, due, primarily, to enzymatic and chemical oxidation of phenolic compounds, represents one of the most unwanted processes that can occur in wine-making. During the crushing of the grapes, the release of laccase from *Botrytis cinerea* affected the beans in the must, thus resulting in a significant reduction of phenolic compounds. The important polyphenols in wine, including their major classes (phenolic acids, catechins, anthocyanins, tannins and stilbenes), are converted to the corresponding quinones, that will pass into dark-colored polymers. These polymers are insoluble in water and aqueous solutions and precipitates from the must and wine. Moreover, the oxidation of the phenolic compounds can adversely affect the sensorial and nutritional properties of the wine.

The storage of beer depends on various factors, such as the haziness, the oxygen content, the temperature. The haziness is caused by small amounts of proanthocyanidins, which are naturally occurring polyphenols and proteins that could cause precipitation. This type of complex is known as “cold haze” and occurs during chilling – can be redissolved at room temperature or at higher temperatures. Even the products that do not have this disorder when packaged, could form it during long storage. The usage of laccase for the oxidation of polyphenols as an alternative to traditional therapy has been tested many times. Also, laccase is used to eliminate the oxygen at the end of the production process of beer (Osma et al., 2010).

Sugar beet pectin is a functional aliment, which can form thermo-irreversible gelatins. These types of gelatin are very interesting and can be used in the food industry because they can be warming while retaining the gel structure. Compared with peroxidase, which is used as a food additive, laccase proved to be more effective and safer for consumption.

One of the biggest problems in the fruit juice processing is the enzymatic and the chemical browning. The color and the taste of the fruit depends on the phenolic compounds, which should be selectively removed from the composition, in order to prevent any alteration of taste, flavor, and color – attributed to oxidation of polyphenols. To prevent the discoloration of the fruit beverages, by replacing the chemical adsorbents, enzymes are being used, such as laccase. This enzyme has the potential to eliminate unwanted phenols responsible for browning and disorder in many beverages, such as fruit juice, beer, and wine.

In the bread-making process, it is known that additives are added to improve bread and bread dough, from which results improved texture and flavor, a larger volume and longer freshness. In recent years, enzymes have been increasingly used as enhancing agents, including laccase. Even if the laccase used in the preparation of doughs could be of any origin, inclusively vegetal, it is preferable to be of microbial origin, because it is easier to handle and produce on a larger scale (Si, 2001).

Dye Decolorization

In the textile industry, there are used numerous chemicals (which vary from organic to inorganic compounds) and a large volume of water. These chemical compounds make the dyes resistant to fading when exposed to water, to light, or to other chemicals. Laccase is able to degrade this kind of dyes, which is why there were created industrial processes based on laccase treatment of the syntethic dyes (Dominguez et al., 2005; Hou et al., 2004).

Blanquez et al. (2004) manipulated *Trametes versicolor* into pellets, with which they treated black liquor, to decompose its aromatic compounds, to reduce its color and its chemical oxygen consumption. Their results showed that the color and the content of aromatic compound was decreased up to almost 80% and the COD (chemical oxygen demand) was reduced up to 60%. Their conclusions were the following: *Trametes versicolor* produces laccase, which is able to completely decolorize five dyes (Acid Red 27, Reactive Blue 15, Congo Red, Reactive Black 5 and Acid Orange 6) without absorbing them and to partially decolorize other three dyes (Remazol Brilliant Blue R, Brilliant Yellow and Brilliant Red) while absorbing them. Also, they discovered that the toxicity of a few of the dyes remained the same, while the toxicity of others decreased all the way to becoming non-toxic.

The laccase-based hair dyes are less irritant and easier to handle that standard hair dyes, because the enzyme replaces the oxygen peroxide in the formula.

Laccase is also used in the dechlorination processes. In the presence of xylidine, which is a laccase inducing agent, that modifies the enzymatic activity by increasing it, the concentration of dissolved oxygen is reduced (Unal et al., 2001).

Romero et al. studied *Stenotrophomonas maltophilia* bacteria and found that it is able to decolorize Congo Red, Toluidine Blue, Methylene Blue, Methyl Green and Methyl Orange (Shraddha et al., 2011).

Bioremediation and Biodegradation

Laccase is used in bioremediation, biosolubilization, and desulfurization, in the production of biosensors and biofuels and also in the production of fiberboards. Laccase is used for degradation of industrial wastes, like paper, oil, leather, pharmaceutical, pesticides because of her oxidizing ability towards phenolic and non-phenolic compound (Senthivelan et al., 2016).

The presence of phenols in the industrial wastewater attracted interest in the application of bioremediation processes and their treatment with laccase. The presence of phenolic compounds in drinking water is a real danger. The distillery wastewater is generated during the production of ethanol by the fermentation of sugar cane molasses. This produces a major environmental impact, due to the high content of soluble organic matter and due to its dark brown color. Fungal laccase showed better properties in the reduction of total phenolic compounds in color than the laccase from other sources.

The intensive use of pesticides and the fast industrialization cause the pollution of the soil, the water and the air. Chemical substances persists in the environment and can be removed with high difficulties. *Trametes versicolor* and *Pleurotus ostreatus* are used in the bioremediation of the soil and in the degradation of polychlorinated biphenyls, pyrocatechines, protocatechuic acid and benzoic acid (Udayasoorian et al., 2005).

Ahn et al. (2002) conducted an experiment with two types of soil and *Thermopsis villosa* laccase, each sample with different percentage of water in it. They used free laccase and immobilized laccase (on montmorillonite) for this research. *Thermopsis villosa* laccase has proved to be able to degrade dichlorophenol

from the soil, most likely from the immobilized form.
The laccase from another source, *Cerrena unicolor*, had the capacity of decreasing the lignin amount from sugarcane bagasse, up to almost 40%, within 24 hours (D'Souza-Ticlo et al., 2009).

Pulp and Paper Industry
The industrial manufacture of paper involves the separation and degradation of lignin, by treating the wood pulp with ozone or chlorine dioxide. To reduce the pollution of chlorine-based processes, an alternative involved laccase-based processes, that provided a milder and cleaner delignification (Kunamneni et al., 2007).
TEMPO oxidation by laccase is used in the production of hydrophobic cotton fibers and hydrophobic jute fibers (with dodecyl gallate in this case).

Medical applications
The biosensors used in biomedical engineering are also produced with laccase. In the determinations of chemical compounds, in nanobiotechnology and biomedicine and cosmetics is used laccase too.
The effect of poison ivy dermatitis, which is caused by urushiol, can be reduced with laccase treatment. It has been shown that laccase can oxidize the urushiol to a quinone derivative, that is innocuous (Madhavi et al., 2009).
Some important drugs, like anti-cancer drugs, antioxidants, hormones and hormone derivatives are prepared with the help of laccase (by oxidation) and are added to some cosmetics (Senthivelan et al., 2016).
Laccase can also oxidize iodide to iodine, which is used as a disinfectant.

CONCLUSIONS

Laccases are the versatile enzymes which catalyze oxidation reactions coupled to four-electron reduction of molecular oxygen to water. They are multicopper enzymes which are widely distributed in higher plants and fungi. They are capable of degrading lignin and are present abundantly in many white-rot fungi. They decolorize and detoxify the industrial effluents and help in wastewater treatment. They act on both phenolic and nonphenolic lignin-related compounds as well as highly recalcitrant environmental pollutants which help researchers to use them in various biotechnological applications.
They can be used and act as a biosensor in textile industries, paper and pulp industries, xenobiotic degradation, and bioremediation. Laccase has been applied to nanobio-technology which is an increasing research field and catalyzes electron transfer reactions without additional cofactors.
Recently, several techniques have been developed for the immobilization of biomolecules such as micropatterning, self-assembled monolayer, and layer-by-layer technique which immobilize laccase and preserve their enzymatic activity.

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TRENDS ON PHARMACEUTICAL PACKAGING MATERIALS

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Abstract

Filling and packaging are very important processes in the pharmaceutical sector. Packaging is important as it provides protection of the dosage form, from the environment and keeps them safe until is opened by the customer. Environmental sustainability has to be also taken into account in this field. Pharmaceutical packaging materials should be eco-friendly. These materials are derived from natural resources, like plant or animal derived proteins, cellulose, starch etc. A type of primary packaging material is the capsule. It has the advantage that provide a slippery, smooth and a tasteless shell. This article is reviewing various aspects of eco-friendly pharmaceutical packaging materials, as: types of packaging materials like capsules as well as recent trends of pharmaceutical packaging in pharmaceutical market.

Key words*: eco-friendly, packaging material, pharmaceutical products.*

INTRODUCTION

In general, the pharmaceutical formulations have three important constituents as: the active part, excipients and the packaging material. The active ingredient is the pharmacological active substance that is generally preserved by excipients and packaged in a packaging material (Kumar, 2013)

The packaging materials should prevent the product from damage, such as light, foreign particles, temperature, atmospheric gases, microorganisms and moisture.

One of the key characteristics of packaging materials is to protect the pharmaceutical formulations from leaching, loss of any volatile material and loss or gain of water from the content.

Packaging should prevent mechanical hazards like shock, compression, abrasion, vibration and perforation. The packaging materials should not react with the product and should be able to preserve the product throughout the shelf-life (Kim et al., 2014)

MATERIALS AND METHODS

The primary, secondary and tertiary packaging of pharmaceutical products constitutes an essential element of the technological procedures. The quality control of packaging material has been done by the quality control department. They examine and analyse the sample and report it as approved or rejected.

Primary packaging is the term used to indicate that the packaging is in contact with the pharmaceutical formulations. The stability of the pharmaceutical formulations mainly depends on the packaging material because it is in direct contact (Campbell & Vallejo, 2015). A few examples of primary packaging containers are vials, ampoules and capsules.

The secondary packaging has two goals: to protect the primary packaging and the product, and it never comes in direct contact with the product. The secondary packaging is visible to the consumer and it contains information such as the name of the product, the usage, the ingredients, fabrication and expiration date as blisters, boxes (Kassarjian et al., 2014).

Tertiary packaging is removed by retailers before products are arranged for sale. The consumers don't see the tertiary packaging. The tertiary packaging containers protects the product from the damage which may occur during the shipping transportation from manufacturer to market or drugstore (Kerry, 2014). Examples of tertiary packaging materials: carton box, wooden box.

Monies and Dublane are credited with the invention of the gelatin capsule. In December of 1834 they patented a method for producing a single-piece, olive shaped, gelatin capsule, which was dosed after filling, by a drop of

concentrated warm gelatin solution. Capsules become a popular formulation because of their advantage of use as: slippery, smooth and a tasteless shell. The main advantage of capsules is that they have not unpleasant taste like tablets. Due to an inexpensive production process, they are produced in large quantities and in a wide range of colors. Capsules provide a ready availability of the compounds. Capsules are not usually used for the administration of extremely soluble materials such as potassium bromide, potassium chloride or ammonium chloride, since the sudden release in the stomach could be irritating. The dehydration of compounds could be prevented by using small volumes of inert oils in the powder mixture.

Capsules are pharmaceutical formulations made of coatings containing unit doses of active substances, associated or not with excipients, such as: solvents and lubricants. Capsules are used for oral administration. The gelatin and starch used to prepare the capsule shell as well as the substances used to adjust the consistency of the capsules must fit with the Pharmacopoeia guidelines or other quality standards like ISO. A few examples of other excipients are the opacity agents, surfactants, fragrance substances, coloring agents and preservatives. Capsules can be engraved. The contents of the capsules may be solid, liquid or solid paste. The content must not damage the coat after administration, when is attacked by the digestive juices when the capsule releases the content.

In the formulation, the talc must be at most 3%, stearic acid not more than 1%, magnesium or calcium stearate not more than 1% and aerosol not more than 10% of the weight of the capsule content. Depending on the nature of the coating, there are different types of capsules:

- gelatinous (hard and soft) capsules;
- modified released capsules;
- amylaceous capsules (cassettes).

Hard gelatin capsules (opaque capsules) are prepared from gelatin. Gelatin is a heterogeneous product, derived from treated animal collagen. Common sources of collagen are frozen pork skin and animal bones. Skin and bone collagen are available in most areas of the world. Type A gelatin is derived from a precursor and has the isoelectric point in the region of pH = 9, whereas type B gelatin has its isoelectric point in the region of pH = 4.7. In practice, hard capsules are produced from the mixture of skin and bone gelatins. This gelatin has a high strength and it produces a tough, clear and a firm film. They are in the form of elongated cylinders, rounded to the heads that are inching through the boot. They usually contain mixtures of substances in the form of powders or granules.

Soft gelatin capsules (pearls) which consist of a continuous and soft gelatin coating have a spherical or oval shape. They contain active substances in the form of paste or solids in solution. Gelatin capsules have a thicker coating than hard capsules. The capsule shell is composed of gelatin, a plasticizer, water, preservatives, coloring agents, opacity agents and flavorings. The shell may contain also active substances. Gelatin should not contain more than 15 ppm of iron. In soft gelatin capsules is used as plasticizers glycerin, propylene glycol and sorbitol. Glycerin and propylene glycol cannot be the major constituents of the capsule content, because they have softening effect on the gelatin shell, which can make the capsule more susceptible to extern factors like humidity and heat. Soft gelatin capsules can be classified according to the ratio between glycerin and gelatin in hard (0.4/1), medium (0.6/1) and soft (0.8/1). Gelatin capsules are generally formed, filled and closed by a single operation, but in some cases for extemporaneous use, the shells can be prefabricated. Liquid substances should be homogenous and air-free before they are included in the capsule, and solid compounds are generally dissolved or dispersed in a solubilizer, to obtain a solution or a dispersion of the consistency of a paste. All these substances should be formulated to produce the smallest possible capsule containing the maximum ingredient and physical stability, therapeutic effectiveness and production efficiency. The active ingredients are mostly oily, and they derive from vegetable oils (soybean oil, sea buckthorn oil), mineral oil and fish oil. These ingredients also function as solvents in vitamin capsules. Oily matrix do not retain moisture, water do not pass from the shell of the capsule into the fill material and out during the manufacture and drying of this

capsule. Depending on the nature of the substances (water miscible and volatile liquids) and the contact surface, it can cause a partial migration of the constituents from the content of the capsule in the capsule case and vice versa. Modified released capsules are hard capsules or gelatin capsules, whose contents, coating or both components contain special excipients, or they are made by special methods, in order to change the speed, place or time when the active substance will be released. Modified released capsules include extended release capsules and delayed release capsules.

Amylaceous capsules (cassettes) are solid preparations, made of a hard shell. These cassettes contain one or more active substances prepared from starch. They are flat cylinders whose diameters, slightly different in size and allow their closure by overlapping and gentle pressing. They contain substances or mixtures of powdered substances. Before administration, the cassettes are soaking in the water for a few seconds and then put on the tongue and swallow with water.

Constant innovations in the pharmaceutical industry have a direct impact on the packaging. Traditionally, medicines on the pharmaceutical market could be found in different shapes like tablets or capsules packed in blister packs or bottled into plastic pharmaceutical bottles.

Oral tablets are available in a wide range of different shapes, colors and sizes as we can see in Figure 1.

Figure 1. Oral tablets as: hard gelatin capsules colored in white, beige and green; soft gelatin capsules colored in red-garnet

Humidity and light are factors which degrade the packaging materials and also the capsule and active ingredients. Packaging of oral tablets should be easy to dispense, child resistance but in the same time adult-friendliness. Packs must also be easily recognizable by aspect, functional and hermetically sealed.

In our lifestyle the blister packs ensure hygiene and they offer convenience. Blister packs are ideal in our quick rhythm lifestyle and because of that there has been a large increase in their use across the years. Indeed, blister packaging has provided the best worldwide growth among all pharmaceutical packaging products. Pharmaceutical producers confront with cost pressures during the packaging process and also with the production process. It should be a challenge to build efficient, user friendly and easy to operate packaging machines (Kunal, 2012)

All packaging materials must be tested on stability studies. Packaging of pharmaceutical products plays a very important role in the maintenance of their quality. Eco-friendly packaging materials are packaging that uses environment safe materials in its production. These eco-friendly materials should not harm the environment and should be marked with eco-labels (Bird, 2009). For example, eco-friendly packs include paper which is recycled and corn starch because is biodegradable in nature.

Corn starch is used as an eco-friendly pack in different products including bags, boxes and trays. Corn starch is an alternative material to plastic because it has similar functional properties and it's biodegradable. When the product is in the market it is very difficult to be rebuilt so the changes should be made in an early stage of the development (Edward, 2009).

Greener packaging designs accomplish the needs of most pharmaceutical producers without sacrificing our environment. Packaging materials are manufactured and designed to allow recycling. The percentage of recycling depends on the weight or on the minimum calorific value. Eco-friendly packaging should be modernized, biodegradable, well-designed, easily recyclable or reusable (Bird, 2009).

Eco-friendly pharmaceutical packaging materials have two concepts: the first is that the material should be recyclable and the second is

that the material should be biodegradable. Some products are also enhancing their brand image by adopting this type of eco-friendly pharmaceutical packaging because is one of the hottest trends (Hunt, 2010).

Eco-friendly pharmaceutical packaging materials can be classified in several ways: based on their uses, based on the chemical constituent and polysaccharide content.

Based on their uses, the packaging materials should have a barrier protection which provides protection against moisture, light, oxygen and temperature variations, the biological protect-tion provides protection against biological contaminants, the physical protection ensures protection against any physical damage. The information communicated on the packs should offer to the consumer information about the correct usage of dosage forms, their prove-nance, their side-effects and warnings. The eco-friendly pharmaceutical packaging should have security protection from small children and against counterfeiting.

Examples of eco-friendly packs:

Starch is an eco-friendly polysaccharide and a widely available raw material. Starch is obtained from various sources like cereals and legumes. The most known source of starch is potato, corn, wheat and rice (Weber, 2000). Starch is used for flexible or rigid packaging, bags and sacks. Since starch packaging mate-rials are fragile in nature, when a high concen-tration of starch is used, various biodegradable plasticizers like glycerol and other low-molecular weight polyhydroxy compounds, polyether and urea are added. Plasticizers inhibit the microbial growth by lowering the water activity. These four types of starch based on the polymers types are thermoplastic starch products, starch synthetic aliphatic polyester blends, starch-Polyvinyl alcohol blends and starch polybutylene succinate (PBSA) polyester blends (Edward, 2009)

Cellulose is a linear polymer which is found abundantly in nature. Cellophane is the most common cellulose-based biopolymer. Methyl and ethyl cellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl cellulose and cellulose acetate are cellulose derivatives used for packaging. These are widely used in pharmaceutical packaging (Weber, 2000).

Xylan is naturally found in plant cell walls and algae**.** It forms a group of substances called hemi-cellulose. Xylan is a biodegradable, compostable and eco-friendly derivate. Xylophane is thus used as an environment friendly packaging material.

Chitin is the second most abundant polysaccharide resource after cellulose. It is found in the exoskeleton of invertebrates. Chitin is used in packaging because it has antimicrobial property, protects the product from unnecessary microbial growth and it maintains the preservative action. Heavy metal ions are absorbed by the chitin. Chitin is mostly used as packaging material in edible coatings (Srinivasa & Tharanathan, 2007)

Protein. A protein is formed of repeating units of amino acids. Protein based materials are derived from agricultural materials or agro-packaging materials that are renewable and biodegradable and they are used in edible packaging. Agro-packaging concept refers to the use of renewable products and control of the end products. Numerous animal proteins and plant proteins are commonly used as raw material for agro-packaging materials. Thus proteins can be split into plant origin proteins (e.g. soy, gluten, pea, potato etc.) and animal origin proteins (e.g. casein, whey, collagen, keratin etc.) (Platt, 2006).

The point of eco-friendly packaging materials is the decrease of the amount of packaging material which ought to be effectively biodegradable, nontoxic, reusable and inert. Recycling of materials like aluminum, paper and glass creates less or no waste and they are environmentally safe. Incineration of pharmaceutical packaging is recommended to eliminate the contaminated packs. The plastic materials that cannot be recycled are therefore incinerated. The recycled materials (glass and metal) are considerate safer for formulations against microorganisms (Marsh & Bugusu, 2007). Renewing the materials is the property of eco-friendly packaging obtained from renewable natural resources that can be reprocessed into new packaging, e.g. thermoplastic. Repurpose the materials the property of eco-friendly packaging material to be molded in another new forms with another pharmaceutical purpose in mind.

Agro-based materials are renewable and biodegradable, and they contribute to development of pharmaceutical sustainable packaging and this reduces their environmental impact. Such biodegradable packaging materials are suitable for single use disposable packaging applications.

The primary idea of eco-friendly packaging materials depends on its biodegradable viability. The biodegradation of pharmaceutical packaging materials mechanism is the initial scission of the enzyme forming a chain. Then the metabolized portions are leading to the enzymatic dissimilation of the macromolecule from the chain ends. The oxidative cleavage of macromolecules constitutes the basic skeleton of pharmaceutical packaging material. This is often leads to better metabolization of the fragments. These fragments are converted by microorganisms because they are smaller enough. The decomposed of eco-friendly pharmaceutical packaging material is made in a bio-waste collection. This bio–waste collection is composted into environment friendly products like carbon dioxide and water (Petkewich, 2003).

Another mechanism is the photo degradation of pharmaceutical packaging material. The photo degradation role is to make smaller disposable materials which do not create any environmental hazard. When biodegradable pharmaceutical packaging material are exposed to chemical based aqueous solutions they rapidly dissolute it. After the rapid dissolution the materials suffers a microbial digestion. These packaging materials disintegrate when they are exposed to aqueous solutions, which are used for the transport and disposal of pharmaceutical wastes (Petkewich, 2003).

RESULTS AND DISCUSSIONS

Organizations such as ISO (International Organization for Standardization) and WHO (World Health Organization) have officially set the standards for protected and effective packaging materials and technologies which ought to be pursued. Therefore, the Pharmaceutical sector has to be specific when is using these eco-friendly materials for packaging because the packaging materials should protect the product against damages produced by external factors as: light, foreign particles, temperature, atmospheric gases, microorganisms and moisture. One of the key attributes of packaging materials is to protect the pharmaceutical formulation from draining, loss of volatile substances and misfortune or increase of water from the content. Packaging ought to avert mechanical dangers like shock, abrasion, compression, perforation and vibration. The materials ought not react with the product and ought to have the capacity to preserve the product throughout the shelf life. Eco-friendly packaging materials should be packaging that uses environment safe materials in its production and it should not harm the environment. This types of environmentally friendly pharmaceutical packaging materials are marked with eco-labels. Eco-friendly pharmaceutical packaging materials should have two concepts the first is that the material should be recyclable and the second is that the material should be biodegradable. For example, starch is an eco-friendly polysaccharide and it's a widely available raw material. It is obtained from various sources like potato, corn, wheat and rice and it is used for flexible or rigid packaging, bags and sacks. Cellulose is another eco-friendly material which is found abundantly in nature. Xylan is also a biodegradable, compostable and an eco-friendly derivate. Xylophane is used as an environment friendly packaging material. Chitin is used in packaging because it has antimicrobial property, protects the product from unnecessary microbial growth, it maintains the preservative action and it has the unique property to absorb heavy metal ions. Protein based eco-friendly materials are derived from agricultural materials or agro-packaging materials and they are renewable and biodegradable.

CONCLUSIONS

Nowadays, packaging of pharmaceutical products plays a very important role in the pharmaceutical sector. The pharmaceutical industry need to focus on the development of new biodegradable materials, regarding the generation of environmental friendly packaging material that adds value to the pharmaceutical

products as well as it creates a new outlook into the concept of eco-friendly pharmaceutical packaging materials. The objective for pharmaceutical organizations is to concentrate on the development of a single eco-friendly packaging material that acknowledge the combined characteristics of glass, plastic, paper, metal and rubber. Starch, cellulose, xylan and chitin could be used as eco-friendly packaging materials because they have antimicrobial properties and because they are compostable and biodegradable.

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HIGHER UTILIZATION OF WASTE FROM THE FOOD INDUSTRY THROUGH BIOTECHNOLOGICAL METHODS

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Abstract

Until now, the most important way to get rid of food waste was to use it in animal feed. Four methods are used to remove food waste: recovery in agriculture and animal husbandry, incineration, anaerobic fermentation, composting. This paper aims to evaluate new methods of recycling waste from the food industry through biotechnological methods. The wastes that were the subject of the research were the following: apple pomace, bakery waste, milk whey. All these have been processed using the strains of the Biotechnology Faculty Collection in order to determine the potential to obtain useful products of high economical value like bioethanol or probiotics. The experimental yield of 0.26-0.24 l of bioethanol/1000 g of food waste was at the minimum data mentioned in the literature (0.265 l) but justifies the capitalization of this food waste.

Key words*: apple pomace, whey, bioethanol, probiotic, biomass.*

INTRODUCTION

The food industry is subject to increased pressures to improve environmental performance, both on the part of consumers and on the part of the legislation, which, in turn, responds to consumer pressure. A series of "friendly and clean" food processing technologies have been designed precisely to enable manufacturers to better understand the effects of their activities on the environment and to adopt practical measures to achieve sustainable production (Gavrila, 2007).

The two crucial issues related to food technologies are energy management and waste management. The production of food is done with high energy consumption, and relatively large quantities of waste result from the process. Waste from the food industry can be divided into three categories: waste from production processes, food waste from municipal waste and packaging.

Current methods of waste use have been developed along with traditional production lines, being closely linked to the agricultural origin of raw materials (Manimehalai et al., 2007). Traditional methods used in the past and beyond for the use of food waste, were: animal feed and fertilizer on farmland (Gavrila, 2007).

The following general methods (Russ et al., 2007) may be used to remove solid waste in general: recovery in agriculture or animal husbandry; incineration; anaerobic or aerobic fermentation; composting. In the case of liquid waste, the following methods can be used: ground application; various methods of physico-chemical treatment, fermentation.

In the case of waste with more than 50% humidity, anaerobic fermentation with the production of biofuels is much more appropriate (Gavrila, 2007). If cellulosic polysaccharides can be decomposed, the rate of decomposition is low, defining the limit of fermentation by producing biogas (Akpan et al., 2008).

Food industry waste should be viewed as a raw material source for the production of high added value products rather than as waste (Shalini et al., 2010).

For example, monosaccharides can be obtained by selective hydrolysis of whey, lactose and oligopeptides can be obtained from whey protein concentrate by peptide hydrolysis. Enzymatic conversion of cellulose-rich waste can produce ethanol. Pectin can be recovered from effluents from fruit juice production (Pap et al., 2000). There is practically no "waste" of the food industry that can not be used as a raw material to get value-for-money products.

This paper aims to evaluate new methods of recycling waste from the food industry through biotechnological methods. All food waste, apple pomace, bakery waste, milk whey, have been processed in order to determine the potential to obtain useful products of high economical value, bioethanol, probiotics, using strains from the Collection of the Faculty of Biotechnologies from UASMV Bucharest.

MATERIALS AND METHODS

Raw materials

The main raw materials were: apple pomace, bakery waste and milk whey.

The whey was provided by a private manufacturer and stored at 4°C until processing.

The apple pomace was obtained in the laboratory, after the preparation of apple juice with a fruit-maker.

Bakery wastes were purchased from a local pastry and consisted of specific product scraps.

Microorganisms

To obtain bioethanol and probiotics, strains of *Saccharomyces cerevisiae* SC2 and *Lactobacillus bulgaricus* L16 from the Microorganism Collection of the Faculty of Biotechnologies were used.

Microbial strains were grown on their specific media, respectively YPG medium for yeast and MRS for lactobacilli.

Processing of waste

The whey was first centrifuged for semipurification by removing solid residues and grease (8000 rpm, 15 min, 3°C). Next, filtration was performed for removing residual impurities left after centrifugation.

Apple pomace, bakery waste were suspended in water in a ratio of 1: 2 g/v and boiled for 5 minutes.

The products obtained were used as the single nutritional source for microorganisms.

Obtaining of bioethanol and probiotics

For the biosynthesis of bioethanol and probiotics each processed waste was inoculated with 10% *Saccharomyces cerevisiae* inoculum.

In addition, milk whey was inoculated with 10% *Lactobacillus bulgaricus* inoculum to obtain the probiotic preparation.

Fermentations with *Saccharomyces cerevisiae* were carried out under the following conditions: 5 days, 25°C, pH = 4.8.

Fermentations with *Lactobacillus bulgaricus* were carried out under the following conditions: 3 days, 35°C, 20 rpm, pH = 5.5.

Analytical control of the fermentation process

Determination of dry cell weight (DCW).

10 ml of whey medium was centrifuged in a centrifuge tube at 4000 rpm for 20 minutes. After the supernatant was removed, the biomass was weighed. Wet biomass was then dried at 105-110°C, to constant weight using a thermobalance.

Post-biosynthesis processing

After the completion of the fermentation process of whey, the first post-biosynthesis operation was to separate the biomass by centrifugation at 4000 rpm for 20 minutes. After centrifugation, wet biomass was collected and weighed, placed in trays and dried in the oven at 105°C. Finally, the total amount of dry lactobacilli biomass was weighed.

In the case of alcoholic fermentation, the media were vacuum filtered on filter paper. The filtrate was distilled using a rotary evaporator to obtain bioethanol.

RESULTS AND DISCUSSIONS

Probiotic yield from milk whey

For probiotic biosynthesis milk whey was inoculated with *Lactobacillus bulgaricus* strain.

Following the post-biosynthesis of the lactobacilli media, 2.8-3.2 g/l of dry biomass was obtained, 36% lower than those mentioned in the literature, which stated amounts of 5-5.1 g/l (Rezvani et al., 2016) (Figure 1).

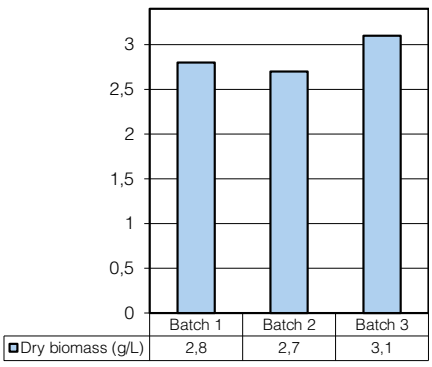
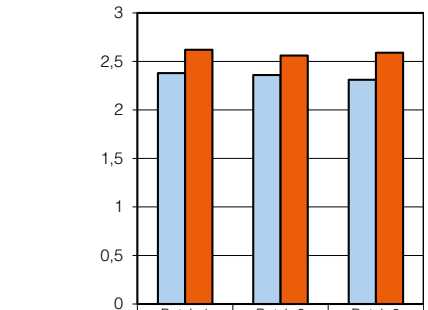


Figure 1. Dry biomass obtained from lactic fermentation

Bioethanol yield from apple pomace and bakery waste

The alcoholic fermentation process was characterized by two important parameters: the amount of dry biomass obtained and the resulting bioethanol volume.

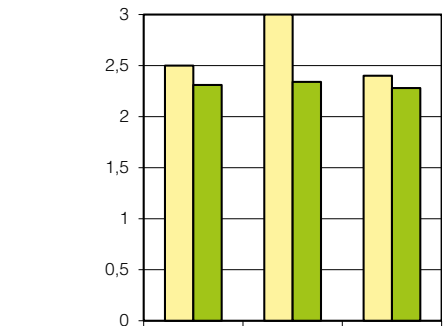
From the data presented in the Figure 2, it can be concluded that apple pomace is a valuable nutrition source for the growth of yeasts in order to obtain bioethanol, achieving productivity of 2.56-2.62 ml/g of solid substrate.



	Batch 1	Batch 2	Batch 3
Dry biomass (g/L)	2,38	2,36	2,31
Bioethanol (mL/g)	2,62	2,56	2,59

Figure 2. Dry biomass and bioethanol obtained from alcoholic fermentation of apple pomace

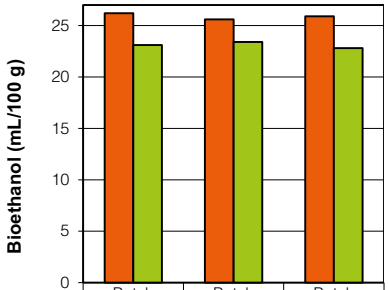
In the case of alcoholic fermentation of bakery waste, the results were slightly weaker than apples (Figure 3). The productivity in bioethanol ranged between 22.8-23.4 ml/100 g of material (Figure 4).



	Batch 1	Batch 2	Batch 3
Dry biomass (g/L)	2,5	3	2,4
Bioethanol (mL/g)	2,31	2,34	2,28

Figure 3. Dry biomass and bioethanol obtained from alcoholic fermentation of bakery waste

By comparing the amounts of bioethanol obtained from the two nutrient sources, apple pomace has a 9-13% higher productivity than bakery waste.



	Batch 1	Batch 2	Batch 3
Apple pomace	26,2	25,6	25,9
Bakery waste	23,1	23,4	22,8

Figure 4. Comparison of the amounts of bioethanol obtained from the raw materials tested

The volume of bioethanol produced from 100 g of food waste was 26 ml. As reported in literature by Mathewson (1980), that a 1 ton of 40%-60% fermentable sugar substrate can produce 265-378 l of bioethanol, this means that substrate of 1000 g can produce a maximum bioethanol yield of about 0.265 l and a minimum yield of 0.378 l.

The experimental yield of 0.26-0.24 l of bioethanol from 1000 g of food waste is well within acceptable range.

The experimental results obtained in this work showed that fermentation processes was at the minimum data mentioned in the literature for production of bioethanol from food waste.

CONCLUSIONS

For lactobacilli, 2.8-3.2 g/l of dry biomass was obtained on whey, 36% lower than data mentioned in the literature,

For bioethanol, from the two nutrient sources, apple pomace has a 9-13% higher productivity than bakery waste.

The experimental yield of 0.26-0.24 l of bioethanol/1000 g of food waste was at the minimum data mentioned in the literature but justifies the capitalization of this food waste.

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EFFICIENT UTILIZATION OF WATERMELON WASTES

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Abstract

The watermelon is a fruit used on a large scale in human nutrition. Cucurbitceae species are a source of many nutrients such as protein,minerals, lipids as well as ingredients for medicine. The main non-edible parts are the seeds and the rind that make them agro-wastes. Watermelon seeds and rind may represent an appropiate tool for the treatment of various diseases in those countries that use a more classical therapeutic approach. In this article is made a brief analysis of the ways to recover the resulted wastes from the cultivation of watermelon. The most promising directions are obtaining the bioactive compounds, biofertilizers and biogas.

Key words*: watermelon, Citrullus lanatus, wastes.*

INTRODUCTION

Watermelon (*Citrullus lanatus* L.) is a fruit crop part of the Cucurbitaceae family, along with the cucumber, zucchini and melon. It grows in temperate regions of Africa, Turkey, China, Egypt, Iran, Mexico, South Korea, India and some parts of the United States. The fruit of the *Citrullus lanatus* L. is usually used as food and in the pharmaceutical industry for the nutritional factors of the fruit and as well as seeds.

Present review is a collection of information about the phytochemistry, pharmacology and utilization of the watermelon wastes.

BOTANICAL CLASSIFICATION

Kingdom: Plantae
Division: Magnoliophyta
Order: Cucurbitales
Family: Cucurbitaceae
Genus: Citrullus
Species: lanatus

BIOLOGY

Citrullus lanatus known as watermelon, is an annual herbaceous vine with up to 10 m stems creeping or liying on the ground. Leaves are 3-20 cm, deeply palmately lobed with 3 to 5 lobes. Leaf stalks are between 2 and 19 cm long. Male flowers on 1.2-4.5 cm long pedicels. Flowers 1-2.5 cm long and coloured in pale green. Flowers are monoecious, solitary, on pedicels long up to 4.5, with pale green united petals. Fruit of wild plants are nearly spherical, in diameter 1.5- 20 cm, green combined with darker green. The fruit of the cultivated plants are up to 20x60 cm, weight between 3 and 5 kg, the form ellipsoidal or spherical, colour is green or yellowish, or variously mottled or striped. The fruits vary in colour, taste and odour (Dane F., 2007).

Seeds are numerous, 6-10 mm long, compressed, pyriform, black or dark brown. They continue to mature as the fruit rind lightens in colour.

Botanical name: *Citrullus lanatus*

PRODUCTION TECHNOLOGY

Watermelon is cultivated in all over the world in the warmer parts, but is trully native from the Tropical Africa where are sandy and dry areas.

As climate *Citrullus lanatus* needs a dry and warm climate and to develop and grow successfully must be cultivated at a 30-35°C temperature. The flowering as the fruit development are helped by high light intensity and temperature. The areas with high humidity during the fruit formation stage and more rain at maturity are not going to thrive.

It is compatible with all types of soil but most suitable are the sandy soils with a pH range from 5.5-7.5. The soil must be drained.

COMPOSITION

Table 1. Seed nutritonal values (per 100g)

COMPONENT	VALUE	AUTHOR
Protein	34.1 g	Gopalan C., 1971
	35.66 g	Tarek A. El-Adawy, 2001
	35%	Okunrobo O., 2012
Fat	52.6g	Gopalan C., 1971
	50.10g	Tarek A. El-Adawy, 2001
	50%	Okunrobo O., 2012
Arginine	900 mg/g of N	Gopalan C., 1971
	1161.25 mg/g	Tarek A. El-Adawy, 2001
Calcium	100 mg	Gopalan C., 1971
	150 mg	Tarek A. El-Adawy, 2001
	16.8 mg	Okunrobo O., 2012
Phosphorus	937 mg	Gopalan C., 1971
	1279 mg	Tarek A. El-Adawy, 2001
Zinc	10.6 mg	Tarek A. El-Adawy, 2001
	1.2 mg	Okunrobo O., 2012
Magnesium	11.4 mg	Okunrobo O., 2012
Fiber	5%	Okunrobo O., 2012
Potasium	7.8 mg	Okunrobo O., 2012
Sodium	5.7 mg	Okunrobo O., 2012

Chemical components

The watermelon seeds are free from glucoside and alkaloids. The resin has cucurbitol ($C_{24}H_{40}O_4$) and a small amount of phytosterol. The shell of the seed it's 48.7% of the entire seed weight. Another fatty acid present but in small amount and in the shell is the arachidic acid (Biswas R. et al., 2016).

Anti-nutritional factors from watermelon seed

Citrullus vulgaris has a source of protein inhibitors from the watermelon seeds are the amino acid sequences of two trypsin inhibitors that contain Arg5-Ile6 bond at their reactive site.

In the seed kernel flour of *Citrullus lanatus* phytic acid and trypsin inhibitor were found in considerable amount (Biswas R. et al., 2016).

Antioxidants – Polyphenols and flavonid content

The methanolic extract from seeds of *Citrullus vulgaris* have a high antioxidants activity and a high content of flavonoids and polyphenols.

The n-hexane extract of *Citrullus lanatus* seeds showed the highest antioxidant activity and total phenolic content where the ethanol extract had highest total flavonoid content.

Antioxidant activity of *Citrullus lanatus* of chloroform, ethyl acetate and methanol, mesured by DPPH method. Methanolic extract of *Citrullus lanatus* seed showed maximum antioxidant potential (Naresh Singh Gill, 2011).

PHARMACOLOGY

Laxative activity of aqueous fruit pulp extract at doses depending on the body weight (250, 500, 1000 mg/kg), was based on the weight of the faeces matter. The laxative activity was seen after administered orally and on treatments with loperamide to induce constipation. Another thing is that happened is a significant increase of the intestinal transit (in comparison with castor oil (Swapnil Sharma, 2011).

Antimicrobial activity of chloroformic, hexane and ethanolic extracts of leaves, stem, fruits and seeds against bacteria (*E. coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis and Proteus vulgaris*) and fungi (*Aspergillus niger, Candida albicans*). The activity was tested by cup-plate diffusion methods and disc diffusion method. The highest antimicrobial results was in using the chloroform extract of the fruit and it showed results against *Bacillus subtilis* (38 mm), *Escherichia coli* (37 mm), *Staphylococcus aureus* (36 mm), *Proteus vulgaris* (23 mm) and *Pseudomonas aeruginosa* (19 mm). The highest antifungal activity was shown using ethanolic extract of the fruit pulp and stem on *Candida albicans* (41 mm). *Aspergillus niger* on the chloroform extract of the seed was very sensitive and also on the ethanolic extract of the leaves (37 mm), in comparison with drugs as clotrimazol and gentamicin (Loiy Elsir Ahmed Hassan, 2011).

Cucurbitacin L 2-O- -glucoside pure and Cucurbitacin E isolated from *Citrullus lanatus* var. *citroides* have a strong antigiardial activity against *Giardia lamila in vitro* (IC = 2 and 5 μg/ml after 5 days). The best of all

examinated extract was the ethyl acetate extract and then petroleum ether followed by butanol with IC50 0.1, 0.2 and 0.5 µg/ml) (Loiy Elsir Ahmed Hassan, 2011).
Anti ulcer activity of *Citrullus lanatus* seeds of methanolic extract was tested by pyloric ligated and water immersion stress induced ulcer models on rats. The rats trated with methanolic extract in 300 mg/kg have shown a significant decrease in the gastric volume, free acidity and total acidity for the pyloric ligated ones and an inhibition of ulcer by decrease in ulcertive index (Altas S. et al., 2011; Biswas R. Et al., 2016).

Anti-inflammatory activity of *Citrullus lanatus* seed oil *in vivo* and *in vitro* evaluation
In vitro anti-inflamatory activity was screened for human red blood cell membrane stabilization method and *in vivo* by carrageenan-induced paw edema in rat model. The oil had a significant reduction of the edema maximum at 3 hours (the percentage in the reduction of the paw volume 44.44%, 44.56% and 63.11% with 50 mg/kg, 100 mg/kg of *Citrullus lanatus* seed oil and 10 mg/kg with diclofenac. (Madhavi P. et al., 2012)

Anti-hyperlipidemic on hypercholesterolemia induced atherosclerosis in mice
Mice that consumed *Citrulls lanatus* extract had very increased plasma citrulline concentrations, lower body weight and fat mass, a significant decrease plasma cholesterol concentration by the reduction of lipoprotein cholesterol. The consumption of the extract resulted in reduction of atherosclerosis in aortic arch and thoracic regions (Poduri A. et al., 2012).

***In vivo* activity of watermelon seeds**
Effect on growth
Watermelon seed full-fat and watermelon seed meal samples were analyzed for composition and introduced in the diet of broiler chicks. The full fat increased the weight gaining ($P<0.05$), the protein consumption and feed intake. The feed intake was increased linearly with increasing levels of watermelon seed meal. This shows that watermelon seed full-fat can be used as food ingredients in the diets of broiler chicks at up to 20%. Male albino rats fed with *Citrullus lanatus* seeds had higher weight gain ($P<0.01$) and protein efficiency ratio than the group of rats fed with stock diet.

Anti-diabetic effect
Citrullus colocynthis seeds aqueous extracts in streptozotocin induced in diabetic rats had a anti hyperglycemic effect, glucose homeostasis and body weight maintenance.

Cardioprotective effect
A group of male albino rats fed with *Citrullus vulgaris* had the level of serum triglyceride and VLDL-C significantly decreased ($P<0.05$). Serum total cholesterol, LDL and atherogenic index decreased where HDL was increased.

Anti-obesity and anti-arthritic effect
At the highest dose level of 2000 mg/kg with alcoholic and aqueous extract of *Citrullus vulgaris* seed no mortality or abnormal behavior happened at mice. The seed extracts with 200 mg/kg and 4000 mg/kg had a significant result with body weight lose, reducing the food intake, reducing serum glucose, trygliceride, cholesterol levels with an increased HDL levels in CD and AD induced obese rats (Biswas R., 2016).

VALORIFICATION OF WATERMELON WASTES

Yeast cultivation
Using the water extracts from watermelon, cabbage, a mix of residual biomass of tropical fruits and green salads can be used as substrate for yeast cultivation. Vegetables and fruit processing wastes cantain organic acids and soluble sugars, that can be used by some microorganisms, most by yeast, to produce a high contented protein biomass with no need to add any nutrients. Is possible to increase the economic value and nutrients of yeast by adding aprox. 5 µg/ml of selenium (Stabnikova O., 2005).

Carbon source for carotene production
A main carbon source for carotene production can be made using solid agro-food wastes

such as watermelon rind, cabbage and peach peels and a heterothallic fungus, *Blakeslea trispora*. This fungus was able to use different origin agro-food waste, almost equivalently, for carotenoids production with a yield of over 76% in all examinated cases (Papaioannou E.H. et al., 2012).

Cutin source

A new alternative cutin source can be found in agro industrial wastes as inducers of cutinase production by *Fusarium oxysporum*. The cutin that is isolated from watermelon peels can yield 6.77 U/ml and the cutinase using apple cutin 9.64 U/ml. The Fourier transform infrared spectroscopy and C cross polarization-magic angle spinning nuclear magnetic resonance spectra solid state NMR studies show the nature of watermelon to be an aliphatic polyester of polyhydroxy fatty acids. During submerged fermentation the ester linkages in watermelon were completely hydrolyzed.

The GC-MS indicate the critical structural feature of the hydroxyl groups at ɯ-position and middle of the fatty acid chain. X-ray diffraction of the watermelon show thats it has amorphous nature. Differential scanning calorimetry indicates two endothermic transition peaks, one of the broads appear at 30-60°C and the other one at 145°C. Thermogravimetric analysis of watermelon shows that it can be thermally stable up to 200°C. Use of watermelon peels as a cutin source in the production of cutinase can receive commercial value and the industries of processing the watermelons can financially boost (Sandeep A. et al., 2015).

Producing biofertilizer

In farming situation the agro-wastes is often useless and will be discarded. The accumulation may cause environmental, health, esthetic and safety concern and that is why it requires safe disposal.

Produceing a biofertilizer using agro-wastes represents a simple and cost-effective method. Using wastes from watermelon, papaya, pineapple, banana and citrus orange and solid-state fermentation method it can produce biofertilizer and then apply on vegetable plantation. The watermelon biofertilizer had the biggest pH level (5.15) compared to the banana one that was 4.52, papaya 4.45, pineapple 4.42 and citrus the lowest, 4.08. The potassium content was the biggest at the banana fertilizer with 3.932 g K/l, followed by pineapple with 2.828, papaya 2.245, watermelon 1.529 and the least citrus orange 0.472.

The efficiency of the biofertilizer is reflected by crop growth rate. After applying on the first batch biofertilizers on the Mustard plant, *Brassica juncea* var. *rugosa*, the average weight showed that the biofertilizer had the biggest impact of growth from the watermelon one with a value of 0.100 g and the lowest from the citrus orange with 0.026 g. The second batch the best weight was still from the watermelon fertilizer with 0.208 g, the papaya 0.103 g, banana 0.072 g, citrus orange 0.059 g and the least weight 0.051 g. The untreated plant had 0.027 g. This shows shows that the acidity content in the biofertilizer affects the growth of the plant.

The average lenght of longest root in the Mustard plant sample with watermelon biofertilizer was 40.6 mm after the first batch and the second batch 70.8 mm, papaya with 31.8 mm and 47.7 mm, pineapple 28.0 mm and 37.4 mm, banana 31.6 mm and 25.8 mm and citrus orange 19.8 mm and 13.6 mm. The untreated plant had 20.1 mm.

The untreated mustard plant had the average number of leaves 3.5. The watermelon fertilizer grew 5.2 leaves and 5.6, papaya 3.8 and 5.1, banana 4.2 and 3.4, pineapple 3.6 and 2.7, citrus orange 3.6 and 2.5.

This shows that agro-wastes biofertilizers from watermelon, papaya and banana are suitable to be used as biofertilizers using solid-state fermentation (Soh-Fong Lim, Sylvester Usan Matu, 2015).

Biogas generation

Nowadays energy represents a very important factor and an inadequat energy energy supply can affect socio-economic activities and limit economic growth. Because of the rising prices of the crude oil and the degradation of the environment we have to find alternative energy sources that are renewable. Some of these sources are represented by biofuels like biogas, biodisel and bioethanol because they are feasible energy source, compatible with

current combustion engine technology and distribution networks that already exist.

Watermelon rind and pineapple peels were each co-digested with food wastes in ratio 1: 1 while using rumen contents of cattle as inoculum. The generated gas had 68% Methan, 20% Carbon dioxide, 6% Nitrogen, 2.5% Hydrogen, 1.5% Hydrogen sulfide and 1% Oxygen. In terms of pathogen treatment the anaerobic digestion is found efficient, and five logarithmic units were reached with the reduction of coliforms. The establishment of using these substrates for biogas plants will reduce solid wastes and ensure a low-carbon and safe environment (Dahunsi S.O. et al., 2015).

Biosorbent for removal of trivalent chromium from aqueous solution

One of the most toxic heavy metal ions is Chromium that has adverse effects on humans and aquatic life. There are many conventional treatment processes like precipitation, ion exchange, filtration, membrane filtration, electrochemical treatment and reverse osmosis, but they have disadvantages like less efficiency, high treatment and disposal costs. Adsorption is cost effective and more efficient technique for the removal of heavy metals from wastewater.

The watermelon rind was evaluated as economical sorbent for the removal of Cr^{3+} from aqueous solution. By using varying pH, contact time, adsorbent dose and initial metal ion concetration was performed a batch mode adsorbtion. Watermelon rind maximum loading capacity was 172.6mg g^{-1} for Cr^{3+} ions at pH 3. The removal is found as rapid and the equilibrum was reached in aprox. 30 minutes and follows pseudosecond order kinetic model. Desorption studies show that watermelon rind could be used without any decrease in efficiency (Nimmala Anvesh Reddy et al., 2014).

Sorbent for removal of nickel and cobalt from aqueous solution

The most used sorbent for metal removal from effluents is activated carbon, but its application is limited due to high costs for activation and incomplete regeneration and thats why agricultural waste and industrial by-products may be low-cost effective and have better sorbents.

In process effluents we find Ni^{2+} and Co^{2+} ions from battery manufacturing, electro-planting and mineral processing and for because is toxic to humans and animal life we have to remove them. The watermelon rind is rich in polymers like citrulline, proteins, cellulose and carotenoids with functional groups such as amine (proteins), hydroxyl (cellulose) and carboxylic (pectin) and can easily bind metal ions. The maximum sorption capacity of watermelon rind was found to be 35.3 and 23.3 mg g^{-1} for Ni^{2+} and Co^{2+} ions, respectively. Ni^{2+} ions showed higher affinity and adsorption rate compared with Co^{2+} ions under the experimental conditions. Extraction of Ni^{2+} and Co^{2+} ions was significantly affected by the presence of other metals due to competition (Lakshmipathy R. et al., 2013).

Biosorbent for adsorption of methylene blue

Various industries release effluents containing dyes and cause negative impact for the aquatic organisms and humans. These are one of the causes of eutrophication and pollution, toxic and because of their aromatic complex structure and syntethic nature and potetially carcinogenic due. Most of the widely used basic dye for cotton, silk and wool is represented by methylene blue. Is known to cause dysfunction of the liver, central nervous and reproductive system, brain and kidney.

The most effective technique for the removal of methylene blue is adsorption because of its simplicity, flexibility and without generating hazardous secondary products.

Physicochemical characterization of the watermelon rind show that the carboxyl and hydroxyl groups have an important role in the adsorption of methylene blue.

The adsorption capacity of the watermelon rind for the methylene blue was 188.68 mg/g at 303K. The best dossage was 0.06 g and superior methylene blue adsortion capacity was in pH 5 solution. Acording to the thermodynamic adsorption parameters, watermelon rinds was exotheric and spontaneous (Ali H. et al., 2018).

Adsorbent for zinc removal

Waste water from mosaic industry has zinc in concentration range of 350 mg/l to 450 mg/l. Biosorption had some advantages over conventional treatment methods wich are high efficiency, low cost, minimization of chemical and biological sludge, regeneration of biosorbent and the possibility of metal recovery. Biosorbents from fruit rinds consists in citrulline, pectin, proteins, cellulose and carotenoids that have groups of hydroxyl, amine and corboxylic. These can easily bind to metal ionsby changing their hydrogen ions for metal ions or giving an electron pair coplexes with the metal ions.

The silica from the structure o the watermelon rind can enhance the biosorption process.

The optimum pH was 8, biosorbent amount 1.5 g, zinc concentration 400 mg/l and contact time was 30 minutes (Othman N. et al., 2014).

CONCLUSIONS

There are lots of parts of the watermelon that are not consumed by the human because they are considered not edible. The seeds are a source of nutrients such as minerals, proteins and lipids that are used in medicine. The watermelon seeds have low molecular weight polypeptides like albumin, globulin, glutein and lots of glutamic acid, aspartic acid and serine. Because they are rich in protein they could be used in food formulations as ingredients. They have a good impact on the human health as anti-diabetic effect, cardioprotective, anti- ulcerogenic and anti-obesity. In most of the countries there are lots of products that have incorporated watermelon seed oil although is not considered an oil seed.

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MAPPING GEOGRAPHICAL DISTRIBUTION OF ROMANIAN ORCHIDS AND THEIR BIOLOGICAL ACTIVE SUBSTANCES

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Abstract

The present review aims to map the various species of orchids in Romania, in search of reported biological active substances. Several authors have reported that the main biological active compounds of this botanical group are tannins, volatile oils and phenanthrene quinones, which are not water soluble. The review will pay a special attention to autochthonous Orchidaceae species. In Romania most of these plants were identified in the Iron Gates Natural Park and surroundings. The data analysis will also cover the polyphenolic profile of the underground parts of Orchidaceae group to evaluate their antioxidant and antimicrobial potential, as well as to identify some other biochemical reported compounds as source for new biotechnological applications.

Key words*: Orchidaceae, biologically active compounds.*

INTRODUCTION

The *Orchidaceae* family, despite all beliefs, is one of the most widespread families of plants in the world. Total number of species counts almost 26,567 species worldwide (World Checklist of Selected Plant Families, Kew WCSP, 2011). These species are mainly known as ornamental ones, but numerous orchids are known for their healing properties, also as a reliable food source, fragrance and flavor usage. Due to the wide spreading, the orchids have many uses in many different cultures. In Southern Africa, the orchid tubers are used for a meatless type of sausage called "chikanda", representing a cheap source of food. The high consumption of orchid tubers affected very much the orchid population, because of the wide consumption of this kind of food, especially in Zambia, bringing the species almost to extinction in that area. A Turkish kind flour, called "salep", is made using mainly *Orchis mascula* or *Orchis militaris* plants, but also related species can be used. Salep, with small recipe change, is encountered also in Greece ("salapi"), United Kingdom ("saloop") and Middle Eastern countries (Arabian "sahlab"). It is well known as a predecessor of coffee because of his rejuvenating properties. Salep has also powerful aphrodisiac effects, a treatment for the gastrointestinal tract, diarrhea and bilious affections. The main component of orchid flour is glucomannan, a polysaccharide, comprising of mannose and glucose (Kaya & Tekin, 2001). The plant that vanilla is made from is also an orchid: *Vanilla planifolia*. This species contains a substance, vanillin, that is the main responsible for the famous flavor. Two other orchids are known that contain vanillin, *V. pompona* and *V. tahitensis*, but the quality of the flavoring substances is very low and they are not effective from industrial usage. In Bhutan, orchids are used to make a kind of dip or sauce from *Cymbidium hookerianum* called Olatshe. Another Bhutanese delicacy made from *Cymbidium* is Olachoto.

In the USA, *Dendrobium* genus is used as an edible food decoration, but the orchid species used is a hybrid one, not found in nature. *Dendrobium* is also used for making deferent kinds of sauces or dips in Singapore, Thailand and Japan. Australian aborigines use *Dendrobium kingianum* as food, mainly the stems (pseudo-stems). We encounter orchids usage even in Hawaii as salads, *Dendrobium salaccense* is used as a condiment in Malaysia. In Nepal and China *Dendrobium* orchids are used for tea. In China, there is even an Orchid Wine produced, and it is very popular. Faham tea is made in Mauritius and Isle de Reunion.

Although used for their remarkable beauty, Orchids can be found in cosmetic products and play a role in herbal medicines. In other countries, they are used for their therapeutically properties. Most of them have been listed in folk medicine. In the cosmetic industry, the plants from *Orchidaceae* family are valued for anti-ageing potential and skin moisturizing agent.

In Romania, Orchids are mainly use for their ornamental beauty, although there are almost 58 species identified in our country. Almost all the studies concerning the Romanian Orchids, aimed to identify the species, the treats and a proper conservation, if needed.

The aim of this review is to update and to present a comprehensive analysis of traditional and folklore uses, pharmacological reports and phyto-constituents isolated from the *Orchidaceae* family, found on Romanian soil.

MATERIALS AND METHODS

The information on *Orchidaceae* family from Romania was gathered from worldwide accepted scientific databases via an electronic search (Google Scholar, Web of Science, ScienceDirect, ACS Publications, PubMed, Wiley Online Library, SciFinder, CNKI). Information was also obtained from The Plant List, Chinese pharmacopoeia, Chinese herbal classics books, PhD and MSc dissertations, unpublished materials, and local conference papers on toxicology. Plant taxonomy was confirmed to the database "The Plant List" (www.theplantlist.org).

RESULTS AND DISCUSSIONS

As we can see from Table 1, Orchids reports in Romania are more numerous in the National Park Nera Gorges-Beuşniţa, Iron Gate National Park and Piatra Craiului National Park, and, unfortunately, few of them are Red listed species.

Many of the epiphytic Orchids are used as traditional medicine. Chemical components and pharmacology have been studied in recent 15 years. Medicinal orchids, in general, are not subject to detailed pharmacological studies. Extracts and metabolites of these plants, particularly those from flowers and leaves, possess useful pharmacological activities. Particular attention has been given to diuretic, antirheumatic, anti-inflammatory, anti-carcinogenic, hypoglycemic activities, antimicrobial, anticonvulsive, relaxation, neuroprotective, and antivirus, activities.

A comprehensive account of chemical constituents and biological activities is presented and a critical appraisal of the ethnopharmacological issues is included in view of the many recent findings of importance of these orchids. A large number of orchids have been empirically used for treatment of different diseases, thus, several studies have been undertaken to provide scientific proof to justify the medicinal use of various plants in treatment of diseases. The orchids extracts have been used for their diuretic, relaxation, anti-rheumatic, hypoglycemic activities, anti-inflammatory, antimicrobial and antivirus, anticarcinogenic, anticonvulsive, neuroprotec-tive properties.

A wide range of orchids' **chemical compounds** are presented in Table 2, including alkaloids, bibenzyl derivatives, flavonoids, phenanthrenes and terpenoids which have been isolated recently from orchid species. Phytochemicals are substances produced by every plant for various reasons as: reproduction, defense, rejecting other plants or animals etc. In orchid's case, there are few studies made about these substances, and for some of them we do not know at all their biological function.

We can group the phytochemicals encountered in orchids as: flavonoids, alkaloids, antho-cyanins, carotenoids and sterols.

Table 1. Orchids from Romania: Localization

Ref. No.	Area	Species	References
1.	Babadag-Codru Forest - Tulcea County	*Orchis simia*	
2.	Bicaz Gorges - Hăghimaş National Park	*Cephalanthera damasonium*	(Romanescu G. et al, 2013)
3.	Bucegi Mountains	*Chamorchis alpina*	(Biţă-Nicolae, 2011)
4.	Bucegi Mountains - Prahova and Braşov County	*Epipogium aphyllum*	Fotograful de orhidee din Bucegi (Galerie FOTO). "Clubul Rotary Valea Prahovei" Association. Retrieved from https://www.rotarybvp.ro/fotograful-de-orhidee-din-bucegi-galerie-foto/
5.	Bucegi Mountains, Călimani Mountains - Pietrosu, Red Lake, Retezat Mountains, Giumalău Mountains	*Coeloglossum viride*	(Irimia, 2011)
6.	Buzău County	*Ophrys scolopax* ssp. *cornuta* var. *banatica*	(Atanasiu, 2014)
7.	Buzăului Mountains	*Nigritella nigra, Nigritella rubra*	Margoi, D. (2015, June 20*).* Montaniarzi: Sangele voinicului, orhideea salbatica din Carpati. Retrieved December 22, 2018, from http://www.montaniarzi.ro/sangele-voinicului-orhideea-salbatica-din-carpati/
8.	Ciucului Mountains, Bucegi Mountains	*Nigritella nigra, Nigritella rubra*	Puşcarciuc, M. (2012, June 17). Munte&Flori: *Nigritella nigra* (2013, January 11). Retrieved from http://www.muntesiflori.ro/nigritella-nigra-foto/
9.	Defileul Dunării/Porţile de Fier	*Anacamptis pyramidalis*	Photo-hunting: La vânătoare de orhidee sălbatice prin Banat. S.C. Tymes Globetrotter S.R.L. Retrieved from http://www.tymestours.ro/RO/X/X-orhidee-banat-excursii/01-orhidee-banat-excursii.htm
10.	Dobrogea	*Liparis loeselii*	(Sârbu et al., 2006)
11.	Glodeasa Forest - Prahova County	*Herminium monorchis*	
12.	Grohotiş and Hăşmas Mountains - Prahova County	*Orchis ustulata*	Munte&Flori*: Orchis ustulata.* Retrieved from http://www.muntesiflori.ro/orchis-ustulata-foto/
13.	Hăghimas National Park - Neamţ County	*Orchis coriophora, Orchis morio* ssp. *alba, Orchis morio* ssp. *picta, Orchis tridentata, Traunsteinera globosa*	(Romanescu G. et al, 2013)
14.	Iron Gates Nature Park	*Orchis mascula, Orchis militaris, Orchis pallens, Orchis papilionacea, Orchis purpurea, Platanthera chloranth, Pseudorchis albida, Spiranthes spiralis*	(Milanovici, 2014)
15.	Jibou City Area - Sălaj County	*Orchis purpurea*	(Szatmari, 2016), page 34
16.	Măcinului Mountains-Dobrogea County	*Orchis purpurea*	Zana Florilor. Flori din Padure: Orhidee alba in Muntii Macinului *(Orchis purpurea sau gemanarita). (2017, September5).* Retrieved from http://zanaflorilor.eu/flori-padure-orhidee-salbatice-muntii-macinului-orchis-purpurea-sau-gemanarita/
17.	Mădăraş Mountain - Harghita County	*Dactylorhiza fuchsia*	Munte&Flori: Orhideele genului *Dactylorhiza.* Retrieved from http://www.muntesiflori.ro/genul-dactylorhiza/
18.	Meseş Mountains - Sălaj County	*Platanthera bifolia*	(Szatmari, 2016, page 35)
19.	Mic Mountain – Caraş-Severin County,	*Dactylorhiza fuchsia*	Milanovici, S. (2016, November 2). The orchid flora of the Muntele Mic (Caraş-Severin County, Romania). *Journal of Biological Sciences.* Retrieved December 22, 2018 from http://journal.pmf.ni.ac.rs/bionys/index.php/bionys/article/view/185
20.	National Park Nera Gorges – Caraş-Severin County	*Epipactis microphylla, Himantoglossum hircinum, Limodorum abortivum, Liparis loeselii , Listera ovata, Neottia nidus-avis, Ophrys apifera, Ophrys scolopax* ssp. *cornuta* var. *banatica, Orchis coriophora, Orchis mascula, Orchis morio* ssp. *alba, Orchis morio* ssp. *morio, Orchis morio* ssp. *picta, Orchis pallens, Orchis papilionacea, Orchis simia, Orchis tridentata, Platanthera bifolia*	(Bâtea, 2014)

Ref. No.	Area	Species	References
21.	Only mentioned - no exact area defined	*Epipactis palustris, Orchis x gennarii (hibrid)*	(Gubandru-Tomescu, 2018)
22.	Ortelec Hills - Zalău City	*Orchis militaris*	(Szatmari, 2016, page 35)
23.	Piatra Craiului National Park - Brașov/Argeș County	*Anacamptis pyramidalis, Cephalanthera rubra, Chamorchis alpina, Corallorhiza trifida, Dactylorhiza cordigera, Dactylorhiza cordigera* ssp. *siculorum, Dactylorhiza incarnata, Dactylorhiza maculata, Dactylorhiza majalis, Dactylorhiza saccifera, Epipactis atrorubens, Epipactis helleborine, Epipactis microphylla, Gymnadenia conopsea, Gymnadenia odoratissima, Gymnoleucorchis x strampfii* (hibrid), *Herminium monorchis, Himantoglossum hircinum, Leuchorchis albida, Limodorum abortivum, Liparis loeselii, Listera cordata, Listera ovata, Neottia nidus-avis, Nigritella nigra, Nigritella rubra, Ophrys apifera, Ophrys scolopax* ssp. *cornuta* var. *banatica, Ophrys sphegodes, Orchis coriophora, Orchis mascula, Orchis militaris, Orchis morio* ssp. *alba, Orchis morio* ssp. *morio, Orchis morio* ssp. *picta, Orchis pallens, Orchis papilionacea, Orchis purpurea, Orchis tridentata, Orchis ustulata, Platanthera bifolia, Platanthera chlorantha, Pseudorchis albida, Traunsteinera globosa*	(Gubandru-Tomescu, 2018)
24.	Piatra Mare Mountains - Brașov County	*Epipactis helleborine, Epipactis palustris*	(Ardelean et al., 2018)
25.	Piatra Mare Mountains - Brașov County	*Orchis mascula*	(Ardelean et al., 2018)
26.	Plopiș Mountains-Bihor/Sălaj County	*Ophrys apifera, Ophrys sphegodes*	(Szatmari, 2016)
27.	Postăvaru Mountains - Brașov County	*Cephalanthera damasonium*	Romfilatelia: Frumusețea florilor rare pe timbre: Orhidee sălbatice din România. Retrieved from http://www.romfilatelia.ro/ro/frumusetea-florilor-rare-pe-timbre-orhidee-salbatice-din-romania/
28.	Postăvaru Mountains - Prahova County	*Platanthera bifolia*	(Ardelean et al., 2018)
29.	Rarău-Giumălău Mountains - Suceava County	*Goodyera repens*	(Oprea & Sîrbu, 2012)
30.	Retezat Mountains - Hunedoara County	*Herminium monorchis*	(Benedek & Dragulescu, 2006)
31.	Rosia Montana Area - Alba County	*Listera ovate*	(Roman & Cristea)
32.	Sibiu County	*Orchis militaris*	
33.	Solovan Hill - Maramureș County	*Dactylorhiza sambuccina*	(Goja, 2014)
34.	Stânișoara Mountains-Neamt/Suceava County	*Listera cordata, Listera ovata, Neottia nidus-avis, Orchis coriophora, Orchis laxiflora* ssp. *elegans, Orchis laxiflora* ssp. *palustris, Orchis militaris, Orchis morio* ssp. *alba, Orchis morio* ssp. *morio, Orchis morio* ssp. *picta, Orchis purpurea, Orchis tridentata, Orchis ustulata, Platanthera bifolia, Traunsteinera globosa*	(Oprea & Sîrbu, 2012)
35.	Strâmtura - Maramureș County, Hășmaș Mountains - Maramureș County, Cucului Mountains - Harghita County	*Cypripedium calceolus*	Schlesinger, A. (2012, 27 May). Munte&Flori: *Cypripedium calceolus.* Retrieved from http://www.muntesiflori.ro/cypripedium-calceolus-foto/
36.	Tazlăului Subcarpathians, Măcinului Mountains	*Cephalanthera longifolia*	Zana Florilor. Flori din Padure: Orhidee alba in Muntii Macinului *(Cephalanthera longifolia*). (2017, August 5). Retrieved from http://zanaflorilor.eu/flori-padure-orhidee-alba-muntii-macinului-cephalanthera-longifolia/
37.	Sugău Cave - Harghita County	*Corallorhiza trifida*	(Flaviu-Crisan et al., 2014)
38.	Tarcăului Mountains, Vrancei Mountains	*Cephalanthera longifolia*	Photo-hunting: La vânătoare de orhidee sălbatice prin Banat. S.C. Tymes Globetrotter S.R.L. Retrieved from http://www.tymestours.ro/RO/X/X-orhidee-banat-excursii/01-orhidee-banat-excursii.htm
39.	The National Park Nera Gorges-Beușnița	*Dactylorhiza maculata, Dactylorhiza majalis, Dactylorhiza saccifera*	(Bâtea, 2014)

Ref. No.	Area	Species	References
40.	Trotuşului Mountains - Bacău County	*Dactylorhiza incarnata*	Marelena şi Radu Puşcarciuc - Orhidee din Munţii Trotuşului, floră - https://sites.google.com/site/romanianatura56/home/carpatii-rasariteni/trotusului/orhidee-din-muntii-trotusului-flora
41.	Turda Gorge - Cluj County	*Platanthera bifolia*	Nagy, Z. (2014, July 29). Lesser Butterfly-orchid *(Platanthera bifolia)* in the forests around Turda Gorge, Romania. Retrieved December 22, 2018, from https://www.naturepl.com/stock-photo-lesser-butterfly-orchid-platanthera-bifolia-in-the-forests-around-image01469679.html
42.	Umbrăreşti Village - Galaţi County	*Cephalanthera rubra*	

The most important of them is the alkaloid, a type of substance containing carbon, hydrogen and nitrogen atoms (with additional atoms of oxygen and sulfur). Quinine is an alkaloid found in different orchid species and has a pharmacological importance in antimalarial medicines. The most studied substances from Orchids, from a pharmacologically point of view, are: alkaloids, phenanthrenes, terpenoids, bibenzyl derivatives, and flavonoids. They can be found in whole plant, but also in leaves, flowers and roots.

Other secondary metabolites

Organic compounds that are either specific to the plant family or xenobiotic can be transformed in tissue culture. Many orchid species produce secondary metabolites which are either isoprenoid compounds, including sterols (Hills et al., 1968; Wan et al., 1971), or derivatives of shikimic acid. Tissue cultures of *Cymbidium* 'Saint Pierre', *Dendrobium phalaenopsis, Epidendrum ochraceum* maintained *in vitro* on media used for other orchids transformed some isoprenoids (Kukuczanka % Wojciechowska, 1983; Kukuczanka, 1985; Mironowicz et al., 1987), primarily the hydrolysis of (±)-menthyl acetate to menthol (75-85%), the hydrolyzation of phenol acetates, aromatic-aliphatic alcohols and acatates of racemic aromatic-aliphatic alcohols (Mironowicz et al., 1993). Phenanthrene derivatives have been found to be potent phytoalexins, while others act as endogenous plant growth regulators (Gorham, 1980; Majumder et al., 2001). Convallarioides nudol, eranthridin, sitosterol, erianol were isolated from *Eria convallarioides* (Majumder & Kar, 1989). Sitosterol, betulinic acid and some perfumery constituents were isolated from *Luisia indivisa* (Majumder & Lahiri, 1989). Flavone C-glycosides and flavonols were the most common constituents found in 53 and 37%, respectively of 142 species (75 genera) leaves (Williams, 1979). Bulbophyllanthrone: a cytotoxic phenanthraquinone from *Earina autumnalis* (Hinkley & Lorimer, 1999). The addition of

Table 2. Active compounds

Species	Bioactive compound	References
Anacamptis pyramidalis	Phenenathrene quinone: Marchantine A=Orchinol	(Reinhold et al.,1980)
	Mucin content and Orchinol, and p-hydroxybenzyl alcohol	(Teoh, 2016)
Cephalanthera longifolia	Alkaloids, Quercitin and Kaempferol-O-glicosides	(Teoh, 2016)
Cephalanthera rubra, Cephalanthera damasonium	Loroglossin, alkaloids and quercitin	(Teoh, 2016)
Chamorchis alpina	Phytoalexin Orchinol and p-hydroxybenzyl alcohol	(Pridgeon et al., 2001)
Coeloglossum viride	Phenenathrene quinone	(Reinhold et al.,1980)
Cypripedium calceolus	Fatty acid derivatives, isoprenoids, and phenyl derivatives	(Teixeira da Silva, 2013)
	Alkaloids, saponins, sugars, essential oils, phenolic compound,tannins, anthocyanins,lipds, coumarins, luteolin, arbutin	(Jimenez & Pourhashemi, 2014)
Dactylorhiza fuchsii, Dactylorhiza incarnata,	Seven anthocyanins (chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin I/II, serapianin)	(Teixeira da Silva, 2013)
Dactylorhiza maculata, Dactylorhiza majalis, Dactylorhiza saccifera, Dactylorhiza sambuccina	cyanidin 3-oxalylglycosides	(Soare et al., 2011)

Species	Bioactive compound	References
Epipactis atrorubens - Dumbrăviță roșcată	Metabolite: Ophrysanin	*Epipactis atrorubens.* PhytoChemical Interactions DB. Retrieved from https://www.genome.jp/db/pcidb/kna_species/12930#metabolite
Epipactis helleborine	A series of four mannose(Man)-, three *N*-acetylglucosamine (GlcNAc)n-, ten N-acetylgalactosamine/galactose(GalNAc/Gal)-, one 5-acetylneuraminic acid(α-2,3-Gal/GalNAc)- and one 5-acetylneuraminic acid(α-2,6-Gal/GalNAc)-specific plant agglutinins were evaluated for their antiviral activity in vitro	(Bazarini et al., 1992)
Epipogium aphyllum	Carotenoids: neoksantin, lutein, violaxanthin	(Jimenez & Pourhashemi, 2014)
Goodyera repens	Loroglossin	(Pridgeon et al., 2003)
	Alkaloids, rutin, loroglossin, Kaempferol-3-0-rutinozid, gudayerin, izoramnetin-3-0-rutinozid	(Jimenez & Pourhashemi, 2014)
Gymnadenia conopsea	Anthocyanins Gymconopin A, Gymconopin B, Gymconopin D, and 3,3'-Dihydroxy-2,6-bis(4-hydroxybenzyl)-5- methoxybibenzyl, Antiallergic phenanthrenes and stilbenes	(Pérez Gutiérrez, 2010)
Herminium monorchis	Glucomannans, Hydrophylic carbohydrates of high viscosity are found in tubers	(Teoh, 2016)
Himantoglossum hircinum	Phytoalexin orchinol (2,4-dimethoxy-7-hydroxy-9, 10-dihydrophenanthrene) and *p*-hydroxybenzylalcohol, 4-methoxy-2,5-dihydroxy-9,10-dihydrophenanthrene (Phytoalexin hircinol), Loroglossin (phenolic glicoside). The chemical composition of the flowers(E)-3-methyl-4-decenoic acid (Z)-4-decenoic acid and lauric acid.	(Carey & Farrell, 2002)
Liparis loeselli	Alkaloids	(Teoh, 2016)
Listera ovata	A series of four mannose(Man)-, three *N*-acetylglucosamine (GlcNAc)n-, ten N-acetylgalactosamine/galactose(GalNAc/Gal)-, one 5-acetylneuraminic acid(α-2,3-Gal/GalNAc)- and one 5-acetylneuraminic acid(α-2,6-Gal/GalNAc)-specific plant agglutinins were evaluated for their antiviral activity in vitro	(Bazarini et al., 1992)
Nigritella nigra	Phenenathrene quinone: Marchantine A	(Reinhold et al., 1980)
	Seven anthocyanins (chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin I/II, serapianin)	(Teixeira da Silva, 2013)
	cyanidin 3-oxalylglycosides	(Soare et al., 2011)
Nigritella rubra	Seven anthocyanins (chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin I/II, serapianin)	(Teixeira da Silva, 2013)
	cyanidin 3-oxalylglycosides	(Soare et al., 2011)
Ophrys apifera, Ophrys scolopax ssp. *Cornuta* var. *banatica, Ophrys sphegodes, Orchis coriophora, Orchis laxiflora* ssp. *elegans, Orchis laxiflora* ssp. *palustris*	Seven anthocyanins (chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin I/II, serapianin)	(Teixeira da Silva, 2013)
	cyanidin 3-oxalylglycosides	(Soare et al., 2011)
Orchis mascula, Orchis militaris, Orchis morio ssp. *alba, Orchis morio* ssp. *morio, Orchis morio* ssp. *picta*	Phenenathrene quinone	(Reinhold et al., 1980)
	cyanidin 3-oxalylglycosides	(Soare et al., 2011)
	Seven anthocyanins (chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin I/II, serapianin)	(Teixeira da Silva, 2013)
Orchis pallens, Orchis papilionacea, Orchis purpurea, Orchis simia, Orchis tridentata, Orchis ustulata, Orchis x gennarii (hibrid)	Seven anthocyanins (chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin I/II, serapianin)	(Teixeira da Silva, 2013)
	cyanidin 3-oxalylglycosides	(Soare et al., 2011)
Platanthera bifolia	Volatiles from flowers:benzyl benzoate, benzyl salicylate, cinnamyl alcohol, lilac aldehydes, methyl benzoate and methyl salicylate	(Plepys et al., 2002)
	Liliac aldehyde	(Baxter et al., 1998)
Pseudorchis albida	Phytoalexin orchinol (2,4-dimethoxy-7-hydroxy-9,10-dihydrophenanthrene) and *p*-hydroxybenzylalcohol. Flavonoid glycones content of the leaves of P. albida revealed the presence of quercetin and kaempferol The scent was rich in terpenoid compounds, and most of the dominant compounds (e.g. 4-oxoisophorone, β-myrcene, limonene, β-phellandrene, and verbenone) are frequently found in various orchid species	(Jersáková et al., 2011)
Traunsteinera globosa	Ophrysanin	KNApSAcK Metabolite C00006794. PhytoChemical Interactions DB. Retrieved from https://www.genome.jp/db/pcidb/kna_cpds/6794#species

glyphosate (as RoundUp®) resulted in the production of orchinol, a phenolic compound, in *Orchis morio* liquid culture (Beyrle et al., 1995). Dihydrophenanthrenes and bibenzyl synthase are produced in the rhizomes of orchids after wounding, their induced formation depending on wounding and the extent of fungal infection (Gehlert & Kindl, 1991).

Species that has no active substances studied or known: *Corallorhiza trifida, Epipactis microphylla, Epipcatis palustris, Gymnadenia*

odoratissima, Gymnoleucorchis x strampfii (hibrid), *Leuchorchis albida, Limodorum abortivum, Limodorum abortivum Listera cordata, Neottia nidus-avis, Platanthera chlorantha*, *Spiranthes spiralis*.

As we can see in Table 3, there are many medicinal uses for orchids, but not all species encountered in Romania have one. Species without a medicinal use known or studied are: *Cephalanthera rubra, Cephalanthera damasonium, Chamorchis alpina, Corallorhiza trifida, Dactylorhiza cordigera, Dactylorhiza cordigera* subsp. *siculorum, Dactylorhiza fuchsii, Dactylorhiza majalis, Dactylorhiza saccifera, Dactylorhiza sambuccina, Epipactis atrorubens, Epipactis microphylla, Epipcatis palustris, Gymnadenia odoratissima, Gymnoleucorchis x strampfii* (hibrid), *Leuchorchis albida, Limodorum abortivum, Listera cordata, Neottia nidus-avis, Nigritella nigra, Nigritella rubra, Orchis pallens, Orchis papilionacea, Orchis purpurea, Orchis tridentata, Orchis x gennarii* (hibrid), *Platanthera chlorantha, Pseudorchis albida, Traunsteinera globose.*

Table 3. Medicinal uses

Species	Medicinal uses	References
Anacamptis pyramidalis	Demulcent	Rich., L. (N/A). *Anacamptis pyramidalis* Pyramidal Orchid PFAF Plant Database. Retrieved December 23, 2018 from https://pfaf.org/user/Plant.aspx?LatinName=Anacamptis+pyramidalis
	Skin whitener, neuroprotective	(Teoh, 2016)
Cephalanthera longifolia	Appetizer, tonic, it heals wound	(Pant, 2013)
Coeloglossum viride	Memory impairment in mice	(Zhang et al., 2005)
Cypripedium calceolus - Papucul doamnei	Antispasmodic, anxiety, astringent, cramps, delirium tremens, diaphoretic (promotes sweating), diarrhea, enhancing recovery from surgery or illness, hypnotic, hysteria, insomnia, menorrhagia (heavy menstrual bleeding), mood (elevate), muscle spasms, nervousness, pain, pruritus (severe itching), sedative, stimulant, stress, styptic (stops bleeding), tension (emotional), tooth pain.	(Liu D., Ju J.H., Zou Z.J., et al., 2005)
Dactylorhiza incarnata	Demulcent, nutritive	Soó, L. (N/A). *Dactylorhiza incarnata* Marsh Orchid PFAF Plant Database. Retrieved December 23, 2018 from https://pfaf.org/user/Plant.aspx?LatinName=Dactylorhiza+incarnata
Dactylorhiza maculata - *Poroinic*	Impotence treatment, genital vasodilatation	(Bivolaru, 2001)
Epipactis helleborine	Relief of moderate an severe pain - oxycodone	
	Highly inhibitory to human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2), and showed a marked anti-human cytomegalovirus, respiratory syncytial virus and influenza A virus activity	(Bazarini et al., 1992)
Epipogium aphyllum	Restorative, pain reliever, tonic	(Jimenez & Pourhashemi, 2014)
Goodyera repens	Improve the appetite and treatment of colds, kidney and bladder problems	*Goodyera repens* Creeping Lady's Tresses, Lesser rattlesnake plantain PFAF Plant Database. Retrieved from https://pfaf.org/user/Plant.aspx?LatinName=Goodyera+repens
	Roots and leaves were used medicinally, by native Americans of British Columbia to treat cancers, ulcers, lupus,colds, burns, rheumatism	(Pridgeon et al., 2003)
	Emollient, detoxification, to imrpove appetite, as a remedy for snake bites, diseases of the stomach, bladder	(Jimenez & Pourhashemi, 2014)
Gymnadenia conopsea	Demulcent; Nutritive.	*Gymnadenia conopsea* Fragrant Orchid PFAF Plant Database. Retrieved from https://pfaf.org/user/Plant.aspx?LatinName=Gymnadenia+conopsea
	Tubers are used as aphrodisiac. Alcohol extract of rhizomes of *Gymnadenia conopsea* showed effect on the collagen synthesis in rat lungs exposed to silica under the influence on antioxidase activities. The extract can ameliorate silica-induced pulmonary fibrosis by increasing activities of antioxidase and alleviating damage of lipid peroxidation to the lungs. Methanol extract from the tubers of *Gymnadenia conopsea* showed an antiallergic effect on ear passive cutaneous anaphylaxis reactions in mice. Inhibit antigen-induced degranulation.	(Pérez Gutiérrez, 2010)

Species	Medicinal uses	References
Herminium monorchis	Treat kidneys and stomach,and also used for nervous breakdown, insomnia, confusion, anorexia.	(Teoh, 2016)
Himantoglossum hircinum	The antifungal activity of orchinol and hircinol against *Candida lipolytica*	(Carey & Farrell, 2002)
Liparis loeselli	Activity against bacteria and fungi suggesting that there may be a basis for their usage to treat superficial infections. Pyrrolizidine alkaloids possess anti-oxidant activity	(Teoh, 2016)
Listera ovata	Skin	The use of Orchid for medicinal purposes. Retrieved from http://www.orchids-world.com/evergreen/med.pdf
	inhibitory to human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) in MT-4, and showed a marked anti-human cytomegalovirus (CMV), respiratory syncytial virus (RSV) and influenza A virus activity in HEL, HeLa and MDCK cells, respectively	(Bazarini Et al., 1992)
	Tubers were used as a tincture for Stomach disease. Externally skin tone	(Pérez Gutiérrez, 2010)
Ophrys apifera	Aphrosidiac	(Pant, 2013)
Ophrys scolopax ssp. *cornuta* var. *banatica*	Demulcent; Nutritive.	*Ophrys scolopax.* Woodcock Orchid PFAF Plant Database. Retrieved from https://pfaf.org/user/Plant.aspx?LatinName=Ophrys+scolopax
Ophrys sphegodes	Aphrosidiac	Pant, B., 2013. Medicinal orchids and their uses: Tissue culture a potential alternative for conservation, pages 450-453
Orchis coriophora	Antiflatulent; Demulcent	*Orchis coriophora* . Bug Orchis PFAF Plant Database. Retrieved from https://pfaf.org/user/Plant.aspx?LatinName=Orchis+coriophora
Orchis laxiflora ssp. *elegans*	Diarrhea, bronchitis and convalescence	(Singh & Duggal, 2009)
Orchis laxiflora ssp. *palustris*	Diarrhea, bronchitis and convalescence	(Singh & Duggal, 2009)
Orchis mascula	Aphrosidiac	(Pant, 2013)
Orchis militaris	Demulcent	*Orchis militaris.* Military Orchis PFAF Plant Database. Retrieved from https://pfaf.org/USER/Plant.aspx?LatinName=Orchis+militaris
Orchis morio ssp. *alba, Orchis morio* ssp. *morio, Orchis morio* ssp. *picta*	Gastroenteritis, antistringent	*Orchis morio Herb Uses, Cures, Side effects, Nutrients.* Herbpathy.com. Retrieved from https://herbpathy.com/Uses-and-Benefits-of-Orchis-Morio-Cid3278
Orchis simia	Aphrosidiac	(Pant, 2013)
Orchis ustulata	Antiflatulent	*Orchis ustulata.* Dark-Winged Orchis PFAF Plant Database. Retrieved from https://pfaf.org/user/Plant.aspx?LatinName=Orchis+ustulata
Platanthera bifolia	Demulcent	*Platanthera bifolia.* Butterfly Orchid PFAF Plant Database. Retrieved from https://pfaf.org/user/Plant.aspx?LatinName=Platanthera+bifolia
Spiranthes spiralis	Relieve the symptoms of burns.	*Spiranthes autumnalis- Spiranthes spiralis* root liquid. (2009, January 5). U.S. National Library of Medicine. Retrieved from https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=01ec3c41-fa6d-4f10-87d8-d1032662cfa3

CONCLUSIONS

The Orhidaceae family represented in Romania by 58 species, have not only an ornamental value, but also an etnopharmacology importance which was overlooked. In different countries various Orchids species were used in the herbal medicine for their therapeutic activities.

Extracts and metabolits of Orchids plants have have etnopharmacological properties, that should be included in future studies. So far, these plants have been used empirically for the treatment of different diseases, for pain relief, for problems concerning skin, the reproduction organs, and respiratory system.

Clinical trials with phytochemicals from orchids from Romania, are lacking, so therefore medical recommendation are not to be taken into consideration, at the moment. However, it is hoped, that newer explorations of these botanical species, will be ongoing to design pharmaceutical products, since Orchids should be seen as a therapeutic plant, not only as an ornamental one, by the public ant the medical community.

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PRELIMINARY CHARACTERIZATION *IN VITRO* OF *Bacillus licheniformis* STRAIN FOR USED AS DIETARY PROBIOTIC

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Abstract

The purpose of this paper was to provide a direction for evaluating the probiotic potential and safety of a microbial strain in order to use in weaning piglets. Bacillus licheniformis ATCC 21424 was analyzed morphologically, culturally, biochemically, for hemolytic activity and enzymatically (amylase and protease screening). In vitro, some probiotic properties were study as: resistance to pH by simulated gastric juice (pH 2 and 3) and bile salts (simulated intestinal fluid). The biochemical characteristics was performed by catalase assay, API 50 CHB Biomerieux strips, apiweb API 50 CHB V 4.0 soft (B. licheniformis, % ID 99.9) and ABIS online (~90.6% similarity). The hemolytic activity was assayed on blood agar medium. The strain was grown in nutrient medium, in two ways: static incubation (37℃, 24 h, 4 x 10^8 CFU/ml) and under constant agitation (37℃, 24 h, 120 rpm, 1.56 x 10^{10} CFU/ml). To screen out, the most favorable carbon source was included in the basal medium (1% w/v, pH = 7) glucose (12.11 ± 0.1), fructose (11.73 ± 0.67), lactose (12.03 ± 0.14) and starch (12.51 ± 0.27). The strain is a Gram-positive, rod-shaped bacteria, arranged in short chains or in small irregular pairs with ability to produce spores. The endospores were central, paracentral and subterminal. The strain growth was aerobic and was non – hemolytic. The enzymatic process was observed by appearance of distinct zones around strain colonies. The strain presents relatively good viability at pH 3 and tolerated oxgall (0.3%). In conclusion, the results suggested that the strain present some probiotic traits and can be further assessed for other probiotic characters as antibacterial activity, induction of local immune response etc.

Key words*: Bacillus licheniformis, biochemical characters, probiotic properties, hemolytic activity, enzymatic screening.*

INTRODUCTION

The administration of live microbial preparations as "dietary supplements" provides a strategy for animal's breeders to raise up meat production, quality and animal health status etc. (Dumitru et al., 2018a; Habeanu et al., 2016; Duc et al., 2004).

Since 2006, the European Union has banned the use of antibiotics in food-producing (European Union, 2006). The probiotics administration which are called direct-fed microbials (DFM) (Chen et al., 2006), have been demonstrated to be useful when are ingested in a sufficient amount and can be an alternative with high positive effects on the animals' health, by maintaining intestinal ecosystem and their performance (Kaewtapee et al., 2017; Dumitru et al., 2018a).

Microorganisms used in animal feed in the EU are mainly bacterial strains of Gram-positive bacteria belonging to the genus *Bacillus, Enterococcus, Lactobacillus, Pedicoccus, Streptococcus* and strains of yeast belonging to the *Saccharomyces* and *Kluveromyces*.

From the all types, *Bacillus* spp. occurred the most attention (Cutting, 2011); spores of these microorganisms can resist to unfavorable conditions, present potential probiotic properties as resistant to heat, radiation, enzymatic degradation during to the animal gastrointestinal tract (GIT) and stomach's acidic medium (pH, acid tolerance, bile resistance etc.) (Lee et al., 2012; Dumitru et al., 2018a). *Bacillus* spp. represent an importance sources for produce various extracellular enzymes that enhance feed digestibility, improving growth performance, feed

conversion ratio and meat quality of animals (Pant et al., 2015).
According to Upadhaya et al. (2015), dietary supplementation of *B. licheniformis* have positive effects on pigs' growth performance. The probiotics activity is influenced by diet composition (Blank et al., 1999); a high protein content in diet can affect the piglet microbiota in the first 14 d after weaning (Wu et al., 2015).
The present study describes *in vitro* some probiotic properties of *B. licheniformis* ATCC 21424 strain as morphological, cultural, biochemical characteristics, hemolytic ability, enzymatic production (amylase and protease screening), viability at pH 2 and 3 and bile resistance by simulated intestinal fluid as a preliminary investigation of probiotic potential in order to use it in piglet nutrition.

MATERIALS AND METHODS

Characterization of bacterial strain, growth media and enumeration of spore counts

Morphological and cultural properties of *B. licheniformis* ATCC 21424 strain was investigated according to the methods described in Bergey's Manual of Systematic Bacteriology (1957) (Ludwig et al., 2012). The strain was grown in nutrient broth and agar medium (Merck), 90 mm in Petri dishes, to evaluate the cultural traits. Serial dilution (1: 10, in 0.85% sterile physiological serum - SPS) was done (10^5 - 10^{10} - fold), for counts number (CFU/ml) in broth culture, incubated static (37°C, 24 h) and under a constant agitation (37°C, 24 h, 120 rpm). An aliquot of 1 ml from each dilution was homogenized and spread on nutrient agar plate. At least three replicas were done for each dilution. The strain was stored at room temperature, 4°C and -80°C with 20% sterile glycerol, until will be establish the preservation viability. Bacteria viability will be assessed every 2 years.
The strain was deposited in the Collection of National Research Development Institute for Biology and Animal Nutrition Baloteşti (INCDBNA), Romania, under the code IBNA 80.

Preservation of bacterial strain

The medium preservation (months) was done by culture in nutrient agar medium tubes. The strain viability was evaluated after 3 and 6 months. Long-time preservation (years) was done at -80^0C, with addition of glycerol 20%. Bacterial viability will be assessed every 2 years (Sorescu et al., 2019).

Biochemical test

The strain was tested for biochemical characters (catalase assay, API 50 CHB Biomerieux strips) and identified by API 50 CHB V4.0 and ABIS online soft (Stoica & Sorescu, 2017).

The catalase test

The catalase test was done according to the protocol described by Dumitru et al. (2018b, 2017). The method consists in highlighting the enzyme catalase from a bacterial culture by using hydrogen peroxide (H_2O_2) in a concentration of 3%. The catalase enzyme facilitates the breakdown of H_2O_2 into oxygen and water ($2H_2O_2$ + Catalase → $2H_2O + O_2$); the reaction was positive when a small inoculum is introduced in the H_2O_2 and visible effervescently bubbles were observed by the rapid elaboration of oxygen.

The API 50 CHB test

API 50 CHB strips were used for evaluated the carbohydrate acidification of *B. licheniformis* ATCC 21424 according to the manufacturer's protocol (BioMerieux) described by Dumitru et al. (2018a). The results are interpreted using database system API 50 CHB V4.0 and ABIS online software.

Hemolysis production

Blood agar plates [Trypticase soy agar (TSA, Sanimed) containing 5% (w/v) sheep blood], were used to test hemolysis activity (Jeon et al., 2018, cited by Dumitru et al., 2018a). Interpretation was followed after incubation at 37°C, for 24 h.

Specific Growth of *B. licheniformis* ATCC 21424 in minimal medium containing different carbon sources

To investigate the effects of various carbon sources, *Bacillus licheniformis* ATCC 21424 strain was grown in nutrient broth flash (pH 7), 24 h at 37°C in a rotary shaker at 120 rpm, with different substrate addition (1% w/v) in the basal medium: glucose, fructose, lactose and starch (Mageshwaran et al., 2014). Loop full of

culture of *B. licheniformis* ATCC 21424 was inoculated into separate flasks, with continuous agitation of 120 rpm, containing nutrient broth with different carbon sources used as substrate. Viable cells were determined by serial dilutions (10^{12}-fold) of the culture in SPS on nutrient agar by plates incubating at 37°C, 24 h. The number of bacteria was calculated according to the standard of ISO 7218 (2007).

Screening of amylase producing bacteria

According to the protocol instructions (Singh et al. 2015, cited by Dumitru et al., 2018), the bacterial strain was screened for amylolytic properties by starch hydrolysis test, on starch (1% w/v) agar plate.

Screening of protease producing bacteria

Bacillus licheniformis ATCC 21424 was screened for proteolytic activity. The bacteria strain was inoculated on the agar plates containing casein (1% w/v) and milk powder (1% w/v), incubated at 37°C, for 48 h. According to Josephine et al. (2012), it was following the protocol and data interpretation.

Acid tolerance test

The resistance of *Bacillus licheniformis* ATCC 21424 strain was investigated under simulated gastric juice (SGJ) by following the method described (Lee et al., 2012) with some modification: 1 ml of culture grown in nutritive broth for 24 h at 37°C, 120 rpm, representing about 10^9 colony forming units (CFU/ml), was transferred to 9 ml of SGJ [0.5% NaCl, 0.3% pepsin (from gastric mucosa, Sigma), 0.1%v peptone (BD Science)], whose pH was adjusted to 2 and 3 with a Portable meter (Waterproof, pH 7+DHS) using HCl 1 N, then incubated for 0, 30, 60, 90 and 120 minutes at 37°C, 120 rpm. Viable cells of the culture were enumerated by plating 10-fold dilutions [1:10, in NaCl 0.85%] on nutrient agar and plates incubating at 37°C, 24 h.

Bile resistance test

Bile tolerance was determined as previously described (Lee et al., 2012) with the following modifications: 10 ml of culture strain (about 10^9 CFU/ml) grown in nutritive broth (pH 7) for 24 h at 37°C on a rotary shaker (120 rpm), was spun down at 5,000 x g, 20 min, at 4°C. Cell pellets (biomass) were washed with PBS, collected by centrifugation (5,000 x g, 20 min, at 4°C) and resuspended in nutrient broth (pH 7) containing 0.3% oxgall (BD Science). Bacterial suspensions were incubated for 0, 1, 2, 3, and 4 h at 37°C on a rotary shaker at 120 rpm. Viable cells were counted by plating 10-fold dilutions of the culture in SPS on nutrient agar and plates incubating at 37°C, 24 h. The number of bacteria was calculated according to the standard of ISO 7218 (2007).

Data analysis

The analytical data were compared using variance analysis (ANOVA) with STATVIEW for Windows (SAS, version 6.0). The results were expressed as mean values and standard error of the mean (SEM), the differences between means considered statistically significant at $P<0.05$, using Fisher's PLSD test for untitled compact variable.

RESULTS AND DISCUSSIONS

Morphological and biochemical characterization

The taxonomic classification of strain was performed by culturally (aerobic growth), morphologically (Gram-positive, spore forming rods), and biochemically traits (positive catalase test). On nutrient agar after 24 h at 37°C, under aerobic conditions, the *B. licheniformis* ATCC 21424 presents opaque colonies, whitish with rough matte surface, irregular edged (type R) and diameter 0.7-0.9 mm (Figure 1).

Figure 1. Cultural aspect on agar plate for *Bacillus licheniformis* ATCC 21424

After growth in the nutrient medium, the tested strain at microscopic observation appeared as Gram positive rods shaped, arranged in diploid form, in long chains (nutrient agar) or in small irregular pairs (nutrient broth) (Figure 2 a, b).

The sporulation capacity of genus *Bacillus,* determine them a high stability to survive at low pH; the strain produced oval endospores located central, paracentral or subterminal without distorting the vegetative cell.

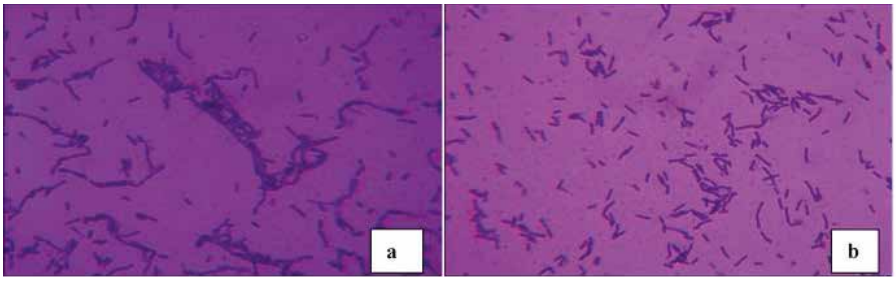

Figure 2. Microscopic observation of *Bacillus licheniformis* ATCC 21424 strain (1000x): a. culture on agar plate; b. culture from nutrient broth

Bernardeau et al. (2017) reported that the feed supplementation with specific *Bacillus* strains can provide numerous benefits including improvement in digestibility, the gut microbiota, immune modulation and growth performance.

These positive effects can be sustained by the ability of bacilli to produce spores, considering a direction in a long storage of processing feed, involving resistant to survive at environmental conditions. More *Bacillus* strains can remain viable for hundreds of years (Liao & Nyachoti, 2017).

The strain sporulation is necessary to regain after entering in the host, the metabolism is reactivated and benefits positive effects will be observed (Cutting, 2011).

The strain was catalase positive, gas bubbles was observed at addition of 3% H_2O_2; this is a characteristic that differentiates *Bacillus* spp. from the anaerobic spore-forming, for example *Clostridium* spp. (Barbosa et al., 2005). The catalase production can stimulate, according to Hosoi et al. (2011), the growth and viability of *Lactobacillus* spp. from GIT.

Results obtained from the API 50 CHB tests indicated that used test was able to confirm *B. licheniformis* ATCC 21424 around 99.9% ID (very good percentage identification) and ABIS online (~90.6% similarity, Figure 3).

The fermentation capacity of carbohydrate was observed by the discoloration of the basal medium, from red to yellow, as positive answer (Figure 4).

Figure 3. Strain identification by ABIS online (www.tgw1916.net)

Figure 4. API 50 CHB strips inoculated with *Bacillus licheniformis* ATCC 21424, before and after incubation (24 h, 37°C)

The results by API 50 CHB were registered as final interpretation after 48 h, at 37°C (Table 1). From the analysis of Table 1, is observed that, the strain fermented esculin, glycerol, salicin, D-cellobiose, D-arabinose, D-maltose, L-arabinose, D-lactose, D-ribose, D-melibiose, D-xylose, D-saccharose (sucrose), D-trehalose, D-raffinose, starch, D-glucose, D-fructose,

glycogen, D-mannose, D-turanose, inositol, D-tagatose, D-mannitol, D-sorbitol, methyl-αD-glucopyranoside, amygdalin and arbutin. The strain did not ferment of erythritol, D-arabinose, L-arabinose, D-lactose, D-melibiose, L-xylose, D-adonitol, inulin, methyl-βD-xylopyranoside, D-galactose, D-melezitose, xylitol, L-sorbose, gentibiose, dulcitol, D-lyxose, D-fucose, L-fucose, methyl-αD-mannopyranoside, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, potassium 5 ketogluconate and N-acetylglucosamine.

Table 1. The results obtained with API 50 CHB for *Bacillus licheniformis* ATCC 21424

Biochemical tests	Interpretation				
	24h	48h		24h	48h
Control	-	-	Esculin	+	+
Glycerol	+	+	Salicin	+	+
Erythritol	-	-	D-cellobiose	+	+
D-arabinose	-	-	D-maltose	+	+
L-arabinose	+	+	D-lactose	-	+
D-ribose	+	+	D-melibiose	-	+
D-xylose	?	+	D-saccharose (sucrose)	+	+
L-xylose	-	-	D-trehalose	+	+
D-adonitol	-	-	Inulin	-	-
Methyl-βD-xylopyranoside	-	-	D-melezitose	-	-
D-galactose	-	-	D-raffinose	-	+
D-glucose	+	+	Starch	+	+
D-fructose	+	+	Glycogen	+	+
D-mannose	+	+	Xylitol	-	-
L-sorbose	-	-	Gentibiose	?	-
L-rhamnose	-	+	D-turanose	-	?/+
Dulcitol	-	-	D-lyxose	-	-
Inositol	+	+	D-tagatose	+	+
D-mannitol	+	+	D-fucose	-	-
D-sorbitol	+	+	L-fucose	-	-
Methyl-αD-mannopyranoside	-	-	D-arabitol	-	-
Methyl-αD-glucopyranoside	+	+	L-arabitol	-	-
N-acetylglucosamine	-	-	Potassium gluconate	-	-
Amygdalin	+	+	Potassium 2-ketogluconate	-	-
Arbutin	+	+	Potassium 5-ketogluconate	-	-

- = negative; + = positive; ? = doubtful, weakly positive.

The API 50 CHB test gives information about the strain capacity to act on the substrate and to fermented it; also, this analyze represents an alternative to understand the strain's enzymatic equipment and can be observed by discoloration of the basal medium.

Hemolysis production

The hemolytic evaluation was determined on TSA agar plates supplemented with 5% sheep blood. The assay is based on the ability of strain to lyse blood cells of culture medium.

The safety of *B. licheniformis* ATCC 21424 to be used as a possible probiotic in piglets' feed was confirmed by non-hemolytic activity on 5% sheep blood agar plate (γ-hemolysis, Figure 5). A clear zone around colonies on TSA medium indicate a complete hydrolysis (β-hemolysis), the strain must to be eliminated for utilization as a probiotic in animal nutrition. According to Prieto et al. (2012) and Seker (2010), non-hemolysis (γ-hemolysis) and α-hemolysis (a green zone around colony) are considered to be safety.

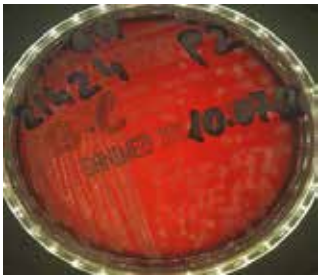

Figure 5. Hemolysis assay of *Bacillus licheniformis* ATCC 21424, at 37°C, 24 h

Growth of the bacterial strain

The growth of *B. licheniformis* was investigated after 24 h incubation at 37°C under static conditions and under a constant agitation, by incubating culture flasks at 150 rpm. After static incubation number cells of *B. licheniformis* were approximatively 4 x 10^8 (CFU/ml), while it in case of agitation were amounted 1.56 x 10^{10} (CFU/ml).

The experimental results, in Figure 6, showed that agitation is a better parameter for growing bacteria, compared with the static incubation. The result was expressed as logarithm of colony forming units/ml (log_{10} CFU/ml).

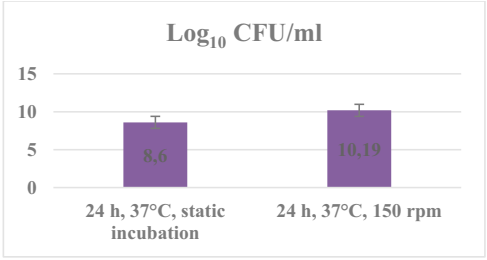

Figure 6. The viability of *B. licheniformis* ATCC 21424 at different conditions

Specific Growth of strain in minimal medium containing different carbon sources

To screen out, the most favorable carbon source was included in the basal medium: glucose, fructose, lactose and starch (1%). The bacterial growth on different substrates was registered in Table 2.

Table 2. Log_{10} CFU/ml of *B. licheniformis* ATCC 21424 on minimal medium containing different carbon sources

Carbon sources	Log_{10} CFU/ml
Control	12.11 ± 0.16^{b}
Starch	12.08 ± 0.14^{a}
Fructose	11.73 ± 0.67^{ab}
Glucose	12.11 ± 0.1
Lactose	12.03 ± 0.022

Results represent the mean ± standard deviation of three experiments (n=3).
[a, b] Values which differ significantly at P <0.05.

The maximum growth rate of *B. licheniformis* was registered in agar medium containing glucose, following by starch, lactose and fructose (Figure 7). The medium with starch addition was selected to put in evidence the enzymatic capacity of strain.

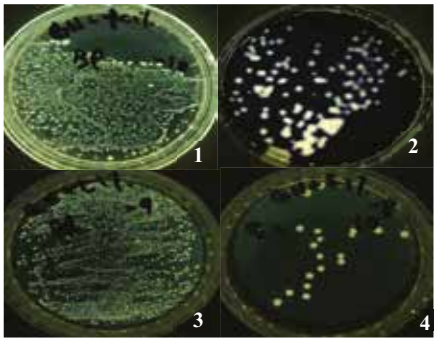

Figure 7. Growth of *B. licheniformis* ATCC 21424 in nutrient agar medium containing different carbon sources (1: glucose, 2: starch, 3: lactose, 4: fructose)

Amylases break down starch; by increasing starch digestibility, amylases potentially permit pigs to extract more energy from the feed, which can be efficiently converted into meat production. In young pig diets, amylases provide benefits by supplementing an immature digestive system where low feed intake post-weaning is associated with a slow maturation of amylase secretion. In addition, amylase also tolerates the use of less cooked grain in the diet, with benefits in feed cost reduction, without compromising young pig performance after weaning (Barletta, 2011).

Screening of amylase enzyme

The amylase method, as qualitative assay, was based on the reduction in blue color intensity resulting from enzyme hydrolysis of different quantity of starch addition in the basal medium (1%, 2% and 3%, w/v). Production of this enzyme was studied after 24 h of incubation at 37°C and pH 7. After addition of Lugol solution, a clear zone of hydrolysis on starch was observed on agar plates (Figure 8). On the plate with 1% starch addition was registered the maximum zone of hydrolysis and this concentration will be selected for other investigations.

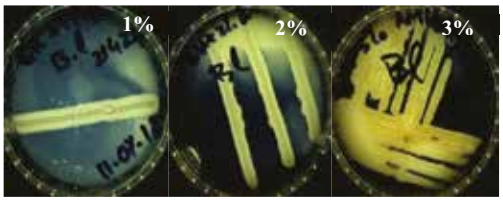

Figure 8. Screening of hydrolysis amylase of *Bacillus licheniformis* ATCC 21424:
1%, 2%, 3%: the quantity of starch addition in the basal medium

The capacity of selected *Bacillus* strains to produce and secrete large quantities of extracellular enzymes has placed them the most important industrial enzyme producers (Manabe et al., 2013). Dumitru et al. (2018a), reported similar data about the screening for amylase producing by a strain from *Bacillus* spp.

The addition of microbial enzymes as DFM, can aiding the digestion process of young animals (piglets, broilers etc.), whose enzymatic system is incomplete development.

Improving feed efficiency, nutrients supplementation, palatability, health in the stressful piglet period by decrease the numbers of pathogenic bacteria from GIT and increase the benefic bacteria colonization, DFM with enzymatic action represent a systematic role in young animal life (Van der Aar et al., 2016).

Screening of protease enzyme

Bacillus licheniformis ATCC 21424 strain was screened for extracellular protease production,

on agar plates containing casein (1% w/v, left photo) and milk powder (1% w/v, right photo). The strain hydrolysis capacity can be observed by appearance of a clear zone around bacteria growth, at addition of TCA 25% (Figure 9).

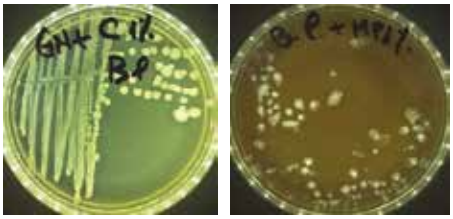

Figure 9. Screening of hydrolysis protease of *Bacillus licheniformis* ATCC 21424

Strains from the genus *Bacillus* produce a huge variety of extracellular enzymes, proteases occurring an important role in animal nutrition. Feed supplementation with specific enzymes improves the nutritional value of raw compound's feed, increasing the efficiency of digestion. Using bacterial strains as sources of extracellular proteases, provide a serial of benefits by break down the proteins from various raw materials, releasing bound energy that can be digested by the animal body (Barletta, 2011).

According to Merchant et al. (2011) the *Bacillus* spp. can be used as DFM in animal nutrition because the pH value in the small intestine is 6 to 7, which is optimal for spores to germinate, grow and produce enzymes and, also, to resist of the enzymatic degradation and low pH of the gastric barrier (Barbosa et al., 2005).

Preservation of bacterial strain

In Table 3 are presented the results of strain viability test which was preserved at 4°C and at room temperature.

Table 3. Testing the viability of *Bacillus licheniformis* ATCC 21424 strain preserved at 4°C and room temperature

Strain	Viability at 4°C	Viability at room temperature
Bacillus licheniformis ATCC 21424	+/3 months; +/6 months	+/3 months; +/6 months

+ = positive, - = negative.

The strain viability will be tested at 9 months, until will be establish the long-time preservation.

Resistance to pH

Bacillus licheniformis ATCC 21424 strain, conserved on nutrient agar tubes at 4°C and room temperature (6 months), was tested for resistance to simulated gastric juice (pH 2 and 3), under a constant agitation (37°C, 24 h, 120 rpm, Table 4).

The strain preserved in different conditions, presents a best survival rate, during 120 min incubation at pH 2 and 3. In Table 4, can be observed the strain resistance when was exposed to simulated gastric juice.

The strain preserved at 4°C, pH 2, present significative different between all times of incubation according to Lee et al. (2012) method. The pH 2 reduced slowly the cell numbers at 4°C and room temperature preservation, compared with pH 3 at 4°C, where the survivability did not differ significantly between the all incubation times.

Table 4. The effect of synthetic gastric juice (pH 2 and pH 3) on the *Bacillus licheniformis* ATCC 21424 viability during 120 min under constant agitation exposure

Strain	pH of synthetic gastric juice	0 min	30 min	60 min	90 min	120 min	SEM	P value
B l ATCC 21424	pH 2/4°C	10.803^{a}	8.393^{ab}	10.15^{bc}	8.593^{ac}	8.393^{ac}	0.283	<0.0001
	pH 3/4°C	10.353	10.16	10.067	10.1	10.047	0.060	<0.5548
	pH 2/room temperature	10.487^{a}	10.16^{ab}	10.083^{ac}	9.177^{abcd}	8.697^{abcd}	0.182	<0.0001
	pH 3/room temperature	11.533^{a}	9.913^{ab}	9.6^{ac}	8.687^{abc}	8.45^{abc}	0.301	<0.0001

Viable counts (log_{10} CFU/ml) of strain at 30, 60, 90 and 120 min was compared with counts at 0 min.
Results represent the mean of three experiments (n = 3). $^{a, b, c, d}$Means in the same row differ significantly at P <0.05.

In addition, Lee et al. (2012), reported low values of analyzed strains from *Bacillus* spp. during 30 min incubation at pH 2. The *Bacillus licheniformis* strain can be a possible probiotic candidate in piglet nutrition, because it maintained high viability during 120 min incubation, at 2 and 3 pH.

According to Nhi and Huong (2016), the numbers of *Bacillus* spp. strains have reduced prominently, comparative with our result, where the strain is capable to survival at low pH (2 and 3).

Bile resistance test

The strain was resistant to oxgall bile salt (Table 5). During incubation at 4 h in medium with 0.3% bill salt, the strain presents a high level of bile tolerance with a slow loss of viability.

Table 5. Effect of oxgall bile salt on the viability of *Bacillus licheniformis* ATCC 21424 strain during 4 hours exposure

Preserved conditions	Viable count (log_{10} CFU/ml) of *Bacillus licheniformis* ATCC 21424						
	0 h	1 h	2 h	3 h	4 h	SEM	P values
4°C	9.187^{a}	9.06^{b}	9.043^{c}	9.437^{d}	8.44^{abcd}	0.112	0.0332
Room temperature	10.153^{a}	10.347^{b}	9.527^{abc}	8.91^{abcd}	8.28^{abcd}	0.213	0.0001

Viable counts (log_{10} CFU/ml) of strain at 1, 2, 3 and 4 h was compared with counts at 0 h. Results represent the mean of three experiments (n=3). [a, b, c, d] Means in the same row differ significantly at P <0.05.

The *Bacillus licheniformis* ATCC 21424 pre-servation at 4°C differ significantly compared with room temperature preservation, suggesting that the spores are able to germinate without to be inhibited by the presence of bile salt in the small intestine (Guo et al., 2006).

CONCLUSIONS

The results suggested that the *Bacillus licheniformis* ATCC 21424 strain presents some probiotic properties. This strain has the capacity to secret amylase and protease enzymes, resists 6 months at 4°C and room temperature, does not show hemolytic activity (γ- hemolysis) on TSA medium confirming that is not pathogenic.

The strain spores were able to tolerate bile salt and survives at low pH in the conditions of stimulate gastric juice after 120 minutes.

This *in vitro* study can represent an alternative to improve animal health by increasing nutrition digestibility. Further experiments on animals are necessary to confirm the probiotic potential and to validate the importance of bacterial strain as source of feed additive.

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RESEARCHES CONCERNING THE USE OF CAMELINA OIL IN THE COMPOSITION OF COSMETIC PRODUCTS: REVIEW

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Abstract

Camelina (Camelina sativa) is a flower plant belonging to the family Brassicaceae, originating in Eastern Europe. Vegetable oil is not only for food use, it can also be used in many fields, such as the cosmetics industry. The oil obtained from Camelina seeds has a varied content of fatty acids with an intake of 50-60% unsaturated fatty acids, 35-40% omega 3 content and 15-20% omega 6. It has a high content of omega 3, being one of the richest vegetable sources compared to this fatty acid.
In this review, we discuss how some of the latest scientific advances such as the high content of tocopherol (E vitamin), the ability to extract lecithin from Camelina oil and the likelihood of being a replacement for the castor oil. Other approaches demonstrate the stability of Camelina oil both in the presence of synthetic and natural antioxidants. Finally, we discuss the potential of Camelina oil to be used in the cosmetics industry.

Key words*: Camelina oil, fatty acids, tocopherol, lecithin, stability, antioxidants.*

INTRODUCTION

Ever since ancient times, emollients based on animal fats and vegetable oils have been used. Technology has advanced today no longer uses vegetable fat or oil as such, but incorporates into more complex preparations for a much better effect, called cosmetics. Many vegetable oils such as coconut oil, avocado oil, castor oil, argan oil, wheat germ oil, saffron oil, and hemp oil are used in the cosmetic industry (Berdick, 1972). In today's cosmetics science, the role of natural ingredients is very important because on the one hand their structure is compatible with human physiology, having no toxic role and very low allergenic capacity, and on the other hand, the interest is more and more for the protective properties and beneficial reactivation for the skin (Rigano et al., 2006). Camelina oil is the main compound obtained from Camelina seeds, and its yield is between 30-40% DM (Budin et al., 1995; Rode, 2002; Zubr, 2003). Camelina oil has been used since ancient times for medical purposes as well as for lightening gas, but in the cosmetics industry, although its use is attempted, its true potential has not yet been decimated. It is part of the *Brassicaceae* family being a plant that does not require a special and complicated cultivation technology (Waraich, 2013).
With seed oil content (36-47%) twice that of soybean (18-22%) and a fatty acid profile (with >90% unsaturated fatty acids) suitable for making jet fuel, biodiesel and high-value industrial lubricants, *Camelina sativa* has tremendous potential to serve as a viable and renewable feedstock for multiple industries.
One of the most important used who has beed study was for sustainable bio-kerosene production.
The technology of Camelina oil was optimizated and can be valued all parts of the plant: roots, leaves, straw, silicules and the proteic part of the seed (included in camelina meal). Additionally, due to exceptionally high levels of α-linolenic acid (32-40% of total oil content), *Camelina sativa* oil offers an additional source of essential fatty acids.
The residual essential fatty acids combined with low glucosinolate levels in *C. sativa* meal make it desirable as an animal feed (Kagale et al., 2014).

GENETIC STRUCTURE OF *Camelina sativa*

Camelina sativa represents the first crop species to be sequenced from lineage I of the *Brassicaceae*. The well-preserved hexaploid genome structure of *C. sativa* surprisingly mirrors those of economically important amphidiploid Brassica crop species from lineage II as well as wheat and cotton (Kagale et al., 2014). study was undertaken to characterize two genes in the fatty acid biosynthesis pathway, fatty acid desaturase (FAD) 2 and fatty acid elongase (FAE) 1, which revealed unexpected complexity in the *C. sativa* genome. Genetically, *Camelina sativa* is closly to the model of *Arabidopsis thaliana*, and the regions downstream of CsFAD2 and upstream of CsFAE1 demonstrate co-linearity with this (Hutcheon et al., 2010).

EXTRACTION OF CAMELINA OIL

In a study made of Moslavac et al. (2014) they evaluated the oil extraction process from *Camelina sativa* (L.) Crantz seeds by screw pressing followed by extraction with supercritical CO_2. In pressing experiments, the response surface methodology (RSM) was conducted in order to study the effects of temperature, frequency and nozzle size on oil recovery and quality parameters. The cake resulting from pressing at optimal conditions was extracted with CO_2 in a new designed and built a homemade supercritical fluid extraction system. The residual oil in the pressed cake was almost totally extracted by supercritical CO_2 (Moslavac et al., 2014). Another procceding of extraction like SC-CO_2 extraction of Camelina seed oil was reported and compared with traditional extraction methods. The conditions used for this study was: pressure (35-45 MPa), temperature (50-70°C), and time (90-250 min). Oil yield increased with pressure and time, but not temperature. Oil yield was further increased to 31.6% at the RSM-optimized conditions by increasing the SC-CO_2 extraction time to 510 min. Soxhlet (hexane) and cold press methods yielded 35.9% and 29.9% oil, respectively. Extraction method did not have a significant effect on the fatty acid composition and tocopherol content ($P > 0.05$); however, phytosterol content of the cold pressed oil was significantly lower than that of SC-CO_2 and Soxhlet (hexane) ($P < 0.05$). Conclusion of the study was that the oil yield of SC-CO_2 extraction was higher than that of cold press (Belayneh et al., 2015).

CHARACTERIZATION OF CAMELINA OIL

Herbal vegetable oils are not only a non-polluting renewable source but also provide a wide range of fatty acids with various applications (Kumar et al., 2016). The chemical composition of Camelina oil is suitable for many branches of the industry, the one covered by this review is the cosmetics industry. The main characteristic of Camelina oil is its composition in linolenic fatty acid, 20-40% - essential and very rare omega 3 (Aldivia, 2007). The oil obtained from Camelina seeds has a varied content in fatty acids with an intake of 50-60% unsaturated fatty acids, 35-40% omega 3 content and 15-20% omega 6. Recently in a study, new proportions of component oils have been identified, namely 22,31-26,57% linolenic acid, 21,25-24,05% linoleic acid and 19,46-21,47% oleic acid (Ergönül, 2018). The process of extracting it is always improved to keep it as high as possible. The optimum condition for obtaining the best recovery oil and the best oil quality were recorded at 52°C, 20 Hz and 9 mm ID (Moslavac et al., 2014).

BIOCOMPOUNDS FROM CAMELINA OIL

The antioxidant activities of *Camelina sativa* methanolic extracts were evaluated by various chemical tests: reduction power, 2,2-diphenyl-1-picrylhydrazyl (DPPH) test, beta-carotene whitening method and metal chelating activity analysis (Terpinc, 2012). This study revealed that after pressing the oil, most of the phenolic compounds remain in seed residues, only a few compounds have been identified in the oil (Terpin, 2012). An interesting biocomponent found in camelina oil is lecithin. It is not found as such in camelina or oil seeds but is obtained by processes called enzymatic degumming and water degumming (Balayneh et al., 2018).

Lecithin obtained by enzymatic degumming contains a higher amount of lisophospholipids, generating a more stable emulsion. Camelina oil analyzed from this point of view promises to be a good alternative of emulsifier (Balazneh et al., 2018).

A comparison was been made regarding the total phenols content between safflower oil and camelina oil. The total phenol content of safflower oils was higher (272.20-525.30 mg GAE/kg) than Camelina seed oils (25.90-63.70 mg GAE/kg). Apigenin, luteolin, tyrosol, siringic acid, 3-hydroxytirozole, p-coumaric acid and synaptic acid have been detected in seed oils. Camelina seed oil was rich in tocopherol (144.11-168.69 mg/100 g). γ-Tocopherol was the predominant tocopherol in Camelina seed oils, consisting of 80% total tocopherol (Ergönül, 2018).

Another study was made to investigate the effects of protein extraction methods on the adhesion performance of different camelina protein fractions. Two Camelina protein fractions, globulin and glutelins, were isolated from defatted Camelina meal using three different methods resulting in total of six protein fractions including globulin 0-2 and glutelin 0-2. Dry adhesion strength of camelina protein adhesives exhibited nearly 100% wood cohesive failure at the curing temperatures of 150-190°C, except glutelin 2 and globulin 0. Glutelin had higher protein aggregation than globulin, as indicated by higher crystallinity, higher thermal stability, and dense protein aggregation (Ningbo Li et al., 2015).

CAMELINA OIL STABILITY

The stability of vegetable oils depends very much on chemical and physical factors. An important impact for maintaining stability is the method and storage conditions. The storage changes in tocopherol content, phenolic content as well as the presence of primary and secondary oxidative compounds wew studied (Abramovic, 2007). By the oil storage at 50°C and 60°C, respectively, the total content of phenolic compounds was reduced to 72% of its original value and 21% of its initial value (Abramovic, 2007). Camelina oil has been found to have a much lower oil stability index and higher p-anisidine storage rates compared to rape or sunflower oils. We have tried to stabilize Camelina oil with 21 antioxidants, both natural and synthetic, based on the Oil Stability Index (OSI). The stability index of Camelina oil was higher than that of rapeseed oil with TBHQ and its formulation with citric acid and above the sunflower oil with EGC, EGCG, carnosic acid, propyl gallate, extract of rosemary with ascorbyl palmitate or gallic acid. Accordingly, stabilized Camelina oils with TBHQ/citric acid and rosemary/ascorbyl palmitate extract were more stable than rapeseed and sunflower oils, respectively in terms of OSI induction times and p-anisidine rates (Frohlich et al., 2011).

A natural antioxidant that protects Camelina oil very well against oxidation is Rosmarin extract, as studies have shown (Moslavac et al., 2014).

THE BEHAVIOR OF CAMELINA OIL COMPARED TO OTHER VEGETABLE OILS

The behavior of Camelina oil has been analyzed compared to other vegetable oils, such as linseed oil, rapeseed oil, and sunflower oil. Several parameters have been analyzed, depending on the study. In order to make this comparison with linseed oil, a series of compounds, namely the fatty acid composition, the peroxide value, the acid value, the anisidine value, the chlorophyll pigments, the carotenoid pigments were analyzed. It was highlighted that they adhere to the Codex Alimentarius (2009) parameters for cold-pressed oils, even though there were differences between the two oils. Significant differences between the two were recorded for chlorophyll content and chlorophyll pigments. Regarding oxidative stability, Camelina oil proved to be more stable than the flax (Raczyk et al., 2015).

The cosmetic particle composition can essentially comprise *Camelina sativa* seed oil which is 100% completely hydrogenated, being in the form of strong cosmetic particles and transformed into soft cosmetic particles after introduction into a topical formulation. The transformed cosmetic particles can be adapted to have a soothing effect on the skin, hair and/or nails of a mammalian subject or other target. A study that had the purpose of building a patent, has fully utilized fully hydrolysed

Camelina oil. Camelina seed oil has at least 17% of its total fatty acid weight greater than 18 carbon atoms. Oil obtained from Camelina seeds is relatively inexpensive compared to many other seed oils, so the cosmetic particles formed with them are relatively cheap natural products (Kleiman et al., 2013).

CONCLUSIONS

The researches emphasized the high content of tocopherol (E vitamin), the ability to extract lecithin from Camelina oil and the posibility of being a replacement for castor oil, the stability of Camelina oil both in the presence of synthetic and natural antioxidants. In conclusion, Camelina oil has a potential to be used in the cosmetics industry.

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FOOD SAFETY

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ANTIOXIDANT CAPACITY IN DONKEY MILK (*Equus asinus*) DEPENDING ON LACTATION

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Abstract

Donkey milk is used as an alternative source in the diet of young children and newborns due to the similar composition to breast milk. The donkey milk is considered to be a functional food by the chemical composition it presents, thus being beneficial in the nutrition of people suffering from food allergies. The chemical composition and antioxidant capacity of the donkey milk are significantly influenced by lactation and also by the lactation period. Donkey milk has a low fat content compared to other species and high lactose content. These parameters are influenced by the variables such as: lactation, animal age, and feeding. The purpose of this study was to determine the effect of lactation on the antioxidant capacity and physicochemical compounds in the donkey milk.

Key words*: donkey, milk, lactation, antioxidant, fat, protein, lactose.*

INTRODUCTION

Donkey milk is used in different directions: for dairy products, creams, soaps and supplements for people suffering from food allergies (Piovesana et al., 2015; Coroian, 2018). It has a high a high antimicrobial activity and benefits the gastrointestinal and immune system. Due to its composition, it can be used against microbial infections and in the diet of people suffering from food allergies (Gubic et al., 2015; Longodor et al., 2018; Carminati et al., 2014; Cavallarin et al., 2015). Donkey milk has a rich chemical composition very similar to maternal breast milk, so it can be used in newborn nutrition (El-Hatmi et al., 2015; Polidori et al., 2009). The physico-chemical composition of milk varies according to species, individual, race, feed, lactation, health, season, climate and age (Coroian et al., 2016). Donkey milk can be used in the diet of people with atherosclerosis and hypercholesterolemia (Chiofalo et al., 2011). The composition in fatty acids suggests its consumption as a functional food for infant nutrition as well as in the diet of adults and susceptible persons (Martemucci et al., 2012; Martini et al., 2014). The chemical composition of milk and colostrum is influenced by feed, climatic conditions, lactation, animal health and housing conditions (Marchiş et al., 2018; Longodor et al., 2018; Coroian et al., 2016; 2017; Diaconescu et al., 2002; Martini et al., 2014). The physicochemical and amino acid composition of donkey milk according to lactation was studied by different authors (Guo et al., 2007; Polidori et al., 2009). Aspects related to the influence of nutrition and lactation on the composition of donkey milk are also shown in the studies of (Salimei et al., 2012). Compared to other species, donkey milk has a higher content of lysozyme. Moreover, lysozyme helps to reduce the number of bacteria, so it can be used to prevent intestinal infections in infants (Polidori et al., 2009). Ascorbic acid is present in donkey milk, which is also present in colostrum and breast milk. Ascorbic acid has a multitude of biochemical functions, such as maintaining a natural barrier against microbial infections (Vincenzetti et al., 2011).

Donkey milk was widely researched in various aspects, such as: mineral content at the different stages of lactation (Bilandzic et al., 2014); protein from donkey whey compared to other species (Brumini et al., 2015). Murua et al. (2013) studied the bactericidal activity of donkey milk, while (Monti et al., 2008) evaluated the influence of donkey milk on nutrition of children suffering from food allergies. Immunological aspects of donkey milk and the effect in preventing arteriosclerosis were studied by (Tafaro et al., 2007), while hygiene and health of donkeys was evaluated by (Pilla et al., 2010). Donkey milk contains nutrients such as proteins, fats, lactose and minerals that can also be detected in milk from other species. The difference between species is given by the different distribution of these nutrients. Protein content is influenced by breed, food, climate, season and udder health (Gubic et al., 2014; Hosoi et al., 2005; Yamawaki et al., 2005). The health of the animal and the udder influences the quality and quantity of milk. The highest milk production is realized in the third lactation. Between individuals and breeds there may be considerable variations in both productive and nutritional value of milk (Popelka et al., 2002). The purpose of this paper was to characterize the composition of donkey milk and antioxidant capacity under the influence of lactation.

MATERIALS AND METHODS

The donkey milk was harvested from lactating animals (lactation I-IV), from small farms in Cluj and Sălaj. Milk samples were collected individually in sterile containers and kept cool until physico-chemical analysis and antioxidant capacity were performed. Samples were harvested in the winter season.

Physico-chemical analysis of donkey milk

The physico-chemical parameters of donkey milk (fat, protein, lactose, water content and pH) were determined using Lactoscan apart.

Determination of the antioxidant capacity of the donkey milk

The ACL method (antioxidant capacity of lipid-soluble compounds) is used to determine the antioxidant capacity. The photochem V02 was used to measure the antioxidant capacity. The calibration and measurement of samples was based upon the inhibition of free radicals. After each measurement two purges of the apparatus were performed using ultrapure water. Determination of the amount of antioxidant capacity was achieved by establishing measurement curves that were compared to the measurement curves obtained for the standard solution. The calibration curve evaluation principle consists of: determining the integrated calibration curve. All the abovementioned calculations were made automatically using a software program called PCL soft.

RESULTS AND DISCUSSIONS

Physico-chemical composition of donkey milk

The physico-chemical composition analysed for donkey milk corresponds to the reference values for this species. The results are similar to those reported by (Longodor et al., 2018). Figure 1 show the fat content, which varies according to the harvesting and lactation area, as follows: 0.98% (L I) - 2.81% (L IV) for Cluj area and lower values for Sălaj area, 0.92% (L I) - 2.77% (L IV).

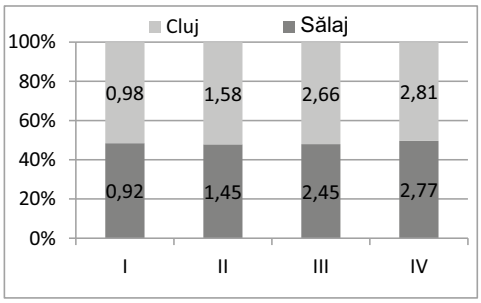

Figure 1. Fat content of donkey milk (%) (I-IV lactation period)

The average fat content of the donkey milk (0.3-1.8%) is similar to the observed values in horse milk (0.3-0.5%) and is much lower compared to other mammals (3.5-4-4% for human milk, 3.5-3.9% for cow's milk) (Guo et al., 2007; Polidori et al., 2009). One of the main causes of a lower fat content in milk is due to the incomplete removal of milk from the udder (Doreau et al., 1989; Caroprese et al., 2006). The amount of fat also varies during the lactation period, being higher in colostrum, and

decreases to the end of lactation (Gibbs et al., 1982). The protein content is influenced by lactation: (L I) - 1.68% and (L IV) - 1.94%, with the highest values in lactation IV (Figure 2). The protein in the donkey milk decreases from one month to the next, averaging 2%. The milk protein content was not influenced by the breed. On the contrary, milk protein content varied strongly during lactation and had a decreasing trend until 1.72 (g/100 g).

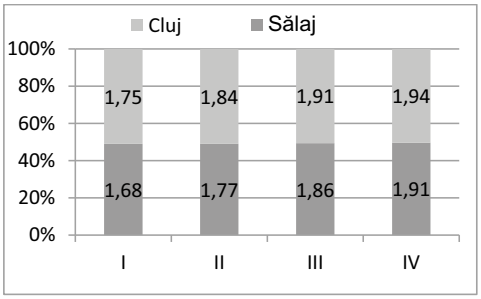

Figure 2. Protein content of donkey milk (%)
(I-IV lactation period)

Whey protein content remains constant during lactation. Whey protein content in the donkey 0.68 (g/100 g) is close to human and horse (Alabisio et al., 2005). In the present study, the protein content was lower in donkey milk than values reported by (Ling et al., 2008).

Lactose is the major disaccharide in milk, being an important parameter for donkey milk also, especially if we use milk to produce dairy products.

Donkey milk has a high content of lactose, with variation between 6.71% (L I) - 6.88% (L IV), for donkey milk in the Cluj area and 6.62% - (L I) and 6.91% (L IV) for Sălaj donkey milk (Figure 3).

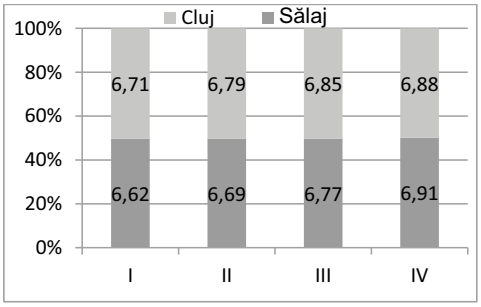

Figure 3. Lactose content of donkey milk (%)
(I-IV lactation period)

Swar et al. (2012) reports for donkeys and horses, that water has the highest content in the composition.

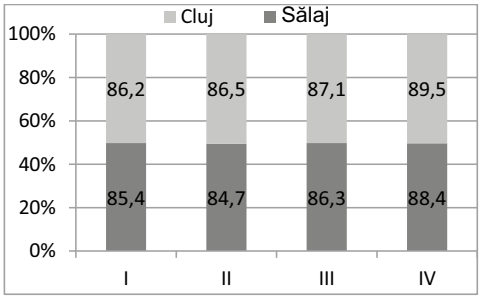

Figure 4. Water content of donkey milk (%)
(I-IV lactation period)

From the data reported by Salimei et al. (2004) and Guo et al. (2007), donkey milk has a pH between 7.14-7.22 and does not vary significantly during lactation compared to horse milk (Mariani et al., 2001).

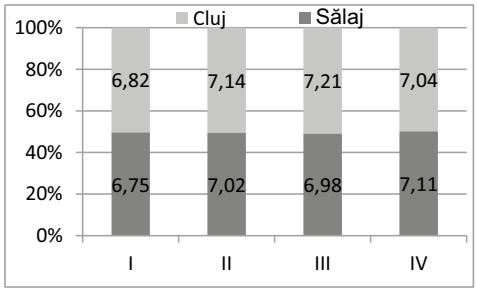

Figure 5. pH of donkey milk
(I-IV lactation)

The average pH (7.18) of the donkey milk is higher than that of cow's milk (6.6-6.7).

Antioxidant capacity of donkey milk

Donkey milk was considered a substitute for breast milk due to nutritional value and antioxidant properties, which can reduce disease (asthma, bronchitis, diabetes, anabrosis, gastritis, gastric ulcer) and oxidative stress (Beghelli et al., 2016; Lu et al., 2006; Ma et al., 2005; Nazzaro et al., 2010).

Figure 6 shows the mean values for the antioxidant capacity of donkey milk during four lactations in both areas (Sălaj and Cluj). The total antioxidant capacity in donkey milk varies between 16.02 U/ml and 17.55 U/ml (lactation IV), in the Cluj area.

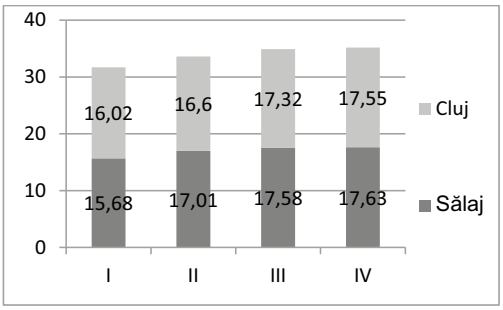

Figure 6. Total antioxidant capacity donkey milk (U/ml)

The antioxidant capacity of donkey milk in Sălaj area varied between 15.68 (U/ml) (L I) and 17.63 (U/ml) in the fourth lactation. In both studies areas, the third and fourth lactations showed the highest values.

These results are in the same line with those reported by Bucevic-Popovic et al. (2014) and Ling et al. (2018) in donkey milk. Donkey milk has a high antioxidant capacity; therefore it is used in the case of allergies, cardiovascular diseases and diabetes mellitus (Ling et al., 2018).

CONCLUSIONS

The physico-chemical composition of donkey milk was influenced by lactation. The highest content of fat, protein and lactose were observed in third and fourth lactation. In both studied areas (Sălaj and Cluj), the antioxidant capacity was influenced by lactation; the lowest content was observed in first and second lactation, while the highest in third and fourth lactation.

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PHYSICO-CHEMICAL COMPOSITION AND ANTIOXIDANT CAPACITY OF BUFFALO MILK

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Abstract

Buffalo milk due to its high fat content from the energy point of view is more valuable compared to cow's milk. The percentage of fat varies depending on the stage of lactation, season, individual, health, animal age and diet. The study of the chemical composition and the antioxidant capacity of buffalo milk is required for both scientific and technological considerations, given the importance of this type of milk in the consumer's diet. The purpose of this study was to evaluate the antioxidant capacity and the physico-chemical composition of buffalo milk by lactation. Buffalo milk can have different qualities, so the quality of buffalo milk is also determined by its content in its components (protein, fat, lactose, vitamins, fatty acids, water content, antioxidant capacity). The physicochemical parameters and antioxidant capacity were influenced by lactation, presenting the highest values in lactations III and IV. During lactation I, parameters such as fat, protein and lactose showed a content of 7.88, 4.35 and 4.71%, respectively. Furthermore, in lactation IV, fat, protein and lactose had increased, showing the content of these parameters as follows 9.53, 4.68 and 4.77%. Antioxidant capacity of buffalo milk showed the highest numbers in lactation III (360.1) and IV, 358.9 μg/ml. From all of the analyzed parameters, only total dry substance content presented the most increased values 18.9% in lactation I.

Key words*: buffalo milk, antioxidant capacity, lactation, fat.*

INTRODUCTION

Nowadays, more and more consumers are concerned in nutritious, good-tasting and high-quality food products containing bioactive compounds that ensure beneficially health impact (Shah et al., 2000). Generally, milk is regarded as an indispensable food product particularly for children and infants diet (Koletzko et al., 2011.) From all of the ruminant species, buffaloes are taken into consideration as second main milk producers at the global scale after cows, respectively. The significance of the Buffalo is attributed by prolonged longevity of the animals, an increased dry matter of milk, bioactive fatty acids content and development of nutritionally dairy products on markets compared with cows. (Bainbridge et al., 2016; Coroian et al., 2013; Cazacu et al., 2014; Diaconescu et al., 2002; Diaconescu et al., 2013). In addition, buffalo milk is appearing rarely in the market squares and is a potentially valuable source of essential minerals and vitamins that have a positive effect on human health (Ahmad et al., 2013; Pasquini et al., 2018; Cazacu et al., 2014). Natural antioxidants from milk, are a valuable asset for a healthy alimentation and represents a basic concern of interest for researchers from different fields such as pharmacology, biotechnology, biochemistry, physiology and so on. Antioxidants are acting as chemical scavengers neutralizing free radicals. Reactive oxygen species (ROS) are precursors of oxidative stress and are commonly associated to induce cell damage, altering DNA, proteins, and triggering various human diseases (Lobo et al., 2010; Mann et al., 2016). Sources of antioxidants include tocopherols, polyphenols, vitamins, flavonoids, carotenoids, amino acids, fatty acids, minerals, proteins, some Maillard reaction products,

sterols, peptides and phospholipids (Carocho et al., 2016). Considering antioxidant characteristics, buffalo milk has a higher antioxidant capacity compared to cow milk, meaning that buffalo milk is rich in bioactive compounds that can benefit a healthier diet (Khan et al., 2017). In addition, milk possess two antioxidant systems, lipophilic and hydrophilic. Lypophilic antioxidant fraction also known as fat soluble, is mostly consisted of vitamins A, E, phospholipids and fatty acids (Khan et al., 2017). Both antioxidant systems perform an essential role for the human body namely supporting antioxidant and pro-oxidant homeostasis by disrupting the activity of reactive oxygen species (Grażyna et al., 2017). Also, fat-soluble antioxidants present higher thermal stability, remaining active in most of the milk products, compared to hydrophilic antioxidants. Hydrophilic antioxidant system (water soluble) is mainly comprised of minerals, trace elements, proteins, vitamins and bioactive peptides (Basilicata et al., 2018; Grażyna et al., 2017). On one hand elements from milk such as zinc (Zn), copper (Cu), selenium (Se), iron (Fe), and magnesium (Mg) play a crucial role in the development of human growth. On another hand trace elements, such as mercury (Hg), lead (Pb), cadmium (Cd) and aluminum (Al), in high concentrations can affect human wellbeing. These heavy metals, exert a serious threat due to their toxicity, bioaccumulation in food products (Babu et al., 2018). Unfortunately, ruminants that are raised especially near industrial polluted sectors can cumulate high concentrations of heavy metals through the intake of contaminated feeds (Meshref et al., 2014). Thereupon, animals are secreting those contaminants in milk, presenting a hazard for human health. (Tunegova et al., 2016; Younus et al., 2016). Heavy metals have a bioaccumulation potential, inducing harmful effects in living organisms as chronic toxicity, decreasing fetal development, damaging the DNA and so forth (Zhang et al., 2019; Govind et al., 2014). Interestingly, the absorption of Pb occurs faster in children compared to adults, accumulating mainly in soft tissues and bones (Norouzirad et al., 2018). The element as trivalent Cr (III) presents low toxicity, while hexavalent Cr (VI) is considered carcinogenic, associated with embryotoxicity and fetotoxicity in animals. (Govind et al., 2014; Samiee et al., 2019). Moreover, the composition of milk is rich in antioxidant enzymes and non-enzymatic antioxidants. Superoxide dismutase (SOD), catalase, glutathione peroxidase (GSHPx) are antioxidative enzymes that have the potential to prevent the formation of radicals such as hydrogen peroxide, superoxide anion, and other peroxides (Lindmark-Månsson et. al., 2000). Based on these findings and to the fact that Buffalo milk has a rich source of antioxidants, proteins and other bioactive compounds that may exert beneficial effects on human health, photochemiluminescence method was applied in order to evaluate antioxidant capacity of milk samples. Therefore, the aims of this study were to evaluate the physicochemical composition and antioxidant capacity of buffalo milk influenced by lactation period.

MATERIALS AND METHODS

Milk sampling

Samples of buffalo milk were individually harvested from buffaloes according to lactation. Five samples were collected for each lactation. It was taken from a small farm in Buciumi commune, Salaj county. Samples were harvested in sterile containers and stored at 4ºC until analyzes were performed (Coroian et al., 2013; Marchis et al., 2018). The buffaloes in the study are of Romanian buffalo breed and are in lactation (I-IV). The buffaloes received the same feed and had the same maintenance site.

Physico-chemical analysis

Lactoscan (Milk analyzer Lactoscan) device was used for physico-chemical analysis, a method also reported by Marchis et al. (2018). The following parameters were determined: fat, protein, lactose and total dry substance %.

Antioxidant capacity analysis

Antioxidant capacity of milk samples was done by the photochemiluminescence method, according to (Popov & Lewin, 1996) and protocol of (Photochem producer), for the measurement of lipidsoluble substances (ACL). PHOTOCHEM® instrument (Analytik Jena AG, Jena, Germany) was used to measure the

antioxidant capacity. The principle of the ACL method: free radicals are produced by irradiating a photosensitizing substance (luminol). Then they are partially removed from the sample by the chemical reactions that occur between the existing antioxidants in the sample and free radicals released by the photosensitizer. TROLOX equivalent in the case of lipid soluble and in ascorbic acid equivalent in the case of water soluble antioxidant capacity. Thus, the antioxidant capacity obtained is measured in equivalent standard units. The reagents used for this assay were the following: reagent 1 - methanol, reagent 2 - buffer solution, reagent 3-250 µl/ bottle stock solution PS-2 (photosensitizer and detection agent), and reagent 4-standard calibration solution for the quantification of lipid-soluble antioxidants, equivalent to TROLOX. The working solutions were prepared according to the following protocol: 1st reagent - methanol without dilutions, 2nd reagent - ready to use, 3rd reagent is obtained by defrosting the vial with the basic solution and adding an amount of 750 µl of 2nd reagent, 4th reagent - stock solution - is obtained by adding 500 µl of 1st reagent to 4th reagent, and 5th reagent- The previously obtained reagent is diluted with the 1st reagent in a ratio of 1: 100; 10 µl of the solution thus obtained will contain 1 nmol of standard TROLOX calibration solution. All calculations were performed automatically using a software program called PCL soft. All measurements were done in triplicate. Five samples were analyzed for the antioxidant capacity of buffalo milk for each lactation. The ANOVA (JMP 12, SAS) program for data analysis was used.

RESULTS AND DISCUSSIONS

Different versatile methods of measuring the antioxidant capacity of milk products are presented in the literature and the most mentioned are FRAP and DPPH methods. Moreover (Zulueta et al., 2009) using $ORAC_{FL}$ assay, observed that total antioxidant capacity is especially attributed to casein. Deproteinized milk and whey showed statistically significant differences of (TAC) obtained from UHT-treated and pasteurized milk. Total antioxidant capacity values were not significantly different for UHT and pasteurized milk. Photochemiluminescence assay used in that study demonstrated many advantages among the other techniques, due to the fact that is fully automatic, sensitive and quick. Moreover, this methodology doesn't need complicated sample preparation steps or time-consuming procedures that are required for example in the case of $ORAC_{FL}$ assay, DPPH method or FRAP method (Sielicka et al., 2014). Photochemiluminescence method is widely used in various studies for antioxidant capacity analysis of samples such as blood, fruits, berries, various plants as well as honey, dairy products and so on (Moffarts et al., 2005; Hegedüs et al., 2011; Balogh et al., 2010; Prasad et al., 2012; Zielińska et al., 2007; Wesołowska et al., 2017). Figure 1 shows the mean values for the antioxidant capacity of buffalo milk by lactation. The lactation stimulates both the physico-chemical composition of buffalo milk and the antioxidant capacity. The antioxidant capacity in lactation 1 showed the lowest mean values of 325.6 (µg/ml). As the number of lactations increases, it can be noticed that the values for antioxidant capacity are higher. Lactation 3 and 4 show the highest values, 360.1 (µg/ml) and 358.9 (µg/ml). These values are similar to those reported in the literature: Physical and chemical parameters for buffalo milk are shown in Figure 2. Studies on the composition of buffalo milk and cow milk were in line with Hamad and Baiomy in 2010.

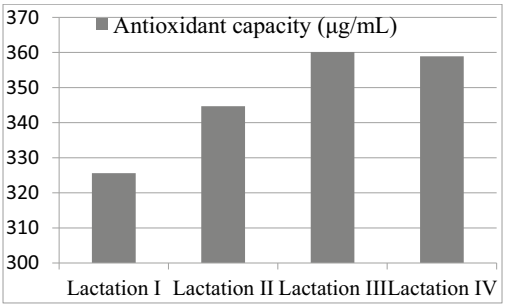

Figure 1. Antioxidant capacity (µg/ml) of buffalo milk by lactation (lactation I-IV)

Many studies have shown that milk proteins have antioxidant action, for example, peptic digestion of casein exhibited to the release of radical scavenging active peptides (Suetsuna et al., 2000; Virtanen et al., 2006). In addition,

peptides derived from whey proteins have also exhibit antioxidant activity. They can be released through fermentation of the milk and enzymatic hydrolysis (Park et al., 2007). Figure 2 shows the mean values for physicochemical profile of buffalo milk influenced by lactation. Buffalo milk fat fraction presented decreased mean values for the lactation I 7.88 (%), it can be observed as the number of lactations grows up, the means are increasing constantly presenting values for lactation II 8.52 (%), lactation III 9.02 (%) and the lactation IV comprising 9.53 (%) of fat, respectively. The same observation is attributed to the protein content, presenting low protein values in the lactation I 4.35 (%) and the highest in the last lactation IV 4.68 (%). Lactose variable presented the highest values during the lactation III with the mean values 4.8 (%) followed by lactation IV 4.77 (%), lactation I 4.71 (%) and lactation II with the lowest content of lactose 4.69 (%). Banu et al., in 1998, in the study of buffalo in our country obtained a mean value for fat of 7.80%, similar values being reported by Georgescu et al., in 2000.

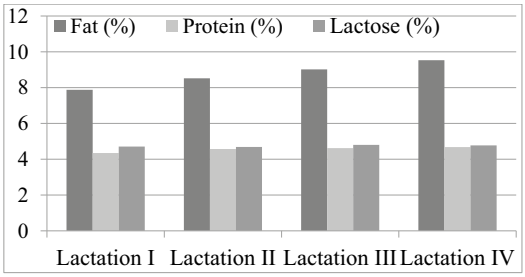

Figure 2. Physico-chemical composition of buffalo milk by lactation

Physico-chemical composition of milk is reported in various studies. The results of the present study are in line with (Smet et al., 2008) showing fat, protein and lactose content of different types of milk. Full-cream milk presented a fat content of 3.26 (%), protein 3.18 (%) and an elevated level of lactose 5.47 (%). PUFA enriched full-cream milk presented the highest fat fraction (3.73%), followed by lactose (4.64%) and protein content (3.35%). Low-fat milk, have the lowest content of fat presenting values of 1.61 (%), lactose 4.72 (%) and having the most increased protein values 3.38 (%) compared to full-cream milk and enriched full-cream milk (Smet et al., 2008). The chemical composition of raw, pasteurized and boiled buffalo and cow milk was reported by Khan et al., 2017. The fat content of raw, pasteurized and boiled buffalo milk showed values (6.45 ± 0.16), (6.42 ± 0.08) and (6.53 ± 0.07), respectively. Cow milk had a lower content of fat comprising values (4.17 ± 0.13), (4.14 ± 0.05) and (4.21 ± 0.11). The protein concentration of raw, pasteurized and boiled of buffalo milk presented values (3.82 ± 0.14), (3.80 ± 0.05) and (3.88 ± 0.12), whereas cow samples revealed significantly lower protein content (3.22 ± 0.09), (3.19 ± 0.03) and (3.26 ± 0.02), respectively. Lactose differences among the milk samples were not so significant depending on the thermal treatment applied. Presenting values for cow milk as follows (4.54 ± 0.19), (4.52 ± 0.23) and (4.61 ± 0.17), whereas buffalo milk showed (4.85 ± 0.26), (4.87 ± 0.12) and (4.94 ± 0.25), respectively. Our results regarding proteins, fat, lactose and total dry substances are in concordance with Khan et al. (2017) presenting similarities of measurements with those from our study. In addition, the same method was used for physicochemical determination of samples (Lactoscan). Figure 3 indicates the total dry substance from buffalo milk samples according to lactation, revealing the most elevated dry content during the lactation I 18.9 (%), it can be noticed that over lactation II this variable is decreasing presenting the lowest values 18.55 (%). Furthermore, during the progression of lactation III and IV, the content of this parameter is increasing again presenting values of 18.64 (%) and 18.72 (%), respectively.

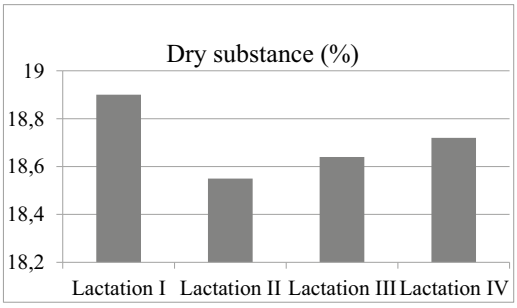

Figure 3. Dry substance level according to different stage of lactation

According to Khan et al. (2017) findings, total dry substances from cow and buffalo raw, pasteurized and boiled milk presented an average means of (12.7 ± 0.34), (12.6 ± 0.28) and (12.9 ± 0.30) (%) instead, the buffalo milk demonstrates the significantly higher values (16.21 ± 0.43), (16.05 ± 0.24) and (16.29 ± 0.18) (%) compared to cow milk.

Interestingly, according to Khan et al. (2017) remarked a suggestion that pasteurized milk preferentially may be consumed within three days for better antioxidant activity effects, also according to their results buffalo milk has a higher antioxidant capacity than cow milk. Animal diet is influencing directly antioxidative properties of milk, increasing compounds as α-Tocopherol and β-Carotene.

Grass-clover silage fed animals excreted milk with higher concentrations of α-Tocopherol and β-Carotene, comprising values of (472 ± 33) g/l and (440 ± 23) g/l whereas cows that received hay roughage diet had a lower means of these antioxidants (504 ± 48) and a decrease (from 445 to 264) g/l, respectively (Havemose et al., 2006).

The fatty acid content of milk is well-known to impact oxidative balance stability (Barrefors et al., 1995; Havemose et al., 2004, 2006). In addition, fatty acids unsaturation degree contributes to lipid hydroperoxides cumulation (Havemose et al., 2006). Unfortunately, goats infected with mastitis demonstrated decreased values of antioxidative properties of milk. Using FRAP method (Silanikove et al., 2014) reported lower values of antioxidant properties in contaminated goat milk (305 ± 36) μM compared to uninfected samples with significantly increased values (427 ± 35) μM. Moreover, subclinical mastitis affects antioxidant/oxidant balance of milk, reducing the total antioxidant capacity (TAC), presenting values of (mmol Trolox Equv./l) 0.54 ± 0.051 for healthy cows and 0.42 ± 0.047 for mastitis infected, as well as (TOC) total oxidant capacity (lmol H_2O_2 Equv./l) 15.91 ± 0.57 for healthy animals and 20.88 ± 0.90 for infected (Atakisi et al., 2010).

Interestingly, that administration of selenium in the nutrition of dairy cows significantly increases catalase activity (CAT) and (TAC), compared with inorganic selenium supplementation (Gong et al., 2014). Furthermore, the mentioned mineral is elevating selenium levels in the blood and milk of the animals. Additionally, vitamin A administrated at the doses of 220 IU/kg of BW to the cows, can significantly raise the total antioxidant capacity and hydroxyl radical inhibition capability enhancing milk production performance (Jin et al., 2014).

CONCLUSIONS

The buffalo milk has a high fat content in all four lactations. This is influenced by the number of lactations and shows the highest values in lactation IV.

The buffalo milk is a food beneficial to the human body due to its high level of fat, protein, lactose and antioxidant capacity. Antioxidant capacity has the highest content in lactation III and IV.

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CHARACTERIZATION OF MERLOT DRY RED WINE COMPOSITION ACCORDING TO THE YEAR OF PRODUCTION

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Abstract

Knowing the physico-chemical composition of the wine allows it to be authenticated and to detect the frauds that can be encountered in marketed wines. The physico-chemical analysis of wine is the basis for controlling and observing the technological flow of wine production. It is also necessary to evaluate the organoleptic characteristics of wine as there is a close link between the sensory characteristics and the chemical composition of the final product. The raw material and the harvest year significantly influence the final composition of the wine. The temperature and precipitations of the raw material harvest year significantly influence the chemical composition of the wine. Tartaric acid varies between 2.51 ± 0.05 and 2.82 ± 0.04 for red Merlot dry wines. The mean values for malic acid are between 1.01 ± 0.04 and 1.57 ± 0.03. The average citric acid values range from 0.19 ± 0.01 to 0.24 ± 0.01 for red Merlot dry wines. These values correspond to the average values reported by other authors in the literature. The purpose of this study was to characterize the content of tartaric, malic, citric and lactic acid in dry red Merlot wine, as well as sensory and physico-chemical properties according to the year of production.

Key words*: Merlot red wine, malic acid, acidity, pH.*

INTRODUCTION

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. Making wine is a long and laborious process in which the total efficiency of all technological operations ultimately determines the quality and price of the finished products. In recent years, the popularity of red wine consumption has increased worldwide. Due to the increased content of phenolic compounds, primarily anthocyanins, leuco-anthocyanins, flavonols (quercetin) and flavan-3-ols (catechin and epicatechin) red wines are characterized by a high biological value (Liu et al., 2018; Motilva et al., 2016). Consumers demands for red wine quality the necessity of continuous improvement of technological processes of wine production. Specifically, control of extraction processes is required in order to extract optimal amounts of coloring and other groups of phenolic substances and anthocyanins for the achievement of the desired color, preventing their oxidation, and excessive astringency (Shirahigue et al., 2010; Francesca et al., 2014). One of the essential factors determining the specificity of red wines is the accumulation of phenolic compounds formed in the grape berries which are undergoing biochemical changes in the processing of wine raw materials. Phenolic compounds play an important role in the addition of the organoleptic properties of various foods (Cheynier et al., 2012). They are contributing to bitter and astringent properties to the taste of fruits and fruit juices, due to the interaction of phenolic substances mainly procyanidins and glycoproteins. As well, these compounds are the main substances that can attribute the color differences in wines, juices, and products made from fruits and vegetables (Liu et al., 2018). Most of the components of the phenolic complex from grapes and wine belong to the group of biologically active substances, making red wines increasingly used to treat numerous

diseases (Giacosa et al., 2013; Biasi et al., 2014; Tresserra et al., 2015). Grape-derived polyphenols are possessing cytoprotective properties and antioxidant properties, thereby effectively binding free radicals and preventing from the diseases such as the risk of cardiovascular disease, metabolic disorders and certain types of cancers (Jiang et al., 2019; Zadnipryany et al., 2017; Agouni et al., 2017; Biasi et al., 2014). Moreover, polyphenols from red wine possess a significant anti-inflammatory action protecting the intestinal barrier integrity (Nunes et al., 2019). For most popular Merlot and Cabernet Sauvignon wines the total flavonoid, phenolic, and anthocyanin content are increasing with the high-altitude. Also, that content is varying due to such factors as climate and soil conditions (Jiang et al., 2019; Jin et al., 2017). Another significantly important characteristics of wines are pH, Brix and the acid composition also known as titratable acidity (TA) composed of acids such as lactic, citric, malic and tartaric acids. An improper harvest treatment can negatively influence these parameters (Casassa et al., 2019). According to (Casassa et al., 2013), Brix from Merlot grapes presented means between (20.18 and 24.9) and pH showed variations between (3.17 and 3.70), respectively. Organoleptic characteristics of the wine are strongly correlated with organic acids and sugars (Coelho et al., 2017). Unwanted quantities of sugars and organic acids presented in grape must or wine can have a negative influence on the taste and Bouquet balance of beverage (Silva et al., 2014). Acetic acid and butyric acid presence in wines can be a precursor of undesirable microbiological alterations that affect the quality of the final product (Lima et al., 2014; Kučerová et al., 2011). Tang et al. (2013) demonstrated cardioprotective effects of malic and citric acids on myocardial ischemia, due to anti-inflammatory and antiplatelet aggregation properties of organic acids. Total amounts of malic and tartaric acids can consist of <80% of the overall amount of acids in grape berries and must. In addition, concentrations of the acids may be different because of factors such as level of maturation, the variety of grapes, climate and so forth (Coelho et al., 2017). Interestingly to the fact that red wines can resist reduced acidities, due to phenolic compounds that have the potential to increase acidity, and also play role in wine maintaining during the aging processes (Kučerová et al., 2011). Therefore, the aims of this study were to evaluate the content of organic acids in Merlot dried wine, as well as sensory and physicochemical parameters.

MATERIALS AND METHODS

Wine description

Commercially Merlot wines from Asconi winery were used in the present study. Asconi company owns about 500 hectares of vineyards located near Geamana village, in the county of Anenii Noi, Moldova. Cultivated varieties of grapes are Merlot, Cabernet Sauvignon, Sauvignon Blanc, Chardonnay and Muscat Ottonel and so on. Grapes are harvested only manually, which prevents the fall of leaves or pieces of a vine in the buckets. Moreover, only matured grapes are harvested. In addition, wines produced in particular harvest year which have benefited from favorable climatic conditions have special qualities that are highly appreciated by consumers but are also reflected in the terms of marketing price.

Wine analysis

Merlot red wine samples were collected according to the year of production (2013-2017). A number of 10 samples were used for each year. FOSS Wine Scanner device that measures many parameters of grape must and wine, based on Fourier Transform Infrared (FTIR) technology to scan a sample of liquid (sample - 50 ml). The measurement results are obtained in only 30 seconds, analysing critical quality control variables. The analyser is connected to a computer, for data interpretation. Facilities regarding software platform allow improved parameter control by measuring and monitoring the results in documents. Final test results help to define the strategy for the next harvest.

RESULTS AND DISCUSSIONS

Wine is not only rich in alcohol but is also rich in content of acids. Acids in wine are arising from grapes as a result of alcoholic

fermentation as a by-product or as a result of the treatments, operations of wine caring and clarifying. Grapes derived acids such as (acidic acid, malic acid) have the highest contribution, therefore it is said that acidity of the wine is born in grape must (Ţârdea et al., 2007; Gheorghita et al., 2002; 2006; Bulancea et al., 2009). Table 1 and Table 2 shows the mean values for the main analyzed acids in high quality Merlot red wine.

Table 1. Content in tartaric and malic acid in red Merlot dry wine - superior quality according to year of production

Year	Tartaric acid		Malic acid	
	X±sx	V%	X±sx	V%
2017	2.60±0.11	1.3	1.18±0.15	1.22
2016	2.51±0.05	4.79	1.28±0.06	1.58
2015	2.44±0.06	5.27	1.01±0.04	1.94
2014	2.61±0.05	4.30	1.11±0.17	4.81
2013	2.82±0.04	3.32	1.57±0.03	4.29

(n = 5; sx - standard deviation; V- variability; X - average value)

Table 2. Merlot dry red wine content of citric and lactic acid

Citric acid		Lactic acid	
X±sx	V%	X±sx	V%
0.23±0.02	1.19	0.30±0.01	1.31
0.19±0.01	4.23	0.33±0.01	3.63
0.24±0.01	2.03	0.32±0.02	3.20
0.21±0.02	3.04	0.39±0.02	5.37
0.24±0.01	2.29	0.38±0.03	6.32

(n = 5; sx- standard deviation; V- variability; X - average value)

Tartaric acid in analyzed samples varies between (2.51 ± 0.05) and (2.82 ± 0.04). The mean values for malic acid are between (1.01 ± 0.04) and (1.57 ± 0.03).

The average values for citric acid are between (0.19 ± 0.01) and (0.24 ± 0.01). These values correspond to the average values reported by other authors in the literature (Casassa et al., 2019; Peres et al., 2009).

Figures 1-3 show average values for pH, color intensity and total acidity in superior Merlot dry red wine, depending on the year of production. The values presented for these parameters are consistent with other studies (Casassa et al., 2013; Jin et al., 2017; Jiang et al., 2012; Casassa et al., 2019).

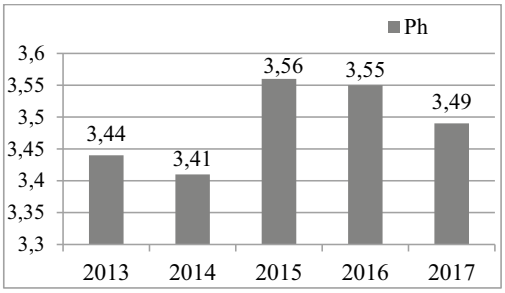

Figure 1. Average pH values for red Merlot red SQ wine

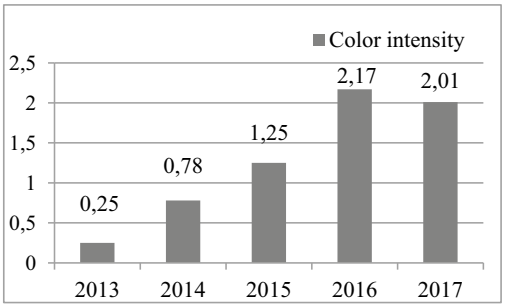

Figure 2. Color intensity for red Merlot red SQ wine

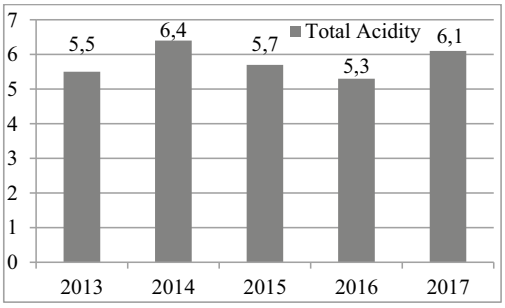

Figure 3. Mean values for total acidity for Merlot red dry wine SQ

Total acidity is the acidity determined by neutralizing acidic functions with a known concentration of (NaOH) solution (alkaline). For this reason, it is also called titratable acidity (TA). The end of dosing is also currently appreciated by a color indicator, such as bromothymol blue, which turns to pH = 7 or phenolphthalein, which turns to pH = 9 (Ţârdea et al., 2007; Gheorghiţă et al., 2002, 2006; Bulancea et al., 2009). According to (Petropulos et al., 2015) chemical composition

of Macedonian red wines (Merlot) demonstrated a total acidity of (5.6-6.0) g/l, and color intensity values as it follows (11.41, 12.35, 21.08, and 24.20) in accordance to (OIV 2014). In addition, another study revealed that Macedonian merlot wines can exert maximum antioxidant activity as a consequence of longer exposure to grape pomace (Kostadinović et al., 2012). Total acidity in organic and conventional Merlot wines from different regions of Italy, according to (Garaguso et al., 2015) comprised means between 6.19 and 5.74 g/l, respectively. Organic acid concentrations for Brazilian Merlot wine for tartaric, malic, lactic and acetic acid presented values between (1495 ± 6), (2243 ± 23), (35 ± 4) and (269 ± 20) (mg/l), respectively. Moreover, Merlot red from 2001, showed increased values of lactic acid (7306 ± 35) and acetic acid (671 ± 13) (mg/l), using a capillary electrophoresis method (Peres et al., 2009). Regarding, Merlot grape chemistry content by (Casassa et al., 2013) they found out tartaric acid differences between the maturity levels during 2011 and 2012. Early harvested grapes from 2011 and 2012 season presented significantly higher levels of tartaric acid with the values range of 7.66 and 8.14 g/l. Later berries harvests showed lower values, 5.69 and 6.37 g/l, respectively. Also, the pH of earlier season grapes from 2011 and 2012 showed values of 3.47 and 3.17, followed by late harvest berries with the values between 3.70 and 3.66. Furthermore, Merlot wines from 2011 and presented values of tartaric acid, pH, malic acid and lactic acid as follows: 5.31 and 5.75 g/l, 3.73 and 3.47, 39 and 27 mg/l, 0.84 and 0.97 g/l, respectively. In addition, extended maceration of Merlot wines presented tartaric acid content of 5.55 g/l with the pH 3.63, malic acid concentration of 39 mg/L and lactic acid 0.92 g/l. (Casassa et al., 2013) find similar results when they finished Merlot red wines at bottling presenting average values of titratable acidity between 5.4-5.6 g/l tartaric acid, with pH 3.76-3.82, malic acid and lactic acid, 0.10-0.13 g/l and 1.26-1.53 g/l, respectively. According to Jin et al. (2017), red Merlot wines from Northern China regions presented values of tartaric acid with a range between 5.38-6.44 g/l, with a pH 3.48-3.30 and color intensity of 7.9-9.4 AU (absorbance unit). Merlot grapes composition obtained from Uruguay showed total acidity (61.2 ± 2.0) in meq l^{-1}, pH of 3.57 and sugar content (221.5±1.9) g l^{-1} (González-Neves et al., 2015). Jiang et al. (2012) compared general composition of Merlot wine and musts from different regions of China, founding out that titratable acidity for wine varied from 6.7 to 8.3 g/l with a pH from 3.0 to 3.6 and for the must, 6.9 and 8.9 g/l, with 3.0 and 3.3 pH, respectively. In addition, a recent study regarding the chemical composition of Merlot grapes from China, presented values for titratable acidity varying from 6.7 to 8.3 g/l having a pH from 3.0 to 3.6. Additionally, tartaric acid of Cabernet Sauvignon grapes was differing (from 6.3 to 7.3), depending on the region of China (Jiang et al., 2019). Recently (Casassa et al., 2019), performed a physicochemical analysis on Merlot wines from Argentina, using three different harvests of grapes (from 3 Feb., 27 Feb., and 29 Mar.). Wines made from the first harvest showed a pH of 3.43 ± 0.09, titratable acidity 5.57 ± 0.03, tartaric acid 2.32 ± 0.03, citric acid 0.31 ± 0.05, malic acid 0.50 ± 0.06 and lactic acid contents of 1.53 ± 0.03, measurements units where expressed in g/l. Second harvest obtained wines presented a pH of 3.60 ± 0.07, TA 5.53 ± 0.17, tartaric acid 1.73 ± 0.10, citric acid 0.37 ± 0.05, malic acid 0.93 ± 0.06, and lactic acid 1.49 ± 0.06. Beverages obtained third harvest (29 Mar.), demonstrated a pH of 3.58 ± 0.09, TA (5.08 ± 0.12) was significantly lower compared to previous harvests, tartaric acid (2.11 ± 0.06), citric acids presented a significantly decrease (0.05 ± 0.02), malic acid (0.50 ± 0.11) and a slight decline in lactic acid content (1.33 ± 0.03) (g/l) compared to the first and second harvests was observed, respectively. Altogether, our results provide some useful information on high-quality Merlot wine production.

CONCLUSIONS

In conclusion, obtained results showed that these wine varieties fall below the maximum admissible limit for parameters such as total acidity, and pH indicating that these wines are of superior quality. Thus, the year of production influences the chemical composition, namely the total acidity, and the physicochemical composition of wines.

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AN OVERVIEW ON HUMAN POTENTIAL EXPOSURE TO BISPHENOL A FROM FOOD-CONTACT MATERIALS USED IN FRUITS PACKAGING AND PROCESSING

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Abstract

Food contamination during the migration process from food contact materials is an important food safety issue and many researches are focused on this topic in the last decades.
Bisphenol A is a hazardous chemical compound used as intermediate in the production of polycarbonate-based materials and epoxy resins, used to obtain plastic food containers for packaging and storage, but also for inner surface coatings of cans. Through contaminated products, this compound can reach the human body where it causes a number of adverse health effects. As a result of this consumption, Bisphenol A can cause diseases of the cardiovascular system, endocrine system, reproductive system, metabolic system. The aim of this review was to investigate Bisphenol A levels from worldwide fruits and fruits products, fresh or processed, packed in different food contact materials. Despite the fact that the values found in the literature are not high, it poses a risk to human health because it can accumulate in the body. To prevent this hazard, the European Food Safety Authority recommended a tolerable daily intake (TDI) of 50 µg/kg body weight/day. As a conclusion the authors try to investigate differences between canned and non-canned fruit products and to establish which material can leach more Bisphenol A and what factors influence this process.

Key words*: Bisphenol A, exposure, fruits, human health.*

INTRODUCTION

Bisphenol A, BPA, is a chemical compound used as an additive in the process of obtaining polycarbonate plastics and epoxy resins with estrogenic activity, being a known endocrine disrupter (Chouhan et al., 2014). Estrogenic endocrine disruptors are exogenous chemicals that mimic or antagonize the effect of estrogenic hormones (Posnack, 2014).
People are exposed to this compound mainly by eating food contaminated with this compound which is able to migrate from food packaging materials in food products under certain time, temperature and pH conditions, but there may be other sources of exposure such as air, dust, contaminated surface water (Michalowicz, 2014).
BPA entered in the body by ingestion, inhalation or dermal contact (Rochester et al., 2013) may undergo bioaccumulation and bioconcentration processes in different tissues or organs (Zielinska et al., 2019) associated with a number of side effects.
The aim of this review is to summarize, on the basis of literature, the level of BPA contamination of worldwide fruits, both fresh and processed, as well as products derived therefrom, but also a number of side effects that may occur as a result of the ingestion of this compound.

BISPHENOL A EFFECTS ON HUMAN HEALTH

Studies have shown that BPA can bind to estrogen receptors, act as an anti-estrogen and block their activity. It can also bind to the receptors of the thyroid gland, perturbing its function, and other organs and systems in the body (cardiovascular system, endocrine system, nervous system) (Rochester et al., 2013). Regarding the reproductive system, it has been observed that BPA can affect the reproduction function (Muhamad et al., 2016; Li et al.,

2010), reduces sperm quality (decrease in sperm count, changes in morphology and motility) (Li et al., 2011), can lead to polycystic ovarian syndrome (PCOS) (Muhamad et al., 2016), decreases fertility (decreases the number of mature oocytes) (Ehrlich et al., 2012), and affects prostate function and morphology (Tang et al., 2012).

Increased levels of BPA in the body could also be related to a number of metabolic disorders such as increased incidence of obesity cases (Muhamad et al., 2016) and type 2 diabetes (Kim et al., 2013). Concerning the effects of BPA on pancreatic cells, studies have suggested that this compound produces insulin resistance (Almeida et al., 2018). Also, some correlations have been established between high levels of serum BPA and elevated LDL and HDL cholesterol levels (Olsen et al., 2012). Experimental studies demonstrated its association with altered liver or thyroid function (Muhamad et al., 2016). On the cardiovascular system it has been shown to increase the risk of cardiovascular disease such as heart attack, hypertension or angina pectoris (Almeida et al., 2018) when exposed to BPA. Exposure of the body to low doses of BPA may also result in cardiovascular system dysfunctions such as cardiac arrhythmia, increased blood pressure, atherosclerosis, and a negative impact on ventricular contractility and intracellular calcium circulation (Poscnack et al., 2014b). Also, high levels of BPA in the body could be correlated with increased myocardial infarction (Patel et al., 2015).

High levels of this compound in blood or urine could also be correlated with other side effects, such as premature birth (Cantonwine et al., 2010), lower birth weight (Chou et al., 2011), impaired immune function, the ability to produce oxidative stress and inflammatory processes, effects on the expression of genes (Muhamad et al., 2016) and DNA stability (Almeida et al., 2018).

LEGISLATION ON BISPHENOL A MIGRATION

To reduce the risk of ingestion of this compound from food packed in different materials, a specific BPA migration limit for plastics has been imposed by European legislation, as well as a daily intake value (TDI).

In the European Union, the use of this compound has been regulated for the first time by EC Directive no. 90/128, where the specific migration limit for this compound was 3 mg/kg, later reduced to 0.6 mg/kg (EC Directive No 72/2002). Regulation 10/2011 replacing Directive No. 72/2002, maintained this value of 0.6 mg/kg until 2018, when through EU Regulation No. 213/2018, which is an amendment to EU Regulation No 10/2011, the value was reduced to 0.05 mg/kg.

At the same time, during the years, tolerable daily intake has undergone significant changes. If in 1986 the TDI was 0.05 mg/kg of body weight/day (Scientific Committee for Food, 1986), in 2002 this was 0.01 mg/kg of body weight/day (Scientific Committee for Food, 2002). In 2006, in an EFSA report, TDI increased to 0.05 mg/kg of body weight/day (EFSA, 2006). Between 2006 and 2015, this value remained constant, and in 2015 this value would fall to 0.004 mg/kg of body weight/day, a value that has remained unchanged to date (EFSA, 2015).

Another regulation on BPA across the European Union was to prohibit its use in articles for infants and young children (Directive No 8/2011) because it produces different behavioural or developmental disorders, and because this category is one of the most sensitive and vulnerable categories of the population (Almeida et al., 2018).

The first countries that renounced the use of this compound in packages for infants and children up to 3 years were Denmark and Austria in 2011, Belgium and France in 2012, and Sweden in 2013 (Ludwicka et al., 2015; Boudalia & Oudir, 2016; Almeida et al., 2018).

In countries like Algeria, BPA is an unknown substance, with no known health effects, and this is why there is no legislation on this compound (Boudalia & Oudir, 2016).

BPA IN FRUIT AND FRUITS PRODUCTS

As it can be seen in Table 1, the literature review shows that the highest values of BPA were obtained in canned samples because these food products are the most exposed to BPA which is mostly present in coating lakes

Table 1. Bisphenol A concentrations in fruit and fruit products

Food category	Origin/commercial area	Packaging	Range	Method	Reference
Citrus, apple, strawberry	Florida	Fresh fruit	2.0 ± 1.4 – 9.0 ± 4.9 µg/kg	GC-MS/MS	Lu et al., 2013
Apple, pear, pitaya	Hetey	Fresh fruit	1.056 - 2.35 µg/kg	DSMIP-SPE- HPLC	Li et al., 2014
Fruit mix, Peaches, Pears	Belgium	Can	10.1 - 20 ng/g in content 6.4 - 14.3 ng/ml in syrup	SPE-SIM-GC-MS	Geens et al., 2010
Pineapple, apple sauce	Belgium	Glass	0.3 - 1.28 ng/g	SPE-SIM-GC-MS	Geens et al., 2010
Pineapple	Belgium	plastic	0.11 ng/g	SPE-SIM-GC-MS	Geens et al., 2010
Apple sauce, Orange juice, tropical juice	Belgium	can	0.2 - 4.73ng/g	SPE-SIM-GC-MS	Geens et al., 2010
Fruit and vegetables juice	Belgium	PET	<0.02 ng/ml	SPE-SIM-GC-MS	Geens et al., 2010
Apple juice	Belgium	Tetra Pak	<0.02 ng/ml	SPE-SIM-GC-MS	Geens et al., 2010
Pineapple, peaches	Spain	canned	< 2.9 - 13 ± 1 µg/kg	HPLC/FLD	Alabi et al., 2014
Pineapple, Organic pineapple, Almond jelly, Sliced peaches, Fruit cocktail	SUA	canned	< 2 - 19 ng/g	HPLC-MS/MS	Noonan et al., 2011
Fresh peach, Fruit and vegetable juice	Texas	Non-canned	0.24 - 0.41 ng/g	LLE- HRGC/LRMS	Lorber et al., 2015
Oranges, peach slices	Texas	canned	0.31 - 2.03 ng/g	LLE- HRGC/LRMS	Lorber et al., 2015
Passion fruit pulp, Mango pulp, Papaya in syrup, Pears in syrup, Mango in syrup, Pineapple in syrup, Peach in syrup, Fruit cocktail in light syrup	Thailand, Indonesia, Kenia, European Union	canned	< 1.0 - 10.2 µg/kg	QuEChER-DLLME-GC/MS	Cunha and Fernandes, 2013
Fruits and vegetables	Thailand	canned	<0.0015 - 0.0087 mg/kg	GC-MS	Poovarodom et al., 2017
Walnuts, chestnuts, jujubes, plums, hawthorn, raisins	China	-	< 0.01 - 132 ng/g	HPLC – MS/MS	Liao, 2014
Tropical fruits, Apple in syrup, Mandarin in syrup, Mango in syrup, Pineapple in syrup, Peach in syrup, Grape juice, Cocktail fruits, Coconut milk, Apple juice	Thailand, China, Indonesia, Tokyo	canned	< 1 - 200 ng/g	GC – MS	Kawamura, 2014
Pineapple in syrup, Peach in syrup	Iran	canned	3.7 ± 0.3 - 7.7 ± 0.8	SPE-DLLME-SFO-HPLC	Sadeghi et al., 2016
Pineapple chunks in pineapple juice, Apple juice from concentrate	Texas	can	< 0.20 - 0.49 ng/g	LLE-HRGC/LRMS	Schecter et al., 2010
Organic cinnamon apple sauce	Texas	plastic	< 0.20 ng/g	LLE-HRGC/LRMS	Schecter et al., 2010
Fruit juice, Peach juice	Mahdia	Cardboard box with plastic cap and metal foil	< 0.002 mg/kg	UPLC-MS/MS	Beltifa et al., 2017
Orangeade	Dongguan, China	can	6.16 ± 0.13 µg/L	UA-ULME-MSD	Mo et al., 2017
Orangeade	Dongguan, China	Plastic bottle	< 1.10 µg/L	UA-ULME-MSD	Mo et al., 2017
Nectar juice, cocktail, guava	Egypt	Tetrapak, aluminium pouch	2,23 - 26,4 ng/ml	HPLC	El-Dars et al., 2018
Pomegranates fresh juice, mango juice drink, strawberry jam, fig jam	Egypt	plastic	0.46 - 82.97 ng/ml	HPLC	El-Dars et al., 2018
Jam	Norway	Glass with plastic or metal cap	0.38 µg/kg	SPE-UPLC–MS/MS	Sakhi, 2014
Juice	Norway	Cardboard box with plastic cap and metal foil	< 0.020 µg/kg	SPE-UPLC–MS/MS	Sakhi, 2014

GC-MS/MS – Gas-chromatography tandem mass spectrometry; **DSMIP-SPE-HPLC** – Dummy surface molecularly imprinted polymer - solid - phase extraction - high performance liquid chromatography; **LLE-HRGC/LRMS** – Liquid/liquid extraction - High Resolution Gas Chromatography/Low Resolution Mass Spectrometry; **HPLC/FLD** - High Performance Liquid Chromatography with Fluorescence Detection; **HPLC-MS/MS** - High Performance Liquid Chromatography tandem mass spectrometry; **UPLC-MS/MS** - ultra performance liquid chromatography tandem mass spectrometry; **SPE-DLLME-SFO-HPLC** - Solid-Phase Extraction–Dispersive Liquid-Liquid Microextraction - Solidification of Floating Organic Drop - high-performance liquid chromatography; **SPE-UPLC–MS/MS** - solid phase extraction - ultra performance liquid chromatography - tandem mass spectrometry; **HPLC** - High Performance Liquid Chromatography; **UA-ULME-MSD** - Ultrasound-assisted upper microextraction and molecular fluorescence detection; **QuEChER-DLLME-GC/MS** - the Quick Easy Cheap Effective Rugged and Safe - Dispersive Liquid–Liquid Microextraction – gas-chromatography mass spectrometry.

(Michalowicz, 2014). This may be mainly due to the sterilization process and storage conditions. Studies have shown that for cans, between 80-100% of the compounds present in the coating lakes can migrate during sterilization (Almeida et al., 2018).

This process is also influenced by the composition of the packaged product, so that in products high in sugar, vegetable oils (corn, soybean, olive oil) or salt, the BPA values found in the product were significant (Almeida et al., 2018; Sungur et al., 2014).

Differences in BPA content from the same product type but from different lots were observed (Sungur et al., 2014), because changes in the composition of can coatings may occur (Almeida et al., 2018; Noonan et al., 2011).

The migration process is also influenced by the type of product packaged, so in the case of foods containing solid and liquid fractions, syrup for example, BPA can migrate in both components, but especially in the solid fraction (Geens et al., 2010; Noonan et al., 2011; Almeida et al., 2018).

In the case of plastic packaging, this compound can migrate from packages through two different processes: diffusion of residual BPA resulted from the manufacturing process of packages or hydrolysis of polymers containing ester bonds (Aschberger et al., 2010; Hoekstra et al., 2013; Ludwicka et al., 2015).

For food packaged in polycarbonate plastics, the migration process is amplified over time due to the hydrolysis of polymers in the packaging material, while for reusable plastics the migration process tends to stabilize (Almeida et al., 2018).

From the factors that can influence the migration of BPA from plastic materials, temperature is the main one, so by exposing the material to about 100°C, the migration rate can be up to 55 times higher than exposure of the same type of material at temperatures of 20°C (Le, 2015; Almeida et al., 2018).

Also, in Table 1, it can be seen that for products packed in glass jars with a lid, the values of BPA are much lower than in the case of cans and plastics. This fact is mainly due to the possibility of migration of BPA only from the lid, which contains inside epoxy resins used as coating paints. In this case, the contact between the food product and the lid is occasional, by shaking the packaging during handling, transport or inadequate storage (Cao et al., 2011; Almeida et al., 2018).

In case of tetra Pak or cardboard boxes laminated on the inside with plastic foil and plastic cap, the possibility of contamination of products with BPA is lower. For this type of packaging, the values obtained for this compound in the product were below the detection limit of the apparatus (Table 1) (Geens et al., 2010; Sakhi, 2014). Also, when packaged products are kept in proper conditions, the migration of BPA from food contact materials takes place at a much lower rate and the concentration found in products are insignificant and do not represent a danger to human health (Almeida et al., 2018).

CONCLUSIONS

From this literature review, it was concluded, that through packaging, food products are predisposed to chemical contamination with a multitude of compounds, among which bisphenol A occupies an important place.

Out of the packaging materials used in the food industry, it can be stated that food packed in cans is more susceptible to be chemically contaminated with BPA, when compared to the rest of the packaging materials. This fact is due to a large number of factors related to the technology of cans manufacturing (type and quality of inner coatings - epoxy-phenolic resins, polyvinyl chloride organosol resins) (Poovarodom et al., 2017), the temperature to which the food is subjected, or composition of the food products (pH, salt content, sugar) (Sungur et al., 2014).

As a preventive measure to reduce this type of contamination, the inner coatings could be replaced with materials that don't contain BPA and have better resistance to different processing temperatures.

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TRENDS IN REFRIGERATION TECHNOLOGIES USED FOR FOOD PRESERVATION – A REVIEW

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Abstract

Food is a perishable commodity. The primary objective of food preservation is to prevent or slow down the growth of microorganisms including moulds, yeasts and bacteria as their growth causes spoilage of food but also to reduce the speed of enzymatic reactions which take place during postharvest or post-slaughter life of raw material or during food product shelf-life. Refrigeration has become an essential part of the food chain. It is used in all stages of the value chain, from farm, food processing, to distribution, retail and final consumption at home. The food industry employs both chilling and freezing processes where the food is cooled from ambient to temperatures above 0°C in chilling and between -18°C and -35°C in freezing to slow the physical, microbiological and chemical activities that may cause deterioration in foods. The use of refrigeration for food production and its preservation is undoubtedly the most extensive technique. The aim of this study is to present the latest trends in refrigeration techniques used for food preservation.

Key words*: refrigeration technologies, food preservation, chilling, freezing.*

INTRODUCTION

Food spoilage represent a continuous threat along the entire food chain, consequently suitable and efficient conservation methods are needed. For food products that cannot be deposited at room temperature, the technologies currently applied are in general represented by chilling and freezing which, while effective, are energy consuming (Santos et al., 2015). New technologies have been presented lately to improve food quality during storage along the cold chain. Despite their advantages, there is still needed the improvement of some factors that may compromise food product's thermal stability and distribution and furthermore causing direct impact on food quality and energy efficiency (Tsang et al., 2017).

Refrigeration is omnipresent in daily life, for example in homes, and vehicles, as well as in less obvious applications, such as cryogenic cooling. The large need for refrigeration on multiple levels of different temperatures has created a vast variety of refrigeration and freezing techniques. For most applications, vapor compression cycles and absorption cycles are used. The latter are used for large scale applications, while the former is used widely in applications encountered on a daily basis (Zink et al., 2010). In terms of food industry, refrigeration represents an essential part of the food chain, being used in all stages from processing to distribution, retail and final consumption at consumer's home (Tassou et al., 2009).

The aim of this study was to provide a brief review of some refrigeration techniques that are or could be used in food industry.

Air cycle refrigeration

The advantages of air as a refrigerant are obvious: it is free, safe for environment and operators and, above all, available everywhere (Shengjun et al., 2011; Giannetti & Milazzo, 2014). Using air as refrigerant has great advantages over conventional refrigerants, many of which have negative environmental effects, are toxic or flammable (Shengjun et al., 2011). This technique allows continuous charge/discharge to the ambient, a unique feature among refrigerant fluids, eliminating the need for a sealed circuit. The circuit can be open to the ambient or to the refrigerated space. In both cases, irreversibility related to heat transfer and flow friction, as well as cost,

weight and volume, are avoided. Regarding the second case, elimination of the evaporator has many advantages in terms of frost avoidance on cold surfaces. If the cold air is introduced at an acceptable speed in the cold space, the electric fan integrated on the evaporator can be eliminated as well (Giannetti & Milazzo, 2014).

An air circulation system is mostly composed of a compressor, expander, indoor and outdoor heat exchanger. In an ideal condition, this refrigeration cycle includes two isobaric and two isentropic processes. The circulating air gets into the compressor and becomes compressed (isentropic process) and the temperature and pressure rise; the compressed air of high temperature and high pressure gets into the outdoor heat exchanger and lets out heat (isobaric process) and the temperature falls; the air flow then gets into the expander and expands to the demanded temperature and pressure; the air flow of low temperature and low pressure gets into the indoor heat exchanger and absorbs the indoor heat (isobaric process), then the air flow gets into the compressor and the new refrigeration circulation begins (Xing et al., 2016).

Foster et al. (2011) build and tested a closed air cycle cooling and heating equipment to be used in the food industry. The equipment used a bootstrap unit developed for aircraft air conditioning in a system that has been modified to run at low temperature necessary for food freezing, by using a parallel compressor. This approach allowed temperatures as low as -140°C leaving the turbine and as high as 234°C leaving the bootstrap compressor. The system has been shown to produce cooling at cryogenic temperatures, with waste heat capable of cooking and boiling water, being a good alternative to current systems used in food industry (Foster et al., 2011). Air cycle technology could be used in food industry in processes like fast chilling and/or freezing, cold storage, refrigerated transport or refrigerated storage cabinets (Tassou et al., 2009).

Ejector refrigeration system

The ejector, as a key device in the ejector refrigeration system, is used to create a region with low pressure in the evaporator (Liu et al., 2018).

Ejectors are widely used in the refrigeration system, fuel cell system, vacuum equipment and desalination plant due to its simple structure, long service life and low maintenance cost. The ejector refrigeration system was studied in recent years due to its promising ability to use the low-grade thermal energy such as solar energy. Nevertheless, compared with the traditional vapor compression refrigeration system, the ejector refrigeration system has relative lower efficiency and presents a complex thermodynamic behaviour due to the supersonic flow phenomenon inside the ejector chamber (Liu et al., 2017).

Ejector refrigeration system has many advantages over traditional compressor-based systems, such as simplicity, the ability to work with different types of refrigerants, low installation and low operating costs. Furthermore, the direct correlation between the peak cooling load and peak solar radiation makes solar ejector cooling systems more advantageous. However, low coefficient of performance (COP) and dependency on the environmental conditions are the main drawbacks of this type of systems (Aligolzadeh & Hakkaki-Fard, 2019). One way to improve COP is to include a booster refrigeration system with ejector which modifies the conventional booster refrigeration system by adding an ejector between evaporator and compressor, which can effectively enhance the system COP when the ambient temperature is high according to Huang et al. (2018).

Ahammed et al. (2018) conducted a thermodynamic analysis of transcritical CO_2 systems that use ejector instead of throttle valve for chilling and heating of milk in the process of pasteurization in a dairy plant. The analysis showed that for pasteurization process, energy savings of about 10% can be reached by replacing the conventional throttle valve with an ejector. Compared to the conventional pasteurization process that use separate heating disposition, use of ejector based transcritical CO_2 system yields a primary energy savings of about 29% even under conservative values of energy conversion (Ahammed et al., 2018).

Applications in the food sector could be for example in areas where waste heat is available to drive the ejector system. Such applications can be found in food processing factories

where the ejector refrigeration system can be used for product and process cooling and refrigerated transport (Tassou et al., 2009).

Sorption-adsorption refrigeration

Sorption technology is used in thermal cooling methods. An absorption machine consists of four main components: a desorber, an absorber, a condenser and an evaporator (N'Tsoukpoe et al., 2014).

Sorption technology can be classified as either open sorption system or closed sorption system. Open systems refer to solid or liquid desiccant systems that are used for either dehumidification or humidification.

Principally, desiccant systems transfer moisture from one airstream to another by using sorption and desorption processes. In closed sorption technology, there are two primary methods: absorption refrigeration and adsorption refrigeration (Sarbu & Sebarchievici, 2015).

Solid sorption refrigeration cycle, which is driven by low-grade heat, is extensively used for air-conditioning, freezing, sub-cooling for vapor compression systems to get for example extreme low temperature energy. It shows promising alternatives for application due to its properties for saving energy, decreasing pollution, and beneficial to sustainable development. For solid-gas sorption refrigeration, chemical working pairs generally have the advantage of high sorption capacity and volume cooling density over physical sorbents (Li et al., 2012). Because the sorbents generally have extremely low thermal conductivity, the conventional solid sorption refrigeration systems were mostly driven by hot water or oil, which present much higher heat transfer coefficient than gas. This way the system will be compact, but having the drawbacks of complicated structure for the heat recovery in exhaust gas due to additional heat transfer process between the gas and the liquid, which is required (Gao et al., 2018).

The existing systems for producing cold using solar thermal energy are mainly based on sorption technology: the process by absorption liquid–gas and the process by adsorption solid–gas (Fan et al., 2007). Solar sorption refrigeration technologies are considered as a promising way to meet the growing refrigeration needs for thermal comfort, food products preservation, and vaccines and medicines conservation (Fan et al., 2007; N'Tsoukpoe et al., 2014). These technologies are attractive for refrigeration applications in remote or rural areas of developing countries where the access to electricity is impossible (Fan et al., 2007).

For freezing application that needs temperature below 0°C, like icemaking or frozen storage; an absorption chiller, an adsorption chiller, or a chemical reaction chiller can also be used. Overall, the lower cooling temperatures demanded, the higher generation temperatures are needed for driving a sorption refrigeration system (Fan et al., 2007).

Stirling refrigeration cycle

The Stirling refrigeration cycle is one of the major cycle models in cryogenics. It consists of two reversible isothermal processes and two reversible constant co-ordinate processes such as constant volume or isomagnetic processes. The working matter of a Stirling refrigeration cycle can be a gas, a magnetic material etc. For different working substances, the Stirling refrigeration cycle will have different regenerative properties such as its coefficient of performance is not only dependent on the temperatures of the two heat reservoirs but also, in general, on the specific properties of the working substance (Chen & Yan, 1993). The duplex Stirling refrigerator is an integrated refrigerator consisting of a Stirling cycle engine and a Stirling cycle refrigerator used for cooling. The equitability of the work generation of the heat engine to the work consumption of the refrigerator is the main limitation of the duplex Stirling (Erbay et al., 2017).

Gadelkareem et al. (2019) performed a study regarding the optimization of different parameters of the Stirling refrigerator/heat pump cycle for a drinking water cooler/heater. In this sense, a mathematical model was developed, based on Schmidt analysis, to evaluate the equipment performance taking into account the flow losses and the regenerator efficacy. Some of the main conclusion of the authors are: (i) the Stirling refrigerator/ heat pump has a great potential to be used for drinking water cooler/heater; (ii) the presented Stirling cycle for water dispensers consumes the minimum electric power compared to the

other commercially available technologies; (iii) the presented Stirling cycle can produce hot water at 95°C without using heating elements, which consumes high electric energy (Gadelkareem et al., 2019).
A study performed by Sun et al. (2008) investigated the reverse Stirling cycle for use in refrigeration processes. This type of cycle is referred to as Stirling cooling or Stirling cooler. An experimental free-piston Stirling cooler (FPSC) was developed and the effects of the device parameters in relation to the performance of the cooler were studied; the equipment was then experimentally applied to churning butter. The results indicated that by churning butter using the Stirling cooler, coagulation of the butter occurred more rapidly compared to when the control was used in the process; the water content of the obtained butter was lower, having a higher fat content when the Stirling cooler was used, showing that the feasibility of using the Stirling cooler for churning butter is high (Sun et al., 2008). The Stirling cycle equipment can have many applications in food industry due to the fact that can it work down to cryogenic temperatures.

Thermoacoustic refrigeration
Thermoacoustic technology received a great attention due to the fact that it uses simple devices without mobile mechanical parts compared with conventional technologies (Belaid and Hireche, 2018). Thermoacoustics is a domain that focuses on the interaction between thermodynamics and acoustics. The thermoacoustic effect represents the energy transformation of acoustic work absorbed to transport heat (thermoacoustic refrigerator TAR) or the energy conversion of the heat supplied to produce acoustic work (thermoacoustic Stirling heat engine TAE) (Alcock et al., 2018), resulting in a system that can operate on waste heat and does not contain refrigerants or moving parts (Zink et al., 2010).
Heat-driven thermoacoustic refrigeration presents great advantages of high reliability and external heat-driven mechanism. In a system like this, heat can be first of all converted into acoustic power and then the acoustical power operates a refrigerator to generate cooling effect without any moving mechanical components (Wang et al., 2019).

Thermoelectric refrigeration
Thermoelectric machines are composed of semiconductor materials that can directly convert heat into electricity. Thermoelectric machines which convert a temperature difference into electrical power are called thermoelectric generators (TEGs). This form of energy conversion is called the See beck effect. The conversion of current to temperature that occurs when an electric current flow through a thermoelectric device (Enescu, 2018) called thermoelectric coolers (TECs) is called the Peltier effect. Although the efficiency of TEC is not very high when compared with conventional refrigeration equipment, it is irreplaceable. TEC is portable, quiet, environmentally friendly and it has high temperature-controlling capacity (He et al., 2017). Recently, thermoelectric devices are taken into consideration in a practical power generation, refrigeration and energy recovery applications. This fact is thanks to remarkable features and properties of these devices, including simplicity, small size and weight, lack of moving parts, ability to heat and cool with the same module, precise temperature control, absence of working fluid and solid-state operation, high reliability and environmentally friendly operation (Hadidi, 2017).

Trigeneration
Polygeneration energy systems are shown to be a reliable, competitive and efficient solution for energy production. The recovery of otherwise wasted energy is the first reason for the high efficiency of polygeneration systems (Urbanucci et al., 2019). Polygeneration can be defined as the combined production of two or more energy services from a common resource. cogeneration, or combined heat and power, is the simplest form of polygeneration, and generally refers to the joint production of electricity (and/or mechanical energy) and heat from a common resource. A typical extension of cogeneration is trigeneration, also known as combined cooling, heating and power, which usually refers to the combined production of electricity, heat, and cooling (Pina et al., 2018).
The principle of operation for a trigeneration system is the conversion of the heat taken from a high temperature process by the heat engine into mechanical work achieving a maximum

efficiency equal to the Carnot cycle. Mainly, trigeneration systems integrate various devices such as refrigeration units, heat engines, heat pumps, hydrogen production units and desalination units (Leonzio, 2018).

Depending on the user's refrigeration needs, direct or indirect activation can be used between the cogeneration and the absorption chiller in food applications, having good results. Direct activation consists of the direct use of exhaust gases to drive the absorption chiller.

Indirect activation uses the exhaust gases to produce hot water or steam in a heat exchanger that is afterwards used as a heating environment for the chiller (Marimón et al., 2011).

CONCLUSIONS

The food industry relies mainly on the vapor compression refrigeration cycle for food preservation and processing (Tassou et al., 2009). To reduce environmental impact, there were developed and tested many technologies based on air cycle refrigeration, ejector refrigeration system, sorption-adsorption refrigeration, Stirling refrigeration cycle, thermoacoustic refrigeration, thermoelectric refrigeration or trigeneration, technologies that can reduce the quantity of used energy, that can use solar energy instead of conventional ones or that can use refrigerants which are environmentally friendly.

In the food industry, refrigeration technologies are applied in all stages from raw materials storage and food processing to distribution, retail and final consumption at consumer's home and from hundreds of years now compression refrigeration cycle is used everywhere.

However, the potential to save energy, resources and significant amounts of money requires more than simply using new technologies.

It requires an entirely new approach to engineering the whole system with which a facility operates, not just the refrigeration system and components themselves. More research and developments are needed to replace the old techniques at industrial level.

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THE MOUNTAIN PRODUCT - THE VISIT CARD OF THE MOUNTAIN AREAS

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Abstract

The paper presents the path of the linear economy to the circular economy for sustainable development and growth. Moving to a circular economy generates opportunities such as: reduced environmental pressures; increased security of the supply chain of raw materials; increased competitiveness; innovation; growth and development of new jobs. In this way, small farmers and producers who use the results of research studies in the production process, in the manufacture of products or the packaging process gain added value, which enables them to be better value on the domestic or foreign markets. The sustainability and resilient development of mountain rural communities can be made by increasing the use of local resources to mitigate/stop village depopulation and local natural heritage degradation requires the identification of existing structural and functional connections between existing resources and their use by local communities. It is thus possible to establish measures for the sustainable use of natural resources, reducing environmental pressures, enhancing the security of raw materials supply; use of biotechnology's advantages; increased competitiveness, increasing the quality of life of the population.

Key words*: bioeconomy, circular economy, mountain product, sustainable development, small farmers, agriculture.*

INTRODUCTION

Providing consumers with high-quality food products, from mountain areas, has always provided a support to small-scale farmers to develop and ensure working conditions and life make them continue their activity in these areas, keep and pass on to future generations the knowledge, customs and traditions of these areas, while complying with food safety and security requirements.

Food products that are produced in the mountain areas, labelled with the phrase "mountain product", guarantees the quality of the raw materials used, thus meeting the requirements of citizens and consumers around the world, increasingly demanding both quality products and traditional products, being also concerned with maintaining the diversity of agricultural production, meeting the demands of consumers, has shown the need to create a legislative framework that allows small farmers to develop and promote their products. This situation generates a demand for food products with certain identifiable characteristics, especially with regard to the geographical origin of the ingredients and their quality (Muscalu et al., 2015).

THE CONCEPT OF CIRCULAR ECONOMY

The circular economy is increasingly becoming a way to resource management, including in the agri-food industry. It is essentially linked to the environment, industrial ecology, technological innovation, re-use, and resource recycling. This has to be addressed in the general context of organizational development, focusing on business and operational management principles, technological innovation promoted by all means, leading to efficient resource management, but also to the role that the state has in creating a framework of regulations to boost the implementation of the circular economy (Muscalu & Mateescu, 2016).

Figure1. Mountain area - Vatra Dornei, Suceava county
Source: Personal archive

Bioeconomy offers alternative solutions to manufacture products other than using fossil fuel or energy and can contribute to the circular economy.

In order to prevent food waste production and to cope with situations that can vary from one country to another and from one region to another, it is essential to take action across the value chain.

In this context, Romania has achieved targets at European and international levels, with regard to: the circular economy, the reduction of food waste (by 50% by 2030) and the reduction of carbon footprint.

The transition to a low-carbon economy is one of the objectives of rural development, the achievement of which contributes to the Europe 2020 strategy for smart, sustainable and favourable growth, focusing on the following:

The efficiency of water use in agriculture;

- the efficiency of energy uses in the agri-food sector;
- the facilitation of the supply and use of renewable energy sources, by-products, waste and residues and other non-food raw materials, in order to achieve bio-economy;
- reducing greenhouse gas and ammonia emissions from agriculture;
- promoting sequestration and carbon sequestration in agriculture and forestry.

In order to cope with global population growth, rapid resource exhaustion, increased environmental pressures and climate change, in line with the EU Bioeconomy Strategy, Europe has to radically change its approach to production, consumption, processing, storage, recycling biological resources (Zaman, 2019).

By the specifics of the activities carried out, the research, development and the innovation in the field of bioeconomics at the national level should develop on the same priority directions, consistent with those enumerated at the community level, namely investing in research, innovation and skills for bio-economy;

The Europe 2020 strategy advocates bio-economy as an essential element for smart, green growth in Europe. Progress in bio-economy research and innovating will enable Europe to improve the management of its own renewable bio-resources and open up new and diversified markets for food and bio-products. The establishment of bio-economy in Europe has special advantages: the ability to maintain and generate growth and jobs in rural and industrial areas to reduce dependence on fossil fuels and to increase the economic and environmental sustainability of primary production and manufacturing (Suciu, 2015).

AGRICULTURE OF THE MOUNTAIN AREA

In Romania, about 30% of the country's territory is classified as a mountain area, amounting to 656 administrative-territorial units (ATUs). The mountain range is recognized for its low pollution level, which gives the food coming from this area more value.

On 05.02.2019, it was published in the Official Gazette, Part I, no. 90, Order of the Ministry of Agriculture and Rural Development no. 49 of 14.01.2019 amending and supplementing the annex to the Order of the Ministry of Agriculture and Rural Development no. 52/2017 approving the procedure for checking the conformity of the data contained in the tender dossier in order to grant the right of use of the "mountain product" option and to verify the compliance of the European and national legislation by the economic operators who have obtained the right of use of that mention (Mountain Product, Retrieved from

www.madr.ro/industrie-alimentara/sisteme-de-calitate-europene-si-indicatii-geografice/produse-agricole-si-alimentare/produs-montan.html).

The mountain farming is the result of the:

- developing markets and competitiveness in the bio-economy sectors through a sustainable increase in primary production through the conversion of waste streams into value-added products and through mutual learning mechanisms to improve production and resource efficiency;
- enhance policy coordination and stakeholder involvement, by setting up a bio-economy group, a bio-economic observer, and by organizing stakeholder conferences on a regular basis.

Figure 2. Logo "mountain product"
Source: National Legislation, Order of the Ministry of Agriculture and Rural Development no. 49/14.01.2019

Products that possess the optional label "mountain product" are marked with a national logo (logo, trademark, symbol). The "mountain product" logo is used exclusively on the labels of products that meet the requirements of Regulation (EU) no. 1151/2012, Delegated Regulation (EU) no. 665/2014 (Regulation (EU) No 1151/2012 of the European Parliament - EUR-Lex, Retrieved from https://eurlex.europa.eu/LexUriServ/LexUriServ.

A number of sectors such as mountain farming face specific challenges in the context of the cyclical economy, given the particularities of their products or their value chains, their environmental footprint or their dependence on materials outside of Europe. A clearly targeted approach is needed in this sector to ensure that interactions between the different phases of the interaction between traditional culture, gastronomy and the livelihoods of peasant farms in the mountainous countryside. It is imperative to develop optimized models on product groups as well as the analysis of economic, social and cultural aspects that contribute to the capitalization through short chains of production, acquisition, storage and marketing of mountain products for reviving and increasing household profitability and of mountain agricultural holdings (Isachi & Chitiga, 2016).

PROMOTING AGRICULTURAL PRODUCTS

Due to the global economic climate, Romanian agri-food products have to cope with strong competitive pressure from imported products, which in some cases do not meet the high standards of European food quality and safety, being delivered at prices below the production costs of Romanian products, thus creating a competitive gap to the detriment of Romanian farmers (Alexandri, 2016).

The marketing of locally grown food, short chains, and local markets should become an important component of the agri-food sector in Romania.

Supporting and encouraging the development of short supply chains is necessary for terms of opening up market opportunities to active farmers by promoting and selling products in the vicinity either individually or jointly. In this context, the marketing of agri-food products can make a significant contribution to the development and relaunch of the agricultural sector, based on a product promotion policy, and consequently on exports tailored to the real needs of the Romanian agricultural sector.

Combined with the promotion of offensive interests on third-country markets, and taking into account the crisis situations that persist in certain sectors of agriculture at national and European level, especially in the dairy, pig meat cycle are fully taken into account throughout the value chain (Gavrilescu et al., 2016).

For this purpose, for better visibility and valorification of the mountain area potential, it is necessary to develop guides for processors regarding the use of modernized traditional

technologies to ensure the reproducibility and food safety of the mountain products made.
Action plans for local authorities to create local fairs/markets for consumer information and awareness on the quality and safety of Romanian agro-food products, and last but not least, awareness-raising campaigns to change consumer behaviour.

Figure 3. Promoting agri-food products from the mountain area to the Green Week in Berlin, 2019
Source: Personal archive

Thus, the emphasis will be on increasing the presence of Romanian products and improving/ strengthening the position on the markets of interest at European and National level.
Also, the implementation of trade and cooperation agreements with third countries will create a competitive advantage for the agri-food sector in Romania by obtaining tariff concessions on imports in these countries, thus favouring the growth of agricultural exports and high value-added exports with impact positive and fruit and vegetables sectors, Romania must maintain a high degree of protection for imports of agri-food products, mainly for the following products: beef, poultry and pig meat, sunflower and rapeseed, cereals (wheat, corn and barley), grain and oil soybeans, sugar, tobacco and cigarettes, in order to protect the domestic production and the competitiveness of Romanian farmers on the European and international market.
Due to the global economic climate, Romanian agri-food products have to cope with strong competitive pressure from imported products, which in some cases do not meet the high standards of European food quality and safety, being delivered at prices below the production costs of Romanian products, thus creating a competitive gap to the detriment of Romanian farmers. (Rosu, 2016).
In this context, Romania needs to register as many agri-food products as possible on European and National quality schemes, in order to capitalize on the potential of our country.

Figure 4. Romanian food products
Source: Personal archive

CONCLUSIONS

Romanian agriculture must reach a level of development comparable to that of other European Member States that already have modern agriculture, steps must be taken to achieve sustainable growth. These stages depend on economic growth, creating new jobs and improving competitiveness at international level.
Romania needs to focus on manufacturing high value-added products that meet environmental, animal health and animal welfare and quality requirements in line with European standards.
In addition to national quality legislation, Romania implements European legislation on quality systems. These systems allow consumers to identify products that have specific qualities due to their origin and/or production methods.
In order to make consumers trust the legitimate nature of label labels, compliance with the

product specification is monitored by public authorities or a private certification body.
Regulation (EU) No. 1151/2012 of the European Parliament and of the Council of 21 November 2012 on quality systems for agricultural and food products and Regulation (EU) No. 1169/2011 of the European Parliament and of the Council of 25 October 2011, on information to consumers on foodstuffs, amending Council Regulation (EC) 1924/2006 and (EC) No. 1925/2006 of the European Parliament and of the Council and repealing Commission Directive 87/250/EC, Directive 90/496/EC Council Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council the European Parliament and the Council, Commission Directives 2002/67/EC and 2008/5/EC; Council Regulation (EC) No 608/2004 of the Commission.

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REVIEW ON LEGAL, SOCIAL AND ECONOMIC ASPECTS OF THE NEW BREEDING TECHNIQUES

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Abstract

The paper aimed to present a review on the social and economic aspects of NBTs, the studies on two different species of plants, subjects of NBT's. The plants variants generated by NBTs are more readily accepted in the market and for crop improvement. In this article we will present briefly the benefits, application and expected developments, regulatory status of NBTs in and outside the EU. It was developed a system for the detection of a broad spectrum of GMOs for analysis of food/feed matrices by the characterization of transgene flanking regions and the typical combinations for transgene constructs. We will describe two different species of plants, subjects of NBTs: 1) Tomatoes for carotenoid sequestration mechanisms and the carotenoid biosynthesis. The carotenoid accumulation and changes in carotenoid profiles suggest that the plastid can adapt to changes in carotenoid content through plastid differentiation and preferential sequestration; 2) Edited maize genome by biolistic delivery of pre-assembled Cas9-gRNA ribonucleoproteins into maize embryo cells and regeneration of plants with both mutated and edited alleles. As a conclusion, CRISPR/Cas9 is the most used technology for genome editing due to its simplicity and efficiency. In this article, we aim to highlight the application of CRISPR/Cas9 technique system, like the powerful genome editing tool for crop improvement.

Key words*: CRISPR/Cas9, genome editing, crop improvement, NBTs.*

INTRODUCTION

In the next 37 years, by 2050, the world population is estimated to grow by about 200,000 net people per day to a total of 9.6 billion people (Populaire Reference Bureau, 2012).

Among the benefits of genetic engineering in agriculture are increasing crop yields, reducing costs for food or feed production, reducing the need for pesticides, increasing nutrient composition and food quality, resistance to pests and diseases, and the benefits of food intake for the growing population world.

In support of these goals, agricultural and food scientists have developed new plant breeding techniques (NBT) in recent years, including CRISPR/ Cas9, the simplest and most effective technique for crop improvement, known as genome editing. This is a technique that allows the plant genome to be precisely modified by removing unwanted genes or byspecifying which genes can get new functions (Wolt et al., 2016). The genome-generated varieties are similar to the naturally occurring variations. It is less time-consuming and easier to accept on the market.

MATERIALS AND METHODS

The research methodology used in this paper has the following main aspects:

- bibliographic study of the national and international literature;
- collecting the concrete information within the researched area;
- ordering, processing and presenting of results in synthetic form;
- analysis and interpretation of results, formulation of conclusions and recommenddations.

RESULTS AND DISCUSSIONS

The site-specific nucleoside-based genomic editing system can be classified into three categories: the zinc nucleus finger (ZFN), the transcriptional effector nucleases (TALEN), and the intermediate short-acting palindromic groups that have been associated with the

binding protein of Cas9 on specific DNA sequences (CRISPR/Cas9). The main differences between categories consist in their mechanism of inducing the double-catenary break and their efficiency in targeting the desired sequences.

In plants, the administration of Cas9-RNA complexes (Figure 1.) has been demonstrated through protoplasts of several species. Protoplasts are "bare" cells generated by the enzymatic removal of cell walls and have a unique property of cell wall reforming and regeneration in plants. Protoplasts have been successfully used to edit the genome in a variety of plants such as tobacco, salad, rice and some flowers.

For most monocotyledonous species such as corn, wheat, rice, barley and sorghum, plant regeneration in protoplasts is less effective.

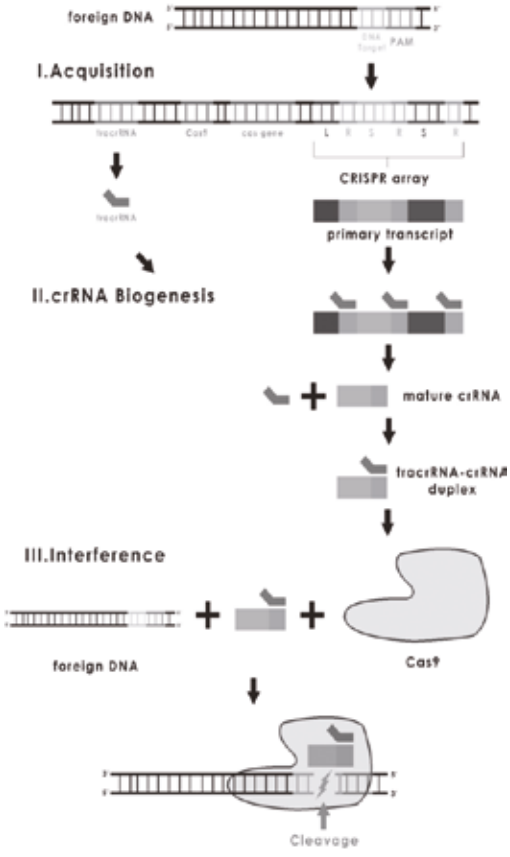

Figure 1. The CRISPR/Cas9 system involves three steps - acquisition, crRNA biogenesis and interference at DNA target cleavage
Source: www.intechopen.com

RNA-guided *Streptococcus pyogenes* Cas9 endonuclease was successfully used to modify the genome in several plant species. In most of the experiments, guideline RNA (gARN) as well as selectable marker genes and Cas9 were delivered to plant cells using either T DNA (*Agrobacterium tumefaciens* infection) or plasmid DNA (particle bombardment).

The supplied DNA integrates relatively easily into the target genome, but can lead to various side effects, such as gene disruption, plant mosaicism, and potential off-site cutting.

To alleviate potential negative effects, pre-integrated Cas9 nucleosus plants were generated and used to administer RNAs in the form of RNA molecules.

This approach requires time and resources for the development and characterization of pre-integrated lines (Abdallah et al., 2015).

New genome editing techniques may be accompanied by some unintended effects (cellular damage if repair mechanisms are imprecise, cleavage and mutation to similar unwanted genomic sites, mutations outside the target to genomic edited plants).

The genomic editing techniques, in general, show a much smaller number or a lack of unintentional mutations compared to organisms obtained by conventional reproductive techniques (Table 1.).

The absence of unintentional, potentially harmful effects can be verified with the whole genome sequencing (SAM, 2017).

The main modes of action in genome editing, as seen in Table 1, were:

- gene disturbance,
- genetic regulation,
- gene insertion.

Among the main attributes and expressed functions of the modified genes were:

- biosynthesis of important nutritional and health substances, as well carotene, inositol, tartaric acid, phytic acid, acetolactate, gibberellin,
- growth regulators,
- RNA regulation of biogenesis,
- initiating factor of translation,
- luorescence,
- cereal dormancy regulator,
- growth of root hair factors,
- auxin response factor.

Table 1. List of plants based on genome modification via CRISPR Cas9 system
Source: www.intechopen.com

Species name	Target gene	Gene function	Description	Action mode
Arabidopsis thaliana	BR11, JAZ1, GAI	growth regulators	displayed retarded growth	Gene disturbance
Brassica oleracea	BolC.GA 4.a	biosynthesis of gibberellin	displayed dwarf phenotype	Gene disturbance
Citrus sinensis	CsPDS	carotenoid biosynthesis	displayed albinism expression	Genetic regulation
Cucumis sativus	eIF4E	Initiating factor translation	developed resistance toward a broad range of virus	Gene disturbance
Glycine max	Bar, GmFE11 GmFE12	Growth of root hair factors	displayed higher root hair growth induction	Genetic regulation
Hordeum vulgare	HvPM19	Cereal dormancy regulator	displayed signs of dormancy	Gene disturbance
Marchantia polymorpha	ARF1	Auxin response factor	showed no response toward auxins	Gene disturbance
Medicago truncatula	GUS	Fluorescence	displayed no signs of staining	Gene disturbance
Nicotiana benthamiana	NbPDS	Carotenoid biosynthesis	displayed albinism expression	Gene insertion
Nicotiana tabacum	NtPDS	Carotenoid biosynthesis	displayed albinism expression	Gene disturbance
Oryza sativa	OsPDS, OsMPK2 OsBAD2	Carotenoid biosynthesis, growth regulator	displayed albinism and dwarfism	Gene disturbance
Petunia hybrid	PDS	Carotenoid biosynthesis	displayed albinism expression	Gene disturbance
Populus tomentosa	PtoPDS	Carotenoid biosynthesis	displayed albinism expression	Gene disturbance
Solanum lycopersicum	SlAGO7	Involved in the RNA regulation of biogenesis	displayed needle-like or lacking lamina leaves	Gene disturbance
Solanum tuberosum	StALS1	Acetolactate biosynthesis	showed increased resistance on herbicides	Gene insertion
Sorghum bicolor	DsRED2	Fluorescence	showed signs of red fluorescence	Gene insertion
Triticum aestivum	TaINOX, TaPDS	Inositol and carotenoid biosynthesis	displayed albinism expression	Gene disturbance
Vitis vinifera	IdnDH	Tartaric biosynthesis	showed no signs of tartaric acid in their fruits	Gene disturbance
Zea mays	ZmIPK	Phytic acid biosynthetic pathway catalyst	showed reduction of phytic acid level	Gene disturbance

Global acceptance of plant biotechnology

The genome editing with modified nucleases has evolved as a highly specific and efficient tool for crop improvement, with the potential to quickly generate new phenotypes.

Figure 2. Global scheme of biotechnology acceptance
Source: www.nbtplatform.org

It arise the question how genetically modified plants with the desired characteristics will be received by the public and regulated under GMO legislation.

According to a recent study comparing the views of researchers and citizens on a range of scientific, engineering and technology issues (Funk and Lee, 2015), 37% of the general public responded that genetically modified foods are safe for consumption and 88% of scientists interviewed recognize genetically modified foods as safe (Wolt et al., 2016).

With all the studies done so far, it is undeniable that the CRISPR Cas9 system is about to change the pace and course of the agricultural industry.

Judgment of the European Court of Justice (C528/16ECJ)

As stated in the European Law, the definition of GMO means an organism except human beings where the genetic material has been altered in a way that does not occur naturally by mating and / or natural recombination.

The European Commission has stressed that the decision to include or exclude a technique within the scope of Directives 2001/18 and 2009/41 EC depends only on the interpretation of the definition of genetically modified organisms and genetically modified micro-organisms and the conditions for exemption laid down in the two directives (Laaninen, 2016).

There are regulatory authorities such as the German Consumers Protection Association or known as the Verbraucherzentrale Bundesverband (VZBV) and Swedish scientists

who call for the exclusion of such a "genetic modification" from the GMO Regulation if such crops do not contain any "foreign DNA" (Ammann, 2016).
On 25 July 2018, the European Court of Justice delivered its judgment in Case C-528/16, holding that organisms obtained by mutagenesis must be considered as GMOs, binding on national courts. The judgment of the above-mentioned European Court of Justice says that organisms created by new gene editing techniques (such as CRISPR Cas9 and related methods) are GMOs - Directive 2001/18/EC. The Directive requires organisms produced by genome editing to be detected as such by testing laboratories. EURL-GMFF has developped a draft report in this area that is not public yet but discusses detection issues and suggests potential ways to detect these products.

Examples of legal status of new breeding techniques outside the EU
Argentina is one of the first countries in the world to establish a regulatory framework and to underline the legal heterogeneity that characterizes cultures derived from New Breeding Techniques in Resolution 173/2015. It establishes a case-by-case assessment if a product falls under the category of a GMO or not.
Brazil, in line with the new regulatory resolution 16 (NR 16) published on 16 January 2018, the Brazilian National Biosafety Technical Commission may exempt new products from the same assessment of the GMO regulation, which has an annex to the BNT procedures that can create a product that is not considered GMO.
The United States of America, through the United States Department of Agriculture (USDA), has confirmed that it does not intend to impose new or additional regulations on crops developed by new breeding techniques, such as gene editing. The agency says new breeding methods can introduce new plant features faster and more accurately, saving years or bringing farmers new varieties.

The position of other world stakeholders interested in new plant breeding techniques
Jan Plagge, President of IFOAM in the EU: "The European Court of Justice's confirmation that the new GMOs will be traceable and labelled is good news for organic farmers, farmers and processors, but also for all European producers and consumers, the freedom to avoid such genetically modified products and the protection of the environment against the potential risks of these new technologies."(IFOAM EU Press Release: New genetic engineering techniques will be regulated as EU-EU GMOs welcomes the ECJ decision, 2018).
Franziska Achterberg, EU Greenpeace Director for Food Policy: "The Court clarifies that plants and animals derived from genetic publishing are subject to the same safety and labelling requirements as other genetically modified organisms. These requirements exist to prevent harm and to inform consumers the release of these new GMOs into the environment without adequate safety measures is illegal and irresponsible, especially given that gene editing can lead to unintended side effects. The European Commission and European Governments need to ensure that all new GMOs are fully tested and labelled and that all field trials are brought into line with GMO standards.", she said. (EURACTIV: Industry shocked by the European Court's decision to put the genetically engineered technique in place with GM law, 2018).
USDA Secretary Perdue Statement on the ECJ judging genomic publishing: "We encourage the European Union to seek the contribution of the scientific and agricultural communities and its trading partners to determine the proper implementation of the decision of innovations in biotechnology, such as genome editing, include their benefits include healthier, high-quality foods at affordable prices. For farmers, they include improvements in productivity, plant and animal health and environmental sustainability."(USDA Press, Release No. 0155.18, 2018).

Socioeconomic aspects of new breeding techniques in plants
Based on the position expressed by various stakeholders, such as farmers' associations, researchers and plant breeders, the ECJ's decision is expected to have a profound impact on European agriculture, research and trade.

Benefits for plant growth:
• increased yield,
• reducing the use of natural resources,
• reducing dependence on chemical protection
• contribution to biodiversity,
• adapting to changing conditions.

Benefits for farmers
• Increased resistance to biotic and abiotic stress factors (reduced pesticide treatment).
• Reducing the impact on the environment.
• Improving land and investment efficiency.
• Improving crop efficiency and product diversity.
• Rapid adaptation to climate change.
• Longer preservation time, so less food waste.
• Development of the plant breeding sector (selection of edible species from wild populations, selection of plant species with desired characteristics) (Figure 3).

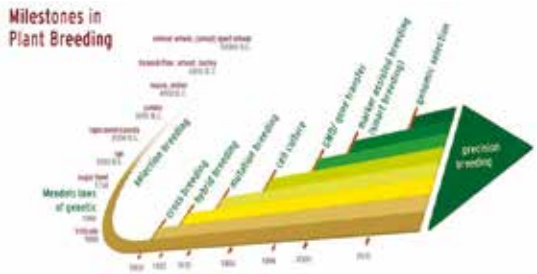

Figure 3. Highlights for plant breeding
Source: www.nbtplatform.org

Problem of detected genome edited
Modifications of the DNA sequence introduced by genomic editing methods are not presently identified, as compared to DNA sequence changes produced by natural processes or conventional mutagenesis. The exception is when genome editing is used to introduce more than two base pair changes into DNA at a single location, these being less likely to be natural or mutagenic.
If there is no information about the changes introduced (no known target), it is difficult to detect the changes. Detection could only be possible with a suitable reference genome for comparison (Lusser et al., 2011).

Ways suggested to detect the genome edited
• If a body with the genome edited has undergone a documentation process (example an authorization) detailing the modified DNA sequence of the gene region being edited and providing complete details of the organism itself (example variety), it would be possible To identify if an unknown sample corresponds to the edited variety. This clearly shows that the unknown sample originates from a gene with an edited genome, but it is not a direct proof that the sample was from an edited genome.
• If the suspect sample originates from a cultivated plant, the sample genome could be compared to a genomic reference database of non-genomic varieties of that plant to identify DNA sequence differences. Differences would be modifications by genome editing. This is a poor assumption because new mutations could have occurred (naturally or by mutagenesis) in any variety in each generation. The proposal to set up a database for genomic comparisons would be a very large economic effort. There are 14,442 varieties of wheat bread, Durham wheat, maize, soy beans, barley, rape, rape and potatoes registered in the EU, as shown by the European Commission's plant varieties database.
From Wikipedia, there are 7500 apple varieties and 10,000 varieties of tomatoes, without their varieties. This very costly way may be impossible to put into practice and would provide relative proofs.

Editing corn genome through ribonucleo-protein complexes
The biolistic introduction of Cas9-gARN ribonucleoproteins pre-assembled as ribonu-cleoprotein complexes into maize embryo cells by particle bombardment and plant regene-ration with mutant alleles and edited was successfully performed, using this method, DNA-tagged DNA mutagenesis is also obtained in maize (Zhang et al., 2016).
Four genomic regions, liguleless1 (LIG), acetolactate synthase (ALS2), and two male fertility genes (MS26 and MS45) were targeted by pre-assembled Cas9 protein pre-assembled with in vitro transcribed gRNAs. Cas9-gRNA complexes were delivered to corn embryonic cells on gold particles (0.6 μm) using a helium gene gun. Total genomic DNA was extracted and fragments surrounding the target sequences were amplified by PCR and analysed by

sequencing (Table 2). Mutations were found at all target sites where the Cas9-gARN complexes were provided (Svitashev et al., 2016).

Table 2. Mutations in corn embryonic cells
Source: www.ncbi.nlm.nih.gov

Target site	Target site sequence with PAM	Cas9 (%)	DNA (%)	Cas9 g ARN (%)
LIG	GCGTACGCGTACGTGTGAGG	0.004	0.56	0.57
ALS2	GCTGCTCGATTCCGTCCCCATGG	0.020	0.51	0.45
MS26	GCACGTACGTCACCATCCCGCCGG	0.004	0.43	0.21
MS45	GGCCGAGGTCGACTACCGGCCGG	0.002	0.34	0.69

To measure the frequency of plant-level mutations, 60 bombarded embryos were placed on growth medium and 36 segments of herbicide-resistant callus segments, which were tested for mutations, were regenerated. Of the 36 events, 17 (47%) contained mutant alleles and 19 (53%) had only wild type alleles. The ability to provide Cas9-gARN complexes on gold particles in corn cells combined with the high frequency of mutant plant recovery without selection makes this approach practical for genome editing in cultured species. The results obtained on maize provide new opportunities for advancement of agricultural reproductive practices for any species of plants subject to biolytic delivery (Svitashev et al., 2016).

Differential accumulation of carotene in tomatoes by chromoplasts

Carotenoids are high-value compounds for the food industry. The global market for these substances will grow to $ 1.95 billion by 2025, based on an annual growth rate of 5.1% (Accuray Research LLP, 2017).

Tomato (*Solanum lycopersicum*) is the plant model for carotenoid-related studies because the fruits contain high levels. The study of a tomato line specifically designed for a higher capacity of carotenoid accumulation via the PSY1 gene (psy1 - the carotenoid biosynthesis enzyme) has led to the observation of chloroplast differentiation in chromoplasts in immature fruits.

The PSYsense overexpression line has a phenotype in which the associated carotenes accumulate at the onset of fruit growth, resulting in a pink-orange colour of the mature green fruit (Fraser et al., 2007).

Because of their unique characteristics, carotenoids can function as modulators of membrane structures, a hypothesis that has been tested in bilateral lipid models mixed with different carotenoids *in vitro*.

The way in which carotenoids are captured (Figure 4) in the membrane depends on the trans or cis configuration and leads to different ways of membrane integration (Widomska et al., 2009).

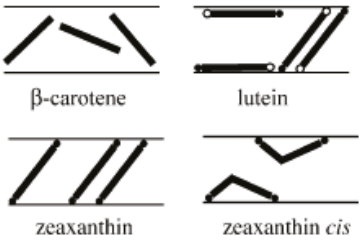

Figure 4. Carotenoid lipid interactions
Source: www.pure.royalholloway.ac.uk

DNA for the coding sequences of the genes of interest was obtained from *Escherichia coli* DH5a genomic DNA samples and *Rhesus capsicum annuum* (sweet pepper) RNA samples for plastid extraction (Cheng and Jiang, 2006).

An optimized vector used for overexpression of bacterial carotenoid genes crtZ and crtW was used as a source for promoter and terminator parts of the constructs created (Misawa and Shimada, 1998).

Qualitative confirmation for expression of the coding sequences was performed by PCR method.

The selected lines were mutant lines disrupted in the overexpressed carotenoid biosynthesis of the psy1, crt-iso, LCY-b and crt1 genes (Ailsa Craig). Sub-chromoplastic fractions show the accumulation of specific carotenoids, which may have changes at the grip sites. An important modifier of the grip preference is the "cis" or "trans" (Widomska et al., 2009).

These structural changes specifically modify the membrane integration of carotenoids (Gruszecki and Strzalka, 2005).

The significantly higher capacity of chromoplast accumulation allows them to function as a storage reservoir for carotenoids compared to chloroplasts (reviewed by Egea et al., 2010).

The increase in carotenoid content occurs simultaneously with chloroplast differentiation

in chromoplast, because the chromoplastic structures are found in the immature fruits of the PSY-1 sense line (Fraser et al., 2007).

Figure 5. Different accumulation of carotenoids
Source: www.pure.royalholloway.ac.uk

Early activation of the PSY1 enzyme results in the accumulation of carotenoids and the differentiation of chloroplasts in chromoplasts in immature fruits that have the green crown, but the tissues are orange pink.

Was observed two-fold increase in total carotenoid content (lycopene and β-carotene) for the PSY-1 sense line compared to control. This growth is explained by the occurrence of phytotecine (6.4 μg), phytophluene (4.3 μg), z-carotene (4.7 μg) and lycopene (2.0 μg) and lutein growth (1.5 times) carotene and xanthophils (1.5 times). (Nogueira et al., 2013).

Over-expression of psy1 in the PSY-1 sense line gives a similar response to immature fruits. Chromoplastic differentiation in tomato fruits occurs at the onset of maturation and regulates carotenoid biosynthesis at transcriptional level (Llorente et al., 2016; Toledo-Ortiz et al., 2014).

CONCLUSIONS

There are still many uncertainties about the use of plant genome editing. Therefore, in-depth studies are needed to ensure that these new plant breeding technologies will have zero risks, while maximizing benefits. The idea of editing the genome could also raise ethical questions from the public, and they should be approached appropriately by scientists who are well-trained in genome engineering. Educational discussions or workshops on genomic editing should also be offered to non-scientists to ensure that the benefits of this technology are well understood by consumers, the true beneficiaries of research and innovation in the agro-food industry.

More regulation will be needed to apply new plant breeding techniques to ensure that they are used responsibly without slowing down development and research. Generally, new mutagenesis techniques are faster and cheaper than conventional reproductive techniques. There are already several plants generated with the new mutagenesis techniques that are near or in the phase of field testing or marketing.

By editing the genome, mutations can be targeted. Depending on the technique, non-specific mutations (allele insertion) or specific mutations (oligonucleotide genetic mutation, targeted insertion) can be rapidly introduced into a desired gene, creating a desired donor allele. In polyploid plant species (wheat), all homologous copies of a gene may be targeted at the same time, so that traits that may be obtained with great difficulty through tradetional mutagenesis can be obtained.

Mutagenesis would allow farmers to combat the pests of plants resistance of pesticides, increase yields and improve the nutritional content of crops, and allow Europe to remain competitive with other major agricultural powers, such as the US and Brazil.

The results obtained in plants such as corn and tomatoes open new opportunities to develop new reproductive practices in a wide variety of cultured species. The ability to provide Cas9-gARN complexes on gold particles in corn cells combined with the high frequency of mutant plant recovery without selection makes this approach practical for genome editing in cultured species. Tomato mutant lines appear due to overactive or inactive key steps in carotenoid synthesis, and the significantly higher capacity of chromoplast accumulation allows them to function as a reservoir for carotenoid storage.

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INFLUENCE OF LACTATION ON COMPOSITION OF MARE'S MILK

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Abstract

Mare's milk is valuable because of its nutritional properties. It is used in human food and tartamente for various diseases. Mare milk changes its composition depending on the lactation. It has the lowest values in first and second lactation in lactating 3, the highest values. The fat content varies between 1.88 g/100 g (lactation 1) and 2.17 g/100 g (lactation 3). Milk protein varies between 1.74 g/100g (lactation 1) and 1.92 g/100 g (lactation 3). For colostrum, affecting all day postpartum physico-chemical parameters. The highest values for these parameters are within 3 days postpartum. On day 5, the low values are observed. The fat content of the colostrum varies between 2.85 g/100 g, on day 1, and 2.13 g/100 g, on day 5.

Key words*: mare milk, colostrum, fat, protein, lactose.*

INTRODUCTION

Mare's milk is used in food, cosmetics and medical. It is used for various diseases (tuberculosis, liver disease, gastritis, allergies) (Doreau et al., 1989; Salhanov et al., 1979; Malacarne et al., 2002). Mare's milk composition (high content of protein and globulin, essential amino acids, fatty acids) it is advisable to cancer patients, people with low immunity, heart problems, those suffering from atherosclerosis (Kharitonova et al., 1978, Mao et al., 2009; Chen et al., 2010; Jirillo et al., 2010). Studies on the milk and colostrum from the mare are: the influence of lactation on the chemical composition of mare's milk; influence on nutrient colostrum postpartum day; mare's milk benefits; caseins in milk distribution; mare's milk protein (Wells et al., 2012; Salamon et al., 2009; Mateja et al., 2014; Solaroli et al., 1993; Malacarne et al., 2000; Klemen et al., 2011). Quality, composition and production of mare milk are influenced by: feed, race, maintenance conditions, the process of milking, milk preservation and thermal processes applied (Doreau et al., 1989; Uniacke-Lowe et al., 2010; Orlandi et al., 2003; Markiewicz-Keszycka et al., 2015; Cosentino et al., 2015; Caroprese et al., 2007). Aim of the study was to evaluate the influence of lactation and postpartum day on milk and colostrum from the mare.

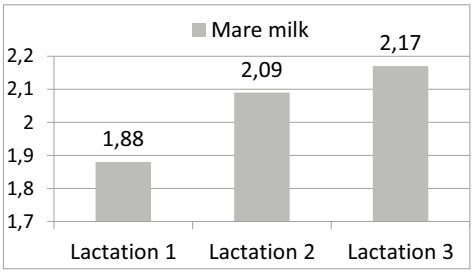
MATERIALS AND METHODS

Sampling. Milk was collected from a total of 5 copies per lactation. And samples were collected according to the postpartum period (day 1, 3, 5). Were studied animals breed Semigreu Românesc. It was chosen race, Semigreu Românesc because heavy breeds have a higher milk production. Sampling was performed manually in sterile containers and stored in the cold until analysis.

Physico-chemical analysis. Analysis of physico-chemical parameters of milk and colostrum from the mare were analyzed with the device Lactoscan S (Figures 1 and 2).

Physico-chemical parameters are analyzed milk: fat, protein, lactose, and pH. Colostrum we have examined: fat, protein, lactose, dry matter and pH.

RESULTS AND DISCUSSIONS



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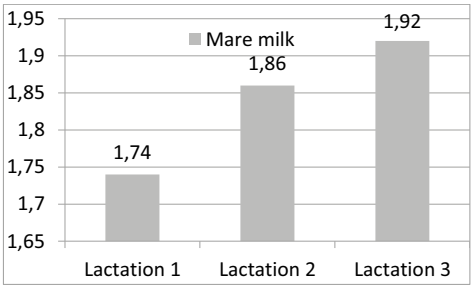

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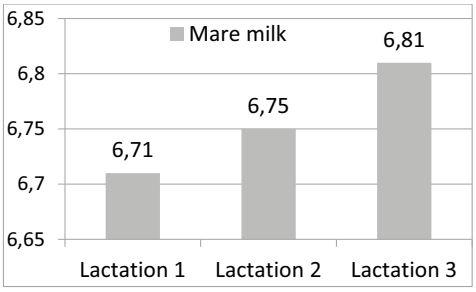

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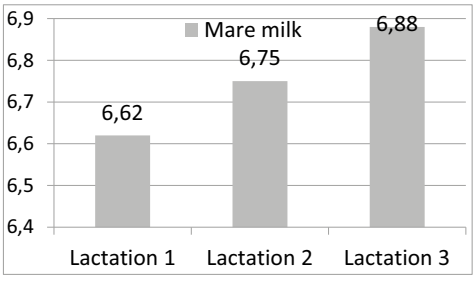
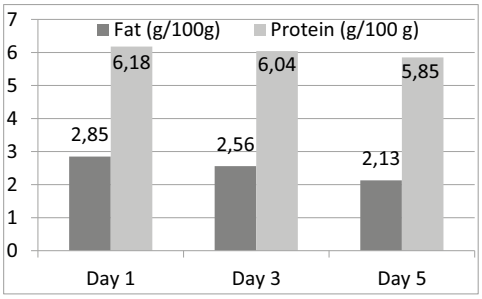


d

Figure 1 (a-d). Physico-chemical composition of the mare milk on three lactations: a- fat; b-protein; c-lactose; d-ph



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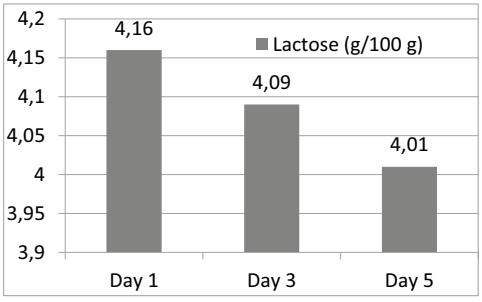

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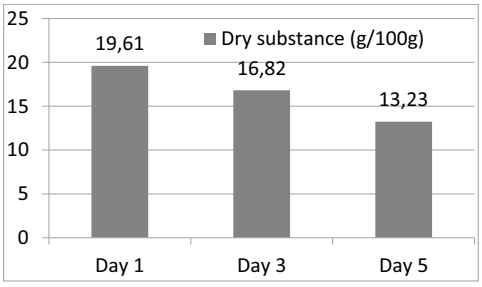

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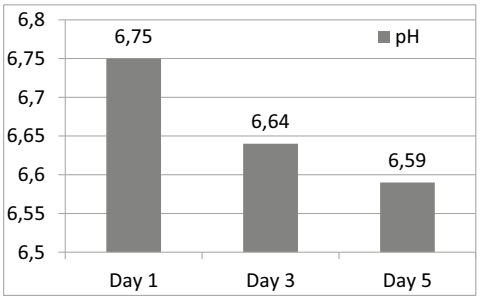

d

Figure 2 (a-d). Physico-chemical parameters of colostrum

Figure 2 (a-d) are given values for physico-chemical parameters of influence colostrum postpartum day (day 1, 3 and 5).

Mare's milk

The amount of fat varies depending on the lactation stage of lactation and in increasing quantities in colostrum (Gibbs et al., 1982). The amount of the higher fat is 2.17 g/100 g, in 3 lactation. Diet influences the composition of the milk, a diet rich in fiber such oil and causes a higher fat content (Hoffman et al., 1998). The amount of protein is influenced by the properties of the month lactation and changes from one month to another (Gibbs et al., 1982). Similar values are reported for the protein of (Gibbs et al., 1982). Aspects of physicochemical parameters change depending on the time period of lactation and colostrum are reported (Ciesla et al., 2009; Oftedal et al., 1983; Pagliarini et al., 1993). Similar values for physico-chemical composition of mare's milk are reported Leaflet et al., 2012. Lactose in mare's milk varies between 6.71 g/100 g, (lactation 1) and 6.81 g/100g (lactation 3) (Figure 1, c).

Colostrum

The fat content is influenced by the postpartum day (Figure 2a). The fat content of the colostrum is higher than milk. Similar appearance was also reported (Pikul et al., 2008). Salamon et al., 2009 reports the influence of the race on the chemical composition of colostrum according to the postpartum day. Values similar to those obtained for the colostrum are also reported by Salamon et al. (2009). The colostrum phase significantly influences the protein content in the early postpartum days (Ullrey et al., 1996). Lactose shows higher values in the early postpartum days and decreases towards the end of the colostrum (4.16 g/100g day 1 and 4.01 g/100g day 5). The dry substance in colostrum is 19.61 (g/100 g) on day 1, postpartum and 13.23 (g/100 g), 5th day postpartum.

CONCLUSIONS

The physico-chemical parameters of colostrum show the highest values in the early postpartum days, and decrease towards the end of the colostrum. The physicochemical parameters of milk change its properties according to lactation.

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THE NUTRITIONAL AND LIFESTYLE HABITS OF THE STUDENTS' POPULATION AT THE UNIVERSITY OF BIHAĆ AND PRESENTS THE RISK FACTORS FOR COLORECTAL CANCER

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Abstract

Colorectal cancer is the second leading cause of death due to malignant diseases in developed countries, and the fourth in the world, despite the fact that this disease can be cured by surgery if the diagnosis is set at an earlier stage. Risk factors include diet, lifestyle, habits and genetic factors. People with the highest risk are those who eat low-fiber foods and many proteins, fat and carbohydrates. People with Chron's disease also fall into a risk group. The aim of this research was to determine the nutritional and lifestyle habits of the students' population and on the basis of the obtained results to determine whether there are risk factors for colorectal cancer in the same population. Our results are showing the presence of a large number of risk factors associated with colon cancer. These are a high prevalence of increased body mass (12%) and obesity (9%), high smoking prevalence (42%), low physical activity level (54% inactive), alcoholic beverages consumption (53% once or more weekly, 3% daily). Among nutritional habits of the studied population of students has noticed a high intake of meat and meat products, and a low intake of fish and fruits and vegetables.

Key words*: colorectal cancer, risk factors, students, nutritional habits.*

INTRODUCTION

Cancer is one of the leading cause of death over the world, and colorectal cancer there is in the third place of the malignant diseases in the world in the men and women. The incidence of cancer increases with the age of the population, starts at the age of 40th, significantly grows in 50th, and later grows with geometric progression (Marković Bergman, 2015).

According to registered data of the Institute for the public health of the Una-Sana Canton, in the period of 2012-2016, the number of incidences of malignant diseases colorectal cancer were 93 suffering from this illness, 53 men and 43 women (data of the Institute for the public health of the Una-Sana Canton, 115/18).

The risk factors associated with etiology to colorectal cancer can be divided into two categories.

The first is the risk factors which can be controlled, and they are in the relationship with nutritional and lifestyle habits, and the second is the immutable factors such as age and family anamnesis (Banjari & Fako, 2014). Though the risk factors can influence on the development of cancer, most of them don't directly influence the development of cancer. It is very important to note that the nutritional and lifestyle habits overcome the genetic predisposition of the person for the development of colorectal cancer (Johnson et al., 2013).

According to data of the World Cancer Research Fund International (2007), almost one-third adult in the whole world isn't enough physically active. The physical activity protects the human body of the development of colorectal cancer.

Some studies showed that smokers have increased the risk for the mortality causes with colorectal cancer opposite nonsmokers (Leufenks et al., 2011).

There are the shreds of evidence which showed that the consummation of the alcoholic beverages increased the risk of the following types of carcinoma: intestine (colorectal),

breast, mouth, pharynx and larynx, esophagus (carcinoma of the laminar cells), liver and stomach.
The persons who consume in average 2 to 4 alcoholic beverages per day, they have 23% more highest risk of colorectal cancer, compared to those who consume less of one alcoholic beverage per day (ASC, 2011).
The students have a bad nutritional habits, because of different obligations, they have the tendency of the skipping some meals, less choice of the food, more often they consume unhealthy food for the snack, and they consume the inadequate meal in terms of nutrition, what can negatively affect on the mental activity and possibility of the learning.
With bad nutritional habits, there are bad lifestyle habits, such as decreasing of the physical activity, the consummation of alcoholic beverages and smoking (Banjari et al., 2011.)
The aim of this work was the establish the nutritional and lifestyle habits of the students' population at the University of Bihać and on the basis of the obtained results, determine which the risk factors there are present for the appearance of colorectal cancer.

MATERIALS AND METHODS

The examination was carried among the students at the University of Bihać during October - December 2018 year.
The experiment included 100 respondents, 46 men, and 54 women. For the purpose of this experiment, it was developed an anonymous questionnaire which consisted of four parts. In the first part of the questionnaire, there were the general characteristics of the students population (age and sex), in the second part, there were the questions about life habits of the students (consumption of water during a day, consumption of alcoholic beverages, smoking, and physical activities), the third part was established on the family anamnesis, and in the fourth part was the nutritional habits of students (for example, the number of meals, the frequency of some food consumption).
The participation in this examination was on a voluntary basis, and before the respondent filled in the questionnaire, he or she was informed about the purpose of this examination, and they got the instructions for the filling of the questionnaire. Anthropometric data about body mass and height filled the respondents ourselves.
From these data, it was calculating the body mass index (BMI), and regarding obtained results, all respondents were categorized in the four groups following the world standards.
The body mass index was calculated that the body mass of the respondent (kg) divided with the square of the height (m) (Grujić, 2002).
The given results were analyzed by applying the corresponding mathematical-statistical methods, and it was assessed the significance of the obtained results.
The level of significance $p<0.05$ was used for all comparisons and for the discussion of the obtained results. For comparing the data inside and outside the groups used the Fisher's test. The differences between two independent groups were tested with nonparametric Mann-Whitney U test.

RESULTS AND DISCUSSIONS

The average age of the respondents (Table 1) was 21 years for the women and 22.09 for the men. The range for the men was from 18 to 32 years, and the range for the women was from 18 to 27 years.

Table 1. The average age of the students

Student	n	%	Average ± SD	Range (min.-max.)	p
Male	46	46	22.09 ± 3.02	18-32	0.06015
Female	54	54	21.00 ± 2.33	18-27	

p - Mann-Whitney U test

In Table 2 there are the results of the respondent's body mass index, and in Table 3 there is the distribution of the respondents by categories of the nutritional status in relation to body mass index.

Table 2. Body mass index

Student	n	%	Average ± SD	Range (min-max)	p
Male	46	46	25.15 ± 3.77	17.3-38.7	5.276×10^{-5}**
Female	54	54	22.33 ± 2.64	17.3-28.9	

Mann-Whitney U test, ** statistical significance p = 0.01

Table 3. Categories of respondents according to body mass index

Category		n		%		%	p
Malnutrition		M	F	M	F	M+F	
M	F						
19.15 ± 1.22	18.15 ± 0.58	6	4	13.0	7.4	10	0.1066
Min. = 17.3	Min.= 17.3						
Max.= 20.5	Max.= 18.6						
Normal body mass		n		%		%	
M	F	M	F	M	F	M+F	
24.22±1.56	22.1±1.84	24	45	52.2	83.3	69	7.171×10^{-5}**
Min. = 20.8	Min.= 19.1						
Max.= 26.3	Max.= 25.7						
Increased body mass		n		%		%	0.1611
M	F	M	F	M	F	M+F	
27.34±0.41	27.0±0.36	9	3	19.5	5.5	12	
Min. = 26.2	Min.= 26.6						
Max.= 27.8	Max.= 27.3						
Obesity of 1. degree		n		%		%	0.4642
M	F	M	F	M	F	M+F	
30.66 ± 3.69	28.6 ± 0.42	7	2	13.0	3.7	9	
Min = 27.9	Min= 28.3						
Max= 38.7	Max= 28.9						
Obesity of 2. degree		n		%		%	
M	F	M	F	M	F	M+F	
38.7	0	1	0	2.17	0	1	

n - number of respondents, M- male, F - female, Mann-Whitney U test
**statistical significance p = 0.01

Table 4. Life habits of respondents

Question	The offered response	n		%		%	p
		M	F	M	F	M+F	
1. How often do you drink coffee?	Never	20	14	43.4	25.9	34	0.18829
	1 to 3 times a day	21	33	45.6	61.1	54	
	More than 3 times a day	5	7	10.9	12.9	12	
2. Do you smoke cigarettes?	Yes	18	24	39.1	44.4	42	0.68546
	No	28	30	60.9	55.5	58	
3. How often do you drink alcohol?	Every day	3	0	6.5	0.0	3	0.04383*
	1 to 3 times a week	27	26	58.7	48.1	53	
	Never	16	28	34.8	51.9	44	
4.How physically active you are?	I am totally inactive	20	34	43.5	63.0	54	0.14065
	I recreate 30 minutes a day for the whole year	17	14	37.0	25.9	31	
	I'm actively involved in sports	9	6	19.5	11.1	15	

n - number of respondents, M-male , F - female, Fischer's exact test, * statistical significance p = 0.05

Table 5. Family history respondents

		n		%		%	p
		M	F	M	F	M+F	
1. Has anyone in the immediate family been suffering from colon cancer?	Yes	2	4	4.3	7.4	6	0.68792
	No	44	50	95.7	92.6	94	
2. Are you on long-term antibiotic therapy?	Yes	1	1	2.2	1.9	2	1.0
	I was	1	2	2.2	3.7	3	
	No	44	51	96.5	94.4	95	
3. Did anyone in your family have, or have the following diseases?	Diabetes	22	20	47.8	37.0	42	0.54268
	Thyroid disease	6	10	13.0	18.5	16	
	Increased blood pressure	20	24	43.5	44.4	44	
	Increased fat in the blood	11	14	23.9	25.9	25	
	Some other form of cancer	3	9	0.07	0.17	12	

n - number of respondents, M-male, F - female, Fischer's exact test

In the first category of the malnutrition persons, there are 13.0% men and 7.4% women, until, in the second category of the normal body mass, there are 52.2% men and 83.3% women. Most of the students have a normal body mass (total 69%), but there is a prevalence of the increased body mass and obesity (12% students have increased body mass, and 9% students have the obesity of first degree). There are 19.5% men with increased in body mass, compared to women (5.5%), while in the 1. degree obesity there are 13.0% of men and 3.7% of women. Increased body mass is an important risk factor for colorectal cancer (Banjari & Fako, 2014). According to Kushi et al. (2006), in the USA, one third from 572,000 carcinoma deaths during the year, can be attributed to nutrition and physical activity, including increased body mass and obesity, and the other deaths were caused by exposure to tobacco products.

54% of students drink coffee 1 to 3 times a day (61.1% women and 45.6% men). 43.4% of men never drink coffee, opposite 25.9% of women (Table 4). With caffeine, coffee contains numerous polyphenols that act as antioxidants. In the case of colon cancer, cafestol and kahweol from coffee can be reducing the risk of illness by reducing the secretion of bile acids into the colon (Naganuma et al., 2007). At the other side, these substances increase the level of serum cholesterol, which is a risk factor for the development of cardiovascular disease (Urgert et al., 1995).

According to the results of this survey, 42% of respondents are an active smoker, with a higher percentage of women smokers, 44.4%, with regard to 39.1% men. Smoking is a significant risk factor for the illness of colorectal cancer (Banjari & Fako, 2014). Smokers have an increased risk of mortality due to colon cancer with regard to non-smokers (Leufkens et al., 2001; Colorectal cancer, 2008).

According to the results of these surveys, 6.5% men drink alcohol every day, however, a large percentage of people who consume alcohol once or three times a week have been recorded (58.7% men and 48.1% women). Men are more likely to consume alcohol than women. Colić Barić et al. (2003) points out that the higher percentage of men who consume alcohol and strong alcoholic drinks more often than women.

Regard to physical activity, the conclusion is that students are inactive, as many as 63.0% of women and 43.5% of men (both 54%) is totally physically inactive. Physiological characteristics, such as obesity, decreased physical activity and increased body mass index (BMI) can increase the risk of colorectal cancer. Healthy body weight, physical activity, and proper nutrition reduce the risk of cancer. With a change in lifestyle, the risk of colon cancer can be reduced by as much as 60-80%. Physical inactivity may also be one of the causes of a weaker bowel discharge, and thus increases an exposure time of the organism to potentially toxic metabolites, which also represents one of the risk factors for the appearance of colon cancer (Cummings & Bingham, 1998; Banjari & Fako, 2014). A positive family history of colorectal cancer was found in 4.3% of men and 7.4% of women (6% both) (Table 5). However, we do not ignore the high incidence of diseases that have been associated with studies with an increased risk for colon carcinoma, such as diabetes (47.8% men and 37% women; 42% both), high blood pressure 44% both respondents. Also, inflammatory bowel disease poses a risk for colon cancer (Johnson et al., 2013).

90% of established cases of colon cancer are in direct connection with nutritional habits (Banjari & Fako, 2014). The results of this research shown in Table 6, show that one meal daily eats 6% of respondents, then 2 to 4 meals a day consumed 80% of respondents, and 5 and more meals daily consume 14% of respondents. The home-cooked meal is most commonly consumed by 84% of students. Men (10.9%) less consume fast food than women (16.7%).

According to the results of this survey, the conclusion is that students have a lower daily intake of water than the recommended amount, 2300-2700 grams of water per day (Grujić, 2000). 62% of students drink 0.5 to 1.0 L water a day, women 70.4%, and man 52.2%. 32% both of students drink recommended amount 2 to 3 L water a day, 39.1% men, and 25.9% women.

Table 6. Nutritional habits of respondents

		n		%		%	p
		M	F	M	F	M+F	
1. How many meals do you consume during the day?	5 and more	8	6	17.4	11.1	14	0.37835
	2 - 4	34	46	73.9	85.2	80	
	1	4	2	8.7	3.7	6	
2. What do you eat most often?	A homemade meal	39	45	84.8	83.3	84	0.22998
	Bakery, Fast food	5	9	10.9	16.7	14	
	Restaurants	2	0	4.3	0.0	2	
3. How often do you eat fast food?	1 or more times a day	11	5	23.9	9.2	16	0.14812
	Up to 5 times a week	28	40	60.9	74.1	68	
	Never	7	9	15.2	16.7	16	
4. How much water do you drink during the day?	0,5 – 1,0 L	24	38	52.2	70.4	62	0.14851
	2,0 – 3,0 L	18	14	39.1	25.9	32	
	More than 3 L	4	2	8.7	3.7	6	
5. How often do you eat fermented dairy products?	1 to 2 times a day	17	20	37.0	55.6	37	0.00112**
	Up to 5 times a week	28	16	56.5	29.6	42	
	Never	3	18	6.5	14.8	21	
6. How often do you eat fresh fruits?	1 to 2 times a day	18	28	39.1	51.9	46	0.44346
	Up to 5 times a week	25	24	54.3	44.4	49	
	Never	3	2	6.6	3.7	5	
7. How often do you eat vegetables?	1 to 2 times a day	15	15	32.6	27.8	30	0.79858
	Up to 5 times a week	24	28	52.2	51.8	52	
	Never	7	11	15.2	20.4	18	
8. How often do you eat fresh vegetables in the form of salads?	1 to 2 times a day	18	16	39.1	29.6	34	0.32631
	Up to 5 times a week	22	25	47.8	46.3	47	
	Never	6	13	13.1	24.1	19	
9. How often do you eat potatoes?	1 to 2 times a day	9	11	19.6	20.4	20	1.0
	Up to 5 times a week	35	41	76.1	75.9	76	
	Never	2	2	4.3	3.7	4	
10. How often do you eat meat?	1 to 2 times a day	13	15	28.3	27.8	28	1.0
	Up to 5 times a week	33	38	71.7	70.4	71	
	Never	0	1	0.0	1.8	1	
11. How often do you eat pate, chicken, salami, etc?	1 to 2 times a day	15	14	32.6	25.9	29	0.28108
	Up to 5 times a week	21	33	45.6	61.1	54	
	Never	10	7	21.8	13.0	17	
12. How often do you eat fish and/or sea fruits?	1 time a day	5	3	10.9	5.6	8	0.03408*
	2 to 3 times a week	18	35	39.1	64.8	54	
	Never	23	16	50.0	29.6	39	

n - number of respondents, M-male, F - female, Fischer's exact test, *statistical significance p = 0.05
**statistical significance p = 0.01

According to the results of the frequency of consuming fermented dairy products, it was found that 14.8% of women never consumed this type of product, also 6.5% of men.

The components in dairy products that protectively affect against colon cancer include calcium and vitamin D (World Cancer Research Fund, 2007). Specific cultures of lactic acid bacteria which used in the fermentation of milk, fall into antimutagenic and anticarcinogenic substances (Strnad & Babuš, 1997). Fermented dairy products contain *Lactobacillus* strains that produce lactic acid.

Some studies suggest that probiotics produce short-chain fatty acids in the colon, which can reduce the content of the pro-carcinogenic enzymes (Divisi et al., 2006).

18% of respondents (20.4% of women and 15.2% of men) never consume vegetables, and 19% are never consumed fresh vegetables in the form of salads. 49% of respondents consume fruit up to 5 times per week, and 46% of total respondents consume fruits 1 to 2 times a day. On the other side, fruits are more consumed than vegetables (only 5% of the total number of respondents never eats fruits). Low intake of vegetables, ie. vitamins, and minerals, as well as dietary fiber, is associated with increased risk for colon cancer (Banjari & Fako, 2014).

The components of fruit, that can provide the protective role of colon cancer are carotenoids, vitamin C, flavonoids, isothiocyanates and glucosinolates (Turner et al., 2004). A diet rich in red meat, and poor in fruits and vegetables increases the risk of developing colon cancer.

Flavonoids there are in citrus fruits, apples, onions, green leafy spices (celery, parsley, and nuts), teas, black wine, soy, cherry, strawberries. Polyphenols may have anti-inflammatory, antiallergic and anticancer activity (Jakobek et al., 2008).

Nutrition rich in vegetables, especially cauliflower, broccoli, apricot and cabbage (*Brassicaceae* family), tomato and legumes, suggests a preventative effect from the development of digestive system cancer. Vegetables from a *Brassicaceae* family is rich in nutritional carotenoids, vitamins C, E and K, minerals and dietary fiber (Marti et al., 2016.)
71% of respondents consume meat up to 5 times per week. 28% consume meat once or twice a day, and never consumes only 1.8% of women. Other meat products (pate, salami, etc.) are also often consumed. It has been shown, that is approximately 15% to 20% greater risk of colon cancer by consuming 100 grams of red meat, or 50 grams of processed meat per day.

CONCLUSIONS

The nutritional state of the students' population of the University of Bihać with regard to lifestyle and nutrition indicates a negative trend in the form of increase of body mass. 12% of respondents have increased body mass, and 9% show the obesity of the first degree.
The prevalence of increased body mass in the population of students can be considered as a significant risk factor for colon cancer, in particular in male patients.
The living habits of the students show negative results such as high percentage of coffee consumption, a high percentage of cigarettes and alcohol consumption, and lack of physical activity. Among respondents, 54% consumed coffee once or three times a day, 42% of respondents are smokers, and 3% of students consume alcohol every day, while 53% of those who consume it once, or more than once a week. Regard to the exercise of physical activity, it was found that 54% of students were totally inactive.
Most of the surveyed students have a positive family history that manifests itself or in the diseases involved in the development of colon cancer (eg. elevated blood pressure 44% of respondents, fat in the blood 25% of respondents), or colon cancer (6% of respondents).
Bad nutritional habits were identified among the examinees, which is one of the major risks of colon cancer. Consumption of pate, salami, and similar products on a daily basis was recorded in 29% of students; 71% of students consume meat five times a week, fast food consumed once or more times during the day by 16% of students.
In order to avoid the increased risk of colon cancer, it is necessary to follow the recommended preventive steps in everyday life: limit the intake of red meat to 500 grams a week, increases the intake of fruits and vegetables, as well as whole grains and legumes.

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DEBUNKING MISINFORMATION ABOUT FOOD

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Abstract

Not only the general public, but particularly also politicians and opinion makers judge the safety and desirability of technologies on information that comes free from many media, often provided by self-proclaimed experts. Few people take the time and effort to check the facts. The result is often that beneficial technologies are dismissed while these benefits have the potential of increasing the standards of living. While in some parts of the world the benefits are seen and the technologies accepted and applied, on other parts they are not, even in cases where these technologies have the potential of solving huge problems. The use of genetically modified (GM) food and the use of irradiation to preserve food for example, are heavily emotionalised and in parts of the world antis tend to win the battles.

Key words*: irradiation, food technology, genetic modification, misinformation, safety.*

INTRODUCTION

Why misinformation is believed, even trusted, by so many.

The book "Before you know it: The unconscious reasons we do what we do" has been written by John Bargh, PhD, a reputable psychologist working at Yale University, a top university in the USA (Bargh J., 2017). He summarises the evidence that demonstrates that most of what we do in our life is decided by our unconscious mind. The unconscious mind has knowledge from human history (that is why even babies shy away from danger) and is learning continuously by experience. Try to think of every step you take when you walk or even better, when you descent the stairs. If you consciously want to decide where to place your feet with every step, you either go very slowly or you would fall. Healthy people need not to think about where to put your spoon or what to do when confronted with a sudden danger. When awake, the unconscious brain is constantly deciding what you need to do, your conscious brain is too slow and busy with other things. As a consequence most of the energy spent by the brain is for its unconscious tasks. While 100,000 years ago a man would jump away when a ferocious animal wais approaching him at high speed, today when suddenly a car races at you, you would do the same, without thinking about it. Thinking would have killed you.

Ancient mechanisms and fears are used by antis to influence public opinion, similar to shameless liars like many politicians and contemporary presidents. They tell you what bad things may happen if you do not follow them or their advises. Because of their positions, their appearance (they may be good looking, be friendly, seem to care or be tough), or because they are frequently seen on television, many automatically follow them, even if they know that they are lying.

Misinformation finds its way in popular publications and is hard to be countered by peer-reviewed scientific publications. Most people have the feeling that there is no smoke without a fire. Publications like "Horrible chemicals in our food" (Thomson, C.K., 2014) and "Don't eat cancer" (Cohen, S.D., 2014) sell well. Barbara H. Peterson, an activist with a Bachelor's degree in Business, leads a personal revolution against GM food. On her website she writes (Peterson, B.H., 2010):

> *"We are already having to deal with food that is injected with foreign genes (GMOs), blasted with pesticides, irradiated beyond recognition, pasteurized, homogenized, scraped off a slaughterhouse floor, and making us sicker by the minute, and now Codex guidelines are about to set the minimum and maximum levels of so-called "nutrients" we are allowed to have. If it doesn't meet the minimum NRV guideline, just add a little more GMO such as golden*

rice, chock full of artificial inserted "vitamins" to the food supply and force feed it to the public via stealth, free trade sanctions and the SPS agreement, and by all means make sure that the upper nutrient level wouldn't keep a hamster alive. And if a company or nation doesn't meet Codex guidelines? Then it is creating a barrier to free trade and can be prosecuted under the law.

And we just go along with it. Better to run to the corner store and get more of those genetically engineered foods and vitamins designed to strip us of our humanity and alter our DNA so that big pharma can keep us in its death grip and suck the remaining life out of our bones by "treating" the diseases created by our "new and improved" lifestyles with even more "new and improved" designer drugs."

On the photograph shown on the website, Barbara Peterson looks distinguished and convincing. She possibly believes what she writes and that believe made her an apostle for the cause. She, however, clearly lacks the scientific background to judge what she has picked up from the media.

Critics

Not all antis are liars, some have good reasons to be critical and may provide valid arguments supported by good data. Being critical is a fundamental requirement for scientific development. That is why evidence is so important and why food scientists need be aware of facts and know where to find the scientific information to debunk or support opinions. If critics have valid questions and they cannot or not yet be answered, further research should be done. If anything has been shown to be wrong, people need to know and want to know and measures to counter the wrongness should be taken.

Irradiation

Energy can be transferred by electromagnetic waves, the shorter the wavelengths (or the higher the frequency) the higher the power of the waves and thus the more energy can be transmitted in a certain time. Wavelengths (λ) between 400-700 nm does not have power enough to cause serious harm, unless the exposure is very long. UV light, however, with λ between 10 and 400 nm, has enough power to cause significant chemical changes, reason why exposure to sunlight does change the colour of the human skin. The shorter λ, the more energy is transferred and therefore the more damage can be done. Below 10 nm we talk about x-ray and γ-radiation, such radiation frees electrons from atoms and molecules and is therefore called ionising radiation. In particular γ-radiation (having the shortest wavelength and therefore the highest power) can deliver enough energy to ionise atoms and molecules, produce radicals and make significant changes to the irradiated substance. For a clear picture of the differences between radiations, see https://upload.wikimedia.org/wikipedia/commons/9/99/EM_Spectrum3-new.jpg (accessed 15 July 2019).

For λ-radiation usually either Cobalt 60 or Cesium 137 are used. These materials are radiating continually and therefore require expensive safety measures to protect operators. Electron beam (e-beam) radiation is also ionising and therefore can also be used but the technology is different. While λ-radiation is electromagnetic radiation, e-beam uses high-speed electrons and can deliver the same energy as the Cobalt and Cesium isotopes. The important difference is that an e-beam is produced by an electronic device that can be switched on and off. When off, there is no radiation. The isotopes cannot be switched off and when not in use must be stored and protected in a dedicated space. Therefore, although installations for both methods can be operated safely, from an occupational point of view, e-beam technology is more attractive.

Irradiation of food

Food irradiation is the exposure of food to ionising radiation to cause chemical changes that harm microorganisms, including viruses and parasites, as well as insects, to the extent that they cannot reproduce anymore. The same irradiation also causes chemical changes in food that may result in slowing down ripening and does prevents sprouting of some vegetables. That way irradiation can be used to increase shelf life of food.

The quantity of chemicals resulting from the radiation treatment, however, is very small and mostly less than the chemical changes caused by heat treatments aimed at prolongation of

shelf life. The safety of the consumption of food treated with radiation has been thoroughly investigated for decades (Smith and Pillai, 2004). The only chemicals that rose concern were benzene and its derivatives, and 2-alkylcyclobutanones (2-ACBs). Extensive research, however, showed that the amounts formed, compared with the consumption from other food, were too low to be of concern (McNeal et al., 1993).

Similar to chemicals, radiation may harmful, depending on the dose. Too much radiation like too much of a chemical such as Vitamin A will do harm and thus must be avoided. Irradiated food, however, is not radioactive food, contrary to what anti-irradiation activists state, such as in "Zapped! Irradiation and the death of food" (Worth and Hauter, 2008). A customer wrote a review:

> *"Everybody is focused on GMO and pesticides, and meanwhile government (or those behind it to be exact) quietly give orders to irradiate our food. If some of us still manage to find out and ask, they just say it is safe and not radioactive. We are talking about amounts of radiation, that are equal to 5 billion times more of the chest x-ray. And no, they are not safe. They are changing our DNA in as little as 2 weeks. Studies with animals showed, that first generation eating those foods was sicker, but somewhat okay, second was very sick, and ...you guessed it -there was no third generation." (https://www.amazon.in/Zapped-Irradiation-Death-Mark-Worth/dp/1567513689; accessed 16 July 2019).*

Radiation and radioactivity

Food irradiation dose not make food radioactive. Similar to that fruit exposed to sunlight (= sun radiation) will not cause sunburn (does not make the fruit radiate sunlight) (Figure 1).

Another misconception is that many consumers often see in the Radura symbol (Figure 2, left) a warning that the product is radioactive while the symbol is meant to show that the product has been irradiated to make it safe (Ehlermann, D.A.E., 2009). Antis persist in telling the public that the Radura symbol just replaces the Radioactivity warning symbol (Figure 2, right) and that the Radura one has been developed to hide the danger.

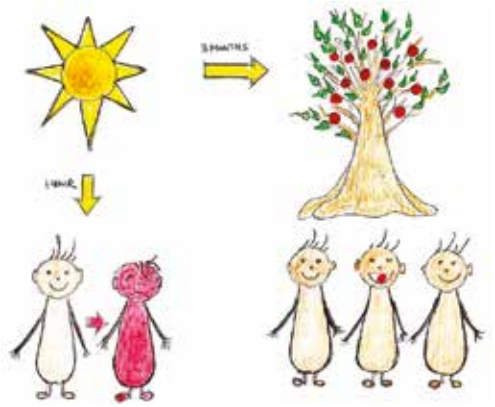

Figure 1. Exposure to sunlight for just one hour may cause sunburn. The consumption of fruit exposed to the same sunlight for months does not cause sunburn

Figure 2. Radura symbol (left) used on packaging of irradiated food and the radioactivity warning symbol (created in 2006 by Cary Bass; https://commons.wikimedia.org/wiki/File:Radioactive.svg (accessed 16 July 2019)

Bevelacqua and Mortazavi (2019) illustrated in a very clear way the influence of the environment and history on the thinking of people. If society applied the radiophobia logic to cooking food, it would be viewed as a negative technology. A hypothetical example illustrates applying the radiophobia mindset to cooking food with thermal radiation:

> *"Scientists have developed a new technology called thermal radiation (e.g., infrared radiation) as a method that is alleged to improve the taste and edibility of foods. Thermal radiation proponents claim that it kills known pathogens and prolongs the food's shelf life. Unfortunately, thermal radiation has a number of negative side effects that suggest its use is potentially harmful. Thermal technology produces carcinogenic materials in meat, reduces the vitamin content of fruits and vegetables, and produces hazardous chemical compounds in eggs. Therefore, cooking foods with thermal radiation should be avoided and restricted by regulations until detailed research proves that it is not harmful to human health."*

Irradiation of food or food ingredients is practised in many but by far not all countries and nowhere for all food. In many countries a permission is still required. Countries where irradiation of food is at least partially approved: Australia, China, European Union (28 countries), India, Indonesia, Japan, Malaysia, Mexico, South Africa, Thailand, USA, Vietnam. If occupational safety requirements are met and the applied dose is in accordance with the levels proven to be safe, based on scientific data irradiation could be allowed everywhere. There is global scientific consensus that irradiated food is safe to consume; nutritionally adequate and has the same sensory properties as non-irradiated food (Koutchma et al., 2018).

The bottom line is that irradiation is a technology that is suitable to prevent insects and microorganisms to make food unfit for consumption, reducing food wastage. It may do so in particular where other technologies cannot be applied.

GM food

Definition of GMOs

WHO: Genetically modified organisms (GMOs) can be defined as organisms (i.e. plants, animals or microorganisms) in which the genetic material (DNA) has been altered in a way that does not occur naturally by mating and/or natural recombination (https://www.who.int/foodsafety/areas_work/food-technology/faq-genetically-modified-food/en/; accessed 16 July 2019).

Why GMOs?

There always have been parts in the world where people were starving. Technology has helped mankind to improve the production of food and successfully such that in the western world there is no shortage of food. In many other parts of the world it the production of food has been and still is insufficient to feed the ever growing population. The global food production in the world is enough to feed everyone, if that food would be in the right places. Much of the staple food today actually is GM food and already for decades although most people do not know. Without this, probably there would not be enough food and certainly not everywhere.

Potential benefits of GMOs

Resistance to diseases

By changing tiny bits of the genetic makeup of plants, they can be made and have been made resistant to herbicides, insects and microorganisms. Corn (maize), staple food in South Africa used to be infested by insects causing damage to the skin of the corn and thereby facilitating the growth of moulds that produced mycotoxins and made about 50% of the corn toxic. Today, 85% of the maize and also 95% of the soy in South-Africa is produced with genetically modified species (Groenewald, 2019). Central and eastern Africa depend largely on banana as a staple food. In Uganda the government supported the development of banana that are resistant to the mould *Mycosphaerella fijiensis* that otherwise causes black Sigatoka, a disease that can decrease the yield of banana by up to 50% (Namanya, 2019).

Essential nutrients

Many people in developing countries suffer from vitamin A deficiency (VAD), causing blindness of hundred thousands of children every year and killing millions of people. In many developing countries rice is the staple food, but rice does not contain ß-carotene, the precursor that the body converts to vitamin A. The successful insertion of genes for the production of ß-carotene into wild rice makes it possible to alleviate the problem. Because of the orange colour of ß-carotene, the GM rice too is orange and has the nickname "Golden Rice". Research in underway to make staple food also producing other essential nutrients that many people are lacking due to a very one-sided availability of food (Hefferon, 2015).

Stress resistance

There are many areas in the world where growing food is impossible, due to lack of or too much water; too high or too low temperatures; too salty or too acid soil. There is, however, vegetation in these areas, specialised to cope with the prevailing situation. Obviously, modification of staple food crops to make them resistant to stresses where such crops are important has the potential of solving hunger problems. This probably can be done by using genes from stress resistant plants. Work is done on making crops drought resistant is reviewed by Liang et al. (2016).

There are many more possibilities and if they have the potential to solve serious problems, they should be investigated and tested for safety. If safe their use should not be prevented by activists who simply are "against", whatever the product or technology, because being against can be profitable and that whole populations are deprived from badly needed solutions is not their problem.

Can GM food be unsafe and cause diseases?

The public is incessantly bombarded with by activists and activist organisations claiming to know with certainty that GMO food is bad and dangerous to eat. Hundreds of examples of how they do this can be found on https://nl.pinterest.com/pin/344666177711821774/. Because every knew technology and every new product may have undesired and even dangerous properties, before being allowed on the market the safety of new products should be thoroughly investigated. A potential danger of GM food that has been made resistant to microorganisms to which there originally was no resistance, might result in microorganisms resistant to antimicrobials, including antibiotics. That will be undesirable and hence this should be very carefully investigated. If GM food produces proteins that have not been part of the modified food, it must be investigated if that protein may cause allergy although thorough review of research in this did not indicate that GM food is more allergenic than their conventional counterparts (Dunn et al., 2017). Although GM food has now been on the market in many countries for more than a decade and probably at least a billion (1000 million) consumers eat GM food daily, there have been no indications that there is a difference in the incidence and types of cancer between people who do and those who do not eat GM food regularly.

Safety of GMOs

The EU funded many projects to investigate whether there is any indication that GM food would be less safe that non-GM food and 10 years of research did not find any evidence that it would (European Commission, 2010).

The National Academies of Sciences, Engineering, and Medicine established a Committee on genetically engineered crops: past experience and future prospects, with the task to examine evidence regarding potential negative effects and benefits of genetically engineered crops as well as the potential benefits and negative effects of future GE crops. The findings have been reported in 2016. Twenty experts reviewed more than 1,000 studies, concluding, based on epidemiological data on incidence of cancers and other human-health problems, that there is no evidence that foods from GE crops are less safe than foods from non-GE crops (National Academies of Sciences, Engineering and Medicine, 2016).

GENERA is a project of Biology Fortified, Inc. (BFI), an independent non-profit organization incorporated in Middleton, Wisconsin, USA. In 2017 they reported that currently there are near 2000 peer-reviewed reports in the scientific literature that document the general safety and nutritional wholesomeness of GM foods and feeds (Nicolia et al., 2014).

The most recent review is that of Delaney (2018), who concluded that "Decades of testing food and feed products from insect resistant, herbicide tolerant and stacked traits of previously approved single traits, and other types of GE crops in laboratory and livestock animals have shown that the technology used to produce them is not inherently hazardous. No adverse effects have been observed to date".

The success of activists and activist organisations - people die needlessly

Activist organisations go very far with their actions, among which are the destruction of and experimental fields of genetically modified Golden Rice in the Philippines in 2013 (https://slate.com/technology/2013/08/golden-rice-attack-in-philippines-anti-gmo-activists-lie-about-protest-and-safety.html; accessed 16 July 2019); GM wheat in the UK in 2012 (https://www.independent.co.uk/hei-fi/news/scientists-plead-with-anti-gm-protesters-not-to-destroy-crop-7788322.html; accessed 16 July 2019) and of corn in Hungary in 2013. It is claimed that the destruction in Hungary was strongly supported by Hungary's Minister of Rural Development (https://www.abcplus.biz/GMO_6-26-13_Hungary_Torches_GM_Corn; accessed 16 July 2019). The Hungarian government is one of those that are blindly following anti-GM

activists. Greenpeace claim to know that GM food is dangerous. They never provide evidence, obviously because such evidence does not exist, but they also do not need to, because their claim is that they know that they are right and moreover that all scientists should know too. By frequent and persistent repetition of their claims they successfully convince a large part of the general public well as many politicians. There are many books about the dangers of GM food, blaming governments to approve GM food only to help the food industry to increase profit and that at the expense of the misled consumer/taxpayer. An example of such s book is "Altered genes, twisted truth" (Druker, 2015). The subtitle summarises the contents: "How the venture to genetically engineer our food has subverted science, corrupted government, and systematically deceived the public". In the book "Genetic Roulette: The documented health risks of genetically engineered foods" (Smith, 2007) 65 claims are presented that GM food causes harm in many ways. Academics Review debunked each of the claims based on peer-reviewed evidence and provides all the references (http://academicsreview.org/reviewed-content/genetic-roulette/; accessed 17 July 2019). Academics Review is an association of academic professors, researchers, teachers and credentialed authors from around the world who are committed to the unsurpassed value of the peer review in establishing sound science (http://academicsreview.org/about-academic-review/purpose/; accessed 17 July 2019).

NGO's in rich countries, where they have enough food, have successfully convinced the governments in poor countries that GM food is unsafe. These organisations, lead by GreenPeace, can be held responsible for the death of millions of people, annually. They lie to the officials in the suffering countries, who generally lack the capacity to deal with the scientific information and trust the large international organisations from the developed rich countries.

The NGOs cleverly do not refer to the reports of the scientific organisations in the those countries that have repeatedly and clearly described that GM food is not less safe than non-GM food (Paarlberg, 2014).

During the massive famine in Southern Africa, in 2001, several governments in the region objected to genetically modified (GM) grain, especially Zambia and Zimbabwe, the countries hardest hit by the drought. Citing health and environmental concerns, Zimbabwe blocked the GM food aid from entering the country. In Zambia, where some GM grain had already arrived, the government placed it under lock and key, banned its distribution and then blocked another 40,000 tonnes that were in the pipeline. Source: Africa Renewal, Vol.16 #4 (February 2003), page 5 . This is the result of overwhelming activities of antis, in particular in Europe, who claim with no evidence that GM food is dangerous. The reality is that hundreds of millions of people consume GM food daily and there is not a single report of a health incident related to GM food. The local governments choose to let their citizens starve to death rather than giving them GM food.

Letter of Nobel Laureates to Greenpeace:

On the 29th of June 2016 Nobel Laureates in medicine, chemistry, physics and economics sent a letter to Greenpeace, the UN and Governments around the world. They ask Greenpeace to cease and desist in its campaign against Golden Rice specifically, and crops and foods improved through biotechnology in general. They ask governments to reject Greenpeace's campaign against Golden Rice specifically, and crops and foods improved through biotechnology in general; and to do everything in their power to oppose Greenpeace's actions and accelerate the access of farmers to all the tools of modern biology, especially seeds improved through biotechnology. Opposition based on emotion and dogma contradicted by data must be stopped. The concluding question is "How many poor people in the world must die before we consider this a 'crime against humanity'?" (Nobel Laureates, 2016).

Essential knowledge for everybody

What everybody should be made to realise - and here education at all levels could play an important role - is that genetic modification is done by nature, since life started. Nature does so now and will continue do so in the future. Nature, however, does not do it for the benefit

of mankind. To survive, everything living in nature tries to kill competing living things, including man. Mankind has evolved and survived using gathered knowledge.
Why would "natural" be better than “modified by man”? Farmers explored - be it unknowingly - mutations by cross-breeding, trying and selecting crops with improved traits. They did so long before Gregor Mendel found out how it worked. Since scientists do it, based on knowledge and experience, enormous hurdles have been created.
The most recent developments, using CRISPR-Cas9 enzymes (and similar) can make desired DNA changes very accurately, eliminating the chances that the results can be harmful, moreover this is done with much less effort than before (Lemay and Moineau, 2019).
Many countries are exploring this technology but amazingly, thanks again to the efforts of the activist organisations, it is not allowed in the EU without going through the same time-consuming and expensive procedures that apply to the methods of the decades past.

Labelling

What information is useful on a label?

The answer is that it should have what consumers *need* to know about the product and thus *should want to know* and what many of them would ask if they would have sufficient reliable information about food.
In the past decade self-proclaimed experts have told that food has become a great risk and one must be very careful because food today contains chemicals and chemicals are dangerous.
After the European commission had decided that the safety of chemicals added to food should have been proven safe and that to help consumers to find out about these additions, E-numbers had been introduced, making it easier to look the information up.
One would not need to type in "ethyl ester of beta-apo-8'-carotenic acid" but just E160f to find all information about the substance. Promptly you are told that E-numbers have been invented to hide that there are chemicals in the food. When as a response manufacturers went back to using the chemical names, the message became that chemical names are used to hide E-numbers.
In "Swallow This: Serving Up the Food Industry's Darkest Secrets " (Blythman, 2015) you may read:

> *"How clean is your label? Pick up some rustic-looking salami and even the most guarded shoppers might relax when they notice rosemary extract on the ingredients list. But rosemary extracts are clean label substitutes for old guard of techie-sounding antioxidants. Manufacturers use them to slow down the rate at which food go rancid. Rosemary extracts do have an E number (E392) but manufacturers prefer to label them more poetically as ‘extract of rosemary’, and loose off ending E. because that way they sound like lovingly made Slow Food ingredients."*

or

> *"Not sure what to have for dinner? How about a chicken noodle dish? If you noticed that it contained an amino acid such as L-cysteine E910, your enthusiasm might wane."*

Toxicity of chemicals

It is time that children already in the Kindergarten learn that everything is chemical and that chemicals need not scare them. They need to know that water and air are chemical and become resistant to scary misinformation. At the basic school they may be shown nice labels developed by James Kennedy, a chemistry teacher in Melbourne, Australia (Figure 3). His intention is to demonstrate that “natural” products are usually more com-plicated than anything created in a laboratory. And he omitted the thousands of minority ingredients, including DNA.
What everybody should know is that chemicals as such are not toxic, but that it is the amount of a chemical that may make it toxic, as discovered and explained a few hundreds years ago by Paracelsus (Bombastus ab Hohenheim, 1658): *"Poison is in everything, and no thing is without poison. The dosage makes it either a poison or a remedy"*. For many substances the situation is as Paracelsus discovered: if the dose is too high, damage is done. However, too low a dose of the substance may also be a health risk, as is the case with vitamins and minerals. Without them we get ill and may die, but too high a dose has the same result (Figure 4; from GHI, 2016).

Figure 3. All the ingredients on this list are 100% natural in a non-GM banana. None of them are pesticides, fertilisers, insecticides or other contaminant and the label is not complete, there are also another thousands of minority ingredients

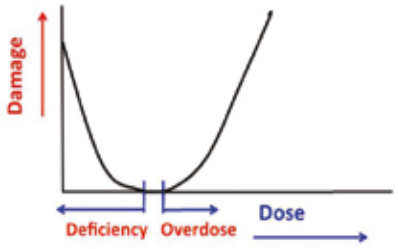

Figure 4. This graph illustrates what applies to most chemicals in our food. Not enough may lead to damage, such as blindness in the case of vitamine A, but too much will also do damage, the amount will make even vitamins toxic

The message is that all food contains potentially toxic substances, substances that like any substance, will become toxic if the amount consumed is larger that the body can handle. In many cases the body needs these substances, but not in excess. With not enough vitamin A you may turn blind and eventually die if it lasts too long. With too much vitamin A you will die too. Here is a short list of examples of chemicals present in normal food, harmless, unless consumed in excess.

- Caffeine, theobromine, theophyline (coffee, chocolate, tea)
- Coumarin (cinnamon, peppermint, green tea, chicory, blueberries)
- Cyanogenic glycosides, such as amygdalin (almond, laurel) and linamarin (cassave)
- Enzyme inhibitors (soy, peas, beet, cereals)
- Glucosinolates such as sinigrin, progoitrin (cabbage, broccoli, brussels sprouts, cauliflower, turnip, radish, horseradish, mustard, rapeseed)
- Lectins (or hemaglutinins) (pulses)
- Oxalates (rhubarb, spinach, parsley, chives, purslane, cassava, amaranth, chard, taro leaves, radish, kale, monstera fruit)
- Piperidines (black pepper)
- Saponins (peanut, soy, spinach, broccoli, potato, apple)
- Solanine (potatoes, tomatoes, aubergines)
- Tomatine (tomatoes)

The good news is that, at least in developed countries, with normal but not monotonous eating habits it is unlikely that any component of food will be consumed in too high or too low quantities, perhaps with the exception of vitamin D in winter or in case of adverse medical conditions.

Organic food

All food is organic, people that market "organic food" provide misleading information, suggesting that other food is not organic. The food that is labelled "organic" would be of

better quality and healthier. There is, however, again no evidence that "organic food" is any better than other food. Insects are as keen as humans to eat, therefore insects need to be kept away from the food intended for humans. For food grown in greenhouses this is possible to a large degree but for food grown in the open, this is not possible. For that reason insecticides are used, also on so-called organic food. The difference is that, while the synthetic ones used on normal food have been thoroughly tested for safety, those used on organic food are not, because they are organic. When plants are stressed or damaged, such as during a pest attack, they may greatly increase their natural pesticide levels, sometimes even to levels that can be toxic to humans. Americans consume with their food about 10,000 times more pesticides than synthetic pesticide residues (Ames et al., 1990). Although if properly applied the amount of pesticides, natural or synthetic, in or on food products is so low that they will not make the food unsafe, it would in principle be safer to eat food with the thoroughly tested synthetic pesticides than the not tested organic ones. For more and detailed information on this topic, consult Swirsky et al. (1997).

Knowledge that should be on the label

Based on the information discussed above, it is concluded that what is needed, in addition to information about storage and preparation, a declaration of constituents that

- may be harmful if too much is consumed (such as sugar and oxalic acid)
- are essential nutrients and may be lacking in a monotonous diet (such as vitamins)
- may give allergic reactions
- may be unsuitable for a significant part of the population (such as gluten and lactose)

This will already occupy much space and more information will not be helpful while shopping. For more information the manufacturer should provide an internet link or QR-code.

Diets

Unless there are medical disorders, by sticking to a decent varied diet, you may have control over your weight and stay healthy. The wheel of five, shown in Figure 5, is a good guide (Brink et al., 2017). Diets that cut out food groups may result in deficiencies and that obviously is not healthy.

A gluten-free diet makes sense only for people with celiac disease or gluten sensitivity (Van Buul and Broun, 2013) and that are not as many people as the many who believe they suffer from these disorders (Capannolo et al., 2015).

Vegetarian diets are healthy provided sufficient protein is consumed from vegetarian sources. Vegan diets are also healthy provided care is taken that in addition sufficient essential nutrients are consumed.

Claims that probiotics are good for health are at least doubtful (Zmora, 2018).

Figure 5. The wheel of five, developed by the Netherlands Nutrition Centre

Varying menus will provide all nutrients needed for healthy people. The recommendation is to pay attention to the wheel of five; not to eat too much; not to add more than a little salt; not to add sugar; consume 2 litre of water per day (but that is including the water present in the food) and last but not least: enough physical activity. In case of weight problems that cannot be solved by these points: consult a reliable nutritionist.

Recommendation

To fight misinformation, it is recommended to use the information in this article to teach students, discuss with colleagues, management, politicians and whoever else you may be able to influence.

If surprising information about food, food safety and food security is encountered, always

look for peer-reviewed scientific evidence. Also, in meetings with officials and politicians, address regulations that are morally and scientifically wrong and harm people.

Books that provide peer-reviewed scientific information

Genetically Modified and Irradiated Food - Controversial Issues: Facts versus Perceptions Editor: Veslemøy Andersen. Elsevier, 2019. ISBN: 9780128172407

Ensuring Global Food Safety - Exploring Global Harmonization. Editors: Christine Boisrobert, Aleksandra Stjepanovic, Sangsuk Oh and Huub Lelieveld. Elsevier/Academic Press, 2009. ISBN: 9780080889306

Regulating Safety of Traditional and Ethnic Foods. Editors: V. Prakash, Olga Martin-Belloso, Larry Keener, Siân Astley, Susanne Braun, Helena McMahon and Huub Lelieveld. Elsevier/Academic Press, 2016. ISBN: 978-0-12-800605-4

Nutritional and Health Aspects of Food in Nordic Countries. Editors: Veslemøy Andersen, Eirin Bar and Gun Wirtanen. Elsevier/Academic Press, 2018. ISBN: 978-0-12-809456-3

Global Food Legislation: An Overview. Editors: Evelyn Kirchsteiger-Meier and Tobias Baumgartner. Wiley, 2014. ISBN: 978-3-527-33555-8

EU Food Law Handbook. Editor: Bernd van der Meulen. Wageningen University Press, 2014. ISBN: 978-90-8686-246-7

Genetic Modification and Food Quality: A Down to Earth Analysis. Robert Blair and Joe M. Regenstein. Wiley, 2015. ISBN: 978-1-118-75641-6

Global legislation for food contact materials. Editor: Joan Sylvain Baughan. Elsevier / Woodhead Publishing, 2015. ISBN 978-1-78242-014-9

The Use of Nanomaterials in Food Contact Materials - Design, Application, Safety - Editor: Rob Veraart. DEStechpublications, 2017. ISBN: 978-1-60595-136-2

Hygiene in Food Processing. Editors: Huub Lelieveld, John Holah and David Napper. Elsevier / Woodhead Publishing, 2014. ISBN: 9780857094292

Handbook of Hygiene Control in the Food Industry. Editors: Huub Lelieveld, John Holah and Domagoj Gabrić. Elsevier / Woodhead Publishing, 2016. ISBN: 978-0-08-100155-4

Hygienic Design of Food Factories. Editors: John Holah and Huub Lelieveld. Elsevier / Woodhead Publishing, 2011. ISBN: 978-1-84569-564-4

Food Safety Management – A Practical Guide for the Food Industry. Editors: Yasmine Motarjemi and Huub Lelieveld. Elsevier/Academic Press, 2013. ISBN: 9780123815057

Les invisibles. Yasmine Motarjemi. Elstir Editions, 2010. ISBN 2970051257; 9782970051251

English translation: Invisible things. Sara Andersson. CreateSpace Independent Publishing Platform, 2012. ISBN-13: 978-1469985718

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CHEMICAL COMPOSITION AND ANTIOXIDANT ACTIVITY OF *Hyssopus officinalis* L. SELECTIVE FRACTIONS OBTAINED BY DIFFERENT METHODS

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Abstract

The aim of this study was to obtain selective fractions of Hyssopus officinalis L. by different methods and to investigate a possible correlation between their chemical content and antioxidant activity in order to establish a potential effect of this species on counteracting diseases associated with aging processes. HPLC analysis and quantitative determination of active principles from nine selective fractions show that the values were well correlated with the ones resulted by spectrophotometrically methods. The selective fractions have a total flavonoid content expressed as rutin from 1.236 to 19.060 % and respectively 0.126 to 16.783% polyphenolcarboxylic acids expressed as rosmarinic acid. It has been observed that the fractions with high content in polyphenolcarboxylic acids and flavonosides exhibit maximum antioxidant activity. There are selective fractions containing only one of the classes of compounds (flavonosides or polyphenolcarboxylic acids) in a higher amount that show great antioxidant activity. A strictly correlation between the flavones content of the selective fractions and antioxidant activity can not be made by this method.

Key words*: antioxidant activit , flavonosides, Hyssopus officinalis L., polyphenocarboxylic acids.*

INTRODUCTION

In two previous studies we presented the results regarding the antioxidant action of some selective fractions obtained from the plant species cultivated in Romania with potential effect on the counteracting diseases associated with aging processes (Ashok & Rashid, 1999; Babovic et al., 2010).

In this paper are presented the results of researches regarding the obtaining of selective fractions of *Hyssopus officinalis* L. and their antioxidant action.

The species *Hyssopus officinalis* L., the *Lamiaceae* family, is known in the Romania in the traditional medicine for the therapeutic effect. Modern medicine has confirmed that, due to existing classes of active compounds in aerial parts, this species is beneficial in the treatment of certain diseases.

Classes of compounds with demonstrated therapeutic effects are flavonosides (apigenin, quercetin, diosmin, luteolin and glucosides thereof), polyphenolcarboxylic acids (chlorogenic, ferulic, caffeic and rosmarinic acids), volatile oil, calchones, triterpenes (ursolic and oleanolic acid), β-sitosterol and bitter principles (marubin). These compounds are responsible for the stomachic, carminative, antispasmodic, antiasthmatic, anticatarrhal, antiseptic, healing, antimicrobial and antioxidant effects (Colceru-Mihul et al., 2016; 2017).

Among chemical compounds derived from plant species, rosmarinic acid, caffeic acid or other compounds belonging to polyphenolcarboxylic acids class; diosmin, diosmetin or other flavonoidic compounds, are very well known for their antioxidant properties (Fathiazad & Hamedeyazdan, 2011; Istudor, 2001).

Compounds with antioxidant activity are regarded as basic elements of the anti-aging strategy because free radicals are considered the main responsible agents of premature aging and also of diseases associated to aging status (Marin et al., 1998).

MATERIALS AND METHODS

The plant material consisting of aerial parts of *Hyssopus officinalis* L. (Hyssopi herba) was

obtained from culture, dried and ground as a fine powder (sieve VII).

Selective fractions obtainment:

Method I consisted of repeated extraction - two times of the active substances from 200 g Hyssopi herba, with 50% ethylic alcohol v/v (vegetal material/solvent ratio = 1/10 m/v for the first extraction and 1/5 m/v for the second extraction) at boiling temperature of the solvent for 1 hour per extraction with continuous mechanical stirring, followed by cooling and filtration of the extracts. The reunited hydroalcoholic solutions were rota-evaporated for alcohol removal. The resulting aqueous solutions were spray-dried and selective fractions HI were obtained.

Method II consisted of active principles extraction from 300 g Hyssopi herba, with 50% alcohol (plant/solvent = 1/10 m/v ratio) at boiling temperature for 1 hour with continuous mechanical stirring, followed by cooling and filtration of the extracts. Hydroalcoholic extract solution was evaporated to a volume of 1/1 m/v plant/solvent mixture and centrifuged. A precipitate (which was labeled as HII0 after drying) and an aqueous solution were obtained. In order to obtain selective fractions, aqueous solution was further processed by:

- 3 successive liquid-liquid extractions with ethylic ether, followed by solvent removal from the reunited etheric by rotaevaporation, resulting HIIA.
- 3 successive liquid-liquid extractions with chloroform, followed by solvent removal from the reunited chloroformic extracts by rotaevaporation, resulting HIIB.
- 3 successive liquid-liquid extractions with ethyl acetate, followed by solvent removal from the reunited ethyl acetate extracts by rotaevaporation, resulting HIIC.
- 3 successive liquid-liquid extractions with n-buthylic alcohol, followed by solvent removal from the reunited buthanolic extracts by rotaevaporation resulting HIID.
- adding acetone in a 2/1 v/v acetone/aqueous extract ratio resting at 4-6^{0}C for 24 hours, filtration and drying the precipitate resulting HIIE. This is shown in Figure 1.

Method III consisted of repeated extractions - two times of the active substances from 200 g Hyssopi herba, with methylic alcohol (plant/solvent ratio = 1/10 m/v for the first extraction and 1/5 m/v for the second extraction) at boiling temperature of the solvent for one hour per extraction with continuous mechanical stirring, followed by cooling and filtration of the extracts. Methanolic solutions were reunited, the solvent removed by rotaevaporation resulting HIII selective fractions.

Method IV consisted of macerating 200 g Hyssopi herba in acetone (plant/solvent ratio = 1/7 m/v), removing the solvent from acetone solution and re-extracting the residue in methanol. The active substances were extracted from moist plant material with 20% ethanol (plant/solvent ratio = 1/10 m/v) at boiling temperature of the mixture for 2 hours, followed by hydroalcoholic solution evaporation to an aqueous extract. Methanolic and aqueous extract were reunited and filtered. The resulting precipitate was dried and selective fractions HIV were obtained.

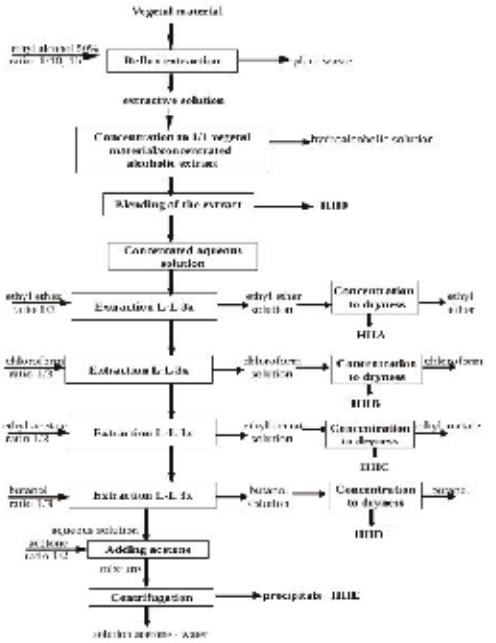

Figure 1. Selective fractions obtainment - method II

HPTLC analysis of selective fractions was performed using Silica Gel 60F_{254} as stationary phase and a mixture of ethyl acetate - acetic acid - formic acid - water (100: 11: 11: 27 v/v/v/v) for chromatographic elution. The plates were scanned under 360 nm after the

derivatization with NP/PEG. The reference compounds for HPTLC analysis were from Sigma-Aldrich: caffeic acid, rosmarinic acid, chlorogenic acid, rutin, hyperoside and diosmin (Reich & Schibli, 2008; Romanian Pharmacopoeia, 1993).
HPLC analysis of selective fraction consisted in chromatographic separation on a Purosher ODS column (250 x 4.6 mm, 5 μ) at 40^0C, using a gradient elution (both mobile phase and flow). The mobile phase was a binary gradient: water with orthophosphoric acid (pH = 2.5) and methanol. The eluent absorbance was monitored at 330 nm. The reference substances were from Sigma-Aldrich: caffeic acid, rosmarinic acid, rutin, diosmin and luteolin.
Quantitative determination of active principles from selective fractions consisted of determination of flavones by a colorimetric method based on their property to form intensely yellow complex with Al_3^+ and of determination on polyphenolcarboxylic acids by a colorimetric method based on the property of phenols to form nitrocompouds or nitro oxime with nitrous acid which give red stain when dissolve in alkaline solutions due to their weak acid character. For the quantification of flavones, rutin was used as reference substance and for polyphenolcarboxylic acids quantification rosmarinic acid was used as reference substance (Sanchez Moreno et al., 1998).

Analysis of antioxidant action

DPPH assay: In each reaction tube 100 μl vegetal extract of different concentrations was mixed with 3900 μl of 0.0025 g/l DPPH at room temperature for 30 min. 50% methanol solution was used as control. The reduction of the DPPH free radical was measured by reading the absorbance at 515 nm. Rosmarinic acid (from Sigma-Aldrich) was used as positive control. Inhibition ratio (percent) was calculated from the following equation (Wagner & Bladt, 1996):

% inhibition = [(absorbance of control – absorbance of sample)/absorbance of control] x 100

DPPH radicals react with suitable reducing agents losing color stoichometrically with the number of electrons consumed which is measured spectrophotometrically at 515 nm (Wagner & Bladt, 1996).
For determination of antioxidant activity, the selective fractions were chosen according to the yield obtained from 100 g plant and depending on the flavones and polyphenolcarboxylic acids content.

RESULTS AND DISCUSSIONS

Nine Hyssopi herba selective fractions were obtained by experimental methods mentioned above. The quantities of product obtained from 100 g plant are shown in Table1.
Flavonoids (rutin, hyperoside, diosmin) and polyphenolcarboxylic acids (rosmarinic acid, caffeic acid, chlorogenic acid) were identified by HPTLC in most of selective fractions.
The content of caffeic acid, rosmarinic acid, diosmine, rutin and luteolin in each fraction was determined by HPLC method. The values obtained from individual assessment by HPLC were well correlated with the values obtained by the spectrophotometrically methods mentioned above. For example, rosmarinic acid content from the selective fractions, determined by HPLC, correlates with the polyphenolic acids content expressed in rosmarinic acid, determined by the headline method.

Table 1. The content of active principles of Hyssopi herba selective fractions

Bioactive product	Product yieldt from 100 g plant	Flavonoids expressed as rutin % g/g	Polyphenol-carboxylic acids expressed as rosmarinic acid % g/g
HI	19.21 g	4.556	3.182
HII0	2.90 g	2.513	3.888
HIIA	0.41 g	10.347	1.138
HIIB	0.65 g	2.326	0.074
HIIC	1.15 g	19.060	14.655
HIID	4.10 g	2.853	1.030
HIIE	6.29 g	2.178	2.726
HIII	15.10 g	2.734	16.783
HIV	6.21 g	1.236	3.962

The flavonoid content expressed as rutin and polyphenolcarboxylic acids expressed as rosmarinic acid of Hyssopy herba selective fraction are shown in Table 1.

The most affluent fractions in polyphenolcarboxylic acids expressed as rosmarinic acid are HIII (16.783%) and HIIC (14.655%) followed by HIV (3.962%), HII0 (3.888%) and HI (3.182%). HIIE contains 2.726% and the fraction with most low content in polyphenolcarboxylic acids are HIIE (2.726%), HIID (1.030%) and HIIB (0.074%).
The most affluent fractions in flavonosides expressed in rutin are HIIC (19.060%) and HIIA (10.347%) folowed by HI (4.556%). HIID, HIII, HII0, HIIB and HIIE containing 2.853%, 2.734%, 2.513%, 2.326% and 2.178%. The fraction with most low content in flavonosides expressed in rutin are HIV (1.236%).
Antioxidant activity of selective fractions is shown in Figure 2.

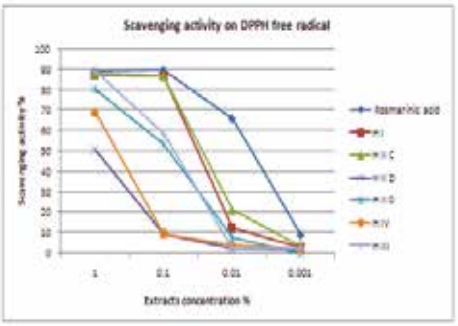

Figure 2. The antioxidant activity of the selective fractions from *Hyssopus officinalis*

Using the method for the analysis of the antioxidant activity described, it can be noted that rosmarinic acid in a percentage of 1, 0.1, 0.01 and 0.001% exhibits an antioxidant activity of 88.99%, 89.84%, 66.15% and 9.14%.
The selective fraction in a percentage of 1, 0.1, 0.01 and 0.001% exhibits an antioxidant activity.
The selective fraction HIII containing 16.783% polyphenolcarboxylic acids has a similar antioxidant activity as rosmarinic acid in a dilution of 1% and a much weaker activity in the dilution of 0.1% while the selective fraction HIIC containing 14.655% polyphenol-carboxylic acids has a slightly lower antioxidant activity compared to rosmarinic acid activity in both dilutions.
Some selective fractions with lower polyphenolcarboxylic acids content such as HI (3.182%), exhibit over 80% antioxidant activity in dilutions of 1% and 0.1%. Other selective fractions, such as HII0 (3.888%) show over 80% antioxidant activity only at 1% dilution.
Even the polyphenolcarboxylic acids content of the selective fraction HIV (3.962%) is similar to the content of HI, HII0 (which exhibits a good inhibitory potential), these fractions show a weaker antioxidant activity.
Comparing the antioxidant activity of selective fractions and the polyphenolcarboxylic acids expressed as rosmarinic acid and flavones expressed as rutin content it can be concluded that when the concentration of polyphenolcarboxylic acids increases the antioxidant activity also increases, though not an exact correlation can be made.
A correlation between the flavones content of the selective fractions and antioxidant activity can not be made by this method.

CONCLUSIONS

From *Hyssopus officinalis* L. aerial parts (Hyssopi herba) nine selective fractions enriched in flavones and polyphenolcarboxylic acids were obtained by different methods.
Out of six selective fractions tested for antioxidant activity, three of them exhibited a scavenging activity comparable with the rosmarinic acid.
It can be concluded that a high content of polyphenolcarboxylic acids expressed as rosmarinic acid lead to a higher antioxidant activity but a precise correlation can not be made.
The antioxidant activity of the flavones was not highlighted by the method used in this study for the antioxidant activity evaluation.

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COLOUR AND ORIGIN OF POLLEN PELLETS FROM TWO FRESH BEE POLLEN SAMPLES – A PRELIMINARY ANALYSIS

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Abstract

Food supplements are marketed in large numbers in Romania at present, they are the products of a dynamic and profitable industry. Bee pollen is a food supplement with variable composition and properties, reflecting the floral biodiversity used as a source. This product is collected for human use and at the same time it is indispensable for the survival of bee colonies. In the present work fresh bee pollen samples were obtained from two commercial sources in Bucharest (CS1 and CS2) and pellets were sorted by colour and analysed microscopically on unacetolised fresh mounts, one pellet at a time using an optical microscope (magnification 400×; 1000×). The colour of pollen pellets was variable, in the first sample (CS1) there were for example: white (Pinaceae – Pinus sp.), light yellow (Rosaceae – Malus sp.), lemon (Brassicaceae – Brassica sp.), orange (Asteraceae – Taraxacum officinale), firebrick (Geraniaceae – Geranium sp.) and black (Fabaceae – Trifolium pratense) pellets compared to the second sample (CS2) which contained only yellow and orange shades, for example: lemon (Brassicaceae – Brassica sp.), pale yellow (Cucurbitaceae – Cucumis sativus), orange (Asteraceae – Taraxacum officinale).

Key words*: bee pollen, pellet colour, pollen source.*

INTRODUCTION

Palynological studies of bee pollen are used to determine the botanical origin of the pollen which is important in assessing the nutritional quality of this product and at the same time indicate the foraging selectivity of the bees in a geographical area with a high floral diversity.

For example, for the Transilvania region (Romania) the analysis of 35 bee pollen samples showed the predominant plant sources and their influence on the polyphenol and carotenoid content of bee pollen (Stanciu et al., 2016).

Similarly, the botanical origins of selected honeys from Romania were determined by analysing the frequencies of the pollen grains found in their composition (Dobre et al., 2013).

Theoretically color identification of corbicular pollen could be a very useful tool for macroscopic pollen identification if combined with collection time and floral composition of an area but in practice it is not possible to sort pellet samples into plant species based on color alone since pellets from different melliferous species can have the same colour, mostly shades of yellow (Mărghitaş, 2002; Newstrom-Lloyd et al., 2009; Spulber et al., 2017).

MATERIALS AND METHODS

The analysis of the bee pollen samples was carried out at the Laboratory of Biology of the Faculty of Biotechnologies, University of Agronomic Sciences and Veterinary Medicine of Bucharest. Pellet external colour was estimated by color-matching using a standard colour chart (Reiter, 1947), then the microscopic analysis of the pollen was carried out for each pellet separately without acetolysis, on wet mounts (sometimes toluidine blue (TB) was added for better contrast) using a Micros Austria optical microscope with ocular micrometer (calibration ratio was 1 μm for objective 100×, 2.5 μm for objective 40×, 10 μm for objective 10×). Microscopic images of monofloral pellets have been photograped with a Sony Cyber-shot® digital camera (Carl Zeiss Vario-Tessar 5× zoom lens) and were later used to describe the grains. Several morphological characteristics were studied such as the shape, the size, the apertures and the surface patterns. Classification of pollen according to size was based on values from Popescu & Meica (1997). For the present study, the pollen descriptions were compared to those

found in the literature, for example Tarnavschi et al. (1981; 1987; 1990), Şerbănescu-Jitariu et al. (1994), the Pollen-Wiki site (Pollen-Wiki - Der digitale Pollenatlas, Stebler Th., https://pollen.tstebler.ch/MediaWiki/index.php), the PalDat - Palynological Database (www.paldat.org) or the Pollen Atlas of the Medical Faculty of Wiena (www.pollenwarndienst.at).

RESULTS AND DISCUSSIONS

White pollen pellets

White pellets were present in one of the two samples (CS1) of bee pollen that were analysed and they were composed of large size (~ 70 μm) bisaccate pollen, found in members of the family *Pinaceae*, possible *Pinus* (Figure 1).

Figure 1. Large size bisaccate pollen found in white pollen pellets in the present study, granular cytoplasm can be seen

Light yellow pollen pellets

Medium size 3-colporate striate pollen grains (~ 36 μm), oblate in equatorial view, with large elliptic pores (~ 23 μm height, ~ 13 μm width) and thick exine (~ 1 μm) were seen in light yellow pollen pellets found in CS1. This pollen is similar to pollen found in Family *Rosaceae* (Figure 2).

Pale yellow pollen pellets

Pale yellow pollen pellets were seen in CS2. The microscopical images showed medium size 3-porate, spheroidal pollen grains (polar axis ~ 40 μm, equatorial axis ~ 40 μm), elliptical pores and psilate, thick exine (~ 1 μm). Some pollenkitt was present (Figure 3). This pollen could be *Cucumis sativus* (cucumber), Family *Cucurbitaceae*.

Figure 2. Medium size 3-colporate pollen seen in light yellow pollen pellets (TB)

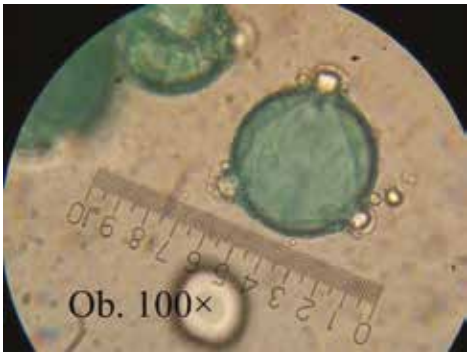

Figure 3. Medium size 3-porate spheroidal pollen grain seen in pale yellow pollen pellets (TB)

Lemon pollen pellets

Lemon coloured pellets were found in both CS1 and CS2 pollen and showed the same medium size 3-colpate, reticulate pollen grain (~ 35 μm) that is similar to that of Family *Brassicaceae* (*Brassica* sp.). Figure 4 shows a triangular (convex) shape in polar view, with 3 angular colpi and a thick exine (~ 2-3 μm). The granules have pollenkitt.

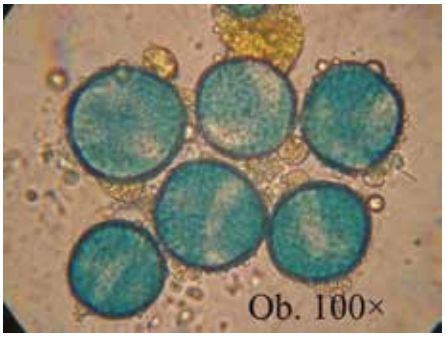

Figure 4. 3-Colpate reticulate medium size pollen grains found in lemon coloured pollen pellets (TB)

Orange pollen pellets

Orange pellets were also present in CS1 and CS2 bee pollen samples. Microscopic images showed medium sized (~ 30 μm) 3-aperturate (porate) spheroidal grains, fenestrate, echinate and surrounded by a lot of pollenkitt (Figure 5). This pollen is *Taraxacum*-type pollen found in the *Asteraceae* Family, most likely it is dandelion pollen (*Taraxacum officinale*).

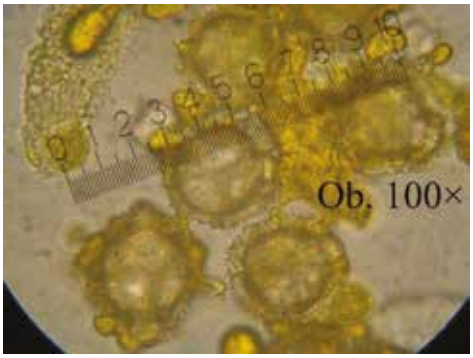

Figure 5. Fenestrate pollen grains with large drops of pollenkitt (*Taraxacum officinale*)

Firebrick pollen pellets

Large size 3-porate pollen grains (~ 70-80 μm) were seen in firebrick coloured pollen pellets that were found in CS1 pollen. The shape of this pollen in polar view is triangular convex, with pores on the corner of the grain. Equatorial view shows elliptic (tall) pores, the size of the polar axis is ~ 50 μm, the size of the equatorial axis is ~75-90 μm (Figure 6). There is a baculate surface pattern (Figure 7). This pollen could be *Geranium* sp., Family *Geraniaceae.*

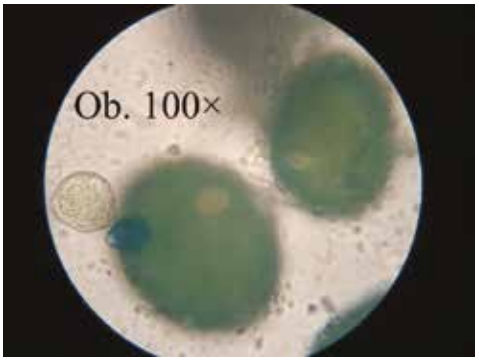

Figure 6. Large size 3-porate pollen grains from firebrick pollen pellets (side view) (TB)

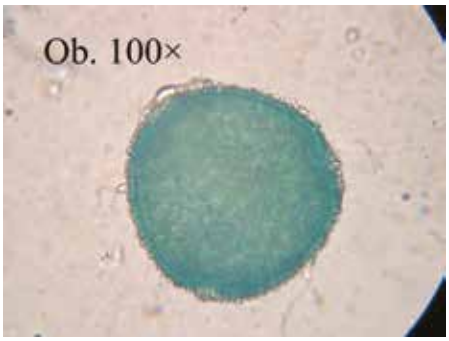

Figure 7. Polar view of 3-porate pollen grain from firebrick pollen pellets, the image shows the surface pattern (TB)

Black pollen pellets

Large size (~ 50 μm) 3-colporate, reticulate, prolate pollen grains were found in CS1 pollen. The apical view shows the angular position of the apertures and a triangular (convex) contour, lateral view shows long colpi that intersect oval pores that have annullum (Figures 8, 9). The pollen could be from Family *Fabaceae*, for example *Trifolium pratense* (red clover).

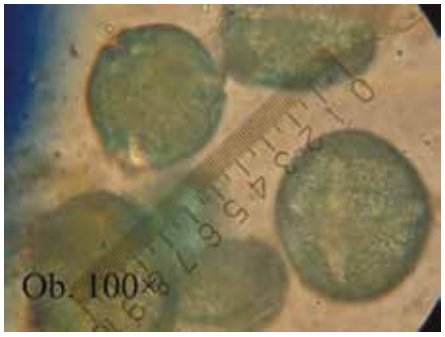

Figure 8. Large 3-colporate pollen grains seen in black pollen pellets, the image shows grains in polar and equatorial view (TB)

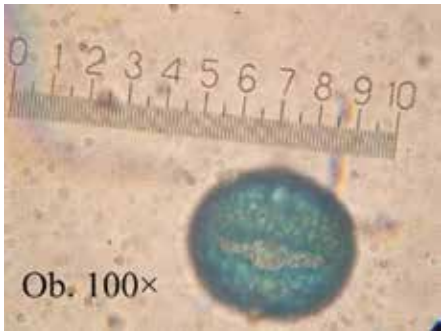

Figure 9. Reticulate 3-colporate, prolate pollen grains, side view (TB)

CONCLUSIONS

The current work provided some information on the pollen composition of the bee pollen samples that were analysed. Several plant families were suggested: *Asteraceae, Brassicaceae, Cucurbitaceae*, for CS2, and *Asteraceae, Brassicaceae, Fabaceae, Geraniaceae, Rosaceae, for CS1,* as well as the anemophilus Family *Pinaceae*.

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INITIAL STEPS TOWARDS THE ESTABLISHMENT OF A POLLEN COLLECTION AT USAMV BUCHAREST: THE STUDY OF ALLERGENIC POLLEN

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Abstract

Pollen allergy (polinosis) is one of the most common allergic seasonal respiratory diseases in Romania. Allergenic pollen is generally anemophilous, it is produced in large quantities, it is light and can be transported by the atmospheric currents and it contains major allergens. The detection of aeroallergens, the inventory of plant species with allergenic potential and the knowledge of their flowering period provide valuable information for both allergy sufferers and allergy physicians. Thus, several European countries have developed aerobiological surveillance networks that make daily bulletins containing data needed to prevent exposure to local allergenic pollen. Considering the theoretical and practical importance of this subject and the current need to develop a network of aerobiological monitoring laboratories in our country, the aim of the present work was the morphological study of the pollen with allergenic potential found in Romania by microscope analysis and the establishment of a collection of pollen images at our laboratory. Such a collection is needed for comparisons and further identification of pollen grains from the air.

Key words*: pollen allergy (polinosis), allergenic pollen, optical microscope.*

INTRODUCTION

Pollen grains are the most important cause of outdoor allergies. The pollinic allergens are water-soluble proteins or glycoproteins found in the cytoplasm of the pollen grains but they can be released and after contact with the airway mucosa or the conjunctiva of allergic individuals specifically sensitised they can trigger an IgE antibody-mediated allergic reaction in seconds (Taketomi et al., 2006).

According to RNSA (2019) the type of pollinic allergen determines the allergenicity potential of a particular pollen, but the allergy risk is due to two more factors: the size of the pollen grain, since smaller pollen are lighter and will stay longer in the air and the quantity of the pollen that is released by a plant, since that influence the risk of exposure too.

During the last several decades airborne pollen and fungal spores started to be recorded permanently by special pollen monitoring stations with air sampling equipment distributed in numerous European contries (Thibaudon & Monnier, 2015). This analysis is coupled with the mapping of the allergenic pollen vegetation to help prevent environmental exposure which is important for the whole population, but mostly for allergy sufferers and atopic children. The aerobiological monitoring is based on the microscopic counting and identification of pollen grains at regular intervals and needs to be carried out by trained specialists (aerobiologists) who apply specific methods and standards (Galán et al., 2014; Garcia-Mozo, 2017). Because of the difficulties and the time consuming nature of this manual analysis, the aerobiological monitoring is carried out in certain centers only and is not able to provide information for the whole territory of a country.

According to Raţiu (1971) the research in aeropalynology has started in Romania in the 60s with the first iatropalynological study having the title "Determination of the degree of pollen infestation of the atmospheric air in Bucharest and the sensitizing value of some allergens prepared from pollen" (Bulla et al., 1963), this was followed by other studies (Seropian et al., 1963; Popescu et al., 1965; 1966; 1969; Capetti et al., 1969). Thus, it was found that 1-25% of the medically investigated allergies are pollinoses, being caused mainly by *Poaceae* pollen, by pollen of some *Asteraceae*, by poplar (*Populus*) and linden (*Tilia*) (Raţiu,

1971). Also, some studies indicated a higher incidence of polynoses in the sub-Carpathian regions, which have a different climate, but have similar vegetation represented by deciduous forests dominated by oak, beech, poplar, birch and other species, as well as plains with grasses, *Asteraceae*, *Plantaginaceae* etc. (Popescu & Capetti, 1971).

More recently, microscopic identification of pollen from allergenic species was carried out in Oradea area (Pallag et al., 2011) and the first centre for aerobiological study of pollen was opened at the Biology Department of the West University of Timișoara (1999-2012) (Ianovici & Faur, 2004; Ianovici, 2007a; 2007b; 2016) and the second at the Colentina Clinical Hospital in Bucharest (2014-2016) (Leru et al., 2018).

MATERIALS AND METHODS

The vegetation that produces allergenic pollen found in Romania includes species of trees, grasses and weeds and is spread throughout the country (Table 1) (Berghi, 2012). High allergenicity potential have the *Poaceae*, *Betula*, *Corylus*, *Quercus*, *Platanus*, *Ambrosia*, *Artemisia* and *Parietaria* (RNSA, 2019). *Poaceae* pollen is the most important allergenic pollen in Europe where it has a large distribution and there are high sensitisation rates among allergy suferers (McInnes et al., 2017). Working with allergenic pollen should follow specific safety regulations, any exposure, even if very small, entails some risk of sensitisation at any age.

The present study was carried out at the Laboratory of Biology of the Faculty of Biotechnologies, the University of Agronomic Sciences and Veterinary Medicine of Bucharest, using fresh plant inflorescences collected during spring 2018 and the herbarium of the laboratory.

The analysis of pollen from the herbarium: from the 12 species of *Poaceae* that are considered important due to their allergy risk, 4 species were found with flowers in the laboratory collection, namely: *Hordeum vulgare*, *Lolium perenne*, *Phleum pratense* and *Poa pratensis*; of the 8 weed genera that are considered important due to their allergy risk only two were found with flowers in the laboratory collection, namely *Plantago lanceolata* and *Urtica* sp. (*U. dioica* and *U. urens*). Instead of *Amaranthus* sp., *Celosia cristata* was used since its inflorescence was very well preserved, this genus has very similar pollen grains with *Amaranthus retroflexus* and with *A. graecizanus* (Tarnavschi et al., 1981, pp. 44).

Table 1 Grasses, trees and weeds that produce allergenic pollen found in Romania

	Plant family	Genus/species
Grasses	*Poaceae*	*Alopecurus pratensis* (meadow foxtail) *Anthoxantum odoratum* (sweet vernal grass) *Avena sativa* (oat) *Dactylis glomerata* (cock's-foot) *Festuca rubra* (red fescue) *Holcus lanatus* (meadow soft grass) *Hordeum vulgare* (barley) *Lolium perenne* (perennial ryegrass) *Phleum pratense* (timothy-grass) *Poa pratensis* (blue grass) *Secale cereal* (rye) *Triticum aestivum* (*T. vulgare*) (common wheat)
Trees	*Aceraceae*	*Acer* sp. (maple)
	Betulaceae	*Alnus* sp. (alder) *Betula* sp. (birch) *Carpinus* sp. (hornbeam) *Corylus* sp. (hazel)
	Cupresaceae	*Juniperus* sp. (junipers) *Thuya* sp.
	Fagaceae	*Castanea* sp. (chestnut) *Fagus* sp. (beech) *Quercus* sp. (oak)
	Juglandaceae	*Juglans* sp. (walnut)
	Moraceae	*Morus* sp. (mulberry)
	Oleaceae	*Fraxinus* sp. (ash) *Ligustrum* sp. (privet)
	Platanaceae	*Platanus* sp. (plane trees)
	Salicaceae	*Populus* sp. (poplar) *Salix* sp. (willow)
	Tiliaceae	*Tilia* sp. (lime tree/linden)
	Ulmaceae	*Ulmus* sp. (elm)
Weeds	*Asteraceae*	*Ambrosia elatior (A. artemisiifolia)* *A. trifida* (ragweeds) *Artemisia vulgaris* (mugwort) *Xanthium strumarium* *X. commune* (cockleburs)
	Amaranthacee	*Amaranthus* sp.
	Plantaginaceae	*Plantago lanceolata* (plantain)
	Polygonaceae	*Rumex* sp. (dock, sorrels)
	Urticaceae	*Urtica* sp. (nettle) *Parietaria officinalis* (common pellitory)

Pollen was analysed from fresh flowers in the case of *Acer pseudoplatanus, Betula verrucosa (B. pendula, B. alba), Corylus avellana, Fraxinus excelsior, Juglans regia, Ligustrum vulgare, Platanus* sp.*, Quercus robur, Salix caprea, Tilia* sp. and wild grasses.

Pollen wet mounts with or without staining (toluidine blue - TB) were analysed using an optical microscope Micros Austria. To measure, an ocular micrometer was used, the calibration ratio was 1 µm for ob. 100× and 2.5 µm for ob. 40×. Microscopic images of the pollen grains were photographed with a Sony Cyber-shot® digital camera (Carl Zeiss Vario-Tessar 5× zoom lens) and were later used to describe the grains. Comparisons were made with pollen descriptions found in the literature (Tarnavschi et al., 1981, 1987, 1990; Şerbănescu-Jitariu et al., 1994) or on various Internet sites (Pollen-Wiki - Der digitale Pollenatlas, Stebler, 2019a; the PalDat - Palynological Database or the Pollen Atlas of the Medical Faculty of Wiena, Berger, 2019). The classification of pollen according to size is from Stebler (2019b).

RESULTS AND DISCUSSIONS

A selection of images of the allergenic pollen grains analysed in the present study are presented in Figures 1-18. There were:

- triporate pollen grains: *Betula verrucosa*, *Corylus avellana*, *Urtica* sp.;
- tricolpate pollen grains: *Acer pseudoplatanus, Fraxinus excelsior, Platanus* sp.*, Quercus robur*;
- periporate pollen grains: *Juglans regia, Celosia cristata, Plantago lanceolata;*
- tricolporate pollen grains: *Ligustrum vulgare, Salix caprea, Tilia* sp.;
- monoporate pollen grains: *Poaceae.*

The triporate pollen grains of the two *Betulaceae* that were analysed have similar grain shapes, exine pattern and protruding pores (onci), however these are larger in *Corylus* than in *Betula* and in *Betula* a vestibulum is present (Figures 1-4). Birch pollen is small (~ 25 µm) while hazel has medium size pollen (~30 µm).

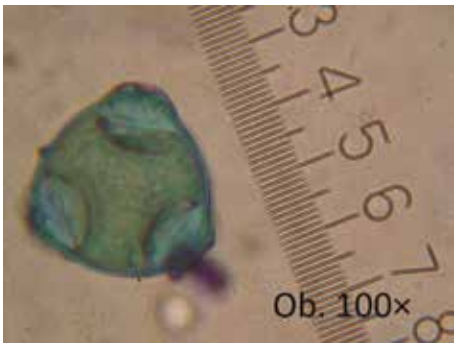

Figure 1. *Corylus* pollen, polar view (TB)

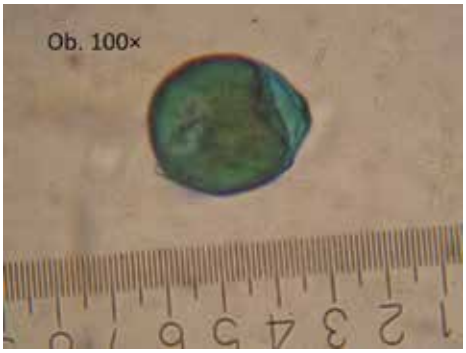

Figure 2. *Corylus* pollen, lateral view (TB)

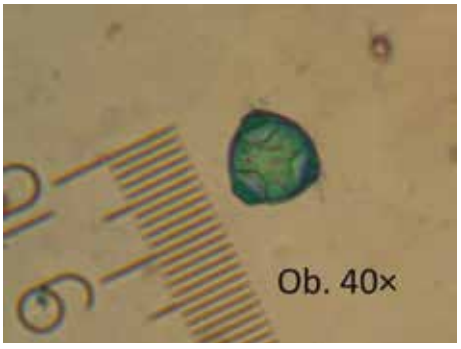

Figure 3. *Betula* pollen, polar view (TB)

Figure 4. *Betula* pollen, optical section (TB)

Triporate *Urtica* pollen (both *U. dioica* and *U. urens*) is similar to the pollen of *Parietaria,* and has small size and psilate exine (Figure 5).

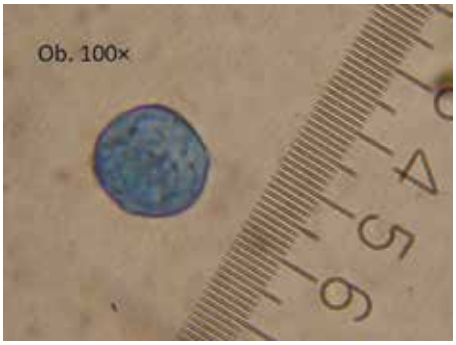

Figure 5. *Urtica* pollen, polar view (TB)

The four tricolpate pollen grains that were analysed (maple pollen, oak pollen, plane tree pollen, ash pollen) could be difficult to distinguish, they have similar polar and equatorial shapes and small to medium sizes (Figures 6-11). Therefore the colpi length, width, shape of the colpi apex and the surface pattern of the grain must be considered.

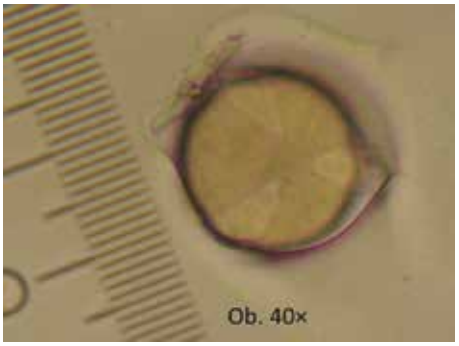

Figure 6. *Acer* pollen, polar view

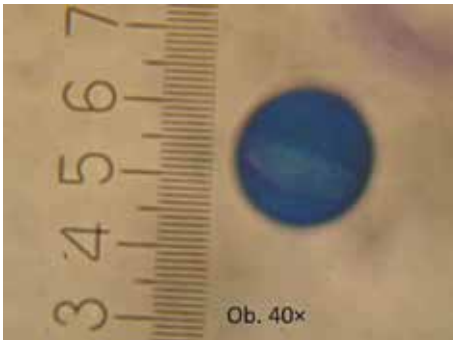

Figure 7. *Acer* pollen, lateral view (TB)

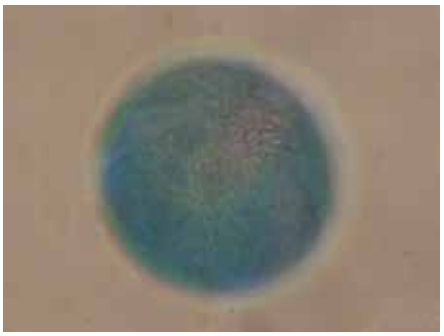

Figure 8. *Acer* pollen, striate exine (TB)

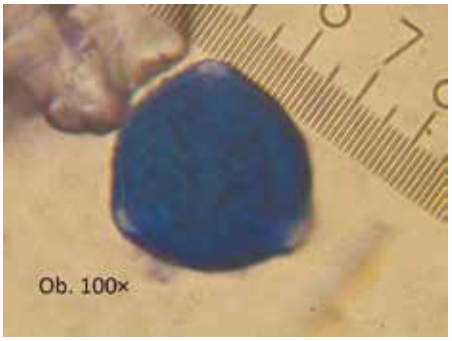

Figure 9. *Quercus* pollen, polar view (TB)

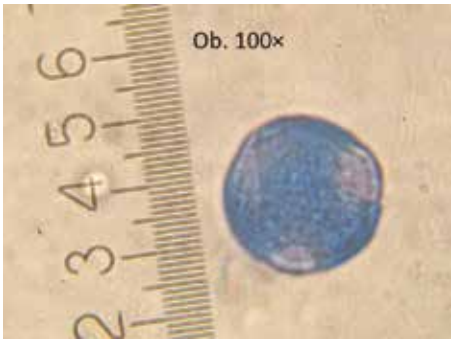

Figure 10. *Platanus* pollen, polar view (TB)

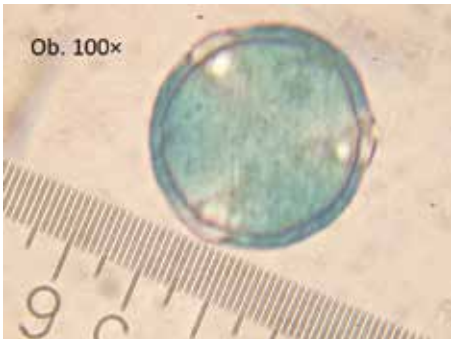

Figure 11. *Fraxinus* pollen, polar view (TB)

The periporate pollen grains that were analysed (*Juglans regia, Celosia cristata, Plantago lanceolata*) have a medium size and a spheroidal shape. Both *Plantago* and *Celosia* pollen are pantoporate (Figures 12, 13), but *Juglans* pores can be distributed unevenly and have a lenticular thickening around them (onci) (Figure 14).

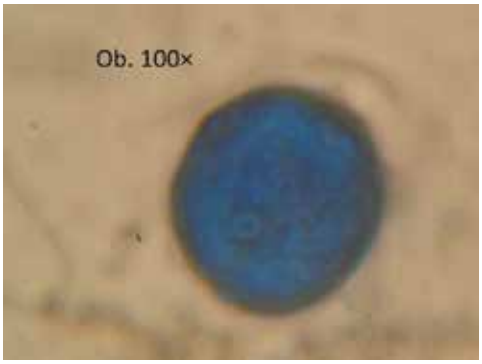

Figure 12. *Plantago* pollen, pantoporate, operculate (TB)

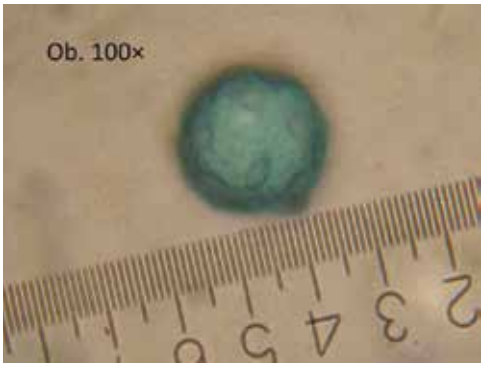

Figure 13. *Celosia* pollen, pantoporate, verrucate exine (TB)

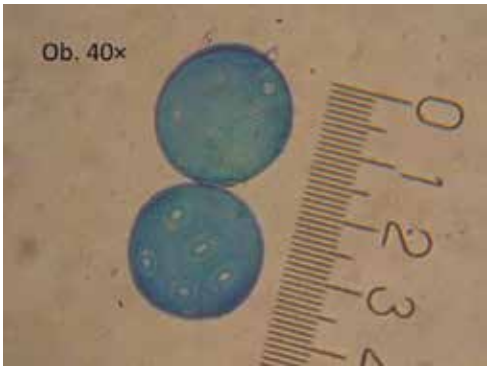

Figure 14. *Juglans* pollen, size > 40 µm (TB)

The analysis of the tricolporate pollen grains, namely *Ligustrum vulgare, Salix caprea* and *Tilia* sp. included grains that have different shapes and characteristics. The pollen of *Ligustrum vulgare* is medium size, has a triangular convex shape in apical view, it is oblate in equatorial view and on the surface has a reticulate pattern (Figure 15).

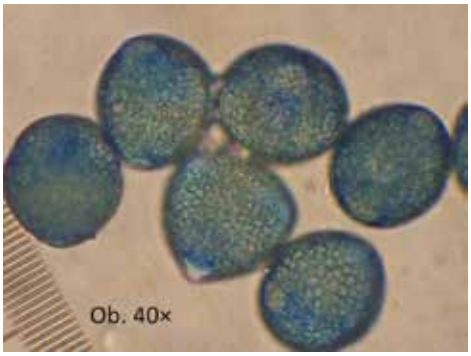

Figure 15. *Ligustrum* pollen, polar and lateral view, ~45 µm (TB)

Tilia pollen is medium size, the polar shape is triangular convex with mid-wall apertures that have a thickening around them, the equatorial shape is oblate (Figure 16).

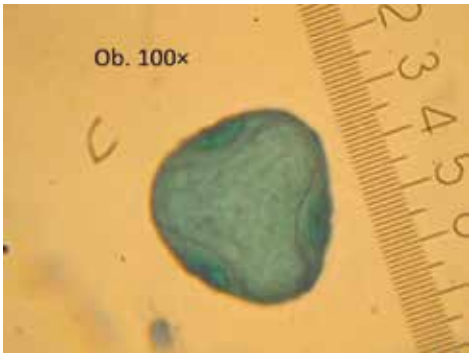

Figure 16. *Tilia* pollen, polar view, ~35 µm (TB)

Salix caprea has a small pollen, is circular with a reticular pattern (Figure 17).

The fresh *Poaceae* pollen, similar to the one from the herbarium showed the characteristics of the pollen of this family: monoporate, annulate, heteropolar and of small or medium size (only some cultivated *Poaceae* have large size pollen grains) (Figure 18).

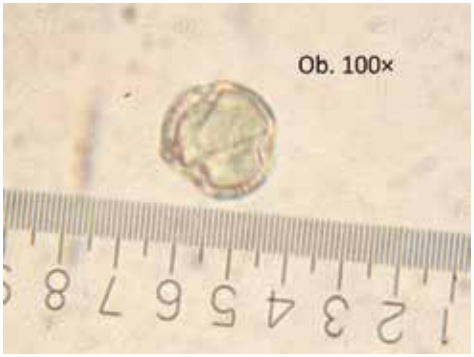

Figure 17. *Salix* pollen, ~18 µm

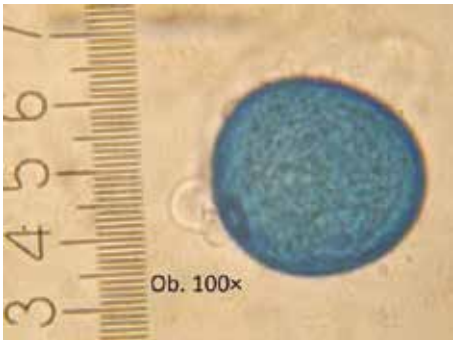

Figure 18. Medium size *Poaceae* pollen

CONCLUSIONS

Although the number of anemophilous plants is high, there are only about 20 allergenic pollen types that are being monitored. The knowledge and experience of their morphological forms alow that a visual pollen counting could be achieved at magnification 400×.

Knowledge of the potential allergy risk that some plants have is important in landscaping, especially in the case of trees and ornamental grasses, but also in the prevention of growth of invasive allergenic species such as *Ambrosia.*

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