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SUMMARY

SUBSECTION I: AGRICULTURAL BIOTECHNOLOGY

SUBSECTION II: FOOD BIOTECHNOLOGY

SUBSECTION III: MEDICAL AND PHARMACEUTICAL BIOTECHNOLOGY

SUBSECTION IV: ENVIRONMENTAL BIOTECHNOLOGY

AGRICULTURAL BIOTECHNOLOGY

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GENETIC DIVERSITY AMONG SOME PEARS GENOTYPES FROM WEST PART OF ROMANIA ON THE BASE OF RAPD MARKERS

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Abstract

An important tool in fruit tree breeding programs is genetic variation. In present work were studied the genetic diversity of some pears genotypes from West part of Romania. For this way we used 10 randomly amplified polymorphic DNA (RAPD) primers. Five out of the 10 primers used in this study amplified clear and reproducible bands. The RAPD primers produced 62 bands, and 46 of them were polymorphic, with an average of 12.4 amplicons/primer. The polymorphic bands, registered per primer ranged from 6 (OPA18) to 10 (primer OPA12). Total polymorphism generated by a certain primer (PIC), registered values between 0.39 P11 0.45 OPA18. The discrimination index (PI), presented values among 2.72 for the primer OPA18 and 5.38 for P-25 primer, which had the highest capacity to generate polymorphic bands to genotypes being studied. Results showed the suitability of RAPD analysis in genetic diversity studied of pear landraces.

Key words*: genetic diversity, RAPD, pears.*

INTRODUCTION

Rosaceae is one of the large and diverse family of plants that includes a multitude of important plant species, such as some fruit trees that are economically important. This family is the third most important plant family in temperate zones with over 100 genera and 3,000 species (Dirlewanger et al., 2002; Shulaev et al., 2008; Lo et al., 2012). *Pyrus communis* is the most commonly cultivated pear species in Europe, America, and Africa (Bell, 1990). In previous decades, the identification and assessment of pear species was based on botanical and chemotaxonomic characters (Challice and Westwood, 1973). In recent decades, the introduction of modern and more productive varieties of pears grown in special orchards has led to a drastic decline in diversity and genetic erosion of ancient varieties. Local varieties of pears "took refuge" on the hills and at the foot of the mountains, where it stubbornly continues to grow in people's orchards, in small clumps and compact, forming a real "resistance movement" in the face of "invasion" very productive improved hybrids and varieties. In horticulture Randomly amplified (RAPD) polymorphic DNA techniques have been used extensively for germplasm identification and progeny testing (Gogorcena et al., 1994; Polito et al., 1994; Tancred et al., 1994; Bartolozzi et al., 1998). Sustainable use and conservation of plant genetic resources is a necessity for future food security. Advances in biotechnology have created new opportunities for genetic resources, conservation and use (Rao, 2004). The environment has an influence on botanical characteristics of plants, making this technique (molecular biology) more valuable because it is based directly on genetic structure (Morell et al., 1995). Attempts to characterize pear genetic variability by DNA molecular markers have been rare. In fruit tree RAPD have been used to analyse different aspects of plant genomes, including taxonomic classification and genetic diversity (Chaparro et al., 1994; Lisek et al., 2010; Oliveira et al., 1999) construction of genetic maps (Liebhard et al., 2003),

identification of progenies of cross pollination (Zamani et al., 2010), marker-assisted selection (Zhang et al., 2014), population structure (Walisch et al., 2015). These multilocus markers are not just fast, simple, and sensitive, but also universal, making useful for comparing related genera and species at the DNA level. For future food security conservation and sustainable use of plant genetic resources is a necessity. New opportunities for genetic resources, conservation and use, have been generated with the help of advances in biotechnology (Rao, 2004). DNA marker based on (PCR) - are versatile tools in different aspects of genomic studies. It is possible to analyse, closely related genera in order to evaluate their phylogenic relationships by using DNA molecular markers. The aim of the study was to investigate at the molecular level some of the pear landraces from different county to obtain more information about the genetic relationships of these trees.

MATERIALS AND METHODS

In this study were used a total of 10 pear landraces belonging to three county from West part of Romania (Table 1).

Table 1. Biological material

Landraces	County	Landraces	County
1. Par rosu	Caras-Severin	6. Lubenicarka	Mehedinti
2. Lubinite	Caras-Severin	7. Par de Balvanesti	Mehedinti
3. Malaiete	Caras-Severin	8. Par de Malovat	Mehedinti
4. Albe de Sf. Petru	Timis	9. Mici galbene	Timis
5. Limunka	Mehedinti	10. Marganesc	Caras-Severin

Leaf samples of pears were stored at -80ºC until use. Genomic DNA was extracted from pear leaves according to CTAB method (by Doyle, 1987). The PCR reaction was performed with the following protocol: two minutes for 94°C, for 40 cycles. Each cycle consisted of 1 min at 95°C, 10 sec at 50°C, 15 sec at 45°C, 20 sec at 40°C, 1 min at 35°C, 30 sec at 45°C and 1 min 45 sec at 72°C and a final extension step for 5 min at 72°C. Finally, the mixture was kept at 4°C until electrophoresis. Following amplification, the PCR products (10 μL) were loaded in 2% agarose gels, stained with ethidium bromide in Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), and separated by electrophoresis, and photographed on an ultraviolet trans illuminator. Each gel was analysed, by scoring the presence (1) or absence (0) of bands for the genetic relationship analysis. A dendrogram was constructed based on the similarity matrix. In view of the potential characterization of different molecular marker systems to evaluate inter population variability in the studied genotypes, different parameters were calculated:

- the total polymorphism generated by a certain primer (PIC = Polymorphic Information Content) which indicates its discriminatory power.

$$PIC = 1 - \sum_{i=1}^{n} P_{ij}^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2$$

Pi - frequency of allele i; Pj - frequency of allele j; Pij - frequency of allele i for locus j; n - the total number of loci.

- the discrimination index (PI), which certifies the effectiveness of a particular primer in detecting polymorphism.

$$PI = \sum PIC$$

Genetic similarity among genotypes studied calculated through coefficient Jaccard, which was recommended to be used for dominant markers ISSR, RAPD, taking in view that the absence of a bands was associated to a homozygous loci. JC= a/(a+b+c), where a, b, c, represented the commons and un-commons of those genotypes (Dangi et al., 2004). On base of genetic similarity matrix among landraces, it was made the dendrogram using the method of clusters average.

RESULTS AND DISCUSSIONS

For the analysis of the genetic polymorphism of the pears landraces included in the study, 10 RAPD primers were tested. Of these primers, 5 primers produced clear, reproducible, and informative patterns were scored. A total of 63

fragments (loci) were generated. The fragments length ranged from 100 to 1750 base pairs (bp). This is in accordance with the results reported by (Teng et al. 2002). Each primer generated a number of bands which varied from 11 in OPA18 to 14 in OPA12 respectively, with an average of 12.6 bands/primer (Table 2). These findings are also in line with those reported by Oliveira et al. (1999) and Monte-Corvo et al. (2000) in studies related to pear identification using RAPD markers. Among the 63 amplified loci, 46 showed polymorphism with an average of 9.2 polymorphic bands/primer that was higher than those reported by Cho et al. (2012), while lower than what was reported by Monte-Corvo et al. (2000). The total polymorphism generated by a particular primer (PIC) recorded values between 0.392 for the P11 primer and 0.453 for the OPA18. The efficiency of a particular primer in detecting polymorphism is given by the discriminatory index (PI), which recorded values from 2.72 for the OPA18 primer and 5.38 for the P25 primer, which has the highest capacity to generate polymorphic bands.

Table 2. Polymorphism rate through RAPD primers

Primer	Primer sequence 5'- 3'	Length of the fragments	Number of amplified fragments	Number of polymorphic fragments	% de polymor phism	PIC $\overline{x} \pm s_{\overline{x}}$	PI
P25	GCACTGAGTA	175-1200	14	12	85.71	0.448±0.046	5.38
P11	GCTGCTCGAG	150-1050	13	10	76.92	0.392±0.057	3.92
OPA 13	CAGCACCCAC	150-1500	11	8	72.72	0.412±0.061	3.3
OPA 12	GGGTAACGCC	150-1750	14	10	71.42	0.418±0.053	4.18
OPA18	AGGTGACCGT	100-1450	11	6	54.54	0.453±0.073	2.72
	Total		63	46			
	No bands/primer		12.6	9.2			

Based on genetic similarity, pears landraces were classified hierarchically into two clusters, with an average diversity of about 37%. The first group has a complex structure and includes four landraces that own about 68% of the common alleles of the five primers. The 78% genetically similar Limunka and Mărgănesc landraces make up a first subcluster, along with the St. Peter's White landraces, which differs by about 26% from the two previously presented. The populations in the first subcluster have flattened yellow spherical fruits ripening at the end of August. The population of St. Peter collected from Timis county has greenish yellow pear-shaped fruits. The second cluster is composed of the landraces from Mehedinti County, Malovăţ pear and Lubenicarka, between which there is a genetic similarity of 73%, together with the landraces of Mălăieţe and Lubiniţe from Caras-Severin. The populations of this cluster are characterized by green-skinned fruits with red spots and red flesh. The fruits are small, pear-shaped. There is an average diversity of about 35% between the populations in these two clusters. Bălvăneşti pear and Small yellow landraces represent a separate group that owns approximately 75% of the common alleles of the five primes. These populations, even if they come from different areas, have fruits with similar shapes and color, small yellow. The interpopulation similarity for the alleles of the five RAPD markers had values ranging from 53.97% between Păr roşu and Lubiniţe to 79.37% between Limunka and Mărgănesc.

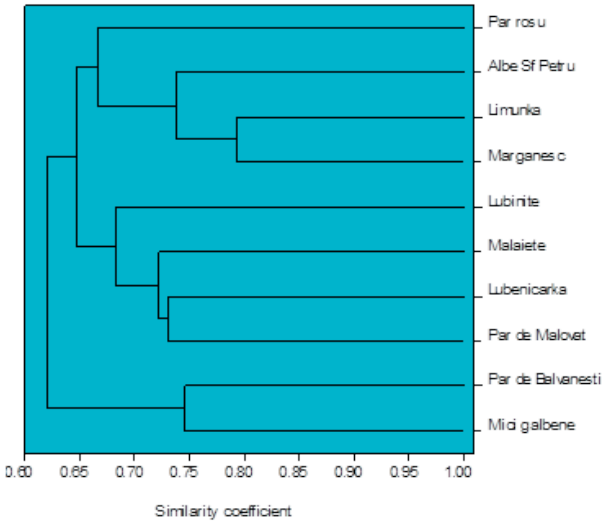

Figure1. UPGMA clustering of pear landraces using RAPD primers

Table 3. Similarity matrix between pear landraces using RAPD primers

Landraces	1	2	3	4	5	6	7	8	9
1. Par rosu	1								
2. Lubinite	0.5397	1							
3. Malaiete	0.6190	0.6984	1						
4. Albe de Sf.Petru	0.6508	0.6032	0.6508	1					
5. Limunka	0.7825	0.6032	0.5873	0.7460	1				
6. Lubenicarka	0.6825	0.6349	0.7143	0.6190	0.6825	1			
7. Par de Balvanesti	0.6190	0.6032	0.5873	0.6508	0.6825	0.5556	1		
8. Par de Malovat	0.6349	0.7143	0.7302	0.6349	0.6984	0.7302	0.5714	1	
9. Mici galbene	0.5873	0.7302	0.7460	0.6508	0.5556	0.5556	0.7460	0.5714	1
10. Marganesc	0.6667	0.6508	0.6984	0.7302	0.7937	0.7302	0.7467	0.7143	0.6032

CONCLUSIONS

RAPD markers can be used in studies concerning the genetic variability in pears. The RAPD primers generated 63 bands, and 45 of them were polymorphic, with an average of 12.4 amplicons/primer. The polymorphic bands, registered per primer ranged from 5 (OPA18) to 10 (primer OPA12). Total polymorphism generated by a certain primer (PIC), registered values between 0.39 to P11 and 0.54 to OPA18. The discrimination index (PI), presented values among 2.72 for the primer OPA18 and 5.38 for P-25 primer, which had the highest capacity to generate polymorphic bands to genotypes being studied. Results showed the suitability of RAPD analysis in genetic diversity study of pear. The interpopulation similarity for the alleles of the five RAPD markers had values ranging from 53.97% between Păr roşu and Lubiniţe to 79.37% between Limunka and Mărgănesc. The obtained results will be useful for plant breeding programs.

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PATHOGENICITY OF *Beauveria bassiana*, *B. pseudobassiana*, AND *Metarhizium anisopliae* INDIGENOUS ISOLATES AGAINST *Plodia interpunctella* AND *Galleria mellonella* IN LABORATORY ASSAYS

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Abstract

Using two different bioassay methods, the pathogenicity of three isolates of Beauveria bassiana, one of B. pseudobassiana and one of Metarhizium anisopliae, was evaluated against two model insects, Plodia interpunctella and Galleria mellonella in larval stage. In laboratory conditions, the insects were treated by immersion in a conidial suspension of 1x10^8 UFC/ml and by dusting. Larval mortalities were recorded daily, 14 days post-exposure. All the fungal isolates have been shown to be pathogenic to test insects. Thus, two isolates of B. bassiana determined the highest mycosis percentages for test insects both by immersion and dusting. One B. bassiana isolate (BbTd1) killed P. interpunctella larvae in the shortest time (6 days), and the other isolate (BbTd2) determined the highest mycoses percentage, followed by B. pseudobassiana isolate (BpPa) by both treatment methods. The highest mycosis percentage was determined by BbTd2 and BPa isolates in the dusting treatment. G. mellonella larvae proved to be the least sensitive to fungal treatments applied by immersion (MST>50%). In the dusting treatment, the BbTd1 and MaF isolates induced the highest percentage of mycosis of G. mellonella larvae. All isolates have pathogenicity against test insects, indicating their possible use for biocontrol.

Key words*: Beauveria bassiana, B. pseudobassiana, Metarhizium anisopliae, immersion, dusting.*

INTRODUCTION

By 2050, the number of people to be fed will be as significant as nine billion (Hawkins et al., 2018). Sustainable food production for all populations should be considered, and crop protection has an essential role in maintaining soil and crop yields healthy (Godfray et al., 2010). Pests, pathogens, and unfavorable growing conditions are responsible for up to 26% of crop losses, estimated at over $470 billion worldwide (Bamisile et al., 2021). The evolution of pesticide resistance of pests and pathogens in agriculture and concerns for sustainable agriculture has led to a reassessment of pest control options. In recent years, awareness of the impacts of chemical plant protection products on the environment, soil, biodiversity, and human health has resulted in struggles to reduce dependence on chemical pesticides (Committee on the Environment, Public Health and Food Safety of European Parliament, 2019).

Entomopathogenic fungi are currently used as biological control agents (BCA) in biological control programs and integrated crop protection strategies (IPM) against pests, being an environmentally friendly alternative to chemical pesticides. The idea of using entomopathogenic microorganisms to control harmful insects appeared about two hundred years ago (Tanada and Kaya, 1993). After discovering the entomopathogenic character of some fungi, numerous studies on the interaction with arthropods have been conducted (Steinhaus, 1975). To date, more than 700 species have been described and have been characterized as insect-pathogenic fungi (Khachatourians & Qazi, 2008). The most studied entomopathogenic fungal species are *Beauveria bassiana* (Balsamo-Crivelli), Vuillemin, *Isaria fumosorosea* Wize,

Metarhizium anisopliae (Metchnikoff) Sorokin, and *Lecanicillium lecanii* (Zimmerman) Viegas (Li et al., 2011; Chen et al., 2015). The most common mycopesticides are products formulated from *B. bassiana*, *M. anisopliae*, *B. brongniartii*, and *I. fumosorosea* (Bamisile et al., 2021).

In recent years, an increasing body of molecular evidence has shown that the *Beauveria* genus is divided into cryptic lineages, and new species were described, *B. asiatica*, *B. australis*, *B. kipukae*, *B. pseudobassiana*, *B. sungii*, and *B. varroae* (Rehner et al. 2011).

B. pseudobassiana has many morphological similarities to *B. bassiana* (Wang et al., 2020) and probably it has been confused with this species before. Maybe this is the reason for only a few studies available on host diversity and virulence of *B. pseudobassiana*. Recently *B. pseudobassiana* has been shown to have great potential in the biocontrol of numerous insect pests (Wang et al., 2020).

The path from discovering a BCA in a natural outbreak to its commercialization as a bioinsecticide goes through many laboratory, field, and greenhouses experiments (Dent, 1998).

To be used as a mycoinsecticide, it is necessary to establish the pathogenicity, virulence, temperature ranges, and humidity requirements for germination, infection, sporulation, etc., for each fungal isolate.

The Indian meal moth *P. interpunctella* (Lepidoptera: Pyralidae) is a common cosmopolitan household principally on stored food products and processed food commodities. It is one of the most used lepidopterans as a test insect (Takov et al., 2020) for different research topics such as sexual selection (Gage, 1998; Lewis et al., 2011) and host-parasite dynamics (Sait et al., 1994; Knell et al., 1996).

G. mellonella is widely used as a model insect to evaluate bacterial pathogenesis and virulence (Jønsson et al., 2017; Morales et al., 2019). The size of the larvae makes their manipulation easy. Monitoring survival is also very timely because larvae acquire a dark color due to strong melanization when they die (Contador & Zaragoza, 2014).

G. mellonella and *P. interpunctella* are known to be susceptible to entomopathogenous fungi like *B. bassiana* and *M. anisopliae* so they are used as model host insects in pathogenic investigations (Būda & Pečiulytė, 2008; Hussein, 2011; Baydar et al., 2016; Vertyporokh et al., 2020) and also relatively easy to grow in the laboratory.

This paper aimed to evaluate the pathogenicity of some *Beauveria bassiana*, *B. pseudobassiana,* and *Metarhizium anisopliae* indigenous isolates recovered from insects against the two model test insects, *Plodia interpunctella* and *Galleria mellonella.*

MATERIALS AND METHODS

Test insects: *Galleria mellonella* and *Plodia interpunctella* larvae were obtained from cultures held at Research-Development Institute for Plant Protection (RDIPP) using insect colonies maintained in the laboratory at 23±2°C, in sterile glass gears, 14:10 h, L:D. The larvae were reared on Hydak medium amended with bee wax (350 ml/kg medium).

Fungal material: Three isolates of *Beauveria bassiana*, one isolate of *B. pseudobassiana*, and one isolate of *M. anisopliae* were used in the experiments (Table 1).

Table 1. Origin of fungal isolates

Code	Species	Host insect	Region of isolation
BbTd1	*Beauveria bassiana*	*Tanymecus dilaticollis* (adult)	Tulcea
BbTd2	*Beauveria bassiana*	*Tanymecus dilaticollis* (adult)	Ilfov
BbIt	*Beauveria bassiana*	*Ips typographus* (adult)	Botosani
BpPa	*Beauveria pseudobassiana*	*Pyrrhocoris apterus* (adult)	Giurgiu
MaF	*Metarhizium anisopliae*	*Anoxia villosa* (larva)	Ialomița

They were isolated from natural infected insects and maintained as pure cultures in the Culture Collection of Entomopathogenic Fungi, Department of Useful Organisms (RDIPP Bucharest).

All fungal isolates were grown on sterile barley grains in polypropylene bags (30 x 50 cm). The aerial conidia of *Beauveria* and *Metarhizium* isolates were produced by two-stage and one-stage techniques, respectively (Mascarin and

Jaronski, 2016). After 30 days of incubation at 25˚C, 50 grams of barley kernels colonized by fungus (0.8-9 x10^9 UFC/g) was washed with 40 ml sterile distilled water containing Tween 80 (0.01%). After homogenization, in order to remove coarse impurities, the suspension was filtered through sterile cotton wool. Conidial concentrations were determined using a Burker hemocytometer, at a 400x magnification. The adjustment of the suspension titer was made by dilution with sterile distilled water containing Tween 80 (0.01%).

Bioassay: Batches of 15 or 20 larvae were pre-sorted into separate 80 ml pp tubes (4cm Ø) with snap-top lids just before the testing. The larvae were exposed to the fungus by immersion or by dusting.

In immersion treatment, each batch of insects was transferred on the bottom of a Sartorius funnel covered with filter paper connected with vacuum pump over which 30 ml of each fungal suspension was applied. Sterile-distilled water with Tween 80 was used for control experiments. After 10 seconds, the liquid was absorbed and after one minute, the larvae were transferred individually in sterile, compartmentalized plastic boxes to avoid cross-contamination. Equal amounts of diet were distributed for each insect. There were three replicates (boxes) per treatment and insect species and 15 to 20 individuals per box. Insects were incubated at 25°C. Larval mortalities were recorded daily, for 14 days, after-exposure.

In dusting treatment, groups of 15 larvae of *P. interpunctella* or 20 larvae of *G. mellonella* were sorted into sterile plastic tubes with a capacity of 80 ml (4 cm diameter) provided with a lid over which barley kernels colonized by fungus were inserted to cover the larvae completely and left in contact an hour, after which they were individually transferred to sterile compartmentalized boxes with lids. Each insect was distributed equal portions of food. Insects were incubated at 23± 1ºC, 16:8 L: D, and 50-60% humidity. Mortality of larvae was recorded daily, during 14 days after inoculation. The dead larvae were removed from the box and placed in Petri dishes provided with moistened filter paper and incubated at 25°C for 3-5 days to stimulate the appearance of fungal mycelium. Death due to fungus infections were confirmed by conidia formation on cadavers.

Statistical analysis: the effectiveness of fungal isolates was expressed as a cumulative percentage of mycosis. The virulence of each isolate on each insect species, was estimated by the values of median survival time (MST), calculated by Kaplan Meyer survival curves which were modelled using GraphPadPrism V7 and a log-rank (Mantel-Cox) test was applied to the significance threshold $p < 0.05$ (GraphPad Software, San Diego California USA). The individuals who survived until the end of the observation period were considered censored.

The percentages of larval mortality were transformed (arcsin) and analyzed using variant analysis. The averages were compared using the Tukey test and were considered statistically different at a signification level of 5%.

RESULTS AND DISCUSSIONS

Both insect species have manifested diseases caused by treatment by immersion in conidial suspensions or by direct contact with barley kernels colonized by fungus (dusting). The mycelium that covered the treated larvae confirmed that they died from fungal infection. There were no deaths due to fungal infection in control.

The effect of treatments on the larvae of *Plodia interpunctella*

Immersion treatment

The lowest percentage of survival (the highest mortality) was recorded in the case of larvae treated with *B. bassiana* isolate (BbTd2) after ten days of incubation, followed by the isolate of *B. pseudobassiana* (BpPa), after eight days of incubation (Figure 1).

The comparison of survival curves showed a significant difference in susceptibility of *P. interpunctella* larvae to immersion treatment (X^2=14.56, p=0.0057) with different fungal isolates. All fungal isolates resulted in a significantly lower percentage survival of *P. interpunctella* larvae compared to the control demonstrated by log-rank analyses (Table 2). The laboratory results of the virulence bioassay show the median survival time (MST) for the larvae of test insect, *P interpunctella* assessed at 14 d is presented in Table 3.

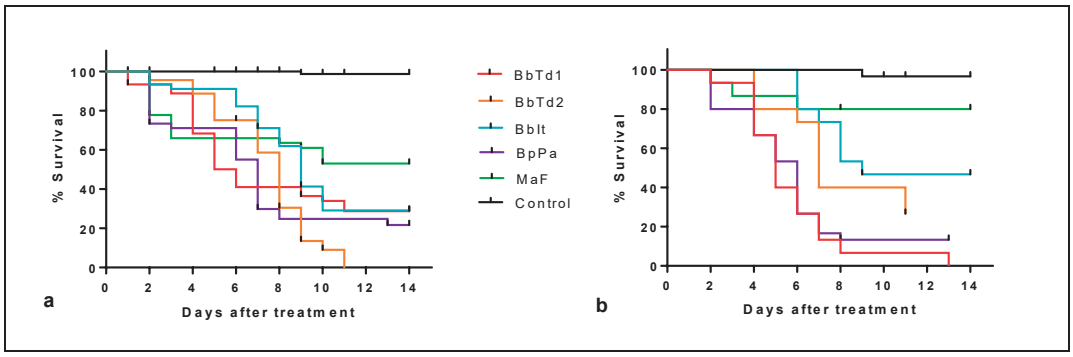

Figure 1. Percentage survival of *Plodia interpunctella* larvae after immersion (a) and dusting (b) treatments with three fungal isolates of *Beauveria bassiana* (BbTd1, BbTd2, and BbIt), one isolate of *B. pseudobassiana* (BpPa), and one isolate of *Metarhizium anisopliae* (MaF), recorded after 14 days

Table 2. Pairwise treatment comparisons using Log-Rank (Mantel-Cox) test based on Kaplan-Meir survival analysis of *Plodia interpunctella* larvae after immersion and dusting treatment

Immersion										
Isolate	**BbTd1**		**BbTd2**		**BbIt**		**BpPa**		**MaF**	
	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P
BbTd1	-	-								
BbTd2	0.50	0.47	-	-						
BbIt	1.64	0.199	9.19	0.002	-	-				
BpPa	0.08	0.769	0.23	0.631	5.81	0.015	-	-		
MaF	2.75	0.096	10.25	0.001	1.91	0.166	7.24	0.007	-	-
Control	15.70	<0.0001	27.58	<0.0001	15.56	<0.0001	20.58	<0.0001	9.04	0.002

Dusting										
Isolate	**BbTd1**		**BbTd2**		**BbIt**		**BpPa**		**MaF**	
	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P
BbTd1	-	-								
BbTd2	5.66	0.0170	-	-						
BbIt	16.10	<0.0001	1.38	0.238	-	-				
BpPa	0.51	0.4735	4.54	0.033	11.04	0.0009	-	-		
MaF	17.89	<0.0001	5.98	0.0145	2.412	0.12	14.87	0.0001	-	-
Control	32.39	<0.0001	17.2	<0.0001	9.308	0.002	22.77	<0.0001	2.86	0.09

Table 3. Median survival time (MST) of the *Plodia interpunctella* larvae treated with isolates of *B. bassiana*, *B. pseudobassiana,* and *Metarhizium anisopliae* for 14 days

Isolate	MST (days)	
	Immersion	Dusting
BbTd1	6	5
BbTd2	8	7
BbIt	9	9
BpPa	7	6
MaF	-	-
Control	-	-

MST= Median Survival Time

The shortest median survival time was registered for larvae treated with suspensions of *B. bassiana* isolate BbTd1 (6 d) followed by *B. pseudobassiana* isolate, BpPa (7 d). The log-rank analyses indicate that the two treatments are statistically similar in terms of survival percentage (X^2=0.08, p=0.769). Because survival of larvae over 14 days was more than 50% (Figure 1), the MST for *P. interpunctella* untreated control and MaF treatment could not be determined. This is according to another experiment, where more than 50% of *P. interpunctella* larvae survived during 14 days after being sprayed with *Paecilomyces farinosus* conidial suspension (2.6×10^6 conidia ml^{-1}) (Būda & Pečiulytė, 2008).

Regarding the percentage of mycosis, statistical analysis indicated that there are no significant differences between treatments. However, the highest rate of mycosis was recorded in the case of treatment with *B. bassiana* BbTd2 isolate (82.2%), and the lowest in the case of treatment with *M. anisopliae* MaF isolate (44.4%) (Figure 2, a). Similar results were

registered by Mantzoukas et al. (2021), who noticed an 86.5% and 50% mortality of *P. interpunctella* larvae when were sprayed with $1x10^8$ conidia ml^{-1} of *Isaria fumosorosea* and *Gnomoniopsis castaneae*, respectively.

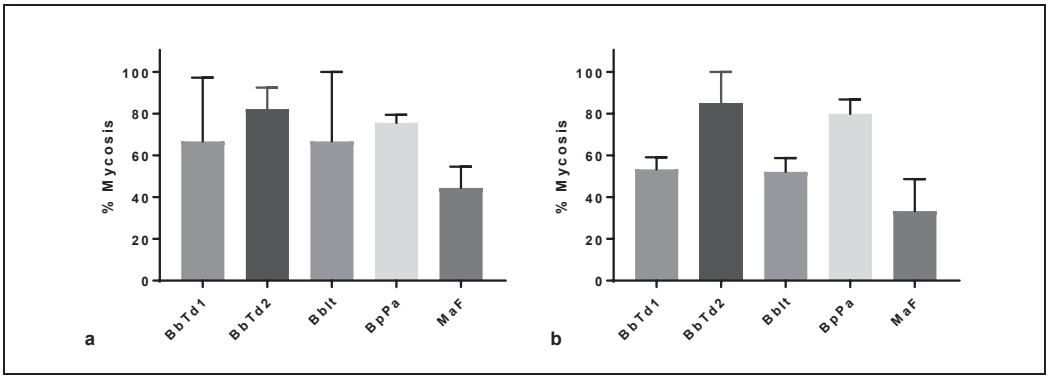

Figure 2. Cumulative percentage of mycosis (±SD) (before arcsin transformation) of *Plodia interpunctella* larvae treated by immersion (a) and dusting (b), with three different isolates of *Beauveria bassiana* (BbTd1, BbTd2 and BbIt), one isolate of *B. pseudobassian* (BpPa) and one isolate of *Metarhizium anisopliae* (MaF)

Dusting treatment

The lowest survival percentage was recorded in the case of larvae treated with BbTd1 followed by BpPa, after eight days of incubation (Figure 1). The log-rank analyses indicate that the two treatments are statistically similar in terms of survival percentage (X^2=0.51, p=0.473). As in the immersion treatment, those two isolates also determined the shortest MST of 5 and 6 days, respectively.

The comparison of survival curves indicated a significant difference in the susceptibility of *P. interpunctella* larvae to treatment with different fungal isolates (X^2=33.34, p=0.0001) by dusting. All fungal isolates resulted in a significantly lower survival percentage of *P. interpunctella* larvae compared to the control demonstrated by log-rank analyses (Table 2).

The method of treating the larvae of *P. interpunctella* by dusting highlighted again the effectiveness of the BbTd2 isolate, which determined the highest percentage of mycosis (85%) (Figure 2, b).

The effect of treatments on the larvae of *Galleria mellonella*

Immersion treatment

The larvae of *G. mellonella* proved to be less sensitive to fungal treatments applied by immersion than the larvae of *P. interpunctella*, the percentage of survival being more than 50% (Figure 3, a), which is why the average survival time could not be calculated. All fungal isolates resulted in a significantly lower percentage survival of *G. mellonella* larvae than the control demonstrated by log-rank analyses (Table 4). Mycosis percentages were also reduced, with the highest being induced by the strains BbTd1 (43.3%) and MaF (37.7%) (Figure 4, a).

This could be due to poor conidial attachment of conidia from aqueous suspension to the insect cuticle. This is in contrast with results from other similar experiments in which *G. mellonella* proves to be more sensible to *Beauveria* action. In a study of genes involved in *B. bassiana* infection to *G. mellonella*, Chen et al. (2018) obtained 90% G. *mellonella* larvae mortality after 99 h post treatment. In another experiment evaluating the virulence of various isolates, a mortality of 100% of *G. mellonella* larvae was due to *B. bassiana* and *M. anisopliae* treatment at concentrations of 10^5 or 10^6 conidia ml^{-1} (Hussein et al., 2011).

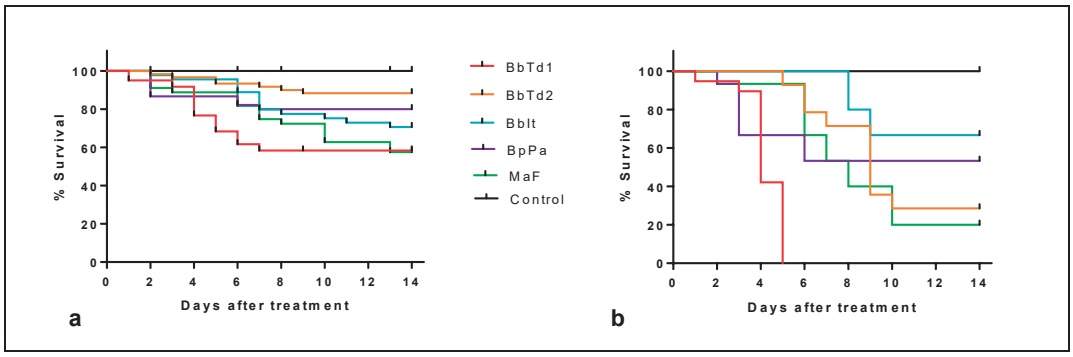

Figure 3. Percentage survival of *Galleria mellonella* larvae after immersion (a) and dusting (b) treatments with three fungal isolates of *Beauveria bassiana* (BbTd1, BbTd2 and BbIt), one isolate of *B. pseudobassiana* (BpPa) and one isolate of *Metarhizium anisopliae* (MaF) recorded after 14 days

Table 4. Pairwise treatment comparisons using Log-Rank (Mantel-Cox) test based on Kaplan-Meir survival analysis of *Galleria mellonella* larvae after immersion and dusting treatment

Immersion										
Isolate	BbTd1		BbTd2		BbIt		BpPa		MaF	
	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P
BbTd1	-	-								
BbTd2	14.29	0.0002	-	-						
BbIt	2.86	0.090	4.78	0.028	-	-				
BpPa	4.85	0.0270	1.51	0.218	0.65	0.418	-	-		
MaF	0.14	0.699	12.20	0.0005	1.62	0.202	3.97	0.046	-	-
Control	35.73	<0.0001	8.26	0.004	22.63	<0.0001	15.06	0.0001	35.49	<0.0001

Dusting										
Isolate	BbTd1		BbTd2		BbIt		BpPa		MaF	
	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P
BbTd1	-	-								
BbTd2	27.3	<0.0001	-	-						
BbIt	31.45	<0.0001	3.84	0.049	-	-				
BpPa	8.21	0.0042	0.23	0.626	1.38	0.239	-	-		
MaF	24.95	<0.0001	0.50	0.475	7.71	0.005	1.08	0.296	-	-
Control	31.45	<0.0001	16.2	<0.0001	5.81	0.015	8.86	0.002	20.14	<0.0001

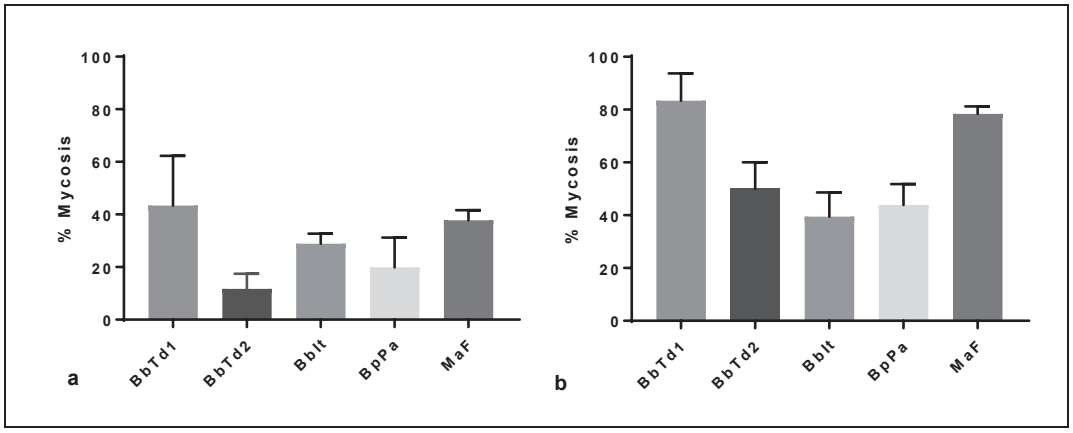

Figure 4. Cumulative percentage of mycosis (±SD) (before arcsin transformation) of *Galleria mellonella* larvae treated by immersion (a) and dusting (b), with three different isolates of *Beauveria bassiana* (BbTd1, BbTd2 and BbIt), one isolate of *B. pseudobassian* (BpPa) and one isolate of *Metarhizium anisopliae* (MaF)

When Oreste et al. (2012) evaluated the pathogenicity of 23 isolates of *B. bassiana* and four of *M. anisopliae* against *G. mellonella* and *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae in laboratory assays, a mortality of 80-100% after 17 days was reported, using 2×10^6 conidia ml^{-1} fungal suspensions, and that two *B. bassiana* isolates killed *G. mellonella* larvae within 2.2 and 2.3 days, respectively. But Kryukov et al. (2018) reported 63% mortality after 12 days due to mycosis of *Galleria* larvae treated with 10^8 conidia ml^{-1} of *B. bassiana* occurred. The authors state that the increase of the mycosis percent was due to the transmission of conidia between wax moth larvae in clusters. Other entomopathogenic species like *Purpureocillium lilacinus* prove to be highly infectious for *G. mellonella,* causing 100% larval mortality within seven days post-immersion with 1×10^8 conidia ml^{-1} and a median lethal time (LT50) value of 1.83 days (Demirci & Altuntaş, 2019). Using the agar surface technique, *P. lilacinus* demonstrated very low infectivity of only 30% larval mortality on *G. mellonella* in 10 days and an LT50 value of 16.16 days (Baydar et al., 2016).

Dusting treatment

The comparison of survival curves (Figure 3) indicated a significant difference in the susceptibility of *G. mellonella* larvae to treatment with various fungal isolates (X^2=55.01, p<0.0001) by dusting.

The comparison of survival curves indicated that *B. bassiana* isolate BbTd1 is significantly more effective than all the other fungal isolates (Table 4) killing the larvae treated by dusting within the shortest time (4 d) (Table 5).

Table 5. Median survival time (MST) of the *Galleria mellonella* larvae treated with isolates of *Beauveria. bassiana*, *B. pseudobassiana* and *Metarhizium anisopliae* for 14 days

Isolate	MST (days)	
	Immersion	Dusting
BbTd1	-	4
BbTd2	-	9
BbIt	-	-
BpPa	-	-
MaF	-	8
Control	-	-

MST= Median Survival Time

For the BbIt and BpPa isolates, the average survival times could not be calculated. Dusting treatments with BbTd1 and MaF isolates induced the highest percentage of mycosis of *G. mellonella* larvae of 83% and 78%, respectively (Figure 4).

About the efficacy of treatment related to the kind of bioassay (with "dry" conidia by direct contact or dusting and "wet" conidia by dipping or spray), our study shows that it depends on the target pest, *G. mellonella* being more susceptible to fungus infection applied by dusting than by immersion. The treatments of *Rhynchophorus ferrugineus* with dry conidia of *B. bassiana* induced significant adult mortality compared to the dipping method (Ricaño et al., 2013; Güerri-Agulló et al., 2010). The laboratory and greenhouse trials results found dry conidia of *M. anisopliae* to be more effective than wet conidia in infecting mosquitoes (*Culicoides nubeculosus*), causing 100% mortality after five days (Ansari et al., 2011). In contrast, the mortality of 1st larval instar of both *H. variegata* and *C. undecimpunctata* and pupal stage of *C. undecimpunctata* were significantly increased with the spray method only (Sayed et al., 2021).

CONCLUSIONS

The obtained results demonstrated the efficacy of pathogenicity screening to isolate entomopathogenic fungi to control insect pests, and the availability of indigenous virulent entomopathogenic fungi, which can be exploited for the development of sustainable crop protection strategies.

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STIMULATION OF SEABUCKTHORN (*Elaeagnus rhamnoides*) MICROBIAL SYMBIOSES

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Abstract

This paper reviews the present knowledge regarding the stimulation of the microbial symbioses of the sea buckthorn (Elaeagnus rhamnoides syn. Hippophae rhamnoides). Sea buckthorn is an actinorhizal plant, developing nitrogen-fixing symbioses with the actinobacteria from the Frankia genus. At the same time, sea buckthorn roots can form endomycorrhizal symbioses with various arbuscular mycorrhizal (AM) fungi. AM symbiosis increases nutrient uptake and nutrient use efficiency, especially on phosphorus and micro-elements. Due to these microbial symbioses, sea buckthorn is an efficient colonizer of marginal lands and a suitable crop for low-inputs organic/ecological farming. Helper bacterial strains were demonstrated to promote microbial symbiosis between Frankia actinobacteria or AM fungi with roots of other host plants. Only scarce and indirect information suggests the involvement of gram-positive, endospore-forming bacteria as a helper of microbial symbiosis for sea buckthorn. Also, there is no information regarding the role of rhizosphere signals in promoting the sea buckthorn microbial symbioses. The finding of this paper highlights the need for future works focused on the stimulation of sea buckthorn symbioses.

Key words*: sea buckthorn symbioses, Frankia actinobacteria, arbuscular mycorrhizal fungi, bacterial helper, rhizosphere signals.*

INTRODUCTION

Sea buckthorn, *Elaeagnus* (synonym *Hippophae*) *rhamnoides* (L.) A. Nelson cultivation is continuously expanding due to its high economic value and significant ecological benefits (Ciesarová et al., 2020). *E. rhamnoides* is a deciduous, thorny shrub native to different regions of Europe and Asia, with high resistance to cold and drought.

Eight subspecies of *E. rhamnoides* were described: *E. (H.) rhamnoides* spp. *fluviatilis* Soest, from Alpes, radiating also in Apennines and Pyrenees; *E. (H.) rhamnoides* spp. *rhamnoides*, from northwestern Europe; *E. (H.) rhamnoides* ssp. *carpatica* Roussi, Carpathian mountains, low Danube basin, northwestern shore of the Black sea; *E. (H.) rhamnoides* ssp. *caucasica* Rousi, between Black and Caspian sea; *E. (H.) rhamnoides* spp. *mongolica* Rousi, from the Altai Mountains, Lake Baikal basin, and Outer Mongolia; *E. (H.) rhamnoides* spp. *sinensis* Rousi, from western China and Inner Mongolia; *E. (H.) rhamnoides* spp. *turkestana*, from the northern part of Himalaya; *E. (H.) rhamnoides* spp. *yunnanensis*, from western China, Yunnan, western Sichuan (Swenson & Bartish, 2002).

In its wild state, the sea buckthorn has been recognized for millennia as a plant with health benefits in different parts of the world - in Europe, by the ancient Greeks, and in Asia, in early Chinese Pharmacopeia and ayurvedic medicine (Suryakumar & Gupta, 2011; Wani et al., 2016).

In the last decades, sea buckthorn started to be domesticated (Li & Schroeder, 1996). Various chemometric and molecular genetic techniques were developed to characterize the affiliation of different domesticated cultivars to different subspecies: *E. rhamnoides* subsp. *carpatica*, from Romania (Buzoianu & Socaciu, 2014); *E. rhamnoides* ssp. *sinensis*, *E. rhamnoides* ssp. *yunnanensis*, *E. rhamnoides* ssp. *turkestana* and *E. rhamnoides* ssp. *mongolica*, from China genetic pool (Liu et al., 2018), *E. rhamnoides*

ssp. *mongolica* from Russia, China, and Mongolia (Ruan et al., 2004), *E. rhamnoides* ssp. *fluviatilis* from Latvia (Lacis & Kota-Dombrovska, 2014).

The economic and ecological benefits of sea buckthorn are directly related to its ability to form microbial symbioses, with nitrogen-fixing actinobacteria and with arbuscular mycorrhizal (AM) fungi. This paper aims to review the present knowledge related to the stimulation of the sea buckthorn symbioses and the importance of such technological intervention to enhance further the economic value and the ecological benefits of sea buckthorn cultivation.

Our goal is to highlight the existing gap in knowledge. The perspectives of the better exploitation of sea buckthorn symbioses by their stimulation through technological intervention are also considered and discussed.

BENEFITS OF SEA BUCKTHORN CULTIVATION

The high economic value of the sea buckthorn is determined by the beneficial effects on human health of the active ingredients from its fruits and leaves (Gatlan & Gutt, 2021).

Fruits are used to produce a nutritious, healthy beverage and seed oil (Beveridge et al., 1999). The pulp remaining from the mechanical extraction of juice and oil is further used for the production of food additives (X. Guo et al., 2019) and tocopherols (Kitrytė et al., 2017).

The healthy beverage produced by mechanical squeezing of the berry pulp retains most of the hydrophilic antioxidants (i.e., hydrophilic polyphenols/flavonoids and vitamin C/ascorbic acid) and the lipophilic bioactive ingredients, i.e., carotenoids, tocopherols, flavanols, and (mono)unsaturated fatty acids, including ω-7 palmitoleic acid (Bal et al., 2011; Ciesarová et al., 2020).

Due to this unique combination of active ingredients, sea buckthorn healthy beverage is highly efficient as a dietary supplement in various health conditions (Ursache et al., 2017). Sea buckthorn is efficient against metabolic disorders and associated cardiovascular diseases (Olas, 2016). Sea buckthorn has demonstrated antiproliferative effects on human liver and colon cancer cell lines (Grey et al., 2010) and on prostate, breast, and gastric adenocarcinoma (Boivin et al., 2007).

The antioxidant effect of sea buckthorn juice is involved in the prevention of both cancer and cardiovascular diseases (Olas & Skalski, 2022; Olas et al., 2018). Scavenging of the reactive oxygen species (ROS) and the resulting modulation effect on ROS level are also related to the immunomodulatory and anti-inflammatory effects (Ren et al., 2020).

E. rhamnoides is one of the few natural sources of the rare palmitoleic acid (16:1 n-7), a monounsaturated fatty acid (MUFA) with high physiological significance (Dąbrowski et al., 2022). Palmitoleic acid prevents and reverses the metabolic syndrome by increasing insulin sensitivity (Hu et al., 2019), mainly due to its lipokinine function, i.e., an endo-signal of adipose tissue (Frigolet & Gutiérrez-Aguilar, 2017). The carotenoids from sea buckthorn healthy beverage synergize the palmitoleic acid reversing effect on metabolic syndrome (Marcelino et al., 2020; Matsumoto et al., 2021). Carotenoids level is very high in sea buckthorn beverages, their specific color being related to this high carotenoids content (Pop et al., 2015).

The hydrophilic antioxidants from the sea buckthorn healthy beverage, ascorbic acid, and polyphenols also target metabolic syndrome and type II diabetes (Liu et al., 2019; Zheng et al., 2020). The flavonoids existing in the healthy beverages that are obtained from *E. rhamnoides* berry, ursolic acids (Grey et al., 2010), flavonoid aglycones, including quercetin, isorhamnetin, and kaempferol (Guo et al., 2017) and flavonol glycosides (Enkhtaivan et al., 2017), were proven to have pro-apoptotic and antiproliferative activity of the sea buckthorn extracts.

The oil obtained from sea buckthorn seeds is emollient and it is used in various cosmetic products (Beveridge et al., 1999). The dried sea buckthorn pomace has a high nutritional value due to its high content in lipids with essential fatty acids, proteins with high content of essential amino acids, and prebiotic fibers (Nour et al., 2021).

The leaves of sea buckthorn are used for the production of tea (Ma et al., 2019), and various types of extracts are used for cosmetic and

dietary supplements (Asofiei et al., 2019; Criste et al., 2020).

The health effects of sea buckthorn are enhanced by organic farming. Organic farming has been proved to increase the polyphenols and flavonoids content on sea buckthorn leaves (Heinäaho et al., 2006) and fruits (Heinaaho et al., 2009). Sea buckthorn is an appropriate plant to be cultivated in organic farming because of its microbial symbioses with nitrogen-fixing actinobacteria and mycorrhizal (AM) fungi (Li et al., 1996; Tian et al., 2002). These symbioses significantly support fertilization and plant protection organic management.

The ecological benefits of sea buckthorn crops are water conservation and soil formation (La, 2020), marginal land colonization and reclamation of the degraded land (Enescu, 2014), and soil decontamination by immobilization of potentially toxic elements (Nowakowska et al., 2017). The ecological benefits of the sea buckthorn cultivation are also significantly promoted by its symbioses with nitrogen-fixing actinobacteria and AM fungi (Constandache et al., 2016; Zhao et al., 2013).

BENEFITS OF SEA BUCKTHORN SYMBIOSES

The symbioses between sea buckthorn and actinobacteria from *Frankia* genera generated nitrogen-fixing nodules. The difference between actinorhizal nodules and legume nodules is in hosting bacterial symbiont. In legume nodules, rhizobia differentiate in organelle-like structures, called symbiosomes, and in actinorhizal nodules, the bacteria remain not-differentiated (Holmer et al., 2017). The actinorhizal nodules are nitrogen-fixing nodules, the bacteria fixing atmospheric oxygen based on the carbohydrates supplied by the sea buckthorn (Nguyen & Pawlowski, 2017).

The sea buckthorn roots form endomycorrhizal symbioses with various arbuscular mycorrhizal (AM) fungi. AM fungi are essential for the mobilization of soil phosphorus and phosphorus acquisition by the plant (Smith et al., 2011). Besides the phosphorus, AM fungi also increase the bioavailability and uptake by the plant root of the microelements (Willis et al., 2013).

The benefits of the microbial symbioses are not related only to improved nutrient acquisition (Figure 1). Due to extended microbial symbioses, sea buckthorn is an excellent colonizer of marginal and/or degraded lands (Enescu, 2014). Both actinorhizal and AM symbioses immobilize potentially toxic elements (e.g., Cu ions), by different mechanisms - by detoxification by metallophore in the case of *Frankia* (Mohr et al., 2021) and by immobilization in the fungal mycelium by AM fungi (Cabral et al., 2015).

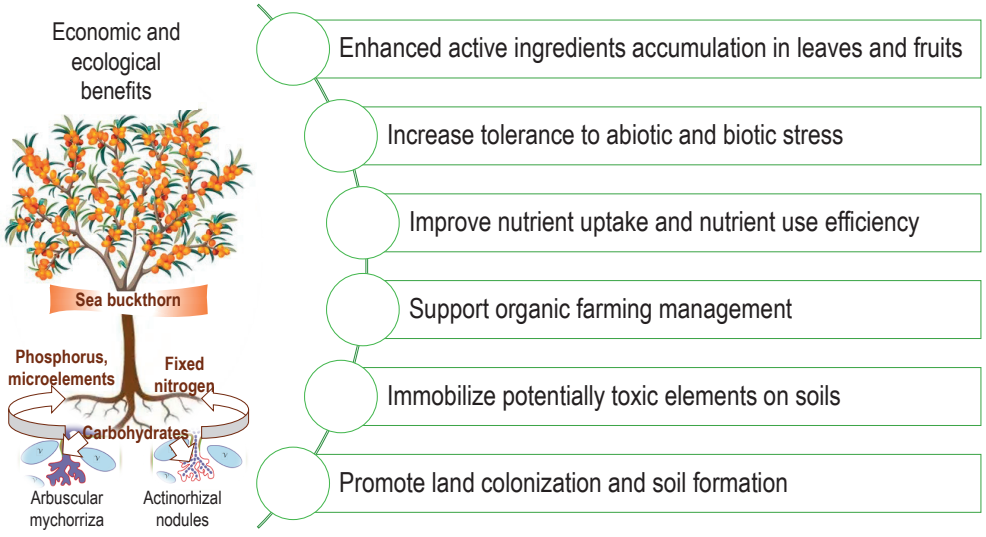

Figure 1. Illustration of the benefits of the microbial symbioses for the sea buckthorn

The benefits of microbial symbiosis for sea buckthorn are not limited to the support for the colonization of marginal and poor lands. The nitrogen fixation and improved phosphorus and microelement nutrition support organic farming management (Kalia et al., 2011). The AM symbiosis determines and improves nutrient uptake and nutrient use efficiency, leading to higher mineral content in sea buckthorn leaves (Jaroszewska et al., 2016). The actinorhizal symbiosis increase plant tolerance to abiotic stress (Diagne et al., 2013). Symbiosis with AM fungi also determines an increased tolerance to abiotic stress (Begum et al., 2019).
Several of the effects of microbial symbiosis on sea buckthorn performances are presented in Table 1. Actinorhizal and mycorrhizae symbioses enhance sea buckthorn performance, both in terms of economic added value and ecological services.

Table 1. Effects of the microbial symbioses on sea buckthorn performances

Symbiosis	Effect	Reference
Actinorhizal symbiosis	Ability to grow in soil with low available nitrogen	(Li et al., 2014)
Actinorhizal symbiosis	Detoxification of the potentially toxic element ions – e.g., Cu^{2+}	(Mohr et al., 2021)
Actinorhizal symbiosis	Enhancement of the content of the active ingredient	(Kanayama et al., 2008)
Mycorrhizae symbiosis	Enhanced growth in marginal land	(Zhang et al., 2020)
Mycorrhizae symbiosis	Increased level of mineral and bioactive ingredients in sea buckthorn leaves	(Jaroszewska & Biel, 2017; Jaroszewska et al., 2016)
Mycorrhizae symbiosis	Increased level of mineral and bioactive ingredients in sea buckthorn fruits	(Jaroszewska et al., 2018)

The microbial agents that form symbioses with the sea buckthorn have the characteristics of the microbial plant biostimulants. By definition, plant biostimulants enhance/benefit nutrient uptake, increase tolerance to abiotic stress and improve crop quality (du Jardin, 2015). AM fungi were included for almost a decade in the category of microbial plant biostimulant (Rouphael et al., 2015). Symbiotic nitrogen-fixing bacteria from *Frankia* genera were not yet considered plant biostimulants. However, the rhizobia that produce nitrogen-fixing nodules in legumes are already included in this category of microbial plant biostimulants (Hendriksen, 2022). These microbial plant biostimulants need better exploitation in the sea buckhorn farming system, especially in the organic farming system.

TECHNOLOGICAL INTERVENTION FOR STIMULATION OF SEA BUCKTHORN SYMBIOSES

Despite the importance of microbial symbioses for sea buckthorn cultivation, the technological interventions intended to stimulate/amplify the formation and development of such symbioses are insufficiently studied.
The microorganism from sea buckthorn symbioses interact in a synergic manner (Zhou et al., 2017), and dual inoculation stimulates plant growth and development and nitrogen fixation (Tian et al., 2002).
Actinobacteria from *Frankia* genera and AM fungi use common rhizosphere exo-signals to detect their host - e.g., flavonoids (Abdel-Lateif et al., 2012). The exchange of exo-signals between microbial symbionts and their host is promoted by the humic and fulvic acids (Capstaff et al., 2020; Gryndler et al., 2005).
Several rhizobacteria enhance the formation of symbiosis - helper bacteria for mycorrhizae and nitrogen-fixing bacteria (Frey-Klett et al., 2007; Ghodhbane-Gtari et al., 2021; Teng et al., 2015). These stimulation means could be included in technological interventions intended to enhance the formation of microbial symbioses (Table 2).

Table 2. Technological interventions that enhance microbial symbioses

Technological intervention	Target	Reference
Application of flavonoids to rhizosphere	Amplification of communication between microbial symbionts and their hosts	(Sugiyama, 2021)
Application of humic acid to the soil	To facilitate the exchange of exo-signals between microbial symbionts and their hosts	(Capstaff et al., 2020; Gryndler et al., 2005)
Application of polyamine rich material to the soil	Improvement of host reaction to symbiotic agents	(Atici et al., 2005; Cheng et al., 2012)
Inoculation with helper bacteria AM fungi	Support communication and interaction between hosts and AM fungi	(Frey-Klett et al., 2007)
Inoculation with helper bacteria for Frankia	Enhance tolerance to abiotic stress of the symbioses partners	(Ghodhbane-Gtari et al., 2021)

These stimulation means could be considered as a second plant biostimulant, intended to synergize the microbial sea buckthorn

biostimulants - *Frankia* nitrogen-fixing bacteria and AM fungi. These could lead to the development of next-generation plant biostimulants with synergistic biostimulatory action (Rouphael & Colla, 2018). Figure 2 illustrates the concept of such synergist of microbial sea buckthorn plant biostimulant, leading to enhanced economic and ecological benefits from sea buckthorn cultivation.

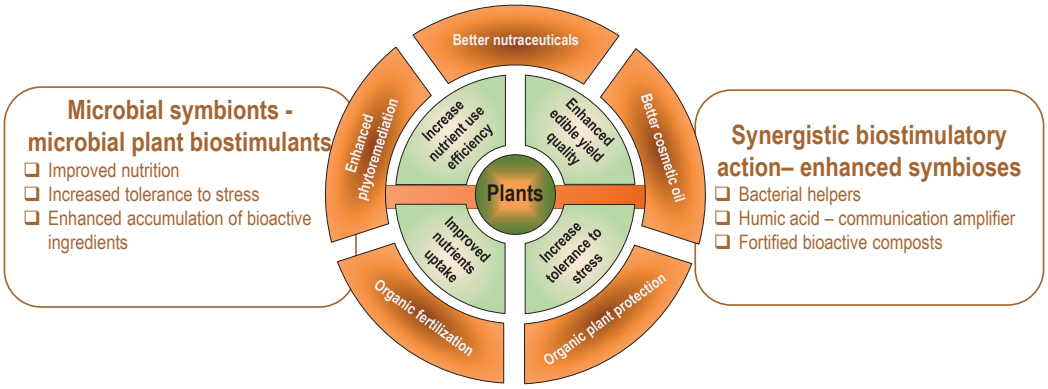

Figure 2. Illustration of the enhancers of the sea buckthorn symbiosis as next-generation plant biostimulants, with synergistic biostimulatory action

One integrated technological intervention is the application of bioactive composts fortified with AM fungi/*Frankia* bacteria helper. Bioactive composts include a large quantity of humic acids (Guo et al., 2019). Humic acid support communications between microbial symbiont and host plant roots (Shah et al., 2018).

The by-products from sea buckthorn berries harvesting, branches and leaves, are a good substrate for bioactive and biofortified compost production. The high flavonoid content of branches and leaves could further support the formation of sea buckthorn symbioses (Yang et al., 2009). Utilization of the by-products from sea buckthorn berries harvesting to produce a complex sea buckthorn biostimulant is an example of the circular bioeconomy, with direct economic benefits and ecological service (Xu & Geelen, 2018).

CONCLUSIONS

Sea buckthorn forms actinorhizal nitrogen-fixing symbioses with bacteria from *Frankia* genera and multifunctional symbioses with AM fungi. The sea buckthorn symbiotic microbes fulfill the characteristics of microbial plant biostimulants.

The stimulation means of sea buckthorn microbial symbionts represent next-generation plant biostimulants with synergistic biostimulatory action.

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RESEARCH ON BIOCONVERSION OF LIGNOCELLULOSIC WASTE FOR THE CULTIVATION OF BIOCOMPOUNDS PRODUCING MACROMYCETES

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Abstract

Macromycetes are a topic of great interest for researchers around the globe and in our country, mushrooms being well-known for their nutritional, gourmet and medicinal values. In line with current ecological trends, a sustainable solution for environmental protection is to produce mushrooms by bioconversion of some lignocellulosic waste/by-products of agro-forestry origin and therefore to examine the qualitative and quantitative impacts of multiple substrate recipes upon mushroom production. Assiduous research has improved the biotechnologies for the production and propagation of mycelium used for seeding spawn, in parallel with obtaining and characterization of extracts rich in bioactive compounds from both mycelium and fruiting bodies of edible and medicinal species of macromycetes. This review proposes a current presentation of the knowledge at the intersection of these research directions, focusing on their applications, targeting the species of the genus Pleurotus.

Key words*: biocompounds , bioconversion, macromycetes, mycelia, Pleurotus spp.*

INTRODUCTION

A future-oriented bioeconomy, based on renewable resources for replacing petroleum materials and chemicals, can fulfill important environmental, social and economic requirements for a sustainable development of modern society. Many innovative products and materials derived from renewable sources have already been developed in the context of the green economy concept. A sustainable waste management strategy might be successfully implemented by cultivating the well-known group of oyster mushrooms for the bioconversion of agro-industrial wastes achieved by myco-remediation in an economically efficient manner (El-Ramady et al., 2022). Lignocellulosic matter, found in agricultural, industrial and forest residuals accounts for more than half of all vegetal biomass produced on the planet and is used in biotechnological applications including cosmetics, medicines, foods and feeds, biofuels, biopesticides, biofertilizers and a variety of other products. This scientific overview is focused on the recovery of lignocellulosic residues from different industries for the generation of nutritious and suitable substrates for the cultivation of edible and/ or medicinal macromycetes.

Edible mushrooms have been a component of human civilization from the ancient period. They've made a significant contribution to mankind's history due to their organoleptic and appealing culinary traits, edible species gaining enormous popularity. Mushrooms are trendy these days due to their multiple nutritional and health benefits (Fulgoni and Agarwal, 2021). Mushroom industrial cultivation, as a notable biotechnological process, displays a worldwide expanded and economically valuable industry that generate protein rich foods by fungal bioconversion of cellulosic materials. *Pleurotus* species are the second most widely cultivated edible mushroom in the world after *Agaricus* spp. Many edible species of macromycetes with significant therapeutic, biotechnological, gourmet and environmental applications are present in this genus (Comandini and Rinaldi, 2020; Melanouri et al., 2022). In recent years, the worldwide production of *P. ostreatus* was estimated to be more than 4.1 million tons, being cultivated on a variety of lignocellulosic based substrates (Hřebečková et al., 2020).

Unlike some other species, *Pleurotus* spp. (oyster mushrooms) are the simplest, fastest and least expensive to cultivate, with more than 100% biological efficiency, which combined with its distinct flavor, aroma and excellent drying and preservation qualities, secures its status as a delicacy. The cultivation of *Pleurotus* mushrooms for the recovery and reuse of lignocellulosic biomass provides an opportunity to use renewable energy sources in the production of protein-rich aliments that might ensure food security for people in underdeveloped countries. Mushrooms cultivation is one of the most economically effective methods for the bioconversion of lignocellulosic wastes (Cohen et al., 2002; Sanchez et al., 2002).

Mushrooms are rich in high-quality protein, have a large amount of dietary fibers and contain numerous vitamins and minerals. The presence of secondary metabolites isolated from both mushroom fruiting bodies and mycelia is responsible for the multidirectional health promoting and therapeutic applications. The biologically active compounds found in oyster mushrooms include polysaccharides, peptides, proteins, terpenes, fatty acid esters, polyphenols etc. This biocompounds exhibit anti-diabetic, anti-neoplastic, antioxidative, immuno-stimulatory and a plenty of other human health-enchasing properties (Alam et al., 2009; Jayakumar et al., 2011; Wasser, 2014, Chilanti et al., 2022). This review summarizes the current state of knowledge regarding the recovery of lignocellulosic waste of agro-forestry origin through the cultivation of edible and/or medicinal macromycetes of the genus *Pleurotus*, an overall picture of the *in vitro* mycelium manipulation biotechnology and the production of fruiting bodies, the current level of industrialization of the entire process of obtaining the spawn and its fruiting potential in the substrate, all of that proceeding in parallel with an increased attention to the main biologically active compounds.

LIGNOCELLULOSIC BIOMASS

Because billions of tons of lignocellulosic biomasses are collected worldwide each year, researchers are increasingly interested in its recovery, being extremely essential to capitalize on the bioconversion and valorisation of these materials as efficiently and productively as possible. Improper waste disposal can have negative environmental, health and socio-economic consequences. In recent years, there has been a serious interest in converting these resources into value-added goods. Nevertheless, the potential of these materials is restrained due to the complex and rigid structure of lignocellulosic waste, which necessitates advanced depolymerisation of the three main components: lignin, cellulose and hemicellulose to break them down into victual units. These polymers are associated with each other in a hetero-matrix of varying proportions and different composition depending on the type, species and the source of the biomass (Chandra et al., 2007; Carere et al., 2008). Lignocellulose is the main constituent of both woody and non-woody plants such as grass or weeds and is a substantial source of renewable organic matter, making them a valuable source for biotechnological substrates (Table 1).

Table 1. Composition of grain straws (source: Tian et al., 2018)

Lignocellulosic biomass	% dry matter		
	Cellulose	Hemi cellulose	Total lignin
Wheat straw	30	22	17
Rice straw	31	22	13
Corn stover	38	26	17
Barley straw	34	22	14
Rye straw	31	22	25
Oat straw	39	27	18

Lignin is the most abundant natural organic polymer found in plant cell walls; an hetero polymorphic network of phenyl propane units (p-coumaril, coniferyl and synaptic alcohol) which limits the action of enzymes in the hydrolysis of lignocellulosic raw materials. Lignin is the most challenging polymer to process in the lignocellulosic biomasses preventing the enzymatic and microbiological hydrolysis of the biomass due to its tight interaction with cellulose microfibers. Chang and Holtzapple (2000) revealed that reducing lignin improves biomass digestibility. Because lignin is hydrophobic, it prevents water from penetrating the cell walls, therefore protecting cellulose and hemicellulose (Mokhothu and John, 2015), being resistant to chemical and enzymatic degradation and responsible for the preservation of lignocellulosic biomass against

bacterial activity (Mussatto and Teixeira, 2010; Isikgor and Becer, 2015). Because of its challenging structural breakdown, lignin is very difficult to be depolymerized by enzymatic hydrolysis, which further impedes the decomposition and utilization of cellulose and hemicellulose. The most significant constraint to the large-scale industrial implementation of biological conversion is lignocellulose's low degradation efficiency. Because lignocellulose is very resistant to decomposition directly by microorganisms, a pretreatment is required to breakdown the structure of lignocellulose matter and subsequently depolymerize lignin (Bhatia et al., 2020), fortunately the cultivation of macromycetes does not require such treatments due to the very efficient enzymatic system of mycelia.

Cellulose is a polymer formed of ß-D-glucopyranose linked by -1,4 glycosidic bonds, the main component of the plant cell walls that provides structural support, also being found in bacteria and fungi. In nature, the degree of polymerization of cellulose chains ranges from 10 to 15 thousand units of glucopyranose. Cellulose molecules are consistently organized into microfibers, which are then grouped into cellulose fibers. The structure of cellulose is mostly determined by the existence of covalent and hydrogen bonds along with the Van der Waals forces. Hydrogen binding in a cellulose microfibril determines the linearity of the chain, but the inter-dependence of hydrogen bonds induce the structure (crystalline) or disturbance (amorphous) of the cellulosic structure. Cellulose is primarily used in the manufacture of paperboard and paper. Smaller amounts are transformed into a broad range of derivative products, including cellophane and rayon. As a sustainable energy source, cellulose from crop residues is being converted into biogas and ethanol. Because of its advantageous properties including its biocompatibility, hydrophilicity and reactive hydroxyl groups, cellulose is a versatile resource for derivate materials such as films, composites, fibers, fuels and chemicals (Baig, 2020).

Hemicelluloses are a mixture of polysaccharides and vegetable gums found in plant cell walls along with cellulose and lignin. Hemicellulose are firmly connected to cellulose microfibrils and lignin, composing a complex network of covalent linkages that provide structural support (Speight and Radovanovic, 2020). Hemicelluloses are made up of a variety of sugars including: xylose, arabinose, glucose, mannose, galactose and rhamnose. The proportion of the compounds is different depending on the type of the source, which is mostly xylan in hardwood and glucomannan in softwood. Hemicelluloses have a random, amorphous structure, with lower breakdown resistance. They are easily hydrolysed by diluted acids or bases, as well as a wide variety of hemicellulase enzymes. Because of their structural diversity, hemicelluloses can be used as thickeners, emulsifiers, stabilizers and binders in a wide range of industries including foods, cosmetic, pharmaceutical and agricultural (Spiridon and Popa, 2008). The amorphous nature of hemicellulose, as well as its low degree of polymerization and pretreatment processes, make it ideal for usage in a variety of industrial applications such as hydrogels, drug carriers and cosmetics (Ashokkumar et al., 2022).

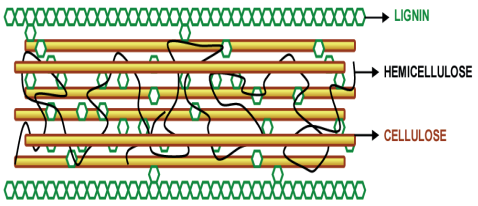

Figure. 1 Lignocellulose structure showing cellulose, hemicellulose and lignin fractions (source: Mussatto and Teixeira, 2010)

Fungi's ability to efficiently degrade lignocellulose (Figure 1) containing materials is due to their highly efficient enzymatic system. The hydrolytic system, which includes cellulase and hemicellulase that are accountable for polysaccharide breakdown, together with the ligninolytic enzymes are responsible for the transformation and degradation of the lignocellulosic biomass. The bioconversion of residues is based on the mechanism characteristic of basidiomycetes to produce enzymes called ligninases: phenol oxidases (laccase) or hem-peroxidases (mangan peroxidases and lignin peroxidases) and thus can solve the complex problem of high-cost degradation of cellulose, hemicellulose and lignin (Adebayo and Carrera, 2015; Gutiérrez-

Soto et al., 2015). The ligninolytic enzymes referenced above have applications in food processing, cosmetics, biosynthesis of fine chemicals and the production of biofuels (Kumar and Chandra, 2020).

MUSHROOM CULTIVATION

Because of their economic importance, *Pleurotus* species have been the most dynamic group of mushrooms in terms of culture expansion and production volume reported globally over the last decade. An impressive evolution of the biotechnologies involved in the production of the spawn, the biological material needed for "seeding", was implicated among with the economical worth in the spectacular development of the *Pleurotus* cultivation. According to the current systematics, *Pleurotus* species are part of the most evolved phylum of fungi - *Basidiomycota*, order *Agaricales*, family *Pleurotaceae*, genus *Pleurotus*. The main cultivated species and varieties are: *P. ostreatus, P. pulmonarius, P. columbinus, P. florida, P. citrinopileatus, P. eryngii* and *P. sajor-caju.* These lignivorous/xylophagous macromycetes sprout in nature on dead wood waste and, along with other *Pleurotus* group members, are highly effective agents for the biological recycling of a very numerous lignocellulosic agro-forestry waste or by-products from the food, textile, paper and other industries. By cultivating them, as with other edible/therapeutic mushroom species, organic matter from lignocellulosic by-products is directly converted into human food via a sustainable biotechnological process. Since the second half of the past century, the worldwide popularity of mushrooms has grown due to the development of the cultivation technique, an increased knowledge of their therapeutic benefits supported by scientific studies and the new trends in healthy diets that have required the use of rich alternative protein sources. It is intended that mushroom cultivation would become a significant industrial activity in rural development programs, resulting in economic growth for the communities. Given the technological advances, the worldwide mushroom business is in the phase of a high-tech sector in several affluent nations of Europe, Asia and America, with extremely high levels of industrialization. Edible grown mushrooms have become a symbol of recovering protein from lignocellulosic residues. Wood decomposing fungi easily consume this biomass which is linked to their ability to breakdown lignin. *Pleurotus* species have been shown to be one of the most effective lignocellulose degrading variety of white rot fungi, numerous agro-industrial by-products being used as a suitable substrate for the cultivation of *Pleurotus* mushrooms, based on the agricultural wastes that are locally available.

Mushrooms cultivation involves two main phases: obtaining the seeding spawn and the fruiting bodies production. Performance in mushroom cultivation, as in other horticultural crops, can only be achieved with the application of a high-quality biological material - spawn (commercial mycelium) - that provides high and consistent yields of quality mushrooms. The manufacture of commercial mycelium is the first and most important component of the intensive-industrial system of mushrooms production. Spawn is the vegetative mycelium produced on a suitable medium such as wheat grains, pearl millet, rye, sorghum etc. to produce the biological “seeding" material. The nutrient substrate/type of support on which it grows, as well as the technique employed to produce it, have a major influence on the quality of the spawn, along with microclimate parameters such as temperature, O_2/CO_2 saturation, humidity and light conditions. This process involves preparing of a pure culture of mycelia from tissues or spores, commonly maintained on many agar media, followed by inoculation on sterilized grains (premixed with calcium salts) and further colonization of the grains. The quality of the spawn is vital for the efficient mushroom cultivation. The success of production and productivity is strongly dependent on the quality and biological purity of the spawn. For the production of the spawn, the most highly advanced technological model is being applied in numerous major laboratories around the world, benefiting from current, high-capacity industrial machinery as well as top mechanization of the whole mycelium production chain. Cereal grains, water and calcium salts ($CaCO_3$ and $CaSO_4$) are

combined in massive double-walled stainless steel mixers. Calcium carbonate and sulphate form a thin layer at the surface of grain caryopsis or substrate, preventing clogging and material conglomeration, therefore avoiding the initiation of fermentation processes, as well as the possibility of expanding the mycelium's growth front. $CaCO_3$ is used to raise the pH (increasing alkalinity) by neutralizing acids, and $CaSO_4$ is used to supply calcium and sulfur. The mixture is sterilized, cooled and injected with pure mycelium cultures, all in the same place. The sterilized and inoculated grains are then distributed in polypropylene bags/sacks equipped with microbiological filters and placed for incubation in dedicated rooms under rigorous air sterility control. Within the upgraded technologies applied in some laboratories provided with modern equipment, some firms use "liquid" mycelium as an inoculation source for generating commercial mycelium (spawn). Submerged cultures provide an increased rate of mycelium growth into the substrate that reduce the duration of the production, hence, a reduction of time and costs, lowering the contamination risk and ensuring the biological purity. Different growing techniques and substrate types are employed for submerged cultivation of medicinal and edible species depending on the physiological and morphological properties of the mycelia and their behavior in different environmental situations.

A successful growth of submerged cultivated mycelium can be ensured by increasing the accessibility of nutrients from the culture medium and enriching it with sources of organic nitrogen, carbon and minerals. To generate submerged mycelial biomass from most macromycetes, cultivation media including glucose, peptone, yeast extract, minerals and vitamins in various forms are frequently applied. This implicitly leads to a rise in the nutrient content of the mycelium, which has a good impact on fruiting, harvest quantity and quality. After complete colonization, the spawn is stored under refrigerated conditions, regardless of the technique used for production. The spawn is sent directly to the mushroom growers in the same container in which it was grown and obtained, transforming the technological recipient into commercial packaging, the risk of contamination during distribution preparation is reduced, the technological process is standardized and the production cost of the biological product is less expensive. Mushrooms are highly effective biological recyclers of various lignocellulosic residues from agro forestry, food, textile, paper and other industries.

Table 2. Agro-industrial wastes chemical composition (source: Sadh et al., 2018)

Agro-industrial wastes	Wastes chemical composition (% w/w)				
	Cellulose	Hemi-cellulose	Lignin	Ash	Moisture
Sugarcane bagasse	30.2	56.7	13.4	1.9	4.8
Corn stalks	61.2	19.3	6.9	10.8	6.40
Sawdust	45.1	28.1	24.2	1.2	1.12
Barley straw	33.8	21.9	13.8	11	
Cotton stalks	58.5	14.4	21.5	9.98	7.45
Oat straw	39.4	27.1	17.5	8	
Sunflower stalks	42.1	29.7	13.4	11.17	_
Wheat straw	32.9	24.0	8.9	6.7	7

For the fruiting bodies production, residues (Table 2) such us cereal straws, corn cobs, cotton stems, various grasses and plants, maize or sorghum stover, sugarcane bagasse, corn husks, coffee pulp/husk, cotton and sunflower seed hulls, rice husks, sawdust and woodchips are some examples of residues and by-products that can be recovered and transformed to higher value and suitable substrate components degraded by mycelia (Pandey et al., 2000b; Webb et al., 2004; Sadh et al., 2018). Substrates for the cultivation of edible mushrooms require varied degrees of pretreatment, heat treated done by pasteurization or sterilization, to assure the exclusion of other organisms for the mycelium to grow. The substrate must be rich in essential nutrients that are accessible for the mycelium to feed on. After obtaining commercial mycelium, the technological flow proceeds with its seeding in the substrate and fructification, including the following phases of the process:

1. Raw and auxiliary materials are typically processed/ chopped into adequate sizes using a suitable machine.
2. The lignocellulosic materials are then moistened with water. This procedure can be performed out in different ways:

a) by laying the materials in compacted and irrigated layers, with the possibility of draining and recirculating the liquid flow; 24-48 hours;

b) by total immersion in a container; 24-48 hours;

3. Depending on the technological system used and the availability of steam, thermal disinfection or sterilization of lignocellulosic materials can be achieved in a variety of ways:

a) hot water treatment: sunken lignocellulose material can be brought to and maintained at 75-85ºC for a few hours before cooling progressively;
b) large amounts are treated by direct steam action in thermally insulated pasteurization chambers/ tunnels equipped with ventilation and temperature monitoring systems; the steam treatment lasts 12-24 hours depending on the temperature to which the cellulose mixture is exposed (60-80ºC);
c) sterilization at 121-123ºC for 90-100' for experimental batches or for certain species who require substrate microbiological purity.

4. The next stage is to administer the calcium amendments: chalk (4-6%), plaster (3-5%) or in some cases whitewash (2-3%).

5. "Seeding" is performed by the combination of substrates and commercial mycelium. The average spawn inoculation rate is 2-4%.

6. The inoculated substrate is dispersed in polyethylene or polypropylene bags using modern equipment, which maximizes performance and diminishing contamination risk. Bags of various sizes and capacities can be used, with either opaque or clear foil. The bags are pierced to enable gas exchange between the substrate and the external environment and subsequently fruiting at this level. To avoid overheating of the seeded substrate during incubation, the diameter of the bags should not exceed 50 cm.

7. The optimum temperature for incubation is in the range of 24-26ºC within the substrate and will be 3-5ºC higher than the air in the room throughout the incubation due to the rapid metabolism of the developing mycelium. As a result, special care is necessary to control the temperature in the incubation room within normal ranges in order to avoid heating of the substrate (temperatures beyond 29-30ºC), which would result in the demise of the *Pleurotus* spp. mycelium. Ventilation is less necessary at this stage since the mycelium is encouraged to grow by a higher CO_2 content in the air, but vigorous air quality control is required. Light is not required at this stage of development. The incubation period spans from 15 to 22 days, depending on the species and cultivated strain, as well as the microclimate factors, particularly the temperature.

8. Unlike *Pleurotus ostreatus*, where a negative shock is required for the appearance of primordia, hybrids do not require a thermal shock to promote fruiting. Bags/ sacks are placed in fruiting conditions for the appearance of primordia by either keeping them in the same spaces where the incubation and significant modification of the microclimate conditions (monozonal system) or transferring them in facilities with climate specific to the stage of fruiting-harvesting (bizonal system). The climate conditions needed during this phase vary based on the species or strain cultivated, have the following features and values on average: RH of 92-95% at the primordia appearance, 80-85% in full harvest wave and 85-90% between flushes; light with an intensity of 150-250 lux during waves, conversely 50-100 lux between waves; vigorous ventilation during waves to keep CO_2 concentration below 1200 ppm in the air. The best time to harvest *Pleurotus* mushrooms is during the carpophores development period, when the pileus is still slightly bulging or flat, before the margins twist upwards.

A balanced diet is a major concern in developed countries around the world in order to ensure and maintain the health and good functioning of the human body. A number of scientific studies have shown that a controlled diet can regulate human bodily processes, thereby contributing to the maintenance of health or homeostasis, which is required to lower the risk of many chronic diseases. Because mushrooms contain a higher concentration of celluloid substances, including dietary fiber, they are used as a low-calorie diet with a higher therapeutic value for diabetic patients.

Figure 2. *Pleurotus* mushrooms (*P. ostreatus, P. citrinopileatus, P. eryngii, P. columbinus*) - "Mushroom laboratory" - RDIVFG

Mushrooms (Figure 2) began to be used increasingly frequently as dietary supplements in the treatment of various diseases and health problems. Many of these actions and new applications have been developed over the last decades. Edible mushrooms contain high-quality protein that can be synthesized with greater biological efficiency than animal protein. They are high in fiber, minerals, vitamins and have a low crude fat content, with a high amount of polyunsaturated fatty acids relative to total fatty acid content. These qualities are important contributors to mushrooms longstanding status as healthy foods. Macronutrient content of *Pleurotus ostreatus* are shown in Table 3.

Table 3. Macronutrients of *P. ostreatus* (source: Khan et al., 2010)

Nutrients Content (g/100 g dried mushroom)	
Proteins	17-42
Carbohydrates	37-48
Lipids	0.5-5
Fibers	24-31
Minerals	4-10
Moisture	85-87%

Mushrooms are an inexhaustible source of immunomodulatory biologically active compounds with clinically established effects in the treatment of tumor, infectious and immunologic illnesses. Currently, over 270 fungus species are known for their varied qualities (antimicrobial, antioxidant, anti-inflammatory and hepatoprotective).

Mushrooms possess biocompounds (Table 4) such as glucans and protein-polysaccharide complexes with therapeutic qualities including antitumor, antioxidant, hypoglycemic, anti-inflammatory and antimicrobial (Dufosse et al., 2021; Jovanovic et al., 2021). Vitamins are present, particularly several of group B vitamins (thiamine, riboflavin, folic acid), mushrooms being the only non-animal source of ergosterol. Mushrooms are high in K and P, but low in Ca and Na, which is ideal for hyposaline diets. Potassium is an extremely vital mineral that controls arterial blood pressure and keeps cells operating properly. The zinc content of *Pleurotus* species is often the highest. Selenium is an antioxidant that contributes in the neutralization of free radicals, avoiding cell damage and decreasing the risk of cancer and other disorders. Mushrooms have the highest selenium content of any food. They also contain vital elements such as phosphorus, zinc and magnesium (Deepak and Deepika, 2016).

Table 4. Nutrients Content of *Pleurotus* mushrooms (source: Golak-Siwulska et al., 2018*)*

Bioactive compounds	Species	References
β-glucans	*P. ostreatus*	Jedinak et al., 2010
α-glucan	*P. ostreatus*	Lavi et al., 2006; Wu et al., 2011
proteins	*P. ostreatus* *P. nebrodensis*	Wang and Ng, 2000 Lv et al., 2009
polysaccharides proteoglycans lectin	*P. ostreatus* *P. ostreatus* *P.citrinopileatus* *P. ostreatus*	Tong et al., 2009 Sarangi et al., 2006 Li et al., 2008 Wang et al., 2000
polysaccharides heteroglycan	*P. ostreatus* *P. cornucopiae* *P. ostreatus*	Shamtsyan et al., 2004 Devi et al., 2013
lovastatin ergosterol	*P. ostreatus* *P. sajor-caju* *P. ostreatus*	Alam et al., 2009 Khan et al., 2011 Dissanayake et al., 2009
D-mannitol peptides	*P. cornucopiae* *P. cornucopiae*	Hagiwara et al., 2005 Jang et al., 2011

The fruiting bodies of *Pleurotus* mushrooms contain lovastatin, which belongs to the class of statins that influence cholesterol metabolism. These chemicals reduce LDL cholesterol oxidation. They are anti-inflammatory, anti-oxidative and anticoagulant (Golak-Siwulska et al., 2018). Antioxidants are protective

compounds with a wide range of structures and biological functions that serve as free radical scavengers. As a result, an antioxidant is described as a substance that can prevent or slow down the oxidation of other molecules (Bita et al., 2022). Polyphenols and structural polysaccharides such as β-glucans are among the key bioactive substances produced by macromycetes.

Basidiomycetes contain an abundance of other therapeutic and beneficial metabolites such as alkaloids, flavonoids, saponins and steroids. Because of their presence, these mushrooms are valued both for their consumption and for the industrial applications in the production of new pharmaceuticals. The discovery of the synergistic action of these compounds in the human body would allow for the full utilization of oyster mushrooms' health-promoting and medicinal potential.

CONCLUSIONS

Lignocellulose, found in agricultural, industrial and forest residuals accounts for more than half of all vegetal biomass produced on the planet and is used in biotechnological applications including cosmetics, medicines, foods and feeds, biofuels, biopesticides, biofertilizers and a variety of other products. Macromycetes have the ability to convert lignocellulosic waste materials into a wide range of products that offer multiple benefits for humans such as food, tonics, medications, feeds, fertilizers and for safeguarding and regenerating of the environment. Mycelia can secrete enzyme complexes that directly attack/degrade the lignocellulosic residues and by-products. Mushroom cultivation can not only transform these massive lignocellulosic biomass wastes into human food, but it can also produce remarkable nutraceutical products with numerous health benefits. Furthermore, mushroom production has the potential to generate equitable economic growth. Mushroom cultivation can be labour intensive and as a result the activity has the potential to create new jobs, particularly in tropical or less developed countries. Mushrooms, with their pleasant flavour, high protein content, tonic and therapeutic properties, are without a doubt one of the world's biggest emerging supplies of healthy and appetizing food for the future. A number of scientific studies have shown that a controlled diet can regulate human bodily processes, thereby contributing to the maintenance of health or homeostasis. Mushrooms began to be used increasingly frequently as dietary supplements in the treatment of various diseases and health problems.

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BIOSYNTHESIS OF INULINASES BY *Aspergillus terreus* USING ORANGE PEELS POWDER AS A POTENTIAL SUBSTRATE

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Abstract

Inulinases are an important class of enzymes used in many fields, especially in the food and pharmaceutical industries, to produce fructose syrups. Microbial inulinases are important in the hydrolysis of inulin to produce fructose syrup and FOS. These enzymes are produced by various strains of microorganisms, of which Aspergillus sp. and Kluyveromyces sp. are the most commonly used strains for inulinase production. The goal of the study was to biosynthesis inulinase using the Aspergillus terreus ICCF 262 strain, with inulin and orange peel powder as carbon and energy sources, the enzyme being isolated from the fermentation medium by fractional precipitation with ammonium sulfate followed by purification on DEAE-Cellulose using ion exchange chromatography. Within 7 days of cultivating the fungal strain on a mineral medium containing inulin and orange peel at a final concentration of 2% in the fermentation medium, yields of biotechnological interest were higher than those previously reported in the literature. Through the procedure of isolating and purifying inulinase from the fermentation medium results a specific activity of between 164.6 - 396.4 U / mg protein.

Key words*: inulinases, Aspergillus terreus, biosynthesis, inulin, orange peel powder.*

INTRODUCTION

In the past few years, due of an increase in metabolic problems (cardiovascular disease, obesity, diabetes, hypocalcemia, gout), researchers have turned their attention to creating natural polysaccharides. Thus, the production of high purity fructose syrup was studied by enzymatic hydrolysis of inulin to D-fructose, using immobilized inulinase. Fructose is useful to diabetics, obese people, boosts calcium absorption and bifidobacteria growth, and promotes iron absorption in children (Chi et al., 2009).

Inulinases are an important class of enzymes used in many fields, especially in the food and pharmaceutical industries, to produce fructose syrups. The inulinases characterized to date (five inulinases) show considerable variability in biophysical and biochemical characteristics. These enzymes hydrolyze the inulin chain's β-(2,1) links to create fructose and fructooligosaccharide (FOS) units. They are known as 2, 1-D-fructan fructanohydrolases and are divided as endo- or exo-inulinases based on the mode of action of the inulin. Endo-inulinases (fructano-hydrolases 2, 1-β-D-fructan; EC 3.2.1.7) are inulin specific and hydrolyze it by breaking the bonds between fructose units far from the polymer chain ends to create oligosaccharides. Exo-inulinases (β-D-fructohydrolases; EC 3.2.1.80), cleave successive terminal units at the non-reducing end of the inulin molecule.

In addition to inulin, exo-inulinases also hydrolyze sucrose and the rest of the fructose in raffinose (Henrissat, 1991; Henrissat & Bairoch, 1993; Pons et al., 1998). Microbial inulinases are important in the hydrolysis of inulin to produce fructose syrup and FOS.

These enzymes are produced by various strains of microorganisms (fungi, yeasts and bacterial strains), such as those of the species *Penicillium, Kluyveromyces, Streptomyces* and *Aspergillus*. Of these, strains belonging to the genera *Aspergillus* and *Kluyveromyces* are the most commonly chosen for inulinase production (Gao et al., 2007; Santisteban et al., 2009; Gern et al., 2001)

Because of their thermostability, bacterial strains are used to produce inulinase. Information on inulinase biosynthesis using

bacterial strains is limited, mainly referring to end-inulinases. *Streptomyces* spp. has been found to be a good producer of inulinase, for example Sharma et al. (2006) used garlic as a substrate to produce an inulinase with an activity of 524 IU/L.

Yeasts are used in the production of inulinase because they grow easily compared to bacteria. *Kluyveromyces* spp., *Pichia* spp., and *Candida* spp. are yeasts with a high potential for producing inulinase, as evidenced by high yields and activity. Gao et al. (2007) screened over 400 marine yeasts and discovered that some of them can secrete large amounts of inulinase.

In order to optimize the fermentation process, a number of researchers studied inulinase production parameters (agitation, aeration, carbon supply, fermentation medium composition, fermentation time) on *Kluyveromyces* spp. strains. Thus, Santisteban et al. (2009) investigated the effects of carbon and nitrogen sources, using sucrose at a concentration of 20 g/L as a carbon source and obtaining an inulinase activity of 208 U/ml. Inulinase purification from *Kluyveromyces marxianus* was studied using an ethanol precipitation method followed by ultrafiltration (Golunski et al., 2011), yielding a specific activity of 262.9 U/mg. By purifying the enzyme obtained from *Kluyveromyces marxianus* var. *bulgaricus* using ion exchange chromatography methods, Kalil et al. (2010) obtained an inulinase with an activity of 194.1 U/ml.

Of the sixteen fungal strains of *Aspergillus* spp. (Gern et al., 2001), a favorite species for inulinase production, a maximum inulinase activity of 100 U/ml was obtained with the *Aspergillus niger* DSM 2466 strain, using sucrose S-770 added to the fermentation medium in a concentration of 6 g/L as substrate. Kumar et al. (2005) obtained a maximum inulinase activity of 176 U/ml with the stem *Aspergillus niger* using a fermentation medium containing 5% (w/v) inulin. Other *Aspergillus* spp. strains described for inulinase production in the literature include A. *fumigatus* (Gill et al., 2006), A. *awamori* (Nagem et al., 2004), A. *ochraceus* (Guimaraes et al., 2007), A. *ficuum* (Chen et al., 2011), and A. *parasiticus* (Ertan et al., 2003b).

The mentioned microorganisms use natural substrates from pure inulin to agro-industrial residues as carbon sources, registering different activities and properties, depending on the producing strain and substrate. Inulin can be found as a reserve of carbohydrates in the tubers and roots of plants such as chicory, turnips, dahlias, dandelions, garlic, onions, rye, barley, bananas, wheat, etc.

As microbial inulinase is an inducible enzyme, in the biosynthesis process a substrate rich in inulin can be used resulting from agro-industrial residues (cassava, corn cobs, oats, rice straw, sugar cane, wheat bran), which leads to streamlining the enzyme biosynthesis process (Singh & Chauhan, 2016).

Inulin is a natural fructose polyglucide with functional properties related to the length of the molecular chain, and it belongs to the fructans class of carbohydrates. Inulin is a generic term for all linear (2-1) fructans with polymerization levels ranging from 2 to 60. Because inulin is not recognized by digestive enzymes in the small intestine, tiny molecules can pass through the cell wall, whereas long-chain molecules (10-65 monomers) reach the colon intact, resulting in bacterial fermentations (Sarote et al., 2007)

Natural materials rich in inulin are preferred as substrates for obtaining inulinase, but lately, agro-industrial residues have gained the attention of researchers. In nature, inulin can be found in the tubers and roots of plants such as chicory, turnips, dahlias, dandelions, garlic, onions, rye, barley, bananas, wheat, and others as a carbohydrate reserve (Chi et al. 2011).

As a result, agro-industrial wastes and plant extracts appear to be a good source of inulinase. Glucose, sucrose, cassava flour, corn cob, oatmeal, rice straw, sugar cane and wheat bran were used to test the effect of carbon source on inulinase biosynthesis by the *Aspergillus ochraceus* strain (Guimaraes et al., 2007). When cane was used as a carbon source, the highest amount of extracellular inulinase activity (108 units of activity) was produced. Sharma et al. (2006) used various substrates to produce inulinase, namely: rye, barley, bananas, garlic, wheat, pure inulin, chicory, onion and dahlia.

The focus of this research is on inulinase production by *Aspergillus terreus* ICCF 262, including bioprocess optimization and inulinase purification. In order to optimize enzyme biosynthesis, we tested various substrates (inulin and orange peels) and optimization strategies

(nitrogen sources, temperature, pH, bioprocess duration).

MATERIALS AND METHODS

The microorganism *Aspergillus terreus* belongs to the Collection of Microorganisms of Industrial Importance of the National Institute for Chemical-Pharmaceutical Research - Development, ICCF. *Aspergillus terreus* ICCF 262, was cultivated on specific media and maintained at 4ºC in vegetative preserves. During the experiments, weekly/monthly passages were made to maintain their viability. Reagents used for the research, such as: organic solvents, analytical reagents and mineral salts, were purchased from Merck and Sigma-Aldrich.

Media culture and cultivation conditions

Preinoculum phase. The strain was grown in tubes on solid agar medium, PDA (potato dextrose agar), and incubated for 24-48 hours at 28-29°C.

Inoculum phase. After washing the preinoculum tubes with 2 ml of sterile inoculum medium, they were inoculated into 500 ml Erlenmeyer flasks containing 100 ml of liquid medium. For 24 hours, *Aspergillus terreus* ICCF 262 was grown on an inoculum medium containing 1 percent glucose, 0.3 percent malt extract, 0.3 percent yeast extract, and 0.5 percent peptone, with stirring at 220 rpm.

The bioprocessing phase. As shown in Table 1, the inoculum was used at a final concentration of 2% to inoculate 500 ml Erlenmeyer flasks containing 100 ml of the fermentative medium, which consisted of M2 or M3 (fermentation media) medium supplemented with 2% (g/v) inulin or/and orange peels as inductive substrates.

Table 1. Composition of the fermentation media utilized to create the experimental model

No.	Component	Concentration
M2		
1	Inulin/Orange peels	2.0%
2	Yeast extract	2.0%
3	NH_4NO_3	0.3%
4	$(NH_4)_2$ HPO4	0.4%
5	KH_2PO_4	0.1%
6	$MgSO_4$	0.05%
M3		
1	Inulin/Orange peels	2.0%
2	Corn steep liquor	2.0%
3	NH_4NO_3	0.3%
4	$(NH_4)_2$ HPO4	0.4%
5	KH_2PO_4	0.1%
6	$MgSO_4$	0.05%

The cultivation conditions were 28-29°C, an initial pH of 6.5, and 7 days of agitation on a rotary shaker with 220 rpm and a 2 cm agitator eccentricity. The mycelia were then separated by centrifugation and the supernatant was tested for enzyme activity.

Enzyme Purification

The final fermentation medium is centrifuged at 8000 rpm, for 20 minutes at 9°C. To extract inulinase, the supernatant is fractionally precipitated with ammonium sulfate. This phase is accomplished by adding 60% $(NH_4)_2SO_4$, centrifuging, and extracting the precipitate containing ballast protein; $(NH_4)_2SO_4$ is then added to the supernatant from the first fractionation process until saturation is reached at 80%. The precipitate containing 95% inulinase is centrifuged and stored at 4ºC. The precipitate is dissolved in distilled water and dialyzed for 24 hours against water before being dialyzed for another 24 hours against 20 mM phosphate buffer, pH 7.

The purification of inulinase is performed through the use of ion exchange chromatography. The dialysate precipitate is applied to a column of DEAE-Cellulose that has been pre-equilibrated with 20 mM phosphate buffer at pH 7. The enzyme is eluted with a NaCl solution in a gradient of concentrations ranging from 0.1 to 1.0 M in the same buffer, at a flow rate of 60 ml/h, in fractions of 3 ml. The fractions with enzyme activity were collected and lyophilized after they were analyzed. (Singh & Chauhan, 2016; Pessoni et al., 2007)

Analytical Methods

Miller (1959) described the following method for determining inulinase activity: the enzyme solution (0.1 ml) was mixed with 2% inulin (0.9 ml) in a 0.1 M acetate buffer, pH 5.5; at 50°C, the process takes 15 minutes. The reducing sugars were determined using the dinitrosalicylic acid method. 2 ml DNSA reagent was added to each tube and placed in boiling water for 5 minutes to stop the enzyme activity. Each sample's temperature was brought to room temperature. A spectrophotometer was used to measure the absorbance at 540 nm. Under test circumstances, one activity unit is defined as the quantity of enzyme necessary to create one micromole of reducing sugar per minute

(Petrescu & Eremia, 2018). Using BSA as a standard, the protein content was measured using the Lowry technique. (Lowry et al., 1951).

RESULTS AND DISCUSSIONS

1. Inulinase microbial biosynthesis and cell growth in conventional media

In previous research, the fungal strains *Aspergillus* sp. were reported to have the maximum amount of gas created in the cell formation process by metabolizing inulin from the environment in the Durham experiment (Durham, 1952). *Aspergillus awamori, Aspergillus niger, Aspergillus nigricans,* and *Aspergillus terreus* strains had higher cell activity and growth than the other strains examined, indicating that these strains may be able to use inulin as their only carbon source (Eremia & Petrescu, 2021).

The *A. terreus* strain was chosen to continue the research into the type of inoculum (Ig - inoculum with a single carbon source of glucose and Ii - inoculum with a single carbon source of carbon inulin) and the preferred source of organic or inorganic nitrogen to optimize inulinase biosynthesis conditions (Figure 1).

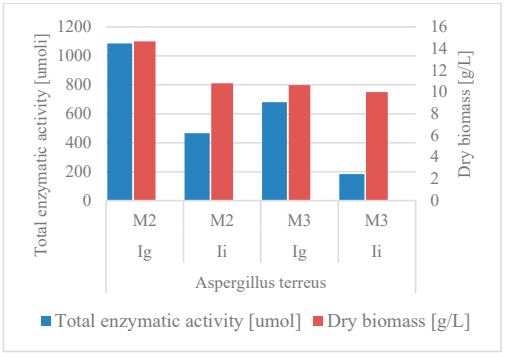

Figure 1. Bioprocess optimization

The optical density, dry biomass, final pH value, and enzymatic activity of extracellular inulinase were used to track inulinase production by the evolution of cell growth.

The *Aspergillus terreus* strain had a relatively uniform distribution of biomass development on the chosen medium variants, and enzymatic activity was higher on the Ig-M2 variant, with a correlation between biomass development and enzymatic activity on the same culture medium.

For the study of biomass development, the growth curves of the *A. terreus* strain were performed in static and agitated fermentation for 7 days in optimal conditions for the development of cultures on liquid media.

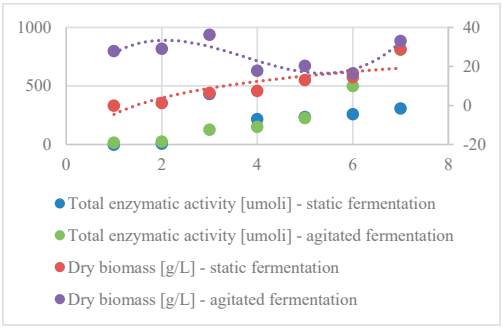

Figure 2: Growth curve of *Aspergillus terreus* strain and enzymatic activity

Along with the biomass study development over time, the effect of aeration on biomass development and enzymatic activity were also analyzed (Figure 2). In agitated fermentation, the strain developed significantly faster; more biomass accumulated in the static fermentation medium only on the seventh day. Stirred fermentation increased enzymatic activity as well, with a significant difference on the fifth day. The highest enzymatic activity was determined after 6 days of bioprocessing.

2. The effects of different Carbon sources on cell growth and enzyme production

On the variant of medium containing inulin and orange peel as a carbon source, the strain produced the highest biomass and had a higher enzymatic activity than when inulin or orange peels was used as a carbon source (Figure 3).

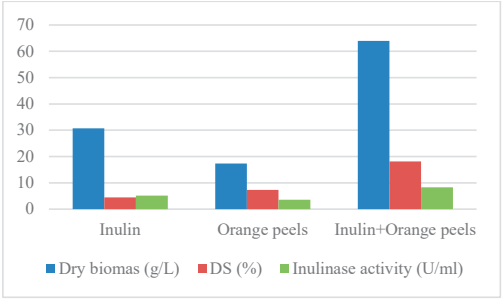

Figure 3. The effect of C source on *Aspergillus terreus* inulinase production

On the environmental version with inulin, the strain progressed well in terms of biomass, but considerably improved enzymatic activity on the environmental variant with orange peel and inulin as carbon sources.
It's worth noting that using agri-food waste as a substrate boosts the strain's bioproductivity.

3. Inulinase purification

The supernatant obtained by centrifugation of *A. terreus* culture, with inulinase activity, was purified by two successive steps: ion exchange chromatography on DEAE-Cellulose after fractionated precipitation with ammonium sulphate (Table 2).
Following the purification stage, a precipitate with higher inulinase activity is formed (approximately 76% of the initial activity).
The eluted inulinase was purified over 37.16 times with a yield of 52.3% in the second phase, which consisted of purification on a DEAE-Cellulose column (Table 2 and Figure 4).
Following the chromatographic purification study, increasing the degree of purification results in an improvement of the specific activity.

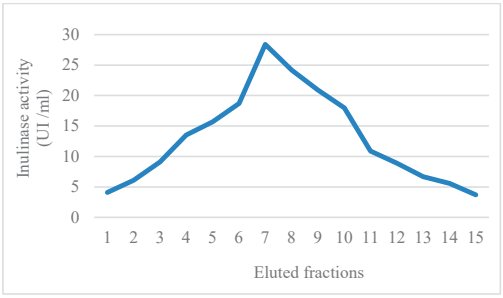

Figure 4: Ion exchange chromatography for inulinase purification using a DEAE-Cellulose column and a gradient of 0.1-1 M NaCl in pH7 acetate buffer in 3 ml fractions

The *Aspergillus terreus* strain was identified as the most important in terms of inulinase bioproductivity on diverse nutritional substrates. These results are equivalent to those reported in the literature using the *Aspergillus niger* strain, as a model (Rawat et al., 2015; Cruz et al., 1998; Singh & Chauhan, 2016).

Table 2. Purification of inulinase from *Aspergillus terreus*

Stages	Volume (ml)	Activity (U/ml)	Total activity (U)	Protein (mg/ml)	Specific activity (U/mg protein)	Purification factor
Fermentation supernatant	170	11.67	1984	1.09	10.62	1
Dialyzed precipitate	33	45.7	1508	0.64	71.4	6.73
Eluted from DEAE-Cellulose Purification	27	29.2	788.4	0.074	394.6	37.16

CONCLUSIONS

The *Aspergillus terreus* strain was identified as the most important in terms of bioproductivity of inulinases on diverse nutritional substrates among the *Aspergillus* sp. strains previously tested according to the stage of biomass and inulinase production.
It was performed with several carbon sources, namely inulin and agri-food by-products (orange peels), to study the optimization of the bioprocess for producing inulinase with *Aspergillus terreus* ICCF 262 strain performed on enzymatic and specific activities. These tests revealed that inulinase is more active when a mixture of approximatively 2% inulin and orange peel powder is used as a carbon and energy source rather than just inulin.
After post-biosynthesis processing of the fermentation medium, a solution with a 29.2 U/ml inulinase content and a yield of 39.7% was obtained.
It should be highlighted that using agri-food waste (in our case, orange peel) as a substrate enhances strain bioproductivity by supplementing the fermentation environment, with an increase in inulinase production yields, also having a favorable impact on their production.

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EVALUATION OF THE IMPACT OF THE *Trichoderma* TREATMENT IN BUZĂU BELL PEPPER (*Capsicum annuum*) CULTURE 10

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Abstract

In the experimental field of the Buzău Vegetable Research and Development Station, research was carried out on the effect of Trichoderma T85, administered at planting, in granular form, for the culture of bell peppers, Buzău 10 variety, created by SCDL Buzău. In this regard, the growth and development of bell pepper plants, the Buzău 10 variety, the monitoring of the phytosanitary condition of the plants and the harmful and useful fauna from the soil were determined and monitored in dynamics. 5 experimental variants were set up in randomized blocks. This fungal inoculant was applied to V5 - three granules at planting. The obtained results confirm the data presented in the literature: Trichoderma prevents the growth of other pathogenic fungi, very widespread, such as: Alternaria, Botrytis, Colletotrichum, Fusarium, Phythophtora, Pytium, Sclerotinia, Xanthomonas. At the same time, this fertilizer is an environmentally friendly option because it does not pollute groundwater.

Key words: *Trichoderma, Capsicum annuum, fertilizer, pepper fruit production.*

INTRODUCTION

Trichoderma is often found in soil microflora of various ecosystems, such as agricultural fields, forests, wetland areas in all climatic zones. Root colonization by *Trichoderma* strains leads to root growth and nutrient uptake and utilization, increased production, and increased resistance to abiotic and biotic factors. Field crop productivity can increase up to 30% after treating seeds or soil with different *Trichoderma* species (Benitez T., Rincon A.M., Limon M.C., Codon A.C., 2004). The purpose of this study was to evaluate the effect of applying application and evaluation of the impact of the treatment on the fat pepper culture Buzău 10.

The main action of *Trichoderma* is the microbial antagonism that is usually manifested by various mechanisms of action such as: when considering the interactions of *Trichoderma* fungi with plants, it was found that these fungi have an advantageous effect on plants. Stimulation of plant growth and yield takes place thanks to this interaction and the advantageous effects are seen in the production of vitamins, the increased availability of biogenic elements (nitrogen, phosphorus), the mobilisation of nutrients from the soil and from organic matter, and the enhanced intensity of mineral uptake and transport.

Furthermore, *Trichoderma* fungi are capable of producing zeaxanthin and gibberellin, i.e. compounds accelerating seed germination. Many *Trichoderma* strains produce acids, e.g. gluconic, citric, and coumaric acids, causing the release of phosphorus ions and microelements, which subsequently become available to plants (Harman et al. 2004).

Trichoderma thus prevents the growth of other pathogenic fungi, very widespread, such as: *Alternaria, Armillaria, Botrytis, Colletotrichum, Fusarium, Phythophtora, Pytium, Rhizoctonia, Sclerotinia, Xanthomonas*, etc. It has an important activity of nutrition and phytostimulation and induces an increase in productivity.

In the southern part of Romania, more precisely in the Buzău vegetable basin, the pepper met favorable conditions for development and currently occupies a leading place among cultivated vegetables.

The strains were grown in Potato Dextrose Agar (PDA) plates and incubated for 10 days at 28°C to obtain abundant sporulation. Spore suspen-

sions of *Trichoderma* sp. were prepared by scraping the spores from cultures using 15 mL distiled water and after added Tween 80, 0.1% solution to reduce surface tension and facilitate spore release. The concentration of spore suspension was counted using haemocytometer and was adjusted to two concentration 107 spores/mL and 108 spores/mL.
Trichoderma strains (Td85 and Tal12) which have been used in this experiment were obtained from the collection at the Research and Development Institute for Plant Protection.

MATERIALS AND METHODS

Within Vegetable Research and Development Station Buzău, research was carried out on the influence of *Trichoderma* administered at planting, in cubes, for seedlings, on the bell pepper plants of the Buzau 10 variety.
The culture technology used was the culture technology specific to this species, adapted to the climatic conditions of 2019, in a conventional system.
In 2019 an experimental field of bell pepper was organized, in which the rules of experimental technique were applied in terms of regarding the size of the plot, the number of repetitions, the observations made, the necessary analyzes, the calculation of the results, their statistical interpretation.
The seeds were sown in alveolar paddles of 70 cubes, with a volume of 50 ml, in blond peat partially decomposed and with the addition of trace elements (Figure 1).
The arrangement of the experiment was in randomized blocks, with 5 variants and 4 repetition. Below we see the planting scheme of bell pepper Buzău 10 (Table 1).

Table 1. Planting scheme - bell peppers Buzău 10

V5R1	V2R2	V4R3	V1R4
V4R1	V1R2	V3R3	V5R4
V3R1	V5R2	V2R3	V4R4
V2R1	V4R2	V1R3	V3R4
V1R1	V3R2	V5R3	V2R4

The emergence period was between 11.04-24.04, and the percentage of rise ranged from 94.3-98.6%.

The application dose was 3 granules/cube, at the time of planting on 04.06.2019.

Figure 1. Peppermint seedling Buzău 10

The biological material used to establish the crop was the Buzau 10 bell pepper variety, created by Vegetable Research and Development Station Buzău.
Characterization of biological material:
- vigor medium;
- fruits average weight 90-120 g;
- shape trapezoidal;
- height 9 cm;
- diameter 6 cm;
- average fruit width 5.58;
- pulp thickness 6-7.5 mm;
- color at consumption maturity medium yellow to red;
- color at physiological maturity intense red with luster;
- maturation period early;
- number of lobes 3;
- tolerance high in VMT and potato virus Y (PVY);
- good tolerance to pathogens of economic importance;
- storage capacity very good;
- production potential 35-40 t/ha. (Catalog Buzău Vegetable Research and Development Station, July 2013).

It can be grown both in solariums and in the open field for fresh consumption and industrialization (Figure 2).

Figure 2. Detail fruit - bell peppers Buzău 10

Phytosanitary treatments were applied during the vegetation. Here we have the treatment scheme during the vegetation periods (Table 2).

Table 2. Treatment schema

The damage agent: Disease/harmful agents	The name of the product	Dose used
Phytophthora capsici, Thrips, Aphids, Spider	Dithane, Mavrik, Milbeknock	0.2%; 0.5%; 0.75%
Micoze, Spider, Caterpillar	Ortiva Top, Afirm, Envidor	1 l/he
Thrips, Aphids, White fly	Actara, Karate zeon	0.2 kg/he; 0.015%
Bacteriosis, Vascular disease, Spider, Thrips, Aphids	Dithane, Topsin, Nissorun, Mospilan	2 kg/he; 1 kg/he; 0.4 kg/he

Production destination - fresh consumption, but also industrialization.
In the culture of bell pepper Buzău 10 was used the culture technology specific to this species, adapted to the climatic conditions of 2019 where we had. The average temperature varied:
- in April between 9.5-14.1°C;
- in May between, 9.1-15.4°C;
- in June between 19.6 - 23.0°C;
- in July between 21.08 and 25.0°C;
- in August between 22.6 and 24.7°C;
- in September between 17.4-22.2°C.

Between April and September were the following precipitations:
- April - 0.3 l/sqm;
- May - 27.7 l/sqm;
- June - 67.4 l/sqm;
- July - 83.0 l/sqm;
- August - 33.4 l/sqm;
- September - 3.4 l/sqm.

The soil within Vegetable Research and Development Station Buzău, the agrochemical analysis found a weak alkaline pH (8.20), medium supplied in humus (2.57) soil favorable for vegetable cultivation through a value of total nitrogen (0.151%), total phosphorus (0.183%) and mobile potassium ppm (> 268) bioavailable to plant growth. (Vegetable Research and Development Station Buzău report, internal document).
Two variants were placed in the field: V1 untreated control and V5 treated with *Trichoderma*, at the time of planting. The repetition plot had 7 sqm, and the variant plot 28 sqm.
The land was modeled in raised furrows, with a height of 94 cm at the canopy and a width of 1.40 m. 2 rows of seedlings were planted on the furrow, with 70 cm between rows and 25 cm between plants per row.
Trichoderma is a genus of fungi in the Hypocreaceae family, which is present in all soils, where the most common fungi are. Many species of this genus can be characterized as opportunistic avirulent symbionts of plants. (Anil K. Sharma, Pratibha Sharma, 2020)
Fungi from the genus *Trichoderma* are commonly found in all climatic zones. The most typical habitats of these fungi include soil and rotting wood (Druzhinina I., Kubicek C.P., 2005). These fungi may be found on sclerotia and other propagating forms of fungi in the soil environment. They colonise the grain, leaves, and roots of plants. They were also isolated from such unusual sources as marine bivalves, shellfish, and termites. Fungal species from the genus *Trichoderma* are characterised by rapid growth and abundant production of conidial spores as well as the capacity to produce sclerotia (Stan N., Munteanu N., Stan T., 2003). These species produce several pigments, ranging from a greenish-yellow up to a reddish tinge, although some colourless specimens are also present. The conidia may also have diverse colouration, ranging from colourless to different hues of green or even grey or brown tinges.
Trichoderma spp. Is a saprophytic (non-pathogenic) fungus, one of the strongest in the category of antagonistic microorganisms, characterized by a high capacity for adaptation and rapid growth.

Trichoderma introduced into the soil improves the health of plants and increases disease resistance without eliminating other beneficial microorganisms.
Trichoderma also stimulates plant growth.

RESULTS AND DISCUSSIONS

The results recorded following the statistical calculation are as follows (Table 3).

Table 3. Statistical calculation - Number of fruits on bell peppers Buzău 10

No	Var.	No. fruit	No. fruit relative %	DIF	t	P %
1	V1 Mt.	6.29	100.00	0.00	0.00	-
2	V2	8.20	130.47	1.92	1.19	25.10
3	V3	8.01	127.49	1.73	1.08	29.10
4	V4	7.27	115.71	0.99	0.61	56.00
5	V5	8.09	128.64	1.80	1.12	29.10

After a long period of rain, in June and July where the water quantities were: 67.4 l/sqm and 83.0 l/sqm, the meteorological conditions favored the appearance of some diseases specific to pepper *Phytophthora capsici* (Figure 3), which is an oomycete plant pathogen that causes rot and rot of pepper fruits.

Figure 3. *Phytophthora capsici* in the cultivation of bell peppers

Later, this oomycete plant pathogen that causes rot and rot of pepper fruits was stopped with the treatments in the table above.

The administration of *Trichoderma* has had a positive influence on the culture of Buzău 10 bell pepper and is a non-polluting solution for the environment. The use of *Trichoderma* in field crops benefits the growth and production of fruit. The fruits are larger, of superior quality, in the variant treated with Trichoderma, the results being at the limit of the statistical significance threshold. (Jaimin Pandya, 2020).
Trichoderma spp. are free-living fungi that are common in soil and root ecosystems. Recent discoveries show that they are opportunistic, avirulent plant symbionts, as well as being parasites of other fungi. At least some strains establish robust and long-lasting colonizations of root surfaces and penetrate into the epidermis and a few cells below this level. They produce or release a variety of compounds that induce localized or systemic resistance responses, and this explains their lack of pathogenicity to plants. These root–microorganism associations cause substantial changes to the plant proteome and metabolism. Plants are protected from numerous classes of plant pathogen by responses that are similar to systemic acquired resistance and rhizobacteria-induced systemic resistance. Root colonization by *Trichoderma* spp. also frequently enhances root growth and development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients (Figure 4).

Figure 4. Appearance stem and root of bell pepper Buzau 10

The analysis of the root mass shows that variant 5 (treated with *Trichoderma*) (Table 4) was superior to variant 1 (Table 5).

Table 4. Analysis of bell pepper plants - variant 5

V/R	The plant	Top (g)	Root (g)
V5R3	1	171	33
	2	264	20
	3	152	49
	4	309	39
	5	234	40
	6	141	34
Average		**211.83**	**35.83**

Table 5. Analysis of bell pepper plants - variant 1

V/R	The plant	Top (g)	Root (g)
V1R3	1	185	30
	2	162	23
	3	158	28
	4	172	29
	5	133	22
Average		**162.00**	**26.40**

The weed spectrum was also performed in the field of experience, exemplified below:
- *Convolvulus arvensis*;
- *Portulaca oleracea*;
- *Setaria glauca*;
- *Amaranthus* sp.;
- *Galisoga parviflora*;
- *Chenopodium album*;
- *Poligonum persicaria*;
- *Lamium purpureum.*

The most abundant was: *Portulaca oleracea.*

CONCLUSIONS

Following the treatment with *Trichoderma* administered at planting in Buzău 10 bell pepper culture, a beneficial effect on fruit quality, development and crop production resulted.

At the same time, this fertilization is an environmentally friendly option, because it does not pollute the groundwater.

Trichoderma induces an increase in plant productivity, due in part to inhibiting the activity of toxic compounds in the root zone and increasing the absorption of nutrients. It also increases the efficiency of nitrogen use, as well as an increase in the solubility of nutrients in the soil (Rădulescu A., Coțianu R., 2004).

This fungus induces root formation and stimulates colonization with the rhizosphere and other beneficial microorganisms on the roots. It also has the ability to phytoremediation of plant tissues, caused by some residual (persistent) pesticides in the environment.

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COMPARISON OF DIFFERENT TYPES OF MOLECULAR MARKERS USED IN GENETIC DIVERSITY STUDIES OF BROOMRAPE FROM SERBIA

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Abstract

The purpose of this study was to investigate the utility of the different types of molecular markers in assessing the genetic diversity of seven Serbian broomrape (Orobanche cumana Wallr.) populations, such as ISSR and SSR. The discriminatory potential of SSR markers was on average lower (Rp=2.46) than for ISSR (Rp=13.85), indicating a large interpopulation genetic variability. According to the indices of genetic diversity, a higher intrapopulation molecular variability in the case of SSR markers (PIC=0.58; H=0.63) compared to ISSR (PIC=0.33) was revealed. AMOVA analysis also showed a high genetic diversity within populations with SSR markers (within pops 53% and among pops 47%) and the most genetic diversity among populations with ISSR (34% and 66%, respectively). These results showed a high degree of genetic variations among and within broomrape genotypes from Serbia which favor the evolution of more virulent physiological races of O. cumana. This study can serve as scientific support for future researches in monitoring and developing strategies to improve sunflower crops resistant to this pathogen.

Key words*: microsatellite markers, intra- and interpopulation variability, broomrape, populations.*

INTRODUCTION

Broomrape (*Orobanche cumana* Wallr.) began to affect sunflower fields from Serbia since 1950's, when sunflower cultivation area was suddenly expanded with more productive sunflower foreign varieties (Bošković, 1962). Two dominant races of broomrape have been identified in Serbia, namely race B and E at the moment (Mihaljčević, 1996; Miladinović et al., 2014). Thus, the study of the current situation of *O. cumana* infestation of sunflower fields from Serbia has shown a stability and slower evolution of aggressiveness of this parasite.

In order to the development of effective sunflower breeding programs with broomrape resistance, pathogen control durable strategies, the modern methods of study quite effective as molecular markers are increasingly used (Calderón-González, 2021; Cvejić et al., 2020; Duca & Martea, 2020a; Sukhareva & Kuluev, 2018). At the moment there is a rich variety of molecular markers (RFLP, RAPD, SSR, AFLP, SCAR, ISSR etc.). Each of their have its own advantages and disadvantages, so the usefulness and applicability of a single marker system are not universal. The most appropriate genetic marker depends on the aim pursued, and also the presence of sufficient technical facilities, marker desirable properties (highly polymorphic, reproducible, abundance and evenly distributed throughout the genome etc.), and financial limitations.

In recent years, *Inter-Simple Sequence Repeat* (ISSR) technique based on the polymorphism of the nucleotide sequences in microsatellite regions distributed throughout the genome and with high reproducibility (92-95%) was developed. The ISSR sequences belong to a class of semi-arbitrary and multilocus markers (detect polymorphism in different genes or chromosomes) that are inherited dominantly according to Mendelian laws (Idrees & Irshad, 2014; Tsumura et al., 1996).

Simple sequence repeats (SSRs) represent the specific DNA sequences consisting of short (1-9 bp), tandemly repeated di-, tri-, tetra- or pentanucleotide motifs. SSR markers are multiallelic and codominant, so they can distinguish heterozygotes from homozygotes genotypes. These markers belong to the highly polymorphic due to the fact that the repeats are

from a few to more than ten alleles in each locus (monolocus) and widely dispersed across the genome (Sukhareva & Kuluev, 2018; Vieira et al., 2016).

Both molecular marker systems possess a high level of polymorphism therefore they are widespread applied in developing of genetic linkage map, estimating of gene flow, studying of genetic diversity, analysis of phylogenetic relationships and genetic structure of *O. cumana* populations (Benharrat et al., 2002; Calderón-González et al., 2019; Duca et al., 2019; Duca et al., 2017; Guchetl et al., 2014; Pineda-Martos et al., 2014a; Pineda-Martos et al., 2013).

The superiority of the ISSR and SSR methods over other molecular techniques in determining genetic diversity of the different species (rice, cotton, broomrape, nut, pine, narcissus etc.) in a great number of published works is reflected (Abbasi et al., 2017; Noormohammadi et al., 2013; Duca et al., 2019; Jia et al., 2011; Wu et al., 2004; Zangeneh & Salehi, 2019).

The purpose of this study was to determine and compare the effectiveness of two microsatellite marker systems, which are SSR and ISSR for estimating genetic variability of seven Serbian broomrape populations.

MATERIALS AND METHODS

Plant material. Seven populations consisting from 49 genotypes of *O. cumana* were used as experimental material. Broomrape populations were collected from infected sunflower (*Helianthus annuus* L.) fields in Serbia and kindly provided by Dr. Dragana Miladinović (Institute of Field and Vegetable Crops, Novi Sad, Serbia).

Seeds of *O. cumana* were germinated on sunflower roots in the greenhouse of the laboratory. Fresh tissue samples from each population were collected and they were stored at -80°C until DNA extraction.

DNA extraction. Total DNA was extracted using GeneJET Plant Genomic DNA Purification Mini Kit #K0791 according to the manufacturer's protocol (*Thermo Fisher Scientific*, USA). Quantity and quality of isolated DNA were determined by spectrophotometry (T60 UV-VIS) and, also checked by 1% agarose gel electrophoresis in 1xTAE buffer (Sambrook & Russell, 2001).

ISSR and SSR amplification. Fourteen ISSR primers listed in Table 1 were used. The amplifications were performed with a thermocycler Genset 9700 (*Applied Biosystems*) according to the program: 95°C - 5 min; 35 cycles: 95°C - 30 sec, 45°C - 45 sec, 72°C - 2 min; 72°C - 5 min.

The SSR amplifications were conducted using 15 specific markers (Table 1). The PCR program was: 95°C - 3 min; 35 cycles: 95°C - 30 sec, 57°C - 45 sec, 72°C - 1 min; 72°C - 5 min (Veriti-96 Well Thermal Cycler, *Applied Biosystems*).

The amplification products were separated by 2% agarose (ISSR) and 8% polyacrylamide (SSR) gel electrophoresis. The gels were visualized on transilluminator under UV radiation after staining with ethidium bromide. GeneRuler Express DNA Ladder, ready-to-use SM1553 (ISSR) and GeneRuler Low Range DNA Ladder SM1191 (SSR) (*Thermo Fisher Scientific*, US) were used to estimate the molecular weight of amplified products. The molecular analysis results were documented using gel documentation system Doc-Print VX2 (SXT-F20.M, France).

Data analysis. The DNA amplified fragment analysis and determination of allele sizes were performed with the Photo-Capt V. 15.02 and POPGENE V.1.32 software, and also Microsoft Excel Office 2010. *Analysis of molecular variance* (AMOVA) was carried out using GenAlEx 6.501 and Mantel test by means of XLSTAT version 2014.5.03. The *Polymorphic Information Content* (PIC) of each dominant ISSR marker was calculated according to Roldán-Ruiz I. (Roldán-Ruiz et al., 2000) as:

$$PIC_i = 2f_i(1 - f_i),$$

where f_i is the frequency of the amplified allele (band present), and $(1 - f_i)$ is the frequency of the null allele.

The PIC index for SSR markers was computed according to Botstein D. (Botstein et al., 1980):

$$PIC = 1 - \sum_{i=1}^{n} P_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2,$$

where *Pi* and *Pj* is the frequency of the amplified alleles (*i, j*) and *n* is the number of detected amplicons (https://gene-calc.pl/pic).

Resolving power (Rp) was calculated according to Prevost A. (Prevost & Wilkinson, 1999):

$Rp = \sum Ibi$, where *Ibi* describes the relative amplicon informativeness *i* and it is calculated as: $Ibi = 1 - \left[2 \times \left|0.5 - pi\right|\right]$, *pi* is the proportion of individuals with identified amplicon *i*.

Table 1. Nucleotide sequences of ISSR and SSR markers used in this study

No.	*Inter-Simple Sequence Repeat* (ISSR)			*Simple Sequence Repeats* (SSR)		
	Name	Sequence (5′→3′)	NB	Name	Sequence F 5′→3′ /R 5′→3′	NB
1.	BC807	agagagagagagagagt	17	Ocum-052	catgtctaagcttttggctcg/ caagacttggaacaagcaaatc	21/22
2.	BC810	gagagagagagagagat	17	Ocum-059	tcttgatttgtatatgtctgatgcaat/ atgctacaatagaaatacacaacgaac	27/27
3.	BC835	agagagagagagagagyc	18	Ocum-070	aagctgtaaacaatgcctgaa/ cctcctccagtaccactaggc	21/21
4.	BC841	gagagagagagagagayc	18	Ocum-074	cctaaaattgaaaccttaaggaaa/ actttccgtgagacggagtc	24/20
5.	BC857	acacacacacacacacyg	18	Ocum-075	tgtggatagagtataagctaccagttc/ ttcccgtagcttggagaatg	27/20
6.	$(CAA)_5$	caacaacaacaacaa	15	Ocum-081	ttacaaggtgaaaccaccca/ cagctactgtccgcaagaaa	20/20
7.	$(GACA)_4$	gacagacagacagaca	16	Ocum-087	ttctcgacagctttgggtaaa/ atgccaacttcgagtgatcc	21/20
8.	$(GATA)_4$	gatagatagatagata	16	Ocum-108	tcgttaataagtggttcacgaaaa/ tgactaaaaataaaatgtacgggtg	24/25
9.	$(CA)_6R^2G$	cacacacacacarg	14	Ocum-122	ggaatacatcattaaagtagttgtccg/ gaaggagtcattaaactccgtga	27/23
10.	$(CTC)_4R^2C$	ctcctcctcctcrc	14	Ocum-141	cagcaactgtttcttccatagag/ tccaagaagaggaaaagaagtga	23/23
11.	$(CAG)_5$	cagcagcagcagcag	15	Ocum-160	tgagggtttgtaaagtgggc/ cgtaccttatccctccgtca	20/20
12.	$(CT)_8TC$	ctctctctctctctctc	18	Ocum-174	caaccaacaaacaagtagtgacg/ tcttgcggcaaaaccatt	23/18
13.	$(CA)_6AC$	cacacacacacaac	14	Ocum-196	gtatgtgcgcccgtcttg/ ggggatgactgtgtttcgat	18/20
14.	$(AG)_8Y^3A$	agagagagagagagagya	18	Ocum-197	agagacggcatcatcaatca/ gtgatcgtgcaggcaccta	20/19
15.				Ocum-206	ccgattgctgtttatgttgtatt/ tgtaggagatgcccagttca	23/20

[1]NB = number of nitrogenous bases; [2]R= (A, G); [3]Y= (C, T).

RESULTS AND DISCUSSIONS

Investigation of the genetic diversity among seven *O. cumana* populations based on microsatellite markers has allowed for revealing both the significant differences and similarity of genotypes depending on the analysed population or type of primer.

ISSR genotyping. Fourteen ISSR primers generated a total of 195 amplicons, and each primer had 2-22 amplified fragments with an average of 13.93 bands/primer (Table 2).

The highest number of amplicons was produced by $(AG)_8YA$ (22), followed by BC835 (21), BC810 and BC807 (20), $(CAG)_5$ and $(CTC)_4RC$ (19), and the least by $(GATA)_4$ (2). The size of the amplified fragments ranged from 360 to 5667 bp. Percentage of polymorphism varied from 62.50% $(CA)_6AC$ to 100% (BC835,BC841, $(CAG)_5$, $(CT)_8TC$, $(CTC)_4RC$, $(GATA)_4$) with an average of 90.82% (Table 2).

ISSR markers utility was evaluated by calculating the *Polymorphic Information Content* (PIC) and *Resolving power* (Rp). The PIC values for all ISSR primers ranged from 0.19 for $(CA)_6AC$ to 0.40 for $(AG)_8YA$ (mean value 0.33), and have been within acceptable limits for dominant markers (up to 0.50). *Resolving power* (Rp), which indicates the discriminatory ability of the primer to distinguish the genotypes or individuals, was estimated for each marker. The highest Rp value of 23.71 was observed for primer $(AG)_8YA$ and the lowest of 0.78 at $(GATA)_4$ with an average Rp of 13.85.

According to the same ISSR markers, the similar results of PIC values (mean value 0.33) were identified in *O. cumana* populations from China

and Turkey. Another index like Rp was lower both in Turkish (12.42) and Chinese (7.24) broomrape populations (Duca & Bivol, 2021a; Duca et al., 2021b).

Thus, based on the ISSR markers assessment on their informativeness and efficiency by means of the statistical indices (number of total fragments from 9 to 22, percentage of polymorphism ≥92.86%, PIC ≥0.30, and Rp ≥10.49) for the Serbian broomrape populations the following primers were selected: (AG)8YA, (CTC)4RC, BC807, (CAA)5, BC841, (CAG)5, (CT)8TC, BC857, BC835 (Table 2).

Table 2. Characteristics of ISSR marker system used for evaluating genetic diversity of *O. cumana* populations

Marker	Fragment sizes (*bp*[1])	Total number of fragments (N)	Percentage of polymorphism, %	Polymorphic Information Content (PIC)	Resolving power (Rp)
$(AG)_8YA$	467-5000	22	95.45	0.40	23.71
BC807	585-3000	20	95.00	0.32	21.55
BC810	461-5500	20	80.00	0.23	19.76
BC835	480-5125	21	100.00	0.38	17.76
BC841	360-5385	13	100.00	0.37	14.41
BC857	385-2233	15	93.33	0.30	14.29
$(CA)_6AC$	798-2951	8	62.50	0.19	6.04
$(CA)_6RG$	443-1313	7	85.71	0.28	4.86
$(CAA)_5$	631-3183	14	92.86	0.33	16.08
$(CAG)_5$	623-5267	19	100.00	0.38	18.50
$(CT)_8TC$	897-5000	9	100.00	0.38	10.49
$(CTC)_4RC$	470-5667	19	100.00	0.36	19.22
$(GACA)_4$	1117-4326	6	66.67	0.23	6.41
$(GATA)_4$	1059-2376	2	100.00	0.27	0.78
Total	360-5667	195	-	-	-
Mean(±SD[2])	-	13.93	90.82	0.33(±0.01)	13.85(±1.88)

[1]bp = base pairs; [2]SD = Standard Deviation.

These primers $(CTC)_4RC$, $(CAG)_5$, $(AG)_8YA$, BC807, BC841, and $(CT)_8TC$ have found equally efficient to identify of genetic diversity also in the case with Turkish populations (N: 15-28 amplicons, percentage of polymorphism ≥88,89%, PIC ≥0.36, Rp ≥11.37) (Duca & Bivol, 2021a). But for the Chinese broomrape populations were chosen the lowest number of informative markers: BC841, BC857, $(CAA)_5$, $(CAG)_5$, $(AG)_8YA$ (N: 10-14 amplicons, percentage of polymorphism ≥60%, PIC ≥0.35, Rp ≥7.20) (Duca et al., 2021b).

SSR genotyping. Analysis of the genotyping results of 49 *O. cumana* accessions from Serbia has determined that from all 15 SSR microsatellite markers used in this study, the only Ocum-122 is a monomorphic in contrast to other 14 polymorphic markers. Ocum-122 generated a monoallelic profile for all studied genotypes, represented by one type of allele - 244 bp (Table 3). A total of 68 alleles using all studied microsatellite markers were detected. The number of alleles produced of each marker ranged from 1 (Ocum-122) to 11 (Ocum-197) alleles with a mean of 4.53. The average effective number of alleles per marker was 3.53, ranging from 1.00 at Ocum-122 to 5.81 at Ocum-059.

The genetic polymorphism and discriminatory power for SSR markers were determined using the same indices: PIC, Rp and also *Nei's genetic diversity* – H (relevant index for codominant markers). The calculated minimum values of PIC and H for each marker constituted of 0.08 and 0.08 respectively for Ocum-160 but maximum of 0.81 and 0.83 respectively for Ocum-059 with average values of 0.58 (PIC) and 0.63 (H) (Table 3). According to Botstein D. et al. (1980), the majority of the molecular markers used in the genotyping of Serbian broomrape populations proved to be the most informative (PIC >0.5), with the exception of the moderately informative Ocum-074 (PIC =0.41) and the least informative Ocum-160 (PIC =0.08). The similar PIC values for these markers were reported also by other authors (Duca & Martea, 2020a; Duca et al., 2021b; Pineda-Martos et al., 2014b; Ziadi et al., 2018).

The highest Rp values for a single marker were obtained by Ocum-197, with a value of 8.49, followed by Ocum-059 with a value of 5.67. In the case of this index, unlike the results obtained

Table 3. Characteristics of SSR marker system used for evaluating genetic diversity of *O. cumana* populations

Marker	Allele size range (bp^2)	Total number of alleles/ marker (N)	Effective number of alleles (Ne)	Polymorphic Information Content (PIC)[1]	Nei's genetic diversity (H)	Resolving Power (Rp)
Ocum-052	130-178	5	5.00	0.77	0.80	0.00
Ocum-059	85-136	8	5.81	0.81	0.83	5.67
Ocum-070	100-126	4	2.90	0.59	0.66	2.98
Ocum-074	101-114	3	2.08	0.41	0.52	1.96
Ocum-075	98-135	5	5.00	0.77	0.80	0.00
Ocum-081	90-110	3	3.00	0.59	0.67	0.00
Ocum-087	109-151	6	5.35	0.79	0.81	4.00
Ocum-108	143-196	5	3.82	0.70	0.74	2.00
Ocum-122	244	1	1.00	0.00	0.00	0.00
Ocum-141	192-226	4	3.66	0.68	0.73	2.29
Ocum-160	134-145	3	1.09	0.08	0.08	2.04
Ocum-174	190-211	3	2.99	0.59	0.67	3.43
Ocum-196	196-343	4	4.00	0.70	0.75	0.00
Ocum-197	96-173	11	4.67	0.75	0.79	8.49
Ocum-206	118-131	3	2.55	0.53	0.61	4.00
Total	85-343	68	-	-	-	-
Mean(±SD[3])	-	4.53(±2.42)	3.53(±1.49)	0.58(±0.25)	0.63(±0.26)	2.46(±2.44)

[1]Interpretation PIC: high informative level PIC > 0.5; moderate informative 0.25< PIC <0.5; slightly informative PIC <0.25 (Botstein et al., 1980).
[2]bp = base pairs; [3]SD = Standard Deviation.

at ISSR, five markers (Ocum-052, Ocum-075, Ocum-081, Ocum-122, Ocum-196) with a zero value of Rp were highlighted (Table 3). Thus, these markers did not contribute to the differentiation of Serbian broomrape populations. The discriminatory potential of SSR markers was on average lower (Rp =2.46) compared to that identified for ISSR markers (Rp =13.85).

Thus, based on the obtained results of the statistical parameters, the most efficient SSR markers that can be used in the genetic characterization of *O. cumana* populations were highlighted.

The highest values for all analyzed indices and the best potential in differentiation of the investigated broomrape genotypes were determined by the Ocum-059, Ocum-087 and Ocum-197 markers. At the same time, highly informative loci are also Ocum-052, Ocum-075, Ocum-108, Ocum-196, which characterized a wide genetic diversity (values PIC and H ≥0.7) within populations. It is important to note that Ocum-206 also showed a relevant discrimination capacity (Rp =4.00) for the studied genotypes.

The high level of polymorphism determined by SSR markers within populations and genotypes is due to the codominant and multiallelic nature of these microsatellite sequences. In other words, SSR observed polymorphism are the result of the polymorphism of tandemly repeated DNA sequences, identifying a larger number of alleles at a locus (Mason, 2015; Vieira et al., 2016). Also, SSR sequences detect homo- and heterozygous genotypes within population.

The analysis of molecular variance (AMOVA) based on ISSR data, confirmed that the highest percentage of variation of 66% was found among populations, and the lowest of 34% within Serbian populations with a significant genetic difference (P <0.001) (Figure 1).

AMOVA test, based on SSR data set, revealed that 47% of genetic variability was due to the differences among populations and 53% was due to the differences within populations (Figure 1). In four broomrape populations from Republic of Moldova about a high genetic diversity within populations (69%) and a low genetic diversity among populations (31%) were reported by Duca M. and colab. (Duca et al., 2020b). Similar results for the populations of *O. cumana* from Turkey were described, which demonstrated that the differences within populations (66%) were greater than the differences between populations (34%) (Bilgen et al., 2019). Guchetl S. et al. (2014) also

reported that a high proportion of the genetic diversity of 78% was within populations and the 22% was due to variation between populations (Guchetl et al., 2014).

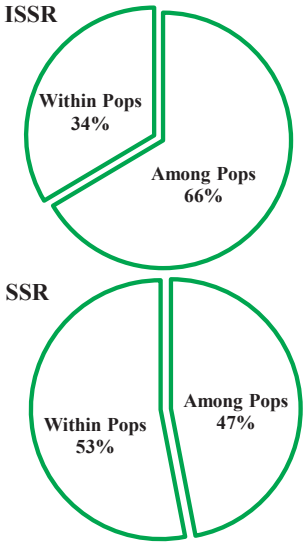

Figure 1. Analysis of molecular variance (AMOVA)

The high polymorphism at interpopulation level, as well as the high power of discrimination identified by the investigated ISSR markers are due to their biallelic and dominant nature.

Also, a significant correlation (r =0.48) was found between ISSR and SSR binary data set by the Mantel test. This correlation reveals the microsatellite nature of both systems of markers and the high level of genetic polymorphism identified by them. The both microsatellite marker sets used have proved a high reproducibility and specificity.

These results showed a high degree of genetic variations within genotypes of *O. cumana* from Serbia which depends directly on environment and the planted lines of sunflower. At the same time, a greater genetic variability determines the evolution of more virulent physiological races of broomrape.

CONCLUSIONS

In the present study the ISSR and SSR microsatellite markers were compared for their efficiency in analyze the genetic diversity of Serbian broomrape populations.

The SSR markers have been more informative in revealing a high intrapopulation genetic variability based for the specific genetic diversity coefficients (H =0.63; PIC =0.58) than ISSR (PIC =0.33). But, the ISSR markers were found more effective in identifying a high interpopulation genetic variability as evidenced by the higher discriminatory potential (Rp =13.85) compared to the SSR (Rp =2.46).

These results were also confirmed by analysis of molecular variance (AMOVA) which showed that for SSR markers 53% of the total genetic variability was due to differences within the populations, and for ISSR markers 66% was attributed to differences among the populations.

Based on the calculated statistical parameters, ISSR markers as: BC807, BC835, BC841, BC857, $(AG)_8YA$, $(CTC)_4RC$, $(CAA)_5$, $(CAG)_5$, $(CT)_8TC$ together with SSR: Ocum-052, Ocum-059, Ocum-075, Ocum-087, Ocum-108, Ocum-196, Ocum-197, Ocum-206 were the most efficient markers and can be successfully utilized in estimating genetic diversity of *O. cumana* populations.

Findings from this research showed evidence for high degree of genetic variations among and within populations of *O. cumana* from Serbia which may lead to change in virulence of parasite and appear more virulent physiological races of broomrape in the future.

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THE ANTIOXIDANT EFFECT OF RAW BEE POLLEN COLLECTED FROM ECOLOGICAL CROPS - MINIREVIEW

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Abstract

Bee products containing bee pollen collected from flowers and harvested by humans from the hive, include both active principles produced by plants and active principles added during collection, processing, and storage by bees. Pollen, is one of the most bee products used in apitherapy, is a valuable source of bioactive substances, as it contains most of the active ingredients that are directly assimilable in the human body (vitamins, minerals, hormones and substances acting as prehormones, enzymes and simple carbohydrates) and have by adding in the diet a wide range of indications, recommendations and applications. As a result, this mini-review systematically presents both the highlighting of the influence of pollen in daily human consumption and the analysis of composition, antioxidant activity, quality parameters and sensory properties of biologically active pollen harvested from organically grown honey crops.

Key words*: antioxidant activity, bee pollen, honey, phenolic compounds.*

INTRODUCTION

Organic beekeeping provides food security and contributes to the quality of life in rural areas, especially in beekeeping practices (Martinello et al., 2021).

Bees are the dominant pollinators of most flowering plants globally and are important for the pollination of many crops. 71% of the plants which generate food worldwide are pollinated by bees (FAO, 2022).

Pollinators are especially important for the conservation of plant biodiversity (Martinello et al., 2021).

Martinello & Mutinelli (2021) pointed out that bees could be considered as active bio-samplers of environmental pollution and possible warning biomarkers for human health through the importance related to human welfare through pollination and production of honey and other bee products.

Most contaminants accumulate in wax (Walsh et al., 2021), but also in other bee products (Zawislak et al., 2019).

The need for ecologic beekeeping farms and organic farming is of vital importance for biodiversity conservation and human health, as this argument has not been fully researched in the scientific community, according to our knowledge.

Rahimi et al. (2020) in the scientific paper "*Organic Beekeeping Practices in Romania: Status and Perspectives towards a Sustainable Development"*, drew attention on the importance of using certain conceptual assessment models in the case of sustainability for the organic beekeeping sector.

This concept is also supported by Kouchner et al. (2018), highlighting the lack of necessary tools for sustainability assessment in this field, and suggesting an assessment system dedicated exclusively to the cultivation of organic plants in order to obtain bee products with perfect antioxidant potential.

Currently, beekeeping is an important sector that is developing a strong focus on organic crops. In general, organic beekeeping is practiced separately from the rest of mainstream agriculture. The clear principles applied to organic farming are also strictly applied in the practice of organic beekeeping and are regulated by legislation (MADR, 2022) and certified by European recognized certification bodies (e.g. Ecocert).

The clear principles of organic beekeeping are to support the health and vitality of bee colonies

with great care and attention, and to minimize negative influences on the environment and especially on consumers of bee products.
First and foremost, the beekeeper must have extensive expert knowledge of natural bee behavior and organic hive methods (Pocol et al., 2021).
For a sustainable development of ecological beekeeping, an important measure is training to increase the level of socio-cultural development of the parties involved in this process: farmer - beekeeper. Education for the growth and development of ecological services with modern technologies targeted at farmers and beekeepers. In addition to these support measures, there is a need for the development of support programmes for stakeholders in organic farming and beekeeping and the ability to understand the legislation, according to Juríčková et al. (2020). Their research (Juríčková et al., 2020) sees organic farming as a 'sustainable' farming system offers a potential solution for sustaining biodiversity by contributing to environmental protection.

THE NUTRITIONAL ANALYSIS OF POLLEN

Modern agricultural systems use both bees and wild pollinators to provide pollination, which have the ability to pollinate in confined spaces or cold temperatures. The decline of bee species due to the increased use of pesticides raises concerns about pollination in agriculture. The number of farmers is quite low in terms of adopting more environmentally friendly practices for pollination. This mini-review aims to identify an eco-economic model at the bee farm level to explore the impact of pollination on organic crop production.
This study evaluates farmers' decisions of adopting and optimizing organic crops with a well-defined role in ensuring pollination and the good development of bee and other pollinators.
Results show that through various organic farming and beekeeping sustainability schemes, effectively for encouraging and implementing new farming practices to ensure pollination and maintenance of organic beehives and viable pollinators, farmers are willing to cultivate organic honey crops for pollen collection maintenance (Kleftodimos et al., 2021).
According to a study conducted by researchers (Feás et al., 2012) it appears that the antioxidant activity of pollen is higher due to the fact that it comes from organic plant crops.
Analysis of the origin of pollen from different geographical areas is especially important to identify the antioxidant value of pollen and the organic plants of origin.
Through palynological analysis, it is possible to identify the floral areas, the vegetation in certain honey bases, very importantly the honey species that have been of interest to bees in certain periods.
The nutritional analysis of pollen collected by bees, made by researchers, has revealed that it is an excellent source of energy (Feás et al., 2012). For example, pine (*Pinus sylvestris* **L.**), corn (*Zea mays*) and common reed *Phragmites australis*) pollen contain: 13.92; 36.59; and 31.93% total carbohydrates, 13.45; 20.32; and 18.90% protein; 1.80; 3.7; and 1.16% lipids and 2.35; 4.90; and 3.80% total ash, respectively. It is obvious that, by analyzing these pollen sources, we can also identify the organic honey plants that honeybees (*Apis melifera* L.) visit predominantly (Feás & Estevinho, 2011).
The increased interest in recent times of researchers in an older ecological plant has been studied (Berti et al., 2016), in the paper *Camelina uses, genetics, genomics, production, and management*, which, according to the researchers, sown in autumn, but also in spring, provides pollen for honeybees as well as for wild pollinators, both in season and between the harvest of the main honey plants. Researchers say that, through this pollination mechanism, the camelina provides an abundance of nectar.
Camelina sown in late fall begins flowering in early May and camelina sown in early spring begins flowering in late June, both of which flower much earlier than soybean, rapeseed, sunflower in the same area (Eberle et al., 2015; Thom et al., 2016).
Camelina sown in late autumn yielded more pollen (100 kg/ha) than rapeseed (*Brassica napus oleifera*) (82 kg/ha) (Eberle et al., 2015).
Camelina flowers are a particularly reliable source of pollen and nectar, providing 100 to 250 kg of honey a year to a family of bees (Axel et al., 2011).
Moreover, organic plants, *Camelina species, C. microcarpa* and *C. alyssum* are visited by

honeybees more frequently (Séguin-Swartz et al., 2009), and according to the Canadian Food Inspection Agency (CFIA, 2012) provides abundant pollen compared to the shepherd's teat (*Capsella bursa pastoris*) a plant with important therapeutic properties.

Numerous specialized studies (Faure & Tepfe 2016) show the importance of antioxidants in camelina oil, high levels of omega-3 lipids, vitamin E, are beneficial to human health (Zubr 1997).

The classical method of collecting raw pollen collected by bees consists of fixing a pollen collector with holes of 5 mm in diameter in front of the hive warp, which has a collecting drawer. Bees need a high variability of pollen sources for the harmonious development of the colony (Abramovic & Abram, 2005).

Honeybees have the ability to produce about 6 different hive products used in apitherapy (Kolayli & Keskin, 2020) honey, pollen, propolis, royal jelly, shepherd's purse, bee venom, and a more recent hive product apilarnil discovered by Ilieșiu in 1980.

Table 1 shows the total content of polyphenolic compounds, flavonoids, phenolic acids, anthocyanins, and carotenoids in polyfloral honey with added pollen according to (Habryka et al., 2021). The higher the pollen addition in honey (Tomczyk et al., 2019), the higher the content of phenolic compounds in the honey analyzed by (Habryka et al., 2021), reaching a value of 178.26 mg GAE/100 g (Table 1) for the sample with the largest addition of grams of bee pollen.

Table 1. The total phenolic, flavonoid, phenolic acid, anthocyanin, and carotenoid content in multiflower honey and honeys enriched with bee pollen.

Addition of Bee Pollen (%)	Total Phenolic Content (mg GAE/100 g)	Total Flavonoid Content (mg QE/100 g)	Total Phenolic Acid Content (mg CAE/100 g)	Total Anthocyanin Content (mg/100 g)	Total Carotenoid Content (mg/100 g)
0	30.75 ± 0.25	2.77 ± 0.29	11.02 ± 0.68	2.01 ± 0.05	0.138 ± 0.001
5	63.33 ± 0.27	5.94 ± 0.25	16.65 ± 0.19	4.02 ± 0.05	0.311 ± 0.004
10	89.42 ± 0.61	8.38 ± 0.19	17.08 ± 0.23	5.57 ± 0.38	0.934 ± 0.001
15	136.63 ± 0.44	12.11 ± 0.48	20.32 ± 0.52	7.60 ± 0.19	1.404 ± 0.002
20	156.13 ± 0.92	14.25 ± 0.27	21.26 ± 0.39	9.16 ± 0.09	1.726 ± 0.001
25	178.26 ± 1.13	16.39 ± 0.16	24.44 ± 0.17	11.32 ± 0.10	2.333 ± 0.001
$LSD_{0.05}$	0.83	0.36	0.51	0.22	0.003

Table source: Habryka et al., 2021.

According to the study Kocot et al. (2018), the phenolic acid content of pollen can even reach 190 mg/100 g, with gallic acid being the most important representative of that.

The medicinal properties of these products were recognized thousands of years ago by ancient civilizations, although in modern times they have limited use.

Hive products are complete sources of bioactive compounds, macro- and micronutrients, which in a comprehensive way confer multiple biological actions to these by-products, such as, for example, antimicrobial, antioxidant, and anti-inflammatory properties (Giampieri et al., 2022).

Bee products, represent an inexhaustible source of natural antioxidants, including phenolic acids, flavonoids and terpenoids, as well as numerous other phytochemicals, which are able to ameliorate the effects of oxidative stress underlying cause of many diseases (Martinello et al., 2021).

Pollen consists of a multitude of microscopic corpuscles contained in pollen sacs in the anthers of plant flower stamens.

Physiologically, they are tiny granules that constitute the male fertilized elements of the flower (male reproductive part) (Ialomiteanu, 1976).

According to a study by Dr. Rawhi (2012), bees harvest more than 300,000 pollen grains in a single transport, as they have three pairs of limbs, anterior, median, and posterior.

The bee's limbs are morphologically adapted to perform specific pollen-collecting functions

(Beekeepers Association - Beekeeping Research and Development Institute, 2012).
The pollen grain has a diameter of 1:50 000 µm, and the bee collects about 12 - 15 mg of pollen in a single transport, equal to one tenth of its weight (Rawhi, 2012).
The weight of the pollen grain differs according to the atmospheric humidity, floral origin, but also to the orientation ability and vitality of the bee. After harvesting, the pollen is transported to the hive, stored in honeycombs, enhanced with enzymes, enriched with enzymes and a little honey and then the bee presses it into the honeycomb cell.
Collection by the beekeeper is carried out by various techniques before it is stored in the combs (Francis-Baker, 2021).
The pollen collected by the beekeeper holds the color of the flowers visited by the bees. The chemical structure of the pollen varies significantly depending on the weather conditions during harvesting and the development of the floral anthers.
Pollen is considered a rich product of bioactive ingredients, including proteins, carbohydrates, lipids, phenolic compounds, and vitamins (Spulber et al., 2020).
By drying, pollen loses vitamins, aminoacids, volatile substances, biological water, lactic flora with antibacterial activities, lactoferments.
Frozen raw pollen retains all these active elements, as well as the intense taste and pastel colors.
Freshly frozen, raw pollen can be kept at a temperature of -5 to -15°C.
The raw pollen in the collector must be cleaned of impurities and foreign bodies, left to dry in the shade, protected from sunlight, heat, moisture, dust, and insects.
Ultraviolet rays destroy pollen, the atmospheric temperature should not exceed 40-45°C and it is very important not to use air fans.
Fresh raw pollen can be left at room temperature for about 5 days and 14 days in the refrigerator.
An essential factor is that freezing and thawing of raw pollen can be repeated without affecting its quality due to its low water content (8-10%) and the fact that it does not contain pathogenic bacteria.
Freshly frozen pollen retains its qualities for about two years after collection according to Dr Rawhi (2012).
Like any protein-rich food it loses its nutritive and curative qualities within a few days if not stored properly (Spulber et al., 2020).
Raw pollen is recognized as a food with some standardized qualities in countries such as Argentina or Switzerland (Codigo Alimentario Argentino, 1998, Buenos Aires, Argentina: La Canal y Asociados).
In Argentina, for example, the quality standards of raw pollen are well defined:
aerobic microbiological ones should not exceed ISO x 10° CFU/g, fungal 10° CFU/g without pathogenic microorganisms, moisture should not exceed 8%, pH between 4 and 6, protein content should be approx. 15-28% Kjeldahal (N x 6.25) weight of dry pollen (Method developed by Johan Kjeldahl).
Raw pollen must be free of impurities, foreign bodies.
Raw pollen is analyzed in specialized laboratories, the most commonly used being to determine its vegetable origin.
In an article (Spulber et al., 2020) it was pointed out the need for a study at the whole geographical area of Romania in order to identify the type of monofloral pollen and the characteristics of its antioxidant properties.
The biological quality of raw pollen collected by bees is closely related to its plant origin, nutritional quality, nitrogen content and chemical composition.
Physical properties are understood to mean the ovoid or spherical shape of a pollen grain which is about 20-40 microns in diameter and has two envelopes called exine and intine.
The color of the raw pollen indicates its plant origin or geographical area, ranging from golden yellow to black.
Identification of raw pollen is based on morphological characteristics examined with a scanning electron microscope (SEM) and screening of phenolic compounds of raw pollen collected by bees is performed using the capillary electrophoresis method.
Raw pollen samples were collected from bee colonies in the stationary hive. From each type of pollen samples were collected and stored in individual containers at -18°C (Spulber et al., 2020).
However, taking into account the variable composition of raw pollen, one of the important

biological activities is its antioxidant activity (Freire et al., 2012).
Many studies have reported a positive correlation between antioxidant activity and the total phenolic content occurring in the composition of raw pollen collected by bees (Domenici et al., 2015).
The flavonoid content of raw bee-collected pollen can be up to 2.5% being mainly found as glycosides. Kaempferol derivatives, apigenin, luteolin and quercetin derivatives have been detected in raw pollen. The most common phenolic acids found in raw pollen collected by bees include gluconic, chlorogenic and formic, lactic, butyric acids and their derivatives.
Raw bee pollen is also rich in carotenoids and vitamins, including tocopherols and, in smaller quantities, calciferol. In addition, raw pollen collected from bees is a rich source of valuable macro and microelements for the human body (Kocot et al., 2018).
Biologically active compounds present in bee pollen include substances with different properties, e.g., phytosterols, organic acids and enzymes. Compounds possessing antibacterial properties include inhibins and phenolic acids, triterpenes and phytohormones (Pascoal et al., 2014).
Raw pollen collected by bees is a valuable source of vitamin E, which, due to its antioxidant properties, protects unsaturated fatty acids and some vitamins against oxidation.
Raw bee pollen also contains quercetin, an antioxidant that reduces LDL cholesterol in the human body and also has anti-atherosclerotic properties (Denisow & Denisow-Pietrzyk, 2016).
Polyphenols play a significant role in detoxifying the human body after drug or alcohol intoxication. They inhibit the activity of enzymes that are responsible for the formation of inflammation. They also have an antibiotic effect on fungi and bacteria pathogenic to humans.
Raw pollen collected by bees has an anti-atherosclerotic effect as it reduces the content of total lipids, total cholesterol and triacylglycerides in blood serum and also reduces the aggregation capacity of platelets.
Pollen is also of high nutritional value and supplements deficiencies of exogenous vitamins, bio elements and aminoacids (Kocot et al., 2018).
An interesting theory is the identification of resveratrol in raw pollen found in the *Brassica* sp. and in the methanolic extract from raw pollen of *Papaver* spp. Pollen type and luteolin has been identified in high amounts in *Papaver* spp. (Thakur & Nanda, 2020) also reported the presence of resveratrol in different *Brassica* species.
Antimicrobial activity of raw pollen collected by bees has been studied and it has been demonstrated by scientific work that raw monofloral pollen and also raw polyfloral pollen has clear antitumor, immunostimulatory action (Basim et al., 2006; Carpes et al., 2007).

POLLEN RESEARCH AS AN ANTIOXIDANT

According to research by Spulber et al. (2020). 45% of the raw pollen collected by bees is predominantly a single type of raw pollen of floral origin.
Phenolic compounds are regarded as the major constituents of bee pollen responsible for antioxidant activity (Harif et al., 2017).
An important research study in determining the antioxidant potential of raw pollen samples collected by bees (Habryka et al., 2021) was conducted using an ethanol-water extract with an initial concentration equal to 0.2 g/mL. An appropriate amount of sample was dissolved in ethanol: water solution (1:1 v/v), centrifuged (3000 x g; 15 min) and filtered through filter paper. The analysis was performed on a UV-Vis V-630 spectrophotometer (Jasco, Tokyo, Japan).
Research on raw pollen by Habryka et al. (2021), revealed the impact of raw pollen addition on the sensory characteristics of honey. Its color, odor, texture, and taste were evaluated. The mean results of the polyfloral honey colour assessment sensory descriptors were analysed to estimate colour: brightness, clarity, cloudiness, and uniformity. Polyfloral honey was designated as very golden, clear, and very uniform when mixed with pollen (Spulber et al., 2020.)
Furthermore, the analysis carried out did not reveal differences in texture and increasing the addition of raw pollen collected by bees to the honey reduced its brightness. Already an addition of 5% raw pollen collected by bees reduced honey brightness to an average value of

3.07, and with the addition of 25% raw pollen collected by bees, honey brightness was reduced to a level of 1.21 (Spulber et al., 2017).
The addition of pollen also reduced honey uniformity, clarity, and brightness.
In honey samples with added pollen, color uniformity was good and differences between the specimens for honey samples with different amounts of added bee pollen were small.
Raw pollen is considered a complete food, a valuable hive product, and, due to its composition, it can be transformed into a protein food and a complex nutrient (Kouchner et al., 2019). Raw pollen contains most of the vital elements of the human body. It acts in an absolutely miraculous natural synergy with the body, this synthesis cannot be artificially reproduced in the laboratory.
Based on the research work of Spulber et al. (2020) concluded the following hypothesis the pharmacological properties of pollen are different due to the potential source of over 1000 different flowers.
The most important properties of raw pollen, according to scientific research are: antibacterial, anti-inflammatory, antibiotic, antioxidant, antitoxic, aphrodisiac, allergy, anabolic, antisclerotic, antidepressant, antipyretic, diuretic, lowers LDL cholesterol in the blood, reduces the risk of genetic diseases, raw pollen is tonic, hepatoprotective, regulates hormones, is adjuvant against the negative effects of chemotherapy, is beneficial for the prostate (Donadieu, 1981).
Many medical researches, as well as some scientific research works have shown that pollen administered daily performs decongestive actions in various stages of prostate hypertrophy, ensuring preventive effects of prostatism (Donadieu, 1981) in elderly people.
According to Donadieu (1981), reliable results can be obtained by continuous daily administration of two tablespoons of raw pollen.
According to the study carried out by Spulber et al. (2020) raw pollen contains plant pigments, in particular flavonoids which in combination with vitamins C and E become a powerful reducer of free radicals determining the development of atherosclerosis and cardiovascular diseases. Flavonoids in pollen increase the collagen-storing action of vitamin C. (Teixeira et al., 2007).
According to the studies conducted by Dan et al. (2018) for a pollen harvest without *Nosema* spp. spores at the beginning of the winter season of bee families is important the source of honeycomb with food reserve. Examination of *Nosema* spp. spores presence in the food reserve before the winter makes it possible to remove (eliminate) from consumption these sources of infestation, which significantly decreases the morbidity and mortality rate among bees.

CONCLUSIONS

This paper is a summary of previous research on the impact of enriching the daily diet with raw pollen.
By adding raw pollen to the daily diet research studies have shown an increase in the phenolic content leading to an increase in antiradical and antioxidant activities.
A natural and fresh preparation can be a novelty for the end consumer, for the treatment of certain diseases or as a nutritional supplement, it can be a future research topic with favorable implications for a number of fields such as environmental protection, industry, apitherapy.
Romania has a great beekeeping and beekeeping potential, by encouraging and specializing young farmers with financial and informational support, this type of product with added raw pollen collected by bees can be made for the natural apitherapy food industry.
The key to a healthy lifestyle is a reorientation towards nature, a minimum care for the environment, an exceptional care for health through the quality of the products consumed, with a rich nutritional value.

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BIOTECHNOLOGICAL APPLICATIONS OF MYCORRHIZAL PRODUCTS IN INTENSIVE AGRICULTURE

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Abstract

The article analyzes the evolution of mycorrhizal fungi, which have occupied the function of symbiotic partners in association with plants, more precisely with their root system. The advantage brought by the existence of mycorrhizal symbioses for plant nutrition, highlighted the influence that this association has on the growth and development of plants. Mycorrhizae are present in mature ecosystems, ecosystems that show a cyclical and unitary evolution of the components between the biotic and abiotic unit, when mycorrhizal associations have the role of regulating the assimilation of food resources for the plants with which they are associated. In this association, hyphae play an important role in the nutrient cycle, having the function of stopping losses in the ecosystem.
The present study aims to highlight the benefits of associating fungi with plant roots on wheat production. Thus, it was cultivated on a small area of wheat on a land where a fungal suspension was inoculated. Cultivated in parallel the control variant, in order to highlight the benefits of mycorrhiza. The obtained productions, the abiotic factors and the evolution of the plants were analyzed.

Key words: *Mycorrhizae, fungi, symbiosis, nutrients.*

INTRODUCTION

Mycorrhiza is a form of symbiosis between fungi and plants, more precisely a fungus comes in contact with the roots of plants. After studying the fungus-plant relationship, especially the connection with the root system of the plant, it was observed a considerable capacity of them to deliver nutrients to the plant. Thus, it is believed that mycorrhiza can replace inorganic fertilizers in cereal cultivation technologies in an intensive system. The need for crop fertilization and the principles of rational fertilization are summarized in the fertilization plan which is the instrument of control and management of fertilizers. The fertilization plan is based on a foundation made up of the combination of the following parameters: crop rotation, the genetic production potential of the crop, the availability of the nutrient reserve in the soil, the water resource and the dose of fertilizer applied. The dose of fertilizer applied is the result obtained from the calculation of the system made up of three equations: the genetic production potential of the crop, the availability of the nutrient reserve in the soil and the water resource. Basic fertilization is carried out with organic fertilizers and / or complex chemical fertilizers that provide the plants with the necessary nutrients, which they need for the desaturation of the vegetative cycle with a satisfactory result. The role of the *Glomus intraradices* fungus is to form mycorrhiza, and improve plant nutrient absorption from the soil.

In mycorrhizal symbiosis, the main benefit for the host plant is the progressive assimilation of immobile nutrients, especially nitrogen and phosphorus. The vesicular-arbuscular type relationships increase the assimilation of nitrogen in the tissues of the host plant, as a result of the competition of hyphae for mineralized organic nitrogen. The most important role is to minimize chemical fertilizer inputs from the farm management system, optimizing nutrition cycles - with minimal negative environmental impact - by ensuring increased yields. Therefore, these fungi should be considered an important component in sustainable agricultural system.

The shrub was assumed to be a key unifying structure and is the only place where the mushroom acquires carbon. This certainly does

not rule out the role of carbon transfer regions of intercellular hyphae and intracellular loops. There are other difficulties: the definition states that all descriptions of vesicular-arbuscular fungal species must be accompanied by proof that these fungi can form shrubs, which is problematic because, in some symbioses, curly hyphae predominate; there are also fungi in which the development of spores and vegetative morphology are typical but atypical carbon transfer. Leaving aside these issues, the remaining characters used by Morton and Benny (1990) are based on spore characteristics that are qualitatively varied but stable and distinct in each species. Vegetative structures were not used due to the plasticity of their development and their variation in different host plants.

The choice of the host plant was made according to its genotype. The effectiveness of the same species of mycorrhizal fungus can vary greatly between different species of host plants, so some associations are much more effective than others. The effectiveness of symbiosis may vary within the same species depending on the soil conditions in which the species is located (e.g. pH variations). The physiological and anatomical characteristics of the host plant influence the way in which colonization takes place; therefore, plants dependent on a large amount of phosphorus are more susceptible to colonization with vesicular-arbuscular fungi than the less demanding ones. Depending on the amount of absorbent hairs that the plant possesses, colonization can be more or less efficient. Consequently, it was chosen as the host-wheat plant, meeting the primary conditions.

Another parameter that influences mycorrhiza is the soil - the structure of the soil influences the characteristics regarding the water regime, the biochemical processes, the carbon storage, the resistance to erosion. Soil organic matter plays a major role in the formation of soil aggregates, as a result of biological activity in the soil.

Mature ecosystems are characterized by a permanent and cyclical movement of the elements between the biotic and abiotic part of which they are composed. Mycorrhizae have the role of regulating the composition and functioning of plant communities by allocating food resources and influencing the growth of the plants with which they interact.

Fungi interact with nitrogen-fixing bacteria found in the soil. Colonization with vesicular-arbuscular fungi favorably affects the populations of nitrogen-fixing bacteria in the rhizosphere of the plant that colonizes it; and the growth and development of the plant colonized by both organisms is greatly stimulated. The hypothesis that mycorrhizae should be independent at all times from the physiological state of the host plant is difficult to accept. Following an experiment by Azcon et al. (1978), with hormones synthesized by different bacteria (*Azotobacter, Rhizobium, Pseudomonas*), it was found that the formation of mycorrhizal associations is positively influenced by treatments on host roots. Among the hormones used in the experimental stage, auxins are distinguished by the influence they have on the formation of roots and on the relaxation of cell walls; gibberellins act on the formation of leaves and roots, and cytokinins are involved in the basic processes of plant growth.

The aim of the study is to use the benefits of mycorrhizae in the productivity of wheat plants. The experiment of absorption of macronutrients from the soil was performed in relation to the wheat flats. The system designed to highlight the benefits of mycorrhizae was to sow wheat in a chemically unfertilized soil. The necessary food being provided by the connection made between the root system of the plant and the extraradical hyphae of the fungus.

MATERIALS AND METHODS

Experimental works were performed both in the field and the collaborating laboratories of Agricola Berceni SRL. The multiplication of the *Glomus intraradices* fungus was used, on a solid nutritious Bulion type culture medium. After Multiplication, they were stabilized in compliant solutions, later reaching the experimental field cultivated with the host-wheat plant. Two experimental plots of wheat were established in the experimental field. One plot was cultivated as a control, while the second plot was cultivated and treated with mycorrhizae. In order to establish the wheat crop, the technology according to this crop was executed, without compromising on any operation.

Two experimental fertilization variants were used:
Variant 1: Suspension based on fungi - *Glomus intraradices* - Mycorrhiza: with rooting effect it was used for fertilizing the crop. The fertilizer was added to the surface of the crop substrate when the wheat crop was in the needle phase. An average rate of 16 L/ha of dizziness was distributed on the mycorrhizal base. The fertilizer was added in one pass. The same treatment options were applied for the two plots eluted in the analysis. The Mycorrhiza-based compound used was added in order to determine the rapid development of the root system based on the formation of the mycorrhizal relationship optimizing the transformation of nutrients blocked from solin nutrients available to the plant, bringing the elements in a form assimilable by the root system. fortified.
Variant 2. Classical wheat cultivation technology. At sowing it was fertilized by incorporation with NPK complex (15-15-15 active substance). Straw cereals have specific average nutrient consumption (Cs = kg N, P_2O_5, K_2O/1 t production), but are extremely demanding to fertilization, given that they have a poorly developed root system and have low solubilization capacity. nutrients from soil reserves, especially wheat. Later in the winter, at the start of vegetation, in the spring was fertilized with 140 kg N active substance in the form of Urea, being film-coated with neem oil. The presence of neem oil, which is an inhibitor, allows the root system to gradually absorb nitrogen over a longer period, acting as a nitrification inhibitor, preventing leaching, and in the phenological phase of the bellows. applied foliar fertilizer highly concentrated in micronutrients, containing cationic micronutrients (iron, copper, manganese and zinc) that are completely chelated (EDTA).
Later, after the winter period, at the beginning of vegetation, in spring, the active substance in the form of urea was fertilized with 140 kg N, and in the phenological phase of grain formation the phase in which the formation of reproductive elements takes place (micro and macrospogenesis), microgametogenesis, growth of floral components, blades and elongation of the rachis segments. With the appearance of the ear, there is macrogametogenesis and finalization of the formation of all organs of inflorescence and flowers, and flowering occurs fertilization and formation of zygotes-applied foliar fertilizer highly concentrated in micro-nutrients, containing cationic micronutrients and zinc, which are fully chelated (EDTA).

RESULTS AND DISCUSSIONS

A well-developed root system means a good capacity for the absorption of nutrients from the soil followed by the sustained development of the aerial part of the plant, the increase of the vegetative mass and the final increase of the quality of the harvested vegetables. The addition of phytohormonal solutions can add to the growth of the plant.
The purpose of biological fertilization with mycorrhizal compound (*Glomus intraradices*) is to identify an increase in wheat yield. From the first year of cultivation the differences in productivity were observed. The control variant registered in the agricultural year 2019/2020 a production of 3500 kg/ha, while in the variant treated with Mycorrhiza a production of 4950 kg/ha was registered. The production growth trend for the variant treated with mycorrhiza was also maintained in the agricultural year 2020/2021, in pursuit of productivity in the agricultural year 2021/2022. But in addition to increasing productivity, the aspects of physical/ architectural development of the plant were also monitored.
In most types of mycorrhizae, the movement of carbohydrates, produced during photosynthesis, is done from the host plant (autotrophic partner) to the symbiotic fungus (heterotrophic partner). In the case of absorption of nutrients from the soil, the transfer has an inverse direction, from the fungus to the host plant (Jacobsen 1999). The contribution of vesicular-arbuscular fungi to the assimilation of nutrients is the absorption of nutrients (especially phosphorus) from the soil, with the help of extraradicular hyphae - especially from those parts of the soil to which the plant did not have access. The hyphae of the fungus act similarly to the absorbent hairs on the root of the plant; After comparing the diameter of the absorbent hairs (5-20 μm) with that of the mushroom hyphae (3-7 μm), the absorbent hairs would gain the cause, but comparing the length and density of the mushroom hyphae with that of the absorbent hairs - the fungus would be,

because it exceeds the possibilities of expansion of the plant by 10 to 100 times more.

Glomus intraradices increased the concentration and content of macronutrients, especially nitrogen from the root in the plant, this being supported by the coloristic aspect of the foliar apparatus of the plant, taken in comparison with the control variant. It is known that a proper nutrition with nitrogen leads to a coloristic of the foliar apparatus of dark green wheat, while the lack of this element corresponds to a foliar apparatus of green-yellow color.

Concentrations and nitrogen content in the root were significantly higher when the hyphae had a rich water intake.

As can be seen in Figures 1 and 2, the control variant shows a weak development compared to the variant treated with mycorrhizae, both in terms of root system and foliar apparatus. The photo presented above is in the first decade of April.

The abiotic factors that impact mycorrhizae have been studied.

The light. The energy source of the symbiotic fungus is in the plant and depends directly on how it processes photosynthesis and its ability to translocate the products of photosynthesis to the root (Varma, 2008). The lack of light source causes a restriction for the development of the fungus, so its evolutionary process is slowed down, sporulation no longer takes place, and the spread of the mycelium in the soil and root is reduced.

Temperature. In terms of spore germination processes, root penetration by hyphae, and proliferation within cortical cells, temperature may be a limiting factor (Gavito et al., 2005).

Soil pH. The efficiency of the fungus-plant association is determined by the adaptability of the fungal partner to a certain pH level of the soil. The pH affects both the germination of spores and their development. The relationship between soil pH and the effects of mycorrhizae depends on the host species, soil type, phosphorus forms, and fungal species involved.

Salinity. In the case of high salinity, a decrease in the production of propagating structures (propagules) and in the colonization of vesicular-arbuscular fungi was observed (Pfeiffer & Bloss, 1988).

Figure 1. Variant comparison (left-mycorrhiza-treated variant, right control variant)

Figure 2. Variant comparison (left - control variant, right - mycorrhiza-treated variant)

CONCLUSIONS

The general conclusion of the research is to identify the intake of nutrients brought by mycorrhiza for wheat cultivation. The mycorrhizal relationship leads to the solubilization of minerals, the production of plant growth stimulants and the control of

pathogens. The cumulative benefit brought to the plant leads to a high production by substituting chemical fertilize.
It is concluded that it brings a major benefit in the transport of nutrients for plants.

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USE OF HYDROGELS AS A SUSTAINABLE SOLUTION FOR WATER AND NUTRIENTS MANAGEMENT IN PLANT CULTURE

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Abstract

The current climate changes are felt at global level through two primary consequences, namely the increase of day and night temperatures and the decrease of water availability, a fact that determines an important impact in the practices of cultivating plants. Due to this reason, superabsorbent materials such as hydrogels, have been developed to be used especially in plant cultivation systems. The practical relevance of the use of hydrogels in plant substrate could lead to an improvement of water and nutrients management conditions in the field of agriculture. Hydrogels act as a reservoir of water and/or nutrients with gradual release according to plant requirements and in a controlled manner. Thus, a favorable environment for the development of roots and plants is created and maintained throughout their growth. At the same time, this technology brings advantages to the physical characteristics of the soil, by improving the porosity of the soil, thus considerably reducing the undesirable effects observed when applying conventional watering techniques, such as soil drying after sprinkler irrigation and soil erosion during gravity irrigation.

Key words*: hydrogels, water and nutrients management, plant culture conditions.*

INTRODUCTION

Nowadays, the agricultural sector, and therefore the food industry, is facing many problems, but the most important are the challenges due to climate change which causes significant reduction of crop yields (Vicente, 2022); drought affecting the soil being one of the main outcomes of this process. As a result, agricultural production is carried out on arid and semi-arid soils with large pores leading to less water and fertilizer retention, thus affecting their quality and productivity (Oladosu et al., 2022; Singh et al., 2021).

These emerging issues lead to the development of new sustainable and highly efficient agricultural technologies, reducing the negative impact on the environment and providing a suitable growth ecosystem for different plant species (Kassem et al., 2022).

Ecological and sustainable agriculture requires a more intensive use of biological relationships in nature, a more rational use of all natural resources and means of production, and the use of friendly technologies to avoid environmental pollution and increase soil fertility (Liu et al., 2022).

At the same time, organic agriculture, as an alternative to intensive commercial agriculture, is based on a specific agricultural system, which is designed and managed in three directions: ecological, economic and social. Plant cultivation is based on ecological principles such as diversity, stability, equity and productivity, and conventional technological elements are replaced by ecological ones (Ghobashy, 2020).

In this context, the latest development in this field is focused on finding new and sustainable solutions able to respond to these aspects: to assure the water and nutrients for plants with a minimum negative impact on the soil-plants system.

Hydrogels have been defined in various ways and the most precise description refers to hydrogels as water-swollen materials with cross-linked polymeric chains. Also, can be described as materials with remarkable swelling ability that not require structural changes or changes in shape and volume (Oladosu et al., 2022).

In fact, hydrogels are three-dimensional polymer networks, which can be assimilated with a huge macromolecule, with the ability to

incorporate large amounts of water or aqueous solutions (Durpekova et al., 2022; Oladosu et al., 2022). In general, the three-dimensional networks of hydrophilic polymers can absorb the amount of water representing at least 20% of the total weight and if water quantity represents more than 95% of the total weight, the hydrogel is called superabsorbent.

The structure and properties of hydrogels depend on their nature, being synthetic as polyesters (non-biodegradable), or natural, based on proteins (for example, gelatine or collagen), or based on polysaccharides (for example, agarose and alginate) and also of the type of bonds between the macromolecular chains, which can be chemical or physical (Ghobashy, 2020; Oladosu et al., 2022).

Chemical gels present interchain covalent bonds, while physical gels present as interchain connections hydrogen bonds, van der Waals bonds (Skrzypczak et al., 2022).

Physical hydrogels are primarily three-dimensional networks formed by secondary bonds, also known as non-covalent bonds (such as hydrophobic interaction, chain entanglement, hydrogen bonding, and electrostatic interaction), between linear molecules to form physical cross-linking joints. The sol-gel appearance for these hydrogels is usually reversible and because no chemical reactions are involved, they are suitable for biomedical applications; (De Kruif et al., 2015; Oladosu et al., 2022).

But then, chemical hydrogels are produced by chemical reactions - irreversible molecular cross-linking that occurs during their formation. They possessed good mechanical properties and stable properties (Oladosu et al., 2022). However, until now, most available hydrogels are produced based on acrylate, so they are non-biodegradable. Moreover, there are some concerns related to the possible toxic effect after their use in the soil (Oladosu et al., 2022).

The most important property of hydrogels is that they swell in the presence of water and contract in its absence. The extent of swelling is determined by the nature of the polymer chain and the crosslinking density.

The higher water content determines the mechanical, diffusion and adsorption properties of hydrogels, giving them the ability to mimic living tissues. Besides that, the higher water content allows the fraction of biomolecules bound to the surface to increase, thus controlling the interactions between the hydrogel and biopolymer (Guilherme et al., 2015).

The development of smart polymeric and biopolymeric materials can help the agricultural sector because of their high water and mineral retention and releasing capacity (Oladosu et al., 2022; Ramli, 2019). Different types of hydrogels are currently developed and studied by researchers as sustainable and eco-friendly biopolymeric materials for agricultural applications (Durpekova et al., 2022).

MATERIALS AND METHODS

In this paper, a literature review that investigated research articles published in the past decade, regarding the benefits of using hydrogels in order to improve the culture conditions of different plant species was carried out. The review paper presents a synthesis of the properties and characteristics of different types of hydrogels, with special focus on the advantages of their use in relation with their sustainability and eco-friendly benefits. The selection criteria of the reviewed articles were represented by the technologies currently used to obtain hydrogels, their physical characteristics and advantages of the use of hydrogels in improving cultured soil. International databases such as Web of Science, Wiley, Elsevier and Springer were electronically searched for articles and the literature reviews.

RESULTS AND DISCUSSIONS

Modern agriculture required a large quantity of water and because of the changing climate conditions, the water quantity is dropping from year to year (Koupai et al., 2008; Mantovan et al., 2022). Organic polymers have many properties that allow the optimal use of water in agriculture. The first application covers a wide range of polymers in emulsion, powder or block, suitable for any type of irrigation. Under the action of the polymer, fine surface particles form aggregates. This improves soil porosity, thus considerably reducing soil drying effects as a result of spray irrigation and erosion under gravity irrigation (Oladosu et al., 2022).

Hydrogels are synthesized from various monomers, most of them being hydrophilic, due to the presence in their structure of hydrophilic functional groups of the type: -OH, -COOH, -$CONH_2$, -CONH and -SO_3H. However, it was established that by copolymerizing some hydrophilic and hydrophobic monomers hydrogels with increased mechanical resistance can be obtained (Dergunov and Mun, 2009; Ullah et al., 2015).

The need to improve the physical-chemical, biological and mechanical properties of hydrogels has led to the diversification of the range of neutral monomers or carriers of electrical charges (anionic, cationic), as well as with groups susceptible to cross-linking. Obtaining hydrogels by copolymerization (in the presence of a cross-linking agent) is used as a mean of improving the mechanical properties, diffusion through the gel, or limiting the degree of swelling of the obtained gels (Akhtar et al., 2016).

The technologies currently used to obtain hydrogels include different types of polymerizations (in mass, in solution, in suspension, in emulsion), polymerization in the gas phase or induced with plasma, which are very expensive technologies but lead to the formation of very pure and uniform gels (Varaprasad et al., 2017). Another method for hydrogels obtaining is the crosslinking of hydrophilic polymers with crosslinking agents or by exposing them to different types of radiation (Bustamante-Torres et al., 2021).

Hydrogels have a large domain of applicability, in the food (Klein and Poverenov, 2020; Li et al., 2021; Liu et al., 2012; Zhang et al., 2020), biomedical and pharmaceutical domain (Aswathy et al., 2020; Liu et al., 2012; Peppas et al., 2020), agriculture (Koupai et al., 2008; Michalik and Wandzik, 2020; Narjary et al., 2013; Raafat et al., 2012), biosensors (Bae et al., 2020; Herrmann et al., 2021; Sun et al., 2018; Wang et al., 2021) and cosmetics (Mitura et al., 2020; Montesano et al., 2015).

Chitosan based hydrogels are currently used in the agriculture sector in order to deliver valuable bioactive compounds to plants and food products and also to treat the agricultural recycled wastewater (Laftah et al., 2011; Qu and Luo, 2021).

Just as chitosan, alginate is a natural carbohydrate that can be used to produce biodegradable hydrogels. Previous research into the water absorption and retention capacity of alginate-based hydrogels (Davidovich-Pinhas and Bianco-Peled, 2010; Idrissi et al., 2022; Van der Merwe et al., 2022) show potential for using hydrogels in the field of soil amendments. Some key benefits to using alginate as the hydrogel-forming component, as opposed to other synthetic hydrogels such as polyacrylamide, include that alginate is relatively cheap compared to synthetic polymers, alginate is abundant, and alginate is biodegradable within the soil (Michalik and Chojnacka, 2021; Van der Merwe et al., 2022).

Extraction studies, acute systemic toxicity, tissue and blood compatibility, have confirmed the stability and biocompatibility of the hydrogel. The biocompatibility of hydrogels expands the scope of biomedical applications, without risks for the receiving organism (Chai et al., 2017). Much of the research conducted on hydrogels has been directed towards applications such as controlled drug release devices (Gupta et al., 2002).

According to many studies, hydrogels were also obtained from materials such as polyurethane (Tanasić et al., 2021), carboxymethyl cellulose (Bauli et al., 2021), nanocellulose (Li and Chen, 2020), fenugreek galactomannan-borax (Liu et al., 2020), carboxymethyl tamarind kernel gum with sodium-acrylate (Warkar and Kumar, 2019), polyvinylpyrrolidone (Raafat et al., 2012), cassava starch and polyvinyl alcohol (Jungsinyatam et al., 2022) or amphiphilic calcium alginate (Zhang et al., 2022).

Polymer mixtures are composed of two or more polymers, synthetic or natural, which can be thermodynamically compatible or incompatible (Loo et al., 2021).

The possibility of obtaining new materials, with improved properties, by mixing two or more already existing polymers proved to be a much more interesting solution, from an economic point of view, than the design and synthesis of new polymers (Zhang and Khademhosseini, 2017). In the last ten years, the particular importance of a new class of polymers has emerged, that of polymers with biomaterial applications.

The notion of biomaterial mixture of polymers is very broad, but corresponds often to that of a multiphase polymer system (Anstey et al., 2021). The use of these polymer systems to obtain different products requires a processing stage, after which their shape and final structure are fixed. The latter is the one that influences the usage properties of the product (mechanical, optical, dielectric properties, etc.). In this context, the special importance given to the methods of characterizing the morphology of polymer systems is explicable, in order to explain the relationship that exists between the processing parameters, the structure and the properties of the obtained products (Carreau et al., 2021).

The most important beneficial uses for hydrogels made from polysaccharides are the prevention of soil erosion, the controlled nutrient release and the increased water retention properties of sandy soils (Ghobashy, 2020; Oladosu et al., 2022).

Song et al. (2020), studied the effect of a hydrogel obtained from sodium alginate, konjaku flour and lignosulfonate, which was prepared by crosslinking and applied into a soil in order to investigate its influence on the physical-chemical properties of the soil and its degradability. Also, the water holding capacity and retention curve as well as nutrient retention were analysed over tobacco plants under drought stress. The results of the study showed that the hydrogels increased the water capacity of the tested soil and also improved the photosynthetic capability of tobacco plants under drought stress. The proline levels were improved as well and the growth time of the plants were increased.

An eco-friendly and cost-effective hydrogel based on proteins was developed by Hu et al. (2021), by using biomass waste collagen. The scope of the hydrogel was to absorb heavy metals from the soil and have the ability to slowly release nutrients. The results showed that the hydrogels have high water absorbency capacity, are biodegradable and can controlled-release potassium and nitrogen in the soil for a period of up to 40 days. As for the adsorption capacity oh heavy metals, the hydrogels presented good results when teste for Cr (III).

Albalasmeh et al. (2022), studied the effects of a hydrogel based material on corn growth and soil physical properties in laboratory and greenhouse conditions. Ten concentrations of hydrogel were used to evaluate their effect of the hydraulic and physical properties of the soil and four concentrations to monitor the growth of *Zea mays* as a model plant. At a concentration of 0.27% hydrogel, the soil aggregate percentage was 35% and at concentrations of 0.33% hydrogel, the water available in soil was of 49%. The results showed that improvement in soil aggregate percentage was of 35% with 0.27% hydrogel concentration whereas, 0.33% hydrogel concentration increased the soil available water by 49%. Furthermore, water use efficiency was increased for all tested concentrations as well as plant growth.

Hydrogels made of acrylic acid, guar gum and cross-linked with ethylene glycol di methacrylic acid were studied by Thombare et al. (2018), in terms of water absorption and biodegradation properties.

The results showed that the use of hydrogels has significantly improved the porosity, water retention and holding capacity of soil. Furthermore, the hydrogels proved to be biodegradable.

Water-soluble organic polymers are anionic polyacrylamides and their use in improving the culture conditions has the following advantages: up to 95% reduction in soil loss due to leakage; resistance to surface erosion, compaction, and consolidation; the soil is easier to work, especially plowing and the costs are reduced; environmental protection is respected, by reducing the leakage of pesticides and fertilizers; being spread on a surface to be treated, polymers allow the formation of a synthetic mulch that improves both soil cohesion and its permeability (Xiao et al., 2022).

Thanks to the superior hydrophilic properties, the soil is more stable on the surface, so that the seeds germinate more easily and ensures the roots are gripped effectively, they are easy to use and can be spread with seeds and fertilizers and helps to improve soil cohesion and reduce the effects of erosion (Chang et al., 2015; Patra et al., 2022).

A synthesis of the most important results of the literature review regarding the advantages of the use of the hydrogels (with different composition) in different agriculture applications are presented in the Table 1.

Table 1. Characteristics and effects of hydrogels on plant culture and soil conditions

Composition of hydrogels	Plant culture	Effect	Reference
Sodium alginate, konjaku flour and lignosulfonate	Tobacco plants	- Increased the soil water capacity - Improved the photosynthetic capability and reducing sugar - Prolong growth rate	(Song et al., 2020)
Biomass waste collagen	*Ensifer* sp.Y1.	- High water absorbency capacity - Highly biodegradable - Excellent adsorption capacity for Cr (III).	(Hu et al., 2021)
Commercial hydrogel, cross-linked potassium poly-acrylic acid.	*Zea mays*	- Increase the soil available water and water use efficiency - Plant growth improvement	(Albalasmeh et al., 2022)
Superabsorbent hydrogel based on zinc oxide nanoparticles and watermelon peel waste	Pepper plant	- Control of *Fusarium* disease - Decrease the necessary irrigation water quantity	(Abdelaziz et al., 2021)
Ativated-carbon-filled agarose hydrogel	Rapeseed	- Improvement water retention - Swelling ratio of rapeseed seedlings decreased - Enhanced rapeseed growth indexes (germination capacity, root and stem length, fresh and dry weight)	(Cao and Li, 2021).
Cassava starch (CSt)-g-polyacrylic acid /natural rubber /polyvinyl alcohol	Chili plant	- Slow-released fertilizer capacity which facilitated the growth of chili plant - Improve water retention - Plant growth improvement	(Tanan et al., 2021)
Reinforced starch-based hydrogels with natural char nanoparticles	Tomato plant	- Reduced the negative effects of water-deficit stress - Maintain soil moisture content	(Nassaj-Bokharaei et al., 2021)
Whey/polysaccharide-based hydrogel with polylactic acid (PLA)	*Raphanus sativus* and *Phaseolus vulgaris*	- Improve water retention capacity of the soil by 30%. - Better plant growth	(Durpekova et al., 2022)
Carboxymethyl cellulose and nanocellulose / nanoclays	Cucumber (*Cucumis* L.)	- Potential reduction of fertilizer loss during application - Improve water use in agriculture	(Bauli et al., 2021)
Carboxymethyl tamarind kernel gum and sodium-acrylate	Chickpea plants	- Potential use as soil conditioner - Better growth of chickpea plants	(Warkar and Kumar, 2019)
Alginate-carboxymethyl cellulose enriched with chitosan and Cu^{2+}	Cucumber (*Cucumis* L.)	- Chitosan may provide an additional source of nitrogen for plants and exhibit antimicrobial activity	(Skrzypczak et al., 2021)
Chitosan hydrogel cross linked with Ethylene diamine tetraacetic acid (EDTA)	Soybean plants	- Better soybean plant growth (leaf dimensions and number)	(Ritonga et al., 2019)
Chitosan, gelatin and polyvinyl alcohol (PVA)	Chili plants	- Antimicrobial activity on *Phytophthora capsici*	(López-Velázquez et al., 2019)
Carboxymethyl tamarind kernel gum with sodium-acrylate	Chickpea plants	- Potential use as soil conditioner - Significant improvement of the moisture absorption (35%), porosity (7%) and water retention capacity	(Warkar and Kumar, 2019)
Chitin, NaOH/urea aqueous solution, crosslinking with epichlorohydrin	Rapeseed (*Brassica napus*)	- Safe and biodegradable - Promote seed germination and growth	(Tang et al., 2014)
Superabsorbent hydrogel type Suprab A200 (commercial grade)	*Ligustrum ovalifolium*	- Increased residual and saturated water content	(Koupai et al., 2008)
Sodium carboxymethylcellulose and hydroxyethyl cellulose cross-linked with citric acid	Cucumber and sweet basil	- Overall enhancement of plant growth - Increased water retention properties	(Montesano et al., 2015)
Polyacrylic acid, sodium polyacrylate, cellulose, starch	Maize plants	- Enhanced growth of maize - Improved water availability in the soil	(Mazen et al., 2015)
Superabsorbent hydrogel type Belsap (commercial grade)	*Cajanus cajan* seedlings	- Improved growth in both the height and root collar diameter - Increased soil moisture	(Gilbert et al., 2014)

There are important advantages that hydrogels have when used in agriculture domain, such as the ability to stabilize the soil even on steep slopes, to reduce the costs of improving soil characteristics, the ease of use and the possibility to spread them with seeds and fertilizers and also help improve soil cohesion and reduce the effects of erosion (Vundavalli et al., 2015).

CONCLUSIONS

The characteristics of hydrogels, composed from different materials such as chitosan, carboxymethyl cellulose, nanocellulose, polyvinylpyrrolidone, polyvinyl alcohol, etc., combine three properties that are consistent with current priorities regarding the sustainable development requirements of the agricultural system: biodegradability, recycling of residual materials and suitability for multiple processing techniques.

The application of hydrogels at culture substrate level, ensure a sustainable solution for water management in agriculture and controlled fertilization of the soil, thus improving the yield by stimulating the germination of the seeds and the metabolic processes of the plants. Because of these properties adding hydrogels to soil could contribute to produce large and constant harvests in variable environmental conditions.

The beneficial effects of hydrogels use in improving culture conditions of different plant species highlighted by the current research are the improved growth rate, photosynthetic capability and adsorption capabilities. Another advantage of using hydrogels is the reduction of the negative effects of water-deficit stress and slow release of fertilizers.

In addition, acting as a soil conditioner, it contributes to increasing soil water availability and water use efficiency, improving water retention potential and the antimicrobial properties, effects also highlighted in the reviewed research studies.

One major drawback of hydrogels is the lack of mechanical strength, so researchers need to find ways to improve the mechanical integrity and 3-dimensional hydrogels structures. Further research is also needed in the field of application technology which depends on both the structure of the hydrogels and the plant culture and soil type.

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USE OF MICROENCAPSULATION AND NANOENCAPSULATION TECHNIQUES IN DAIRY TECHNOLOGY

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Abstract

Encapsulation is a new technology known as packing of food components, enzymes, microorganisms, cells or different substances, which are found in solid, liquid and gas form, with coating materials such as proteins, hydrocolloids, polymers, polysaccharides and lipids. Encapsulation techniques have the high potential to protect the food systems. They are divided into three categories according to sizes of produced capsule: these are nanoencapsulation, microencapsulation and macroencapsulation. Microencapsulation is commonly used in pharmacy, agriculture, cosmetic industries for encapsulation of solid and liquid oils, vitamins, minerals, flavour components, enzymes, and colouring components used in dairy technology. Nanoencapsulation is especially used in packing systems to ensure food safety and to detect pathogen microorganisms. This review is focused on microencapsulation and nanoencapsulation, which maintain the controlled preservation of dairy products.

Key words: *coating materials, dairy products, encapsulation techniques, microencapsulation, nanoencapsulation.*

INTRODUCTION

In recent years, people have been turning to healthier eating habits. As known, foods are not only important sources for human nutrition but also, they play a crucial role for preventing diseases, which are related to nutrition (Varhan and Koç, 2018; Aspri et al., 2020). Healthy nutrition has been important from both quality and reliability points of view (Onwluata, 2013). Depending on the technological developments, there have been many positive changes related to health and nutrition, such as the search for various functional foods and new food matrices rich in beneficial components. These changes in human history and culture present a promising situation for progress (Şengün and Yahşi., 2021).

The developments in the food industry supply have many advantages especially in microbial systems, food safety, composition of raw materials, sensorial, textural and organoleptic properties of final product. Moreover, these quality parameters are important not only for the consumer but also for the food producers (Iravani et al., 2015).

Encapsulation technology, which includes all these advantages, is used in food technology in addition to many industrial applications such as consumer products, cosmetics, pharmaceutical/medical products and agricultural products. Encapsulation involves the coating or entrapment of a pure material or a mixture into another material. The coated or entrapped material is usually a liquid but can be a solid or gas. The coating material can be of protein, carbohydrate and lipid source (Gökmen et al., 2012). By using this technique, produced encapsulated materials can be protected from moisture, heat or other extreme conditions, thus their stability is enhanced and viability is maintained (Varhan and Koç, 2018). Although the sizes of the produced capsules vary, they are divided into 3 groups: nanocapsule, microcapsule and macrocapsule (Geniş and Tuncer, 2019). In addition, the covering materials should maintain the stability, increase the bioavailability, be nontoxic and inexpensive (Atak and Koç, 2017; Çınkır et al., 2019).

For an effective encapsulation, the suitable encapsulation materials should be selected. The most widely known coating materials among carbohydrate sources are starch, polyols, chitosan, pectin, agar, carrageenan and alginate (Zimet and Livney, 2009; Spada et al., 2012; Çınkır et al., 2019). Various techniques are used

to form the capsules, including spray drying, spray chilling or spray cooling, extrusion coating, fluidized bed coating, liposome entrapment, coacervation, inclusion complexation, centrifugal extrusion and rotational suspension separation (Sobel et al., 2014). The most important techniques are further presented.

Spray Drying

Spray drying is a method based on reducing water activity and thus microbial growth of the product is minimized and the quality of the product is enhanced. Moreover, during storage and transport, the cost is also reduced (Boza et al., 2004). Due to this method, the specific properties of the final product are protected. It is mostly used in milk powder production in dairy industry (Gökmen et al., 2012). Besides these advantages, some disadvantages of this technique are reported. One of them is controlling of particle size which is difficult and another one is degradation of materials due to the differences in heat stability of compounds (Suganya and Anuradha, 2017).

Freeze Drying

Freeze drying is also called lyophilization. This technique is based on the sublimation of water in foods. The applied temperature ranges between -40ºC and -80ºC. Although it is a simple technique, the cost is high. Whey protein, gelatin and pullulan can be given as examples of the most commonly used coating materials in the encapsulation process applied with this technique (Çınkır et al., 2019).

Extrusion Method

Due to its low cost and simple formulation, extrusion provides highly ease of use. It is based on solidification of the solution containing hydrocolloid. The applied temperature is quite low (<100°C). This technique provides an extremely protective feature of products against oxidation. By means of extrusion method it is possible to prevent the loss of compounds in foods and in this way the shelf-life of foods is prolonged. Its disadvantage is that capsule formation rate is low (Geniş and Tuncer, 2019). For example; Harz et al. (2000) determined the following method for the encapsulation of enzymes: all the essential ingredients are mixed in the method and the resulting mixture is fed into the extruder at 40°C. It is rolled under a temperature of 100°C and cooled to 45°C in a few seconds (Harz et al., 2000; Açu et al., 2014). The advantage of extrusion is that the material is totally isolated by the wall material and that any core is washed from the outside. It is mainly used for encapsulation of components such as visible flavor fragments, vitamin C, colors.

Emulsification Method

In the emulsion technique, a discontinuous phase is added into the continuous phase. This mixture is used to form a water-in-oil emulsion, homogenized and the prepared emulsion is kept for a while, so that the non-continuous phase passes into a small gel form without dissolving in the continuous phase. And then the obtained capsules are separated from the liquid solution by filtration (Gökmen et al., 2012). Hydrocolloids such as carrageenan are used as coating material (Gökbulut and Öztürk, 2018).

Spray Cooling

The most important difference between spray cooling and spray drying is the particle formation area. In the particle formation zone, the particles are formed not by evaporation of a solvent, but by cooling and hardening of the droplets (Poshadri and Aparna, 2010; Kanat and Gülel, 2021). Spray cooling is an inexpensive encapsulation technique. However, no matter how small the microparticles are, there is a medium used for possession and storage (Kanat and Gülel, 2020).

Coavercation Method

In this technique, phase separation takes place first to separate the polyelectrolyte and/or polyelectrolyte mixture from a solution, then a coacervate phase is formed and the core is completely coated (Çınkır et al., 2019).

APPLICATION OF ENCAPSULATION TECHNIQUES IN MILK AND DAIRY PRODUCTS

In recent years, most studies have focused on the encapsulation techniques due to their protective properties against the environmental conditions such as O_2, temperature, light, humidity, and providing of controlled release of the active

ingredients, not only in food but also in pharmacy, cosmetics and agricultural industry (Balci-Torun and Özdemir, 2021). In general, the components used for encapsulation applications can be listed as follows: food active ingredients, colorants, enzymes, minerals and vitamins, flavoring agents, additives, probiotic microorganisms etc. Therefore, it is possible to use such encapsulated products in dairy technology especially in yoghurt, ice cream, milk powder, cheese production and in this way the desired quality is maintained and the product stability is ensured for a certain period of time (Peker and Arslan, 2011).

One of the applications areas of encapsulation techniques is the production of fermented dairy products such as cheese, yogurt, kefir and cream in terms of lactic acid bacteria. The encapsulation applications in cheese generally provide benefits in terms of the desired level of taste, flavor and aroma formation, shortening the ripening time of ripened cheeses, by reducing and/or eliminating the problems that may arise from the direct addition of the enzymes, and by preventing rapid and excessive proteolysis. The use of encapsulation techniques in different products is shown in Table 1.

Table 1. Some selected studies carried out using encapsulation techniques

*Encapsulation method	Study	Key findings	References
NE	An extract from *Plinia peruviana* plant was prepared by nanoemulsion and added it into cow's milk and investigated the antioxidant activity and phenolic content of cow's milk.	The antioxidant properties of cow's milk and phenolic substance content in milk increased	Di Maio et al., 2019
ME	*Lactobacillus plantarum* was microencapsulated using serum protein. The spray drying method was used.	There was no decrease in the viability of the bacteria after 56 days of storage. Bacterial cells still maintained their viability.	Eckert et al., 2017
NE	In this study, turmeric was encapsulated with nanoencapsulation technology and prepared by the freeze-drying technique. Turmeric, was added to the ice cream mixture	There were no changes in the sensorial properties of the nanoemulsion added ice cream compared to the control sample, and the encapsulation efficiency was 93.7%.	Kumar et al., 2016
ME	*Lactobacillus acidophilus* was encapsulated with polymerized serum protein, which was used in yoghurt production.	It has been stated that encapsulates, in which polymerized serum proteins are used as coating material during the storage of yoghurt, are more efficient, more effective and more protective.	Jiang et al., 2016
NE	Isolated lactoferrin from camel milk nanoencapsulated with 0.2-0.5% calcium alginate.	Calcium alginates are a very natural tool for the digestion of lactoferrin. Using 0.5% calcium alginate lactoferrin release was actually high.	Raei et al., 2015
ME	In order to accelerate the ripening of Cheddar cheese, microencapsulated aminopeptidase enzyme was added and showed that some properties of the samples with encapsulated enzyme were better	The taste, aroma and textural values of cheeses containing encapsulated enzymes were higher than the control. The total amount of free amino acids during storage was in the cheese sample containing the encapsulated enzyme increased the proteolysis.	Açu et al., 2014
ME	In this study the probiotic *Lactobacillus acidophilus* (La-5) cells either in free & encapsulated form was incorporated into yoghurt-ice cream and their survivability were studied.	Encapsulation of bacteria with fructooligosaccharide protected the live probiotic cells both during the freezing stage and during frozen storage.	Ahmadi et al., 2014

*ME:microencapsulation; NE:nanoencapsulation

In a study performed by Mudgil et al. (2022), the effects of microencapsulation of probiotics (*Pediococcus pentosaceus*) on the stability and viability in Chami cheese were investigated. It

was stated that probiotics that were encapsulated by camel milk proteins and wheat starch had higher cell viability in Chami matrix, especially those using camel milk proteins (Mudgil et al., 2022).

In another study performed by Siyar et al., (2022) the physicochemical and textural properties of cheese were investigated by adding encapsulated saffron extract at varied concentrations in ricotta cheese. It is stated that the saffron extract encapsulated in nanoliposomes provided stability, as well as protecting the bioactive substances of saffron during storage and did not significantly affect most compositional parameters. The study revealed that saffron extract encapsulated in nanoliposomes can be incorporated into a ricotta cheese production.

Jeong et al. (2017) investigated the influence of the addition of powdered tomato extracts on the physicochemical, microbial and sensory properties of Queso Blanco cheese in their studies. It has been reported that the lactic acid bacteria count and lycopene concentrations found in cheeses containing encapsulation were higher when compared to the control cheese group without encapsulation. It was determined that increasing the encapsulated tomato extract concentration in cheese causes the accession of gumminess, chewing and hardness parameters. It was also determined that the addition of these encapsulated additives improve the texture properties of the cheese.

The encapsulation technique was investigated to increase the viability of *L. plantarum* 564 in soft goat cheese. It was stated that spray drying of *L. plantarum* 564 strain can be used successfully to increase the number of viable probiotic cells after cold storage (Radulović et al., 2017).

Rashidinejad et al. (2016) conducted a study in which catechin or green tea extract was encapsulated in soy lecithin nanoliposomes and used in the production of a full-fat cheese. Some analyzes such as the determination of antioxidant capacity after 90 days of storage at 8°C were made and it was stated that the encapsulated green tea extract could be used in the cheese product. During cheese ripening total phenolic content (TPC) and antioxidant activity were measured. It has been stated that an addition of encapsulated bioactive compounds increases the relevant parameters (total phenolic content and antioxidant activity).

The viability of *Bifidobacterium bifidum* BB-12 and *Lactobacillus acidophilus* LA-5 microencapsulated by extrusion-emulsion technique was investigated in white brine cheese production and ripening. Although there was no sensory difference between the control cheese and encapsulated microorganisms added cheese, it was reported that the cell viability was slightly limited. It was observed that both microorganisms encapsulated during the production and maturation of white brine cheese preserved cell viability better than cheeses without capsules (Özer et al., 2009).

In addition, encapsulation techniques often provide benefits in fermented products such as yogurt in the dairy industry. The food industry aims to overcome the problems, which are related with the production of live starter microorganisms, because the yogurt should contain bacteria in live form so that the yogurt bacteria can metabolize lactose to lactic acid and in this way it increases the shelf life of yogurt (Değirmenci, 2017). In order to eliminate these problems and improve the physicochemical properties, it may be preferred to apply encapsulation techniques to yogurt production. By means of the encapsulation technique, the bacteria used as a culture can be protected against the external environmental conditions and increase the vitality rate at 80-95%. It is also possible to add probiotic live cells to increase the functional properties of yogurt. Encapsulation techniques reveal that the encapsulation of microorganisms and probiotics used at this point is a beneficial practice for the continuation of their viability (Altun and Özcan, 2013). According to recent studies, it has been revealed that encapsulating probiotic bacteria with prebiotic foods as well as encapsulating them alone increases the resistance of probiotics to external factors (Peker and Arslan, 2011).

In a study performed by Wang et al. (2018) the effects of microencapsulation on the viability of *Lactobacillus acidophilus* LA-5 on the physicochemical properties of yogurt was investigated. It was found that *Lactobacillus acidophilus* LA-5 remained highly viable in the gastrointestinal tract without damaging as a result of microencapsulation. They also found that the structure of yogurt was improved

significantly and the water release (syneresis) reduced, contributing to the development of the physicochemical properties of the yogurt (Wang et al., 2018).

In a study, which was carried out in probiotic yogurt production trial with encapsulated *Lactobacillus acidophilus* by using whey protein powder, changes in storage of yogurt containing encapsulated *Lactobacillus acidophilus* and yogurt without encapsulated bacteria were observed. It was stated that yeast and mold formation were still not observed in yoghurt samples containing encapsulated probiotics, even after 28 days of storage (Değirmenci, 2017).

Probiotics were encapsulated with sodium alginate as coating material and used in the production of stirred (broken) type fruit yoghurt. Further, the viability of encapsulated probiotic bacteria during transition in gastrointestinal tract was investigated and the study concluded that the number of encapsulated probiotic bacteria were higher than the free cells, which showed more therapeutic activity (Palamutoğlu and Sariçoban, 2013). In another study, *Bifidobacterium breve* R070 was added to yogurt after encapsulation with whey proteins and the microbiological changes during 28 days of storage were investigated. It was reported that *Bifidobacterium breve* maintained its viability 2.6 log more than other microorganisms that were not encapsulated (Kanat and Terzi Gülel, 2021).

Encapsulated *Lactobacillus acidophilus* and *Bifidobacterium bifidum* cultures coated with a mixture of calcium alginate and corn starch were added to yogurt, and then their viability levels were investigated in artificial gastrointestinal conditions. It was stated that the encapsulation did not have a significant effect in terms of resistance within the gastrointestinal tract. However, the encapsulated bacteria lost 0.5 log unit of their viability at the end of the storage period, while the loss of nonencapsulated free cells was 1 log unit (Sultana et al., 2000; Açu et al., 2014).

Altın (2016) encapsulated cocoa shell phenolic compounds with nanoliposomal systems and used them in the production of buttermilk, and compared different encapsulation techniques regarding encapsulation efficiency. The aim of this study was enrichment of ayran with bioactive components and determine the efficiency of encapsulation. The results of the study showed that liposomes in powder form provided high encapsulation efficiency, and the bioavailability of cocoa phenolic components in spray-dried liposomes increased at least 7 times during the shelf life. Encapsulated phenolic components were preserved better *in vitro* conditions before and after digestion than phenolic compounds that did not undergo encapsulation.

Kalkan, (2019) investigated the probiotic kefir microorganism called *Saccharomyces cerevisiae* var. *boulardii* encapsulated by the extrusion technique. The aim is to investigate the fermentation of milk by taking advantage of the benefits provided by probiotic microorganisms. The probiotic kefirs were stored at 4°C for 28 days and the microbiological and chemical properties of kefir samples were compared with each other. According to the results of the research, it was stated that the microbiological and chemical properties of kefir samples containing encapsulated *S. boulardii* were similar to those of kefir without encapsulated *S. boulardii*. It has also been demonstrated that this microorganism can be used as a probiotic in fermented milk products.

Homayouni et al. (2008) carried out the encapsulation of lactic acid bacteria with different encapsulation techniques in ice cream production. The encapsulation of lactobacilli was performed with calcium alginate gel as coating matrix. It was stated that the application of encapsulation techniques ensures that the bacteria in the products are more durable and longer lasting compared to the unencapsulated free bacterial cells in freezing and/or cryopreservation processes (Kınık et al., 2003; Peker and Arslan, 2011).

Similar to this study *Bifidobacterium longum* CFR815j was encapsulated and added into the ice cream formulation to find out whether it had an effect on the viability of the bacteria and on the physiological properties of the ice cream. It was stated that all the properties analyzed were comparable to normal ice cream, and they also revealed that the encapsulation application can maintain the viability of the probiotic *Bifidobacterium*, which has the minimum biological value required for the final product, without affecting the sensory properties of the ice cream (Kataria et al., 2018).

In a study in which barberry, known as an anthocyanin-rich plant fruit, was encapsulated by the ionic gelation method by applying extraction, the obtained capsules were included in the ice cream formulation. As a result of the research, the usability of these capsules for ice cream production and pH, antioxidant activity, etc. were investigated. As a result of the storage analyzes of the capsules added to the ice cream, it was stated that the anthocyanin content of the ice cream with the capsule addition preserved the stability of the product quite well and its usability in ice cream production was positive (Okurkan, 2018).

Milk fat is a valuable component that significantly affects the organoleptic properties of milk and dairy products. Since it is a very sensitive substance compared to other milk components, it has a short shelf life and is susceptible to oxidation (Himmetağaoğlu et al., 2019). However, with the application of microencapsulation by spray drying, it is possible to protect fast perishable foods and to obtain more stable products that are more resistant against to external factors (Himmetağaoğlu et al., 2019). Peker and Arslan (2011) investigated the encapsulation of milk fat by using the spray drying method. As a result of their study, the encapsulation technique was successfully applied and more than 90% encapsulation efficiency was obtained.

CONCLUSIONS

Recently, the encapsulation techniques presented great success in the dairy industry especially in cheese technology. Therefore, it has been possible to offer consumers more nutritious, reliable products with a relatively longer shelf life. It is known that despite the economic feasibility of applying the techniques, it is not very burdensome, limited and still does not require large investments. It is thought that there is a great potential in the future especially in the food industry and that these developments should be increased and more comprehensive researches should be carried out in our country.

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CHARACTERIZATION OF FEED CONTAMINATION BY *Fusarium* sp. - A REVIEW

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Abstract

Certain Fusarium species and strains are potential producers of three most important classes of mycotoxins: fumonisins (FB1, FB2, FB3); zearalenone (ZEA) and trichothecenes, such as deoxynivalenol (DON), *nivalenol* (NIV), or *HT-2 toxin and T-2 toxin. The ingestion consequences of these fungal compounds can lead to a range from acute to chronic diseases with high morbidity. The use of contaminated feed can have serious effects not only on health, but also on the productive potential of livestock and poultry, with high risk of further mycotoxins spreading in the food chain to the final consumer. Therefore, this paper aimed to present information on the main mycotoxins produced by different species of Fusarium contaminants, focusing on the toxicological effects on farm animals. The effects of each mycotoxin type on ruminants, horses, pigs, and poultry are described.*

Key words*: feed, Fusarium, mycotoxins, livestock, poultry.*

INTRODUCTION

Feed can be classified into groups including forages, cereals, compound feeds, products and by-products from the human food and brewing industry. Animal feed usually includes a combination of elements that must meet nutritional requirements at low cost for a good health. Cereals and cereal-based products are usually the most used ingredients in feed production and contain most of the nutrients useful for animal husbandry (Pereira et al., 2019).

Filamentous fungi are microorganisms that can be found everywhere in nature. Although they mostly live saprophytically or in symbiosis with other living organisms, they can also develop infections and contaminations. In the case of mycotoxigenic fungi, the problem is not limited to their presence and development, but also on the mycotoxin contamination which contribute to the depreciation of infected substrates. The genus *Fusarium* includes some infectious pathogenic species for plants, animals, and humans. The spectrum of mycotoxins produced is very varied, however the most studied are: fumonisins, trichothecenes and zearalenone with a particularly serious impact on human and animal health (Ismaiel & Papenbrock, 2015; Fremy et al., 2019).

In this context, the current review is intended to provide a comprehensive summary on important feed contaminants of *Fusarium* species, their toxic metabolites, and effect of mycotoxin exposure on farm animals.

Fusarium contamination in feed

Mycotoxins are highly stable compounds, and due to their persistence in the food network they are of major concern. Moulds and mycotoxins can contaminate feeds at all stages of production, in the field, before harvesting, and also during storage or processing. The main fungal contaminants able to produce mycotoxins are Aspergillus*, Penicillium, Fusarium, Alternaria* and *Claviceps.* The severity of infection and contamination is dependent on the host plant, climatic conditions and agronomic practices (Gallo et al., 2015). Among mycotoxins, the most common are aflatoxins, ochratoxins, fumonisins, paulin, deoxynivalenol and their derivatives, as well as other trichothecenes. The infiltration of

mycotoxins in the food chain is promoted by both biotic and abiotic factors. Temperature and humidity are of high importance, as they influence the biology and ecology of phytopathogenic fungi. Contamination can also be influenced by technological factors, such as the phytosanitary treatments applied in vegetation for the prevention and plant protection against pest and diseases, time and harvesting methods, and storage conditions (Ponce-García et al., 2018).

The contamination with *Fusarium* spp can occur in the field, spreading in the plant, and eventually, continuing the depreciation during storage. In many cases the fungal infection is not limited to quantitative depreciation, and can be worsened due to the mycotoxin contamination, which reduces the quality of the harvest, or even compromise it.

The *Fusarium* genus comprises around 70 well-known species, and as many as 300 putative species, of which only some regularly contaminate feed (Table 1).

Table 1. Recommendations and regulations for safe limits of *Fusarium* mycotoxin concentrations in grains at the European Union[a] (Munkvold, 2017)

Mycotoxins	Fungal species	Limits of mycotoxines concentration in grains	
		for human food	for animal feed
Deoxynivalenol	*Fusarium graminearum F. culmorum*	750 ppb	1750 ppb
Fumonisins B1, B2, B3	*F. vertillioides F.proliferatum*	1000 ppb	4000 ppb
T-2	*F. acuminatum F. langsethiae, F. sporotrichioides*	50-200 ppb [b]	100-200 ppb[b]
Zearalenone	*F. graminearum F. culmorum*	75-100 ppb[c]	100-350 ppb[d]

Legend: [a]Commission Regulation (EC) No 1126/2007 or 576/2006, [b]Varies among grain types, [c]Varies among specific food items, [d]Varies among livestock species, up to 1000 ppb for oats with husks.

Mycotoxins produced by *Fusarium* species

Mycotoxigenic *Fusarium* species have the ability to produce secondary metabolic compounds with toxic effects on humans and animals, especially when they develop on suitable substrates. These substances are characterized by a low molecular weight that can easily be absorbed, ingested or inhaled, causing a wide range of diseases, even death of humans and animals. Moreover, due to their slow metabolism and increased accumulation risk in the body, their negative effects are more harmful to the host (Ferrigo et al., 2016).

Several hundred compounds have been described as toxic or potentially toxic secondary metabolites of *Fusarium* spp. Toxicity of many of these compounds has been demonstrated in bioassays or feeding studies. In this context, many studies are focused on investigating the short and long term exposure effects to these compounds, either as one or as mycotoxin mixture. Several authors have already shown the dynamics of various toxins within the body, their bioavailability and mechanisms of action according to the species involved (Loiseau et al., 2015).

Among the most important mycotoxins produced by *Fusarium* species are the fumonisin (FB1, FB2, FB3); zearalenone (ZEA) and trichothecene (deoxynivalenol, nivalenol, HT-2 and T-2 toxins), while trichothecenes are potent inhibitors of protein synthesis. They can also produce emerging mycotoxins such as fusoproliferin (FUS), beauvericin (BEA), eniatins, moniliformin (MON), fusaric acid, fusarin AD, gliotoxin, butenolite, which are recently discovered and less studied (Stanciu et al., 2017). Mycotoxins produced by species of *Fusarium* genus have acute toxic effects and chronic effects. Due to these factors, *Fusarium* species are considered among most economically important mycotoxin producing fungi. However, the major problem following the consumption of contaminated products isn't the acute sickness episodes, but rather the ingestion of low amounts of toxins that can cause a number of metabolic, physiological and immunological disorders. Symptoms related to mycotoxicosis can occur when mycotoxins are consumed at very low concentrations, even below the limits of detection (Escrivá et al., 2015).

Forage contaminants of *Fusarium* genus

The contamination with *Fusarium* spp can cause plants, animals and humans, diseases / health damages etc. In plants, they have a varied host spectrum. The American Society of Phytopathology estimates that approximately 80% of economically important plant species are affected by *Fusarium* sp. (Moss, 2002). There are many *Fusarium* species that can infect cereals, causing quantitative and/or

qualitative losses to both food and feet grain crops, as well as grasses. For instance, on cereals, the most common phytopathogenic fungi are *F. graminearum, F. culmorum*, *F. avenaceum*, *F. poae* and *F. langsethiae*. Many of these species can be present in soil, as saprophytes, but can become pathogenic, and damage seed, seedlings, mature plants, including the harvest. All cereals are susceptive to the infection, which can be caused either by individual *Fusarium* species, or more commonly, co-occurring species complex (Ferrigo et al., 2016).

Characterization of *Fusarium culmorum*

F. culmorum is associated with stem base rot and ear blight (also known as Fusarium head blight). *In vitro*, colonies are rapidly growing, exceeding 9 cm in diameter after 7 days when incubated on Potato-Dextrose-Agar (PDA) medium. The aerial mycelium is whitish to yellow or tan, while the substrate mycelium and the reverse are carmine to intensely red brown (Figure 1). The optimum temperature for the mycelial growth and sporulation is 20 to 24°C.

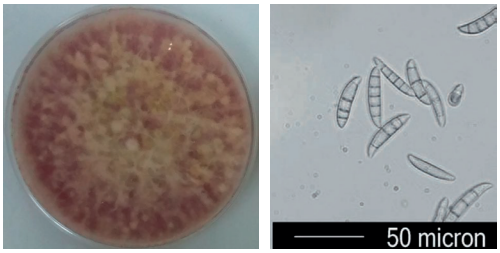

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Figure 1. *Fusarium culmorum*
a. Colony morphology on PDA (original);
b. macroconidia (Pancaldi et al., 2010).

The main mycotoxins produced by *F. culmorum* include trichothecenes, such as deoxynivalenol (DON), nivalenol (NIV), and T-2 toxin, as well as zearalenone (ZEA) and fusarins (Wagacha & Muthomi, 2007). But the major compound produced is DON, also known as vomitoxin. When animals are fed with contaminated feed by DON , this toxin causes vomiting in animals or feed refusal because the feed is unpalatable., especially to pigs (Amaresan et al., 2020).

Phylogenetically, *F. culmorum* is closely related to *F. graminearum*, with who is sharing similar colony morphology. However, they can be distinguished based on macroconidia morphology, some genetic differences, and secondary metabolites profile (Sirbu et al., 2020).

Characterization of *Fusarium avenaceum*

F. avenaceum forms dense, pale orange aerial mycelium on PDA, becoming pinkish white to white, as the culture ages. The other side of *F. avenaceum* can be peach or pale orange to orange. Some cultures produce a darker red mycelium and a red pigment in the agar, as the cultures are ageing (Figure 2).

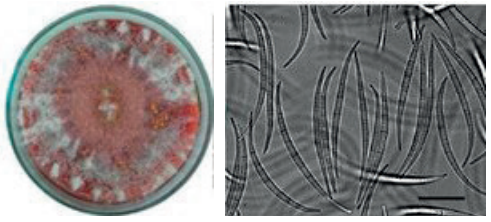

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Figure 2. *Fusarium avenaceum*: a. typical colony morphology on Potato-Sucrose-Agar (after 14 days incubation at 23°C, in dark; b. macroconidia compared to the scale bar of 20 μm (Yli-Mattila et al., 2018)

This fungus is relatively fast growing, the optimum temperature for growth and sporulation being at 20°C. It is found in temperate climates as a saprophyte in the soil but can become a parasite on cereals (such as wheat and barley) and perennial grasses, as well as on vegetables, or carnations.

Characterization of *Fusarium graminearum*

Fusarium graminearum is the anamorph of *Gibberella zeae*. Their colonies grow rapidly, and form dense mycelia of variable colour, from white at the beginning of growth, than pink, as the formation of conidia, turning to reddish-brown with age, with yellow or orange iridescence on the upper mycelium (Figure 3). The optimum temperature range for its growth is between 24-26°C (Dudoiu et al., 2016).

This specie is cosmopolitan, it predominantly infects maize, wheat and barley, but also other annual and perennial grass species (Yli-Mattila et al., 2018). In addition to the quantitative

yield losses, harvested grain sustains qualitative problems of contamination with mycotoxins such as NIV, DON, and ZEA (Tamba-Berehoiu et al., 2012).

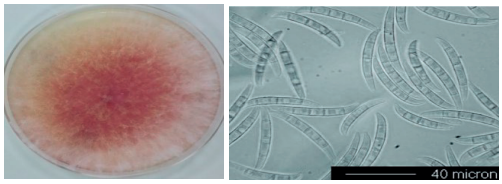

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Figure 3. *Fusarium graminearum*:
a. Colony morphology on PDA after 5 days, at 26°C (original); b. macroconidia (Pancaldi et al., 2010)

Characterisation of *Fusarium poae* species

The aerial mycelium is abundant, and as it forms microconidia, it takes on a powdery appearance. Initially the mycelium is light in colour, but with age darkens to reddish-brown (Figure 4). In the growth medium, it can secrete red (most common) or yellowish pigments. The culture may have a peculiar, sweet odour.

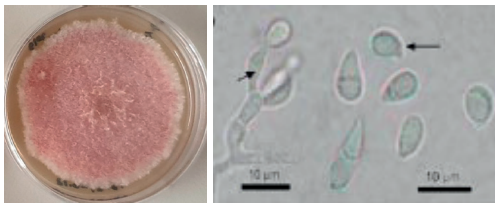

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Figure 4. *F. poae:* a. colony morphology on PDA (original); b. monophialide and microconidia (Morales-Rodríguez et al., 2007).

F. poae is a less important species than *F. graminearum* and *F. culmorum* because this species has been previously considered as a weak pathogen of cereals. although it can infect it. Some isolates of *F. poae* can produce beauvericin, fusarin C, trichothecene diacetoxyscirpenol, nivalenol or T-2 toxin (Pancaldi et al., 2010).

Mycotoxins effect of on animals

Fusarium mycotoxins can induce both acute and chronic toxic effects when ingested. These toxicological effects depend on the mycotoxin type, concentration level, and duration of exposure, exposed animal species and age, as well as other dietary conditions during contamination (Antonissen et al., 2014).

Fumonisins effects on animals

Fumonisins are a class of mycotoxins generally produced by *F. verticillioides* and *F. proliferatum.* However, many other *Fusarium* species are known to produce fumonisinins, but they are of lesser importance in terms of worldwide spread, and incidence in agri-food crops (Bertero et al., 2018).

Among grains, the most contaminated with fumonisinins are maize and maize-based products.

Many fumonisins homologues are known. They are at least 28 types, most of them designed in the A, B, C and P-series. Among these, the B-series is more common and economically important. It includes B1 fumonisin (FB1), which is more studied due to its spread and toxicity impact, followed by B2 fumonisin (FB2) and B3 fumonisin (FB3) (EFSA, 2004). These are commonly found in cereal grains and animal feed, often associated with other mycotoxins. For this reason, the United States Food and Drug Administration (USFDA) has developed certain guidelines which regulates the maximum accepted levels of fumonisin's concentrations in human food and animal feed (USFDA, 2001), ranging from 1 to 50 ppm depending on animal species (Table 2).

Table 2. Fumonisins maximum accepted levels in animal feed as recommended by the US FDA in maize and maize by-products

Animal Class	Maximum Recommended Levels of Total Fumonisins in Maize and Maize By-Products (ppm[1])	Feed Factor[2]	Recommended Maximum of Level of Total Fumonisins in the Total Ration (ppm[1])
Horses[3]	5	0.2	1
Rabbits	5	0.2	1
Catfish	20	0.5	10
Swines	20	0.5	10
Ruminants[4]	60	0.5	30
Mink[5]	60	0.5	30
Poultry[6]	100	0.5	50
Ruminant, Poultry & Mink Breeding Stock[7]	30	0.5	15
All Others[8]	10	0.5	5

Note: [1]Total fumonisins = FB1 + FB2 + FB3; [2] Fraction of maize or maize by-product mixed into the total feed ration; [3]Includes asses, zebras, and onagers; [4] Includes cattle, sheep, goats, and other ruminants that are > 3 months old and fed for slaughter; [5]Fed for pelt production; [6]Includes turkeys, chickens, ducklings and other poultry fed for slaughter; [7]Includes laying hens, roosters, lactating dairy cows and bulls; [8]Includes dogs and cats.

For each animal species the effect of fumonisin B1 can be differ. For example, poultry are defined as less sensitive to fumonisins exposure than pigs and horses, but this does not mean that poultry have immunity against this mycotoxin, or that the presence of fungal toxins in feed does not affect the production of meat and eggs. In 1995, the effects of fumonisins exposure were clearly seen when two-layer farms were seriously affected. The outbreak was characterized by black, sticky diarrhoea. The mortality rate increased to 10%, while egg production decreased by 20%. Overall, only hens are affected when they are exposed to exceptionally high amounts of pollution. Immunosuppression, hepatotoxicity, and nephrotoxicity, as well as a performance reduction, are some of the most evident side effects (Šegvić & Pepeljnjak, 2001).

Horses were found more sensitivity to FB1 toxicity. At concentrations of 0.02 to 0.12 µg/g of feed, FB1 can cause outbreaks, affecting the liver, heart and horses central nervous system. The common manifestations are loss of appetite, weakness, lethargy, allergic reactions, inability to swallow, breading problems, muscle fasciculation, sweating, circling, dilated pupils or absence of a pupillary light reflex (Vendruscolo et al., 2016). In equines, FB1 also induce neurological syndrome and cardiovascular dysfunction (Smith et al., 2002).

Although the fumonisins mechanism of action is not fully understood, it has been shown that in mammalian cells, high concentrations of this mycotoxin alters sphingolipids biosynthesis pathway, which inhibits L-type calcium channels, leading to a decrease in Ca ion release, thus reducing the cardiac activity. Therefore, it can be assumed that leukoencephalomalacia progress may be correlated to a decreased cardiovascular function and damage of the brain vessels (Bertero et al., 2018). Studies on epidermal and dermal cells showed rapidly increase of sphinganine and sphingosine concentrations, associated with disruption of membrane integrity and cell damage (Reisinger et al., 2016). *In vitro* toxicity of FB1 on fresh and frozen semen was also determined. No effects on fresh sperm viability were found after exposure up to 25 µM of B1 fumonisin (Minervini et al., 2010).

Horses and pigs are also very sensitive to fumonisins. In swine, pulmonary edema is one of the typical signs of acute toxicosis triggered by FB1. Pigs sensitivity to fumonisines was seen in several cases of feed contamination, with both *F. verticillioides* or fumonisins fungal metabolites. The main signs were porcine pulmonary edema and reproductive abnormalities, including abortion (Marasas et al., 1988). Due to these observations, several studies were conducted which demonstrate same pulmonary edema caused by B1 fumonisin exposure (Bertero et al., 2018). In swine, as in other species, FB1 causes an inhibition of ceramide synthase (Gumprecht et al., 2001). Hypercholesterolaemia is another sign of FB1 exposure. The necropsy of pigs which died from pulmonary edema, revealed various types of lesions. Beside endothelial lesions, other types of injuries were also described to be caused by chronic intoxication with FB1. Such lesions include basal cell layer dysplasia of the esophagus, sometimes associated with gastric ulceration. These findings also allowed important knowledge for human medicine, as fumonizines ingestion, especially FB1, could be associated with the occurance of human esophageal cancer(Wellington et al., 2000).

Pigs' exposure to FB1 mycotoxin also triggered poor reproductive performance. Therefore, some *in vitro* studies designed to analyse its effect on reproductive functions, using porcine granulosa cell cultures, concluded that at a dose of 10-14 µM the granule cell numbers decreased, but lower doses had no effects. Comparable results have been obtained in other porcine epithelial cell lines and primary cells. On granule cell steroidogenesis, significant effect of FB1 were seen only progesterone production, which was stimulated (Cortinovis et al., 2014).

Ruminants are considerably less sensitive to FB1 compared to monogastric, most likely due to their intestinal microflora activity. However they are not immune to the mycotoxins and can develop biochemical and microscopic liver changes and kidney damage if heavily contaminated feed is consumed. After a prolonged feeding with FB1 contamination, lymphocyte blastogenesis was impaired (Osweiler et al., 1993). Moreover, the short-

chain fatty acids production revealed not to be disturbed by the FB1, therefore neither the ruminal microflora seems to be affected by this mycotoxin (Caloni et al., 2000).

Regarding the reproductive function, FB1 mycotoxin showed no effects on proliferation of granulosa cells and no significant changes on progesterone production. However, at 1 to 3 μM concentrations, the estradiol production seemed to be weakly inhibited (Albonico et al., 2017), thus showing that cattle reproductive function could be affected. But these was not linked to a significant change in CYP19A1 gene expression, as it was seen in porcine granulosa cells by Cortinovis et al. (2014), thus indicating another mechanisms of action that could be involved. *In vitro* studies on the reproductive effects have shown that exposure to multiple mycotoxins plays a key role in cattle and have a strong influence on the entity and type of effects exerted (Albonico et al., 2016; Pizzo et al., 2016).

Deoxynivalenol and its effects on animals

Deoxynivalenol (DON) is the main representative of type B trichothecenes produced by mycotoxigenic *Fusarium* species, most likely by *F. graminearum* and *F. culmorum* but not only (Sobrova et al., 2010). Studies showed that this mycotoxin can contaminate both cereal and their by-products (Bertero et al., 2018). DON seems to inhibit protein synthesis if ingested (Pestka, 2010), and in high doses it can cause emesis, thus also being called as vomitoxin, especially in USA (Wu et al., 2013).

Specific effects of DON in certain animal species sowed that pigs, especially young piglets, are poorly tolerant to this toxin. Absorption and distribution in pigs are generally high, and the excretion is via the urinary and biliary routes. Unlike ruminants, minor metabolism occurs in pigs, and only a limited amount of DON can be detoxified by microflora. The most common signs described in chronically poisoned pigs are reduced feed intake or anorexia due to the stomach and intestine lesions. Other clinical signs also describe lungs and kidneys lesions. Plasma biochemical parameters were also altered (Bertero et al., 2018).

In piglets, DON-contaminated feed altered their innate immune response (Alizadeh et al., 2015) as well as the whole immune system (Pinton et al., 2008), but no clinically relevant impact was observed.

The reproduction system is also affected by DON exposures, even at low and very low doses. At 10 μM of DON, the follicular maturation process was affected, decreasing the follicle reserve and the number of normal follicles (Gerez et al., 2017). Exposed to 0.02 to 2 μM of DON, porcine cumulus-oocyte complexes were either degenerated or dead, with a significant consequence of a reduction in oocytes proportion that reached metaphase stage II (Bertero et al., 2018). Increased number of granulosa cells was reported after 0.034 μM and 0.34 μM DON treatment, with drastic reduction in their number at a dose of 3.4 μM (Ranzenigo et al., 2008). However, the same toxin did not alter bovine granulosa cell proliferation at concentrations between 0.1 and 3.3 μM (Pizzo et al., 2016).

Compared to pigs, cattle are considered less sensitive to DON, due to their metabolism in the rumen, were the microbiota converts it almost completely to a less toxic metabolite called deepoxy-deoxynivalenol (DOM-1). The remains (less than 1%) will be absorbed and find its way into the circulatory system. Although healthy ruminants are less affected due to their intestinal microflora activity, those suffering of acidosis and the young animals could not be able to similarly convert DON in less toxic metabolites, as their ruminal activity is less efficient. Therefore, such animals are considered susceptible to DON toxicosis. The renal route is the main way of excretion as reported by Bertero et al. (2018). The EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) identified no obvious adverse effects (NOAEL) for dairy cows and heifers at a dose of 5 to 18 mg DON/kg feed, as no adverse effects on body weight, feed intake or milk production over a 13-week period (EFSA, 2017). Cows fed with DON contaminated feed at 0.59 to 104 mg DON/dry matter concentrate, showed no change in feed intake and total milk yield, while milk fat decreased in relation to mycotoxin concentrations (Daenicke et al., 2011). Although rumen exposure to DON seems

generally resistant, the *in vitro* studies revealed that the reproductive system could be considerably affected by this mycotoxin (Pizzo et al., 2016).

Like cattle species, poultry appear to have a higher tolerance to DON exposure, even at high doses, their performance and productivity revealing no considerably losses. This is considered due to the low level of absorption and rapid metabolism, which helped mycotoxin removal from the plasma (Broekaert et al., 2017). Acute mycotoxicosis, triggered by DON in broiler chickens, has been characterized by extensive under skin haemorrhages, alteration of the nervous system and inflammation of the upper gastrointestinal tract, but only at extremely high contamination, which are unlikely to occur. Regarding egg production, no adverse effects were reported on yield, egg weight and shell thickness (Sypecka et al., 2004). However, the immune system revealed to be sensitive to DON exposure (Awad et al., 2014).

Given the average concentrations of DON in feed it is less likely for this mycotoxin to cause health concern in horses (Bertero et al., 2018).

Zearalenone and effects on animals

Zearalenone (ZEA), is also known as F-2 toxin. Due to their chemical configuration, ZEA and its derivatives are the only known mycotoxins expressing estrogenic effects (King, 2002). ZEA is a lactonic mycotoxin of resorcylic acid produced by several species of *Fusarium*, especially *F. graminearum*. It can be altered in plants, fungi, and animals by phase I and II metabolism. The modified forms of ZEA found in feed include its reduced phase I metabolites (α-zearalenol and β-zearalenol) and its conjugates in phase II (conjugated forms with glucose, sulphate, and glucuronic acid) (Zhang et al., 2018)

The ZEA mycotoxin is commonly found in feed stuffs (Table 3). The maximum recommended values of ZEA concentration in feed stuffs are regulated according to the European Commission Guidance and United Stated Food and Drug Administration Guidance.

Pigs can rapidly absorbed ZEA if fed with contaminated feed; this draws an increased biliary excretion and entero-hepatic circulation. However, the main route of ZEA excretion in pigs is through the urinary tract. Therefore, the metabolic pathway and the amount of ZEA metabolites are the main reasons for different sensitivity to this toxin observed among animal species.

Table 3. Maximum Recommended Levels of ZEA in feed stuffs according to the European Commission Guidance and US Food and Drug Administration Guidance (Dänicke & Winkler, 2015)

Item	Livestock categories		ZEN (μg/kg)
EU	Poultry		-
	Swine	Sows and fattening pigs	250
		Piglets and gilts	100
	Ruminants		500
FDA	Poultry		No guidance levels
	Swine	Sows and fattening pigs	
		Piglets and gilts	
	Ruminants		

Due to the strong estrogenic activity, ZEA and its derivatives are able alter the reproductive system, acting as an endocrine disruptor (Denli et al., 2017). The induced estrogenic effects are hyperestrogenism, anesthesia, ovarian atrophy, and changes in the endometrium (Bertero et al., 2018). The zearalenone effects depend on several factors: the reproductive status of the animal (prepubertal, cyclist or pregnant), as well as the administration time and dose (Holda & Glogowski, 2014).

Unlike monogastric, ruminants are less sensitive to zearalenone toxicity (Pizzo et al., 2016). ZEA is converted to α- and β-zearalenol and not only by hepatic biotransformation, but also by the rumen protozoa. However, young animals are having a less effective ruminal microflora compared to mature cattle, which make calves and young heifers more sensitive. Clinical signs due to ingestion of zearalenone are very rare and occur only in highly contaminated feed or after prolonged exposure. Due to the estrogenic effect, α-zearalanol is used in some countries as a growth-promoting agent in cattle (Thevis et al., 2011). Studies on α- and β-zearalenol impact on follicular cell function showed they may affect the proliferation and steroidogenesis of bovine granular cells. In addition to supporting the ovarian effect of zearalenone, sheep fed this mycotoxin (3-24 mg/sheep/day) have been shown to significantly reduce their ovulation rates during estrus (Pizzo et al., 2016).

Pigs are considered the most sensitive species, and ZEA has various harmful effects on their health, causing reproductive disorders, increasing oxidative stress, decreased digestibility of nutrients, while reducing the growth rate. The high sensitivity of this species is considered to be related to zearalenone convertion into α-zearalenol, which has a higher estrogenic effect than its parent compound, or β-zearalenol. At 1 to 5 ppm, ZEA can induce in young sows, vulvar edema and hyperemia, even vaginal or rectal prolapse, while in adult animals, at various stages of their oestrous cycle, nymphomania, ovarian atrophy, and endometrium changes are more common (Bertero et al., 2016). Adverse oestrus effects in pigs are also reported by Dai et al. (2016), symptoms being related to the dose and oestrus stage in which the mycotoxin is consumed. The effects of α and β-zearalenone on pig oocytes showed a significant decrease in their maturation rate when exposed to 7.5 μM α-zearalenol for two days, compared to 30 μM of β-zearalenol (Alm et al., 2002). Beside the estrogenic syndrome in pigs, that targets the reproductive tract, the mycotoxic effects are also seen at the mammary gland (Minervini & Dell'Aquila, 2008).

ZEA effects on horses were studied mostly *in vitro*, where it was seen that zearalenone is mainly biotransformed to α-zearalenol, than β-zearalenol, which was two to three-fold lower (Bertero et al., 2018). However, recent studies, demonstrated that ZEA induces severe reproductive disorders in both mares and stallions (Dänicke et al., 2021).

In poultry feed, ZEA is a commonly found mycotoxin. Due to the structural similarity to estrogen, ZEA lead to hyperestrogenism resulting in decreased reproductive performance in poultry. In broiler and leghorn chickens ZEA affects the reproductive system (Chi et al., 1980). It also affects egg quality and can increase embryo mortality resulting in reduced hatchability (Liu & Applegate, 2020).

In fish in addition to hepatotoxic, genotoxic, haematological, and reproduction effects (Pietsch et al., 2015), ZEA is also affecting the expression of several genes involved in the regulation of the immune system (Pietsch, 2017).

T-2 and HT-2 toxins and their effects on animals

T-2 and HT-2 toxins belonging to the type A group of trichothecene, which are produced by various *Fusarium* species predominantly by *F. sporotrichioides.* Contamination can occur mainly in cereals, either in field or during storage. The T-2 toxin production seems to be stimulated by the presence of other contaminants, such as *Aspergillus* and *Penicillium* genera (Köpke eta al., 2007). The toxicity of T-2 and its deacetylated form HT-2 toxin is influenced by various factors, such as livestock category, exposure and dosage, animal age, sex, and general physical condition, as well as simultaneous exposure to other mycotoxins (Stoev et al., 2010).

Ruminants are considered less sensitive to the T-2 toxin. As in the case of other mycotoxins, the negative effects of T-2 toxin are significantly diminished by rumen activity. However, younger animals could experience bloody feces and ulcers if exposed to high amounts or prolong feeding with T-2 and HT-2 toxins, as their incipient microflora is less is efficient in detoxification. To sheep, the T-2 toxin reduces the reproductive performance (Bertero et al., 2018). T-2 toxin effects also reduce the reproductive performance in pigs, this animal category being among the most sensitive among all livestock. Immunological and / or haematological effects were noticed at 0.03 mg T-2 toxin/kg/day. Prolonged pigs' feeding with T-2 toxin contaminated forage results in anorexia, and damages of the oral cavity and esophagus. As T-2 toxin inhibits protein synthesis, the immune response and antibody production are also affected. *In vitro* studies indicate that T-2 toxin may alter the growth of granular cell layer and steroidogenesis, proportional to the tested dose (Caloni et al., 2009). Other studies performed on porcine oocytes confirmed the potential of HT-2 and T-2 toxins to disturb the reproductive performance of pigs (Xu et al., 2021).

In poultry, T-2 toxin is rapidly absorbed into the intestinal tract of chickens, then metabolized and eliminated almost completely (approximately 90%) in a single day, although at prolong exposure toxic effects arise (Young et al., 2007), inducing genotoxic, cytotoxic, and immunomodulatory effects, as well as several

disorders at the digestive system, liver, nervous system and skin. The first signs of T-2 toxicosis are the lower feed intake, reduced weight gain and growth retardation, or lower egg production. The eggs collected from T-2 exposed poultry revealed a reduced weight, thinner eggshells and decreased hatching percentage. The lethal dose of T-2 toxin was noticed at approximately 10 mg/kg body weight of chickens, during a seven-day feeding period (Young et al., 2007). Other symptoms of T-2 toxin poisoning reported in poultry include some neurological signs, leukopenia, oral lesions, cyanosis of the comb, depigmentation of the feet's skin and feather alterations.

On horses, there are only few information regarding T-2 toxicosis. Older research results suggest altered locomotion and animal death after one month exposure. Blood biochemistry revealed leucocytosis and anemia. Prolong exposure revealed skin lesions and liver degeneration (Gabal et al., 1986). More recent studies indicated an increased incidence of colic in horses when exposed to DON and T-2 toxins (Caloni & Cortinovis, 2010). In racehorses, it is a strong belief that mycotoxins exposure, even at low levels, can negatively affect to their performance or breeding activities, although no such signs have been reported (Newman & Raymond, 2005).

CONCLUSIONS

Fusarium is one of the most economically important fungal genera due to its mycotoxigenic potential additionally to its' pathogenicity on a wide host variety. *Fusarium* infections significantly reduce plant yields and quality, and make harvest and by-products unsuitable for marketing, due to mycotoxin contamination of food and feed products. The spectrum of mycotoxins is varied, all causing serious negative impact on human and animal health. Mycotoxigenic *Fusarium* species are capable of producing three of the most important classes of mycotoxins, such as fumonisins, zearalenone, and trichothecene. If ingested, such toxins can produce acute and chronic effects in humans and animals following consumption. Pigs are among the most affected livestock, while ruminants are considered less sensitive, thanks to the metabolism of rumen microbiota. Poultry however are affected on prolong exposure.

Regarding other *Fusarium* mycotoxins, such as eniatins and beauvericin, the toxic profile is not fully understood, despite the emerging interest in it, thus being a challenge for future toxicological studies to be performed.

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BREAD QUALITY IMPROVEMENT BY ADDING DEHYDRATED SOURDOUGH IN THE RECIPE

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Abstract

The production of sourdough, used for thousands of years, it can be considered that is one of the oldest biotechnological techniques. Currently, methods for manufacturing bakery products using sourdough could improve the texture, flavour, and increase the shelf life from a microbiological perspective. The fermentation process is based on the symbiosis between certain lactic acid bacteria and yeast. If desired to integrate the properties of traditional bread in the industrial manufactured, a profitable and simple to use technology is the utilization of dehydrated sourdough in the recipe. The aim of this paper is to examine the influence of dehydrated sourdough addition over the sensorial and physicochemical properties of wholemeal and white bread. The assessed characteristics were: smell, flavour, crumb appearance, total acceptability, elasticity, acidity, volume and porosity. The study demonstrate that the quality of bread obtained with dehydrated sourdough was improved: higher acidity, better elasticity and porosity, higher volume, higher score for total acceptability, more pleasant flavour and taste. Technological aids such as sourdough utilization can be a very helpful instrument to increase bread quality but also bread shelf life.

Key words*: bread; dehydrated sourdough; sensory properties; shelf life.*

INTRODUCTION

Bread is the most common and well-known food in the world, a very old component that is part of human nutrition (Arranz-Otaeguia A. et al., 2018). Studies show that bread is present uninterruptedly in the daily diet. The average consumption of bread per capita is 97 kilograms per year. Given this quantity, it is observed that the average level of consumption in Europe is exceeded (Tamba-Berehoiu et al., 2014). Bread was originally a household product, and for the industrialization of the bakery it was necessary to develop the technology and science of food and first of all it was necessary to discover the microorganisms responsible for the development of the dough and implicitly of the sourdough. The sourdough is in fact an old natural yeast used in baking and which over the years has been replaced by yeast produced at the industrial level and with chemical agents (Nionelli L., & Rizzello C., 2016). This natural fermenting agent has stood the test of time and is still maintained today, used by consumers who are increasingly aware of the nutritional quality of food and of course its impact on health.

Traditional sourdough is the result of a mixture of water and flour where the native lactic bacteria of yeast and flour produce the phenomenon of fermentation. The so-called back-slopping process, which is characterized by the use of small amounts of product in the initial fermentation as the starter culture in the next fermentation process, is the one that promotes proteolysis, synthesis of exopolysaccharides, enzymes, antifungal compounds and organic acids (Brandt M.J., 2019). Both the level of these compounds and their formation in the dough are directly proportional to the selected strains used to start the fermentation, to the activity of the natural microbiota of the flour and to the quality of the raw materials (Păcularu-Burada B. et al., 2020). The interest regarding the improvement of the fermentation process was given by that search to diversify the raw materials considering first of all their nutritional and functional properties (Papadimitriou K. et al., 2019; Reese A. T. et al., 2020; Rizzello C. G. et al., 2019). The

bioavailability and bioavailability of non-nutrients and nutrients are particularly important to ensure adequate nutrition of the fermentation process and the health benefits that the final product can bring (Păcularu-Burada B. et al., 2020; Siepmann F.B. et al., 2018). The glycemic index of bread may be lowered by the sourdough. The sourdough can also release bioactive peptides, improve the properties of the dietary fiber complex, increase the absorption of phytochemicals, minerals and vitamins. If we compare two categories of bakery items: an item containing rye flour or whole wheat flour and another item of highly refined white wheat flour, we notice that the former has higher amounts of vitamin E and B1. Foods can be enriched with vitamins B1 and E by adding additional vegetable ingredients. These additives in the bakery area have been used for diversification, and for enriching the flavor, aiming to be a motivation and a guide to a healthier consumption of food (Gherghina et al., 2015). The lactobacilli present in the dough have a microbial metabolism that produces new active compounds such as potentially prebiotic exopolysaccharides and amino acid peptides and derivatives (Păcularu-Burada B. et al., 2020; Chiș M.S. et al., 2019; Rashmi B. S. et al., 2020). The scientific community is interested in the by-products of microbial metabolism because it is possible to create new products based on maintaining health in case of chronic non-communicable diseases such as diabetes and cancer, autoimmune diseases, irritable bowel syndrome, heart disease, colitis, high cholesterol, irritable bowel syndrome (Bo S. et al., 2017; Diowksz A. et al., 2020; Gobbetti M. et al., 2019; Olojede A.O. et al., 2020; Rizzello C.G. et al., 2017). "Functional foods" are a relatively recent concept. This concept has proven to have developed rapidly in the last few years. This type of food should support the improvement of health and well-being and at the same time should reduce the risk of chronic high diseases, but also degenerative diseases such as cancer, obesity, cardiovascular disease and gastrointestinal disorders (Zamfir et al., 2014).

MATERIALS AND METHODS

The Web of Science database has been used to search the electronic library for articles published in recent decades. This search for literature included research articles and reviews. The keywords used were: bread; dehydrated sourdough; sensory properties; shelf life. The analysis took place in the physico-chemical analysis laboratory (Mass - electronic scale, Volume - Fornet, Porosity - sharp brass perforator, analytical/technical scale, Elasticity - elasticity determination device; Acidity - laboratory determnatons, Moisture - laboratory determinations, Ash - calciner, Fat - Soxhlet, Protein - Kjeldhal) and in the sensory analysis laboratory (in a large room for observing the social distance regarding the situation we are in), with a group of trained examiners (evaluation panel). The samples were sliced shortly before to avoid drying/hardening, each sample being pre-coded.

RESULTS AND DISCUSSIONS

Being considered a long tradition in the production process of bakery products, the use of sourdough plays an important role. We can obtain the dough by spontaneous fermentation of a mixture of water, salt and flour, the dough and in recent years the control of the fermentation process as well as specific cultures has been used. The sourdough is used in baking process and its main qualities such as its ability to improve the quality and prolong the shelf life of bread have been widely studied (Arendt EK et al., 2007; Gocmen D. et al., 2007; Katina K. et al., 2006; Martinez-Anaya M.A., 2003). The impact of these specific processing conditions on the microbial quality of wheat and bread dough was investigated by Debonne et al.

The sensory evaluation panel consisted of 9 people with an average age of 30 years, where 4 bread samples were analyzed coded as follows:

534 - Bread with white flour, without sourdough.

239 - Bread with wholemeal flour, without sourdough.

620 - Bread with white flour, with sourdough.

345 - Bread with wholemeal flour, with sourdough.

The samples were analyzed individually and the neutralizing agent was water for rinsing the mouth. The questionnaire that accompanied the tests included all these characteristics and will be evaluated on a scale from 0 to 5.

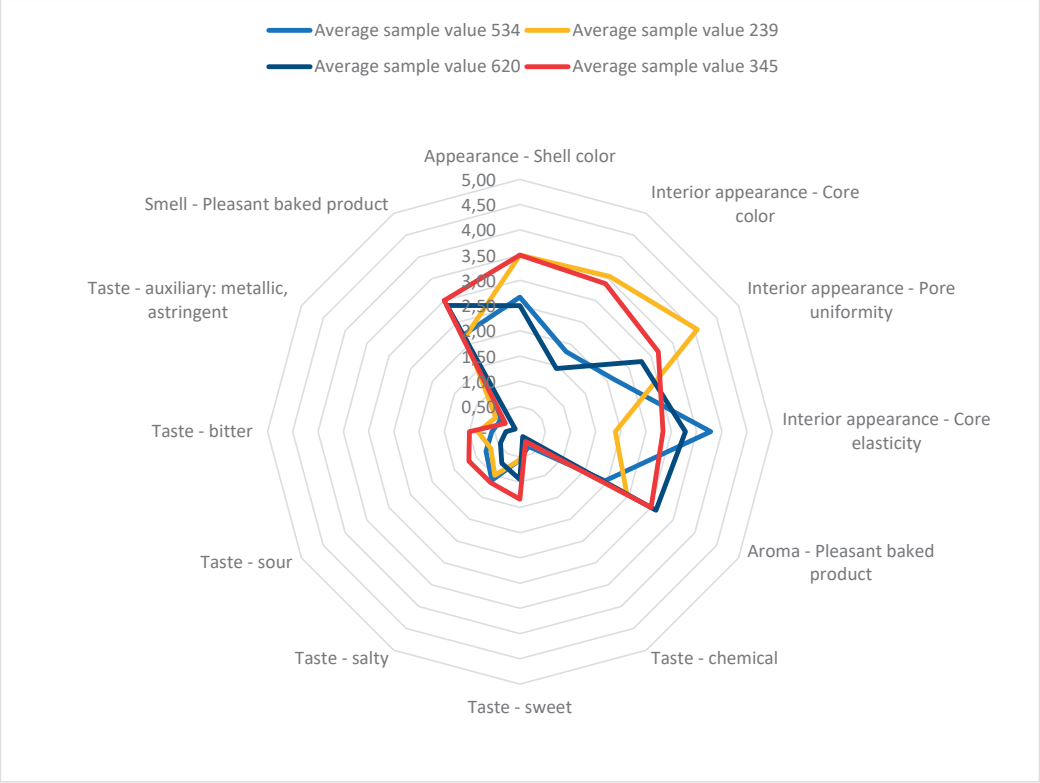

Figure 1. Diagram of the sensory characteristics of bread samples

Figure 2. White flour bread samples

Figure 3. Whole grain flour bread samples

The quality of sourdough largely depends on the type of wheat from which the flour used in the fermented dough is made.

Wholemeal flour is recommended because it has many nutrients or microbial substrate, such as phytochemicals and vitamins, minerals, as well as sterol, phenolic and tocopherol compounds. The main ingredient in sourdough is traditionally wheat flour.

However, different types of flour, whether conventional or unconventional (quinoa, corn, oats, barley, rye, sorghum) can replace wheat in order to increase quality and meet consumer needs.

The use of so-called unconventional flours in the manufacture of doughs can improve some of the beneficial health effects associated with bakery products.

The consumer's perception of the quality of the bread is mainly determined by sensory and health attribution.

Table 1. Sensory analysis results

Characteristics	Average test value 534	Average test value 620	Average test value 239	Average test value 345
Exterior appearance - color	2.66	2.51	3.51	3.51
Interior appearance - color	1.82	1.43	3.55	3.38
Interior appearance - pore uniformity	2.1	2.77	4.05	3.16
Interior appearance - elasticity	3.77	3.27	1.88	2.82
Aroma - pleasant baked product	1.93	3.10	2.43	3.01
Taste - chemical	0.32	0.10	0.21	0.21
Taste - sweet	0.55	0.93	0.55	1.32
Taste - salty	1.10	0.71	1.01	1.16
Taste - sour	0.77	0.43	0.66	1.16
Taste - bitter	0.55	0.27	0.82	1.01
Taste - auxiliary: metallic, astringent	0.43	0.10	0.55	0.32
Smell - Pleasant baked product	2.21	2.88	2.16	3.01
Total acceptability	1.66	0.55	1.10	0.55

Table 2. Physico-chemical results

Sample Test	534	239	620	345
Moisture (%)	43.86	45.47	43.83	38.51
Moisture in breadcrumbs (%)	0.35	0.51	3.75	5.91
Mass (g)	385.97	398.6	386.66	364.67
Acidity (°)	1.0	2.2	1.1	2.1
Total ash (%)	1.88	2.63	1.91	2.67
Protein	14.02	17.57	13.50	16.27
Fat (%)	0.1	0.4	0	0.3
Starch (%)	74	66	71	58
Total dietary fiber (%)	0.32	2.83	0.28	2.46
Salt (%)	0.958	0.87	0.94	0.81
Volume (cm^3 / 100 g)	341	223.5	381.8	252.7
Porosity (%)	81	63	76	68
Elasticity (%)	97	71	96	86

CONCLUSIONS

All aspects regarding quality of bread (texture, flavour, nutritional quality and shelf life) are influenced by the use of sourdough in bread-making process. The sort of cereal flours, the metabolism of fermenting microorganisms, the baking conditions, the characteristics of sourdough preparation are the factors who determine the bread aroma. As an alternative to adding value to sourdough bread, we can use the unconventional alternative flours and in the same time allowing gluten-free bread production. The increased bioaccessibility of non-nutrients and nutrients and the bioactive compounds released from the matrix contribute to the health benefits from the consumption of sourdough bread.

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IMPACT OF COVID-19 PANDEMIC ON THE FOOD SAFETY REQUIREMENTS IN THE FISH AND SEAFOOD CHAIN

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Abstract

Producing safe and high-quality fish and seafood products, for both domestic and export markets must be considered a priority for the entire fish and seafood chain, from fishers and producers towards consumers and food safety competent national authorities, who should update the relevant food safety legislation and ensure compliance with it. The aim of this study is to highlight the rules of hygiene and food safety that are imposed on the fisheries and seafood sector in order to prevent staff illness with Covid-19 and ensure the safety of products. Several practical recommendations are given for completion and improvement of the current preventive measures such as good hygiene practices to which is added specific protocols to safeguard the health of the employees who works in the fish and seafood production and processing sector.

Key words*: fish and seafood products, food safety, good hygiene practices, preventive measures, fish and seafood production and processing sector.*

INTRODUCTION

Coronaviruses are a large family of viruses and a subset of *Coronaviridae* that include a group of viruses which are capable to induce disease in humans and animals, and consist of common cold virus up to more severe pathogens such as SARS-CoV that causes Severe Acute Respiratory Syndrome (SARS), MERS CoV that causes Middle East Respiratory Syndrome (MERS) and SARS-CoV-2 that causes Coronavirus disease (Covid-19) (Ranaei et al., 2020).

The recently coronavirus disease (Covid-19) that emerged in the Wuhan city and then in Hubei province in China has rapidly spread around the world, resulting in the declaration of a pandemic by the World Health Organization (WHO) on March 11, 2020.

After more than 2 years of pandemic, Covid-19 continues to spread and thousands of new cases occur every day worldwide due to the lack of specific antiviral treatments for this virus (Han et al., 2021). The best way to prevent and slow down transmission is to be well informed about the disease and how the virus spreads (WHO, 2022a).

At the time of this review, over 500 million confirmed cases and over six million deaths have been reported globally due to Covid-19 disease (WHO, 2022b). Therefore, many countries have implemented social distancing measures, or more stringent lockdown, doing great efforts to slow the spread of the virus and thus reduce the pressure on the public health system (by reducing the number of hospitalized humans).

The Covid-19 pandemic and subsequent lockdowns are creating health and economic crises, with extensive social and economic effects, changing our habits, affecting the way we live, work, shop, travel and interact (EFSA, 2022a). Some groups and sectors are highly susceptible and vulnerable to the rapid social and economic effects of the Covid-19 pandemic (Bennett et al., 2020). In addition, each country is facing adverse impacts on their economies due to the Covid-19 infection, with marketing problems throughout food supply chains, which is one of the worst-hit areas (Han et al., 2021).

This paper aims to give an overview of potential food safety requirements through additional good hygiene practices to which

some specific protocols could be added in order to minimize the risk of virus transmission and to safeguard the health of the employees who work in the fish and seafood production and processing sector.

MATERIALS AND METHODS

This paper is based on recently published articles, accessing Science Direct from Information platform, also on information available on Food and Agriculture Organization (FAO), Food and Drug Administration (FDA), Centers for Disease Control and Prevention (CDC), Occupational Safety and Health Administration (OSHA), World Health Organization (WHO), European Food Safety Authority (EFSA) websites as well as specific legislation in different countries. First, the used search terms were "covid-19 or coronavirus disease impact" and "seafood or fishery or aquaculture sector", afterwards a selection was made and a database collection was created with the newest items which were focused on responses about Covid-19 pandemic and its effects on fisheries and seafood sectors.
This work could help the specialists from fishery and aquaculture sector to implement protocols necessarily for human health monitoring at the workplace as well as establishing new requirements for good hygiene practices in the fish and seafood processing factories up to the final consumer.

RESULTS AND DISCUSSIONS

The design and the implementation of strict working Procedures and Protocols are necessary so that the activity in the fish and seafood sector can be carried out in the best conditions that will ensure the maintenance of the workers' health and the reduction of virus spread.

Recommendations for primary production and processing sector

Coronavirus disease (Covid-19) is an infectious disease caused by the SARS-CoV-2 virus (WHO, 2022a). Despite some similarities to smaller shocks, the Covid-19 global pandemic has triggered larger, more unpredictable and synchronous impacts felt throughout the entire food supply chains (White et al., 2020), which has been seriously disrupted with impacts occurring at multiple levels and across supply chains (Hobbs, 2020; Global Panel, 2020; Devereux et al., 2020; Chenarides et al., 2020).
Fish and seafood chain has also been affected by the restrictive measures imposed in the context of this pandemic and its impact has been extremely strong (Baldwin & Tomiura, 2020; Zhang et al., 2021). Fishers, processors and sellers also face risks of Covid-19 spread and infection, and thus have to make difficult decisions - feeding their families or risking exposure (Bennet, 2020). Fishing communities and ports could potentially become "hotspots" for rapid infection due to the migratory nature of fishers and frequency of international visitors (FAO, 2020).
Guidance from the Centers for Disease Control and Prevention (CDC) advises that critical infrastructure workers may be permitted to continue to work following potential exposure provided (1) they remain asymptomatic, (2) they do not test positive, and (3) additional precautions are taken to protect them and the community. Factors that may increase risk include duration of contact, type of contact (respiratory droplets from talking, coughing, or sneezing) and housing or living quarters (CDC, 2020).
It is necessary to develop policies and to establish procedures within any onshore or offshore factory in order to minimize the risk of transmitting this virus to the workplace. For this purpose, the employer should identify a qualified worksite coordinator responsible for Covid-19 assessment and control planning. The Covid-19 coordinator must be aware of and follow all applicable regulations and public heath guidelines. Covid-19 coordinator will have the responsibility to establish the communication channels for workers informing on the good hygiene practices and food safety procedures that are established in the unit as a result of the regulations and the public health legislative measures.
Communication in risk management is essential for carrying out the activity at work and can contribute to more efficient management of food safety and also more effective health education activities. Employers must ensure that all workers know how to contact and

communicate with the worksite coordinator via the communications channels that were previously insured. Risk communication is an element of risk analysis, but it also plays a central role in health education in food safety, which is a risk management option. Iterative risk communication takes place between regulatory risks assessors and risk managers in the context of their duties. It also takes place between these authorities and stakeholders of the food chain, including industry, consumers, and others (Motarjemi et al., 2014). One of the recommendations to prevent the disease from spreading is to even quarantine staff before boarding a fishing vessel for a period of time set by the authorities, which employers should consider paid leave or worktime.

In Alaska, seafood processors are implementing quarantines for incoming seasonal workers, modifying processing lines to increase social distancing, providing daily screenings, and procuring medical supplies. These actions are necessary to maintain operations and seafood production while protecting workers and communities (Campbell, 2020).

Some large fishing vessels that process fish at sea may be especially vulnerable to virus transmission, because workers live and work in close quarters for weeks at a time sometimes (CRS report, 2020).

Screening workers for Covid-19 symptoms before they enter the worksite or aboard the vessel as well as periodically monitoring their health status at regular intervals, could be a preventive measure. It is also recommended to test the new entrants into the worksite and those who are re-entering after an absence. This screening may include a questionnaire on symptoms, control of body temperature of employees, rapid tests for virus detection in order to receive results before entering the worksite and, if necessary, PCR tests. It is also necessary to appoint a person in charge of the screening activities of the workers.

An action plan is required when there are workers who have specific symptoms of Covid-19 as well as for employees who decline testing or who are unable to be tested. A number of measures should be taken to this end, such as: (a) providing access to medical care or telemedicine for workers who have symptoms or a positive test result, (b) encourage workers to self-isolate when they have symptoms, (c) ensure communication with health officials, workers in quarantine or isolation and human resources department in order to ensure their replacement in the food factory, (d) providing rapid test for direct contacts at the workplace.

An important issue for employers is to ensure protection measures to reduce the virus spread at workplace, therefore actions like providing appropriate personal protection equipment (PPE) to workers and training them how to properly use it (put on, take off, dispose or clean if reusable) are really necessary. PPE must consist of gloves, gown, eye protection and face mask (an N95 filtering respirator face-piece or more protective ones are recommended), at minimum. Also, a safety measure to limit the virus spread is to ensure adequate ventilation in the work areas through minimizing the use of hard-mounted cooling fans, which can blow potentially infectious droplets from one worker to another. Another issue in designing the safety protocols for worksites is to ensure social distancing, at least 6 feet apart in all directions for workers inside working areas and physical barriers at the workplace. For this purpose, is allowed to use markings and signs to remind workers to maintain social distancing.

When a sick or symptomatic person is on board the vessel, a special procedure should be followed which implies immediate isolation in a specially arranged area with single occupancy quarters and a separate bathroom, if available. In this case a safely transportation of sick workers at home or at hospital, if needed, is recommended as well as cleaning and disinfection of the work areas, equipment, respectively common areas; the access of the other workers in these areas being allowed only after cleaning and disinfection procedures are finished. Workers with Covid-19 must return to work when their state of health allows it and when compliance with all the recommendations imposed by the authorities is ensured.

Table 1. Action plan for food safety requirements to protect fishery and seafood workers from Covid-19

Actions	Measures	Comments
Appoint a qualified employee as coordinator responsible for Covid-19 assessment and control planning	- Ensuring a risk communication procedure; - Ensure that Covid-19 coordinator is up to date with the regulations;	Adequate/Inadequate
Develop specific protocols and procedure for worksite	- Hygiene Protocols; - Personnel Training Programme; - Covid-19 Testing Procedure; - Worksite Cleaning and Disinfection Procedures; - Action plan for sick workers; - Action plan for close contact workers with someone with confirmed or suspected Covid-19.	Adequate/Inadequate
Develop and implement a testing plan	- Preventive testing of all employees once a week; - Testing of new entrants into the worksite and those re-entering after an absence/a holiday; - Establish a procedure for employees who refuse testing or are unable to be tested; - Choosing a quick detection test to be supported by a PCR test when needed.	Adequate/Inadequate
Establish the Priorities by which workers will be tested	Priority for testing workers with symptoms Immediate testing of direct contacts or suspected cases (within 6 feet for a total of 15 minutes or more)	Adequate/Inadequate
Monitoring the health of employees	- Testing the body temperature before every work shift; - Conducting screening interviews; - Conducting rapid test.	Adequate/Inadequate
Develop and implement an action plan for Covid-19 confirmed or suspected workers	- Protect confidentiality of suspected or confirmed Covid-19 workers - Provide an isolation room for sick workers.	Adequate/Inadequate
Manage the health status of the sick workers	- Ensure access to health care system, to hospitals if needed or telemedicine for sick workers; - Provide isolation or quarantine rooms at the worksite (on board fishing vessels for example) if needed, for confirmed or suspected Covid-19 workers.	Adequate/Inadequate
Develop and implement measures to assure social distancing in the workplace	- Assure 6 feet distance between workers in all working areas; - Establish safety protocols during the mealtimes, break times, etc.; - Ensure good airflow in common working areas.	Adequate/Inadequate
Emphasized the important measures for personal hygiene	- Provide regularly good hygiene practices training; - Provide hand washing/sanitizing materials; - Provide personal protection equipment (PPE) for workers (Gloves, face masks, etc.); - develop and implement cleaning, disinfection and sanitation protocols for working areas; - Displaying posters with specific information for safety and hygiene practices.	Adequate/Inadequate
Conducting regular monitoring	- Identify human health exposure risks; - Checking the compliance with hygiene rules; - Ensure implementation of hazard controls.	Adequate/Inadequate

An action plan for food safety requirements to protect fishery and seafood workers from Covid-19 was design and provided in Table 1.

Safety protocols must be implemented in order to reduce crowding in all common areas and keep the social distance during work shifts, mealtimes and break times. Employees must be periodically trained to respect the safety and hygiene protocols and to comply with it during the work time and as much as possible after that. To remind them of the instructions learned during the training the employees can provide visual cues such as floor makings, signs, posters, etc. Posters can be placed at the worksite entrances and in all common areas to reinforce training. The information provided by

the posters can be written in all preferred languages, easy to understand and it can include: (a) information about Covid-19 symptoms, how it spreads, how workers can protect themselves; (b) proper handwashing and use of hand sanitizer; (c) social distancing practices at the worksite; (d) cough and sneeze etiquette; (e) clear instructions about putting on and taking off PPE, gloves, goggles, face shields and face masks; (f) how to proceed if workers become ill, presents Covid-19 specific symptoms or if they are close contact with someone with confirmed or suspected Covid-19. If the employer provides transport for the workers, then he should coordinate this activity so as to ensure their movement in compliance with the conditions of social distancing. While traveling, both the driver and the employees must be encouraged to wear face masks and follow the coughing and sneezing etiquette. Good hygiene practices implemented must be further supported and adapted to the new epidemiological conditions. Regarding the hygiene rules of the staff, regular training for workers should be organized and their awareness in multiple ways, such as the instructions displayed in visible places on washing hands as often as possible with soap and water at least 20 seconds. It is recommended, if possible, to increase the number of hand washing stations, also to provide access to temporary stations equipped with hand sanitizer containing at least 60% alcohol or other appropriate sanitizer, which can be placed in multiple locations including entry, exit, changing rooms, smoking place, time clock stations. In this sense, the permanent supply and availability at work of hygiene products (soap, sanitizer, single-use towels, etc.) and protection materials (gloves, gown, eye protection, face masks, etc.) must be ensured.

Employees can conduct additional training in order to educate workers to to adopt a behavior that limits the spread of the virus such as: (1) to avoid touching their faces, including their eyes, nose and mouth, until after washing hands thoroughly; (2) to wash and sanitize their hands after completing work, removing PPE, removing face coverings, and before and after eating, smoking or touching their face; (3) to ensure that face masks fit over the nose and mouth and fit snugly and comfortably against the side of the face, are secured with ties or ear loops, include multiple layers of fabric, allow for breathing without restriction; (4) to wear clean face masks, do not used if they become damaged, wet or contaminated and change them whenever needed; (5) to handle the face masks as little as possible to prevent transferring it infectious materials.

Best practices recommended for shopping food

Prevention of SARS-Cov2 transmission during shopping of food must also be a priority for retailers and stores. Therefore, they have to implement safety measures for their employees as for customers. A longer operating schedule could prevent stores from crowding along with procedures that prioritize vulnerable consumer segments such as the elderly.

During this pandemic, consumers are getting most of their food from grocery stores, and many stores have modified their operating hours to allow for more time to restock shelves and clean. In addition, many stores are providing special hours for seniors or other high-risk individuals to shop and are offering pick-up and delivery services (FDA, 2020).

In order to limit the transmission of the virus authorities recommend to consumers to follow a few rules such as: (1) to prepare a shopping list in advance so as to shorten the time they spent in the store; (2) to wear a face covering or mask while they are in the store; (3) to wipe down the handles of the shopping cart or basket with their own wipes, or use the ones provided by the store; (4) to practice social distancing while shopping, keeping at least 6 feet from other shoppers, and store employees; (5) to wash their hands with warm water and soap for at least 20 seconds when they return home and again after putting away their groceries; (6) before eating, to rinse fresh fruits and vegetables under running tap water, including those with skins and rinds that are not eaten; (7) to regularly clean and sanitize kitchen counters using a commercially available disinfectant product.

CONCLUSIONS

The Covid-19 pandemic has raised a lot of issues regarding the public health systems capacity and the food chain sustainability

which has led to economic and food crisis all over the world. There was a lack of collaboration and actions between government, agencies, industries and individuals which causes disruption along the food chain from primary producers to final consumers. It is desirable that national and international agencies have a comprehensive and integrated approach through combined and complementary knowledge, skills and expertise, as well as their agility to deliver scientific advice for policy makers. Also, these agencies could be a source of expertise and networks for foresight activities in crisis management for future threats of any kind. These guidelines could be a useful tool in designing a better risk management system for seafood or fishery workplaces and consumers in order to keep under control the risk assessments and risk communications in crisis like pandemics.

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AMINO ACIDS COMPOSITION OF WHEAT-GERMINATED LEGUMES COMPOSITE FLOURS

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Abstract

The aim of this study was to analyze the amino acids content of different legumes types (beans, lentil, soybean, chickpea and lupine) in a raw and germinated form. Also the effect of different levels (0%, 2.5%, 5%, 7.5%, 10%, 15%, 20%, 25%) of legumes addition in a germinated and raw form in refined wheat flour has been discussed. According to our data for the wheat- bean mix, lentil-wheat flour, soybean-wheat flour mix, lupine-wheat flour mix the highest amount of essential amino acid were recorded for the histidine whereas for the mix between chickpea-wheat flour the highest amount of the essential amino acid were recorded for valine. Regarding the amount of non-essential amino acids content the highest levels were obatined for glutamic acid for all the mixes between germinated legumes and wheat flour and the lowest one for glycine.

Key words*: amino acids content, germinated legumes flour, legumes flour, wheat flour.*

INTRODUCTION

Wheat flour is the main raw material for bakery products. However, the proteins from their content are deficient in essential amino acids, such as lysine, tryptophan, threonine, etc. (Laze et al., 2019). Different composite flours can be used to correct this deficit by partial substitution of wheat flour with other grain products such as legumes ones. Their use in wheat flour addition is recommended after some processing techniques in order to minimize their antinutrients contents and unpleasant flavor. Such a technique is the germination one which besides reducing their antinutrients compounds it also improves their nutritional content (Atudorei & Codină, 2020). Various studies have shown that germination increases the availability of nutrients, such as amino acids, minerals, vitamins, etc. (Ohanenye et al., 2020; El-Suhaibani et al., 2020) and at the same time reduces antinutritional compounds in seeds such as protein inhibitors, hemagglutinins, antivitamins, phytates (Singh & Sharma, 2017; Sokrab, Mohamed-Ahmed & Babiker, 2012). The international literature has pointed out that the germination process increases the amount of phenolic compounds, chemical compounds with antioxidant action (Atudorei & Codină, 2020). At the same time, the germination process activates the hydrolytic enzymes in the grains, which promotes the digestion of compounds such as starch and proteins (Han et al., 2016). It seems that germination process activate grains endo-enzymes, such as proteases and amylases, which hydrolises macromolecular substances such as proteins and carbohydrates and improve the digestibility of nutritional compounds from grains. However, the germination process does not significantly affect the content of macromolecular compounds from grains, although the amylose content appears to slightly decrease with the increase of the total sugar amount (Atudorei & Codină, 2020). From the protein point of view, the nutritional value of legumes is improved by increasing their content in essential amino acids available such as lysine, arginine, tyrosine, tryptophan, methionine due to the action of proteolytic enzymes which was synthesized during germination process on proteins (Boye et al., 2010). More, some inhibitors like trypsin, chymotrypsin which may have adverse effects on protein digestibility may be eliminated during germination process. This fact is desirable since protein in these forms has a higher digestibility.

Adding them in a germinated form in wheat flour can lead to a significant increase in the essential amino acid content of wheat flour. This represents the main raw material for bread

making. Bread is the main source of vegetable protein in the human body, which covers about 1/5-1/3 of the total protein requirement and about 2/3 of the body's vegetable protein requirement (Segal, 2002). Due to the inadequate balance of essential amino acids in bread, the biological value of bread proteins is relatively low. This deficiency can be corrected by adding germinated legumes in wheat flour which improves its nitrogen balance and, as a result, increases the assimilation coefficient of amino acids in bread. In our study was used as legumes in a germinated form for addition in a refined wheat flour lentil, lupine, bean, soybean and chickpea. According to the literature, these type of legumes present a higher amount of protein (17-40% dw) compared to the wheat flour (3-7% dw) (Atudorei & Codină, 2020). More, the supplementation of wheat flour with this type of germinated legumes will complement the deficiencies in these amino acids from the cereal-based products (Patrascu et al., 2019). From the amino acid content point of view, lupine is higher in arginine and leucine, whereas the amount of methionine is lower compared to other legumes (Martínez-Villaluenga et al., 2006). Chickpeas has a higher amount of arginine, leucine and lysine and beans of lysine, leucine, phenylalanine, tyrosine. Soy and lentils have a high amount of leucine and lysine (Boye et al., 2010). The aim of this paper was to present an analysis of the evolution of amino acids content in legumes: lentil (LN), lupine (LpN), bean (BN), soybean (SN) and chickpea (CN) at the initial time and after 4 days of germination: germinated lentil (LG), germinated lupine (LpG), germinated bean (BG), germinated soybean (SG), germinated chickpea (CG) and the impact of its addition in a refined wheat flour at different levels in order to improve its quality from the amino acids content point of view.

MATERIALS AND METHODS

Materials

The legumes used in this study (harvest 2019) were the following: bean (*Phaseolus vulgaris*), soybean (*Glycine max* L.), lentil (*Lens culinaris* Merr.), chickpea (*Cicer aretinium* L.) and lupine (*Lupinus albus*). Refined wheat flour (WF) of 650 type (2019 harvest) was provided by S.C. Dizing S.R.L. company (Brusturi, Neamț, Romania). The wheat flour (WF) and legumes in a native and germinated form physical-chemical characteristics have been reported in a previously study (Atudorei et al., 2021).

Legumes germination process

The germination process was made after a method previously described (Atudorei et al., 2021). Shortly, before germination, legumes grains were soaked at a temperature of 20°C for 6 hours in the case of lentil and soybean and for 12 hours in the case of beans, lupine and chickpeas. The germination took place in dark conditions for 4 days on filter paper at 25°C and 80% humidity. In order to reduce their humidity the samples were lyophilized at -50°C, for 72 h, at 4.2 Pa pressure. To obtain wheat-germinated legumes composite flours the lyophilized germinated legumes were grinded and mixed in various amounts in wheat flour.

Amino acid quantification

The amino acids determination was done by using the EZ:Faast kit (Phenomenex, Germany) and consisted of a solid phase extraction step and a derivatization and liquid/liquid extraction step. The solid phase extraction was performed via a sorbent packed tip that bended amino acids while allowing interfering compounds to flow through. Amino acids on sorbent were then extruded into the sample vial and quickly derivatized with reagent at room temperature in aqueous solution. Derivatized amino acids concomitantly migrate to the organic layer for additional separation from interfering compounds. Organic layer was then removed, evaporated, and suspended again in dissolution solvent and analyzed on a Shimadzu GC/MS system (GC MS-QP 2010 Plus, Shimadzu, Kyoto, Japan) with a Zebron ZB-AAA GC column. The essential amino acids obtained from the raw materials in a native and germinated form were: histidine (His), isoleucine (Ile), leucine (Leu), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp), valine (Val). The non-essential amino acids content obtained were: glutamine (Gln), glycine (Gly), proline (Pro), tyrosine (Tyr), alanine (Ala), asparagine (Asp), aspartic acid (Asx), glutamic acid (Glu), serine (Ser).

RESULTS AND DISCUSSIONS

The essential amino acid content of the raw materials used are shown in Figure 1.

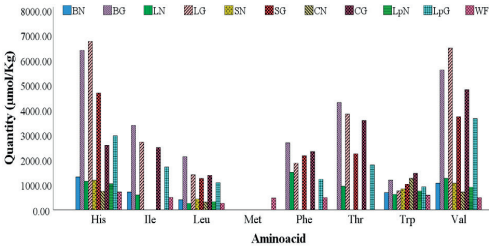

Figure 1. Raw materials essential amino acids contents

According to the data obtained the wheat flour presented lower levels of essential amino acids compared to the legumes flour. Also the legumes flour in a germinated form (after 4 days of germination) presented in generall higher levels of essential amino acids compared to the non germinated legumes. This data are in agreement with those reported by other authors which also noticed an increase of the essential amino acid content through the germination process (Atudorei & Codină, 2020). For germinated legumes flour the highest levels for essential amino acids were recorded for histidine, followed by valine and threonine while the lowest level were recorded for tryptophan. The highest amounts for essential amino acids from germinated legumes were recorded for germinated lentil followed by germinated bean. Also non-essential amino acids content were determined for wheat flour, legumes and germinated legumes (after 4 days) the data obtained being presented in Figure 2.

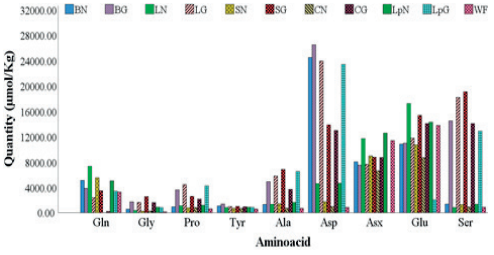

Figure 2. Raw materials non-essential amino acids contents

Generally, among the non-essential amino acids the highest amounts were recorded for the asparagine followed by serine and glutamic acid. The highest amounts for asparagine were recorded for germinated bean and the highest amount for serine for germinated soybean. More, some essential amino acids were not detected in raw legumes but appeared after germination. For example phenylalanine and tryptophan were not present in native bean, soybean, chickpea and lupine but were detected in their germinated form. Similar data has also been reported by Kuo et al. (2003).

Regarding the wheat flour it presented high amounts for essential amino acids such as histidine, isoleucine, methionine, fenilalanine, tryptofan, valine. From the non-essential amino acids the highest amount was obtained for serine and aspartic acid these data being in agreement with those reported by Mustafa et al. (2007). In wheat flour the highest amount obtained was those for non-essential amino acids glutamic acid of which value were higher than those obtained for legumes and germinated legumes flours.

The esential and non-essential amino-acids content of the composite flour for the wheat-bean mixes are shown in Figures 3 and 4.

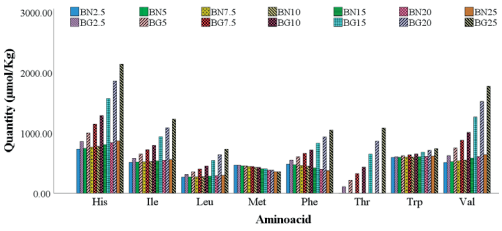

Figure 3. Wheat-bean composite flours essential amino acids contents

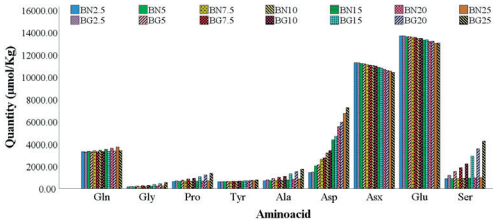

Figure 4. Wheat-bean composite flours essential amino acids contents

As it may be seen the highest amount of essential amino acids were obtained for histidine, fallowed by valine, isoleucine, threonine, phenylalanine, leucine of which value increased with the increase level of germinated bean flour addition in wheat flour.

For non-essential amino acids content the highest value was recorded for glutamic acid followed by aspartic acid, asparagine and glutamine. High levels of glutamic acid for bean have also been reported by Kuo et al. (2003). Although the wheat flour presented a higher level of glutamic acid compared to the bean flour in a raw and germinated form, the amount of this amino acid in the final product decreased by wheat flour substitution with bean flours.
The amino acids content for wheat-lentil mixes are shown in Figures 5 and 6.

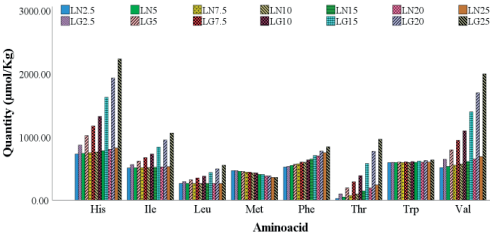

Figure 5. Wheat-lentil composite flours essential amino acids contents

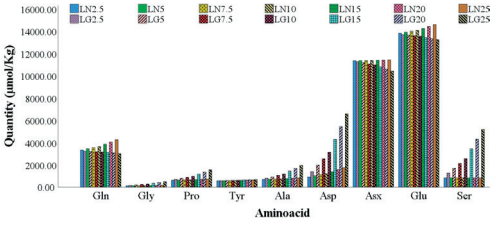

Figure 6. Wheat-lentil composite flours non-essential amino acids contents

According to the data obtained the highest levels for amino acids were obtained for histidine, valine, isoleucine, threonine and phenylalanine. This data are in agreement with those reported by Kahraman (2016) which also found high values for these amino acids type in different lentil varieties. By wheat flour substitution with lentil in a raw or germinated form the value of these amino acids increased. For non-essential amino acids content the highest levels were obtained for glutamic acid, aspartic acid, glutamine, and asparagine. However, the values for aspartic acid and glutamic acid decreased with the increase level of lentil flour addition in wheat flour due to the fact that wheat flour contain high levels of these amino acids compared to the lentil one. More, the mixes with germinated lentil addition in wheat flour presents low levels of these amino acids compared to the mixes with raw lentil flour addition in wheat flour. This is explainable since raw lentil contains high levels of aspartic acid and glutamic acid compared to the germinated one. These data are in agreement with those reported by Kuo et al. (2003) which also obtained a decrease of these amino acids during the germination period of lentil.
The mixes between wheat and soybean flours present the essential and non-essential amino acids content as it may be seen in Figures 7 and 8.

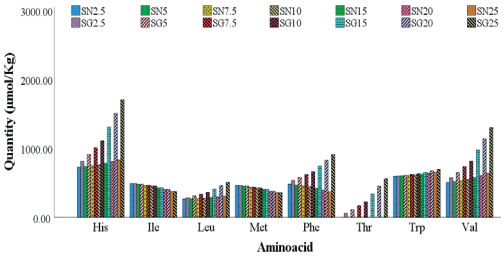

Figure 7. Wheat-soybean composite flours essential amino acids contents

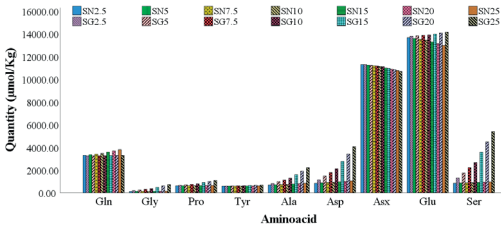

Figure 8. Wheat-soybean composite flours non-essential amino acids contents

For wheat-soybean composite mixes the highest values for essential amino acids were obtained for histidine, valine, phenylalanine, and tryptophan. These values are higher for wheat-germinated soybean mixes than for the wheat-raw soybean mixes. These are due to the fact that during germination these amino acids content increased in soybean (Bueno et al., 2020). For the non-essential amino acids the highest values were obtained for glutamic acid for which these values increased with the increase level of germinated soybean and decreased when raw soybean were added in wheat flour. Similar data has also been reported by Martínez-Villaluenga et al. (2006) which also obtained an increase of this amino acid for germinated soybean. Others high values of non-essential amino acids were obtained for aspartic acid which decreased with

the increase level of soybean addition, serine and asparagine when high levels of germinated soybean were added in wheat flour.
The data for amino acids content for the wheat-chickpea composite flours are shown in Figures 9 and 10.

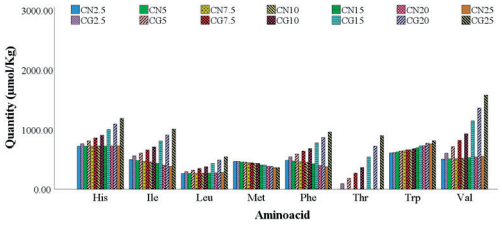

Figure 9. Wheat-chickpea composite flours essential amino acids contents

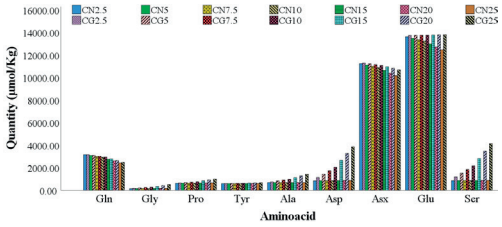

Figure 10. Wheat-chickpea composite flours non-essential amino acids contents

As it may be seen from the data obtained the highest values were obtained for essential amino acids valine, histidine, isoleucine for the mix samples with germinated chickpea addition in wheat flour. More, threonine has only been detected when germinated chickpea was incorporated in wheat flour. The increase of these amino acids values after chickpea germination has also been reported by others (Fernandez & Berry, 1988; Atudorei & Codină, 2020). From the non-essential amino acids point of view the highest levels were recorded for glutamic acid, aspartic acid, asparagine, serine for the mixes between wheat flour and germinated chickpea flour. High levels of glutamic acid and aspartic acid has also been reported for chickpea by Ghribi et al. (2015).
The essential and non-essential amino acid content for the mixes between wheat flour and germinated lupine flour are shown in Figures 11 and 12.
According to the data obtained the highest levels of essential amino acids were recorded for histidine and tryptophan for the mixes between wheat flour and raw lupine flour.

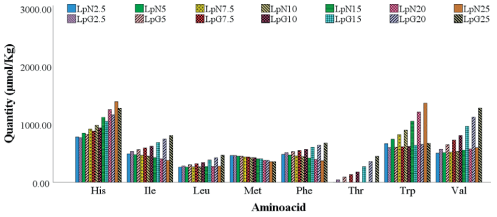

Figure 11. Wheat-lupine composite flours essential amino acids contents

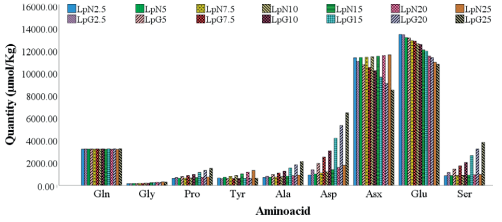

Figure 12. Wheat-lupine composite flours non-essential amino acids contents

Also high levels were recorded for valine, isoleucine and phenylalanine but only for the mixes when germinated lupine flour were incorporated in wheat flour. More, threonine was detected only for germinated lupine flour addition in wheat flour. This fact was explainable since it is well known that lupine is poor in threonine (Ahmed, 2014). The highest levels of non-essential amino acids content were obtained for glutamic acid and aspartic acid for the mixes between wheat flour and raw lupine and for asparagine, serine, glutamine for the mixes between wheat flour and germinated lupine flour. These facts are due to the lupine flour composition which is higher in some amino acids in raw form and lower one in germinated form. Similar data has also been reported by Villacrés et al. (2015) which obtained a decreased of glutamic acid and aspartic acid and an increase of serine for germinated lupine compared to the raw lupine one.

CONCLUSIONS

Compared to the legumes flours, the refined wheat flour presented lower amounts of essential amino-acids like histidine, leucine, tryptofan, valine. Generally, through germination the amino acid content of the legumes increased and even more, some essential amino acid which were not present in

legumes flours were detected in germinated ones. Generally, by wheat flour substitution with germinated legumes flours the amino acids content increased. These values increased with the increase level of germinated legume type flour addition in refined wheat flour. The highest amounts of essential amino acids were recorded for the histidine followed by valine for the mixes formed from germinated bean-wheat flour, germinated lentil-wheat flour, germinated soybean-wheat flour and germinated lupine-wheat flour whereas for the mix between germinated chickpea-wheat flour the highest amounts were recorded for valine followed by histidine. Except methionine, all the essential amino acids of the wheat flour were improved when germinated legumes flours were added in wheat flour.

ACKNOWLEDGEMENTS

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CURRENT STATUS OF THE APPLICATIONS OF PULLULAN AND ITS DERIVATIVES IN BIOMEDICAL FIELD

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Abstract

This review highlights the applications of pullulan in biomedical field, focusing on drug delivery. Pullulan is a microbial exo-polysaccharide produced by yeast like fungus Aureobasidium pullulans and it has been declared safe by FDA in United States and has GRAS status. Pullulan has biocompatible, biodegradable, non-mutagenic, non-toxic, non-carcinogenic, non-immunogenic properties, as well as other functional properties. Furthermore, pullulan can be easily derivatized by several chemical reactions such as etherification, amidification, esterification, oxidation and co-polymerization in order to widen its applications. Due to its unique features pullulan and its derivative is being explored for various biomedical applications like drug and gene delivery, tissue engineering, wound healing, diagnostic imaging, etc. This research was supported through Nucleu project PN 1941-04 01.

Key words*: pullulan, biomedical applications, drug delivery, microbial exo-polysaccharide.*

INTRODUCTION

Pullulan is a microbial exo-polysaccharide produced by yeast like fungus *Aureobasidium pullulans*. There are other microorganisms that produce pullulan, such as: *Tremella mesenterica, Cytaria harioti, Cytaria darwinii, Teloschistes flavicans, Rhodotorula bacarum, Rhodosporidium paludigenum, Eurotium cheyalieri* and *Cryphonectria parasitica* (Singh et al., 2008; Forabosco et al., 2006; Chi and Zhao, 2003; Reis et al., 2002). Pullulan consists of α-(1,6)-repeated maltotriose units via an α-(1,4) glycosidic bond. The molecular formula of pullulan is $(C_6H_{10}O_5)_n$ with a molecular weight ranging between 4.5×10^4 - 6×10^5 Da. It presents like a white to off-white powder, and it is soluble in water, dimethylsulfoxide, formamide, and dilluted alkali.

Pullulan has biocompatible, biodegradable, non-mutagenic, non-toxic, non-carcinogenic, non-immunogenic properties, etc, and it has been declared safe by Food and Drug Administration (FDA) in United States and has GRAS (Generally Recognized as Safe) status. Pullulan has good mechanical strength, film-forming ability, and adhesiveness.

Pullulan has been researched for food, cosmetic, pharmaceutical and biomedical applications (Ran et al, 2017; Leathers, 2003). Some applications of pullulan in food industry are: edible coating material, material in packaging industry, adhesive material (e.g. to bind nuts to the cookies), low-calorie food additive in solid or liquid foods, low viscosity filler in sauces and juices, intensifier in baked foods, confectioneries and beverages, etc (Singh et al, 2019; Trinetta et al, 2011). Pullulan can be used in cosmetic industry specifically in skincare products, because possess good skin adherence and can provide an instant skin-tightening effect (Coltelli et al, 2020). Furthermore, pullulan can be easily derivatized by several chemical reactions such as etherification, amidification, esterification, oxidation, sulfation and co-polymerization in order to widen its applications. Due to its unique features pullulan and its derivatives is being explored for various biomedical applications including drug delivery, gene delivery, tissue engineering, medical imaging, etc.

This review highlights the applications of pullulan and its derivatives in biomedical field, with a special focus on drug delivery.

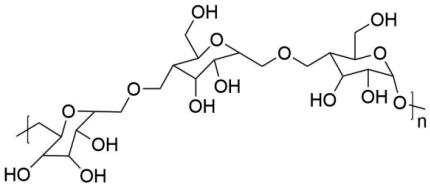

Figure 1. Structure of pullulan

MATERIALS AND METHODS

A bibliometric search was carried out in Scopus database in February 2022, using keywords like "pullulan" and "pullulan drug delivery". The search was conducted taking into consideration title, abstract and keywords of publications. Only papers published in the period of 1990-2021 were included in the evaluation of trends in publications. After the exclusion step, the distribution of papers by year, type of publication, language, top five fields and top 10 publishing journals were extracted directly from Scopus.

RESULTS AND DISCUSSIONS

1. Drug delivery applications

1.1. Trends in publications of pullulan use in drug delivery applications

The evaluation of trends in publications showed that from 4796 papers were published regarding pullulan, a number of 616 publications addressed drug delivery. Majority of these publications were written in English (603) and just a few in Japanese and Chinese. The evolution over time of these publications is presented in Figure 2. The publications increased over the years, especially between the years 2018 and 2020. Papers involving pullulan applications in drug delivery concerned 23 fields of knowledge. The top five fields with the highest number of publications are Pharmacology, Toxicology and Pharmaceutics (291), Biochemistry, Genetics and Molecular Biology (198), Materials Science (176), Chemistry (139) and Chemical Engineering (115). Mainly, the publications were original articles (400), followed by reviews (166) and conference papers (24), as shown in Figure 3. The top 10 journals in which published the papers were: International Journal Of Biological Macromolecules (32), Carbohydrate Polymers (27), International Journal Of Pharmaceutics (20), Journal of Controlled Release (17), Advanced Drug Delivery Reviews (14), Biomaterials (13), European Journal Of Pharmaceutical Sciences (13), Journal Of Drug Delivery Science And Technology (10), Materials Science And Engineering (9) and Current Pharmaceutical Design (8).

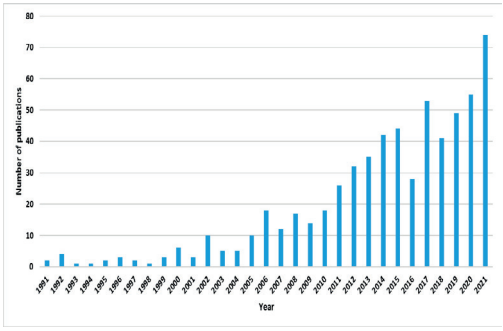

Figure 2. The evolution over time of the papers published regarding pullulan in drug delivery

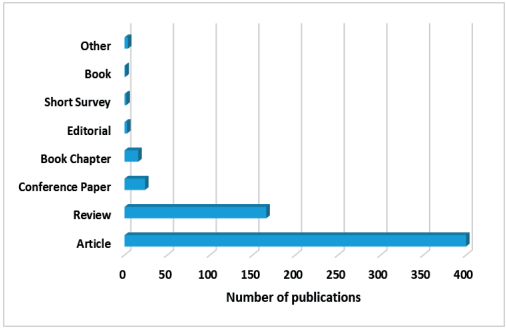

Figure 3. Type of papers published regarding pullulan in drug delivery

1.2. Pullulan based drug delivery systems

Various formulations based on pullulan like hydrogels, micro/nano-particles, nanogels, micelles can be effective drug delivery systems possessing increased permeability and enhanced drug retention capacity. Also, the incorporation of therapeutic agents in pullulan based drug delivery systems reduces their toxicity.

Various pullulan systems acted as good drug carriers to different active substances such as: anticancer agents, doxorubicin (Li et al., 2014; Zhang et al., 2011; Balasso et al., 2017),

docetaxel (Satoh et al., 2008), mitoxantrone (Yang et al., 2014, Yuan et al., 2019), mitoxantrone - doxorubicin (Lu et al., 2009), cisplatin (Wang et al., 2015), paclitaxel (Huang et al, 2017), 5-fluorouracil (Ganeshkumar et al., 2014), antibiotics (Adriamycin) (Guo et al., 2014), epirubicin (Zhang et al., 2009; Zhang et al., 2010), indomethacin (Constantin, et al., 2017), antiinflamators, diclofenac (Constantin et al., 2007) and diclofenac - α-tocopherol (Mocanu et al., 2014), naproxen (Bishwambhar et al., 2012), antiviral agents, lopinavir (Ravi et al., 2014), antipepileptic agents (clonazepam (Jung et al., 2003; Jung et al., 2004), oral antidiabetics, insulin (Lin et al., 2019), antiasthamatic agents, salbutamol sulfate (Xu, et al., 2015), biophosphonates administered for slow down or prevent bone loss, risedronate (Velasquez et al., 2014), natural antioxidant agents for liver health, silymarin (Kumar et al., 2012), etc. Furthermore, pullulan and its derivatives were used in targeted drug delivery for different diseases like cancer, hepatitis C virus, autoimmune diseases, atherosclerosis, graft rejection, ischemia, asthma (Constantin et al., 2007; Masuda, 2001; Satoh et al., 2008; Suginoshita et al., 2002).

Extensive research for the utilization of pullulan and its derivatives was made in cancer therapy. Several papers described stimuli sensitive systems based on pullulan (temperature, pH, charge, redox, light). Liang et al. (2019) designed pH-responsive injectable hydrogels based on chitosan-grafted-dihydrocaffeic acid and oxidized pullulan for localized drug delivery. Lu et al. (2010) prepared pH sensitive pullulan-doxorubicin conjugates which displayed enhanced drug accumulation in tumors and reduced cardiotoxicity. Fundueanu et al. (2010) developed pullulan combined with poly (N-isopropyl - acrylamide - co - acrylamide) microspheres with temperature dependent delivery capability. Mocanu et al. (2011) designed thermosensitive carboxymethyl pullulan nanoparticles for controlled drug release.

Various pullulan systems can be used to target breast, kidney, lung, brain, or ovary cancer (Raychaudhuri et al., 2020). For example, Huang et al. (2017) designed pullulan nanoparticles loaded with paclitaxel for liver-targeting. In another study, Li et al. (2014) designed carboxymethyl pullulan based nanoparticles with pH sensitivity for liver targeting. Also, Satoh et al. (2008) showed the *in vitro* and *in vivo* ability of cholesterol bearing pullulan nanoparticles modified with amine containg docetaxel to target lung cancer cells.

Apart from cholesterol bearing pullulan and carboxymethyl pullulan, pullulan acetate was as well used in cancer treatment. Pullulan acetate micelles have can be used for longer circulation of drugs in blood and their delivery to the targeted tumor cells/tissues. Pullulan acetate combined with carboxymethylated poly(ethylene glycol) possess good delivery properties like enhanced hydrophobic active substances release as well as avoidance of macrophages (Jung et al., 2003). In another study, Suginoshita et al. (2001) described diethylenetriamine penta acetic acid - pullulan for cancer treatment possessing higher uptake of active substances in tumors. Also, Seo et al. (2012) reported poly(L-lactide) and poly(DL-lactide-co-glycolide) grafed on pullulan thermosensitive nanogels with enhanced cytotoxicity against the tumor cells.

Furthermore, folate modified pullulan systems were used for active targeting of cancer cells exploiting the fact that in general folates are taken up into cells by folate receptors *via* receptor-mediated endocytosis. Chenab et al. (2018) described pullulan nano-micelles decorated with folate loaded with doxorubicin and shRNA of Beclin1 effective in tumor targeting in HeLa and HepG2 cells. Zhang et al. (2010) developed folate modified pullulan acetate nanoparticles loade with epirubicin with better drug entrapment efficiency and release properties for active targeting of cancer cells. Li et al. (2013) described pH sensitive folate-decorated maleilated pullulan for cancer therapy with increased cellular uptake of active substances and enhanced cytotoxicity.

Although a large part of the literature regarding drug delivery systems based on pullulan address cancer therapy, there are some papers focused on the treatment of other diseases like Alzheimer's disease, hepatitis, autoimmune diseases, atherosclerosis, graft rejection, ischemia, asthma, etc (Shi and Li, 2005; Masuda, 2001; Suginoshita et al., 2002; Boridy et al, 2009). For example, cholesterol bearing pullulan can be used for insulin delivery with

improved biological activity, better stability, reduced side effects (Shi and Li, 2005). In another study, Boridy et al. (2009) reported the use of hydrophobically modified cholesterol bearing pullulan as a replacement of antibody immunotherapy for Alzheimer's disease treatment.

2. Other biomedical applications

2.1. Gene delivery

Formulations of modified pullulans have significant potential for targeted gene delivery. The genes can be encapsulated inside the pullulan formulations and protected from DNase destruction while being delivered to the desired cells or organs. Pullulan with a cholesterol group within creates a hydrophobic core with self-aggregation characteristics (Singh et al., 2015). Cholesterol-bearing pullulan is used for targeted distribution of various hydrophobic proteins or genes due to these features (Lee and Akiyoshi, 2004). It has also been used to transport proteins to immune cells, such as shortened HER2-147 (Ikuta et al., 2002). Pullulan hydrogels have high plasmid DNA loading efficiency and provide sustained DNA release to cancer cells (Gupta and Gupta, 2004). Using cationic pullulan formulations such as polyethylenimine pullulan, desired genes can be targeted in the liver (Kang et al., 2010). Polyethylenimine pullulan reduces the side effects of DNA or genes and is commonly used to target tumor cells (Ambattu et al., 2015; Kang et al., 2010; Rekha and Sharma, 2011; Wang et al., 2014). Pullulan based on diethylaminoethylamine can be manufactured in tubular or three-dimensional matrices to deliver genes to local arteries or muscle cells while also preserving them from DNase destruction (Juan et al., 2007). Folate modification improves gene silencing and gene transfection effectiveness (Wang et al., 2014). Pullulan spermine has been shown to deliver the intracellular gene and promote dopamine release in the treatment of Parkinson's disease (Nagane et al., 2009). It is also used for neuronal gene delivery and gene targeting to human bladder tumor cells (Kanatani et al., 2006). Pullulan derivative formulations, such as pullulan-g-poly(l-lysine) (Park et al., 2012), pullulan-protamine (Liu et al., 2014; Priya et al., 2014; Yang et al., 2014), and succinylated pullulan (Kim and Nan, 2010) are effective for targeted gene delivery while minimizing cytotoxicity.

2.2. Tissue engineering

From data literature, various derivatized forms of pullulan have been used for tissue engineering applications i.e. phosphorylated pullulan, carboxylated pullulan, pullulan-cellulose acetate, etc. Tissue engineering may be a handle to improve self-healing potential of the harmed tissues or organs by making a reasonable cell environment using a fitting manufactured 3-dimensional (3D) scaffold (Tabata, 2003; Singh et al., 2016). Various biopolymers which have properties same as characteristic tissues can be molded into different shapes like hydrogels, scaffolds, micro-molded frameworks, micro-beads and nanoparticles for tissue engineering application (Mallick and Cox, 2013). The application of these biopolymers in the field of tissue engineering primarily includes surface adjustments. The surface properties of pullulan can be effectively improved by substitution of some specific chemical moieties on its hydroxyl groups (Kumar et al., 2012). Pullulan presents amazing mechanical properties, a high hydration capacity and an excellent cell compatibility (Chaouat et al., 2006; Shingel, 2004). Due to these properties, pullulan based scaffolds play a main role in encouraging cell-based dermal substitution, tissue designing of vascular cells and bone recovery. Therefore, pullulan hydrogel scaffolds help assemble cell-loaded microtissue complexes and encapsulate damaged cells for regeneration and proliferation purpose (Bae et al., 2011), and due to the structure of hydrogels, they support the controlled release of active substances to the target site. Pullulan hydrogels have applications in maxillofacial surgery and orthopedics, because they help stimulate the differentiation of bone cells from mesenchymal stem cells (MSCs). In addition, coating with nanocrystalline hydroxyapatite particles (nHAP) improves the mechanical properties of these 3D hydrogels and also improves the ability of cells to attach to these constructs. They have anti-adhesion properties and help alleviate various problems such as postoperative pain, infertility, and intestinal obstruction (Bang et al., 2016). Also, the innovative pullulan bioconjugate can

be used to selectively treat bone metastases in breast cancer (Bonzi et al., 2015). On the other hand hydrogels have also received considerable attention as wound dressings due to the fact that they have the same physical properties as natural soft tissues and can absorb large amounts of aqueous liquid. Therefore, hydrogels provide a moist wound environment and can protect against bacterial infections (LayFlurrie, 2004; Loke et al., 2000). Pullulan-collagen hydrogels have consistent porosity, replicate dermal structure and successfully integrate stem cells for early wound healing (Galvez et al., 2009), and present numerous features, such as: antioxidant properties, enhance cell recruitment and activation, accelerate wound healing, enhance the expression of monocyte chemoattractant protein 1 at both transcriptional and protein levels through addition of adipose-derived mesenchymal stem cells, etc. (Wong et al., 2011). Phosphorylated pullulan (PPL) provides good adhesion to hard tissue via ionic bonds and helps repair bone defects (Shiozaki et al., 2011) and can act as a carrier for antibacterial agents and promote the regeneration of femoral defects. Carboxylated pullulan combined with human-like collagen and 1,4-butanediol diglycidyl ether can serve as a promising soft filler for tissue engineering (Li et al., 2015). 3D Pullulan Cellulose Acetate Scaffold has cell compatibility to promote cell attachment, diffusion and proliferation for skin tissue engineering (Atila et al., 2015). The cholesterol-bearing pullulan (CHP)-nanogels can encapsulate hydrophobic active substances for tissue engineering, and can create a moist environment for full-thickness wounds and promote controlled release of some prostaglandins (e.g. E1 prostaglandin) for angiogenesis, neoepithelialization, and damage regeneration (Kobayashi et al., 2009).

2.3. Medical imaging

Medical imaging uses fluorescent probes for labelling living cells, like quantum dots. Although quantum dots possesssuitable properties for biomedical imaging (nanometric saize, bright fluorescence, narrow and symmetricemission spectra, broad excitation, good photo-stability), their delivery into body cells is still challenging (Probst et al, 2003). Hasegawa et al. (2005) designed pullulan-bearing cholesterol and amino group-modified cholesterol nanaparticles for the delivery of quantum dots into body cells. Compared with conventional cationic liposomes, the cholesterol bearing pullulan nanoparticles showed better cellular uptake of the quantum dot therefore these nanoparticles could be promising fluorescent probes for imaging. Wu et al. (2010) described hydroxypropyl cellulose-poly(acrylic acid)-pullulan based hybrid nanogels loaded withcadmium selenide QDs with applications inbiomedical imaging.

CONCLUSIONS

This review highlighted the applications of pullulan in biomedical field (drug delivery, gene delivery, tissue engineering, medical imaging). Pullulan, microbial exo-polysaccharide produced by yeast like fungus *Aureobasidium pullulans*, presents biocompatibility, biodegradability, non-mutagenic, non-toxic, non-carcinogenic, non-immunogenic properties, as well as other functional properties. Furthermore, pullulan can be easily derivatized by several chemical reactions such as etherification, amidification, esterification, oxidation and co-polymerization in order to widen its applications. These unique properties of pullulan and its derivatives make them excellent candidates for various biomedical applications.

Due to the interesting activity and effectiveness shown from pullulan and its derivatives especially in the biomedical field, more research need to be done to explore intensive the use of this polymer in applications related to personal care and cosmetics.

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GENETIC APPROACHES TO SELECT L-ASPARAGINASE PRODUCING *Bacillus* STRAINS

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Abstract

An important enzyme for both the pharmaceutical and food industries is L-asparaginase (EC 3.5.1.1). This enzyme is produced by a wide variety of microorganisms. However, their potential use as sources of L-asparaginase at industrial scale is limited if glutaminase and urease are also produced. This is mainly due to the complexity and expenses of the purification process required to obtain l-asparaginase which make the production system inefficient. In order to select L-asparaginase highly producing strains, lacking detectable glutaminase and urease activity different isolation steps were established and rapid tests are the first recommended. However such studies need to be completed with quantitative analysis. Moreover, additional molecular studies can also confer useful information regarding the biotechnological potential of the selected strains. The aim of this paper is to correlate the microbiologic and biochemical tests with genetic approaches in order to improve the selection process of biotechnologically relevant Bacillus strains.

Key words*: L-asparaginase, ansA and ansZ genes, Bacillus.*

INTRODUCTION

Enzymes are proteins that have the ability to catalyse various reactions in which the substrate is transformed into reaction products. (El-Hadi et al., 2019). The L-asparaginase enzyme catalyses L-asparagine hydrolysis into aspartic acid and ammonia (Batool et al., 2016). This reaction is highly important in the medical field, as well as in the food industry.

In medicine L-asparaginase is used as antineoplastic chemotherapy drug especially for patients with acute lymphocytic leukemia (Egler et al., 2016). Although L-asparagine aminoacid is essential for the cells to synthesis important proteins to stay alive (Starkova et al., 2018), it should be mentioned that only normal cells are able to make this aminoacid, while many cancer cells cannot, due to the lack of L-asparagine synthetase (Salzer et al., 2018). Therefore, tumour cells will need this aminoacid to stay alive (Chiu et al., 2020). In the case of such cancer patients L-asparaginase treatment is administrated as chemotherapeutic drug with cytotoxic activity (Kato & Manabe, 2018). L-asparaginase plays a key role in acute lymphoblastic leukemia treatment especially, but has a potential role also in preventing metastases from solid tumours (Brumano et al., 2019). However L-asparaginase is not limited only as an antitumor agent, but it could also be used as antimicrobial agent, in the treatment of infectious diseases, autoimmune diseases, as well as in feline and canine cancer (Vimal & Kumar, 2017).

L-asparaginase used in clinical treatments is mainly obtained from *Escherichia coli* and *Erwinia chrysanthemi*, but new sources of prokaryotic, eukaryotic and even genetic mutations are still being sought to improve the function of this enzyme (Beckett & Gervais, 2019).

The use of L-asparaginase in medicine has some limitations, because it has been shown to cause some side effects to those with history of pancreatitis, thrombosis or allergies. These are mainly due to the secondary activity of glutaminase (Ramya et al., 2012).

In addition to the medical significance, this enzyme is also used extensively in the food industry to mitigate acrylamide formation, a carcinogenic compound that is formed in

carbohydrate-containing foods that are cooked at high temperatures (Muneer et al., 2020).

The importance of L-asparaginase and the applications it may have, maintained a continuing interest over time in order to detect sustainable and safe sources of enzyme production and easier extraction. Studies revealed a variety of microorganisms able to produce L-asparaginase (Ashok et al., 2019), although before, this enzyme was extracted from guinea-pig serum (Lee & Bridges, 1968). Microbial enzyme production is conditioned by kinetic parameters (El-Gendy et al., 2021). Biotechnology was able to improve industrial production of L-asparaginase using selected strains of *E. coli* and *E. chrysanthemi* bacteria. But trends are targeting the use of genetically improved strains (Onishi et al., 2011), or the use eukaryotic microorganisms due to the presupposition of a better compatibility with the human body (Cachumba et al., 2016).

In bacteria there have been seen two types of L-asparaginase: one found in the bacterial cytoplasm (AnsI) and the second (AnsII) found in the bacterial periplasm. The AnsII was shown to have higher affinity for L-asparagine, being the only one used in clinical treatments (Brumano et al., 2019). If AnsI can hydrolase both L-asparagine and L-glutamine, the AnsII is more specific to the substrate, being the one to have anti-cancer activity (Maggi & Scotti, 2019). The AnsII from *E. coli* (named EcAII) is more often used in medical treatments, probably preferred due to the biotechnological plasticity of this bacterial specie; while the AnsII from *E. chrysanthemi* (named ErAII) is used as an alternative if immune reactions occur to EcAII (Maggi et al., 2017).

The AnsI enzyme is encoded by *ansA* gene in various bacterial species, such as *Bacillus subtilis* (Hegazy et al., 2012), *B. tequilensis* (Shakambari et al., 2018), *E. coli* (Maggi et al., 2017), *Corynebacterium glutamicum* (Kalinowski et al., 2003), *Pseudomonas* spp. (Kishore et al., 2015), *Rhizobium etli* (Moreno-Enríquez et al., 2012), *Streptomyces griseus* (Meena et al., 2015), and many others. The AnsII is expresses only in certain conditions such as limited nitrogen levelsss and oxygen stress (Maggi & Scotti, 2019). The encoding genes of L-asparaginase type II could be *ansB* gene, as it was shown in *E. coli* (Jennings & Beacham, 1990), or *ansZ* gene as in *B. subtilis* (Fisher & Wray, 2002). The AnsAB operon, containing the *ansA* and *ansB* genes, can be repressed by *ansR* activity (Sun & Setlow, 1993); while *ansZ* gene expression is activated by *TnrA* or *GlnR* transcription factors. The *trnA* trigger the expression of *ansZ* gene in nitrogen-limited growth, while *GlnR* represses *ansZ* transcription at excess nitrogen exposure (Fisher & Wray, 2002).

In our study we aimed to correlate qualitative *L-asparaginase* tests with genetic approaches in order to improve the selection of AnsII producing *Bacillus* strains, lacking detectable glutaminase, usease and nitrate reductase enzymes.

MATERIALS AND METHODS

Biological material and culture media

Seven strains of rhizobacteria and three endophytes, all belonging to *Bacillus* spp. were analysed for their L-asparaginase potential (Table 1). All the strains are maintained in collection as frozen in 25% glycerol. Conventionally they are grown on Luria Bertani (LB), at 28°C.

Table 1. Microbial strains and culture media

Strains		Microbial Collection
Rhizobacteria	*Bacillus subtilis* ATCC6633	Reference strain from the American Type Culture Collection
	Bacillus subtilis B5	Faculty of Biotechnology, USAMV Bucharest
	Bacillus subtilis B6	
	Bacillus sp. BPA	
	Bacillus sp. BIR	
	Bacillus amyloliquefaciens BW	
	Bacillus sp. OS15	Research-Development Institute for Plant Protection, Bucharest
Endophytic bacteria	*Bacillus* sp. LT MYM1	Plant Biotechnology Laboratory – Faculty of Biotechnology, USAMV Bucharest
	Bacillus sp. LFF MYM1	
	Bacillus sp. LFF MYM5	

DNA extraction

The DNA isolation and purification was performed from fresh bacterial cultures

obtained overnight in LB broth. For extraction a HP PCR Template Preparation kit (Roche LifeScience) was used, starting from 1ml of culture, following the manufacturer's instructions with some modifications. To improve *Bacillus* sp. cell lysis, an additional step of mechanical disruption was added, in which samples were subjected 2 times at bead-beating, for 30 seconds in Mini-Beadbeater-8 (BioSpec). To increase DNA purity, a final RNase A (10 mg/mL) treatment was applied for 15 min at 37°C.

The DNA quantification was performed in 0.5 µl of volume sample using a SpectraMax QuickDrop spectrophotometer. To equalize the DNA concentration among samples dilutions were needed to obtain 100 ng/µl.

Amplification of L-asparaginase genes

The *ansA* and *ansZ* genes are encoding for two L-asparaginase isoenzymes, L-asparagine amidohydrolase I and II, respectively. To detect *ansA* gene the primers pair Bs_ansAf (5′ - CCC AAG GAA GTC TTT TTC CA - 3′) and Bs_ansAr (5′ - AGT GAA GAG GTG CAT GGT ATG A - 3′) that amplify a 1100bp fragment of *B. subtilis* chromosomal DNA containing *ansA* gene was used (Hegazy et al., 2012). The DNA fragment containing the *ansZ* gene was obtained by PCR amplification with the set of primers mentioned by Fisher & Wray (2002).

The PCR reaction was performed in a total volume of 25 µL containing 14 ng/µL of template DNA, 1X Mango*Taq*™ Colored Reaction Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix (Bioline), 0.5 µM of upstream and downstream primers (ThermoScientific LSG), 0.5 U Mango*Taq*™ DNA Polymerase (Bioline) and 12.65 µL nuclease-free water.

The thermal cycling profile for genes amplification involved an initial denaturation at 94°C for 5 min, followed by a number of 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C and 2 min extension at 72°C, completed with a final extension cycle of 7 min at 72°C (Fisher & Wray, 2002). The amplification program was performed in Eppendorf 5331 Mastercycler Gradient equipment.

The PCR products were migrated at 100V for 1 h, in 1% (w/v) agarose gel and 0.5X TBE, after staining with ethidium bromide. The length of DNA fragments was compared to 1KB Ladder (ThermoScientific LSG).

Rapid screening method for L-asparaginase production

The qualitative enzyme assay for L-asparaginase production was carried out on L-asparagine monohydrate (Chinoin Budapest) substrate. The screening was performed on a 96-well plate. The bacteria were inoculated in 200 µL medium/well, in 8 replicates per trial. The medium used was M9 broth supplemented with 0.5% L-asparagine as substrate. The M9 medium contained 0.2% glucose, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.05% $MgSO4 \cdot 7H_2O$, 0.015% $CaCl_2 \cdot 2H_2O$, with a pH of 7.0±0.5 and was enriched with 0.009% phenol red as indicator dye.

Comparative screening assay of L-asparaginase, glutaminase, urease and nitrat reductase

Comparative screening for the production of other relevant enzymes was evaluated is similar matter using three different substrates: L-glutamine (CellPure®, Carl Roth), urea (Carl Roth) and sodium nitrate (Reactivul București). L-glutaminase test was performed in 8 replicates per trial for each bacterial strain; while urease and nitrate reductase were performed in 4 replicates per trial for each strain. Qualitative enzymes assays were repeated two times.

RESULTS AND DISCUSSIONS

Molecular determination of *ansA* gene

The gene encoding for L-asparaginase type I in *B. subtilis* was amplified by PCR, using the primers designed by Hegazy et al. (2012) based on the published *ansA* gene sequences from *B. subtilis* 168 strain.

In our study the primers used were able to amplify a 1100bp DNA fragment corresponding to *ansA* gene. The reference strain, *B. subtilis* ATCC6633, revealed a strong specific band of PCR amplification product, while in four of the tested strains (BIR, BPA, OS15, and LT MYM1) beside the specific DNA of *ansA* gene, various non-specific PCR

amplification products were also obtained (Figure 1).

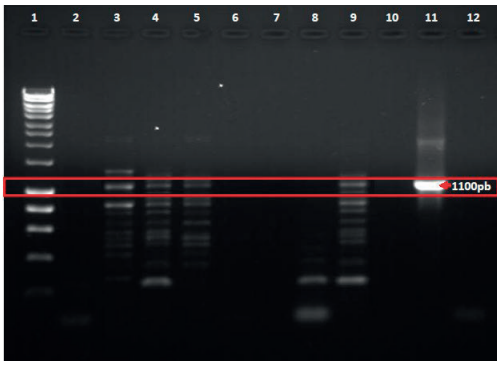

Figure 1. Agarose gel electrophoresis of the PCR products obtained by amplifying the bacterial DNA with the primers set designed for *ansA* gene

Legend: Line1 = 1kb DNA Ladder, Line 2 = BW, Line 3 = BIR, Line 4 = BPA, Line 5 = OS15, Line 6 = B5, Line 7 = B6, Line 8 = LT MYM1, Line 9 = LFF MYM1, Line 10 = LFF MYM5, Line 11 = ATCC6633 reference strain, Line 12 = Negative Control.

The PCR reaction for *ansA* gene performed for *B. amyloliquefaciens* BW, *B.subtilis* B5 and B6 rhizobacteria, as well as *Bacillus* spp. LT MYM1 and LFF MYM5 endophyte bacterial strains did not reveal the presence of targeted DNA fragment. These can be explained as lack of AnsI enzyme production.

Molecular determination of *ansZ* gene

The gene encoding for AnsII enzyme in *B. subtilis* was amplified by PCR. According to Ameen et al (2020), the gene in harbouring a 1128bp DNA fragment, which can be accessed in NCBI database as MN566442 number.

Our results revealed similar amplification products in 7 strains (BW, BIR, BPA, B6, LT MYM1, LFF MYM1 and LFF MYM5) among the ten studied bacteria (Figure 2).

Figure 2. Detail of the agarose gel electrophoresis for DNA fragment of *ansZ* gene amplified by PCR.

Legend: Line1 = 1kb DNA Ladder, Line 2 = BW, Line 3 = BIR, Line 4 = BPA, Line 5 = OS15, Line 6 = B5, Line 7 = B6, Line 8 = LT MYM1, Line 9 = LFF MYM1, Line 10 = LFF MYM5, Line 11 = ATCC6633 reference strain, Line 12 = Negative Control.

Taking into consideration that L-asparaginase I type II enzyme is the only one used in medicine due to its affinity for the substrate (Brumano et al., 2019), we can consider that the *Bacillus* strains revealing *ansZ* gene and lacking *ansA* could be highly valuable for biotechnological processes.

Qualitative determination of L-asparaginase production and other relevant enzymes

The qualitative enzyme assays were able to differentiate the bacterial strains able to produce L-asparaginase, without showing glutamine, urea or sodium nitrate notable digestion.

Enzymes production was observed following the rapid colour change test of the growth medium. According to Shakambari et al. (2019), the phenol red indicator dye is showing the positive reaction due to the bright red colour change of the media.

In our study positive reactions were seen only in L-asparagine containing medium after 3 days of incubation at 28°C (Figure 3a). No activity of glutaminase, urease and nitrate reductase enzymes were visual noticed in the colour dependent reactions (Figure 3b, c).

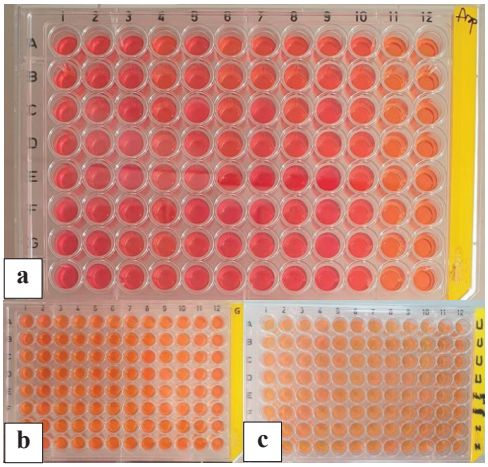

Figure 3. Microplate qualitative assay of enzymes production in various *Bacillus* spp. strains:
a. L-asparaginase; b. glutaminase; c. urease (A, B, C, D rows) and nitrate reductase (E, F, G, H rows) production

Legend: Line1 = BW, Line 2 = BIR, Line 3 = BPA, Line 4 = OS15, Line 5 = B5, Line 6 = B6, Line 7 = LT MYM1, Line 8 = LFF MYM1, Line 9 = LFF MYM5, Line 10 = ATCC6633 reference strain, Line 12 and 13 = Negative Control.

Although *B. subtilis* B5 and B6 strains did not revealed *ansA* and *ansZ* genes they were able to synthesize L-asparaginase (Figure 3).
Two explanations for these results could be identified: either the results are due to the presence of other L-asparaginase encoding genes that have not been subjected to or study, or the affinity between the primers pairs used and the bacterial strains tested (two rhizobacterial strains) is lacking.

CONCLUSIONS

Molecular analysis of *ansA* and *ansZ* genes can provide useful information regarding the biosynthesis of valuable biotechnological products by the selected *Bacillus* strains.
Based on the production of L-asparaginase type II encoded by the *ansZ* gene, which has high affinity for substrate, possible applications in medicine could be developed.
Qualitative enzyme micro-assays were able to confirm the PCR results and differentiate L-asparaginase producing strains lacking detectable glutaminase and urease activity.

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LIMONENE - A BIOMOLECULE WITH POTENTIAL APPLICATIONS IN REGENERATIVE MEDICINE

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Abstract

Limonene is a biomolecule that can be easily obtained from plant sources with remarkable biological effects. It is found in large quantities in citrus essential oils (concentrations up to 98%) and in moderate amounts in essential oils obtained from various species of geranium. The article presents the reasons why this compound may have potential uses in regenerative medicine, especially in dermatological applications, where these compounds or formulations which contain this compound, alone or via intermediary products which result from metabolization (such as perillyl alcohol) can accelerate wound healing, inhibit skin tumors development, or inhibit pathogenic microorganisms such as Staphylococcus aureus or Pseudomonas aeruginosa. Studies performed in vivo, on healthy persons who have tested in time the skin cleaning products which contain limonene or essential oils with limonene, were confirming that the products with Limonene do not give perturbation to normal skin microbiota. These data recommend Limonene as a potential candidate for regenerative medicine applications in the field of dermatology.

Key words*: limonene, Citrus essential oils, regenerative medicine.*

INTRODUCTION

Plant essential oils (EOs) are a mixture of monoterpenes, sesquiterpenes, and oxygenated derivatives. These are used in formulations with antimicrobial, immunomodulatory, antioxidant, antimicrobial, or antitumor properties (Aziz et al., 2018). The citrus genus belongs to the Rutaceae family and is widely important in the world economy. It includes about 17 citrus fruit species, including *Citrus reticulata Blanco* (mandarin orange, tangerine), *C. sinensis* L. (sweet orange), *C. aurantium* L. (bitter orange), *C. lemon* L. (lemon), and *C. paradise* (grapefruit) (Bora et al., 2020)**.** The fruit peel of citrus species contain vesicles with essential oil in which Limonene concentration can attain 97% (Voo et al., 2012; Dugo & Di Giacomo, 2002). A non-volatile fraction from peels contains sterols, fatty acids, waxes, coumarins, psoralens, and flavonoids (Dugo & Di Giacomo, 2012; Tranchida et al., 2012; Chi et al., 2013; Zulaikha et al., 2015). Preclinical studies achieved on lab animals have shown that when administered orally, Limonene is absorbed directly from the intestinal tract and transported to tissues, where it is metabolized to perillyl alcohol (POH), perilic acid, dehydroperyl acid, limonene 1,2 diol, and limonene 8.9 diol (Shojaei et al., 2014; Schmidt & Göen, 2018). One of these compounds, which occurs in the intermediate stages of metabolization, respectively POH, has a stronger antiproliferative effect than limonene, as demonstrated by studies performed on UACC62 melanoma tumor cells. Preclinical studies performed on lab animals with induced melanoma have shown that topical administration of limonene inhibits the development of tumor cells by inhibiting the farnesylation process and disrupting cell signaling by protein kinases (ERKs) (Shojaei et al., 2014). d'Alessio and collab in preclinical studies performed on lab animals with skin lesions induced mechanically or with TPA (12-o-tetradecanoylphorbol-13-acetate) have shown that when treating these wounds with formulations containing limonene or POH, the severity of the lesions is reduced. The continuation of these studies using in vitro

models by the same authors has shown that both d-Limonene and POH induce angiogenesis, accelerate wound healing, and inhibit the formation of proinflammatory cytokines (d'Alessio et al., 2014). Cytotoxicity studies performed *in vitro* by Alipanah and collab on melanoma tumor cell lines A-375 (ARRC CRL 1619), exposed at a different level of nanoformulations that contain Limonene or citrus essential oils (essential oils containing at least 1% limonene), showed that for concentrations between (75-1200 μg/mL, the viability of melanoma tumor cells decreased under 20%. The IC50 cytotoxicity values obtained for nanoformulations applied to melanoma cells in this study ranged from (13.53÷246) μg/mL (Alipananah et al., 2021).

MAIN COMPONENTS OF CITRUS PEEL ESSENTIAL OILS

Generally, many factors, including ripening stage, season, weather conditions, soil type, storage condition, genotype, method of extraction, and analytical process, significantly impact the quality and quantity of essential oil obtained from citrus peels. Citrus peel (CP) contains volatile aromatic compounds, which accounts for about 0.5-5% of the fresh weight of CP. The majority of CP contains monoterpene hydrocarbons, sesquiterpene hydrocarbons, oxygenated monoterpenes, and oxygenated sesquiterpenes. The more critical compound in EOs obtained from citrus plants is Limonene and its concentration in these bioproducts is (32-98)% (Table 1). Compared to citrus plants with thin peels, citrus fruits with thick peels such as sour orange, grapefruit, and bergamot, have a higher concentration of essential oils. The main essential oils found in the peels of mandarin orange, pummelo, sweet orange, grapefruit, and bitter orange were non-terpenoid ester and aldehyde derivatives. Mono and sesquiterpene hydrocarbons are widely distributed in the peels of yuzu, citron, key lime, and bergamot orange (González-Mas et al., 2019). Monoterpene hydrocarbons, such as limonene (92.6%), γ-terpinene (3.39%), β-pinene (1.55%), and α-pinene (1.55%), were found to be the most common essential oil components in orange peels (0.61%). Other EO components include oxygenated monoterpenes (linalool 0.31%), sesquiterpene hydrocarbons (α-humulene 0.08%), and oxygenated sesquiterpenes (cubebol 0.06% and α-sinensal 0.06%).

Table 1. Different concentrations of Limonene from different Citrus plant peels

Source of peel	Limonene content	References
C. sinensis	59.3%,	Oyedeji et al., 2020
C. reticulata	56.76%	Yi et al., 2018
	75.16%	Abdel-Aziz et al., 2019
	80.2%	Fouad, & da Camara, 2017
C. aurantiifolia	38.9%	Fouad, & da Camara, 2017
C. limon	48.48%	Sun et al., 2018
	70.95%;	Dănilă et al., 2018
	46.93%	Moosavy et al., 2017
	52.85%,	Yazgan et al., 2019
	57.65%	Caputo et al., 2020
C. paradisi	93.33%	Deng et al., 2020
C. grandis Osbeck	87.5%	Chen et al., 2018
C. grandis	87.9-90%	Tuan et al., 2019
C. medica	60.44%	Xing et al., 2019
C. bergamia	32.29%	Caputo et al., 2020)
C. myrtifolia	76.83%	Caputo et al., 2020)

Analysis performed by Gas Chromatography coupled with Mass Spectrometry (GC-MS) found eighteen compounds in *C. sinensis* peels. From these, monoterpene represents 63.95%, oxygenated monoterpenes 28.92%, and sesquiterpene 3.54%. The main compounds were limonene (59.3%), terpineol (8.31%), linalool (6.56%), and citronellol (6.21%) (Oyedeji et al., 2020; Yi et al., 2018). Studies performed on mandarin peels essential oil (MPEO) reveal the existence of twelve compounds. MPEO contained two forms of volatile compounds: monoterpene hydrocarbons (81.90%) and oxygenated monoterpenes (18.09%). Limonene was the most abundant monoterpene hydrocarbon in NPEO (75.16%**).** According to Fouad and da Camara, limonene was the key constituent of the *C. aurantiifolia* oil (38.9%) and *C. reticulata* oil (80.2%) (Fouad and da Camara, 2017). Citrus lemon oil (CLO) is a mixture of limonene, hesperidin, δ-gluconolactone, and other compounds extracted from the peels of *C. limon* (Sun et al., 2018). D-limonene (46.93%), γ-terpinene (16.89%), tri-

cyclen (6.67%), 1-β -pinene (4.69%), and 2-β -pinene (3.86%) were the major compounds of lemon peel essential oil (Moosavy et al., 2017). Dănilă and collab. made comparisons between the compounds found in CLO by two extraction methods respectively hydrodistillation (HD) and microwave-assisted extraction (MW). They found that a high quantity of limonene (75,57%) was obtained by extraction with MW (Dănilă et al., 2018). Yazgan et collab. have investigated the antimicrobial effects of nano-emulsified lemon essential oil and raw lemon essential oil on the food-borne pathogens and on the microorganisms implied in seafood spoilage. They found that D-limonene, p-cymene, and β-pinene, the main components of essential oil, are responsible for inhibiting the growth of these microorganisms (Yazgan et al., 2019). Investigation performed on the chemical composition of essential oils extracted from the peels of three citrus species (*C. lemon, C. myrtifolia*, and *C. bergamia*) reveal the existence of 30 components. The key components are limonene (57.65%), γ -terpinene (10.45%), β -pinene (9.31%), and citronellol (8.19%) (Caputo et al., 2020)**.** *Citrus myrtifolia* EO contains thirty-two compounds, and the major compounds were limonene (76.83 %), linalool (10.01%), and α-terpineol (2.66%). These findings were in agreement with results obtained by Plastina and collab. who identified limonene (54.3%) and linalyl acetate (22.9%) as the main constituents of EO (Plastina et al., 2018). The key constituents of EO obtained from *C. bergamia* are oxygenated monoterpenes (51.09%), linalool (33.64%), Limonene (32.29%), linalyl-acetate (9.22%), terpinene (6.39%), α -terpineol (4.62%), and α-pinene (4.29%). Xing et al. performed analysis on EO obtained from *C. medica*, by two extraction methods, respectively Ultrasound-assisted hydro distillation (UAHD) and Headspace solid-phase microextraction (HS-SPME)) have obtained a product with high concentration of limonene in the case of extraction with UAHD (Xing et al., 2019). A high concentration of limonene has been reported in the EO obtained from *C. paradise* peels (93.33%) (Deng et al., 2020). Differences regarding the limonene concentration in essential oils obtained from pomelo peel, are due to soil, climatic factors (temperature, rainfall), extraction method, and the part of the plant from which the EO was obtained. The EO obtained from *C. grandis* Osbeck peels, contains limonene (87.5 %,), myrcene (3.1%), β-pinene (2.7%), α-pinene (6.0 %) (Chen et al., 2018). Another study performed on EO obtained from pomelo peels, demonstrated that it contain Limonene (87.90-89.87%t), β-pinene (2.66-2.83%), and α-phellandrene (1.22-1.38% and low amounts of oxygenated hydrocarbons (0.63-0.89%) (Tuan et al., 2019).

BIOLOGICAL EFFECTS OF LIMONENE

Studies by Kohda and collab. on a model of the human epidermis have shown that when populations of *S. epidermitis* and *S. aureus* are inoculated on the surface of the epidermis, then *S. aureus* growth is inhibited and the release of proinflammatory cytokines are attenuated by the presence of *S. epidermitis* (Kohda et al., 2021). Regarding the effect of Limonene on commensal or pathogenic microorganisms, the studies are divided into two categories: there are numerous studies demonstrating the effectiveness of Limonene alone or in combination with other antibiotics on pathogenic microorganisms that cause skin diseases such as *S. aureus* or *P. aeruginosa*, (Costa et al., 2019; Gupta et al., 2021) and there are studies that have evaluated the effect of hygiene products containing limonene on the skin microbiota. Finally, studies on healthy human subjects with skincare products containing limonene or essential oils with limonene have shown that after exposure to the cleansing agent used, the skin microbiome returns to normal (Wallen Russel, 2019; Hwang et al., 2021). When using formulations with extracts of probiotic bacteria, plant extracts, and sources of Limonene, an abundance of commensal species such as *Cutibactyerium* sp., *Staphylococcus* sp. were found in the skin microbiome, while improving hydration and dermal texture.

Natural bioproducts which contain limonene, such as the EOs obtained from citrus plants, possess many biological activities (i.e. antimicrobial, antitumor, antiviral anti-inflammatory, and antioxidants) presented in the Table 2.

Antimicrobial activity of Limonene

The essential oils of mandarin have been demonstrated to possess potent antimicrobial

activity against different strains of bacteria and fungi (Ambrosio et al., 2017; Mitropoulou et al., 2017; Putnik et al., 2017; Rafiq et al., 2018, Yi et al., 2018). The mechanisms of action of mandarin EOs on microorganisms are due to Limonene and other compounds from it (da Silva Dannenberg et al., 2016), which penetrate the bacterial cell membrane by solubilizing its lipids, destroying it and making it permeable (Dhifi et al., 2016). Three factors are critical in the action of EOs across microbial strains (Djenane, et al, 2015). First, the antimicrobial activity of EO is determined by the functional groups present in their compounds (a major part of them belong to the class of terpenes). Second, some of the minor compounds from EOs are microbiologically effective**.** Thirdly, the presence of EO components can result in additional, adaptive, or even antagonistic antimicrobial activity. Three microorganisms are in connection with a common skin disorder like acne-related inflammation: *P. acnes, P. granulosum*, and *S. epidermidis*. These bacteria are common on the skin microbiome and are often isolated from acne lesions. Many commensal microorganisms (from the normal healthy skin microbiome) such as *S. epidermitis,* which normally stimulate keratinocyte production and limit the invasion of pathogens, (boosting innate immunity), occasionally become pathogenic, being involved in biofilms production. *Staphylococcus epidermitis* activate probiotic microorganisms due to secretion of polyethylene glycol dimethacrylate, with the effect of inhibiting the growth of *S. aureus* MRSA. The introduction of *Lactobacillus reuteri* into the intestinal microbiome (through drinking water) accelerates wound healing twice as fast due to the up-regulation of oxytocin (Johnson et al, 2018). *Propionbacterium acnes* usually produce protecting bacteriocins for other pathogens in sebaceous ducts and are responsible for inducing TLR2 and TLR4 expression in keratinocytes. Occasionally *P. acnes* can become pathogenic, with large populations being associated with the development of acne (Johnson et al, 2018). Moreover, the short-chain fatty acids produced by common skin microorganisms have antimicrobial properties; as a result, the incorporation of these metabolites into the skin treatment suppresses inflammations. Commensal microorganisms protect the epithelial surface against pathogens through competition, the production of antimicrobial peptides (AMP), and proteases. Proteases have a role in cell renewal processes, the production of biofilms, and QS (quorum sensing or quorum signaling). Quorum signaling represents the ability to detect and respond to the density of the microbial population through the secretion of signaling molecules called autoinductors (Bassler, 1999; Boxberger et al., 2021). Self-inducers can be self-inducing peptides for gram-positive microorganisms and N-acyl homoserine lactones (AHL) for gram-negative microorganisms. Normally the skin microbiota interacts with the host's immune cells (T cells) which are trained to respond to pathogenic microorganisms that can colonize the epithelium. Tests performed on lab animals have shown that the expression of 280 genes is modulated in response to increasing microbial populations. Many of the modulated genes have a role in the secretion of proinflammatory cytokines, signaling and localization of the T cells. Many of the commensal microorganisms produce metabolic compounds that have antimicrobial or antitumor effects. Microorganisms from the genus *Malassezia* produce indoles that inhibit yeasts and fungi; *S. epidermitis* is able of producing 6-N-hydroxyaminopurine, a compound that can provide protection against skin cancer (Bassler, 1999; Boxberger et al., 2021). Bioproducts obtained from *S. epidermitis* contain coagulase-negative inducing peptides that inhibit proteases and α-modulin phenol-soluble, secreted by *S. aureus,* which lead to proteolysis of the epidermis and cause skin damage (Williams et al., 2019). A self-inductor peptide (SYNVCGGYF) that inhibits *S. aureus* activity (Williams et al., 2019) has been identified in an isolate from *S. hominis*. These are just a few examples of QS that show how the commensal microbiome of human skin contributes to the homeostasis of the epithelial barrier, inhibiting the production of toxins by *S. aureus.*

S. epidermidis is resistant to current antibacterial reagents such as ciprofloxacin, ofloxacin, fusidic acid, and vancomycin. Infections caused by this microorganism are complicated further by its ability to form biofilms (Gavahian et al., 2012; Moradalizadeh et al., 2013)**.** In their

research, Dănilă and collab. have found that the synergistic role of the EOs mixture in inhibiting *S. epidermidis* biofilms is due to D-limonene (from *C. lemon* EO) and linalool (Dănilă et al., 2018). This study indicates that the mixtures of essential oils containing linalool and D-limonene could be suitable alternatives to antimicrobial reagents, for treating *S. epidermidis* or preventing *S. epidermidis* growth on viable tissues or medical products (Sun et al., 2018). Additionally, *C. lemon* has been shown to possess antibacterial properties against *Streptococcus sobrinus*, by inhibition of glucosyltransferase activity (Liu et al., 2020).

Antioxidant activity

Phenolic compounds, such as limonene, demonstrated antioxidant activity due to their ability of scavenger for the free radicals. Many studies established that limonene was able of ameliorating the effects of oxidative stress *in vitro* and *in vivo*. Limonene exhibit antioxidant activity in diabetic rats, by reducing the oxidative stress in lab animals that received Limonene in food for 45 days (Roberto et al., 2010; Murali et al., 2013; Bai et al., 2016). Similar findings were found *in vitro*, on the BALB/c cells line. As well, Limonene inhibits the expression of apoptotic proteins such as Bax and Bcl-2 which facilitates the apoptotic response to oxygen peroxide (Ahmad & Beg, 2013; Bacanli et al., 2015).

Anti-inflammatory effect

Citrus myrtifolia also known as chinotto is widely distributed as ornamental plant and is probably a mutation of sour orange (*C. aurantium*) (Liu et al., 2012)**.** The primary constituents of chinotto essential oil are limonene, linalool, linalyl acetate, and α-terpinene, which have a significant scavenging activity against DPPH and ABTS (Plastina et al., 2018)**.** Phytochemical studies revealed the presence of geranial, limonene, γ -terpinene, and others in the EOs of *C. Limon*, *C. aurantifolia*, and *C. limonia*. These bioproducts have anti-inflammatory activity, inhibits cell migration and cytokine formation caused by carrageenan. These results were also observed when equivalent concentrations of pure limonene were used. Additionally, *C. aurantifolia* was found to cause myelotoxicity in mice (Amorim et al., 2016)**.** The anti-inflammatory action of *C. lemon* and C. *limonia* (mandarin lime) EO is most likely due to their high limonene content, while the myelotoxicity of *C. aurantifolia* EO is most likely due to its high citral content (Amorim et al., 2016). In this regard, caution should be exercised when working with *C. aurantifolia* EO due to its toxicity. These findings suggest that the essential oils of *C. limon*, *C. aurantifolia*, and *C. limonia* have an important anti-inflammatory effect. Limonene do not have cytotoxicity *in vitro* on neutrophils isolated from the peritoneal cavity of BALB/c mice. When leukotriene B4 was used as a chemoattractant, limonene inhibited neutrophil migrating. *In vivo* assays demonstrated that oral pre-treatment with limonene decreased leukocyte infiltration and the level of TNF-α, whereas the IL-10 levels were unaffected. Limonene inhibited doxorubicin's cytotoxic effect in the kidneys if the food of lab animals was supplemented with Limonene (Kummer et al., 2013; Rehman et al., 2014). Osteoporosis is a common disease characterized by inflammation of the joints and cartilage deterioration. Rufino and collab. have demonstrated in their experiments that limonene exerts an anti-inflammatory effect on human chondrocytes (Rufino et al., 2015)**.**

Antitumor activity

Limonene has been shown to have anti-proliferative, apoptotic, and anti-carcinogenic properties. Chaudhary and collab. were examined the effects of limonene on the growth of skin tumors triggered by 7,12-dimethylbenz (a) anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA). They discovered that applying limonene to the mouse skin reduced significantly the effect induced by TPA (respectively edema, hyperplasia, cyclooxygenase-2 expression). Additionally, the treatment with limonene repaired the reduced glutathione, glutathione peroxidase, glutathione reductase, glutathione S-transferase, catalase, and malondialdehyde activity on the lab animals treated with TPA. The presence of limonene decreases significantly the tumors growth and incidence in skin oncogenesis as compared to DMBA/TPA-treated mice. Additionally, therapy with limonene increased the latency time for tumor growth from four to nine weeks. Limonene

therapy decreased the level of protein kinases as Ras and Raf in DMBA/TPA-induced tumors. Additionally, was observed and an increase of Bax expression in cancerous tissue of mice treated with limonene (Chaudhary et al., 2011).

Antiviral activity of limonene

Limonene is able to suppress viral replication of HSV-1 (Astani & Schnitzler, 2014), inhibit avian influenza (H5N1) (Nagy et al., 2018), and yellow fever virus replication (Gómez et al., 2013), being widely used in dermato-cosmetic formulations (Nazaroff & Weschler, 2004). Due to its savor and fragrance, limonene is generally used in foods, drugs, drinks, and industry (Sun, 2007; Bora et al., 2020)

LIMONENE TOXICOLOGICAL EVALUATION

Limonene is considered a non-toxic natural substance based on the level of lethal dose (LD50) and recurrent toxicity studies.

To assess the limonene safety, toxicological tests were performed *in vitro* and *in vivo*. When is ingested orally, limonene is spread to the liver, kidney, and bloodstream. Dermal exposure to high concentrations of limonene causes skin allergic reactions. This compound possesses irritant properties that differ according to its shape (D or L) and degree of oxidation. Limonene that has been oxidized was found to have the greater sensitizing ability. The oxidation products of limonene include limonene hydroperoxides, R carvone, limonene oxide, perillic acid, and limonene-1,8-diol (Kim et al., 2008)**.** The use of d-limonene in cosmetics is limited in Korea and the European Union (EU), where the peroxide content of limonene must be less than 20 mmol/L. Additionally, the EU Cosmetics Directive 76/768/EEC requires that limonene be listed as an ingredient if its concentration is greater than 0.001 % in "leave-on" products and 0.01 % in "rinse-off" products (Kim et al., 2013).

Table 2. Biological activities of Limonene

Activity	Type of model	Lab experimental models	References
Anti-oxidant properties	*in vitro* *in vivo*	Diabetes of lab animals induced by streptozotocin. Lymphocytes from BALB/c mice	Roberto et al., 2010; Murali et al., 2013; Ahmad & Beg, 2013; Bai et al., 2016
Anticancer properties		Neuroblastoma cell lines (SH-SY5Y); Cells lines initiated of biologic materials of from patients with cancer; Gastric carcinoma cell lines (MGC803); Colon cancer cells line (LS174T) Doxorubicin administrated lab animals	Russo et al., 2013 Miller et al., 2015 Zhang et al., 2014 Jia et al., 2013
Anti-inflammatory properties		BALB/c mice neutrophils; Zymosan-induced peritonitis Chondrocytes stimulated with IL-1β	Kummer et al., 2013 Rehman et al., 2014 Rufino et al., 2015
Antimicrobial properties	*in vitro* *in vivo*	The antimicrobial and antibiofilm efficacy of citrus EO, against acne-related pathogenic bacteria	Yi et al., 2018 Dănilă et al., 2018 Li et al., 2020
Wound healing properties	*in vitro* *in vivo*	Dermatitis Skin damage	A d'Alessio et al., 2014

Additionally, the EU Cosmetics Directive 76/768/EEC requires that limonene be listed as an ingredient if its concentration is greater than 0.001% in "leave-on" products and 0.01% in "rinse-off" products (Kim et al., 2013). The studies of Acute oral toxicity performed by Adams and collab. on Limonene indicated that the LD50 for this compound ranged between 4400 and 6600 mg/kg for rats and mice, while the chronic toxicity was 75 and 300 mg/kg in male and female rats. The chronic toxicity in male and female mice was 250 and 500 mg/kg respectively when Limonene was administrated through oral gavage (Adams et al., 2011). Limonene is registered as a commonly accepted safe (GRAS) material for synthetic flavours under the Code of Federal Regulations (CFR) (Kim et al., 2013). A recent study examined the amount of limonene used in cosmetic products, as well as its cumulative daily exposure to various human bodies. The study findings indicated that (69-95)% are due to the 280

fragrance and antiperspirants products which containing limonene. Additionally, the cumulative exposure to limonene was found to be highest in the face and neck area (Dornic et al., 2018). Studies *in vitro* performed by Rolseth and collab, which have compared the toxicity of limonene and limonene 1,2-epoxide on human lung fibroblasts, concluded that the Limonene is toxic. Additionally, they found that at limonene concentrations of 200-300 μM, the total growth of cells was inhibited. In this study the cell death occurred at concentrations of 350 μM limonene. Regarding mitochondrial activity, the limonene toxicity was significant at levels of 300-500 μM. Whole inhibition of mitochondrial activity was observed at concentrations of limonene above 500 μM (Rolseth et al., 2002). Anderson and collab. have evaluated the toxicity of limonene in the presence of ozone on differentiated epithelial tissue (MucilAirTM) and on epithelial cell line (A549). They discovered that the limonene inhibited cellular proliferation and altered the synthesis of pro-inflammatory cytokines (Anderson et al., 2013). Regarding the genotoxicity of limonene, Fernandez-Bedmar and collab. have investigated the genotoxicity, anti-genotoxicity, and cytotoxicity of citrus juices (lemon juice and orange juice) and their bioactive components (hesperidin and limonene) on *Drosophila melanogaster*. Their experiments revealed that all compounds were non-mutagenic at low concentrations and contributed to the improvement of life expectancy. Additionally, they reported that the limonene and hesperidin have anti-genotoxic properties, inhibiting the hydrogen peroxide H_2O_2 (Fernández-Bedmar et al., 2011).

Hepatotoxicity of limonene

Based on the hepatotoxicity of limonene, the liver was established as a target. Following oral administration of limonene, several researchers have reported the possibility of liver toxicity, after its metabolization. Ramos and collab. have investigated the possible hepatotoxic impact of limonene on Wistar rats. The animals have received between 25-75 mg limonene/kg for 30-45 days. During experiment, biochemical levels of serum alkaline phosphate (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were determined. The scientists reported that the rats treated with 75 mg limonene/kg/day for 45 days had significantly lower ALT levels in comparison with rats from control group (Ramos et al., 2015).

Neurotoxicity of Limonene

Animal studies have revealed the effects of limonene exposure on the central nervous system (CNS), but is still unclear if these findings indicate general toxicity. In studies of acute toxicity made on male mice, limonene epoxide was found to have analgesic and anxiolytic-like effects, (de Almeida et al., 2012). In male Wistar rats subjected to physical stress, the L-limonene has exerted antistress properties by inhibition of the $GABA_A$ receptors (GABA = γ aminobutyric acid) respectively by inhibiting the basal hypothalamic-pituitary-adrenal function (HPA) (Zhou et al., 2009). According to studies performed by Yun, limonene has sedative effects on rats and mice, by modulating dopamine release and serotonin neuronal activity. Additionally, limonene was shown to inhibit methamphetamine (METH)-induced hyperlocomotion (Yun, 2014).

CONCLUSIONS

Studies reported in the scientific literature show that the products which contain Limonene as an active compound (alone or as essential oils from different *Citrus* species) can accelerate wound healing, promote angiogenesis and decrease inflammation via suppressing pro-inflammatory cytokines production. Preclinical studies and studies performed in vitro reveal the ability of this compound to inhibit melanoma. This biomolecule exhibit antimicrobial activities on pathogenic microorganisms implied in skin disorder like *S. aureus* or *P. aeruginosa*. In combination with synthetic antibiotic reagents, Limonene increases the effectiveness of the antimicrobial activity. Studies performed on healthy humans, with cleaning skin products that contain Limonene (alone or as essential oils with Limonene), reveal that this compound does not affect the commensal skin microbiota. In the case in which the cleaning skin formulations contain probiotic and /or plant extract, then the population of skin commensal microbiota increases. Due to their biological effects, the

essential oils with Limonene represent the potential bioproducts which can be used in regenerative medicine. The new research can be initiated with these bioproducts, alone or in association with other drugs, to find new solutions for treating infections with microorganisms with antibiotic resistance or in association with a chemotherapeutic reagent, to suppress the tumor resistence.

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SCREENING OF CULTIVATION MEDIA FOR LDPE BIODEGRADATION BY *Pseudomonas fluorescens*

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Abstract

The research aimed to select the optimal mineral salt medium (MSM) for low-density polyethylene (LDPE) biodegradation by the strain Pseudomonas fluorescens CNM-PFB-01. Four culture media were selected, which differed in salt content, N: P and C: N ratio. After 40 days of submerged cultivation, the following parameters were determined: catalase activity, pH of culture media, biomass accumulation, rate of degradation and the tensile tests of LDPE. It was observed that the strain is catalase-negative both in the control without LDPE and in the presence of LDPE. A weak positive reaction was established only on MSM 4 supplemented with LDPE. In the presence of polyethylene, the pH of the media increased, especially on the MSM 4 - by 0.6 units. The addition of polyethylene to the growth media stimulated the bacterial biomass accumulation by 2-3.6 times. The degradation rate of polyethylene films ranged from 0.37% to 0.86% depending on the culture medium. The tensile test showed increased elasticity of the plastic in the variants treated with bacterial strain. In conclusion, in order to stimulate the biodegradation of LDPE by the strain P. fluorescens CNM-PFB-01, the medium MSM 4 (N: P ratio 4.30: 1 and C: N ratio 0.29: 1) was selected.

Key words*: mineral salt media, catalase activity, LDPE biodegradation, Pseudomonas fluorescens.*

INTRODUCTION

Bacteria of the genus *Pseudomonas*, which are widely spread both in soil and in aquatic environments, are recognized as a valuable source for modern biotechnology, due to their varied metabolic capacities (Wilkes & Aristilde, 2017). Over the decades, where the world is looking for ecological remedies to destroy xenobiotic substances, the metabolic properties of pseudomonads have been used in bioremediation of a wide spectrum of pollutants of different nature, such as organochlorine pesticides, petroleum hydrocarbons, phenolic compounds, heavy metals, plastic polymers, etc. (Wasi et al., 2013).

In terms of plastic bioremediation, besides fungi of the genus *Aspergillus* and *Penicillium*, bacteria of the genus *Pseudomonas* are among the most cited in literature as destructors of different plastic polymers (Kyaw et al., 2012; Wilkes & Aristilde, 2017). Thus, among the bacteria that degrade polyvinyl alcohol, most of them are pseudomonads (Shimao, 2001). There are data that pseudomonads contribute to the degradation of polyethylene succinate (Tribedi & Sil, 2013a), polystyrene (Devi et al., 2016) polypropylene (Arkatkar et al., 2010). Complete degradation of polyethylene glycol was obtained using *Pseudomonas stutzeri JA1001* by Obradors & Aguilar (1991). Also, several strains of pseudomonads including *P. fluorescens*, *P. aeruginosa*, *P. cepacia*, *P. protegens* and *P. chlororaphis* have been found to degrade polyurethane (Cregut et al., 2013).

One of the most used plastic materials in the world economy - polyethylene (PE), is characterized by stability and durability due to hydrophobic carbon backbone, which makes it recalcitrant to the action of external factors, including biological ones. However, strains of pseudomonads capable of degrading PE, from 5% to 50%, have been detected, depending on the structure of the PE, its pretreatment and the type of the *Pseudomonas* strains (Rajandas et al., 2012; Tribedi & Sil, 2013b).

The main strategy in bioremediation of LDPE is based on the use of plastic by the microorganism as sole carbon source. For this, microorganisms synthesize enzymes, which trigger the oxidation processes of carbon bonds. The duration and degree of degradation of PE

depends both on the structure of the polymer surface and on the environmental conditions (pH, temperature, nutrients, minerals, oxygen, humidity etc., as well as on the physiological properties of each individual microorganism) (Dwicania et al., 2019; Iram et al., 2019; Tamnou et al., 2021).

For these reasons, the selection of nutrient media and cultivation conditions, which would ensure the maximum triggering of the physiological processes of the microorganism, aimed to use plastic as a source of carbon, is an important step in bioremediation technologies. Thus, the aim of our research was to select the optimal mineral medium for LDPE biodegradation by the strain *Pseudomonas fluorescens* CNM-PFB-01.

MATERIALS AND METHODS

The object of study was bacterial strain *P. fluorescens* CNM-PFB-01 deposited in the National Collection of Non-Pathogenic Microorganisms of the Republic of Moldova. LDPE sheets used in this work were produced by Kraus Folie Sp.J. Film thickness was 35 µm. Four mineral salt media (MSM) were selected which differed in salt content (Table 1), and N: P and C: N ratio (Table 2).

Table 1. The composition of the mineral media, g/L

Mineral salts, g/L	**MSM 1** (Jamil, 2017)	**MSM 2** (Nakei, 2019)	**MSM 3** (Skariya-chan, 2015)	**MSM 4** (Zajic, 1972)
KH_2PO_4	2.0	1.0	2.0	
K_2HPO_4	7.0	1.0	7.0	1.8
$MgSO_4 \cdot 7H_2O$	0.1	0.2	0.1	0.2
$ZnSO_4 \cdot 7H_2O$	0.001			
$FeSO_4 \cdot 7H_2O$	0.01			0.01
$MnSO_4 \cdot 6H_2O$	0.002			
NH_4NO_3	1.0	1.0		
$CuSO_4 \cdot 7H_2O$	0.0001			
$CaCl_2$		0.02		
$FeCl_3$		0.05		
$(NH_4)_2SO_4$			1.0	
$NaNO_3$				2.0
NH_4Cl				4.0
NaCl				0.1
$Na_3C_6H_5O_7$			0.5	

Table 2. Nitrogen, phosphorus, and carbon content and ratio in the tested mineral media

Mineral media	Nitrogen, g/L	Phos-phorus, g/L	Carbon, g/L	Ratio N: P	Ratio C: N
MSM 1	0.35	1.70	0.40	0.21	1.14
MSM 2	0.35	0.41	0.40	0.86	1.14
MSM 3	0.21	1.70	0.54	0.12	2.55
MSM 4	1.40	0.32	0.40	4.30	0.29

The composition of the selected media was formulated so that the suspended polymer, LDPE, was the sole carbon source for the microorganism. As a growth inducer, 0.1% glucose was added to the medium. The pH of the media was adjusted to 6.5, according to the physiological needs of the bacterium.

Each medium had 2 experimental variants - with LDPE films and without LDPE films (control variant). After 40 days of submerged cultivation at 28°C, on a shaker (180-200 rpm), the following parameters were determined: catalase activity and pH of culture media, quantity of accumulated biomass, the rate of LDPE degradation, optical microscopy of films.

Also, the tensile testing of polyethylene (ISO 20753:2008) was performed using the tensile testing machine CQ-508B (COMETECH Testing Machines Co., LTD). Such parameters as elongation at break (%) and tensile strength at break (N/mm^2) were determined.

The catalase test of culture media was performed by the slide method using 3% H_2O_2.The reaction was interpreted according to the intensity of the formation of oxygen bubbles.

Before adding to culture media, LDPE films were cut into longitudinal and transverse strips, weighed and sterilized by washing with 70% ethyl alcohol for 15 min, and treated with UV-rays for 1 hour twice. After 40 days of cultivation, the LDPE films were recovered, washed with sterile distilled water, air-dried and examined under an optical microscope.

The degradation of LDPE films was determined gravimetrically, by weighing the films before and after incubation. The bacterial cell mass adhering to the polyethylene surface was washed by a 2% aqueous sodium dodecyl sulfate solution for 3 hours and finally with distilled water. The washed LDPE films were

air-dried and weighed. Percentage degradation of polyethylene films was determined by the formula:

Weight loss (in %) = [(Initial Weight − Final Weight) / Initial Weight] × 100.

RESULTS AND DISCUSSIONS

The first stage in the biodegradation of plastic consists in the attachment of microorganisms on the surface of the polymer, followed by the increase of biomass and the synthesis of specific enzymes (Montazer et al., 2020; Wilkes & Aristilde, 2017; Alshehrei, 2017). At the end of the cultivation period of the *P. fluorescens* CNM-PFB-01 strain on different mineral media with and without addition of LDPE films, the catalase activity and the pH of the cultural media were determined (Table 3).

Catalase is a commonly assayed enzyme that degrades hydrogen peroxide into water and oxygen. The presence of the enzyme in the test bacterial isolate or culture medium can be determine by using hydrogen peroxide, which is broken down to bubble-producing O_2 by catalase-positive bacteria (Iwase et al., 2013). The measurement of bacterial catalase activity has been suggested as a method to quantify catalase-positive bacterial content (Serra et al., 2008). There are reports discussed determination of aerobic microbial concentrations based on the correlation between catalase activity and aerobic microbial loads (Ye & Wu, 2011). In our work we used catalase test for rapid assessment of viability of microbial culture. *Pseudomonas* species also typically give a positive result to the catalase test. It was established that the strain is catalase-negative both in the control variants and in the presence of LDPE. The exception is the variant MSM 4 with LDPE, where a weak positive reaction was observed.

The researchers noted that the bacteria *Pseudomonas* grows best in the pH range of 6.3-7.2 (Stoimenova et al., 2009; Gonçalves et al., 2017; Bushell et al., 2019). Over time, the pH of the culture medium changes towards its acidification due to the accumulation of organic acids, as well as metabolic products synthesized by these bacteria during cultivation (Zhou et al., 2017). The evolution of the pH of the culture medium speaks of the metabolic activity of the microbial strain in the medium supplemented with LDPE, and is important for enzymatic activity, since it has been demonstrated that more alkaline pH favors enzyme activity (Dwicania et al., 2019; Iram et al., 2019; Tamnou et al., 2021). Considering physiological requirements for growth of pseudomonads, the pH of media used was initially adjusted to 6.5. After 40 days of cultivation, the pH remained practically unchanged on the MSM 3 (values - 6.3-6.4), but decreased to more acidic values of 5.4-5.6 on the MSM 2, and 5.6 on the MSM 4 without LDPE. Unlike the other media, there was a large difference between the pH values on the MSM 4 with and without LDPE - by 0.6 units (Table 3).

Table 3. Catalase activity and pH of the cultural media after cultivation of *P. fluorescens* CNM-PFB-01 on different MSM with and without plastic

Nutrient medium	Catalase activity		pH of culture media	
	Without LDPE	With LDPE	Without LDPE	With LDPE
MSM 1	-	-	6.1	6.2
MSM 2	-	-	5.4	5.6
MSM 3	-	-	6.3	6.4
MSM 4	-	+	5.6	6.2

N.B.: - No reaction, + Weak reaction

It is well known that the ratio of carbon to nitrogen is an important indicator for the growth and biosynthesis of bacteria (Ginésy et al., 2017). In this work, the source of carbon for the growth of pseudomonads was the polymer LDPE, while glucose at a concentration of 0.1% and sodium citrate (as a component of the MSM 3 medium) served as a growth inducer. Thus, the amount of available carbon was sufficient to initiate bacterial growth, but not enough to accumulate a large amount of biomass, as in the case of growth on media

with a high C: N ratio, as seen in examples from the literature (Hartmann et al., 2004; Onwosi & Odibo, 2012; Veliev et al., 2013). It was observed that the presence of LDPE in the culture media considerably influenced the growth activity of the strain - the amount of accumulated biomass doubled or even tripled (Figure 1).

Thus, on MSM 1 the amount of biomass increased by 2 times, on MSM 2 - by 2.9 times, and on MSM 4 - by 3.6 times. The exception occurred on MSM 3 supplemented with LDPE, where the strain accumulated less biomass than in the control by 13.8%. This medium characterized by C: N ratio 2.55 and N: P ratio 0.12 (limitation on phosphorus).

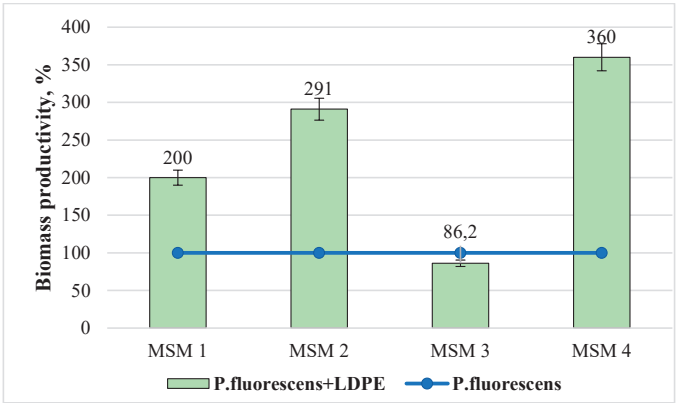


Figure 1. Accumulation of *P. fluorescens* CNM-PFB-01 biomass on different mineral media, with and without LDPE

To assess the degradation of plastic we used such indices as weight loss of the substrate and changes in mechanical properties (tensile strength, percentage of elongation).

The degradation of plastic by bacteria of the genus *Pseudomonas* is intensely studied. In the literature, data showing that the percentage of mass loss of polyethylene varies widely is presented. Thus, *Pseudomonas* sp. AKS2 strain degraded up to 5% LDPE (Tribedi & Sil, 2013b), and *P. aeruginosa*_UMT - 4.8% (Bakht et al., 2020). A series of actively degrading LDPE pseudomonads (with 20% *P. aeruginosa* (PAO1), 11% *P. aeruginosa* (ATCC), 9% *P. putida*, and 11.3% *P. syringae*) were detected by Kyaw et al. (2012). The conditions, nutrition media and duration of cultivation in the listed cases were different from those presented in this paper.

Weighing the LDPE strips after 40 days of cultivation showed that in the presence of *P. fluorescens* CNM-PFB-01 the strips lost mass and the degradation processes of plastic had begun. The percentage of degradation was different, depending on the culture medium, from 0.37% to 0.86% (Figure 2).

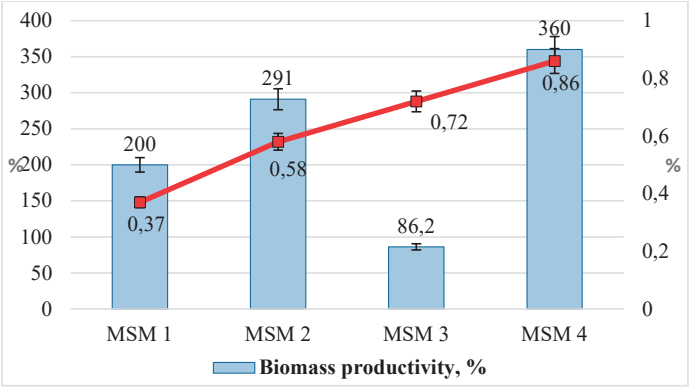


Figure 2. LDPE degradation and the amount of *P. fluorescens* CNM-PFB-01 biomass on different mineral media

It was observed in the case of MSM 1, MSM 2 and MSM 4 that the percentage of degradation is directly correlated with the amount of biomass: the more biomass the strain accumulated, the higher the percentage of degradation was. On MSM 3, although the strain growth was diminished due to limitation on phosphorus, LDPE degradation was found to be more active than on MSM 1 and 2. The most active the LDPE films were degraded on medium MSM 4, with the lowest C: N ratio and the highest N: P ratio. The effect of colonizing the polyethylene surface with microorganisms can also be evaluated by determining the mechanical properties of the film, namely by determining the tensile strength and elongation at break. The higher the tensile strength of the polymer, the better its stability. According to the literature data microorganisms affect the mechanical properties of LDPE leading to a change in tensile strength and elongation at break (Sudhakar et al., 2008; Nowak et al., 2011; Kyaw et al., 2012; Ghatge et al., 2020). In our case, the tensile tests on polyethylene showed that the elasticity of the films increased in the samples processed with microorganisms (Figure 3).

Thus, compared to the untreated LDPE films with pseudomonads, the elongation at break of the transverse stripes changed considerably on all tested media by 21-48% (maximum on MSM 1 - by 48%), and the tensile strength by 4.5-21%. The most visible changes in the elasticity of the plastic were determined on the MSM 1 medium.

The elasticity for the longitudinal stripes has not changed so much. On MSM 1 and 3 media, the values of the elongation at break and tensile strength varied within the control limits. Visible changes of the longitudinal film were established when cultivated on MSM 2, the value of the elongation at break increased by 32% and of the tensile strength by 24%.

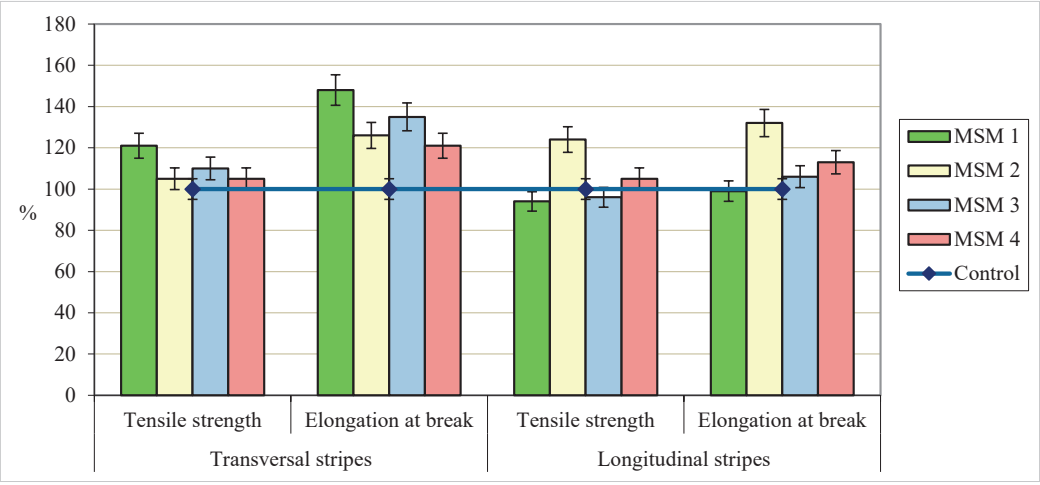
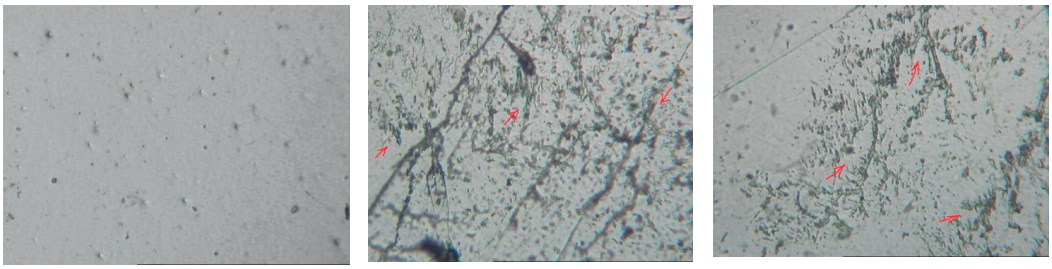


Figure 3. The tensile strength and elongation at break of LDPE films treated with *P. fluorescens* CNM-PFB-01, depending on the culture medium

The process of biodegradation of plastic begins with the colonization of its surface by microorganisms (Kyaw et al., 2012; Ghatge et al., 2020). The microscopy of the LDPE films revealed that the bacterial cells became immobilized on the surface of the films, and the contact damaged the polyethylene surface (Figure 4).



Untreated polyethylene (Control) Traces of damage on the surface of polyethylene

Figure 4. Photo of LDPE films after treatment with *P. fluorescens* CNM-PFB-01

CONCLUSIONS

Of the 4 tested mineral media, it was observed that the most optimal conditions for the active growth of the strain *P. fluorescens* CNM-PFB-01 were created at cultivation on MSM 4, where the C: N ratio was 0.29:1 and N: P ratio was 4.30: 1. Due to the alkalinization of the medium, the synthesis of the catalase ferment was triggered, which in turn favored the initiation of the degradation process of LDPE film. MSM 4 medium was selected for further research.

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BIODEGRADATION OF NOVEL POLYLACTIC ACID BASED BIOMATERIALS BY DIFFERENT METHODS

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Abstract

The extensive production and usage of petroleum based plastic materials represent a great threat to the environment (both terrestrial and marine) due to their properties of slow degradation and landfill accumulation. Therefore, many researches were conducted in order to develop friendly packaging materials, which fulfil the current requirements in terms of biodegradability, bioavailability and compatibility. Among different types of biodegradable materials, polylactic acid (PLA) received more and more attention as green material due to the fact that it is derived from natural and renewable resources and present great physical-mechanical properties. This study aims to present the research conducted for the biodegradability degree determination of some novel biomaterials based on PLA that could be used as packaging materials. The newly developed materials were buried in a natural characterized soil and their biodegradability was determined after 30, 60 and 90 days of maintaining in soil. Furthermore, they were tested from a microbial colonisation and degradation point of view. The results showed low rates of biodegradability for PLA based samples.

Key words*: biodegradation, colonisation, fungi, polylactic acid, soil burial test.*

INTRODUCTION

Petro-chemical based packaging materials present many advantages such as low price, high manufacturing speed, great physical - mechanical and barrier properties (Iordache et al., 2018); however, they accumulate in the environment representing a great concern for both waste management industry and natural environment (Peng et al., 2021).

In recent years, consumer attention has been focused on environmentally friendly products, including packaging materials for their daily use commodities (Turco et al., 2021). Therefore, many studies were conducted for the development of packaging materials derived from natural and renewable resources with high-performing properties similar to conventional materials (Kalita et al., 2020) and environmentally friendly. Aliphatic polyesters in particular, represents the most promising polymers for obtaining biopolymers that fulfil the properties mentioned above (Turco et al., 2021). One of these polyesters is represented by polylactic acid (PLA), which is a renewable material (Jeon & Kim, 2013), considered one of the most promising alternatives to conventional petroleum based plastic materials (Castro-Aguirre et al., 2018). It has good processing properties, high transparency and is easy to process (Janczak et al., 2018). It is also commercially available at a large scale (Mistry et al., 2022), accounting for 25% of the total biopolymer production at a global level (Kalita et al., 2021; Sun et al., 2022). It can be used in various domains, such as food industry, agriculture, textile and medical or pharmaceutical fields (Lv et al., 2017; Freitas et al., 2017), being suitable for conventional plastic materials replacement (Boonluksiri et al., 2021).

Biodegradation of polymers represents a complex process based on the breaking of the molecular structure of the materials (Lammi et al., 2019). Under aerobic conditions two steps occur, namely fragmentation (or hydrolysis of

the polymer in short chains) and enzymes action (produced by microorganisms) which uses as source of energy hydrolysis products such as monomers, oligomers and dimers; following this process results H_2O, CO_2, CH_4 and biomass (Freitas et al., 2017; Janczak et al., 2018; Eslami & Mekonnen, 2022). Soil biodegradation is a method that has been widely applied in the last years on various biopolymers, due to researchers' interest over the biodegradation of different materials in environmental conditions. Another method that could be used for polymers biodegradation is the microorganism development observation on the polymer substrate (Janczak et al., 2018), namely colonisation analysis.
The aim of this study was to asses soil and microbial biodegradation on several polymeric materials based on PLA.

MATERIALS AND METHODS

Soil burial biodegradation test

The samples tested in this experiment are presented in Table 1.

Table 1. Tested samples based on PLA

Sample Code	Description
PLA	PLA film obtained by casting method (control sample)
PLA/Ns	PLA film covered with a nanoemulsion based on polyvinyl alcohol (APV) and nisin (Ns) by electrospinning process
PLA/Ne	PLA film covered with a nanoemulsion (Ne) based on dill essential oil and nisin encapsulated into APV by electrospinning process
PLA/AgNps	PLA film covered by electrospinning process with silver nanoparticles (AgNps) dispersed into APV

An active soil with a water content of (60 ± 5)% of the soil's water retention capacity was used within the experiments. The samples were cut in a rectangular form (3.0 x 1.5 cm). To determine the mass variations, the tested materials were kept at room temperature in a desiccator, until the mass of each sample reached a constant value (approximately after 48 hours), samples being weighed both at the beginning and at the end of the test period. The soil used in these experiments presented the characteristics described in Table 2.

Table 2. Characteristics of the soil used in the experiments

Ph	5.0-7.0 pH units
Total N in dry matter	max. 1.9%
P_2O_2 in dry matter	max. 0.5%
K_2O in dry matter	max. 0.9%
Content of combustion substances	min. 50%
Electrical conductivity	max. 1.2 mS cm^{-1}
Particles over 20 mm	max. 5%
Humidity	max. 65%

The microbiological activity of the soil was determined and analysis showed that the soil was active from a microbiological point of view (Figure 1).

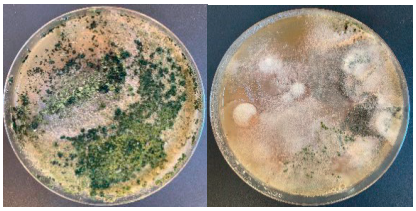

Figure 1. The visual aspect of the development of existing microorganisms in the soil

The polymer samples were buried in soil; the thickness of the layer covering the samples must not be greater than 12.5 cm. To ensure the circulation of oxygen, the containers were not hermetically closed. The containers were then stored at room temperature, which was monitored throughout the experiment (~ 25±1°C), for 30 days, 60 days and 90 days, respectively.
To examine the morphology of the studied samples, a FEI Inspect S50 scanning electron microscope was used, at accelerating voltages of 5 kV, at 200x magnification, in Low Vacuum.

Colonisation and in vitro biodegradation test

The method used was according to SR EN ISO 846:2000. The principle of this method consists of samples exposure to microorganisms' action, for a certain period of time at constant

temperature. Two minimal culture media were used - one without (M I) and one with carbon source (M II) -, both being prepared according to their recipes, sterilized at 121°C for 15 minutes, then cooled at approximate 45°C and poured into Petri dishes. Both *Aspergillus brasiliensis* ATCC 16404 and *Aspergillus terreus* fungi were grown on Potato Dextrose Agar (PDA) medium for 7-9 days and stored at 25°C, before use. The test samples (3.0 x 1.5 cm dimension) were added to the culture media just before the solidifying phase, and then each sample was inoculated in four points with the spore suspension. Two replicates were used for each sample.

The colonization of the samples was monitored by visual observation after 30 and 60 days of incubation in order to evaluate the degree of colonization. Five-grade scale of invasion ranging from 0 to 4 was established as a function of fungi observed on the surface of the films (Table 3).

Table 3. Evaluation of colonization degree over the studied polymeric materials

Growth intensity	Evaluation
0	absence of invasion
1	low attack with a maximum 25 % of the film surface covered with fungi
2	expansion of moderate intensity with a maximum 50 % of the film covered with fungi
3	high degree of colonization over 50 %
4	growth of fungi occupying the whole surface of the specimen

After 60 days of exposure to microorganism's action, the specimens were washed, submerged in ethylic alcohol and dried until a constant weight was obtained.

To determine the ***biodegradation rate*** for both soil burial test and microorganisms' action, the samples were weighed, and the mass variation (degree of biodegradation) was determined using the equation:

$$\Delta M_{biol.} = \frac{\Delta M_i - \Delta M_f}{\Delta M_i} \times 100$$

where: $\Delta M_{biol.}$ represents the variation in the mass of the samples (degree of biodegradation), ΔM_i represents the average of the initial masses of the samples and ΔM_f represents the average of the final masses of the samples (at the end of the maintenance period under controlled conditions).

RESULTS AND DISCUSSIONS

Soil burial biodegradation test - results

At the end of each test period (30 days, 60 days, 90 days), the studied polymeric materials were extracted from the soil, washed with distilled water to remove the soil and dried on filter paper. The samples were dried at room temperature and stored in a desiccator for 48 hours, until a constant mass was obtained.

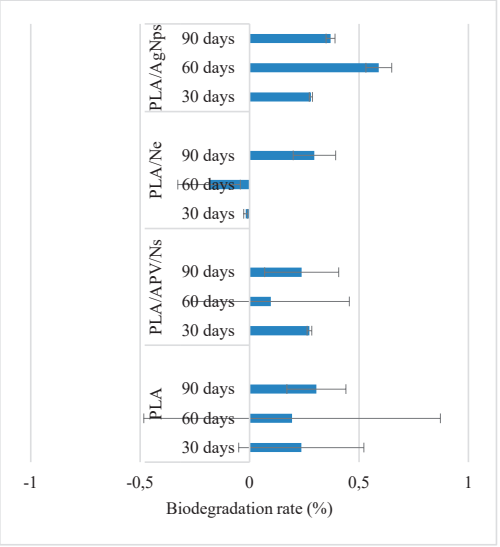

Figure 2. The weight loss values of the PLA based samples at the end of each testing period

Figure 2 presents the values obtained for the biodegradation rate of the PLA based samples. For PLA/Ne, an increase in weight was registered after 30 and 60 days of maintaining in soil, probably due to water absorption of the sample.

The PLA based samples presented low biodegradation rate, between -1.085% and 0.590%. These results are in accordance with other studies, accordingly the biodegradability of PLA taking a long time compared to other biodegradable biopolymers (Pattanasuttichonlakul et al., 2018; Boonluksiri et al., 2021; Mistry et al., 2022). It is mention that the composting is one of the most used methods of biodegradation for this type of material which

can be degraded within 180 days in these types of conditions (Kalita et al., 2021).

The morphology of the studied samples was determined using a scanning electron microscope (SEM). No modifications were observed on the surface of the tested samples at the end of the soil burial test (after 90 days). The analysis showed dense, continuous and smooth structure of the PLA based materials (Figure 3).

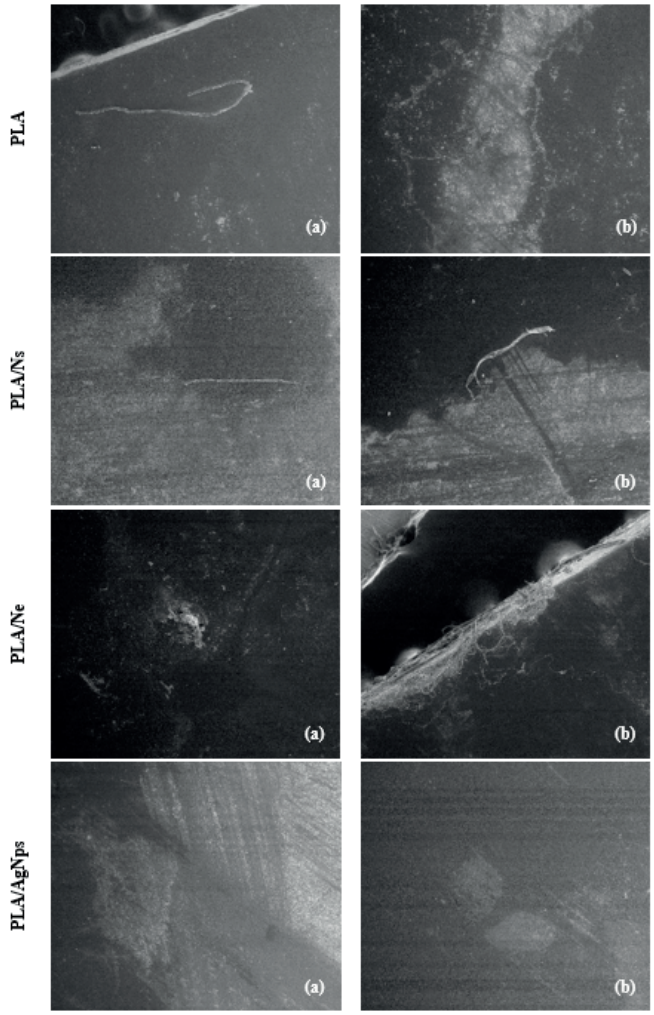

Figure 3. SEM morphology of PLA based samples at the beginning (a) and the end (b) of testing period

Colonisation and in vitro biodegradation test - results

The growth rate of both *Aspergillus brasiliensis* ATCC 16404 and *Aspergillus terreus* fungi on the surface of the studied polymeric samples is presented in Tables 4 and 5.

Table 4. The development degree of *Aspergillus brasiliensis* ATCC 16404 fungus on the surface of the tested polymeric materials

Sample	Incubation time (days)			
	M I		M II	
	30 days	60 days	30 days	60 days
PLA	0	0	0	1
PLA/Ns	1	1	1	2
PLA/Ne	1	1	1	2
PLA/AgNps	0	0	1	1

Table 5. The development degree of *Aspergillus terreus* fungus on the surface of the tested polymeric materials

Sample	Incubation time (days)			
	M I		M II	
	30 days	60 days	30 days	60 days
PLA	1	1	1	1
PLA/Ns	1	1	2	2
PLA/Ne	1	2	1	2
PLA/AgNps	0	0	0	1

The colonisation degree for the studied samples varied between 0 (absence of fungal growth) and 2 (expansion of moderate intensity with a maximum 50 % of the film covered with fungi), depending on the used culture media. The most susceptible samples to be colonised by both fungi were PLA/Ns and PLA/Ne samples. At the end of the testing period (after 60 days) on MII, both samples presented the highest amount of fungal growth on their surface compared to the other tested samples.

The colonisation degree was very low (0 on M I and 1 on M II) for PLA sample when *Aspergillus brasiliensis* ATCC 16404 was used and same values were obtained in the case of PLA/AgNps sample treated with *Aspergillus terreus*. Overall, the colonisation degree was higher for the samples inoculated and incubated on culture media M II, which contained carbon source for microorganism development. This result shows that in order to be colonised, the materials needed support in form of carbon source, found in the culture media. The aspect of the samples after 60 days of incubation is shown in Figure 4.

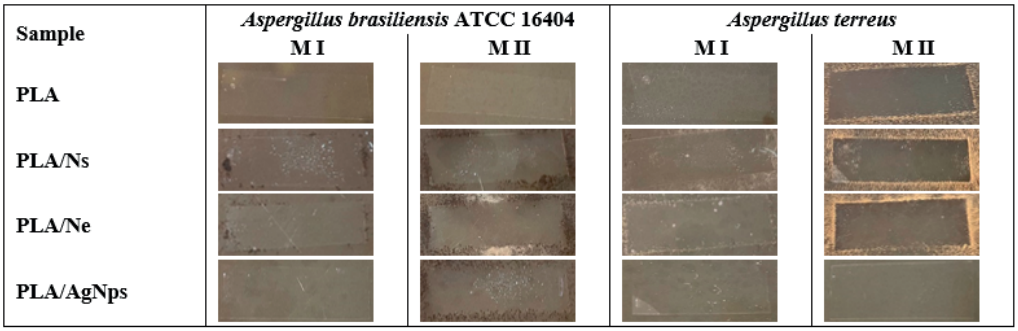

Figure 4. The aspect of PLA based materials after 60 days of incubation for colonisation assessment

At the end of the tested period, samples were taken out of the Petri dishes and cleaned, being stored in a desiccator until constant weight.

The *in vitro* biodegradation rate values for the studies samples are presented in Figures 5 and 6.

The results obtained for *in vitro* biodegradation using two fungal strains are in accordance with the colonisation rate of the studied samples (Table 4 and Table 5). The samples maintained on M II presented higher biodegradation rate compared to the samples exposed to M I, for both *Aspergillus brasiliensis* ATCC 16404 and *Aspergillus terreus*. The highest value for this parameter was determined for PLA/APV/Ns sample, of ~1.42% and ~4.61% on M II when inoculated with *Aspergillus brasiliensis* ATCC 16404 and *Aspergillus terreus,* respectively.

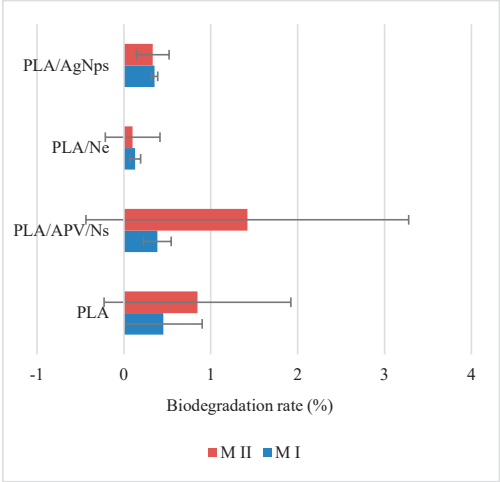

Figure 5. The *in vitro* biodegradation rate of PLA based samples by *Aspergillus brasiliensis* ATCC 16404 fungus

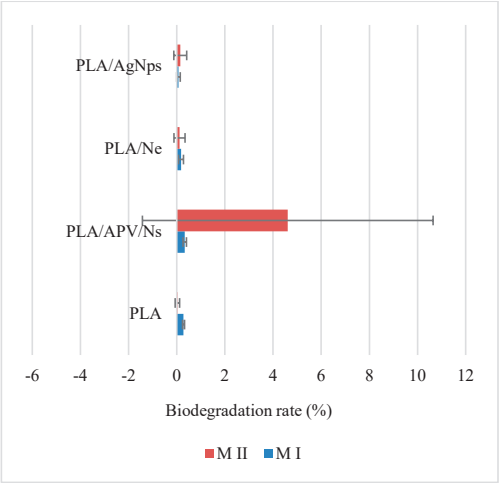

Figure 6. The *in vitro* biodegradation rate of PLA based samples by *Aspergillus terreus* fungus

Within natural environment there are few microorganisms that degrade PLA materials. Therefore, breaking down this polymeric matrix by microorganisms is realized to a lesser extent compared with other biopolymers (Janczak et al., 2018).

CONCLUSIONS

This study aimed at presenting the behaviour and biodegradation aptitude of some PLA based biomaterials approaching two biodegradation methods (soil burial biodegradation and degradation using microorganisms, namely two fungal strains of *Aspergillus brasiliensis* ATCC 16404 and *Aspergillus terreus*). The results are in accordance with other literature studies, PLA being a great biopolymer that could be used to replace conventional packaging plastics. However, its degradation in environmental conditions take a long time, more suitable for PLA biodegradation being composting conditions. The highest biodegradation rate at the end of testing period for soil burial test was obtained for PLA/AgNps sample (0.37%). In the case of *in vitro* biodegradation using fungi, the highest rate of biodegradation was recorded for PLA/Ns (4.61%). The results presented in this study are promising, even though the biodegradation rate is slow, the PLA based materials showed biodegradation properties.

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