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SUMMARY

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COMPARISON OF CLASSICAL VERSUS qPCR METHODS IN THE DETECTION OF *Botrytis cinerea* IN RASPBERRIES

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Abstract

Botrytis cinerea, is a necrotrophic fungus that causes gray mold on fruits and vegetables. This mold ranked second in the list of the top ten fungal pathogens. The aim of this study was to develop a system for rapid and accurate identification and quantification of B. cinerea on fresh raspberry by quantitative real-time PCR (qPCR) and a comparison was made between traditional methods (cell culture) and molecular tool. Raspberry samples were purchased from the local supermarkets. Raspberry diluted samples were cultivated on Dichloran Rose Bengal Chloramphenicol agar selective medium used for the growth of mold, then DNA was extracted from the same samples and qPCR was done. In classical methods (cell culture) the result was negative (no fungal colony growth). By the qPCR technique, B. cinerea was detected in two out of the three samples. In qPCR technology, the test proved to be selective, rapid and sensitive and can be used for the diagnosis of B. cinerea.

Key words*: raspberries, Botrytis cinerea, qPCR*

INTRODUCTION

The fungal genus *Botrytis* contains many species that vary in their biology, ecology, physical characteristics, and host range. About 30 species have been characterized as a result of advancements in molecular genetics, particularly the creation of pertinent phylogenetic markers. Relevant losses in a variety of commercially significant horticultural and floral crops are caused by *Botrytis species* (Poveda et al., 2020). The most often researched polyphagia fungus is *Botrytis cinerea* Pers.: Fr. The *Botrytis* community decided to use *Botrytis cinerea* as the generic name in 2013 at the *Botrytis* Symposium in Bari, Italy, despite the fact that *B. cinerea* is the name of the asexual stage (anamorph) and *Botryotinia fuckeliana* is the name of the sexual stage (Fillinger et al., 2016), sclerotic grow within degenerating host tissues as part of this fungus's life cycle, serving as a crucial survival mechanism. In temperate areas, sclerotic begins to grow in the early spring to produce conidiophores and multinucleate conidia. This fungus reproduces sexually by spermatizing sclerotic, which results in the development of apothecia and asci. Eight binucleate ascosporic are the main source of inoculum inside a crop (Holz et al., 2007). The steps of the *B. cinerea* infection process are typically listed as follows: penetration of the host surface, death of the host tissue/primary lesion formation, lesion expansion/tissue maceration, and sporulation (Choquer el at., 2007). The symptoms caused by this necrotrophic fungus are extremely varied and difficult to generalize to plant organs and tissues. The most common symptoms of soft rots on leaves and soft fruits are collapse, water infiltration of the parenchyma tissues, and the quick emergence of grey masses of conidia. When it comes to many fruits and vegetables, the illness frequently starts on adjacent senescent flowers before spreading as a soft rot and harming nearby developing fruit (blossom-end rot), including raspberries, strawberries and apples. More than 50 hosts have been reported being infected by seed-borne diseases, which frequently start by rotting the herbaceous stems at ground level and also cause additional soft-rot lesions to emerge on leaves and pods (Williamson et al., 2007). In this sense *B. cinerea* is an intriguing model system for necrotrophic diseases, although studying it can be challenging due to the frequent changes in karyotypes among naturally strains (Fillinger et

al., 2016). One of the most thoroughly researched necrotrophic fungal diseases, *B. cinerea*, causes gray mold rot in more than 500 plant species (Williamson et al., 2007; Mercier et al., 2019). Raspberry, grape, strawberry, and tomato are just a few of the commercially significant crops which this disease has a terrible effect on (Dean et al., 2012) and can exist in the stems, leaves, flowers, fruits, and seeds of several plant species. It may manifest overt illness symptoms prior to harvest or after harvest (Fillinger and Elad, 2016). In addition, one of the most significant post-harvest infections in fresh fruits and vegetables has been identified as being induced by *B. cinerea* (Zhang et al., 2014). *B. cinerea* causes annual economic losses that easily surpass $10 billion in the world (Weiberg et al., 2013). *B. cinerea* has been ranked as the second major plant pathogen due to its scientific and economic value (Dean et al., 2012). Because *B. cinerea* has a wide host range, a variety of assault methods, and both asexual and sexual phases that can live in either favorable or unfavorable conditions, it is challenging to control (Fillinger and Elad, 2016). Conidia, the asexual spores of *B. cinerea*, are easily disseminated by wind or water, and sclerotic, the sexual spores of *B. cinerea*, are necessary for survival in adverse environments (Brandhoff et al., 2017). The raspberry is a third-generation fruit with an improved flavor, excellent nutritional and therapeutic properties. It is a non-climacteric variant (McDougall et al., 2017; Xing et al., 2018). But raspberries also have strong respiration rates, delicate fruit tissues, and are vulnerable to mechanical harm when being harvested. This results in softer tissues that are more vulnerable to infection by pathogens after harvesting, which makes shelf life and storage difficult (Tezotto-Uliana et al., 2013; Guerreiro et al., 2015). Researchers have conducted significant research regarding the identification and occurrence of postharvest raspberries diseases, in order to solve the issues of postharvest storage and the management of raspberries fresh supply of (Carisse et al., 2018; López et al., 2016).

The production of organic fruit has steadily expanded over the past few decades, as has its market share in the global food industry. The growth of strawberries, blueberries, and raspberries fruits that are significant exports from Central Europe is driven in large part by rising consumer demand for organic fruit production. Fungi-related infections, which affect plants and fruits from the time of sowing to the point of market sale and result in unanticipated product degradation, are the main worries of food producers. Identifying the causal agents of soil-borne diseases has been accomplished using morphological methods of identification. These conventional techniques are time consuming, are prone to mistakes, and may lead to false results. Due to these drawbacks, more effective approaches are increasingly being adopted, such as using molecular-level analytical techniques. Polymerase chain reaction (PCR)-based techniques enable the rapid amplification of specific DNA fragments (Malarczyk et al., 2019). Experts investigating morphological characteristics of microorganisms, such as colony appearance and the formation of asexual structures on microbiological media or on the host, are classic techniques of identifying fungal pathogens. Samples that have been isolated on suitable agar media can be examined under a light microscope to detect even the smallest features. Colonies are evaluated and counted may be assessed using this procedure (classic techniques), which takes a lot of time. On occasion, colonies must fulfil specific requirements in order to produce conidia, which can impede laboratory work-flow (Frąc et al., 2015). For identification, selective media have been suggested and deployed. Although most species are not host specific and many fungi may live on plants, the identification of external infection symptoms caused by fungus on their hosts can also be utilized to confirm the pathogen. A correct identification based solely on the morphology of the colonies is highly challenging, because to the lack of carrier specificity and symptom changes between plant populations at different latitudes. Interpretations of the pathogen's morphology are also arbitrary and heavily dependent on personal experience. The human aspect could result in erroneous pathogen detection and misguided plant protection measures (Malarczyk et al., 2019). The aim of this study was to compare the classical traditional methods for the detection of *B. cinerea* in raspberry fruits and compare them with qPCR technique, show the pros and cons

and which are better and find the best and fastest methods for the detection of *B. cinerea* in raspberry.

MATERIALS AND METHODS

Raspberry samples were purchased from different food stores of different international origins, taking into account the most popular markets. The samples codes and origin are presented below:
T1: Mega Image - origin: Morocco
T2: Shop & Co. - origin: Portugal
T3: Lidl- origin: Spain
Sample preparation: 5 g of each sample were placed in a sterile vial homogenized with 45 ml of distilled water. They were cultured on Dichloran Rose Bengal Chloramphenicol agar. The total DNA was extracted from the same samples to be used for the qPCR technique.

Morphological characterization

A sample of 1 ml was taken from each sample and diluted 4 times serial dilution (1, 2, 3 and 4) were worked out, then cultured on the selective medium (Dichloran Rose Bengal Chloramphenicol Agar) used for mold growth and placed in the incubator for 72 hours at a temperature of 37°C.

Standard curve for *B. cinerea*

Microbial DNA was extracted from raspberry samples from each sample (T1, T2 and T3). 1 ml was taken from each sample, vortexed and centrifuged for 5 minutes at 10,000 rpm, then *B. cinerea* DNA was extracted and diluted. DNA was quantified using QuickDrop, then DNA extraction was performed with kit Quick DNA Fungal/Bacterial Miniprep kit (Zymo Research Germany).

Real-time PCR amplification

Specific *B. cinerea* primers targeting the ribosomal region between 28S and 18S genes (intergenic spacer) were used: Bc3F (5'–GCTGTAATTTCAATGTGCAGAATCC-3') and Bc3R (5'-GGAGCAACAATTAATCGCATTTC-3'). The DNA sample (5 mL) was mixed in a final volume of 25 mL with *B. cinerea* primer mixture. The program was used: 3 min at 95ºC, followed by 40 cycles of 15 s at 95ºC and 30 s at 62ºC. A melting curve was established by decreasing the temperature from 90ºC by 0.5ºC every 10 s. All reactions were performed in triplicate. The cycle threshold (C_t), or the PCR cycle where fluorescence first occurred, was determined automatically using BIO-RAD software after setting the baseline to 100. The efficiency (E) of the PCR assay was calculated using the formula, $E = [10^{-1/slope} -1] \times 100$, where the slope was extracted from the curve $C_t = f(\log Q0)$ and Q0 is the initial DNA or cell population in the assay. E was expressed as percentage.

Statistics

The mean SD is used to express all values. The statistical program SIGMASTAT 3.0 from statistic was used to analyses the data. Inc. One-way ANOVA was used to analyses group differences. As indicated by Zar, post hoc comparisons were made using the Holm-Sidak comparison test (1996). Statistical significance was defined as a P value of 0.001 or 0.05.

RESULTS AND DISCUSSIONS

Culture-based techniques are sometimes difficult to use because they are laborious and time-consuming and may not be able to detect pathogens in the viable but non-culturable state (VBNC) produced by stress, when they are present in the food (Foddai et al., 2020). The qPCR reaction has a high efficiency, specificity and sensitivity, and it is able to amplify the lowest concentration of DNA. (Si Ammour et al., 2019). Agar with Dichloran Rose Bengal Chloramphenicol has been used to grow *B. cinerea* spores from samples for the detection of fungi. Then qPCR was used to detect the possible presence of *B. cinerea* in the raspberries samples using primers Bc3F and Bc3R. The material was used according to the description of the study (Diguta et al., 2010), (Tanovic et al., 2014).

Culture-based method to detect *B. cinerea*

After plating raspberry samples on Dichloran Rose Bengal Chloramphenicol agar, no mycelium or any indication of *B. cinerea* was detected. Figure 1 shows plates with Dichloran Rose-Bengal Chloramphenicol (DRBC) Agar media without any *B. cinerea* growth. Two hypothesis can be approached: no *B. cinerea*

was present on the sample, or the detection method is not suitable when the contamination level is low.

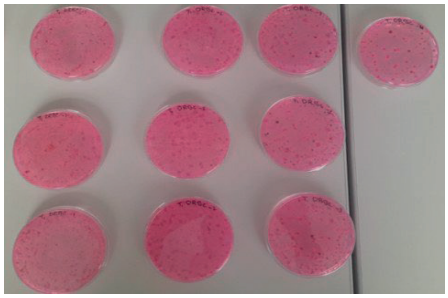

Figure 1. Aspects of total cells charge of raspberries fruits. No *B. cinerea* was detected on DRBC medium on plate

PCR-based method to detect *B. cinerea*

The amplification plots e generated with the Bc3F/Bc3R primer pair is presented in Figure 2.

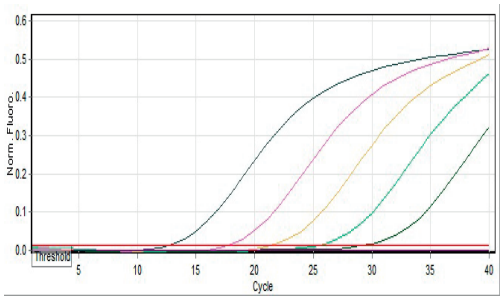

Figure 2. The amplification plots of *B. cinerea*

The standard curve for *B. cinerea* was generated by plotting the Ct value determined by qPCR and the logarithm of the DNA concentration (fg). Linearity was observed throughout the range used and a correlation coefficient (R^2 = 0.9929) indicated very low inter-assay variability. The slope of the standard curve was -3.737, which corresponded to an amplification efficiency of 85.18%. Under the conditions described, the maximum C_t value that could be used was 32, which corresponds to a DNA concentration of 9.8 fg as shown in Figure 3.

The concentration of *B. cinerea* DNA present in each raspberry sample is shown in the Figure 4. In the present experiment, *B. cinerea* was detected in samples 1 and 2. In sample 3, *B. cinerea* was not detected by qPCR (being lower than the established detection limit of 9.8 fg *B. cinerea* DNA).

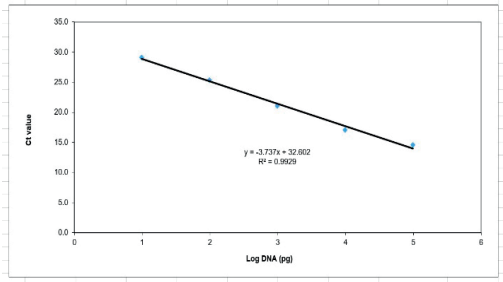

Figure 3. Standard curve generated from the amplification of *B. cinerea* DNA

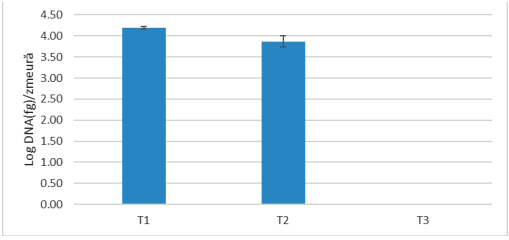

Figure 4. The concentration of *B. cinerea* DNA detected on raspberries by qPCR

The current results were compared with previous studies, where it was compared with (Malarczyk et al., 2019), who diagnosed plant pathogenic fungi that affect berry crops, as well as (Si Ammour et al., 2019), who detected *Botrytis cinerea* in Grapevine Bunch Trash by qPCR. And it was demonstrated that the qPCR reaction has a high efficiency of up to 96%, which indicates excellent sensitivity, and that it is able to amplify the lowest concentration of DNA.

CONCLUSIONS

In this article has been compared a specific and sensitive qPCR protocol with conventional methods for the detection and quantification of *B. cinerea* in raspberries. Conventional methods are useful methods, but they are somewhat inaccurate and require more time and effort compared to qPCR.

Conventional methods require cell counting, as well as the rapid growth of some types of bacteria, which leads to concealment of the main cause, while the qPCR method is characterized by accuracy. It can detect the least amount of *B. cinerea* presented in the sample, obtained accurate results. A commercially available kit was used to isolate fungal DNA, which is an

effective and straightforward technique that enables the routine examination of more samples per day.

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SCREENING OF SSR AND EST-SSR MARKERS FOR BACKGROUND SELECTION IN MUNGBEAN

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Abstract

This study aimed to quantify the recurrent parent genome (RPG) recovery of the new mungbean introgression lines using SSR and EST-SSR markers, and selection of backcross progenies of the crosses between a resistant line (SUPER5) and two recurrent parents (KING and H3). A total of 160 SSR and EST-SSR markers covering 11 mungbean chromosomes were applied for parental polymorphism identification. Among these, 6 polymorphic markers were linked to domestication related traits located on linkage groups (LGs) 1, 3, 7 and 10. Twenty-seven (16.9%) and 23 (14.4%) SSR and EST-SSR markers were found to be distinct and clearly polymorphic between KING and SUPER5, and H3 and SUPER5, respectively. These polymorphic markers were utilized to analyse the RPG recovery in BC_1F_1 and BC_2F_1 progenies. The BC_2F_1 selected progenies with high RPG recovery ranging from 87.2-94.7% and 92.1-97.8% in KING and H3 populations, respectively, were further used to produce BC_3F_1 seeds. These results suggest that SSR and EST-SSR markers can be used in marker-assisted selection (MAS) for background selection to accelerate backcrossing.

Key words*: marker-assisted backcross breeding, molecular markers, polymorphism, recurrent parent genome recovery, Vigna radiata (L.) Wilczek.*

INTRODUCTION

Mungbean [*Vigna radiata* (L.) Wilczek], also known as green gram, is a type of legume that is commonly grown in Asia, Africa, and South America. It is a good source of protein, dietary fiber, and various vitamins and minerals (Hou et al., 2019). Additionally, mungbeans are often used as a cover crop, which can help to improve soil health by adding organic matter and nitrogen to the soil (Kocira et al., 2020). They are also used for forage, as a food for livestock. Overall, mungbeans play an important role in food security and sustainable agriculture. Mungbean is a significant crop in Thailand and grown primarily in the northeastern and northern regions of the country. According to the Chai Nat Field Crops Research Center, Department of Agriculture (2021), mungbean production in Thailand has been steadily increasing in recent years due to increasing demand for the crop both domestically and internationally. However, there are several limitations to mungbean production due to climate challenging because the crop is sensitive to temperatures and humidity. This can lead to reduced yields and increased susceptibility to pests and diseases. Mungbean is susceptible to a variety of pests and diseases, such as leafhoppers, thrips, Cercospora leaf spot (CLS), powdery mildew (PM), which can significantly reduce yields (Nair et al., 2019). Therefore, developing new mungbean varieties that have improved characteristics such as increased yield, improved quality, and resistance to pests and diseases is urgently needed.

Molecular markers have become an essential tool in modern plant breeding and have played an important role in the development of newly improved varieties of plants that have specific characteristics such as disease resistance, yield, and quality. Simple sequence repeat (SSR), also known as microsatellite, is a type of molecular markers that is widely used in genetics and plant breeding. SSR markers are based on the presence of short, repetitive DNA sequences that are scattered throughout the genome. These repetitive sequences can vary in number and size

among different individuals, making them useful for identifying genetic variation. Expressed sequence tag-SSR (EST-SSR) marker is a type of molecular markers that is derived from expressed genes in a genome. ESTs are short, single-pass DNA sequences that are generated from complementary DNA (cDNA) libraries, which are constructed from messenger RNA (mRNA) molecules that are transcribed from actively expressed genes. The marker-assisted backcrossing (MABC) is the backcrossing technique assisted by molecular markers which may accelerate the recovery of the recurrent parent genome (RPG) through two selection steps consisting of foreground and background selection (Ellegren, 2004; Luo et al., 2010; Kalia et al., 2011).

Therefore, the objectives of this study were to identify SSR and EST-SSR markers for background selection and to quantify the percentage of RPG recovery of the new backcross progenies in mungbean.

MATERIALS AND METHODS

Plant materials and crossing scheme

The genotypes used in this study were King originating from Australia, and SUPER5 and H3 lines developed by our research group at Suranaree University of Technology (SUT), Thailand. The seeds of SUPER5 as a donor line (F_9) were planted and the four best plants including SUPER5-1, SUPER5-2, SUPER5-3, and SUPER5-4 with no disease symptoms and growing well were selected to be the resistant source for the F_1 generation. Crosses were made between these resistant lines, which had CLS and PM resistance genes derived from the double cross [(CN72 × V4758) × (CN72 × V4718)] × [(CN72 × V4718) × (CN72 × V4785)] (Pookamsak, unpublished data), and either a susceptible variety with high yield KING or H3 the breeding line resistant to CLS but moderately resistant to PM diseases developed by Papan et al. (2022). Foreground selection was carried out for selecting the F_1 progenies that had a CLS and 2 PM resistance genes. These progenies were further used as male parents for backcrossing to generate BC_1F_1 until BC_2F_1 generations. The parental lines, the selected resistant BC_1F_1 and BC_2F_1 progenies through foreground selection and detached leaf assay were screened by marker-assisted selection (MAS) for background selection.

DNA isolation and PCR amplification

Fresh young leaves of seedlings were collected from parents (SUPER5-1, SUPER5-2, SUPER5-3, and SUPER5-4, KING and H3) and selected resistant lines in BC_1F_1 and BC_2F_1 generations, and the genomic DNA of each genotype was extracted by the cetyl trimethyl ammonium bromide (CTAB) method as described by Lodhi et al. (1994). For use in PCR analysis, the DNA concentration and purity were determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) at the A_{260} and A_{280} and adjusted to a final concentration of 150 ng/µl. The polymerase chain reaction (PCR) of SSR and EST-SSR markers were performed in a 20 µl reaction mix containing 150 ng of genomic DNA template, 1 × buffer (50 mM KCL, 10 mM Tris-HCL, pH 9.1, 0.01% Triton^{TM} X-100), 2 mM $MgCl_2$, 0.2 mM of each dNTPs, 1 unit of *Taq* DNA polymerase, and 0.5 µM each of forward and reverse primers. The PCR reactions were subjected to amplification with initial denaturation at 95°C for 5 min; 35 cycles of denaturing at 95°C for 1 min, annealing at 50-60°C for 1 min, extension at 72°C for 1 min; and a final extension at 72°C for 10 min. This protocol was described by Chen et al. (2015), Arsakit et al. (2017), Poolsawat et al. (2017) and Papan et al. (2021). The PCR products of these markers were electrophoresed on 6.0% PAGE at 200 V for 40-70 min depending on the specificity of primers, and stained with the silver nitrate method according to Sambrook and Russell (2001). All amplifications were repeated at least twice, and only clear bands were considered for analysis.

SSR and EST-SSR analysis

One hundred and sixty SSR and EST-SSR primers that have been reported by Isemura et al. (2012) were used in this study for identifying polymorphism between the parents SUPER5, KING and H3. These primers were associated with domestication traits, putative protein functions and unknown functions in mungbean (Table 1). Out of these 160 markers, 63 markers were linked to the domestication related traits such as 100-seed weight, pod length, stem

length, days to first flower, days to maturity of first pod etc. The polymorphic markers between SUPER5 (SUPER5-2 and SUPER5-4) and KING, and SUPER5 (SUPER5-1 and SUPER5-3) and H3 were then used for background selection of the BC_1F_1 and BC_2F_1 progenies in the KING×SUPER5 (SUPER5-2 and SUPER5-4) and H3×SUPER5 (SUPER5-1 and SUPER5-3) populations, respectively.

Data scoring and analysis

The clearly amplified alleles of each primer were coded using different combinations of 0 and 1. The polymorphic markers were further analyzed for similarity coefficients between various genotypes, in a pair-wise comparison using Jaccard's coefficient, and the similarity matrix was analyzed using the unweighted pair-group method arithmetic average (UPGMA) clustering algorithm; the computations were carried out using NTSYSpc version 2.0 (Rohlf, 2000).

RESULTS AND DISCUSSIONS

Identification of polymorphic markers for background selection

Out of 160 markers, 27 (16.9%) and 23 (14.4%) polymorphic markers were found in KING and H3 populations, respectively. The number of markers/linkage group, length, and the primer name of polymorphic markers were showed in Tables 2 and 3 for KING × SUPER5 and H3 × SUPER5 crosses, respectively. These polymorphic markers were distributed on various linkage groups (Figure 1).

Genetic analysis of backcross progenies derived from KING × SUPER5 cross

In this study, we evaluated genetic profiles of 4 BC_1F_1 progenies derived from crossing between recurrent parent KING and donor parent SUPER5-2 and 3 BC_1F_1 progenies derived from crossing between recurrent parent KING and donor parent SUPER5-4. All of these 7 progenies had one CLS resistance gene and two PM resistance genes identified by using 6 linked marker loci including I16274, I85420, I42PL222, I27R565, VrTAF5_Indel, and VrMLO12_Indel3 markers which were reported in previous studies (Poolsawat et al., 2017; Yundaeng et al., 2020; Tantasawat et al., 2021). One hundred and sixty SSR and EST-SSR primers unlinked to these resistance genes were screened in both parents and 27 were found to be polymorphic (16.9%). The pairwise genetic similarity between all mungbean genotypes studied varied from 0.526 (SUPER5-2 vs K30, K147, K171 and SUPER5-4 vs K30, K147, K171) to 1.000 (SUPER5-2 vs SUPER5-4). The Mantel test in the materials studied with a cophenetic correlation coefficient value of 0.98, indicated that data in the similarity matrix were relatively well presented by dendrogram (Figure 2). The UPGMA dendrogram separated all genotypes into 2 major clusters. Cluster I included KING and all BC_1F_1 progenies including K9, K41, K30, K20, K147, K150, and K171, while cluster II consisted of the 2 donor lines, SUPER5-2 and SUPER5-4. In the cluster I, there were 2 subclusters including IA, IB and one individual, KING. Subcluster IA contained all 4 BC_1F_1 progenies derived from KING × SUPER5-2, while Subcluster IB contained all 3 BC_1F_1 progenies derived from KING × SUPER5-4. In the population of KING × SUPER5-2, the percentage of RPG recovery ranged from 68.8 to 75.0%. The high genetic similarity of 75.0% with KING was observed in one of the four progenies from this cross, K9. In the population of KING × SUPER5-4, one backcross progeny, K147, was found to be most genetically related to KING with the genetic similarity of 77.9% and the percentage of RPG recovery ranged from 75.8 to 77.9%, while the genetic similarity between donor lines (SUPER5-2 and SUPER-4) and KING were distantly related (57.1%) (Figure 2). All of these 7 BC_1F_1 were backcrossed to KING to produce BC_2F_1.

In the BC_2F_1 generation, 15 progenies generated from 7 BC_1F_1 progenies had 3 CLS and PM resistance genes, and were evaluated for their genetic profiles compared with their parents. The pairwise genetic similarity varied from 0.526 (SUPER5-2 vs K9-17, K9-32, K30-239 and SUPER5-4 vs K9-32, K30-239) to 1.000 (SUPER5-2 vs SUPER5-4 and K9-60 vs K150-374). The Mantel test with a cophenetic correlation coefficient value of 0.98, indicated that data in the similarity matrix were relatively well presented by dendrogram (Figure 3).

Table 1. SSR and EST-SSR primers used for screening background selection

Primer name	Linkage groups	Primer name	Linkage groups	Primer name	Linkage groups	Primer name	Linkage groups
CEDCAA001	1	CEDG117	3	cp09781	6	CEDG070	9
CEDG051	1	CEDG176	3	GMES0294	6	CEDG166	9
CEDG074	1	CEDG186	3	GMES0659	6	CEDG259	9
CEDG141	1	CEDG305	3	GMES1156	6	CEDG267	9
CEDG214	1	CEDGAT008	3	GMES3316	6	CEDG290	9
CEDG220	1	GMES0214	3	MBSSR021	6	GATS11	9
CEDG241	1	GMES0294	3	CEDG041	7	GMES0206	9
CEDG256	1	GMES0963	3	CEDG064	7	GMES1216	9
cp04220	1	GMES6583	3	CEDG085	7	GMES3893	9
cp05137	1	VR108	3	CEDG174	7	VCEDG056	9
cp06173	1	CEDC055	4	CEDG176	7	VM27	9
GMES0294	1	CEDG074	4	CEDG186	7	CEDG026	10
GMES1216	1	CEDG088	4	CEDG218	7	CEDG075	10
GMES1604	1	CEDG107	4	CEDG295	7	CEDG097	10
GMES2320	1	CEDG154	4	cp00228	7	CEDG113	10
GMES3316	1	CEDG232	4	cp05941	7	CEDG116	10
GMES4400	1	CEDG269	4	cp06427	7	CEDG150	10
VrD1	1	cp00416	4	cp07863	7	CEDG198	10
CEDAAG002	2	cp00674	4	GMES0856	7	cp02585	10
CEDC050	2	DMBSSR199	4	GMES4101	7	cp05325	10
CEDG006	2	GMES0216	4	GMES6625	7	cp05914	10
CEDG026	2	GMES1124	4	MBSSR008	7	BM149	11
CEDG050	2	GMES1156	4	CEDC031	8	CEDG002	11
CEDG168	2	GMES1216	4	CEDG030	8	CEDG013	11
cp00228	2	GMES3316	4	CEDG040	8	CEDG044	11
GMES0214	2	MBSSR015	4	CEDG059	8	CEDG072	11
GMES0216	2	BM170	5	CEDG071	8	CEDG075	11
GMES0477	2	CEDG008	5	CEDG099	8	CEDG076	11
GMES0856	2	CEDG132	5	CEDG247	8	CEDG098	11
GMES1156	2	CEDG171	5	CEDG257	8	CEDG168	11
GMES3316	2	CEDG184	5	CEDG269	8	CEDG281	11
GMES4137	2	CEDCAA001	6	CEDG271	8	cp00464	11
MBSSR015	2	CEDG037	6	CEDG302	8	cp05096	11
VR0200	2	CEDG041	6	cp06108	8	cp08695	11
CEDAAG004	3	CEDG121	6	GMES0206	8	cp10667	11
CEDC008	3	CEDG146	6	GMES0856	8	GMES0216	11
CEDG010	3	CEDG169	6	GMES5301	8	GMES3893	11
CEDG043	3	CEDG191	6	VM37	8	GMES5007	11
CEDG063	3	CEDG245	6	VR0255	8	GMES5575	11
CEDG084	3	CEDG282	6	CEDG024	9	GMES6098	11

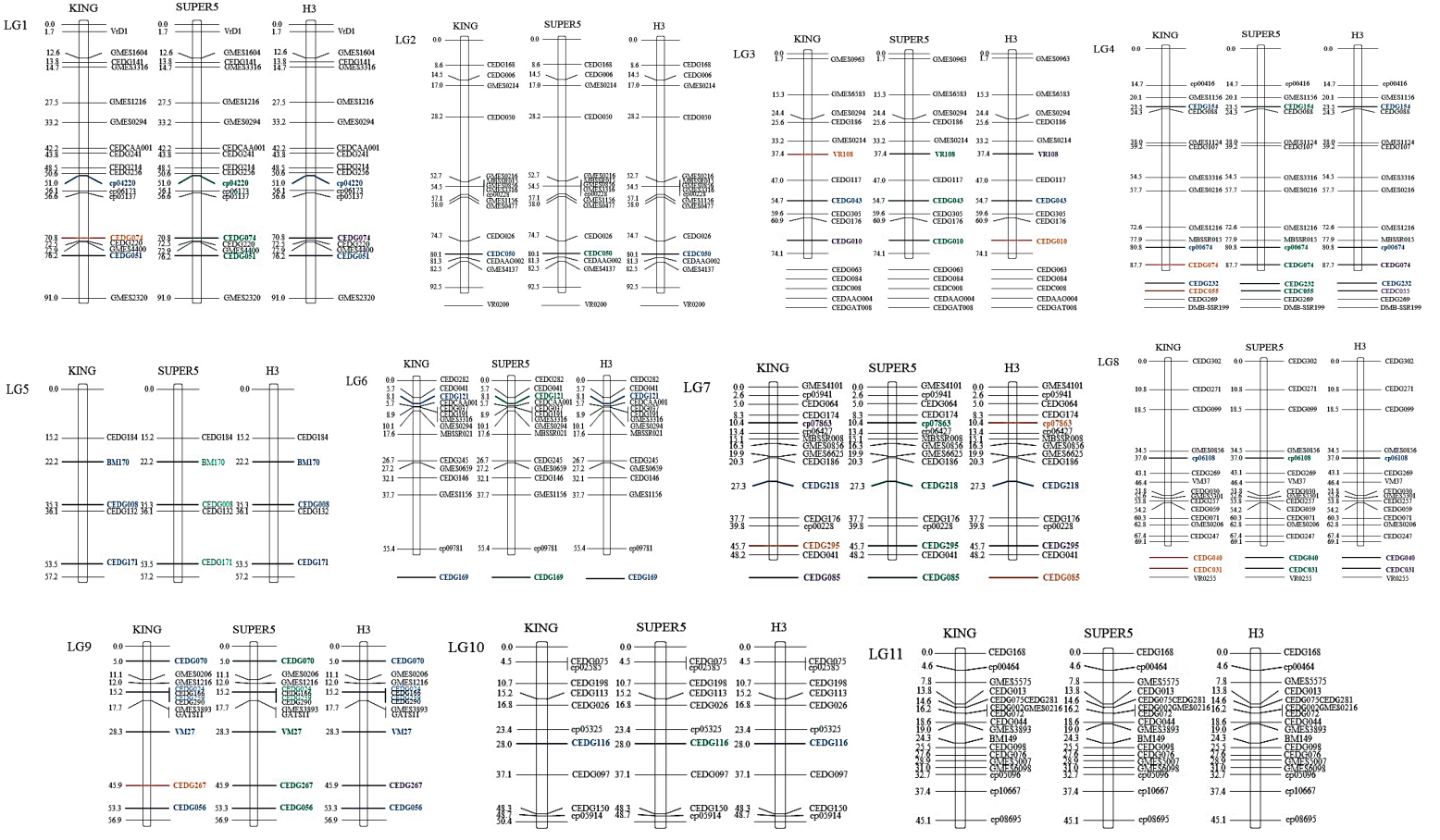

Figure 1. SSR and EST-SSR markers associated with domestication related traits and other putative protein functions or unknown functions in mungbean (Isemura et al., 2012). Different colors demonstrate polymorphism between parental variety/lines

Table 2. Polymorphic SSR and EST-SSR markers used for genetic analysis of backcross progenies derived from KING × SUPER5 cross (Isemura et al., 2012)

Linkage groups	Length (cM)	No. of total markers	No. of polymorphic markers	Primer name
1	91.0	18	3	CEDG051, CEDG074[a], cp04220[b]
2	92.5	16	1	CEDC050
3	74.1	16	2	CEDG043, VR108
4	87.7	16	5	CEDG154, CEDG232, CEDG074, CEDC055, cp00674
5	57.2	5	3	CEDG008, CEDG171, BM170
6	55.4	15	2	CEDG121, CEDG169
7	48.2	16	2	CEDG218[c], CEDG295
8	69.1	17	3	CEDG040, CEDC031, cp06108[d]
9	56.9	12	6	CEDG024, CEDG056, CEDG070, CEDG259, CEDG267, VM27
10	50.4	10	1	CEDG116[e]
11	45.1	19	0	-

[a] 100-seed weight
[b] Pod length
[c] Primary leaf width
[d] Hypocotyl plus epicotyl length
[e] Length of the internodes

Table 3. Polymorphic SSR and EST-SSR markers used for genetic analysis of backcross progenies derived from H3 × SUPER5 cross (Isemura et al., 2012)

Linkage groups	Length (cM)	No. of total markers	No. of polymorphic markers	Primer name
1	91.0	18	2	CEDG051, cp04220[a]
2	92.5	16	1	CEDC050
3	74.1	16	2	CEDG010, CEDG043
4	87.7	16	3	CEDG154, CEDG232, cp00674
5	57.2	5	3	CEDG008, CEDG171, BM170
6	55.4	15	2	CEDG121, CEDG169
7	48.2	16	3	CEDG085, CEDG218[b], cp07863[c]
8	69.1	17	1	CEDG040
9	56.9	12	5	CEDG024, CEDG056, CEDG070, CEDG259, VM27
10	50.4	10	1	CEDG116[d]
11	45.1	19	0	-

[a] Pod length
[b] Primary leaf width
[c] Number of twists along the length of the dehiscence pod when kept at room temperature
[d] Length of the internodes.

Based on UPGMA analysis, 2 major clusters were constructed, cluster I contained all BC_2F_1 progenies and KING and cluster II contained both donor lines, SUPER5-2 and SUPER5-4. Cluster I was further subdivided into 2 subclusters; IA (KING, K9-10, K147-335, K171-448, K9-17, K9-32, K9-66 and K9-78) and IB (K9-60, K150-374, K41-297, K150-364, K20-158, K20-163, K30-239 and K150-396). The similarity of all backcross progenies with KING ranged from 77.9% to 94.7%. Note that 5 backcross progenies including K9-10, K9-17, K9-32, K9-66 and K9-78 obtained from KING × SUPER5-2 were in subcluster IA with KING, all of them were the progenies of the BC_1F_1 K9 (showing 75.0% similarity with KING) and had similarity of 85.1% to 94.7% with KING. The maximum similarity of 94.7% with KING was observed in the K9-10. The remaining 2 progenies K147-335 and K171-448 obtained from KING × SUPER5-4 in this subcluster had similarities of 87.5% and 88.4% with KING, respectively, while other progenies in subcluster IB had similarity of 77.9% to 86.3% with KING. Meanwhile, the percentage of genetic similarity of both donor lines with KING were found at 55.7% (Figure 3). Of these 15 plants, 4 BC_2F_1 plants (K9-10, K9-17, K147-335, and K171-448) that showed high RPG recovery were backcrossed to the recurrent parent to produce BC_3F_1 seeds.

Genetic analysis of backcross progenies derived from H3 × SUPER5 cross

Among the BC_1F_1 progenies of H3 × SUPER5 population, 4 BC_1F_1 progenies derived from crossing between SUPER5-1 as a donor parent and H3 as a recurrent parent, and 4 BC_1F_1 progenies derived from crossing between SUPER5-3 and H3 were selected for PM resistance genes using VrMLO12_Indel3 marker locus (Yundaeng et al., 2020). Twenty-three out of 160 SSR and EST-SSR markers which associated with domestication traits, putative protein functions and unknown functions in mungbean were found to be polymorphic (14.4%). The pairwise genetic similarity between all mungbean genotypes in this generation varied from 0.529 (SUPER5-1 vs H218, H230 and SUPER5-3 vs H218, H230) to 1.000 (SUPER5-1 vs SUPER5-3).

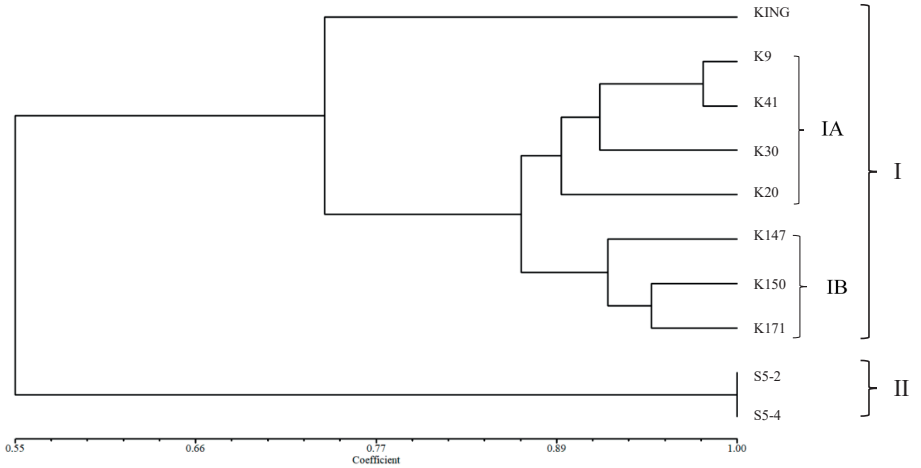

Figure 2. Dendrogram of 7 BC_1F_1 progenies and 3 parental variety/lines in KING × SUPER5 population. Dendrogram shows similarity coefficient and genetic relationships among 10 genotypes of mungbean

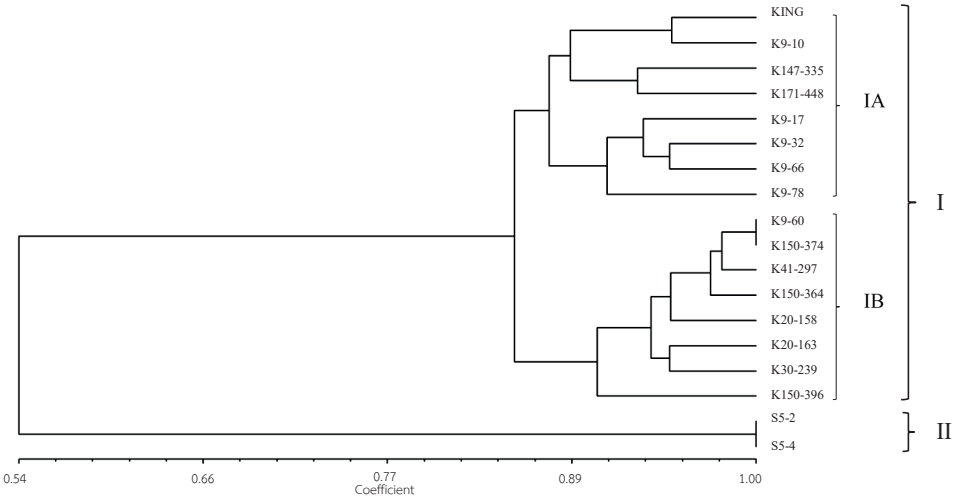

Figure 3. Dendrogram of 15 BC_2F_1 progenies and 3 parental variety/lines in KING × SUPER5 population. Dendrogram shows similarity coefficient and genetic relationships among 18 genotypes of mungbean

The UPGMA dendrogram was carried out for the Mantel test with a cophenetic correlation coefficient value of 0.97 indicating that data in the similarity matrix were relatively well presented by dendrogram (Figure 4). Eleven genotypes were separated into two major clusters by the UPGMA dendrogram. All BC_1F_1 progenies and their recurrent parent were included in Cluster I. Cluster II consisted of the two donor lines, SUPER5-1 and SUPER5-3. The similarity of all backcross progenies in this generation with H3 ranged from 69.8% to 84.1% and the highest of genetic similarity of 84.1% was observed in H207 progeny from the cross between H3 × SUPER5-1. In the cross of H3 × SUPER5-3, two BC_1F_1 progenies H230 and H247 were found to be most genetically related to H3 with the genetic similarity of 74.4%.

While, the similarity between parents, H3 with SUPER5-1 and SUPER5-3 were 56.2% (Figure 4). However, all 8 promising plants were still selected to produce BC_2F_1 seeds.

In BC_2F_1 generation, the Mantel test of H3 × SUPER5 population showed a cophenetic correlation coefficient value of 0.95 indicating that data in the similarity matrix were relatively well presented by dendrogram. The pairwise genetic similarity varied from 0.512 (SUPER5-1 vs H247-552) to 1.000 (SUPER5-1 vs SUPER5-3). The UPGMA dendrogram was constructed using 18 genotypes and it separated into 2 major clusters. Cluster I was further subdivided into 2 subclusters; IA (H3, H207-519, H210-533, H218-537, H206-510, H218-536, H206-514, H210-530, H252-572, and H252-575), and IB (H207-521, H230-540, H248-561, H248-562, H230-544, and H247-552). The cluster II consisted of 2 donor lines (SUPER5-1 and SUPER5-3) that are distantly related to H3 (0.578). BC_2F_1 progenies showed the presence of 84.1-97.8% of RPG recovery in this generation. Among these, the H207-519 and H210-533 in subcluster IA showed the highest genetic similarity of 97.8% with H3. They were the progenies of the BC_1F_1, H207 and H210 (showing 84.1 and 81.8% similarity with H3, respectively) (Figure 5).

Only the BC_2F_1 plants that had higher RPG recovery and produced maximum seeds were backcrossed to the recurrent parent to produce the BC_3F_1 seeds. Among these, H207-519, H210-533, H218-536, and H218-537 were used as male parents to produce BC_3F_1 seeds.

Marker-assisted background selection is useful for obtaining information on the RPG recovery. In this study, we screened a total of 160 SSR and EST-SSR markers. Out of these, 27 (16.9%) and 23 (14.4%) of them were found polymorphic between parents in KING × SUPER5 and H3 × SUPER5 populations, respectively. Among these, 6 polymorphic markers were reported to be linked to domestication related traits i.e., 100-seed weight, pod length, length of the internodes, primary leaf width, hypocotyl plus epicotyl length and number of twists along the length of the dehiscence pod when kept at room temperature, and located on linkage groups (LGs) 1, 3, 7 and 10 (Isemura et al., 2012). When using the polymorphic markers for background selection in two populations (KING × SUPER5 and H3 × SUPER5 populations), the genetics of all BC_1F_1 and BC_2F_1 progenies were similar to their recurrent parents at these 6 loci. Interestingly, the CLS resistance gene is located in LG3 (Arsakit et al., 2017 and Yungdaeng et al., 2020), therefore the polymorphic markers linked to domestication related traits in this LG may help reduce undesirable linkage drag. In the BC_1F_1 generation, the highest RPG recovery was found to be 77.9% in K147 of the KING × SUPER5 population and 84.1% in H207 of the H3 × SUPER5 population, using polymorphic markers in those populations.

All of BC_1F_1 progenies with resistance genes were selected and backcrossed to their recurrent parents to produce the BC_2F_1 generation. When polymorphic markers were used for marker-assisted background selection in BC_2F_1 generation, some progenies of both populations showed higher RPG recovery than estimated contribution by conventional backcross of 94.7 and 88.4% (K9-10 and K171-448) and 97.8, 97.8, 93.3, and 92.1% (H207-519, H210-533, H218-536, and H218-537) in KING × SUPER5 and H3 × SUPER5 populations, respectively.

However, some BC_1F_1 and BC_2F_1 progenies revealed lower RPG recovery than the theoretical means (75 and 87.5%), which may be explained by the "pull" effect, which is an unknown mechanism described by Sundaram et al. (2008). The pull effect might be caused by the genes of interest in the research, which may have favoured the transmission of additional genetic information, resulting in % RPG recovery that is less than the theoretical mean. In the case of linkage group 11 in mungbean, the number of markers that can be identified for polymorphism may be limited due to the short length of the linkage group.

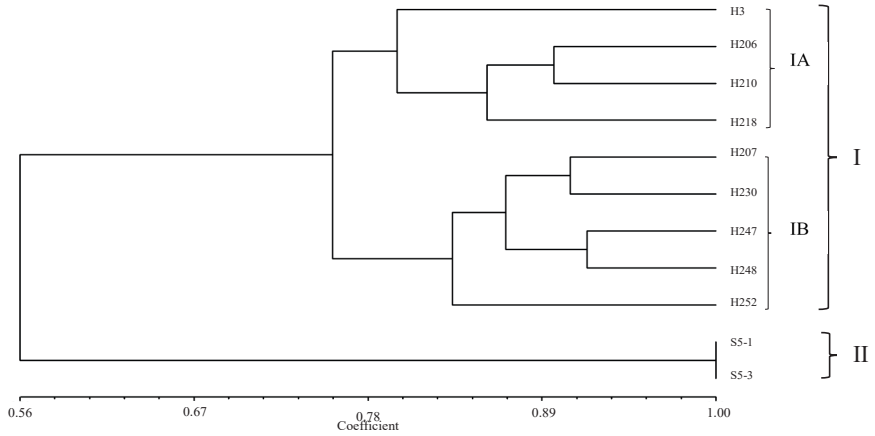

Figure 4. Dendrogram of 8 BC_1F_1 progenies and 3 parental variety/lines in H3 × SUPER5 population. Dendrogram shows similarity coefficient and genetic relationships among 11 genotypes of mungbean

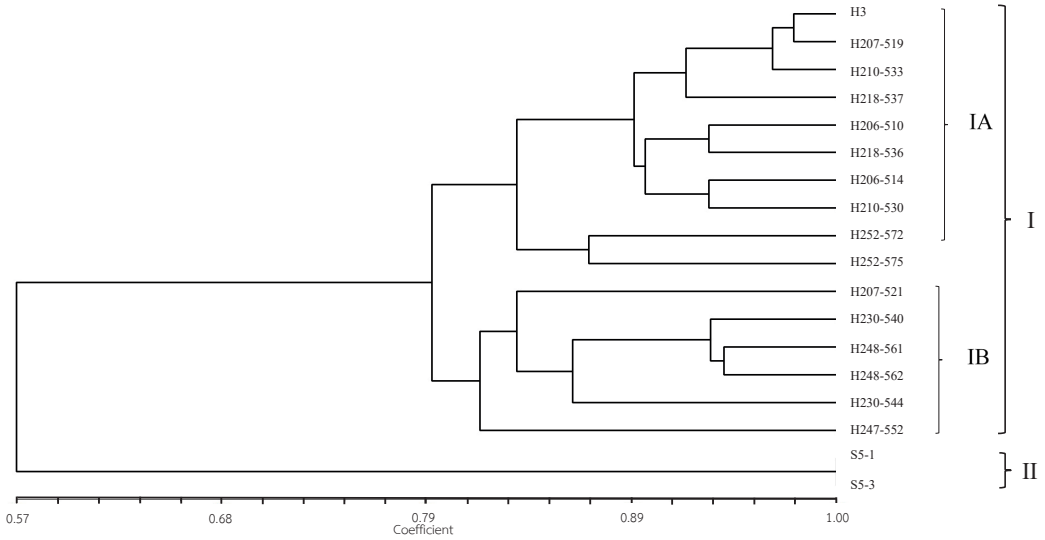

Figure 5. Dendrogram of 15 BC_2F_1 progenies and 3 parental variety/lines in H3 × SUPER5 population. Dendrogram shows similarity coefficient and genetic relationships among 18 genotypes of mungbean

However, some BC_1F_1 and BC_2F_1 progenies revealed lower RPG recovery than the theoretical means (75 and 87.5%), which may be explained by the "pull" effect, which is an unknown mechanism described by Sundaram et al. (2008). The pull effect might be caused by the genes of interest in the research, which may have favoured the transmission of additional genetic information, resulting in % RPG recovery that is less than the theoretical mean. In the case of linkage group 11 in mungbean, the number of markers that can be identified for polymorphism may be limited due to the short length of the linkage group.

CONCLUSIONS

The study found that MABC is a useful method for identifying the best BC progenies. The method effectively reduced the donor parent genome, as seen in this breeding program. The high percentage of the RPG recovery in this study suggests that using SSR and EST-SSR markers for background selection is practical in mungbean breeding. This study supports the idea that MAS has the potential to recover the genetic background of recurrent parents through accelerated backcrossing in mungbean.

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REVIEW ON SECONDARY METABOLITES IN *Salvia* spp.

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Abstract

Salvia genus is the largest member of the Lamiaceae family with around 900 species and is widely distributed in tropical and temperate regions. These species are usually aromatic and have numerous pharmacological and therapeutic applications. They are used in traditional medicine to treat eczema, colds, bronchitis, digestive problems, sore throat, tuberculosis, haemorrhage, some cardiovascular and menstrual disorder. Modern studies suggest that various Salvia species have antibacterial, antifungal, anticancer, antioxidant, and anti-inflammatory properties. These activities are consequence of biosynthesized bioactive substances, including terpenoids, flavonoids, phenolic compounds, etc. These chemical constituents refer to as secondary metabolites and exert multiple therapeutic activities without showing important side effects. The present review summarized the information published in the scientific literature on the secondary metabolites in Salvia spp. In addition, the recent biotechnological approaches, advances in metabolic engineering strategies, successful results and potential problems were presented.

Key words*: bioactive substances, Lamiaceae*, *metabolic engineering, Salvia* sp., *Secondary metabolites.*

INTRODUCTION

The genus *Salvia*, commonly known as Sage, is the largest member of *Lamiaceae* family. Sage includes 900 species of herbs, shrubs, perennials and rarely annuals and biennials that have fragrant leaves and flowers and grow in tropical and temperate regions of the world (Nikavar et al., 2008; Itani et al., 2008). The name *Salvia* is derived from the Latin word "salor" which means "to save". This plant has been of special interest since ancient times, it is the most valuable medicinal plant of the mint family and has important therapeutic properties (Ayatollahi et al., 2009; Smidling et al., 2008; Hamidpour et al, 2014). Sage is used in traditional medicine to treat eczema, colds, bronchitis, digestive disorders, sore throat and tuberculosis. Also, today's studies indicate the antibacterial, antifungal, antitumor, antioxidant and anti-inflammatory properties of different species of this plant. These activities are consequence of biosynthesized bioactive metabolites: Sterols, Flavonoids and other Phenolics**,** di-and triterpenes, etc. (Topçu et al., 2017; Marchev et al., 2014). The production of secondary metabolites through common methods such as collection from nature and cultivation in agricultural fields is not an easy task for several reasons. On the other hand, secondary metabolites may be limited to a specific genus or species, or may be produced only in a specific growth stage, or seasonal conditions, stress and special nutrition. Therefore, in the last few decades, many efforts have been made to develop alternative methods of producing valuable plant compounds, among which the use of cell cultures as a source for the production of secondary metabolites has received considerable attention from researchers (D'Amelia et al., 2017).

MEDICINAL PROPERTIES OR PHARMACOLOGICAL EFFECTS OF *Salvia*

During the Middle Ages, European traditional healers have used *Salvia* for the treatment of constipation, cholera, gout, chronic rheumatism, Alzheimer's, liver disorders, epilepsy, and paralysis. In addition, Sage is a plant that is used as an anticonvulsant, febrifuge, digestive pain reliever and headaches of nervous origin or caused by indigestion, memory enhancement, lowering blood pressure and blood sugar, as well as in migraine and Parkinson's disease (Shafizadeh, 2002).

So far, several species of *Salvia* such *as S. sclarea, S. officinalis, S. hypoleuca* and *S.*

syriaca have been studied and researched (Musarurwa, 2013; Topçu et al., 2017). Almost all species of Sage are used in traditional medicine. Previous studies have shown that the *Salvia* has valuable secondary metabolites such as Terpenoids and Flavonoids in roots, leaves and flowers, which are most concentrated during flowering. Rosmarinic acid and Salvianolic acid are secondary metabolites found in Sage, which belong to the group of flavonoids and are dimer and tetramer of Caffeic acid, respectively. In the mint family, Caffeic acid plays a key role as a component in the production of various phenolic metabolites from simple monomers to various oligomeric products. These secondary metabolites have diverse biological activities such as antioxidative, antiplatelet, antitumor and antiviral (Min-Hui et al., 2008; Sharma et al., 2019). The research carried out considers the presence of some compounds in the essential oil of these plants, such as Thujone, Cineole and Camphene, to be responsible for the antimicrobial, antioxidant and possibly anticancer properties of *Salvia.* This plant also has hypoglycemic action (Zarzuelo et al., 1990). *Salvia* has sedative and hypnotic effects, hallucinogen, skeletal muscle relaxant, anti-pain and inflammation, memory booster, anticonvulsant, neuroprotective effects, anti-Parkinson activity and control of alcohol and morphine addiction syndrome. Studies have shown that a diterpene quinones isolated from the extract of *S. miotiorrhiza* binds to central benzodiazepine receptors. Miltirone, another diterpene present in the extract of this plant, binds to benzodiazepine receptors with greater affinity than GABA (cheng et al., 2010). *Salvia aethiopis* leaf extract has analgesic and anti-inflammatory effects. Aethiopinone, a diterpene compound isolated from the extract, has similar effects to Piroxicam or Ibuprofen in reducing acute inflammation (Hosseinzadeh et al., 2003) (Table 1).

PRODUCTION OF SECONDARY COMPOUNDS

Secondary metabolites are organic compounds that do not directly participate in the growth and development or reproduction of a living organism. Over the years, humanity has benefited from this feature in medicinal plants in various industries such as food, pharmaceutical, and cosmetic industries, etc. Unlike primary metabolites, the absence of secondary metabolites does not lead to the immediate death of the organism, but it may cause a long-term disturbance in the survival of the organism, its fertility or its appearance characteristics, or it may not cause any obvious changes. These compounds often play an important role in the defense system of plants (Musarurwa, 2013).

Table 1. *Salvia* species, active substances and possible mechanism

Effective substance or possible mechanism	**Type of effect**	***Salvia* species**
Cirsiliol and caffeic acid ethyl ester	Sedative and sleep-inducing effect	*Salvia miltiorrhiza*
Neo-clerodane Diterpenes salvinorin A	Hallucinogenic effects	*Salvia divinorum*
Aethiopinone	Pain killer	*Salvia aethiopis*
α-Pinene, Camphor, 1,8-cineole	Strengthening memory	*Salvia lavandulaefolia*
Un known	Anticonvulsant effects	*Salvia haematodes*
Inhibitory effect on superoxide production	Neuroprotective effects	*Salvia leriifolia*
Amphetamine-like and dopamine release induced by k+ stimulation	Inhibition of monoamine oxidase enzyme	*Salvia miltiorrhiza*
Miltirone	Effect on morphine withdrawal syndrome	*Salvia miltiorrhiza*

As mentioned, secondary metabolites are not part of the basic molecular structure of the cell, are found in small amounts and may not play an obvious role in growth and development, and if present, are found in specific tissues, organs or at specific stages of development. Also, their production may be limited on a wide level or to a family or a specific genus or even a specific species of plants. Secondary metabolites are widely used in industrial products and are used in the manufacture of medicine, soap, essential oil, paints, gums, resin, rubber, food and beverage seasoning, etc. These compounds have important functions in the plant itself, such as the function of hormones and growth regulators, removing microbial contamination, attracting pollinating agents, and also driving away herbivores and insects, which by this means reduce the damage of animals and insects and

help the producing plants to survive in their ecosystem. Using these compounds, which belong to different chemical families including Alkaloids and Flavonoids, plants protect themselves against microbial attacks, herbivores, and ultraviolet radiation. Secondary metabolites also play a key role in the attraction of flower pollinating insects (especially Anthocyanin pigment and Terpenoid) (Karuppusamy, 2009).

Metabolites or secondary products, apart from the complex biosynthetic pathway for their production, also have a complex structure, and this has made their study slow. Meanwhile, the high value of these compounds in industries such as medicine necessitates the chemical study of these compounds. The study of secondary metabolites also increases our understanding of their biosynthesis and activity. It should be noted that the structure of these molecules is often too complex for fully efficient chemical synthesis. For this reason, despite the low concentration of these metabolites in plants, plants are still the only economically viable source of many of these valuable metabolites (Pichersky & Gang, 2000; Dewick, 2002; Musarurwa, 2013).

Recently, according to numerous reports regarding the importance and medicinal value of secondary metabolites in *Salvia*, special attention of researchers has been drawn to obtain and apply methods to increase their production capacity in this plant. Important and major medicinal metabolites in Sage plants fall into three main categories, which include: Monoterpenes (Cineole, Thujone, Camphor) and Triterpenes (Ursolic Acid and Oleanolic Acid), Diterpenes, and Phenolic acids. Monoterpenes and triterpenes are present in essential oil and together with Flavonoids, they are more abundant in the aerial parts than other compounds, while Diterpenoids and Phenolic acids are the dominant compounds in the root. The main Phenolic acids in Sage species are Caffeic acid and its derivatives. Caffeic acid usually exists as a dimer as Rosmarinic acid (RA), which is the most important Phenolic acid in *Salvia*. Trimers and tetramers of Caffeic acid are of more interest in terms of treatment. The most important Phenolic acids in *Salvia* are Caffeic acid, dihydroxyphenyl Lactic acid, Chlorogenic acid and Danshensu (Caffeic acid monomers), RA (Caffeic acid dimer), Salvianolic acid A (Caffeic acid trimer), and Salvianolic acid B (Caffeic acid tetramer or RA dimer). Of course, there are many types of Salvianolic acids (SAs), but none of them are as well known and studied as salvianolic acid A (SA-A) and salvianolic acid B (SA-B), because the amount of these two compounds in Sage is more than other types of SAs (Topçu et al., 2017) - Figure 1.

In 2017, Topçu et al. conducted a comprehensive study of bioactive compounds in Anatolian *Salvia* species. By examining about 45 species of Anatolian Sage, they succeeded to isolate 317 biochemical compounds. According to their investigation, most of the extracted compounds had diterpene structure (158 compounds) and the highest degree of diversity among diterpene structures has been seen by using spectroscopic methods. In addition, they managed to identify about 60 Triterpenoids, 15 Steroids, 5 Sesterterpenes, 9 Sesquiterpenes, 41 Flavonoids and 29 other phenolics (Topçu et al., 2017).

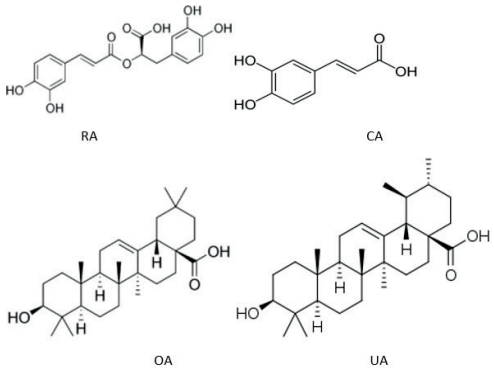

Figure 1. Some important bioactive compounds in *Salvia* species (RA: Rosmarinic Acid, CA: Caffeic Acid, UA: Ursolic Acid, OA: Oleanolic Acid) (Marchev et al., 2014)

TISSUE CULTURE AND CELL CULTURE IN THE PRODUCTION OF SECONDARY METABOLITES

The common method of producing secondary metabolites at the commercial level is extraction from the whole plant. Besides this old method, plant cell culture and tissue culture on a large scale is a significant alternative method that is possible with the help of bioreactors.

Bioreactors provide a sterile environment where environmental factors such as temperature, respiration, oxygen level, movement rate and pH can be controlled, and their use is a reliable method for the propagation of industrial plants (Paek et al., 2005).

Considering the economic importance of secondary metabolites and the limited number of plant species in their natural habitats, cell culture methods are a suitable way to produce valuable chemical compounds. One of the uses of plant cell culture is the production of products with uses such as medicine, fragrances, flavorings and fine chemicals that can limit problems such as slow growth, genetic instability, inability to maintain photoautotrophic growth, and instability of productive cells lines (Paek et al., 2005; Fulzele, 2005). The undifferentiated cells of the hairy roots are of special interest for cell culture, as they are able to continue growing at a high rate by settling in the liquid culture medium, and despite their small size and sensitivity, many bioreactors are able to culture them (Giri & Narasu, 2000). Somatic cells are also a potential source for the mass production of secondary metabolites in bioreactors and because they are small and uniform in size and do not need to be cut for cultivation, they are preferable to traditional micropropagation methods (Paek et al., 2005). The biggest limitation of *in vitro* production is the scale-up of mass production in order to commercialize the product. Hairy roots, callus and suspension cultures face many problems during accumulating. Among the hundreds of secondary metabolites that were mass-produced by undifferentiated cell culture, only Shikonin, Jebensenoside, and Berberine have been produced in high volume, and they have been the most successful samples produced at the commercial level (Paek et al., 2005).

In the *Salvia* species, only *S. officinalis* and *S. miltiorrhiza* are the most widely grown as commercial field crops (Musarurwa, 2013). Many successful studies on the tissue culture of Sage species have been widely conducted. Also, the optimization of micropropagation conditions and increasing the production levels of secondary metabolites have been studied (Santos-Gomes et al., 2003; Mirajalili et al., 2006; Bolta et al., 2013; Musarurwa, 2013).

CELL AND HAIRY ROOT CULTURES

There are some studies on cell suspensions from *Salvia* species as a source of secondary metabolites, but this is clear that due to the abundance of these compounds in the plant, more extensive studies are needed (Marchev et al., 2014). Wang and Wu (2010) investigated callus production using different explants of *Salvia* species. In this study, different explants were able to produce callus on MS medium with the help of growth regulators (Wang & Wu, 2010). One of the common methods of producing secondary metabolites that are created in the root is root inoculation using *Agrobacterium rhizogenes*. Inoculation of plants with microorganisms such as rhizosphere bacteria stimulate specific pathways of secondary metabolites. The production of secondary metabolites in inoculated roots is caused by contamination with *Agrobacterium*, and they usually have the same or higher performance than the metabolites produced in natural plants. This feature makes them suitable for biochemical studies that are difficult on normal plants due to their genetic stability and rapid general growth in a simple culture medium without plant hormones (Ghorbanpoor et al., 2011; Sevon & Oksman-Caldentey, 2002). Due to having a wide level of bioactive compounds, *Salvia* is one of the favorite options of researchers for hairy root culture, however, only limited species of this plant have been used for this purpose (Marchev et al., 2014; Yuan et al., 2009; Xiao et al., 2011; Zhao et al., 2011; Gu et al., 2012; Hao et al., 2012; Grzegorczyk & Wysokińska, 2010). As far as we know, the first successful transformation of tissues from the Sage species was carried out by Hu & Alfermann, 1993. In this study, with the help of different *Agrobacterium* strains, they succeeded in producing transgenic hairy roots in *Salvia miltiorrhiza* (Hu & Alfermann, 1993).

In 2017, Norouzi et al. investigated hairy root induction in some species of Sage. They studied two Iranian endemic species (*Salvia eremophila* and *S. reuterana*) and five non-endemic species (*S. macrosiphon*, *S. multicaulis*, *S. nemorosa*, *S. verticellata* and *S. virigata*) for this purpose. 4 *Agrobacterium* strains 1728, 2659, ATCC-15834 and A4 have been used in this study. According to the obtained results, stem and

petiole explants were unable to produce hairy roots, while all leafy explants produced hairy roots. The results have shown that different strains of *Agrobacterium* and different species of Sage have a significant effect on the number and abundance of hairy roots (Norouzi et al., 2017).

It is obvious that the optimization of cell culture and hair root methods can help to produce biochemical compounds on a large scale. However, the commercialization of the protocols for the production of secondary metabolites in *Salvia* species faces many pitfalls and requires extensive research (Marchev et al., 2014).

ELICITATION

Elicitors are compounds of biological or non-biological origin that cause the biosynthesis and accumulation of secondary metabolites through the induction of defense responses. Biological elicitors include polysaccharides, proteins, glycoproteins or parts of the cell walls of fungi, plants (cellulose and pectin) and microorganisms (glucan and chitin). In general, the use of elicitors in biotechnological studies of plant metabolites pursues two main goals: 1- Obtaining information on the biosynthetic pathways that lead to the formation and regulation of secondary metabolites. 2- Increasing the production of secondary metabolites for commercial use (Zhao et al., 2005; D'Amelia et al., 2017).

Many stimulants such as polysaccharides, glycoproteins, low molecular weight organic acids, fungal cell wall materials, ultraviolet rays and heavy metal salts have been tested to increase bioactive compounds in *Salvia*. As a group of efficient non-biological stimuli, the use of metal ions including Ca^{2+}, Co^{2+}, Ag^{+}, Cd^{2+}, Cu^{2+}, Ce^{3+}, Li^{+}, Mn^{2+} and Zn^{2+} to induce the production of bioactive compounds in plants. Heavy metals have a significant effect on synthesis and secondary metabolism. Many types of heavy metals have been used as stimulants to induce the accumulation of bioactive compounds in the hairy roots of *S. miltiorrhiza*. Among metal ions, silver ion is known as an effective stimulant in improving the production of phenolic acids (Rosmarinic acid and Salvianolic acid B-B) and Tanshinones (Zhao et al., 2010a; Marchev et al., 2014). Another non-biological stimulant effective in inducing the production of phenolic acids in Sage plants is methyl jasmonate. Methyl jasmonate induces the production of secondary metabolites by activating phenylalanine ammonia-lyase (PAL). Jasmonates have been introduced as key messenger compounds in the induction process leading to the accumulation of secondary metabolites (Kuz'ma et al., 2008).

GENETIC MANIPULATION OF BIOCHEMICAL PATHWAYS TO PRODUCE MORE BIOACTIVE COMPOUNDS

Metabolic engineering is the purposeful change of metabolic pathways of an organism in order to better understand and identify cellular pathways and use this information for chemical transport and energy management and supramolecular assemblies. The use of these methods in plants provides the possibility of manipulating biochemical pathways, and transgenic plants are produced, the amount of production of their natural products has been changed according to commercial and agricultural interests and suitable characteristics after harvesting (Lessard, 1996; Kinney, 1998; D'Amelia et al., 2017). In recent decades, plant cell culture has been identified as a powerful tool for the production of commercial metabolites, but despite many efforts in the field of *in vitro* production, since the amount of production is not sufficient for their commercialization, metabolite engineering can be useful methods for Increasing production. Identifying the genes involved in the biosynthesis of bioactive compounds can help to increase the production of these compounds. The biosynthesis of plant metabolites in *Salvia* can be specifically changed through increasing the expression or silencing of genes encoding the production of secondary metabolites (Zhou et al., 2011).

D'Amelia et al. (2017) studied isolated and engineered genes involved in the biosynthesis of Polyphenolic and Terpenoids compounds from different *Salvia* species. Most of these genes have been identified and isolated from *Salvia miltiorrhiza* (D'Amelia et al., 2017) .

PLANT/MICROBE CO-CULTURES

In the production of secondary metabolites, there are three theories about the origin of the metabolism of secondary metabolites in plants. One is that both plants and microbial agents participate in the process of making these natural products. Another theory suggests the transfer of genes between plants and microbes in distant times, and the third theory believes that either plants or fungi produce these secondary metabolites and transfer them to their symbionts (Wink et al., 2005).

The combination of inducing agents in symbiotic plants and fungi increases the accumulation of secondary metabolites in both of them. Therefore, the coexistence and interaction between plants and fungi and their impact on each other during the production of important biological compounds needs further study and can be a good topic for the production of secondary metabolites using metabolic engineering and genetics (Zhang et al., 2009). The studies that have been done so far have shown that among the bacterial species, *Bacillus cereus* has the most impact in increasing the production of plant metabolites, including Tanshinones in *S.* miltiorrhiza (Zhao et al., 2010b). Although few studies in this field have been conducted on the *Salvia*, it is clear that more comprehensive and detailed studies are needed to find more results (Marchev et al., 2014).

FUTURE PROSPECTIVE

The advances made in the field of biotechnology have led to the creation of new methods such as cell culture for the production of biochemical compounds of plants and their commercialization.

The advantage of these methods over the traditional methods of making secondary metabolites under controlled conditions and independent of climate conditions is that they can create reliable renewable resources for the required natural products.

The production of medicinal compounds *in vitro* is considered a promising development in the field of plant sciences, and the use of genetic tools, the identification of the structure of secondary metabolites and their metabolic pathways can help the commercial production of these products. The growing need of the market for natural compounds for the production of medicine, in addition to the lack of raw materials and low production efficiency, helped the emergence of new biotechnological methods for the production of secondary metabolites on a mass level. Until today, there is still no sufficient knowledge about the biosynthesis pathways of chemical compounds in plants and cell cultures, therefore, providing solutions to develop information on the molecular and cellular level of these pathways is a major need.

CONCLUSIONS

Salvia is one of the most valuable medicinal plants, which can be a rich source of plant metabolites due to its wide range of bioactive compounds. The use of modern biomolecular methods and the production of transgenic crops can effectively influence the biosynthetic pathways and will be a big step towards the production of secondary metabolites on a mass and commercial level in this plant.

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PLANT SOMATIC EMBRYOGENESIS - A FOCUS ON THE ROLE OF CALCIUM IN EMBRYO INDUCTION AND DEVELOPMENT

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Abstract

Upon fertilization, plants produce zygote-derived embryos that will develop into new individuals. The study of its regulation is important to overcome the post-zygotic barriers that may prevent hybridization, requiered for the development of new cultivars. The study of zygotic embryo development is challenging, since they are surrounded by a multilayer maternal tissue, and are difficult to reach. Alternatively, plants produce embryos from specific somatic cells under certain in vitro conditions. This process, referred to as somatic embryogenesis, has been established as a model to study plant embryogenesis, due to its similitudes with zygotic embryogenesis and its technical feasibility. Moreover, the production of somatic embryos allows to efficiently propagate plant material. Somatic embryogenesis is regulated by stimuli of different nature. Among others, calcium gradients within cells and tissues are important to achieve a proper consecution of embryogenesis from somatic cells. In this review, we summarize the most relevant advances in different plant species by using multiple approaches that shows the role of calcium in the regulation of somatic embryogenesis.

Key words*: somatic embryogenesis, calcium signalling, ionophore, FRET.*

SOMATIC EMBRYOGENESIS AS A MODEL SYSTEM

Embryos are structures with the capacity of generating a new organism. Plant zygotic embryos are formed upon fertilization of a female egg cell by a male sperm cell, and its development is finely controlled by a specific embryogenic program (Wendrich and Weijers, 2013). Zygotic embryos develop within the embryo sac, surrounded by the nucellus and the ovular teguments. Thus, their study is somehow challenging. Alternatively, under certain in vitro conditions, differentiated cells, both somatic and gametic, can develop embryos with a high morphological similarity to these of the zygotic embryos. Specifically, somatic embryogenesis (SE) results in the production of embryos derived from somatic cells. Somatic embryos are bipolar structures that undergo through the same globular, heart, torpedo and cotyledonary stages as zygotic embryos, although several differences related to the overall morphology, cell and organelle sizes, water status, and biochemical composition, have been detected when comparing both types of embryos in different species (Winkelmann, 2016; Zimmerman, 1993). Although SE can occur in nature, being the case of several species of the *Kalanchoe* genus one of the most fascinating (Garcês and Sinha, 2009), this phenomenon is rare. However, SE can be induced under certain *in vitro* culture conditions. A special case of non-sexual embryogenesis is the formation of seeds without fertilization or apomixis. In this process, which can occur naturally or be induced by different techniques, apomictic seeds are produced either from sporophyte cells of the ovule, or from the megaspore mother cell or nucellar somatic cell contained in unreduced embryo sac cells, and are therefore, genetically identical to the mother plant (Yin et al., 2022).
The first evidence of *in vitro* induced somatic embryogenesis was reported on *Oenanthe aquatic* (Waris, 1957), and carrot (Steward et al., 1958; Reinert, 1958). Steward and co-workers described the formation of roots, shoots, and eventually entire living plants from cell aggregates obtained by liquid culture of carrot root phloem somatic cells (Steward et al., 1958). A closer view to this process showed that initiation of SE is limited to a single or few somatic cells (Backs-Hüsemann and Reinert,

1970), although embryos develop from multicellular proembryogenic cell masses (Goldberg et al., 1989). Due to its valuable biotechnological application (see below), many research groups focused in the study of SE in different species, and currently efficient protocols are available for more than one hundred species, including tobacco (Takebe et al., 1971), woody species (reviewed in Wann, 1988), several palm trees species (reviewed in Ree and Guerra, 2015), maize (Armstrong and Green, 1985), grapevine (Gambino et al., 2011), and the model plant *Arabidopsis thaliana* (Gaj, 2001), among others.

APPLICATIONS OF SOMATIC EMBRYOGENESIS

In vitro culture techniques have helped to increase agronomic features of current crops, including productivity, pathogen and herbicide resistance, and food quality. Such techniques, together with molecular approaches including GMO generation and gene editing, are the basis to further improve plant performance under the uncertain growing conditions imposed by climate change, and to develop new commercial products to satisfy new consumer demands. In this context, SE can be useful at different levels. On the one hand, SE allows for large scale propagation of plant material, which is especially useful for woody species (Guan et al., 2016). Contrary to organogenesis and conventional micropropagation protocols, SE allows for the propagation of green material in a single step, rather than sequential root and shoot regeneration steps. Moreover, SE has a higher multiplication rate than alternative micropropagation techniques, although it depends on the genetic background, and the possibility of scaling up using bioreactors (Egertsdotter et al., 2019). Additionally, the encapsulation of somatic embryos enables their direct delivery as synthetic seeds, thereby facilitating the maintenance of elite cultivars (Onishi et al., 1994). On the other hand, SE is a valuable technique for the regeneration of plants upon genetic transformation. Efficient protocols to produce somatic embryos greatly facilitate the production of high numbers of independent transgenic lines, in order to select the most appropriate in terms of transgene expression, stability and phenotype (Deo et al., 2010). Furthermore, somatic embryogenesis has been used for the elimination of viruses from plant material of different species, including grapevine (Olah et al., 2022; Gambino and Perrone, 2022). Finally, due to the developmental similarities among zygotic and somatic embryogenesis and the absence of maternal tissues surrounding *in vitro* embryos, SE is a very appropriated model to study basic features and regulation of plant embryogenesis. Altogether, the advantages for plant improvement of SE versus other techniques are evident. However, SE has also certain limitations. For example, the non-synchronous formation of somatic embryos within the same explant, the recalcitrance of some species or cultivars to SE induction, the accumulation of undesired mutations due to somaclonal variation along in vitro-cultured generations, and the loss of embryogenic capacity of calli over time (Deo et al., 2010). The study of the basic mechanisms that regulate SE may help us to reduce the negative impact of these constraints, and to establish improved protocols for recalcitrant species.

FACTORS AFFECTING SOMATIC EMBRYOGENESIS

SE is a complex process whose efficiency, generally measured as number of embryos per explant, is affected by several factors of different nature. On the one hand, somatic embryogenesis is highly dependent on the explant type, as many other *in vitro* culture approaches. Somatic embryos have been obtained using different explant types such as leaves (Martins et al., 2022), leaf main veins (Hanh et al., 2022), spikelets (Ornellas et al., 2022), anthers and ovaries (Gray and Mortensen, 1987; Gambino et al., 2007; Perera et al., 2007), flower tepals (de Almeida et al., 2022), roots (Chen et al., 1987), stems (Cuenca et al., 1999), petioles (Rugini and Caricato, 1995), mature and immature embryos (Chan et al., 1998; Fernando and Gamage, 2000; Calabuig-Serna et al., 2023a), seedling hypocotyls (Calabuig-Serna et al., 2023b), and cotyledons (Leva et al., 1995). Not only the explant type, but the explant age determines to a great extent the efficiency of SE, as shown

for coffee (Molina et al., 2002), eucalyptus (Prakash and Gurumurthi, 2010) and soybean (Yang et al., 2009), among others. As for any other in vitro culture-based morphogenic process, SE efficiency strongly depends on the genetic background, not only at the species level, but also within cultivars. For instance, the SE efficiency and the regeneration capability of 25 inbred maize lines were analyzed, observing that some lines were highly efficient on SE production, whereas other were completely recalcitrant (Hodges et al., 1986). Further genetic analysis of F1 crosses with the highly embryogenic line A188, and corresponding F2 plants, demonstrate the genetic dependence of SE induction and plantlet regeneration (Hodges et al., 1986). The genotypic effect has also been studied for other species, such as cotton (Trolinder and Xhixian, 1989), coffee (Molina et al., 2002), soybean (Parrot et al., 1989), and cocoa (Florez et al., 2015), among others. Finally, composition of the *in vitro* culture medium, as well as application of certain abiotic stresses, determines the SE efficiency in different species and genotypes. The nature of the nitrogen source (NO3 versus $NH4^{+}$) in Medicago sativa (Meijer and Brown, 1987), feijoa (dal Vesco and Guerra, 2001) and coffee (Samson et al., 2006), influence both the velocity of the SE process and the efficiency of the process. Specific culture media composition, and incubation conditions for different species has also been described (Fernández-Guijarro et al., 1995; Chen and Chang, 2002). More importantly, the type and concentration of growth factors on growing media are crucial for SE, being auxins a major regulator. Generally, auxin favors the initial formation of callus, whereas SE initiates under auxin-free media supplemented or not with low concentrations of other growth regulators (Chambhare and Nikam, 2022; Su et al., 2009; Filippov et al., 2006). Auxin is considered, indeed, one of the main SE regulators (reviewed in Wójcik et al., 2020; Winnicki, 2020). In summary, SE protocols use to be slightly different among species and even cultivars, including variations in the type of explant, medium composition, and in vitro culture conditions. Therefore, specific protocols have been established for different genetic backgrounds.

These factors affecting SE not only determines the efficiency of the process, but also the degree of de-differentiation of somatic cells prior to the embryogenic process. In this sense, formation of somatic embryos can be either direct from somatic cells or indirect, through an intermediate callus phase, where embryos are formed from cells of the surface of the callus (Sharp et al., 1980). Generally, indirect SE produces more embryos, although the incubation time is longer and the effect of somaclonal variation is higher (Miguel and Marum, 2011). Why a concrete explant under certain conditions produces somatic embryos through a direct or indirect way is not clearly understood, although it may be related to the age of the explant (Horstman et al., 2017b; Merkle et al., 1995), the type of explant and/or the *in vitro* culture conditions. For instance, both direct and indirect SE protocols are established for *Camellia oleifera*, and the difference among them relies in different combination of different growth regulators (Zhang et al., 2021). Similarly, protocols for direct and indirect SE have been established for *Coffea arabica* (Quiroz-Figueroa et al., 2002), carrot (Mizukami et al., 2008; Steward et al., 1958), and maize (Lowe et al., 2018), among many others. Indeed, in some cases, both direct and indirect SE are induced in the same explant (Turgut et al., 1998; Gaj, 2004). An example of the occurrence of both, direct and indirect SE, is arabidopsis, a model species where many molecular tools are developed, and an ideal system to study the molecular regulation of SE (Gaj, 2004). Different protocols and explants are used to induce SE in arabidopsis (Horstman et al., 2017b), although the most extended protocol is based on the application of 2,4-D to immature zygote embryos (IZEs) at the late cotyledonary stage (Figure 1; Wu et al., 1992; Gaj, 2001). IZEs are isolated from green siliques of arabidopsis adult plants, sterilized, and placed on in vitro plates (Figure 1A). Upon 5 days on auxin inductive media a lump, referred as protrusion, is formed on the adaxial side of the cotyledon of responding explants (Figure 1B,C). The embryogenic nature of the protrusion is demonstrated by the expression of embryo identity genes, such as *WOX2* (Godel-

Jedrychowska et al., 2020) and *WUS* (Calabuig-Serna et al., 2023a), and histological studies showed that embryos emerged from protodermal and subprotodermal layer (Kurczyńska et al., 2007). The appearance of the protrusion is the result of an inner proliferating cell mass that, upon 7 days of culture, usually emerges and breaks the explant epidermis (Figure 1D). Finally, upon 14 days of culture, visible embryos arise from the embryogenic cell mass formed (Figure 1E; Calabuig-Serna et al., 2023a).

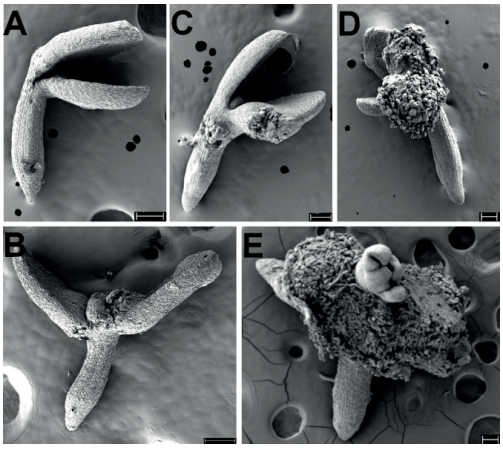

Figure 1. Somatic embryogenesis process from immature zygote embryos of *Arabidopsis thaliana*. A. Freshly isolated immature zygote embryos (IZEs) at the late cotyledonary stage. B, C. IZEs upon 5 days of culture, showing (B) discrete or (C) strong growth of protrusion. D. IZE upon 7 days of culture showing drastic protrusion growth. E. Somatic Embryo growing on the callus-like structure formed on the IZE. Scale bar: 100 μm

GENETIC REGULATION OF SOMATIC EMBRYOGENESIS

During somatic embryogenesis, cells undergo a process of "dedifferentiation" to become embryogenic and, therefore, acquire the capability to develop into a new whole organism. These complex processes require a fine-tuned genetic regulatory network, that has mostly been studied in arabidopsis. The genetic regulatory network controlling SE has been recently reviewed by Horstman et al. (2017b) and Elhiti and Stasolla (2022). Different approaches have been followed to decipher such genetic network. On the one hand, the effect of the ectopic expression of embryo and meristem identity genes on SE activation has revealed their role during SE. For instance, overexpression of *LEAFY COTYLEDON 1* (*LEC1*) induces the formation of embryos in arabidopsis cotyledons (Lotan et al., 1998). Other genes within the same family, such as *LEC2* and *LEAFY COTYLEDON1-LIKE* (*L1L*), are also able to induce SE when overexpressed (Horstman et al., 2017b). Accordingly, *lec1* and *lec2* mutants exhibit low SE response (Lotan et al., 1998; Stone et al., 2008; Gaj et al., 2005). These genes are known to regulate auxin metabolism and signaling, as well as gibberellin/ABA ratios, thus their function during SE can be explained, at least in part, by their role In the hormonal response within the explants (Elhiti and Stasolla, 2022). Transcriptional activation of LEC1 and LEC2 is controlled by *BABY BOOM* (*BBM*; Horstman et al., 2017a). Accordingly, ectopic expression of the transcription factor *BBM*, induces the formation of somatic embryos in leaves and cotyledons, even in the absence of growth regulators (Boutilier et al., 2002). Similar observations were made upon *WOUND INDUCED DEDIFFERENTIATION 1* (*WIND1*; Ikeuchi et al., 2015) and *WUSCHEL* (*WUS*; Chatfield et al., 2013). As a step forward, microarray analysis and chromatin immunoprecipitation experiments helped to the identification of downstream target genes. For instance, microarray analysis of *LEC2* overexpression identified genes related to auxin metabolisms that were important for SE (Stone et al., 2008). On the other hand, the expression of master regulators of SE is controlled by epigenetic signals, such as DNA methylation, chromatin remodeling and, micro-RNA mediated regulation (reviewed in Kumar and van Staden, 2017). First, different methylation patterns during SE have been observed in different species. Whereas high methylation levels were detected during *Daucus carota* (Yamamoto et al., 2005), *Castanea sativa* (Viejo et al., 2010) and *Picea omorika* (Levanic et al., 2009), in some other species, such as *Eleutherococcus senticosus* (Chakrabarty et al., 2003) and *Coffea canephora* (Nic-Can et al., 2013), DNA methylation increased throughout SE development. No matter how, the application of the DNA methylation inhibitor 5-Azacytidine reduces SE in *Daucus carota*

(Yamamoto et al., 2005) and *Coffea canephora* (Nic-Can et al., 2013), which points to the need of controlled DNA methylation stage during SE. Moreover, a specific methylation signature, i.e. H3K27me3, has been described to activate *LEC1* and *BBM1* (Nic-Can et al., 2013). Finally, the pattern of specific non-coding microRNAs changed throughout the embryogenic induction, compared to non-induced calli (Kumar and van Staden, 2017). For instance, arabidopsis microRNA167 controls somatic embryogenesis through regulating its target genes *ARF6* and *ARF8* (Su et al., 2015). In conclusion, SE genetic regulation is a highly complex process that requires both genetic and epigenetic regulation. Some specific genes and gene targets, as well as epigenetic marks that control their expression, have been described. The identification of specific genes involved in SE can help us to obtain genetic backgrounds that produce somatic embryos at a higher efficiency.

CALCIUM SIGNALLING IN PLANT CELLS

Calcium is a microelement needed for proper plant growth and development and is present within cells in its free cationic form (Ca^{2+}), stored or loosely-bound, and covalently bound to macromolecules (Ge et al., 2007). Whereas covalently bound calcium has mainly a structural function, the equilibrium among free Ca^{2+} and stored (loosely-bound) Ca^{2+} plays an important signaling role within cells, and regulates precise cellular responses by its interaction with specific Ca^{2+} sensors or Ca^{2+}-binding proteins such as calmodulin, calmodulin-like proteins, Ca^{2+} dependent protein kinases, and Calcineurin B-like proteins (Tuteja and Mahajan, 2007). Calmodulin (CaM) is a small protein that is present in almost all eukaryotic organisms and, upon Ca^{2+} binding, it is activated and changes its conformation favoring the interaction with diverse targets including specific kinases, which triggers specific signaling cascades (Bredow and Monaghan, 2022). Free intracellular Ca^{2+} is present in the cytoplasm at very low concentrations (50-100 nM) due to its cytotoxicity, although it can reach up to 5 mM when confined in organelles such as the endoplasmic reticulum and vacuoles, or in the cell wall, acting as cellular Ca^{2+} reservoirs. When required, Ca^{2+} is released to the cytosol by selective channels and, upon signaling, it is translocated back to the reservoirs by specific pumps (Pirayesh et al., 2021). These mechanisms allow cells to trigger transient Ca^{2+} signatures (peaks) that act as a second messenger for signal transduction (Ge et al., 2007), avoiding the toxic effects of continuous high Ca^{2+} levels. Detection of cellular Ca^{2+} accumulation during biological processes can shed some light about the role exerted by this substance, and the mechanisms underneath.

Ca^{2+} within cells can be detected by several biotechnological tools (Kanchiswamy et al., 2014). Traditionally, cytosolic Ca^{2+} has been detected using specific, Ca^{2+}-sensitive fluorescent probes, such as Indo-1 and Fura-2 (Bush and Jones, 1990), FluoForte (Rivas-Sendra et al., 2017; Rivas-Sendra et al., 2019), fluo-4/AM (Gee et al., 2000), chlorotetracycline (Tirlapur et al., 1995), and fluo-3/AM (Qiu et al., 2020), among others. However, these techniques, although very informative, require the incubation of the probe with the sample, which allows solely for the detection of Ca^{2+} accumulation at specific time points, precluding the continuous observation of Ca^{2+} changes in living cells and the identification of fast, Ca^{2+} transient peaks. Instead, the use of confocal microscopy to register specific signal of Ca^{2+} binding proteins coupled to a fluorescence resonance energy transfer (FRET) system has been a valuable tool to study in vivo Ca^{2+} dynamics. Briefly, a donor (CFP) and acceptor (YFP) fluorescent proteins are linked by calmodulin bridge in the so-called cameleon constructs (Figure 2A). As the Ca^{2+} concentration increases, calmodulin incorporates Ca^{2+} and changes its conformation, thereby approaching both fluoroproteins and allowing for the FRET emission (Figure 2A; Miyawaki et al., 1997). The availability of these tools allows for the detection for Ca^{2+} signatures throughout biological processes and, therefore, the potential implication of Ca^{2+} on the signaling and regulation of these processes.

Ca^{2+} SIGNATURES DURING SOMATIC EMBRYOGENESIS

Ca^{2+} is an important cation for sexual reproduction in angiosperms, and Ca^{2+} peaks have been observed during pollen germination and pollen tube elongation, pollen-pistil interaction, and gametic interaction and fertilization (reviewed in Ge et al., 2007). At the very initial phase of zygote embryogenesis, it was observed transient elevation of cytosolic Ca^{2+} concentration upon maize *in vitro* egg cell fertilization, that is triggered by gamete fusion (Digonnet et al., 1997). Later, it was demonstrated that Ca^{2+} cellular uptake starts in the fusion site, and then is spread throughout the whole egg cell plasma membrane, resulting in a transient increase of cytosolic Ca^{2+} (Antoine et al., 2000). Moreover, addition of the Ca^{2+}-channel inhibitor gadolinium blocked gamete fusion, what indicates that Ca^{2+} influx is required for sperm incorporation (Antoine et al., 2001). Further *in vivo* experiments showed that different female ovule cell types (i.e. egg cell, synergid cells and central cells) showed specific Ca^{2+} signatures upon fertilization, pointing to a complex regulation of plant fertilization mediated by Ca^{2+} (Denninger et al., 2014). These works demonstrate a prominent role of Ca^{2+} signatures to fine-tune the initial steps of plant zygote embryogenesis, as it has been seen for animal systems (Miao et al., 2012). However, to the best of our knowledge, Ca^{2+} accumulation has not been reported in later plant zygotic embryogenic stages, probably due to the relative inaccessibility of the embryos within the developing seeds.

In alternative systems, such as rapeseed microspore embryogenesis, Ca^{2+} overaccumulation was observed by FluoForte staining in the cytosol, nucleus, and vacuoles of embryogenic microspores, but not in non-embryogenic forms, such as callus-like and pollen-like structures. Mature microspore-derived embryos showed decreased Ca^{2+} levels (Rivas-Sendra et al., 2017; Rivas-Sendra et al., 2019). However, most of the knowledge regarding Ca^{2+} accumulation and signatures during embryogenesis has been obtained in different somatic embryogenesis systems. For instance, measurement of free intracellular Ca^{2+} by different approaches, including incubation with fluo-3, showed a clear increase of Ca^{2+} specific signal, compared to the low signal in proembryogenic masses, in induced embryogenic explants, mostly in the nuclei of cells belonging to the protoderm of late globular to torpedo stages (Timmers et al., 1996). Similarly, high fluorescent signal derived from Fura-2AM was detected in embryogenic *Coffea canephora* embryogenic calli treated with $CaCl_2$, whereas treatment with specific Ca^{2+} channel blockers resulted in a low cytosolic Ca^{2+} accumulation (Ramakrishna et al., 2011). Accordingly, a high expression of calmodulin was observed in sugarcane somatic embryos, compared to non-responsive cells (Suprasanna et al., 2004; Overvoorde and Grimes, 1994). Ca^{2+} measurement by biochemical approaches demonstrates that proembryogenic sandalwood cell clumps transferred to cell differentiation media accumulate more Ca^{2+} than the ones kept on callus proliferation media (Anil and Rao, 2000). Recently, the use of cameleon carrot transgenic lines and confocal FRET signal detection, revealed that Ca^{2+} accumulation was highly dynamic along the different stages of the SE process, being higher in proliferating cells and embryogenic structures, what supports the signaling role of free Ca^{2+} in SE regulation (Calabuig-Serna et al., 2023b). Similarly, FRET analysis of arabidopsis cameleon transgenic lines (Krebs et al., 2012), revealed that Ca^{2+} accumulates in the adaxial side of the cotyledons of the induced IZEs, coincident with the formation of the protrusion (Figure 1B, C; Figure 2B, C), and with the embryo-identity gene *WUS* (Calabuig-Serna et al., 2023a). Ca^{2+} accumulation increased with incubation time in inner cell layers of the cotyledon and was especially high during the protrusion emergence (Calabuig-Serna et al., 2023a).

All these data are coincident on the increase of Ca^{2+} accumulation in induced embryogenic plant somatic and gametophytic cells, what is indicative of a relevant role of Ca^{2+} in the induction of embryogenesis. Whereas Ca^{2+} has been showed to be transiently accumulated at the initiation of zygotic embryogenesis, it seems likely that Ca^{2+} signatures are also important during the following stages, as shown for somatic and microspore embryogenesis.

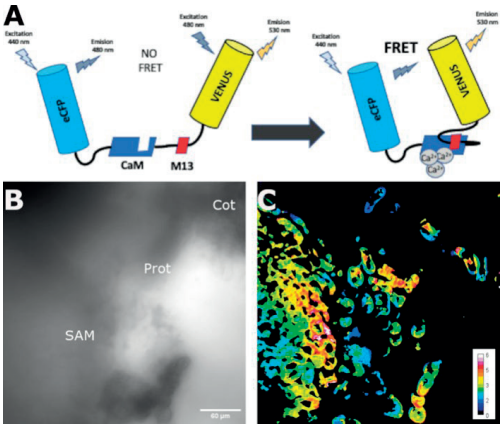

Figure 2. (A) Schematic representation of cameleon system to detect Ca^{2+} accumulation by FRET. Under low Ca^{2+} concentration (left) no FRET signal is detected. Upon Ca^{2+} increase (right), CaM and M13 form a complex that brings closer donor (i.e. eCFP) and acceptor (i.e. VENUS), what results in a FRET signal registration by confocal microscopy. (B) Bright field and (C) FRET assay, of cameleon transgenic line of arabidopsis IZE upon 5 days on embryogenic induction media

Ca^{2+} MODULATION AND EFFICIENCY OF SOMATIC EMBRYOGENESIS

The different works presented in the previous section demonstrate the occurrence of transient Ca^{2+} increases of different duration during specific stages of somatic embryogenesis. This observation, however, does not demonstrate the functional role of Ca^{2+} in this process, as Ca^{2+} increases could alternatively be due to the use of growth regulators (i.e. auxin) in the culture medium, as described for different plant organs and cell types (Vanneste and Friml, 2013). To shed light on this, pharmacological approaches consisting on the application of chemical compounds that induce free Ca^{2+} release within the cytoplasm or avoids Ca^{2+} transport and/or signaling within cells, have been used. The enrichment with $CaCl_2$ of in vitro culture media results in an increase of the SE efficiency and embryo quality in *Hevea brasiliensis* (Etienne et al., 1997), *Manihot esculenta* (Li et al., 2009), carrot (Calabuig-Serna et al., 2023b), and sandalwood (Anil and Rao, 2000). However, in other species, such as *Pinus patula* (Malabadi and van Staden, 2006) and arabidopsis (Calabuig-Serna et al., 2023a), $CaCl_2$ application showed no positive effect on SE. Even a negative effect was observed in *Cocos nucifera* (Rivera-Solís et al., 2018). Moreover, different species within *Musa* genera have different SE responses to increasing $CaCl_2$ concentrations on SE induction media (Marimuthu et al., 2019). Thus, there is not a clear pattern for the effect of Ca^{2+} enrichment in the efficiency of SE, and the reason why SE of different genotypes respond differently to specific Ca^{2+} concentrations on induction *in vitro* medium is not clearly understood. A possible explanation is that, although extracellular Ca^{2+} is higher on $CaCl_2$-supplemented media, Ca^{2+} influx is finely regulated at the plasma membrane level in a genotype-dependent manner. This could result in different species presenting different capabilities to regulate Ca^{2+} cellular homeostasis and, therefore, to accumulate Ca^{2+} within cells. In this scenario, highly responding species would accumulate high Ca^{2+} peaks, that would activate SE, whereas low or non-responsive species, would be more sensitive to intracellular Ca^{2+} toxicity, thus being Ca^{2+} peaks and SE activation low or absent.

Similarly, application of ionophore A23187, a chemical compound that favors the membrane permeability and, therefore, facilitates the cellular Ca^{2+} uptake, results on a higher SE response in carrot (Takeda et al., 2003; Calabuig-Serna et al., 2023b), *Coffea canephora* (Ramakrishna et al., 2011), and *Cocos nucifera* (Rivera-Solís et al., 2018), which points to a positive role of intracellular free Ca^{2+} on SE of different species. However, in other species such as sandalwood (Anil and Rao, 2000) and arabidopsis (Calabuig-Serna et al., 2023a), application of ionophore do not have any effect, or even results on a decrease of SE efficiency at high concentrations. Decreased SE efficiency provoked by the addition of ionophore could be explained by a possible toxic effect of high intracellular Ca^{2+} concentration, a deleterious effect of high ionophore concentration, or both. Moreover, deregulation of Ca^{2+} influx and thus, intracellular Ca^{2+} gradients, may provoke negative effects on SE. As mentioned before for Ca^{2+}-supplemented experiments, different species may have different capabilities for ionophore uptake within cells, or a more fine-tuned mechanism to tolerate high intracellular Ca^{2+} peaks. In any case, all these results

reinforce the notion that specific intracellular Ca^{2+} concentration or the maintenance of Ca^{2+} gradients are required for proper embryo development.

On the other hand, different chemical compounds that block Ca^{2+} signaling, either chelating Ca^{2+} or inhibiting CaM, as well as Ca^{2+} channel blockers, have been used to demonstrate the effect of low intracellular Ca^{2+} and Ca^{2+}-signaling during SE. In this sense, Ca^{2+} chelation by the application EGTA and blocking Ca^{2+}-mediated signaling by the addition of W-7, result on a reduction of SE in sandalwood, but does not reduces calli viability, what points to a specific role of Ca^{2+} on the SE process (Anil and Rao, 2000). Similarly, a reduction of SE efficiency was observed in *C. canephora* (Ramakrishna et al., 2011), arabidopsis (Calabuig-Serna et al., 2023a) and carrot (Calabuig-Serna et al., 2023b) upon media supplementation with Ca^{2+} chelators (i.e. EGTA), Ca^{2+} channel blockers (i.e. verapamil and chlorpromazine), or Ca^{2+} signaling inhibitors (i.e. W-7). Accordingly, reduction of intracellular Ca^{2+} by the application of the Ca^{2+} channel blockers La^{3+} ($LaCl_3$) and EGTA completely eliminated the cold-induced enhancement of SE in *P. patula* (Malabadi and van Staden, 2006). Therefore, inhibition of Ca^{2+} intracellular accumulation and signaling by different chemical approaches results in a reduction of SE in different species, which, again, demonstrates the importance of intracellular Ca^{2+} accumulation and signalling for a proper somatic embryogenic process.

Altogether, the functional role of Ca^{2+} during SE has been demonstrated in different plant species. Increasing Ca^{2+} intracellular concentrations to levels that positively regulates SE might be challenging, since different results have been obtained for different species. Indeed, specific Ca^{2+} signatures (peaks) are needed to regulate embryogenesis (Ge et al., 2007), thus exogenous ionophore application might deregulate Ca^{2+} cellular homeostasis and, therefore, its proper embryogenic activator capability. On the other hand, different studies coincide on the need of a correct Ca^{2+} accumulation and signalling during SE, since Ca^{2+} chelation and inhibition of Ca^{2+} transport and signaling results on a drastic reduction of SE efficiency of different species.

Whether the described effect of Ca^{2+} on SE is specific for this process, or can be extrapolated to other embryogenic processes, either zygotic or microspore embryogenesis, is not fully elucidated. Microspores isolated at the inducible stages from highly embryogenesis-responsive genotypes (i.e. the *B. napus* DH4079 line) accumulate more Ca^{2+} than those from genotypes with lower response (i.e the *B. napus* DH12075 line and the DH36 *S. melongena* line; Rivas-Sendra et al., 2017; Rivas-Sendra et al., 2019), which may indicate a positive effect of Ca^{2+} in the sensitivity to microspore embryogenesis induction. Moreover, pharmacological approaches aimed to study the effect of cytosolic Ca^{2+} in microspore embryogenesis revealed a positive effect of increased Ca^{2+} levels in *Triticum aestivum* (Reynolds, 2000) and Brassica napus (Calabuig-Serna et al., our unpublished data). These observations point to a general effect of Ca^{2+} during embryogenesis. The exact mechanism by which Ca^{2+} regulates SE is unknown. A possible scenario would be a direct or indirect genetic regulation of master embryogenic genes in response to Ca^{2+} signatures, which would be crucial for cells to activate an embryogenic developmental pathway (Sharma et al., 2022). Moreover, callose symplastically isolate somatic arabidopsis cells, prior to the adquisition of totipotency during SE, through its deposition on plasmodesmata (Godel-Jedrychowska et al., 2020), and it has been recently shown that application of 2-desoxy-D-glucose, a compound that inhibits callose synthesis, results in a lower SE efficiency in both carrot (Calabuig-Serna et al., 2023b) ad arabidopsis (Godel-Jedrychowska et al., 2020). Thus, the mechanisms through which Ca^{2+} regulates SE could be related to callose synthesis and deposition. Accordingly, a functional relationship was demonstrated between the embryogenic response of *B. napus* isolated microspores and Ca^{2+} influx and callose deposition at their subintinal layer (Rivas-Sendra et al., 2019), which points to a key role of Ca^{2+} in the establishment of the proper chemical environment for a successful

reprogramming of somatic cells towards embryogenesis.

CONCLUSIONS

Somatic embryogenesis is regulated by a complex genetic and epigenetic network, as well as different growth regulators. Pharmacological approaches to modify Ca^{2+} cellular homeostasis result in altered SE responses, demonstrating the need for a proper cellular Ca^{2+} homeostasis. SE protocols have been developed for many different species due to the biotechnological applications of the process. Specific Ca^{2+} signatures are detected during zygote fertilization and initial zygotic embryogenesis, microspore embryogenesis, and somatic embryogenesis. Due to these and other similarities between in vivo and in vitro embryogenesis, SE is a valuable system to study plant embryogenesis, but there are still many questions to be elucidated. Further research directed to understand the basic cellular and molecular mechanisms that control SE would help us to (1) increase its efficiency and establish new protocols for recalcitrant species, and (2) study factors that determine post-zygotic barriers during intra- or interspecific hybridizations, useful to obtain new cultivars.

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AMINO ACID COMPOSITION AND ANTIOXIDANT ACTIVITY OF BIOLOGICALLY ACTIVE PREPARATIONS OBTAINED FROM WINE YEAST SEDIMENTS

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Abstract

In this study, 4 biologically active preparations obtained from wine yeast sediments are characterized according to their amino acid content and total antioxidant activity.
As a result, it has been established that the preparations contain the full range of essential and immunoactive amino acids, the content of essential amino acids varies between 13.7-287.662 mg/100 ml and immunoactive 13.9-447.33 mg/100 ml. The study of the total antioxidant activity in the biologically active preparations obtained is within the limits of 33.4 ± 0.3-85.9 ± 0.9% inhibition, the maximum being obtained in the amino acid protein preparation SRM-AAP. In conclusion, we can mention that the obtained results indicate that the biologically active preparations obtained from the sediments of wine yeasts are rich in essential and immunoactive amino acids and have a high antioxidant activity, being attractive for implementation in agriculture, especially in animal husbandry, food and cosmetic industry.

Key words*:* amino acid, antioxidant activity, biologically active preparations, wine yeast.

INTRODUCTION

Currently, scientific research is increasingly oriented towards studies that address the problem of industrial waste and that aim to reuse it efficiently and sustainably.
In the context of the Republic of Moldova, the wine industry is an important sector of the economy that has an estimated production of around 11.5 million hectoliters per year and that has recorded constant increases in production in recent years. Following the winemaking process, huge amounts of sediments are obtained that are formed during the fermentation, filtration, centrifugation and maturation stages, which are finally thrown away as waste that pollutes the environment (Devesa-rey et al., 2011).
The main components of wine sediments are yeasts that contain a rich variety of biologically active compounds, including pigments, proteins and amino acids (Baik et al., 2003; Kim et al., 2011). One of the main directions of waste reduction in the wine industry is the development of progressive technological processes for processing and obtaining biologically active preparations with beneficial effects on human and animal health (Tao et al., 2017). To assess the quality, effectiveness and potential benefits of biologically active preparations obtained from wine yeast sediments, the study of amino acid composition and total antioxidant activity is important (Belda et al., 2017; Fidelis et al., 2020).
Research has shown that the amino acid composition of yeast sediments can vary depending on various factors, such as the type of wine, the fermentation process and the strain of yeast used. A study by Li et al. (2015) who analyzed the amino acid composition of yeast sediments from different types of wine found significant differences in their profiles.
Some amino acids were also found to be particularly abundant in yeast sediments, such as glutamic acid, aspartic acid, and alanine (Sarubbo et al., 2015).
These amino acids are known to have antioxidant properties and are involved in various metabolic processes in the body. Research has shown that preparations derived from yeast sediments can have important antioxidant, antimicrobial and anticancer activities (Wu, 2013; Callejón et al., 2014; Zheng et al., 2019).

Based on the above, the relevance of this direction highlights the need to solve the problem of industrial waste that contaminates the environment and remains unused and the prospect of using yeasts in biotechnology for the production of biopreparations with high biological value.

Therefore, this study aimed to determine the amino acid composition and antioxidant activity of biologically active preparations obtained from wine yeast sediments

MATERIALS AND METHODS

The object of study was the yeast biomass (*Saccharomyces cerevisiae*) from the production of the Merlot wine, that was kindly provided by the Cricova winery.

Process for obtaining biologically active preparations from wine yeast sediments

The amino acid protein preparation SRM-AAP was obtained through yeast biomass autolysis using a sodium phosphate buffer (ratio 1: 1) at temperature 45°C for 8 hours. The liquid phase was then separated from the solid residue, and sterilization by tyndalization was performed. The solid residue left over after obtaining SRM-AAP was used to prepare the anthocyanin SRM-AN preparation. The extract was obtained by treating the cell walls with 50% ethyl alcohol, followed by agitation on a shaker at 200 rpm for 30 minutes at room temperature. The resulting mixture was then centrifuged for 15 minutes and sterilization.

The mannoprotein preparation, known as SRM-MP, was obtained from the solid sediment that remained after obtaining the anthocyanin preparation. This was achieved through hydrolysis in a 1N NaOH solution (ratio 1: 5) at a temperature of 80 ± 5°C for 2 hours. The resulting mixture was then sedimented with 96% (v/v) ethyl alcohol at a ratio of 1: 2. Finally, the sediment was dissolved in distilled water, the pH was adjusted to 7.0-7.8, and sterilization by tyndalization was performed (Beșliu et al., 2022).

Finally, from solid residue it was obtained the β-glucan fraction SRM-GL insoluble in alkalis and acids, which was treated with 0.5N acetic acid (at a ratio of 1: 5) at temperature 75 ± 5°C for 1 hour. After the acid hydrolysis, the resulting suspension was centrifuged at 3500 rpm for 15 minutes to separate the phases. The β-glucan fraction was then washed three times with distilled water.

Methods of achieving research

The content of amino acids in the preparations was determined by ion exchange chromatography method described by Garaeva et al. (2009).

The antioxidant activity in the preparations was determined by the spectrophotometric method using the radical cation 2,2-azinobis 3-ethylbenzothiazoline-6- sulfonic acid (ABTS) (Re et al., 1999).

Statistical analysis was done using the MO Excel and Statistics 9.0 software. The results were expressed as the mean, standard deviation and confidence interval at $P \leq 0.05$ from three repetitions.

RESULTS AND DISCUSSIONS

Following the research carried out by fractional extraction, 4 biologically active preparations were obtained from the same volume of processed yeast sediments. Thus, tests to determine the content of amino acids in the amino acid protein preparation revealed that it contains the entire range of essential and immunoactive amino acids. The total amount of essential amino acids (lysine, threonine, phenylalanine, isoleucine, leucine, valine, histidine and methionine) was found to be 287.662 mg/100 ml, while the immunoactive amino acids (glycine, glutamic acid, serine, alanine, arginine and cysteine) was 447.33 mg/100 ml (Figure 1). Among the essential amino acids, lysine was the most abundant with 84.4 mg/100 ml, followed by threonine with 69.0 mg/100 ml, valine with 34.4 mg/100 ml and phenylalanine with 34.3 mg/100 ml. From the range of immunoactive amino acids, maximum amounts of glycine, alanine and serine were determined (Figure 1).

Similar results regarding the amino acid content of autolyzed wine yeast extracts were also presented in the study by Osés et al. (2019) who obtained high levels of essential and non-essential amino acids, with lysine, proline, leucine and glutamic acid being the most abundant (Osés et al., 2019).

The study of amino acid content in the SRM-AN preparation showed lower values compared

to that determined in the amino acid protein preparation. Thus, the sum of the essential amino acids is 168.0 mg/100 ml, and the immunoactive ones are 258.7 mg/100 ml (Figure 2). It was found that the essential amino acids are represented by important amounts of lysine 46.5 mg/100 ml, threonine 35.8 mg/100 ml and valine 20.8 mg/100 ml, and the immunoactive ones by proline 484.6 mg/100 ml, glutamic acid 79.0 mg/100 ml and aspartic acid 38.6 mg/100 ml.

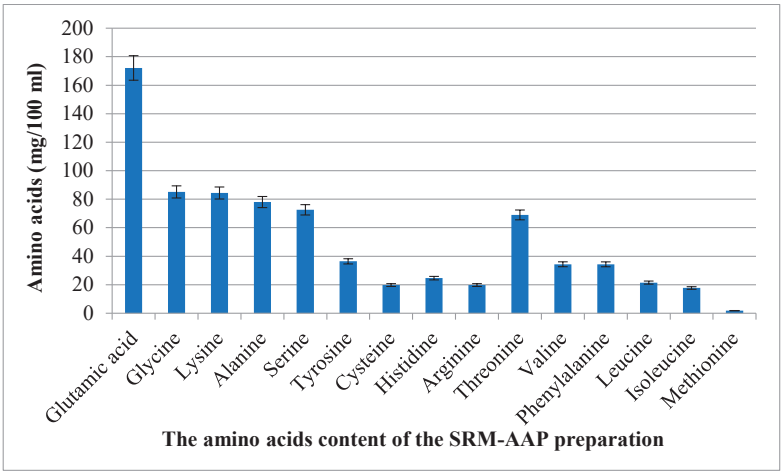

Figure 1. The amino acid content in the SRM-AAP preparation obtained from wine yeast waste sediments

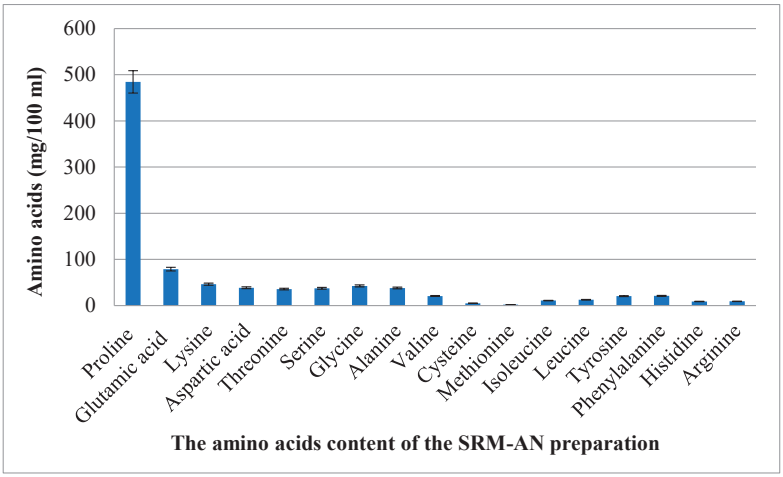

Figure 2. The amino acid content in the SRM-AN preparation obtained from wine yeast waste sediments

Next, to ensure consistent quality and effectiveness, the content of amino acids in the mannoprotein preparation was evaluated. The results are shown in Figure 3. According to the results obtained, it was established that the content of essential and immunoactive amino acids is 13.7 mg/100 ml and, respectively, 13.9 mg/100 ml. Quantitative analysis of essential amino acids established that lysine, leucine, valine and threonine prevail among them. It is also characterized by a high arginine content of 4.39 mg/100 ml, aspartic acid 3.27 mg/100 ml and phenylalanine 1.28 mg/100 ml.

The obtained results indicate that the quantitative composition of amino acids in mannoprotein preparations obtained from wine yeasts can vary depending on the extraction and purification methods used. A similar spectrum of essential amino acids was also obtained in the study by Vivar-Quintana et al. (2004) in which the content of lysine, leucine and valine recorded the highest levels. In contrast, the preparation in the current study had higher levels for arginine only.

These differences highlight the importance of carefully characterizing biologically active preparations to ensure consistent quality and efficacy (Vivar-Quintana et al., 2004).

Finally, the amount of amino acids in the SRM-GL preparation was analyzed.

The findings are presented in Figure 4.

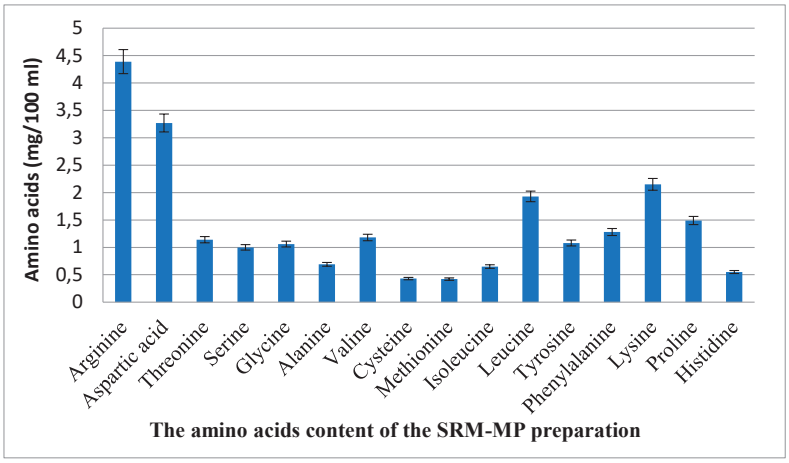

Figure 3. The amino acid content in the SRM-MP preparation obtained from wine yeast waste sediments

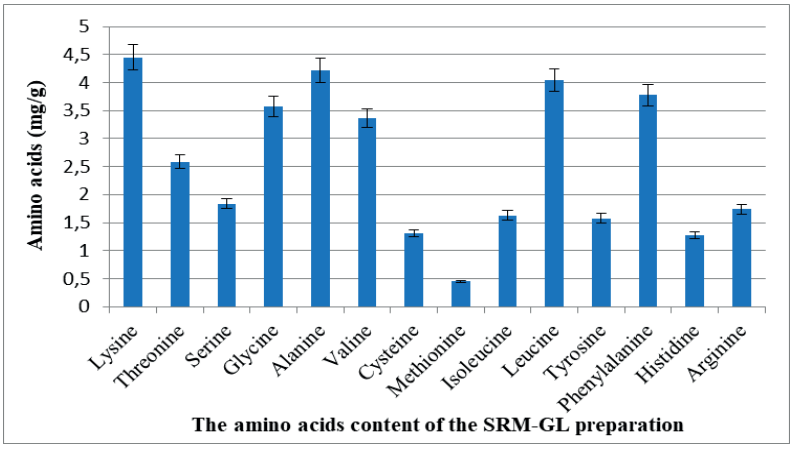

Figure 4. The amino acid content in the SRM-GL preparation obtained from wine yeast waste sediments

It was determined that the preparation contains 51.2 mg/g of proteinogenic amino acids, of which 23.3 mg/g are essential and 27.9 mg/g are immunoactive. Lysine, leucine, phenylalanine and valine are the most abundant essential amino acids, while alanine, serine and glycine prevail among the immunoactive ones. These results are consistent with previous studies in the literature, which report that the total content of essential amino acids in β-

glucans ranges from 22-39 mg/g, with lysine, leucine, and valine being the most abundant (Gobbetti et al., 2010; Barros et al., 2014).
In the later phase of the study, due to the substantial composition of essential amino acids in the preparations, the total antioxidant activity was evaluated. According to the obtained results, it was established that the SRM-AAP preparation is characterized by a very high total antioxidant activity equal to 85.9 ± 0.9% inhibition. Whereas, the activity in the other preparations was within the limits of 33.4-52.2% inhibition (Figure 5).

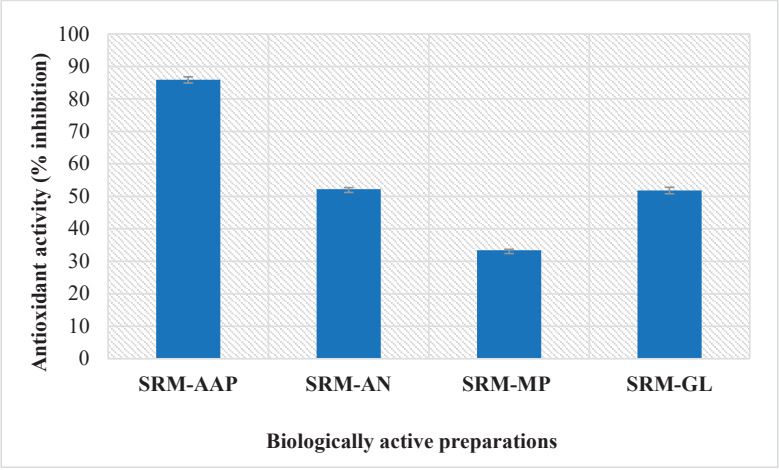

Figure 5. Antioxidant activity in biologically active preparations obtained from wine yeast sediments

Similar results exist in several studies that investigated the antioxidant activity of biologically active preparations obtained from wine sediment waste. Protein and mannoprotein extracts obtained from yeasts were reported to have strong antioxidant activity in vitro, which was attributed to their high content of amino acids and polysaccharides (Kim et al. 2007). Another study by Yao et al. (2020) demonstrates that mannoproteins isolated from wine yeast possess antioxidant activity in vitro and suggested that they could be used as natural antioxidants.
The study by Zhou et al. (2016) found that anthocyanin extracts from waste wine sediments have strong antioxidant activity, which was attributed to their high phenolic content (Zhou et al. 2016).

CONCLUSIONS

The biologically active preparations derived from wine yeast sediments exhibit a rich biochemical composition of essential, proteogens and immunoactive amino acids. Based on the biochemical composition, it can be observed that the preparations also exhibit high levels of antioxidant activity. The antioxidant activity values range from 33.4 ± 0.3-85.9 ± 0.9% inhibition, with the SRM-AAP preparation displaying the highest value.
These preparations have demonstrated a variety of beneficial properties, including nutrition and antioxidant effects, rendering them potentially valuable for a range of applications in several sectors, including animal husbandry, cosmetics and the food industry.

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ANTIMICROBIAL ACTIVITY OF MICROORGANISMS ISOLATED FROM SILT OF THE "LA IZVOR" LAKE SYSTEM (CHISINAU MUNICIPALITY)

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Abstract

Microorganisms are the main source of various bioactive substances used in diverse fields of biotechnology. A current problem in agriculture is the fight against various phytopathogens, which cause crop diseases. Current trends worldwide are green agricultural methods. Actinobacteria, bacteria, and micromycetes are known to naturally associate with plants and have a beneficial effect on their growth. Thus, the groups of microorganisms listed above were isolated from the silt samples of the "La izvor" lake system (Chisinau municipality), and their screening was performed to combat phytopathogenic agents that cause plant diseases. The following strains of microorganism actively inhibit the growth of phytopathogenic fungi: A. alternata - 8 micromycetes, 2 bacteria; A. niger - 1 micromycete, 3 actinobacteria; B. cinerea - 5 micromycetes, 4 bacteria; F. oxysporum - 7 micromycetes, 3 actinobacteria, 2 bacteria. The growth of phytopathogenic bacteria was actively inhibited only by micromycetes: B. subtilis - 15 strains; X. campestris - 8 strains; C. michiganensis - 5 strains; A. tumefaciens - 3 strains; E. carotovora - 5 strains.

Key words*: actinobacteria, antibacterial activity, antifungal activity, bacteria, micromycetes.*

INTRODUCTION

Water ecosystems of lakes in various regions of the Earth provide irreplaceable water resources to humans. The microbiomes of lake ecosystems are suitable bioresources for agriculture, industry and related sectors. An analysis of the microflora presents in the waters of lakes around the world showed that the most common types are *Proteobacteria*, *Cianobacteria*, *Actinobacteria*, *Flavobacterium*, *Firmicutes*, *Acidobacteria*, as well as various representatives of fungi such as micromycetes.

The natural community is an important center of potential microbial resources; only it can serve as a source of microbiological objects for various kinds of research. Diversity of microorganisms is the main source for the creation of new biotechnological processes and products, and screening of microorganisms for potentially useful traits plays an important role. (Borneman & Triplett, 1997; Denisova et al., 1999; Yadav et al., 2018).

Recently, interest has increased in studying the structure and composition of microbial communities, which are one of the key components of aquatic ecosystems. Microorganisms, which are small in size, make up a significant part of the biomass in water bodies. High reproduction rate and wide adaptive abilities enable microorganisms to adapt to various environmental conditions (Bel'kova et al., 2003).

Currently, the world, and especially in developing countries, is experiencing nutritional deficiencies due to a number of causes, including loss of agricultural yields due to diseases of critical crops caused by phytopathogenic fungi and bacteria.

Despite the current trend in the development of ecological farming, the protection of plants from diseases is based mainly on the use of chemical means of protection (pesticides) against pathogens of bacterial and fungal etiology. Intensive and often unregulated use of pesticides leads to environmental pollution due to the accumulation of these substances in soil and natural waters. That is why, much attention is paid to the development of environmentally friendly biological methods for combating plant diseases, which are considered as an alternative to the traditional use of chemical pesticides (Ab Rahman et al., 2018; Burtseva & Sirbu, 2009; Burțeva et al., 2008; Hyakumachi et al., 2014;

Palaniyandi et al., 2013). The biological method of protecting plants from pathogenic microorganisms is based on the use of antagonist microorganisms. Currently, a number of microorganisms have been described that have an antagonistic effect on phytopathogens. Their mechanism of action includes competition for food, effective colonization of the rhizosphere and leaf surfaces, and the synthesis of antibiotic substances (Azizbekyan et al., 2001; Chausaria et al., 2018; Van der Meij et al., 2017).

Microorganisms play a huge role in the control of plant diseases. In recent decades, research into the biological control of plant pathogens has seen a marked acceleration due to the risk of using synthetic pesticides. Therefore, a promising element of modern agroecology is the use of preparations based on microorganisms or their metabolites, which exhibit phytoprotective and growth-stimulating properties, increase plant resistance against pathogens and stress factors (El-Sabbagh et al., 2013; Hata et al., 2015; Hyakumachi et al., 2014; Jeon et al., 2016; Tiwari & Gupta, 2013; Van der Meij et al., 2017).

Biological farming, which is based on the ecological stabilization of agroecosystems, is gaining great popularity in the world. Naturally, there is an increasing interest in microbial preparations to improve plant nutrition, regulate their growth and development, as well as protect against phytopathogens and pests. An important factor in increasing the productivity of agroecosystems is the activation of microbial-plant interaction. Environmentally friendly microbial preparations are being developed and introduced into the system of necessary agrotechnical measures. They contribute to the intensification of physiological and biochemical processes in plants, increase their resistance to diseases and have a positive effect on soil microorganisms. In practice, they are created on the basis of microorganisms isolated from natural biocenoses, including water bodies. They do not pollute the environment and are safe for animals and humans (Andriyuk et al., 2001; Omelyants et al., 2008; Safronova, 2007).

The importance of protecting crops from pests and diseases is becoming a determining factor in increasing the yield and quality of crop products. At the same time, the importance of phytosanitary in the field of environmental safety issues is rapidly increasing. This is convincingly evidenced by the growing volumes of production and use of pesticides in the world. According to Bayer and the Ministry of Agriculture of Japan, in recent years there has been an increase in the resistance of phytopathogenic fungi to fungicides: 20 species of phytopathogens have been found to be resistant to 15 fungicides (Pavlyushin, 2013). In this regard, new strains of actinobacteria, bacteria, and micromycetes were found and included in the lists of agents against plant diseases (Hyakumachi et al., 2014).

The purpose of the research was to screene and finding new strains of microorganisms with antimicrobial properties, which were isolated from the silt sediments of the "La izvor" lake system, Chisinau municipality.

MATERIALS AND METHODS

The following research was conducted within the National Collection of Non-pathogenic Microorganisms of the Institute of Microbiology and Biotechnology of the Technical University of Moldova.

The geographical coordinates of lake system "La izvor" (Republic of Moldova, Chisinau municipality) where samples were collected are: 1) 47°02'44.2"N, 28°47'18.9"E; 2) 47°02'53.7"N, 28°47'42.5"E; 3) 47°02'59.6"N, 28°47'59.3"E. Altogether 11 points were sampled in August 2020. Random samples of silt sediments were collected in sterile containers. The samples were not pretreated. After that, serial dilutions were carried out using distillate water to dilute the samples to 10^{-1}-10^{-6} (Hussein et al., 2018; Yu et al., 2015).

For study actinobacteria, were isolated 8 genera on special selective nutrient media in Petri dishes by inoculation of diluted samples:

Actinomadura - soluble starch (20.0 g/L), K_2HPO_4 (0.5 g/L), $MgSO_4$ (0.5 g/L), KNO_3 (1.0 g/L), NaCl (0.5 g/L), $FeSO_4$ (10.0 mg/L), streptomycin (50 mcg/mL), nystatin (50 mcg/mL), agar, pH = 7.2-7.4 (Zakharova et al., 2003);

Actinoplanes - oatmeal (2.5 g/L), K_2HPO_4 (1.0 g/L), KCl (0.5 g/L), $MgSO_4*H_2O$ (0.5 g/L), $FeSO_4*7H_2O$ (0.01 g/L), streptomycin (50 mcg/mL), nystatin (50 mcg/mL), agar, pH = 7.0 (Zenova & Zvyagintsev, 2002);

Frankia - propionic acid (0.5 g/L), NH_4Cl (0.1 g/L), $CaCl_2*2H_2O$ (0.1 g/L), $MgSO_4*H_2O$ (0.2 g/L), $NaH_2PO_4*2H_2O$ (0.67 g/L), agar, pH = 6.8-7.2 (Semenov, 1990);
Geodermatophilus - yeast extract (1.0 g/L), glucose (1.0 g/L), soluble starch (1.0 g/L), $CaCO_3$ (1.0 g/L), streptomycin (50 mcg/mL), nystatin (50 mcg/mL), agar, pH=7.0 (Semenov, 1990);
Micromonospora - soluble starch (20.0 g/L), K_2HPO_4 (0.5 g/L), $MgSO_4$ (0.5 g/L), KNO_3 (1.0 g/L), NaCl (0.5 g/L), $FeSO_4$ (0.01 g/L), gentamicin (1 mcg/mL), streptomycin (25 mcg/mL), agar, pH = 7.2-7.4 (Zenova et al., 2004);
Nocardia - $NaNO_2$ (2.0 g/L), Na_2CO_3 (1.0 g/L), K_2HPO_4 (0.5 g/L), gentamicin (1 mcg/mL), agar, pH = 7.0 (Semenov, 1990);
Rhodococcus - KNO_3 (1.0 g/L), K_2HPO_4 (1.0 g/L), KH_2PO_4 (1.0 g/L), NaCl (1.0 g/L), $MgSO_4*H_2O$ (0.2 g/L), $CaCl_2*2H_2O$ (0.2 g/L), $FeCl_3$ (0.0001 g/L), yeast extract (1.0 g/L), propionic acid (0.5 g/L), levomycetin (20 mcg/mL), agar, pH = 7.0 (Cheremnykh, 2018);
Streptomyces - glucose (20.0 g/L), KNO_3 (1.0 g/L), NaCl (0.5 g/L), $MgSO_4$ (0.5 g/L), K_2HPO_4 (0.5 g/L), $CaCO_3$ (3.0 g/L), streptomycin (50 mcg/mL), agar, pH = 6.8-7.0 (Semenov, 1990).
The bacteria were isolated by serial dilution technique on nutrient agar medium Liofilchem (Italy): meat extract (1.0 g/L), yeast extract (2.0 g/L); peptone (5.0 g/L), NaCl (5.0 g/L), agar (15.0 g/L), pH = 7.2. Spread plate technique was carried out to isolate the organism form the diluted sample and incubated at 37°C for 24 hours (Kannan et al., 2018).
Fungi are typically isolated by plating a sample on a Petri dish containing wort agar (5.0°B, pH = 5.8-6.0), produced at Rivex SRL Brewery (Republic of Moldova, Chisinau municipality, Gratiesti commune), supporting the growth of a variety of fungi incubated for 7-10 days at 28-30°C (Nevalainen et al., 2014).
The identification of the belongingness of the microorganisms from the samples taken was carried out with optical microscopes (Lomo Mikmed - 2; Optika - B-292) using determinants for bacteria (Birger, 1982; Zarnea, et al., 2004), and fungi (Blagoveshenskaya, 2015; Eremeeva, 2008).
As result were isolated next active microorganisms: 19 strains of actinobacteria group; 12 strains of bacteria group; 15 strains of fungi group. After purification by several passages, the strains were tested for determination of potential antimicrobial activity.
The selected strains were subcultured in Petri dishes to obtain a bacterial lawn with diffusion of antimicrobial substances in agar substrate (Egorov, 2004). Test-cultures are maintained in the National Collection of Non-pathogenic Microorganisms.
Antibacterial efficacy was tested against:
Agrobacterium tumefaciens (*Rhizobium radiobacter*) 8628; *Bacillus subtilis* B-117; *Clavibacter michiganensis* (*Corynebacterium michiganense*) 13[a]; *Erwinia carotovora* (*Pectobacterium carotovorum*) 8982; *Xanthomonas campestris* 8003[b].
While antifungal activity was tested against:
Alternaria alternata; *Aspergillus niger*; *Botrytis cinerea*; *Fusarium oxysporum*; *Fusarium solani*.
Phytopathogenic bacteria tests were subcultured on nutrient agar (pH = 7.0-7.5), and phytopathogenic fungi tests were subcultured on wort agar (5.0°B, pH = 5.8-6.0) (Krassilnikov & Husein, 1974).
The biocidal activities were determined by the disk diffusion method. The tested cultures were subcultured in Petri dishes. The 8 mm agar blocks were cut with a sterile cork borer from the nutrient substrate where the strains of tested microorganisms grew abundantly. The agar blocks were then transferred to prepared cavities in agar nutrient medium with instantly subcultured tests. Petri dishes were kept in a cool place for 1 hour before incubation to allow the diffusion of biocidal substances. The diameter of the growth inhibition zones was measured after incubation at 37°C for 24 h for bacteria, and at 28°C for 72 h for fungi, respectively (Egorov, 2004; Rizk et al., 2007). There were three replications for each test (was applied to the significance threshold $p = 0.05$) and the biocidal assessment was performed twice.

RESULTS AND DISCUSSIONS

In order to evaluate the biocidal activity of the studied microorganisms, various plant pathogens were screened. The selected pathogens cause severe disease and yield losses to many agricultural crops in the Republic of Moldova.

Listed below are diseases caused by test bacteria and fungi:

- *Agrobacterium tumefaciens* (*Rhizobium radiobacter*), which cause neoplastic diseases in plants like crown gall disease of woody plants such as pome (apple, pear, etc.) and stone (cherry, apricot, etc.) fruit, and nut (almond, walnut, etc.) trees (Escobar & Dandekar, 2003; Gohlke & Deeken, 2014; Kado, 2002);
- *Bacillus subtilis*, causative agent of potato disease of baked goods (Zavorohina, 2018);
- *Clavibacter michiganensis* (*Corynebacterium michiganense*), is the main causative agent of bacterial canker on solanaceous crops like eggplant, pepper and tomato (Ansari et al., 2019);
- *Erwinia carotovora* (*Pectobacterium carotovorum*), infects a much broader host of plants and cause soft rot of tomato and potato (Akbar et al., 2014);
- *Xanthomonas campestris*, cause black rot and vascular or leaf spot diseases of brassica species (Vicente & Holub, 2013);
- *Alternaria alternata*, cause *Alternaria* blotch of apple, *Alternaria* black spot of strawberry, and stem canker of tomato, respectively (Gat et al., 2012);
- *Aspergillus niger*, produce mycotoxins during developing on seeds, leaves and other plant organs (Soares et al., 2013; Alkhalifah et al., 2022);
- *Botrytis cinerea*, is a major plant pathogen, causing gray mold rot in a variety of cultures like pome fruits, stone fruits, grapes and berries (Rupp et al., 2017; Kahramanoğlu et al., 2022);
- *Fusarium oxysporum*, caused the *Fusarium* wilt in different fruits, berries and vegetables like tomato, watermelon, strawberry, cabbage, etc. (Gordon, 2017);
- *Fusarium solani*, is a pathogen reported on different crops both in nurseries and in fruit production fields, causing wilt and root rot (Villarino et al., 2019).

Activity of actinobacteria, bacteria, and micromycetes to inhibit the growth of the studied phytopathogenic fungi, is different and mainly depends on the characteristics of the synthesized substances with antimicrobial properties. For example, out of total number of 36 strains of actinobacteria belonging to 8 main genera isolated, 17 strains did not show the ability to inhibit the growth of 5 test cultures of phytopathogenic fungi. Next strains of actinobacteria showed activity against *A. alternata*: 2 of genus *Actinomadura*, 4 of genus *Actinoplanes*, 1 of genus *Frankia*, 3 of genus *Geodermatophilus*, 3 of genus *Micromonospora*, 2 of genus *Nocardia*, 2 of genus *Rhodococcus*, and 1 of the genus *Streptomyces*. While the size of growth inhibition zones varied between 10.0-23.0 mm. The strain of the genus *Actinomadura* N 1.2 showed the best result - the diameter of the growth inhibition zone of the test culture was 23.0 mm. The growth of *A. niger* was inhibited by 14 strains of actinobacteria with a zone diameter of 10.0-35.0 mm. The best result - the growth inhibition zones of this phytopathogen reached 30.0-35.0 mm in size and were noted under the influence of metabolites of *Nocardia* N 6.2 strain (30.0 mm), *Streptomyces* N 8.4 strain (33.0 mm), and *Micromonospora* N 5.5 strain (35.0 mm). The ability to inhibit the growth of *B. cinerea* was not seen among the new strains of actinobacteria. For *F. oxysporum*, 15 strains of actinobacteria were noted that have the ability to inhibit the growth of this test phytopathogen. Actively inhibit the growth by zones of 26.0 m next strains: *Actinoplanes* N 2.4, *Micromonospora* N 5.1, *Nocardia* N 6.2, while in other strains the zones varied between 13.0-21.0 mm. The growth of another representative of the phytopathogens of the genus *Fusarium*, *F. solani*, was inhibited by 10 strains of actinobacteria, and the growth inhibition zones varied in the range of 10.0-22.0 mm (Table 1).

Table 1. Antifungal activity of actinobacteria strains isolated from the silt sediments of the "La izvor" lake system, diameter of growth inhibition zones (mm)

Genus of actinobacteria	Strain No.	*A. alternata*	*A. niger*	*B. cinerea*	*F. oxysporum*	*F. solani*
Actinomadura	N 1.2	23.0±1.1	10.0±0	0	15.0±1.1	14.0±0
	N 1.3	18.0±0	14.0±0	0	20.0±0	10.0±0
Actinoplanes	N 2.2	16.0±0	10.0±0	0	18.0±0	12.0±0
	N 2.3	16.0±0	10.0±0	0	17.0±1.1	16.0±1.1
	N 2.4	0	14.0±0	0	26.0±0	22.0±1.1
	N 2.5	12.0±0	0	0	14.0±1.1	0
Frankia	N 3.2	0	14.0±0	0	18.0±1.1	17.0±0
Geodermatophilus	N 4.1	16.0±0	12.0±1.1	0	17.0±0	18.0±0
	N 4.3	0	11.0±1.1	0	13.0±0	21.0±0
	N 4.5	0	17.0±0	0	16.0±0	0
	N 4.6	10.0±1.1	0	0	0	0
Micromonospora	N 5.1	0	12.0±1.1	0	26.0±1.1	0
	N 5.4	0	13.0±1.1	0	20.0±0	0
	N 5.5	0	35.0±1.1	0	21.0±0	0
Nocardia	N 6.1	10.0±0	0	0	0	0
	N 6.2	0	30.0±1.1	0	26.0±1.1	10.0±0
Rhodococcus	N 7.1	16.0±1.1	0	0	0	0
	N 7.2	10.0±0	0	0	0	18.0±1.1
Streptomyces	N 8.4	0	33.0±1.1	0	16.0±0	0

Note: p = 0.05

The conducted studies showed that out of total number of 22 bacterial strains assigned to 3 genera *Arthrobacter*, *Bacillus*, *Paenibacillus*, 9 bacteria did not show antagonism in relation to 5 strains of phytopathogenic fungi selected as test cultures, in the remaining strains of antifungal activity was ambiguous and was manifested by the formation of growth inhibition zones of a wide range – diameter between 6.0-30.0 mm (Table 2). Thus, the bacteria were able to inhibit the growth of *A. alternata* with zones between 11.0-26.7 mm. As can be seen from the data in Table 2, the metabolites of *Bacillus* 39 and *Paenibacillus* 43 formed inhibition zones with a size of 26.7 and 25.7 mm, while under the influence of metabolites of other strains of bacteria, the zones were between 11.0-21.7 mm in size. The growth of *A. niger* was inhibited only by 5 strains in small zones: 6.0-7.0 mm for bacteria of the genus *Bacillus* and 16.0-19.3 mm for bacteria of the genus *Paenibacillus*.

The studied bacteria inhibited the growth of *B. cinerea* more actively: the size of the zones was noted between 19.3-30.0 mm. Of the 11 bacterial strains marked by the ability to actively inhibit the growth of this phytopathogen, it should be noted *Bacillus* 32 (zones 25.0 mm), *Arthrobacter* 35 (zones 28.3 mm), *Bacillus* 31 (zones 29.9 mm), and especially *Paenibacillus* 47, causing the formation of inhibition zones with a size of 30.0 mm.

Growth inhibition of *F. oxysporum* was noted under the influence of 5 strains of bacteria, and 2 strains of them were active (*Bacillus* 32 and 33), because the zones were 27.7-29.7 mm in size, while 3 strains formed growth inhibition zones of a smaller size - 15.3-19.0 mm. In relation to another representative of the phytopathogens of the genus *Fusarium* - *F. solani*, bacteria isolated from silt sediments showed a different level of antifungal activity: a high activity in two strains (*Paenibacillus* 43 and *Bacillus* 39, respectively), while in other strains the level of activity was lower (zones 15.3-22.0 mm in size). The studied strains of bacteria showed the least antifungal activity against *A. niger* - 5 strains of bacteria formed growth inhibition zones with a diameter of 6.0-19.3 mm.

Table 2. Antifungal activity of bacteria strains isolated from the silt sediments of the "La izvor" lake system, diameter of growth inhibition zones (mm)

Genus of bacteria	Strain No.	*A. alternata*	*A. niger*	*B. cinerea*	*F. oxysporum*	*F. solani*
Arthrobacter	35	21.7±1.73	0	28.3±1.73	0	0
Bacillus	26	0	0	20.7±1.31	0	0
	28	0	0	23.3±3.27	0	0
	31	16.7±1.73	0	29.0±1.13	17.3±0.65	15.3±0.65
	32	19.0±1.13	6.0±1.13	25.0±1.13	27.7±0.65	20.0±1.13
	33	21.3±1.73	7.0±1.13	23.0±1.13	29.7±1.73	17.7±1.31
	39	26.7±1.73	0	21.0±4.08	0	29.3±1.31
	40	0	0	19.3±1.31	0	0
Paenibacillus	37	11.0±1.13	0	24.0±1.96	0	22.0±1.13
	43	25.7±0.65	16.0±1.13	0	15.3±0.65	26.7±1.31
	46	20.7±1.31	19.3±1.31	0	19.0±1.13	20.0±2.26
	47	20.7±1.31	19.3±1.31	30.0±2.26	0	0

Note: p = 0.05

Results of determining the antifungal activity of micromycetes isolated from the silt sediments of the "La izvor" lake system are present in Table 3. The data obtained showed that only one strain out of total number of 16 strains isolated did not show antifungal activity against 5 strains of test cultures of phytopathogens selected for the experiment. For other strains, the results are rather ambiguous: there is a high activity of micromycete strains (growth inhibition zones reach 40.0 mm in diameter) or insignificant (zones with a diameter of 10.7-11.3 mm). The fact of differences in the antagonism of the studied micromycetes in relation to one or another test phytopathogen also draws attention.

Table 3. Antifungal activity of micromycetes strains isolated from the silt sediments of the "La izvor" lake system, diameter of growth inhibition zones (mm)

Genus of micromycetes	Strain No.	*A. alternata*	*A. niger*	*B. cinerea*	*F. oxysporum*	*F. solani*
Penicillium	N 1	18.0±2.26	0	0	14.0±1.13	15.0±1.13
	N 2	18.3±2.36	0	14.7±0.65	10.7±1.31	12.7±0.65
	N 5	17.3±1.31	0	16.7±1.31	0	14.7±0.65
	N 6	17.7±2.85	0	0	0	16.3±1.73
	N 8	16.0±1.13	0	16.3±0.65	0	15.7±0.65
Talaromyces	N 3	39.0±1.13	0	14.3±1.73	18.0±2.26	15.0±1.13
	N 4	39.0±1.13	0	13.3±1.31	11.3±1.31	15.3±0.65
	N 7	17.3±1.31	28.3±1.73	13.3±1.31	26.3±1.31	18.7±1.31
	N 28	27.7±2.85	0	14.3±1.73	27.7±2.85	22.0±2.26
	N 29	22.0±2.26	23.0±2.99	16.3±0.65	18.0±2.26	25.3±0.65
Trichoderma	N 9	28.7±1.31	24.7±0.65	40.0±1.13	29.0±4.08	40.0±2.26
	N 10	40.0±2.26	16.0±1.13	40.7±1.73	40.0±1.13	40.0±1.96
	N 12	40.0±2.26	18.0±2.26	40.7±1.73	40.0±1.13	40.0±1.96
	N 13	31.7±3.27	18.0±2.26	40.0±2.26	40.0±1.96	40.0±2.26
	N 14	40.0±2.26	18.7±1.31	41.3±1.31	40.3±1.73	40.3±1.73

Note: p = 0.05

For example, 8 strains of fungi showed no activity against *A. niger*, while only 2 strains against *B. cinerea*. Those there is a clear selectivity. In addition, we should also note the level of antifungal activity of fungi in relation to each of the test phytopathogens: if micromycetes inhibited the growth of *A. niger* by zones from 16.0 to 28.3 mm, then, for example, the growth of *B. cinerea*, *F. solani*, and *F. oxysporum* fungi inhibited by zones from 13.3 to 40.7 mm, or from 10.7 to 40.3 mm.

Comparing the degree of antifungal activity of the studied micromycetes, it should be noted that it manifested itself least of all in relation to *A. niger*: only one strain *Talaromyces* N 7 had the ability to inhibit the growth of this phytopathogen with zones of 28.3 mm, in other strains, the zone sizes varied between 16.0-24.7 mm.

The growth of *A. alternata* was more actively inhibited by all 15 strains of micromycetes. The activity was higher and the zones varied from 16.0 to 22.0 mm and even were from 27.7 to 40.0 mm in size, and the zones of 39.0-40.0 mm in size formed by 5 strains of micromycetes (Table 3).

An interesting pattern was noted in micromycetes in relation to *B. cinerea*: some strains showed low activity - zones of 13.3-16.6 mm under the influence of metabolites of 8 strains or rather high - zones reached 40.0-41.3 mm under the influence of 5 strains of micromycetes.

For *F. solani*, 5 strains can be considered active antagonists, which formed zones up to 40.0 mm, the remaining 10 strains differed in a variety of low activity values: zones 12.7-18.7 mm or 22.0-25.3 mm in two strains (*Talaromyces* 28 and *Talaromyces* N 29, respectively). In relation to *F. oxysporum*, 4 strains showed active antagonism (zones 40.0-40.3 mm), low activity - 3 strains (zones 10.7-18.0 mm) and medium activity - 3 strains (zones size - 26.3, 27.7, and 29.0 - strains *Talaromyces* N 7, *Talaromyces* N 28, and *Trichoderma* N 9, respectively).

The antibacterial activity of the studied representatives of actinobacteria was, as can be seen when comparing the data presented in Tables 1 and 4, noticeably less than the antifungal activity: the growth inhibition zones of 5 strains of phytopathogenic bacteria selected as test cultures varied between 9.0-16.0 mm. Growth inhibition of *A. tumefaciens* by zones of 9.0-13.0 mm was caused by 6 strains belonging to genera *Geodermatophilus*, *Micromonospora*, *Nocardia*, and *Streptomyces*. Seven strains of actinobacteria retarded the growth of *B. subtilis* by zones of 11.0-16.0 mm, they belonged to the genera *Geodermatophilus*, *Micromonospora*, *Nocardia*, and *Streptomyces*.

Table 4. Antibacterial activity of actinobacteria strains isolated from the silt sediments of the "La izvor" lake system, diameter of growth inhibition zones (mm)

Genus of actinobacteria	Strain No.	*A. tumefaciens* 8628	*B. subtilis* B-117	*C. michiganensis* 13[a]	*E. carotovora* 8982	*X. campestris* 8003[b]
Geodermatophilus	N 4.3	11.0±0	13.0±0	14.0±0	11.0±0	13.0±0
Micromonospora	N 5.1	0	12.0±0	0	0	0
	N 5.4	12.0±1.1	14.0±0	13.0±0	9.0±0	10.0±0
Nocardia	N 6.1	12.0±1.1	14.0±1.1	15.0±0	13.5±0.6	14.0±0
Streptomyces	N 8.1	13.0±1.1	16.0±1.1	16.0±0	12.0±0	15.0±1.1
	N 8.3	11.0±0	12.0±0	14.0±0	14.0±0	14.0±1.1
	N 8.6	9.0±0	11.0±1.1	9.0±0	9.0±0	0

Note: p = 0.05

The growth of *C. michiganensis* was inhibited by 6 strains of actinobacteria by zones of 9.0-16.0 mm. For *E. carotovora* also 6 strains showed weak antagonism - zones 9.0-14.0 mm. The growth inhibition of *X. campestris* was formed by 5 strains of actinobacteria, the zones were also small in size - 10.0-15.0 mm (Table 4).

The results of determining the antibacterial activity of the studied micromycetes are presented in Table 5. It can be seen that 15 strains differently caused the formation of growth inhibition zones against test bacteria. So, in relation to *A. tumefaciens*, 5 strains showed the ability to inhibit the growth of the test culture with zones of 25.3-28.0 mm, other strains against this test bacterium caused the formation of zones by 16.3-24.7 mm in size. In relation to *B. subtilis*, micromycetes quite actively showed the ability to inhibit growth – zones of 30.3-38.0 mm in size were noted. At the same time, zones with a size of 30.3-34.0 mm caused by metabolites of 9 strains, and zones with a size of 35.7-38.0 mm - 6 strains of the studied fungi. The growth of *C. michiganensis* was inhibited by 10 strains of micromycetes (zones between 10.7-23.7 mm), and in 5 strains metabolites formed growth inhibition zones between 25.0-26.0 mm. In relation to *E. carotovora*, 5 strains of micromycetes did not show antibacterial activity, turned out to be higher, which was reflected in the size of growth inhibition zones - their diameter was 28.0-30.7 mm. In relation to *X. campestris*, in 7 strains of micromycetes antibacterial activity was manifested by the formation of zones with a size of 12.7- 23.3 mm, and other 8 strains were distinguished by the ability to cause the appearance of zones of the absence of growth of this phytopathogen with a diameter of 25.3-30.7 mm (Table 5).

Table 5. Antibacterial activity of micromycetes strains isolated from the silt sediments of the "La izvor" lake system, diameter of growth inhibition zones (mm)

Genus of micromycetes	Strain No.	*A. tumefaciens* 8628	*B. subtilis* B-117	*C. michiganensis* 13[a]	*E. carotovora* 8982	*X. campestris* 8003[b]
Penicillium	N 2	22.7±3.46	38.0±1.13	20.7±1.31	20.7±1.31	25.7±0.65
	N 5	25.3±0.65	33.0±4.0	25.0±1.13	24.3±2.36	22.3±2.85
	N 6	25.7±0.65	34.0±1.96	23.3±1.31	0	21.7±3.27
	N 8	21.3±1.31	30.3±0.65	19.3±1.31	0	23.3±1.73
	N 11	28.0±1.96	35.7±1.31	26.0±2.26	30.0±2.26	30.7±1.31
Talaromyces	N 3	16.3±0.65	31.3±1.31	10.7±1.31	0	20.0±2.26
	N 4	18.3±1.73	31.3±1.31	15.0±1.13	28.0±2.26	19.3±1.31
	N 7	22.0±2.26	36.3±1.31	17.3±1.31	17.3±0.65	26.0±1.13
	N 28	25.3±0.65	37.3±1.31	25.7±1.31	0	25.3±0.65
	N 29	23.3±1.31	35.7±0.65	26.0±1.96	0	28.3±1.73
Trichoderma	N 9	21.0±1.13	33.7±1.73	17.3±1.31	20.7±1.31	12.7±1.31
	N 10	24.0±2.26	33.3±3.27	25.7±1.31	30.7±1.31	29.3±1.31
	N 12	24.3±1.31	36.0±1.96	20.7±1.31	28.7±2.61	25.7±3.64
	N 13	24.7±1.73	34.0±1.96	20.7±1.31	23.0±1.96	22.3±2.85
	N 14	26.7±1.73	30.7±1.31	23.7±1.73	28.7±1.73	26.7±1.73

Note: p = 0.05

Analysis of the obtained results showed the dependence of antimicrobial activity in the studied strains of actinobacteria, not only on species characteristics, but also on belonging to a particular genus. Thus, according to the data, it is clear that the strains of these 8 genera are not the same in their ability to inhibit the growth of one or another phytopathogen: out of 5 strains of the genus *Actinomadura*, only 2 strains had antifungal activity (zones between 10.0-23.0 mm), 4 from 5 strains of the genus *Actinoplanes* inhibit the growth of test fungi by zones of 10.0-26.0 mm. Low antifungal activity were in 1 from 2 strain of genus *Frankia* (zones 14.0-18.0 mm). Out of 6 strains from the genus *Micromonospora*, 3 strains showed high antifungal activity (especially, 26.0 and 35.0 mm zones under the influence of metabolites of strains N 5.1 and N 5.5, respectively). The strains of the genus *Nocardia* differed sharply in their antifungal activity: strain N 6.1 had a weak antifungal activity, and strain N 6.2 showed good results - zones against *A. niger* (30.0 mm), and *F. oxysporum* (26.0 mm). Weak antifungal activity was registered for strains of *Rhodococcus*. Only 1 strain out of 6 *Streptomyces* strains showed the ability to actively inhibit the growth of *A. niger* - zones up to 33.0 mm. The obtained results make it possible to choose for further studies a number of active strains of actinobacteria, which were characterized by high antifungal activity against phytopathogenic fungi. The results obtained are consistent with the literature data: out of 8 actinobacteria genera, the highest antifungal activity showed strains of the genus *Micromonospora*, *Nocardia*, and *Streptomyces* (Hata et al., 2015; El-Sabbagh et al., 2013). These strains include some strains of "rare" genera, in particular, *Actinomadura* and *Actinoplanes*, since they caused the formation of growth inhibition zones by 23.0-26.0 mm against *A. alternata* and *F. oxysporum* (N 1.2 and N 2.4). That is, these strains of actinobacteria, due to their antagonistic activity against phytopathogenic fungi, as a biocontrol, can replace chemical fertilizers and pesticides in the future. Moreover, according to the literature, out of 23,000 registered biologically active metabolites, more than 10,000 compounds are produced by actinobacteria, which is 45% of all biologically active metabolites of microbial origin (Aktuganova et al., 2007).

Determination of the dependence of the level of antimicrobial activity on the belonging of new strains of micromycetes to one or another genus showed that for 5 strains belonging to the genus *Penicillium*, a low antifungal activity is generally characteristic of the selected test cultures of phytopathogenic fungi: the strains either did not affect the growth of the test culture at all, or caused the formation of zones of growth inhibition with a diameter of 10.7-18.3 mm (Table 3). Four strains of micromycetes were assigned to the genus *Talaromyces*, of which 2 strains proved to be active antagonists against *A. alternata* (strain N 3 and N 4 - zones 39.0 mm), they did not retard the growth of *A. niger*, and in relation to representatives of the *Fusarium* genus, their activity was either low (zones 11.3-

18.0 mm) or significantly higher (zones 26.3-27.7 mm under the influence of metabolites of strains N 7 and N 28). One of the strains of this genus (N 28) also had the ability to inhibit the growth of *A. alternata* (zones up to 27.7 mm).

The strains of the genus *Trichoderma* were characterized by the ability to actively inhibit the growth of test cultures (except for *A. niger*) by forming zones up to 40.0-41.3 mm or inhibit growth zones by 18.0-24.7 mm (in the variant of experiments with test fungus *A. niger*). That is, micromycetes isolated from silt sediments, assigned to the genus *Penicillium*, *Talaromyces*, and *Trichoderma*, given their active antagonism against the selected test phytopathogenic fungi, can also be considered effective biocontrol.

The growing interest in the application of *Trichoderma* is due to their potential for direct and indirect biocontrol against a wide range of soil phytopathogens. They act through various complex mechanisms such as mycoparasitism, degradation of pathogen cell walls, competition for substrate and space, and induction of plant resistance (Sood et al., 2022; Tyśkiewicz et al., 2022; Guo et al., 2019).

Bacteria isolated from silt sediments and assigned to 3 genera *Arthrobacter*, *Bacillus*, and *Paenibacillus* differed markedly by antifungal activity from actinobacteria and micromycetes: their activity was, in general, higher than that of actinobacteria, but noticeably less than that of micromycetes. As can be seen in Table 3, a strain of the genus *Arthrobacter* No. 35 had the ability to inhibit growth of *A. alternata* and *B. cinerea* (21.7 and 28.3 mm, respectively), a representative of bacteria of the genus *Paenibacillus* No. 37 also had low activity (11.0-22.0 mm and 24.0 mm), while in representatives of the genus *Bacillus* it varied within a fairly wide range - from 6.0, 15.3 to 30.0 mm, and strains that actively inhibit growth of *B. cinerea* (No. 35, 31, and 47 - 28.3-30.0 mm) or the growth of representatives of the genus *Fusarium* (zones 26.7-29.7 mm under the influence of metabolites of strains No. 32, 33, 39, 43). That is, out of 12 strains of bacteria, 7 strains of the genus *Bacillus*, capable of showing antagonism against such phytopathogens that are often found in the Republic of Moldova, such as *B. cinerea* and phytopathogenic fungi that cause various kinds of fusariosis, may be of particular interest. The data obtained are consistent with the literature, which emphasizes the possibility of considering individual bacterial strains as clear candidates for bacterial control agents against fungal pathogens that cause significant damage to agriculture (Grabova et al., 2017; Lemanova, 2019; Pliego et al., 2011).

CONCLUSIONS

The conducted studies have shown that microorganisms (actinobacteria, bacteria, and micromycetes) isolated from the silt sediments of the "La izvor" lake system have a different effect on the growth of phytopathogenic fungi and bacteria, and it should be noted that most of the studied isolates are characterized by a greater degree of antifungal activity than antibacterial.

These new strains of microorganisms, due to their antifungal and antibacterial activity, will replenish the National Collection of Non-pathogenic Microorganisms as a real source of metabolites with antimicrobial properties, as example the substances that contribute to better preservation of agricultural products, or as biopesticides.

The next stage of our research will be determination of the synthesized antibiotic substances by these microorganisms and compare them with well-known antibiotics.

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THE EFFECT OF *Trichoderma* AND DIATOMITE ON THE GROWTH AND DEVELOPMENT PARAMETERS OF SOME BELL PEPPER SPECIES

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Abstract

Within the Vegetable Research and Development Station Buzău, aspects were determined and monitored regarding the effectiveness of products based on Trichoderma T85, administered at planting 3 granules/plant, for bell pepper culture and observations regarding the effectiveness of growth and development parameters for bell pepper, Cantemir (variety approved at V.R.D.S. Buzău). Trichoderma viride T85 is a fungal inoculant that was applied to variant V5 - three granules at planting and solid diatomite, which was incorporated into the soil during the vegetation period at V1, V2, V3, V4. The obtained results confirm the data presented in the specialized literature. Treatments with mixed bacterial preparations, with Trichoderma spp. and with solid diatomite can have a beneficial influence on the growth and development of vegetable species cultivated in organic or conventional systems, according to studies carried out over time, both in the country and abroad. This paper evaluates the effectiveness and benefits of using these bio-stimulants.

Key words*: Trichoderma, Diatomite, Capsicum annuum, fertilizer, pepper fruit, biological control.*

INTRODUCTION

Plant diseases must be controlled for sustainable, sustainable agriculture as they are responsible for the destruction of natural resources. In the agricultural and horticultural sector, these diseases cause huge financial losses to farmers. Plant pathogens can cause major production losses and reduce economic yield worldwide. Leaching into groundwater, persistent residues in soil, and accumulation of heavy metals in food chains are some of the main impacts of pesticides on the environment.
Pesticides have become less active against too many crop pests, which has strengthened farmers to increase the quantity or practice additional influential compounds for pest control. In Pakistan, these soil-borne pathogens occur as underground forms, hence chemical control is not practicable unless highly selective fungicides are available in the market. Chemicals cause unfavorable conditions for plants by affecting microbial activity in the soil, in addition to being very expensive (Chaoui et al., 2003).
Fungi of the genus *Trichoderma* are a large group of microorganisms that have a significant contribution to the environment.
Trichoderma has been largely associated with enzyme production, plant protection and growth promotion, but the importance of this genera goes far beyond this, due to the vast amount of possible applications in agriculture and industry in the form of bioremediation, industrial enzymes, food additives, probiotics, (Chowdhury et al., 2000), antibiotics, pigments, biofuel production and battery components (Briassoulis et al., 2007). Diatomite is a sedimentary rock with a high silica content: 23% quartz and clay minerals up to 77% (Dumitras et al., 2017). By mechanical processing of diatomite are obtained particles that reach submicron dimensions creating amorphous powders with specific properties of nanomaterials. (Atiyeh et al., 2000).
Diatomite has a high silicon content, porosity greater than 80% and very little pollutant, friendly to the environment, so it would be an option to be tried in sustainable agriculture.
The main objective of this research was to evaluate the effect of the application of the two products (*Trichoderma viride* T85 and diatomite powder). At the end, the statistical calculation was performed to determine the impact of the treatment on the bell pepper plants, the Cantemir variety.

MATERIALS AND METHODS

The biological material used was bell pepper, Cantemir variety, creation of V.R.D.S (S.C.D.L.) Buzău (Figure 1). The Cantemir pepper crop (*Capsicum annuum*) is a popular species of the *Capsicum* genus that is part of the *Solanaceae* family. This species turns out to be the most cultivated of all 4 known types of peppers and is of interest to farmers. (Catalog with germplasm collection of Vegetable Research Development Station Buzau, July 2013; www.scdlbuzau.ro)

Figure 1. Appearance of Cantemir bell pepper fruits, fruits in different stages of development

V.R.D.S. (S.C.D.L.) Buzău, owns a *Capsicum* species germplasm base composed of over 305 lines in various breeding stages: stable, advancing and segregating. The research undertaken by the Improvement Laboratory at V.R.D.S. (S.C.D.L.) Buzău on obtaining a new variety of bell pepper adapted to the climatic conditions and the ever-increasing demands of farmers, consumers and processors were completed with the obtaining of a variety, Cantemir. The variety was obtained through repeated individual selection, and the main characteristics have a small margin of variability, which corresponds to the specific objectives proposed for improvement.

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

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.

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.

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.

In the research field of the Vegetable Research and Development Station from Buzău, research was carried out on the influence of the growth and development parameters of bell pepper plants, which were treated with *Trichoderma viride* T85 (Figure 2) (3 granules/plant) which - I administered in the seedling phase and the application of 100 g of diatomite (Figure 3) dust during vegetation.

Figure 2. Granules of *Trichoderma viride* T85

Figure 3. Diatomite dust

The culture technology we applied was the culture technology specific to this species, which was adapted to the climatic conditions of the year 2022, in the conventional experimental field.

The seeds were sown in alveolar pallets of 70 cubes, with a volume of 50 ml, in partially decomposed blond peat with the addition of microelements. The emergence period was in the range of 11-24.04 and the emergence percentage ranged between 94.28-98.57%. (Benitez et al., 2004)

This technology applied in the experimental culture is part of the general technology of pepper cultivation in the open field, adapted to the specifics of the experience.

It is recommended to treat the seeds before sowing. The optimal period for sowing in protected areas takes place in the first decade of January. The planting material was sown in alveolar trays at one of the farms of the Vegetable Research and Development Station Buzău, in order to create optimal conditions for the development of the seedlings.

In order to obtain highly productive, good-quality harvests as early as possible, the quality of the seedlings at planting is essential (Briassoulis et al., 2007).

Sowing pattern: 100 cm between rows and 30-40 cm between plants/row. Density: 55000-65000 pl/ha. Weeding is done post-emergence, then the necessary irrigation and 2-3 mechanical and manual weeding are carried out.

Planting of seedlings was done according to the scheme from experience on variants and repetitions at the optimal time, namely the end of the first decade of May.

The care works applied after planting aimed at the good progress of the culture until its abolition (Cannelas et al., 2002).

The choice of land is made according to the preceding plant. The best precursors are leguminous species (peas, beans).

The experiment was designed by the method of randomized blocks, of 5 variants (V1- Control; V2-with the addition of diatomite in 52.5 g; V3-with the addition of diatomite in 105 g; V4-with the addition of diatomite in 210 g; and V5-*Trichoderma* with addition of 3 granules/plant) and 4 repetitions (R1, P2, R3 and R4), according to the data included in Table 1 and the explanatory legend.

Variant 1, being the control version that was not given any treatment. The amount of *Trichoderma* was established at a dose of 3 granules/cube, at planting, which took place on April 01, 2022.

Table 1. Planting scheme in randomized blocks of bell pepper, Cantemir variety

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

Legend: V1 - Control; V2 - diatomit (52.5 g); V3 - diatomit (105 g); V4 - diatomit (210 g); V5 - *Trichoderma* (3 g)

Bell pepper variety with large fruit firmness, which means that the fruits have a good shelf life;

• The variety has a vegetation period between 110-125 days, falling into the semi-early period;

• The variety shows an average tolerance to the attack of pathogens;

• The diameter of the bush varies from 50.3-68.9 cm, the branching is intermediate and has a number of 3-4 main shoots;

• The fruits have a pleasant commercial appearance and can be consumed both in the green stage and at physiological maturity;

• The taste of the fruits is sweet, aromatic, specific;

• The production potential varies from 38.5 t/ha to 62 t/ha, depending on the culture technology applied;

• Pepper fruits have a high content in vitamin C, a medium fruit of 100 g exceeds the daily requirement of ascorbic acid in human nutrition;

• The fruit has 3-4 lobes;

• The persistence of the pedicel fruit is intermediate, and the persistence of the pedicel with stem is also intermediate;

• Dry matter content varies depending on the culture technology from 6-10%;

• The distribution of production is done according to the diameter, which must exceed 70 mm, in this case, over 90% of the fruits fall into the Extra category, and into the I quality, 6.4%;

• The fruits can be consumed both fresh and for industrialization;

The plant is compact and the bush has medium vigor. The fruits are blocky, with a longitudinally furrowed surface with well-marked furrows.

Morphological characterization aspect of biological material of Cantemir pepper variety, is presented in Figure 4.

Figure 4. Bell pepper plant of the Cantemir variety, detail with ripe fruits

Table 2. Pathogenic agents and treatments applied to them

The damage agent: Disease/harmful agents	The name of the product	Dose used
Phytophthora capsici	Dithane, Mavrik	2%; 5%
Spider	Nissorum	1 kg/ha
Thrips	Actara	2 kg/ha
Bacteriosis, Vascular disease	Dithane, Topsin,	2 kg/ha; 1 kg/ha

Phytosanitary treatments (Table 2) were applied throughout the vegetation to improve the culture. In Table 2, the treatment scheme applied for the studies in this research is presented.

The year 2022 was the third warmest year in the history of meteorological measurements in Romania. The average annual temperature was 11.77 degrees Celsius, and the thermal deviation of 1.55 degrees Celsius.

Average temperatures varied (see list below):

- in April between 11 and 14.1°C;
- in May between, 11.2 and 15.3°C;
- in June between 19.5 and 23°C;
- in July between 21.03 and 25°C;
- in August between 22.6 and 23°C;
- in September between 17 and 22°C.

In the months of April and September, the following precipitations were present:

- April - 0.5 l/sqm;
- May -25 l/sqm;
- June - 63 l/sqm;
- July - 81 l/sqm;
- August - 31 l/sqm;
- September -3 l/sqm).

Predominant are the mollisols from the subsidence plain of Buzau, of the clay-iluvial chernozem type, (46.5%) within farms numbers 2 and 5.

In the Buzau meadow (former minor and major bed), in dammed enclosures with an adequate drainage and irrigation system, we find alluvial soils (38.1%) and alluvial protosols (10.0%).

During the vegetation period, the control of the reported diseases and pests was monitored and ensured, namely bacterial spot *(Xanthomonas campestris*), pepper powdery mildew *(Phytophtora capsici*) and pests that affected the crop, namely aphids *(Aphidoidea*), thrips (*Thrips tabaci*) and the red spider common (*Tetranychus urticae)* (Ciofu et al., 2003)

RESULTS AND DISCUSSIONS

The statistical calculation (Figure 5 and Table 3) recorded the following data: in the months of June and July, significant amounts of water were recorded: 63 l/m^2 and 81 l/m^2 and they favored the occurrence of specific pepper diseases (*Phytophthora capsici*) (Figure 6), it is a disease found in the pepper culture in all phenophases, which cause rotting and rotting of pepper fruits. Later, this pathogen was stopped with the treatments applied based on *Trichoderma viride T85* and diatomite, from the table above (Figure 5 and Table 3).

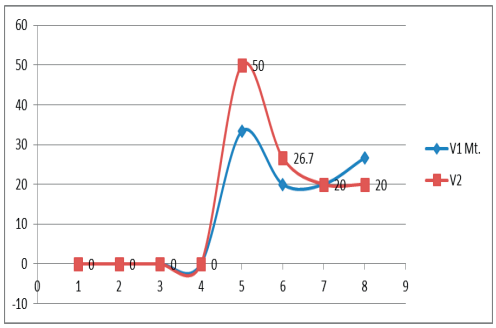

Figure 5. Evolution of growth and development of pepper plants, Cantemir variety

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

No	V	No. fruit	P%
1	V1 Mt.	6.23	-
2	V2	8.21	25.1
3	V3	8.01	29.1
4	V4	7.24	56.0
5	V5	8.08	29.1

The administration of *Trichoderma viride T85* had a positive influence on the culture of bell pepper, the Cantemir variety, the one under study, and it is a very friendly solution for the environment, because it is non-polluting according to specialized studies.

Figure 6. Phytophthora capsici in the cultivation of bell peppers

Use of *Trichoderma* in field crops benefits fruit/plant production. The fruits are larger, of superior quality, in the version treated with *Trichoderma,* the results being at the threshold of statistical significance (Table 4).

Table 4. Average root mass at harves

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
Mediate		211.83	35.83

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

It has an important nutritional and phytostimulating activity. *Trichoderma* brings an increase in plant productivity, due to the inhibition of the activity of toxic compounds in the root zone and the increase of nutrient absorption. At the same time, it increases the efficiency of nitrogen use, as well as an increase in the solubility of nutrients in the soil.

This fungus induces the formation of roots and stimulates colonization with rhizosphere and other beneficial microorganisms on the roots. It also has the ability to phytorepair plant tissues, caused by some residual (persistent) pesticides in the environment (Chaoui et al., 2003).

The analysis of the root mass shows that variant V5 (treated with *Trichoderma*) was superior, compared to variant V1 (Control).

CONCLUSIONS

The treatments carried out had a positive influence and determined a harmonious development of plants, leaf mass and production in the bell pepper culture, Cantemir variety. At the same time, this fertilization is an environmentally friendly option, because it does not pollute the water table.

I propose and recommend to all farmers, local producers the optimized culture technology for bell pepper, technology that can be applied to other biological creations as well.

In the experimental block with peppers, the ecological status of the epigean fauna in all the experimental variants in which there were diatomite 52.5 g (V2), 105 g (V3) and 210 g

(V4) and the antagonistic fungus *Trichoderma* isolated T85 (3 granules administered to the root at planting) it was good, similar to V1 without administration.

The treatment variants with products based on *Trichoderma* T85 and Diatomite did not show phytotoxicity to bell pepper plants.

Following the treatment with *Trichoderma* administered at planting in Cantemir 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.

The effect of the application of diatomaceous earth on the physical and chemical properties of the soil, on the samples taken before and after its application, was analyzed. From a physical point of view, the soil texture was analyzed, finding a clay-sandy texture for all crops. Diatomite was applied in 3 different amounts, and the maximum amount (210 g) was not sufficient to demonstrate significant changes in soil texture. From a chemical point of view, the soil properties meet the requirements of pepper cultivation and have great favorability in the analyzed area. A slightly alkaline pH has been identified and the amount of heavy metals does not exceed the alert threshold. Diatomite applied as an ecological alternative insecticide in the second campaign, did not show significant differences in terms of physical and chemical properties of the soil.

Following the experiences within the Vegetable Research and Development Station Buzău, it was found that diatomaceous earth can also be used to protect plants, to fight diseases and pests, because it offers protection against them.

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COMPARATIVE STUDY ON THE COMPOSITION, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF FENNEL HYDROLATES

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Abstract

The aim of this study was to investigate the composition and biological properties of hydrolates from fennel (Foeniculum vulgare L.*) seeds and their usefulness in food and biomedical applications. Hydrolates were obtained by steam- and hydro-distillation of fennel seeds followed by essential oil separation. GC-MS analysis indicated the presence of volatile compounds in the hydrodistilled extract. HPLC analysis of fennel hydrolates showed a higher concentration of phenolic compounds in the steam distilled extract. The antioxidant activity, determined as Trolox equivalent antioxidant capacity (TEAC) and cupric reducing antioxidant capacity (CUPRAC), was correlated to the total phenolic content of fennel hydrolates, being higher in the steam distilled extract. Thus, besides volatiles, the phenolic compounds could significantly increase antioxidant activity. In turn, higher inhibition of bacterial growth was found for the hydrodistilled extract, due to the significant number of volatile compounds. In conclusion, fennel hydrolates represent a waste of significant interest for valorization within the circular bioeconomy and further application in the food and biomedical industry.*

Key words*: Foeniculum vulgare, by-product, phenolics, antioxidant activity, antimicrobial activity.*

INTRODUCTION

Fennel (*Foeniculum vulgare* L.) is a member of the Apiaceae family, native to the Mediterranean area, but globally widespread due to increased demand for fennel essential oil. Traditionally, the fennel seed oil is used for culinary purposes, due to its flavouring properties, being also a valuable ingredient of cosmetic and pharmaceutical products (Badgujar et al., 2014; Dahmani et al., 2022). Moreover, fennel essential oil has demonstrated insecticidal effects against different species of aphids (Digilio et al., 2008; Pavela, 2018; Dunan et al., 2021), suggesting its potential use as a biopesticide for the protection of plants in culture.

During essential oil production by hydrodistillation or steam distillation, large quantities of floral water residues or hydrolates are generated, but they are generally discarded. However, hydrolates have been reported to contain important quantities of bioactive compounds, such as water-soluble volatile substances (oxygenated monoterpenes, sesquiterpenes, hydrocarbon derivatives) (Acimovic et al., 2020; Gaspar-Pintiliescu et al., 2022a). These compounds are responsible for the antimicrobial activity of fennel hydrolates. Previous studies have reported that fennel hydrolates have exerted strong inhibition on the growth of the *Aspergillus parasiticus* NRRL 2999 strain (Ozcan, 2005). Also, fennel hydrolates were 50 times concentrated using solid phase extraction and exhibited significant antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans* strains, but high resistance in the case of several strains of Arcobacter-like bacteria (Silha et al., 2020). Moreover, the biofilm formation was significantly reduced by the concentrated hydrolate. Another study has reported no antimicrobial activity of fennel hydrolate tested in various Gram-positive and Gram-negative bacterial strains (Sagdic and Ozcan, 2003).

From safety point of view, fennel hydrolate exhibited no cytotoxicity effect in human A549 lung carcinoma cells, suggesting their applicability in the food and biomedical industry (Silha et al., 2020).

Regarding the antioxidant activity of fennel hydrolate, there is scarce information. A study mentioned its ferric reducing antioxidant power (FRAP), due to the volatile compounds content (Lado et al., 2004). No studies on the polyphenolic composition of fennel hydrolates and their valuable bioactivity that could modulate the biological properties were found.

In this context, the aim of this paper was to investigate the chemical composition of Romanian fennel seeds hydrolates obtained by steam- and hydro-distillation and the correlation of both monoterpenes and polyphenols content to their antioxidant and antimicrobial activity, to improve their food and biomedical applications.

MATERIALS AND METHODS

Plant material and chemicals

Fennel (*Foeniculum vulgare* L.) seeds were harvested from ecological cultures of HOFIGAL Export-Import S.A. Romania. They were dried, ground, and stored in a cool and dry place, until the extraction process. All used reagents were of analytical grade and purchased from Sigma-Aldrich (Germany), unless otherwise specified.

Extraction of fennel hydrolates

The hydrolates were obtained from fennel seeds by distillation using a Clevenger-type extraction apparatus (J.P. Selecta, Spain). The ground material (25 g) was moistened in ultrapure water. Two different extraction methods were applied, one based on steam distillation using distilled water as a steam source (Hay et al., 2015) and the other on hydrodistillation (Silha et al., 2020) using ultrapure water (750 mL), at 100°C, for 2 h. At the end of the distillation process, the essential oil was separated from the aqueous fraction, representing the fennel hydrolates, based on density difference. The steam distilled and hydrodistilled fennel hydrolates were filtered and stored in the dark, at 4°C, until analysis. The extraction yield was determined as a percentage of initial dry weight (d.w.).

Gas-chromatography/mass-spectrometry (GC-MS) analysis

Fennel hydrolates were analyzed by GC-MS using Focus GS-type equipment coupled to a mass spectrometer DSQ II (Thermo Electron Corporation, USA), as previously described (Mihai et al., 2021). Briefly, the separation was performed on Macrogol 20000 R capillary column (30 m x 0.25 mm i.d. and 0.25 µm film thickness). The carrier gas was helium at a flow rate of 1 mL/min. The mass spectrometer was operated at 70 eV with a scan interval of 0.5 s and scan range between 40-1000 m/z. The identification of the main constituents was performed by comparing the spectra with NIST mass spectral database.

HPLC analysis

Fennel hydrolates were investigated by HPLC analysis on a reverse phase column C18 Zorbax Eclipse XDB (150 x 4.6 i.d. mm) using an Agilent 1200 HPLC system consisting of a quaternary pump, thermostated autosampler, and diode array detector (Agilent, Germany). A sample (10 µL) was injected and then eluted using mobile phase A consisting of 2 mM sodium acetate, pH 3, and mobile phase B, acetonitrile, using the following gradient: 2-20% B, 0-30 min; 20-30% B, 30-40 min; 30% B, 40-50 min; 30-2% B, 50-60 min (Craciunescu et al. 2012). of Compounds identification was performed by comparison to the retention time of standard phenolic acids (gallic acid, chlorogenic acid, caffeic acid, ferulic acid) and flavonoids (rutin, quercetin 3-O-glucoside (isoquercetin), quercetin, luteolin, apigenin, kaempferol) (Sigma-Aldrich, Germany). The identified compounds were quantified by peak area integration.

Determination of total phenolic and flavonoid content

Total phenolic content was determined by the Folin-Ciocalteu method, as previously described (Gaspar-Pintiliescu et al. 2022b). Briefly, the sample was mixed with Folin-Ciocalteu reagent (1:5, v/v) and incubated in the dark, for 5 min. Then, 2 mL of 12% sodium carbonate were added and the mixture was incubated at room temperature, for 30 min. The optical density (OD) was measured at 765 nm using an UV-VIS spectrophotometer (V-650, Jasco, Japan). The standard curve was built using different concentrations of caffeic acid in

the range of concentrations 0-500 µg/mL. The results were expressed as caffeic acid equivalents (CAE).

Total flavonoid content was determined by the aluminum chloride method, as previously described (Gaspar-Pintiliescu et al., 2022b). Briefly, the sample was mixed with methanol (1: 3, v/v), and then, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M sodium acetate, and 2.8 mL of distilled water were added, and incubation was performed at room temperature, for 30 min. The OD was read at 415 nm using an UV-VIS spectrophotometer (V-650, Jasco, Japan). The standard curve was built using different concentrations of quercetin in the range of 0-150 µg/mL. The results were expressed as quercetin equivalents (QE).

Determination of Trolox equivalent antioxidant capacity (TEAC)

The TEAC assay was performed according to the protocol of Hay et al. (2015) with minor modifications. Briefly, a stock solution was prepared by mixing solutions of 7 mM 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2.45 mM potassium persulfate (1:1, v/v) and then, incubated at room temperature, in the dark, for 16 h. Before the experiments, the stock solution was diluted to an OD of 0.70 ± 0.02 at 734 nm. Different concentrations of the sample were mixed with ABTS solution (1:10, v/v) and incubated at room temperature, in the dark, for 10 min. The OD was read at 734 nm using an UV-VIS spectrophotometer (V-650, Jasco, Japan). The standard curve was built using Trolox, an analog of vitamin E, in the range of concentrations 0-250 µM. The results were expressed as Trolox equivalents (TE).

Determination of cupric ion reducing antioxidant capacity (CUPRAC)

The CUPRAC assay was performed according to an adapted protocol of Georgiev et al. (2019). Briefly, 1 mL of 10 mM $CuCl_2$ solution was mixed with 1 mL of 7.5 mM neocuproine and 1 mL of 1 M ammonium acetate buffer, pH 7. The mixture was vortexed and incubated at room temperature, for 10 min, to develop the complex. Then, 100 µL of sample and 1 mL of distilled water were added and incubation continued at room temperature, for 1 h. The OD was read at 450 nm using an UV-VIS spectrophotometer (V-650, Jasco, Japan). A blank was prepared by sample replacement with distilled water. The standard curve was built using Trolox in the range of concentrations 0.1-1.0 mM. The results were expressed as TE.

Antibacterial activity evaluation by microdilution assay

The antibacterial activity of fennel hydrolates was tested in *S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 9027) strains by serial microdilution assay, as previously described (Saviuc et al. 2017). Briefly, bacterial cultures were grown in brain heart infusion broth at 35°C, for 24 h, and the working culture was adjusted at a concentration of 10^8 UFC/mL to 0.5 McFarland standard. Then, serial dilutions of the sample (0.1-7 mg/mL) were incubated with microbial suspension in the wells of a 96-well microplate, at 35°C, for 24 h. The OD was read at 600 nm using a SPEC-TROstar Nano microplate reader (BMG Labtech, Germany). A bacitracin solution served as positive control. The minimum inhibitory concentration (MIC) was determined as the minimum concentration that inhibited microbial growth, compared to the control.

Statistical analysis

The experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD) (n = 3). Statistical analysis was performed by two-tailed, two-sample equal variance Student *t*-test on control-sample pairs of interest. Differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Preparation of fennel hydrolates

Fennel hydrolates were obtained during the steam- and hydro-distillation process of essential oil preparation, as colored solutions with a pleasant odor. These methods gave extraction yields of 12.33±0.89% (w/w) and 17.68±1.54% (w/w), respectively.

Volatile compounds composition

GC-MS analysis of fennel hydrolates has shown that the extract obtained by hydrodistillation contained volatile substances, while the steam distilled extract presented only traces (not determined). The retention time of the identified compounds and their relative percentage are presented in Table 1.

Table 1. Composition of volatile compounds determined by GC-MS of the hydrolate obtained from fennel seeds by hydrodistillation

No.	Compound	Retention time	% of total peak area
	Monoterpenes hydrocarbons		
1.	α-pinene	6.23	5.16
2.	β-pinene	9.61	0.36
3.	β-phellandrene	10.43	0.11
4.	δ-3-carene	11.84	0.15
5.	α-phellandrene	12.76	4.46
6.	myrcene	13.06	0.47
7.	limonene	14.68	1.50
8.	trans-β-ocimene	17.27	0.30
9.	α-terpinene	17.51	0.53
10.	o-cymene	19.01	0.48
	Oxygenated monoterpenes		
11.	fenchone	26.49	8.09
12.	camphor	33.44	0.09
13.	linalool	36.57	0.12
14.	estragole	43.02	1.36
15.	trans-anethole	51.66	76.82
	Monoterpenes hydrocarbons		**13.52**
	Oxygenated monoterpenes		**86.48**

Fennel hydrolate obtained by hydrodistillation contained 15 volatile compounds, from which monoterpenes hydrocarbons represented 13.52% and their oxygenated derivatives represented 86.48%. The most abundant constituents were trans-anethole (76.82%), fenchone (8.09%), and estragole (1.36%), but also α-pinene (5.16%), α-phellandrene (4.46%) and limonene (1.5%) were present.

Previous studies have reported that trans-anethole, fenchone, estragole, and limonene were the major compounds found in fennel essential oils (Anwar et al., 2009; Damayanti and Setyawan, 2012; Ahmed et al., 2019; Kalleli et al., 2019). However, their proportion varied according to plant maturation stage, harvesting season, climate, and geographical conditions (Telci et al., 2009; Ahmed et al., 2019). Fenchone was proposed as a biomarker of various commercial formulations of essential oil and extracts of *F. vulgare* seeds (Alam et al., 2019). Fennel hydrolates usually contain low quantities of volatile compounds, regardless of the used extraction method. Thus, the hydrolates of Czech fennel seeds obtained by steam- and hydro-distillation were rich in the oxygenated monoterpenes estragole and fenchone (Silha et al., 2020), which were also predominantly found in the composition of the hydrodistilled fennel hydrolate.

Polyphenolic compounds composition

Several polyphenolic compounds were identified and quantified in both fennel hydrolates by HPLC analysis (Table 2).

Table 2. Concentration of polyphenolic compounds in fennel seeds hydrolates obtained by steam- and hydro-distillation, determined by HPLC analysis. The results are expressed as mean ± standard deviation (n = 3)

No.	Compound	Fennel hydrolate obtained by steam distillation (μg/g d.w.)	Fennel hydrolate obtained by hydrodistillation (μg/g d.w.)
	Phenolic acids		
1.	Gallic acid	ND	ND
2.	Chlorogenic acid	6.85 ± 0.31	1.97 ± 0.07
3.	Caffeic acid	10.95 ± 0.45	9.40 ± 0.34
4.	Ferulic acid	8.56 ± 0.38	6.76 ± 0.26
	Flavonoids		
5.	Rutin	ND	ND
6.	Isoquercetin (Quercetin 3-glucoside)	3.20 ± 0.16	2.52 ± 0.10
7.	Quercetin	1.53 ± 0.07	ND
8.	Luteolin	ND	ND
9.	Apigenin	ND	ND
10.	Kaempferol	ND	ND

ND - not determined.

The results have shown that chlorogenic, caffeic, and ferulic acid were the major compounds identified in both fennel hydrolates, but higher quantities were found in the steam distilled extract. Quercetin and its glucoside form, isoquercetin were the main flavonoids detected in the steam distilled extract. Previous studies have also indicated the presence of chlorogenic, caffeic, and ferulic acids, and quercetin in alcoholic extracts of fennel seeds (Roby et al., 2013; Odeh and Allaf, 2017). No HPLC studies on fennel hydrolates were found.

The total phenolic and flavonoid content of fennel hydrolates is presented in Table 3. The results have shown that the values of total phenolic (11.15 mg/100 g d.w.) and flavonoid (2.17 mg/100 g d.w.) content of fennel hydrolate obtained by steam distillation were ~2-fold higher than those found in the hydrodistilled extract. Previous studies have reported values of total phenolic content between 250-4224 mg/100 g for fennel seed ethanolic extracts, according to the geographical area, solvent, temperature, and extraction method (Ahmed et al., 2019; Kalleli et al., 2019; Malin et al., 2022). No reports were found for fennel hydrolates.

Table 3. Total phenolic and flavonoid content and the antioxidant activity of fennel hydrolates. The results are expressed as mean ± standard deviation (n = 3)

Sample	Total phenolic content (mg CAE/ 100 g d.w.)	Total flavonoid content (mg QE/ 100 g d.w.)	TEAC (mM TE/ g d.w.)	CUPRAC (mM TE/ g d.w.)
Fennel hydrolate by steam distillation	11.15 ± 0.39	2.17 ± 0.02	626.12 ± 35.79	365.50 ± 14.48
Fennel hydrolate by hydro-distillation	6.49 ± 0.24	0.79 ± 0.01	375.99 ± 20.79	320.07 ± 23.93

Antioxidant activity of fennel hydrolates

The antioxidant activity of fennel hydrolates was assessed by two different methods, i.e., TEAC assay based on hydrogen atom transfer (HAT) mechanism of free radicals scavenging and CUPRAC assay based on single electron transfer (SET). The results are presented in Table 3. The TEAC of fennel hydrolate obtained by steam distillation (626.12 mM TE/g d.w.) was 1.67 higher than that of the hydrodistilled extract (375.99 mM TE/g d.w.). The CUPRAC of the steam distilled hydrolate (365.50 mM TE/g d.w.) was close to that registered for the hydrodistilled extract (320.07 mM TE/g d.w.).

These data have shown that the antioxidant activity of fennel hydrolates varied directly proportional to the total phenolic content. The volatile compounds, in particular anethole and phellandrene, might be also involved in the antioxidant capacity of hydrolates, as previously reported, due to their ring structure with delocalized electrons (Lado et al., 2004).

Antimicrobial activity of fennel hydrolates

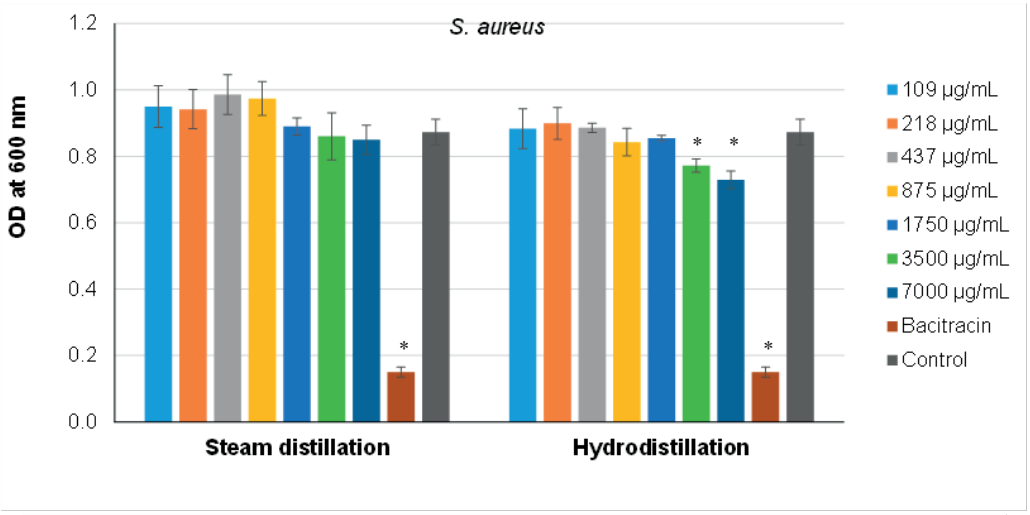
The antimicrobial activity of fennel hydrolates was evaluated in the cultures of the Gram-positive *S. aureus* bacterial strain and the Gram-negative *P. aeruginosa* strain. The results are presented in Figure 1.

The results showed that the steam distilled hydrolate had no antimicrobial activity against any of the tested bacterial strains. The hydrolate obtained by hydrodistillation could inhibit the bacterial growth of *S. aureus* and *P. aeruginosa* strains at a MIC of 3500 µg/mL. A previous study on hydrodistilled fennel hydrolate has shown *S. aureus* inhibition, to a similar extent to fennel essential oil or extract (Silha et al., 2020). This activity was mostly attributable to the volatile compounds present in the fennel hydrolate obtained by hydrodistillation. The compounds containing several functional groups were reported to exert higher antimicrobial activity, due to their affinity to bind the bacterial cell membrane (Basavegowda and Baek, 2021). Thus, oxygenated monoterpenes, such as linalool, eucalyptol, menthol, and thymol, and the monoterpene hydrocarbons pinene, terpinene, and limonene showed the capacity to inhibit bacterial growth (Badawy et al., 2019). In addition, a study on fennel essential oil showed its antifungal activity against *Aspergillus niger* and *Penicillium expansum*, at doses between 14-19 µg/mL (Olaru &Popa, 2019).

CONCLUSIONS

All these data have shown that significant quantities of water-soluble bioactive polyphenolic and volatile compounds were present in fennel hydrolates. The fennel hydrolate obtained by steam distillation had higher total phenolic content, compared to that of the hydrodistilled extract, and good antioxidant activity. The hydrodistilled fennel hydrolate exerted antimicrobial activity, while the steam distilled extract had no activity in *S. aureus* and *P. aeruginosa* cultures. This activity could be correlated to the higher content of volatile compounds found in the hydrodistilled extract. In conclusion, it was demonstrated that the composition of fennel

hydrolates varied with the distillation method and provided different antioxidant and antimicrobial activity. This study provided valuable information on fennel hydrolates for further use in food or biomedical applications.



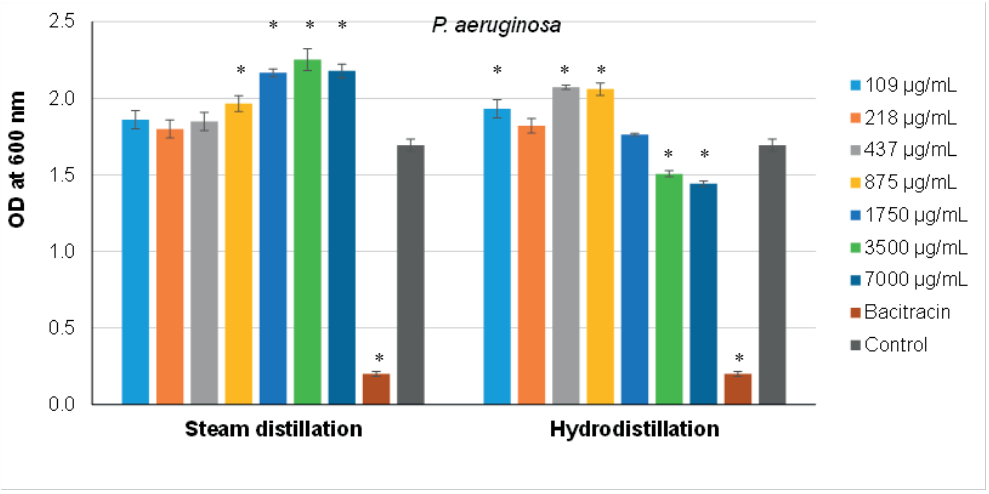


Figure 1. Bacterial growth inhibition of *S. aureus* and *P. aeruginosa* in the presence of fennel seeds hydrolates, after 24 h. The results are expressed as mean ± SD (n = 3). *p<0.05, compared to control.

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THE INFLUENCE OF GROWTH-STIMULATING RHYSOBACTERIA (PGPB) ON THE PHYSIOLOGICAL PROCESSES AND PRODUCTIVITY POTENTIAL OF GRAPEVINES

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Abstract

Environmental contamination, deteriorating socio-economic relations and increasingly frequent weather anomalies have complicated the phytosanitary condition of the vineyard complex. The development of organic farming and application of bacterial inoculants have attracted increasing attention in recent years. In this article are presented the results of research on the influence of bacterial products of the group PGPB (plant growth promoting bacteria) in the realization of productivity potential and some physiological processes in grapevines. It was demonstrated that the application of suspensions of Pseudomonas aureofaciens and Bacillus thuringiensis var. kurstak bacteria with the addition of SiO_2 at different stages of plant development contributed to the increase of photosynthetic pigment content, transpiration, stomatal conductivity, and increased quantity and quality of grapevine harvest.

Key words*: plant growth-promoting bacteria, photosynthetic pigments, productivity, transpiration, stomatal conductivity.*

INTRODUCTION

Nutrient imbalance, drought as well as long-term cultivation of perennial crops are factors that cause intensive soil depletion, leading to considerable losses in plant productivity. Moreover, it should be noted that with the use of pesticides and fungicides against diseases and pests, heavy metals are accumulating in the soil. As a result, the biomass of micro-organisms in soils under vineyards is considerably reduced, which slows down the plant's absorption of available nutrients. Therefore, plants become more vulnerable to pathogens and adverse environmental conditions, and the quality of grapes and wine decreases. One of the main objectives for the development of integrated sustainable agriculture is the development of new biologically efficient technologies.

A new approach to harnessing plant effectiveness is the use of biologically active compounds. The main goal in this direction is to create biopreparations to increase the availability of nutrients to the plant and increase plant resistance to biotic and abiotic stressors. Achieving this goal will increase plant productivity and reduce the chemical load on the ecosystem. Special attention is given to preparations based on one or more bacterial strains, which is conditioned by the positive influence of microorganisms on plants, and to a large extent by their stimulatory effect on metabolic and biochemical processes in plants. But it is also known that the soil under vineyards and orchards is poor in the diversity of microorganism species. The biological preparations used are based on highly productive strains of microorganisms that populate the rhizosphere of healthy plants. These in turn lead to an increase in the quantity of yield as well as an increase in quality. Fertilizations of plants with bacterial preparations has become increasingly widespread in recent years. The mechanisms of action of microorganism strains in the

formation and achievement of plant productivity are insufficiently studied (Asada K., 1990; Avis et al., 2008; Marleny Cadena Cepeda, 2006).

A high level of ecological efficacy and harmlessness of biopreparations does not always have a stable effect on the negative influence of climatic factors on the host plant. To mitigate the negative influence of climatic factors on plants, there are a number of preparations - growth regulators, the use of which mobilizes the plant's defense capacity under outdoor conditions.

This group also includes the preparation silicon dioxide (SiO_2), the use of which is known in many fields as well as in agriculture with the following effects on plants: increased permeability in plant tissues, high adhesion, activation of photosynthesis, capacity as an antidote in combination with pesticides and herbicides, alleviation of stress following plant replanting, wounding or trauma (Avis et al., 2008).

The influence of oxides and SiO_2 in particular on the production of metabolites of non-pathogenic PGPB organisms is known in microbiological practice:

- The introduction of the preparation into the culture medium when growing bacteria of the genus *Pseudomonas* spp increases the amount of phenazine components in bacterial suspensions which influences the antagonistic properties towards pathogens.
- Silicon dioxide falls into the category of potentially promising substances capable of inhibiting insect defense reactions.

MATERIALS AND METHODS

The experiments were set up on the experimental field of the IGPPP (Institute of Genetics, Physiology and Plant Protection) in three repetitions, plot dislocation - according to the block method.

Sauvignon vine plants, young plantation (5-6 years old) served as the object of study. The plants were foliar treated with bacterial metabolites during the growing season five times with an interval of 12-15 days (5 true leaves, 3-4 days before flowering, after flowering, in the phase of intensive plant development, before ripening of the berries). On the basis of pre-elimination research, the strains of *P. aureofaciens* and *B. thuringiensis* var. *kurstaki* were selected. Water-treated plants were used as controls.

Bacteria were grown in liquid nutrient medium for 24 h at 27°C at 10^{10} CFU/ml and applied as metabolites. To acquire the bacterial metabolites, the concentrated suspensions were centrifuged at 8 thousand rpm for 20 min in order to precipitate the bacterial cells and obtain the metabolic products. Silicon dioxide (SiO_2) was also used.

The content of photosynthetic pigments was determined by the acetone extraction method on the UV-Vis spectrophotometer SP-8001. For analysis, the first three developed leaves were taken from the shoot tip (Avis et al., 2008). The net photosynthetic rate, stomatal conductance and transpiration were determined using the LCpro-SD handheld gas analyzer (ADC biotech-scientific Limited, UK). The results obtained were analyzed according to Statistics-7.

RESULTS AND DISCUSSIONS

Plant productivity is the result of the integration of several processes, such as photosynthesis, transpiration intensity, metabolite transport, with photosynthesis playing the primary role. One of the main indicators of plant condition is the amount of photosynthetic pigments in the leaves. The overall process of photosynthesis is a continuous series of interdependent photochemical and biochemical events considered to occur in a linear sequence. The content of photosynthetic pigments in leaves is one of the important indicators of the state of plants during the growing season, as the ratio of the sum of chlorophylls and carotenoids is an indicator of the "damaged" state of plants. From our data, it is observed that foliar fertilization of fruiting bushes with the recommended dose contributed to an increase of total chlorophyll content in grapevine leaves by 20%. The highest amount of assimilatory pigments is in the experimental variant where *P. aureofaciens* + *B. thuringiensis* var. *kurstaki*, rhizobacteria were used, and constitutes 20.4%, mainly due to the increase in the concentration of chlorophyll „b„ which constitutes 37.4% (Table 1) compared to the control variant. The same effect was also observed in the variants

where *P. aureofaciens* + *B. thuringiensis* var. *kurstaki* + SiO_2 were used. In these variants the effect of increasing the sum of chlorophyll a+b pigments is probably due to the synergistic effect of rhizobacteria as well as the stimulatory effect of SiO_2. An optimal level of pigment content can also be seen in the other treated variants, where it is much higher than in the control variant.

The literature data on the effect of treating plants with microbial metabolites on the carotenoid content of plant leaves are quite contradictory (Martínez-Viveros et al., 2010). The results obtained in our experiments show a trend of decreasing carotenoid content in grapevine leaves after each spraying with bacterial products. This is because carotenoids perform a protective function, keeping various organic substances, especially chlorophyll molecules, from light destruction during photooxidation.

Table 1. Determination of the amount of pigments in grapevine leaves, Sauvignon variety field experiment mg/100 g m.p. Field experiment 2020

Experimental variant	Chlorophyll *a*		Chlorophyll *b*		Chlorophyll *a+b*		Carotenoids	
	M±m	Δ, %	M±m	Δ, %	M±m	Δ, %	M±m	Δ, %
10.06.2020								
1.*	0.99± 0.0		0.48±0.01		1.47±0.01		0.21±0.00	
2.	1.33±0.0	13.4	0.99±0.02	20.6	2.31±0.02	15.7	0.18±0.02	-14.3
3.	1.19±0.01	12.0	1.31±0.07	27.2	2.50±0.08	17.0	0.15±0.01	-28.5
4	1.34±0.01	13.5	1.67±0.14	34.7	3.01±0.14	20.4	0.13±0.05	-39.0
5.	1.30±0.01	13.1	1.02±0.04	21.2	2.31±0.04	15.7	0.12±0.02	-42.1
6.	1.35±0.00	13.6	1.11±0.00	23.1	2.45±0.00	16.6	0.15±0.05	-0.28
			26.06.2020					
1.	0.99±0.00		0.49±0.01		1.48±0.01		0.37±0.01	
2.	1.01±0.00	10.2	0.47±0.01	0.22	1.48±0.01	10.0	0.33±0.01	-10.8
3.	1.13±0.02	11.4	0.92±0.06	18.7	2.05±0.06	13.8	0.24±0.02	-35.1
4.	1.14±0.02	11.5	1.00±0.06	20.4	2.14±0.09	14.4	0.21±0.03	-43.5
5.	1.05±0.01	10.6	0.70±0.02	14.2	1.74±0.02	11.7	0.30±0.01	-18.9
6.	1.13±001	11.4	0.79±0.04	16.1	1.93±0.05	13.0	0.23±0.01	-37.8
			29.07.2020					
1.	1.17±0.00		0.45±0,00		1.62±0,00		0.54±0.01	
2.	1.25±0,00	10.6	0.55±0.01	12.2	1.80 ±0.01	11.1	0.53±0.02	-1.8
3.	1.25±0,00	10.6	0.56±0.00	12.3	1.82±0.00	11.3	0.51±0.03	-5.5
4.	1.28±0.00	10.9	0.59±0.00	12.7	1.87±0.00	11.5	0.55±0.00	10.1
5.	1.31±0.00	11.1	0.70±0.00	15.5	2.01±0.00	12.4	0.52±0.00	-3.7
6.	1.13±0.16	9.6	0.74±0.07	16.4	1.87±0.09	11.5	0.50±0.04	-7.4

1. - Control; 2. - *P. aureofaciens*; **3**. - *P. aureofaciens*+ SiO_2; **4. -** *P. aureofaciens+B. thuringiensis* var.*kurstaki*; **5. -** *B. thuringiensis* var. *kurstaki* + SiO_2; **6. -** *P. aureofaciens+B. thuringiensis* var. *kurstaki* + SiO_2

Photosynthesis is an important biochemical process in plants, but also one of the most vulnerable to external environmental conditions. In fertilized plants the photosynthesis process takes place with increased intensity (Table 2). The process of carbon (IV) oxide assimilation under suitable conditions is intensified in plants treated with *B. thuringiensis* var. *kurstaki* + SiO_2 significantly, and constitutes - 15.7% compared to the control variant, compared to the intensity of the process in the control grapevine plants.

Table 2. Determination of the quantityof pigments in grapevine leaves, Sauvignon variety, field experiment mg/100 g m

Experimental variant	Chlorophyll *a*		Chlorophyll *b*		Chlorophyll *a+b*		Carotinoids	
	M±m	Δ, %	M±m	Δ, %	M±m	Δ, %	M±m	Δ, %
15.06. 2021								
1.*	1.14±0.01		0.67±0.02		1.81±0.02		0.28±0.04	
2.	1.22±0.01	10,7	0.73±0.02	20.6	1.95±0.03	10.7	0.33±0.01	11.7
3.	1.08±0.00	9.47	0.52±0.00	27.2	1.61±0.00	8.8	0.33±0.03	11.7
4	1.12±0.02	9.82	0.68±0.01	34.7	1.80±0.03	10.0	0.29±0.04	10.2
5.	1.16±0.01	10.1	0.76±0.02	21.2	1.91±0.02	10.5	0.33±0.01	11.7
6.	1.14±0.01	10.0	0.68±0.02	23.1	1.82±0.02	10.0	0.31±0.04	10.6
7.	1.26±0.05	11.0	0.79±0.02	22.3	2.09±0.10	11.5	0.22±0.10	7.8
17.07.2021								
1.	0.81±0.03		0.69±0.06		1.50±0.08		0.21±0.01	
2.	0.98±0.00	12.2	0.66±0.01	10.22	1.63±0.01	10.8	0.23±0.00	10.6
3.	0.91±0.03	11.2	0.63±0.06	18.7	1.54±0.08	10.2	0.24±0.02	11.4
4.	0.81±0.00	10.0	0.56±0.01	11.4	1.38±0.01	9.2	0.23±0.00	10.6
5.	1.03±0.01	12.6	0.85±0.02	14.2	1.87±0.02	12.4	0.16±0.01	7.6
6.	0.80±0.03	10.4	0.72±0.06	16.1	1.51±0.09	10.0	0.22±0.02	10.4
7.	1.02±0.05	12.3	0.87±0.02	15.6	1.89±0.10	12.6	0.24±0.10	11.4
06.08. 2021								
1.	1.17±0.02		0.45±0.00		1.62±0.00		0.54±0.01	
2.	1.25±0.05	10.6	0.55±0.01	12.2	1.80±0.01	11.1	0.53±0.02	9.8
3.	1.13±0.16	9.6	0.56±0.00	12.3	1.82±0.00	11.3	0.51±0.03	9.4
4.	1.28±0.03	10.9	0.59±0.00	12.7	1.87±0.00	11.5	0.55±0.00	10.2
5.	1.31±0.01	11.1	0.70±0.00	15.5	2.01±0.00	12.4	0.52±0.00	9.5
6.	1.25±0.01	10.6	0.74±0.07	16.4	1.87±0.09	11.5	0.50±0.04	9.2

1. - Control (plants treated with distilled H_2O);2. - Chemical standard; 3. - SiO_2;4. - *P. aureofaciens*.+ SiO_2; 5. - *P. aureofaciens +B. thuringiensis* var.*kurstaki;* 6. - *B. thuringiensis* var. *kurstaki* + SiO_2; 7. - *P.aureofaciens+B. thuringiensis var. kurstaki* + SiO_2

Closing the stomata reduces water loss through transpiration, but inevitably also leads to decreased photosynthesis due to reduced access of carbon dioxide to chloroplasts *(*Avis et al., 2008*).* Analysis of the results obtained using the LCpro-SD portable gas analyzer in the experiments shows insignificant differences in plant response to treatment in the other variants.

Table 3. Influence of rhizobacteria on photosynthesis intensity, transpiration and stomatal conductance in grapevine, Sauvignon variety, Field experiment 2020

Experimental variant	Intensity of photosynthesis, $Mm/m^2/sec$		Transpiration intensity, $Mm/m^2/sec$		Stomatal conductance, $Mm/m^2/sec$	
	M±m	Δ, %	M±m	Δ, %	M±m	Δ, %
1.*	4.25 ±0.24		1.86 ±0.34		0.16 ±0.04	
2.	5.87 ±0.14	13.8	2.15 ±0.31	11.5	0.17 ±0.07	10.6
3.	5.86 ±0.25	13.7	2.43 ±0.26	13.0	0.19 ±0.01	11.8
4	5.43 ±0.18	12.7	2.49 ±0.21	13.3	0.18 ±0.02	11.2
5.	6.71 ±0.15	15.7	3.16 ±0.15	16.9	0.24 ±0.05	15.0
6.	5.58 ±0.34	13.1	2.45 ±0.17	13.1	0.16 ±0.04	10.0

1. Control; 2. *P. aureofaciens*; **3**. *P. aureofaciens* + SiO_2; **4.** *P. aureofaciens+B.* thuringiensis var. *kurstaki*; **5.** *B. thuringiensis* var. *kurstaki* + SiO_2; **6.** *P. aureofaciens+B. thuringiensis* var. *kurstaki* + SiO_2

A rather wide aspect of the response of grapevines to fertilizer application, identified by many researchers, is closely related to productivity (Naoko Ohkama-Ohtsu and Jun

Wasaki., 2010). The quality and quantity of the grape harvest is the defining indicator by which the effectiveness of an agrotechnical method is measured. Harvest records according to the variants of the experiments were carried out using the following indices: average quantity of grapes per plant, average weight of a grape, average yield per plant, quality of grapes.

Analyzing the data obtained (Table 4) it was observed that in the variant where bacteria with *P. aureofaciens*+*B. thuringiensis* var. *kurstaki* strains were used with the addition of SiO_2 the average quantity of grapes per plant is 12.1% higher than in the control variant.

The average weight of a grape was higher in the variant where the bacterial strain *B. thuringiensis* var. *kurstaki* + SiO_2 was used. This effect is due to the growth stimulating effect these bacterial strains have on the plants, and on the assimilation of nutrients.

Table 4. Effect of foliar fertilisation on the quantity of Sauvignon vine yield IGPPP experimental lot, Field experiment 2020

Experimental variant	Average quantity of grapes per plant		Average weight of 1 grape, g.		Harvest		% of sugar	
					Average/Plant, kg.			
	M±m	Δ, %	M±m	Δ, %	M	Δ, %	M±m	Δ, %
1.	12.2±4.18		104.05±1.16		1.3		22.4±0.05	
2.	12.8±6.59	10.4	133.0±1.99	12.7	1.7	13.0	24.3±0.04	10.8
3.	14.0±8.07	11.4	146.5±3.66	14.0	2.0	15.3	24.0±0.04	10.7
4	14.0±5.76	11.4	122.1±3.03	11.6	1.7	13.0	24.7±0.05	11.0
5.	13.8±4.89	11.3	133.7±2.06	12.7	1.8	13.8	24.3±0.03	10.8
6.	14.8±2.72	12.1	126.1±3.02	12.0	1.9	14.6	24.0±0.05	10.7

1. - Control; 2. - *P. aureofaciens*; **3**. - *P. aureofaciens* + SiO_2; **4. -** *P. aureofaciens*+*B.* thuringiensis var. *kurstaki*; **5. -** *B. thuringiensis* var. *kurstaki*+ SiO_2; **6. -** *P.aureofaciens*+*B. thuringiensis* var. *kurstaki* + SiO_2

CONCLUSIONS

It has been shown that the application of suspensions of bacterial strains *P. aureofaciens* and *B. thuringiensis* var. *kurstaki* with SiO_2 addition at critical phases of plant development contributed to increase the photosynthetic pigment content in grapevine leaves, increasing the quality and quantity of the harvest.

The use of suspensions of bacterial strains *P. aureofaciens* and *B. thuringiensis* var. *kurstaki* with SiO_2 addition contributed to increase the intensity of photosynthesis, transpiration and stomatal conductance, as well as the weight gain of grapes in grapevine plants.

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REVIEW ON DIFFERENT APPLICATIONS OF *Lavandula* spp. AS A SOURCE OF BIOACTIVE COMPOUNDS

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Abstract

Lavandula spp., commonly known as lavender, is an aromatic and medicinal plant that belongs to the Lamiaceae family and includes 47 known species of flowering plants of which we mention Lavandula angustifolia, Lavandula stoechas, Lavandula pedunculata, Lavandula dentata, Lavandula multifida, Lavandula latifolia, Lavandula viridis, Lavandula lanata, etc., many of them being hybrids or varieties with important economic value for the pharmaceutical, cosmetic, perfumery, food and agricultural industries. This paper reviews recent literature regarding the characteristics and properties of Lavandula spp., providing a comprehensive view about the use of lavender essential oils and plant extracts in various industrial fields. Variability is discussed by species, geographic area, plant material and extraction methods.

Key words*: biologic compounds, Lavandula spp., various applications.*

INTRODUCTION

The genus *Lavandula* comprises 39 species and numerous hybrids and cultivars (Benabdelkader et al., 2011), growing all over the world: Europe, North Africa, South-West Asia, Arabian Peninsula, India, North and South America (Lis-Balchin, 2012; Messaoud, 2012). Many researches have described the chemical composition and the main components of lavender oils, among which is counted monoterpenoids (linalool, linalyl acetate, 1,8-cineole, β-ocimene, terpinen-4-ol and camphor), sesquiterpenoids (β-caryophyllene) and other terpenoid compounds (Bikmoradi et al., 2017). It has been shown that lavender essential oil (EO) has an effect on the central nervous system (sedative, anticonvulsant, analgesic), and as well has antioxidant, antimicrobial, anti-inflammatory, spasmolytic and carminative activites (Bakkali et al., 2008; Hassiotis et al., 2010). *Lavandula* species, due to their high oil content, are used both as aromatic plants and for medicinal purposes. It represents an important source of raw material for the pharmaceutical, food, perfumery and cosmetic industries, but also for aromatherapy (Plotto & Roberts, 2001; Cavanach et al, 2002; Hyldgaard et al., 2012; Gutierrez, 2008; Adaszynska et al., 2013; Nieto, 2017; Sarkic & Stappen, 2018). Recently, certain compounds obtained from *Lavandula* spp. have been used in ceramics, in paint coatings and in porcelain (Yegorova, 2011).

The literature, as has been specified in this review, shows that the essential oils obtained from *Lavandula* spp. are promising in terms of their biological activity (Adorjan, 2010). They can be the subject of a technological transfer to the pharmaceutical, cosmetic and food industry in order to obtain innovative products, but their practical relevance must be evaluated through *in vitro* tests (Zuzarte et al., 2012).

The main uses of *Lavandula* in a variety of perfumes, soaps, creams are based on scent. *L. angustifolia* is preferred for perfumery, followed by *L. x intermedia* hybrid, which contains linalool and linalyl acetate as the main components. *L. latifolia* can be used in some food and household cleaning products, as well as in medicine (Hart & Lis-Balchin, 2002). Species rich in camphor are mainly used in aromatherapy and phytotherapy (Herraiz-Peñalver et al., 2013). The development of the lavender crops worldwide led to the definition of the international standards to assess the quality

of the EOs (ISO 3515:2002/Cor 1:2004; NF ISO 3515:2004 (T75-301)).

The main purpose of this paper is to provide a systematic view on the lavender essential oil content, its chemical composition and its main uses.

Therefore, this paper is based on recently published articles related to lavender essential oil composition in bioactive compounds and its potential utilization in various industrial fields.

Essential Oil Composition

Currently, in specialized literature there is a lot of research related to essential oil compositions (Cavanagh & Wilkinson, 2005; Bombarda et al., 2008; Giray et al., 2008; Baydar & Kineci, 2009; Danh et al., 2013; Binello et al., 2014; Aprotosoaie et al., 2017; Dris et al., 2017; Tardugno et al., 2019; Detar et al., 2020; Giuliani et al., 2023). All these articles point to the fact that the composition of lavender essential oil is influenced by origin, genotype (Munoz-Bertomeu et al., 2007; Stanev, 2010), the stage of development (Lacusik et al., 2014), agronomic factors (Renaud et al., 2001; Angioni et al., 2006; Pinto et al., 2007; Erbaş & Baydar, 2008, Kara & Baydar, 2013; Camen et al., 2016; Garcia-Caparros et al., 2019; Lyczko et al., 2019; Fascella et al., 2020; Pecanha et al., 2021), parts of plant (Geisel et al., 2004; Lakusic et al., 2014) extraction method, storage and processing of biological material (Chemat et al., 2006; Karapandzova et al., 2014; Babu et al., 2016; Duskova et al., 2016; Salata et al., 2020;). Some authors have shown that there are no significant differences regarding the composition of the oil extracted by various methods. Microwave irradiation greatly accelerated the extraction process but without causing a change in composition. The essential oil contains more than a hundred components, of which the main are linalool (from 9% to 69%) and linalyl acetate (from 1.2% to 59%). The quality of this oil is given by the high content of linalool and linalyl acetate, but also by their proportions (Beale et al., 2017).

In the current literature, there is not a common point of view regarding the correlation between the harvest period and the composition of lavender oil. Some of the differences are visible between the genotypes. If in some situations the highest ratio of linalool was observed in the middle of the flowering period, and the highest percentage of linalyl acetate was detected at the end of flowering, in others cases increased amounts of linalool were recorded from full flowering to the end of the flowering phenophase while the amount of linalyl acetate decreased (Baydar & Kineci, 2009; Cantor et al., 2018; Detar et al., 2021). Other authors showed that linalool, terpinen-4-ol, 1,8-cineole, limonene recorded the highest concentration when the flowers were in buds.

The main constituents of lavender flower are representatives of the terpene compounds (oxygenated monoterpenes, 50-90%), followed by sesquiterpenes (7.5-15.0%) and triterpenoids (Bakkali et al., 2008; Lesage-Meessen et al., 2015; Chrysargyris et al., 2016). The diversity of compounds is highly variable depending on the species. Therefore, regarding oxygennated monoterpenes content, for *L. angustifolia* was reported 36-93%, for *L. x intermedia* 68-93%, for *L. stoechas* 46-93% and for *L. latifolia* 85-94%. High values are also specific for *L. pedunculata, L. luisieri* and *L. viridis*. The richest species in sesquiterpenes are *L. angustifolia, L. stoechas* and *L. luisieri* (over 20%) (Aprotosoaie et al., 2017).

The most common monoterpenoids of lavender EOs reported from flowers are linalool, terpinen-4-ol, a-terpineol, borneol, lavandulol, linalyl acetate, lavandulyl acetate, geranyl acetate, geranyl propionate, camphor, fenchone, thujone and 1,8- cineole. The monoterpenes reported in lavender EOs are limonene, cis-b-Ocimene, trans-b-Ocimene and the sesquiterpenes are b-caryophyllene, b-farnesene, caryophyllene oxide and viridiflorol (Chrysargyris et al., 2016).

Table 1. Composition of lavender essential oils

Origin	Reference	The main compounds of lavender essential oil (%)													
		Linalool	Linalyl acetate	β-Ocimene	1,8-Cineole	Camphor	Limonene	Terpinen-4-ol Gammaterpinene	Borneol	lavandulol acetate	Geranyl acetate/ Bornil acetil	b-caryophyllene	β-farnesene	α-pinene/y cadidene	Eucaliptol/ camphene/ others
Romania	Oroian et al., 2019, LA	18.46-39.5	25.64-29.86	1.63-14.39	0.16-0.74	0.21-0.31	ND	ND	1.72	ND	ND	ND	ND	ND	ND
Romania	Goncearіuc et al., 2018, LA	24.15-50.84	27.29-44.40	0.2-1,8	0.31-1.61	0.2-0.35	0.16-0.92	1.11 – 9.21	0.75-2.2	0 – 2.22	0.64-1.64	1.41-2.40	ND	ND	ND
India	Raina et Negi, 2012, LA	23.6	35.8	1.5	1.8	1.4	0.6	ND	1.4	4.8	ND	1.8	ND	ND	ND
Hungary	Detar et al., 2021, LA	5.1-62.7	2.6-34.9	0.1-8.2	0.2-32.8	0.2-19	0.4-4.0	0.6-23.9	0.6-11.9	0.2-2.17	0.1-1.3	0.1-3.7	ND	ND	ND
France	Beale et al., 2017, LA	9.3-68.8	1.2-59.4	0.2-18.1	0-3.4	0-0.5	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bulgaria	Ognyanov, 1984, LA	30.1-33.7	35.2-37.6	6.8-7.7	2.1-3.0	< 0.5	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bulgaria	Todorova et al., 2023, LA	23.13-35.52	20.79-39.91	0.29-7.19	0.13-8.29	-	0.33-1.31	-	-	4.21 0.41	-	6.09 1.96	Tr-3.99	-	-
Syria	Al-Wassouf et al., 2018, LA	27.3-34.7	19.7-22.4	1.9-2.9	0.2-0.5	0.2-0.3	ND	ND	ND	ND	ND	ND	ND	ND	ND
Serbia	Lakusik et al., 2014, LA	28-37	00.3-3.3	1.1-2.8	20.5-43	8.9-14.2	-	0.6-4.7	10-24.7	0.2-0.3	0.1-0.9	ND	ND	ND	ND
Poland	Walasek-Janusz et al., 2022, LA	15.1-21.77	ND	16.87-34.22	ND	0.56	1.19-2.77	1.79-3.49	1.92-2.45	5.8-8.25	1.9-2.17	0.5-1.47	ND	ND	ND
Poland	Łyczko et al., 2019, LA leaves (dried)	ND	2.21±0.73	ND	ND	2.09 ± 0.29	3.42 ± 1.16	4.09 ± 0.67	4.66 ± 0.69	ND	5.57±0.82	6.11 ± 1.48	ND	10.53± 1.51	7.28 ± 1.06
Italy	Evandri et al., 2005, LA	32.7	43.1	ND	0.8	0.5	0.3	3.1	0.8	ND	ND	4.9	0.8	ND	ND
Italy	Alteriis et al., 2022, LA	39.31	48.45	ND	1.2	0.11	ND	1.4	ND	2.17	ND	2.01	ND	ND	ND
Croatia	Blazekovic et al., 2018, LA	3.97	11.56	0.6	2.94	0.34	0.47	4.7	ND	3.67	1.09	0.29	ND	ND	ND
France	Lawrence, 1993, LA	9.3–68.8	1.2–59.4	0.2–18.1	0–3.4	0.5	ND	0.1–13.5	ND	21.6	ND	ND	ND	ND	ND
China	Xiaotiana et al., 2020, LA	24.30	28.89	ND	ND	0.39	ND	4.4	2.6	ND	0.89	7.89	ND	ND	ND
Algeria	Djenane et al., 2012, LA	22.3	21.8	9.3	1.31	ND	1.18	5.19	2.1	4.99	ND	4.83	ND	ND	ND
Greece	Adam et al., 1998, LA	20.18	18.60	ND	13.1	ND	ND	ND	ND	16.01	ND	ND	ND	ND	ND
India	Fakhari et al., 2006, LA	35.3	13.42	ND	ND	ND	ND	ND	ND	10.90	ND	ND	ND	ND	ND
Cyprus	Chrysargyris et al., 2016, LA	0.17-0.24	ND	ND	58.67-62.28	7.43-11.26	ND	0.6-0.74	9.1-12.34	ND	ND	ND	ND	ND	ND
USA	Wang et al., 2021, LA	29-33	20-49.44	1.15-2.57	ND	0.2-04	ND	ND	2.8-5.6	3.28-4.65	0.4-0.8	3.5-4.84	ND	ND	ND

Table 1. Composition of lavender essential oils (continuation)

Origin	Reference	The main compounds of lavender essential oil (%)													
		Linalool	Linalyl acetate	β-Ocimene	1,8-Cineole	Camphor	Limonene	Terpinen-4-ol Gammaterpinene	Borneol	lavandulol acetate	Geranyl acetate/ Bornil acetil	b-caryophyllene	β-farnesene	α-pinene/y cadidene	Eucaliptol/ camphene/ others
Romania	Marincaş & Feher, 2018, *Lavandula x intermedia* (LI)	21.9 ± 5.27	33.8±8.34	5.62±3.1	ND	0.83±1.72	1.84 ± 1.87	3.13 ± 2.06	2.82 ± 1.63	33.8 ± 8.34	ND	3.67 ± 1.27	ND	ND	ND
Spain	Marin et al., 2016 LO	34.34	34.19	5.05	1.71	0.39	ND	2.43	ND	4.4	0.41	3.83	3.08	ND	ND
Argentina	Martucci et al., 2015, LO	53.5	4.2	-	6.8	8.4	-	7.6	4.7	-	-	-			
Italy	Garzoli et al., 2019, LI	41.6	23	ND	19.8	4.4	3.5	4.8	2.8	3.2	ND	ND	ND	8.7	ND
Poland	Walasek-Janusz et al., 2022, LI	25.53-29,56	ND	18.56-21.05	ND	ND	9.57	3.52-18.08	4.58	3.5-6.9	1.24-3.36	0.75-1.47	ND	ND	ND
Croatia	Blazekovic et al., 2018 LI	57.1	9.83	0.35	8.44	0.12	3.97	3.33	ND	0.2	1.12	0.17	ND	ND	ND
Portugal	Costa et al., 2013b, LV	0.93/L oxid=7/93	ND	ND	7.81	22.4	ND	3.55	2.7	ND	ND	ND	ND	ND	ND
Spain	Méndez-Tovar et al., 2016, LL	30.34 ± 7.69	0.62	0.28	41.96±5.48%	9.27±2.46	ND	0.09	1.67	ND	ND	0.44	ND	ND	ND
India	Al-Ansari et al., 2021, LL	9.1	ND	ND	10.2	13.8	ND	9.1/26.8	ND	9.3	ND	ND	ND	ND	2.32
Morocco	Ez-zoubi et al., 2022, LS	0.74	ND	ND	ND	43.97	0.32	0.22	2.92/1.29	ND	ND	ND	30.39	ND	ND
Brazil	Cossetin et al., 2021 LD	ND	ND	ND	50.73	15.18	ND	ND	ND	ND	ND	ND	17.11	ND	ND
India	Hanamanthagouda et al., 2010, LB	ND	3.37	ND	ND	7.09	ND	ND	ND	ND	ND	3.68	ND	ND	//transcarveol (18.93%), pulegone (8.45%),menthol (5.89%). Pipertone 4.65bicyclogermacrene3.02

[1]LA = *Lavandula angustifolia;* [2]LL = *Lavandula latifolia;* [3]LO = *Lavandula officinalis;* [4]LI = *Lavandula intermedia;*
[5]LV = *Lavandula viridis*; [6]LS = *Lavandula stoechas*; [7] LD = *Lavandula dentata*; [8]LB = *Lavandula bipinnata;*
[9]ND = not detectable.

In the lavender oil obtained from leaves there are predominant the following bioactive compounds: 1,8-cineol (42.17%), p-cymen-8-ol (14.05%), borneol (6,32%), o-cymene (4.38%), bornyl acetate, p-cimene (14.06%), camphor (2.32%) (Giuliani et al., 2023). Perino-Issartier et al. (2013) reported that French lavandin, Grosso cultivar, contains high concentrations of linalool (45.51-47.51%) and linalyl acetate (45.1-48.2%). In *L. stoechas*, the concentrations of linalool and linalyl acetate are very low or absent (Gyrai et al., 2008). 1,8-cineole and camphor are predominant in the essential oils from *L. angustifolia* leaves and stems. Lavandin EOs have higher 1,8-cineole (1.8–47.9%) and camphor contents (2.2–32.7%) (Flores et al., 2014). In *L. latifolia* from Spain, Garcia-Vallejo (1992) reported the hight content in 1,8-Cineole (8.8-71.5%) and camphor in *L. pedunculata* (4.3-84.4%). Fenchone was detected as major compound in *L. stoechas* EOs (3.06-75.50%) (Benabdelkader et al., 2011). Borneol and Terpinen-4-ol and a-Terpineol are abundant in essential oils of *L. angustifolia* (2-14.01% and 2-9.17%). The presence of 4-terpineol (30%) was detected in samples from China, Spain and Italy (Chen et al., 2020). The chemical composition for *L. angustifolia* EOs is under regulation by ISO 3515:2002, respectively ISO 8902:2009 for *Lavandula x intermedia* Grosso, and ISO 4719:2012 for *L. latifolia.* Table 1 presents the main compounds of lavender EOs depending on the country of origin and the considered species.

Utilization of Lavandula spp. EOs and extracts

The *Lavandula* genus is known for its versatility regarding the uses of EOs in different products. Its use continues to be popular in aromatherapy, pharmaceuticals and medical applications (Pistelli et al., 2017). It is known that only the oil obtained from *Lavandula* flowers is used in medicine (European Pharmacopoeia, 2008), but latest research has shown that oil from lavender leaves has also an important role due to the high percentage of camphor (Lyczko et al., 2019). Also, *Lavandula angustifolia*, *L. latifolia*, *L. intermedia* and *L. stoechas* oil and aqueous extracts have been used in perfumery, cosmetics, food manufacturing and in agricultural application (Upson & Andrews, 2004). The scent of lavender is used in home and pet care products and gives a special taste to drinks, sweets, jams, puddings, chewing gum, chocolate, etc. The most common *Lavandula* spp. utilizations are listed in Table 2.

Table 2. *Lavandula* spp. EOs and extracts utilizations

Species	Utilisation	References
Lavandula angustifolia	Therapeutic potential	Bertram, 1995; Buyukokuroglu et al., 2003; Hajhashemi et al., 2003; Woronuk et al., 2011; Raut & Karuppayil, 2014; Koziol et al., 2015; Kivrak, 2018; Malcolm & Tallian, 2018; Bialon et al., 2019; Donatello et al., 2020; Zeinab et al., 2020; Detar et al., 2021; Doha et al., 2021; Firoozeei et al., 2021; Villalpando et al., 2022; Saeed et al., 2023.
	Cosmetic products	Koniger, 1997; Fakhari et al., 2005; Kunicka-Styczynska, 2009; Adaszynska et al., 2013; Kunicka-Styczynska et al., 2015; Saeed et al., 2023.
	Food manufacturing	Fenaroli, 1998; Fakhari et al., 2005; Adaszynska et al., 2013; Fascella et al., 2020.
	Amendment of soils	Yohalem & Passey, 2011.
	Insecticidal effect	Carson & Riley, 1995; Sertkaya et al., 2010; Khosravi et al., 2013; Yazdani et al., 2013; Julio et al., 2014; El Abdali et al., 2022; Ez-zoubi et al., 2022.
	Veterinary products	Wren, 1988; Ercan & Esma, 2019.
	Repellent	Warren et al., 1997.
	Allelopathy	Sidorenko et al., 1995.
	Acaricidal effect	Perrucci et al., 1996; Kaya, 2010.
	Aromatherapy	Lis-Balchin & Hart, 1999; Evandri et al., 2005; Umezu et al., 2006; Michalina et al., 2019; Donatello et al., 2020.
Lavandula coronopifolia	Therapeutic potential	Said et al., 2015; Hasanin et al., 2020; Naseef et al., 2022.
	Phytoremediation of Soils	Shafagha et al., 2012.
	Food manufacturing	Preedy, 2016.
	Veterinary use	Ferguson,et al, 2013.
Lavandula dentata	Therapeutic potential	El Abdali,2022; Bouyahya et al., 2023.
Lavandula stoechas L.	Pharmaceutical and perfume industries	Repici, 2019.
	Therapeutic potential	Bouyahya et al., 2017; Chograni et al., 2021; Rasheed et al., 2023.
	Cosmetic use	Bouyahya et al., 2017.

Table 2. *Lavandula* spp. EOs and extracts utilizations (continuation)

Species	UTILISATION	References
Lavandula x allardii	Fragrance, food, cosmetic and pharmaceutical industries	Chasiotis et al., 2021.
Lavandula latifolia	Food industry	Fenaroli, 1998; Mendez-Tovar et al., 2016.
	Medicinal purposes	Rodrigues et al., 2012; Herraiz-Peñalver et al., 2013; Gayoso et al., 2018; Al-Ansari et al., 2021.
	Insecticidal use	Al-Ansari et al., 2021.
Lavandula officinalis	Medicinal purposes	Meftahizade et al., 2011; Imene, 2012; Et-Touys et al., 2016; Kivrak, 2018.
Lavandula x intermedia	Food	Fenaroli, 1998.
	Anaesthetic	Krasteva et al., 2021; Yigit et al., 2022.
Lavandula luisieri	Antifeedant	Gonzalez-Coloma et al., 2011.
Lavandula multifida	Therapeutic potential	Benbelaid, 2012.

Lavandula* spp. *EOs and extracts properties

Lavandula spp. EOs have demonstrated biological activity in the field of antioxidant, antimicrobial and anti-inflammatory properties. The antioxidant activity of lavender EOs has been evaluated in many studies (Table 3). There are different reports of this activity as a result of the different tests used for their evaluation - the DPPH analysis, respectively the ABTS assays (the relative ability of antioxidants to scavenge the ABTS - 2,2'-azino-bis (3-ethylbenzothiazoline- 6-sulfonic acid) generated in aqueous phase) (Ionita, 2021), and ORAC assays (The Oxygen Radical Absorbance Capacity) (Gupta., 2015; Kasote et al., 2019).

Regarding the antioxidant activity, significant differences have been demonstrated between different authors due to the genotypic variability (Mendez-Tovar et al., 2015), the phenophase of the plant, extraction method (Karabagias et al., 2019) as well as the agro-pedoclimatic conditions and the conditioning of the biological material (Baptista, 2015). The main biologically active compounds of lavender spp. are components of essential oil, phenolic compounds, triterpenes and sterols. *Lamiaceae* Family is a rich source of phenolic acids, especially rosmarinic acid (Zgorka & Głowniak, 2001; Turgut et al., 2016; Costa et al., 2013a). A lower content of polyphenols was reported for *L. angustifolia* than in the case of other plants from the same family (50.6 mg GAE/g compared to 67.8 mg GAE/g in *Origanum vulgare*) (Spiridon et al., 2011). Dobros et al. (2023) presented in an exhaustive manner the most common phenolic acids in *Lavandula* spp.

Table 3. Antioxidant capacity of *Lavandula* spp. EOs and extracts

Species	Extract type/part of plant/extraction method	Antioxidant activity	References
angustifolia Mill.	Aqueous-ethanol extraction/flowers DPPH [µg/mL] Fe2+ chelation assay [µg/mL]	49.93+-0.75 -110.36+-1.40 (according to ssp)	Robu et al., 2012.
	Infusion/ aerial plant/ABTS [mM]	0.72 ± 0.07	Ivanova et al., 2005.
	Inflorescent/DPPH	27.67± 0.56	Blazekovic et al., 2018.
	Shaking/leaves/DPPH [µmol TEAC/g DW]	9.00 ± 3.00, 14.17 ± 9.09	Ahn et al., 2020.
	Shaking/herb/ ABTS [µmol/100 g DW]	22.00 ± 0.00 20.19 ± 2.55	Blazekovic et al., 2010.
	Ultrasonic assisted extraction/ flowers * leaves inflorescence stalks/ DPPH * [µg/mL] TBARS * [µg/mL] Fe2+ chelation assay * reducing power	11.37 ± 0.69 89.36 ± 5.00 319.21 ± 21.96	Blazekovic et al., 2010.
	Ultrasonic assisted extraction/herb/ ABTS [mmol/100 g DW]	10	Dvorackova et al., 2014.
	Pulsed ultrasound-assisted extraction/ flower residues after the distillation of essential oil/DPPH [mg TE/g of dry waste]	107.29 ± 0.05	Turrini et al., 2021.
	Supercritical fluid extraction (SFE)/flowers/ DPPH [%]	50.55 ± 0.7 78.83 ± 1.3	Tyskiewicz et al., 2019.
	flower	95+-3.98 mg AA	Zgorka & Głowniak, 2001.
	Aerial parts/ DPPH%assaySC50(µgmL^{-1})	97.61±0.15-105.08±0.08	Kıvrak, 2018.
	SFE/flower/ DPPH% HD Solvent extraction	63% 48% 12%	Danh et al., 2013.
	Flower/DPPH (mg TE g^{-1} FW) ABTS (mg TE g^{-1} FW TRP (mg AAE g^{-1} FW) FRAP (mg Fe g^{-1} FW)	3.20±0.4 2.54±0.2 0.11±0.02 1.19±0.4	Nikolic et al., 2019.

Table 3. Antioxidant capacity of *Lavandula* spp. EOs and extracts (continuation)

	Aerial plant/(mg g^{-1}) Aerial plant AMF inoculation	38.66±1.3 73.3±1.3	Golubkina et al., 2020.
	mg AA/g	953.98	Spiridon et al., 2011.
	Aerial part/CL-chemiluminescence DPPH/%	88.66 86.693	Bunghez et al., 2015
L. latifolia	Shaking/leaves/DPPH [µmol TEAC/g DW]	6.56 ± 1.13	Ahn et al., 2020
	Aerial parts/DPPH EC50 (mg/mL)	1.85 ± 0.05- 4.56 ± 0.15 (2009) 2.94 ± 0.09- 5.69 ± 0.35 (2010)	Mendez-Tovar et al., 2018
L. × intermedia	Inflorescences/DPPH(mg/mL)	21.58 ± 0.26	Blazekovic et al., 2018.
	Ultrasonic assisted extraction/flowers * leaves inflorescence stalks/DPPH * [µg/mL] TBARS * [µg/mL] Fe2+ chelation assay * reducing power *	17.17 ± 0.33 116.54 ± 9.96 397.71 ± 10.26	Blazekovic et al., 2010.
	Aerial parts/ DPPH assay SC50 (µgmL^{-1})	89.81±0.17 93.20±0.10	Kıvrak, 2018.
L. hybrida	Aqueous-ethanol extraction/ flowers DPPH [µg/mL] Fe2+ chelation assay [µg/mL]	73.53 ± 1.25	Robu et al., 2012.
L. pedunculata	Infusion/flowering stems/DPPH [µg/mL] TBARS [µg/mL] reducing power [µg/mL]	68 ± 0.5–191 ± 2; 14 ± 1–39.1 ± 0.1; 51 ± 1–167 ± 1	Lopes et al., 2018.
	Maceration/aerial parts/TEAC (w, w/e, e) [µmol TE/g extract] ORAC (w, w/e, e) [µmol TE/g extract] TBARS (w, w/e, e) [%] Fe2+ chelation assay (w, w/e, e) [%]	670.95 ± 4.24; 569 ± 1.99; 1530 ± 121; 96 ± 2	Costa et al., 2013.
L. viridis	Maceration/aerial plants/ORAC (w, w/e, e) [µmol TE/g extract], TEAC (w, w/e, e) [µmol TE/g extract]	1502.22 ±39.95 670.95 ± 4.24	Costa et al., 2013.
L. luisieri	Oil aerial parts/ DPPH, TBARS	moderate antioxidant capacity	Matos et al., 2009.
	Randall Extraction/herb/DPPH [µg/mL]	30.66 ± 1.9	Gimenez-Rota et al., 2019.
	Supercritical antisolvent fractionation/herb/ DPPH [µg/mL]	16.17 ± 0.7	Gimenez-Rota et al., 2019.
L. stoechas	Methanol extraction/flowers/ DPPH [mg/mL]	7.05	Karabagias et al., 2019.
	Aqueous extraction/flowers/ DPPH [mg/mL]	1,78	Karabagias et al., 2019.
	Infusion with stirring/ DPPH [%] Fe2+ chelation assay superoxide anion	45 ± 0.0 84 ± 0.0 78 ± 0.0	Gulcin et al., 2004.
	ORAC (µmol TE/mL EO) ABTS (µmol TE/mL EO) DPPH (µmol TE/mL EO)	1.42-2.22 160.9-175.3 0.5661-1.3	Carrasco et al., 2015.
L. officinalis	Oil commercial/FRAP/ TEAC ¥ (mmol/L Trolox)	0.14-0.24+-0.02	Marin, 2016.
L. spica	Oil commercial/ IC50 - mg/L	1828.25	Badr et al., 2021.
L. coronopifolia	Flower/DPPH [µg/mL]	17.8 ± 0.8	Abdelaziz et al., 2020.

Antimicrobial properties of lavender volatile oil

Recently, new non-toxic molecules with antimicrobial effect obtained from essential oils have been developed (Bakkali et al., 2008). This was necessary because the development of antibiotic resistance against MRSA (Methicillin-resistant *Staphylococcus aureus*) limited their effectiveness (Guo et al., 2020). The efficiency of different essential oils against bacteria and fungi, including the essential oil from *Lavandula* spp., have been compared in the literature by analysing the concentrations required to inhibit the growth of the target organisms. To compare the bioactivity of essential oils, most authors used standardized methods such as minimum growth inhibitory concentration (MIC), minimum lethal concentration (LD - lethal dose) minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC), MIC50 and LD50 values (Stanlay & Deans, 2002; Lopez et al., 2005; Raut, 2014; Balouiri et al., 2016; Garzoli et al., 2019). The antimicrobial activity of essential oils can be minimized due to the volatility or decomposition of the compounds subjected to various extreme factors. The works of some authors have demonstrated the effectiveness of *Lavandula* spp. EOs against strains sensitive to drugs, as well as against strains resistant to them, regardless of their potential against biofilms that are tolerant to antibiotics (Galvao et al., 2012).

Regarding the antibacterial and antifungal activity of lavender essential oil, it depends on many factors that affect its composition, such as agricultural technology, harvesting, drying, the part of the plant that is used, the obtaining method for oil and the genetic variation (Walasek-Janusz et al., 2022).

The antibacterial and antifungal effects of *Lavandula* spp. oils are due to the properties of their many components (Jianu et al., 2013). Some authors state that linalool, linalyl acetate, eucalyptol, terpinen-4-ol, β-ocimene, limonene, α-pyrene lavandulyl acetate, borneol, camphor are responsible for antimicrobial properties (Orhan et al., 2011; Lesage-Meessen et al., 2015). Others, such as Shafagha et al. (2012), reported that antimicrobial activities of essential oils of *Lavandula* spp. are difficult to correlate to a specific compound due to their complexity and variability. *Lavandula* spp. EOs has been found to be active against many bacteria, predominantly against Gram-positive, but also against Gram-negative bacteria, including multidrug-resistant bacteria (Teixeira et al., 2013; Gismondi et al., 2021) and fungi (Bouzouita et al., 2005). Some work reports the antifungal activity of *Lavandula* EOs against yeast, dermatophyte and *Aspergillus* strains responsible for human infections and food contamination (Zuzarte et al., 2012). Many studies have demonstrated the effectiveness of lavender EOs against *Enterococcus faecalis*. Also, genus *Bacillus* has been shown to be susceptible to lavender volatile oil in a number of studies (Deans & Ritchie, 1987). Growth inhibition in Gram-positive bacteria was observed at lower concentrations than in Gram-negative bacteria, and yeasts showed significantly greater sensitivity to the *Lavandula* spp. EOs than Gram-positive and Gram-negative bacteria (Walasek-Janusz et al., 2022). Some authors appreciate that EOs can be used as food preservatives due to the phenolic compounds responsible for the antioxidant properties (Zeng & Wang, 2001). Although many studies have been conducted regarding the use of EOs as preservative agents, there are limitations regarding this use. The main limitations are based on the fact that EOs are strong flavorings and are not acceptable from a sensorial point of view for some foods. Also, the concentrations added to the products are reduced and are not sufficient from the point of view of the antimicrobial effect, which implies the combination with other antimicrobial agents (Marin, 2016).

EOs are used to control phytopathogenic microorganisms in the agricultural sector (Al Ansari et al., 2021). EOs extracted from *L. latifolia* and *L. stoechas* demonstrated effectiveness against *F. oxysporum, R. solani, A. nidulans*. EOs extracted from *Lavandula* showed a least activity against *Aspergillus flavus* (Angioni et al., 2006). *Lavandula angustifolia* EOs demonstrated antifungal activity against *T. mentagrophytes, A. nidulans,* whereas *L. stoechas* EOs was effective against *Sclerotinia sclerotiorum* and *Leptosphaeria maculans* (Angioni et al., 2006; Moon et al., 2007). *Lavandula dentata* EO has inhibitory activity against fungi, such as *C. albicans, P. notatum, A. niger*, and also against gram positive bacteria, like *E. faecalis, B. subtilis, S. aureus, Micrococcus* spp. and gram-negative bacteria, *P. aeruginosa, E. coli* (Hanamanthagouda et al., 2010). Stanojevic et al. (2011) has demonstrated inhibitory activity of lavander EOs against *Aspergillus niger, Candida albicans*, *Klebsiella pneumonia* and *Salmonella enteritidis*. Generally, the essential oil showed better antibacterial activity than antifungal activity (Table 4).

Table 4. Antimicrobial activity of *Lavandula* spp. EOs and extracts

Species/MIC	Microorganisms	Inhibitory zone	References
L. luisieri		MIC (µg/L)	Zuzarte et al., 2012
	C. albicans,	0.64-2.5	
	Cryptococcus neoformans,	0.64	
	Aspergillus strains	0.16-0.32	
L. spica		(mg/L)	Badr, 2021
Conc. 250, 500, 1000, 2000, and 3000 mg/L	*S. typhimurium,*	3000	
	S. aureus,	3150	
	A. flavus,	EC50 = 1145.13	
	A. niger	905.43 mg/L	
L. tenuisecta		MIC (µg/L)	Sayout et al., 2020
	Staphylococcus aureus,	6.25	
	Enterobacter aerogenes,	6.25	
	Pseudomonas aeruginosa,	16.66	
	Klebsiella pneumoniae,	12.5	
	Klebsiella oxytoca,	12.5	
	Acinetobacter baumannii,	12.5	
	Enterobacter cloacae	25	

Table 4. Antimicrobial activity of *Lavandula* spp. EOs and extracts (continuation)

Species/MIC	Microorganisms	Inhibitory zone	References
*Lavandula multifid*i Conc. 4 to 0.007 mg/mL	 *Enterococcus faecalis* ATCC, *Escherichia coli* ATCC, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 70603, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10231, *Listeria monocytogens* ATCC 19115, *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633	µl/ml 0.2500 0.5000 10.00 0.5000 0.2500 0.0625 0.2500 0.0312 0.0312	Khadir et al., 2012
L. stoechas subsp. *luisieri*	 *Aspergillus niger,* *Candida albicans,* *Candida guillermondii,* *Cryptococcus neoformans,* *Rhodotorula rubra,* *Saccharomyces cerevisiae,* *Trichosporon cutaneum*	µg/mL) 15.5 >100 62.5 15.5 >100 31 31	Baptista et al., 2015
L. pedunculata	 *Aspergillus niger,* *Candida albicans,* *Candida guillermondii,* *Cryptococcus neoformans,* *Rhodotorula rubra,* *Saccharomyces cerevisiae,* *Trichosporon cutaneum*	MIC (µg/mL) >100 >100 62.5 15.5 62.5 >100 62.5	Baptista et al., 2015
L. bipinnata Conc. 0.5 to 2.0 µg/µl bacteria 2 to 4 µg/µl fungi	*E. coli* ATCC 25922, *P. aeruginosa* ATCC, *Sh. dysenterie,* *E. faecalis* ATCC, *S. aureus* ATCC, VRE ATCC 51299, *B. subtilis,* *Micrococcus,* *A. niger,* *P. notatum,* *C. albicans*	< 0.5 < 2 < 1 < 2 < 1 < 2 < 1 < 0.5 < 2 < 4 < 4	Hanamanthagouda et al., 2010
Lavandula officinalis 20-40 µL	 *Listeria innocua.*	MIC-mm 13.25-17.00	Marin, 2016
L. officinalis, stem and leaf, Conc. 30 µL	*B. subtilis,* *S. epidermidis,* *S. aureus,* *E. faecalis,* *K. pneumonia,* *P. aeruginosa,* *E. coli,* *A. niger,* *C. albicans,* *S. cerevisiae*	11.7-22.1 8.9-25.6 7.2-21.5 9.2-23.3 12.6-16.1 8.4-10.8 7.5-20.3 8.2-15.6 9.9-16.1 9.5-16.9	Shafagha et al., 2012
L. latifolia conc. 0.025 to 10%	 *A. flavus,* *A. nidulans,* *T. mentagrophytes,* *L. maculans,* *R. solani,* *F. oxysporum*	µg/mL 2.5 ± 0.225 >10 0.125 5 ± 0.065 2.5 >10	Al Ansari et al., 2021
Lavandula × *intermedia* Conc. 40, 20, and 2 µL	 *E. coli,* *A. bohemicus,* *P. fluorescens,* *B. cereus,* *K. marina*	MIC 20 µL <2 >40 20 20 20	Garzoli et al., 2019
L. angustifolia Conc. 20, 10, 5, 2.5, 1.25, 0.6, 0.3, 0.16, 0.08, and 0.04 mg/mL	 *Staphylococcus aureus* ATCC, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* ATCC 10240, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 10876, *Salmonella typhimurium* ATCC 14028, *Proteus mirabilis* ATCC 12453, *Bordetella bronchiseptica* ATCC 4617, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans,*	µg/mL 5-10 2.5-10 10 2.5 5 5 10 10 2 10 10 0…3-1.25	Walasek-Janusz et al., 2022

Table 4. Antimicrobial activity of *Lavandula* spp. EOs and extracts (continuation)

Species/MIC	Microorganisms	Inhibitory zone	References
	Candida auris, *Candida lusitaniae* ATCC 3449	1.25 1.25	
Lavandula angustifolia Mill. Conc. 0.39-12.480 µg/mL	*C. albicans.*	MIC µg/mL 512	Khoury et al., 2016
Lavandula viridis	*C. parapsilosis,* *C. tropicalis*	MIC µL/mL 1.25 1.25-2.5	Zuzarte et al.. 2011

CONCLUSIONS

The results obtained from the literature review of *Lavandula* EOs are promising in terms of obtaining natural products that have action against pathogenic fungi and bacteria. Overall, results of the current study suggest that *Lavandula* EOs are worth of further investigations for potential use in combination with antibiotic therapy in order to minimize the lowest effective dose of drugs and to minimize the resistant bacteria development. *Lavandula* EOs have a good potential for antioxidant activity, which is related to phenolic compounds, and can be used in preserving foods. Furthermore, it is necessary to evaluate the practical relevance for pharmaceutical, cosmetic and food industries.

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PREPARATION OF COMPOST FROM SEA BUCKTHORN BRANCHES BY USING A MULTIPURPOSE *Trichoderma* STRAIN

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Abstract

This paper presents a process for preparing from sea buckthorn branches sawdust of a compost by using a multipurpose Trichoderma strain (T. harzianum Td50b). The used strain accelerates the degradation of the lignocellulosic material and has biocontrol and plant biostimulant characteristics. The branches, resulting as a by-product of harvesting sea buckthorn berries and leaves, were grounded and extracted to recover bioactive ingredients - flavonols, flavones, phenolic acids, proanthocyanidins, triterpenoids, hydrolysable tannins, serotonin. The resulting sawdust, depleted in polyphenols with potential anti-fungal activity, was normalized to 90% water activity with ammonium nitrate solution 0.2M, supplemented with 10% eggshells powder, and inoculated with 2% alginates beads granules containing 10^9 spores T. harzianum Td50b per gram. After incubation for 50 days at room temperature in aerobic conditions, the survival of Trichoderma was analyzed by cultivation on selective media. The Trichoderma re-isolated from sea buckthorn branch compost maintained its antagonistic and plant biostimulant characteristics.

Key words*: compost, Trichoderma, antagonist, plant biostimulant, sea buckthorn harvested branches.*

INTRODUCTION

The market for products of the sea buckthorn, *Elaeagnus* (synonym *Hippophae*) *rhamnoides* (L.) Nelson, berries, leaves, and derived nutraceuticals, raised significantly in the last years, exceeding USD 2 billion (Janceva et al. 2022). The significant ecological benefits also drive the expansion of the cultivated area with this thorny shrub (Ciesarová *et al.* 2020).

Sea buckthorn berries lack an abscission layer and are harvested biannually, by cutting the whole branches and recovering the berries and leaves after deep freezing. The harvesting process generates a significant amount of by-products. Leaves, rich in antioxidant polyphenols, are used for infusion with health benefits similar to green tea (*Camelia sinensis*) infusions (Cho et al., 2014; Ma et al., 2019). Branches also have a high content of active ingredients - flavonols, flavones, phenolic acids, proanthocyanidins, triterpenoids, hydrolysable tannins, serotonin (Tkacz et al., 2021). The extraction processes valorizing these bioactive components were published (Andersone et al., 2023; Janceva et al., 2022). However, a large amount of extracted material remains after the bioactive ingredients extraction - and new approaches are needed to valorize it.

A possible utilization of the extracted material resulting from sea buckthorn branches is to produce compost, fortified with microorganisms beneficial for sea buckthorn, useful for further improvement of the cultivation (Giurescu et al., 2022). The horticultural functions of the fortified compost are direct, due to nutrients content and

beneficial microorganisms (Sanchez et al. 2017), and indirect/mediated, due to the large quantities of humic acids formed during composting process (Guo et al*.,* 2019) that improve soil structure and properties (Shah et al., 2018), acts as plant biostimulants (Canellas et al., 2015) and support communications between beneficial microorganisms and cultivated plant roots (Shah et al., 2018). Fungi from the *Trichoderma* genera are multipurpose plant-beneficial microorganisms (Woo et al., 2023). *Trichoderma* strains are active against soil-borne pathogens (Dutta et al., 2023; Rodrigues et al., 2023), including *Verticillium* (Kowalska 2021) and *Fusarium* (Ben Amira et al., 2017), fungal pathogens that produce wilt/drying of sea buckthorn shrubs (Drevinska & Moročko-Bičevska, 2021). *Trichoderma* strains are also recognized for their plant biostimulant effects on horticultural crops (López-Bucio et al., 2015). Their application improved flowering, quality traits, and nutrition status in sea buckthorn (Andrzejak & Janowska, 2022).

This paper presents the preparation of a carrier compost for a multipurpose *Trichoderma* strain from our collection (*T. harzianum* Td50b), from extracted sea buckthorn harvested branches. The survival of the *Trichoderma* population, formed from the propagules released from an alginate beads formulation, during the composting process, and the preservation of the antagonistic and plant biostimulant characteristics of *Trichoderma* population from resulted compost were also analyzed.

MATERIALS AND METHODS

Materials

The *T. harzianum* strain Td50b, NCAIM (P) F 001412, is a multipurpose strain. It was proved to have antagonistic activity against soil-borne plant pathogens, mainly determined by the production of volatile bioactive compounds, including 6-PP, 6-pentyl-2H-pyran-2-one (Oancea et al., 2017a; Răut et al., 2014). Td50 strain was also proved to have a plant biostimulant activity on cultivated shrubs (Şesan *et al.* 2020) and to accelerate the degradation of lignocellulosic material (Oancea et al., 2017a). The plant biostimulant effects of (re)isolated *Trichoderma* was tested on *Arabidopsis thaliana*, cv. Columbia (WeberSeeds, Vaals, Netherland). The branches were harvested from sea buckthorn (*E. rhamnoides*, cv. Mărăcineni), organically cultivated in the BioCătina orchard from Valea Marea, Dâmbovița, Romania - 44°46′44″N latitude, 25°14′18″E longitude, 218 m altitude. The BioCătina orchard is established on molic-vertic preluvosol soil, with a high content of coarse sand, slight acidic reaction (pH of 6.4. in water) and a rather low humus content - 3.16% in the upper (0-20 cm) soil horizon. The multiannual average values (1975-2020) of temperature, total precipitation, sunshine daily duration, and wind speed for the BioCătina orchard are 10.1°C, 512.1 mm, 7.1 h and 3.8 $m.s^{-1}$, respectively. The chicken eggshells used as an additive for supplementation with calcium of the extracted sawdust were obtained from the production of the embryonic extract (Hipocrate 2002, Bucharest, Romania). The ingredients for *Trichoderma* cultivation media were supplied by Scharlab (Barcelona, Spain). For *Trichoderma* selective media chloramphenicol, streptomycin, pentachloro-nitrobenzene (quintozene) were supplied by Sigma-Aldrich (Merck Group, Darmstadt). The source for propamacarb was Proplant 72.2 SL fungicide (Adama, Ashdod, Israel). The Murashige and Skoog Basal Medium used to cultivate *A. thaliana*, sodium alginate (30-40 kDa average mass) and calcium chloride used for alginate beads formulation, the reagents for determination of total polyphenols and total flavonoids contents, including gallic acid (≥ 99%) and quercetin (≥ 95%) used as references, the dimethyl formamide used for chlorophyll extraction and the Breathe-Easy® sealing membrane were purchased from Sigma-Aldrich (Merck Group, Darmstadt, Germany). As protective filler for *Trichoderma* spores was used diatomaceous earth (DE) from the Sibiciu quarry (Sibiciu, Pătârlagele, Buzău). This DE was formed as deposit by marine diatoms in Oligocene and contain nanoporous diatom frustules and small amount of clay, illite and kaolinite (Moale et al., 2021).

Sea buckthorn branch extraction

The harvested branches from BioCătina organic orchard were frozen at -39°C in an industrial

freezer for 2 days. The berries and leaves were separated by gentle shaking. The empty branches were cut into small piece by an electric shredder (UD2500, Makita, Aichi, Japan). The cut branch pieces were finely grinded in cryogenic mill (Antylia Scientific, Vernon Hills, IL, USA). The extraction of the resulted saw-dust was intensified by using microwave (Sharma et al., 2008). Portion of 100 g of sawdust were introduced with 1000 mL absolute ethanol in a 2 L flat bottom flask with ground glass joint. The flask was introduced in a microwave extractor Minilabotron 2000 (Sairem, Décines-Charpieu, France). The neck of the flask was taken out from the microwave oven and a condenser feed with cold water was mounted in its ground glass joint. The extraction was done for 12 min, at 1000 W, 2.45 GHz microwave, in the temperature control mode. At the end of the extraction cycle the sawdust was separated from ethanol by filtration and dried in a vacuum oven (VD23, Binder, Tuttlingen. Germany). In ethanolic extract was determined the total polyphenols content (TPC) and total flavonoids content (TFC). The TPC was determined by a miniaturization of the Singleton and Rossi method (Michel et al., 2012), using 96 well plate and reading the absorbance at 765 nm in a microplate reader (CLARIOstar, BMG LabTech, Ortenberg, Germany). The reading were expressed as mg of gallic acid equivalent (GAE) per g of dried weight (d.w.), by using a calibration curve made with gallic acid. The TFC was determined by a miniaturization of the aluminum chloride method (Cacique et al., 2021), using quercetin equivalents (CQ) to transform spectrophotometric readings at 425 nm into flavonoids content.

***Trichoderma* inclusion in the alginate beads**

Td50b strain was cultivated in an optimized medium with the following composition: 34.2 g/L glucose, 0.37 g/L ammonium sulfate, 0.8 g/L yeast extract, 2.7 g/L soymeal, 1.2 g/L K_2HPO_4, and 1.7 g/L KH_2PO_4. This medium was shown to promote chlamydospores formation (Zamfiropol-Cristea et al., 2017). The growing conditions were also chosen to promote strain sporulation - aerated and agitated medium in an orbital shaker (Unimax 2010, Heidolph, Nuremberg, Germany), at 25°C maintained in a transparent hood, with additional light for 16 h per day. The initial inoculation was done with 10^7 spores per ml, recovered from the sporulated culture on PDA medium. The Td50b strain was grown for 2 weeks in the aerated and agitated medium. At the end of the growing cycle, the fungal suspension was aseptically poured through a cotton tissue and the spores were concentrated by centrifugation (Oancea et al., 2016). The alginate beads were produced by coagulation in a 0.25M $CaCl_2$ bath of the droplets resulted from a mixed suspension, 2% DE and 10^8 Td50b spores, in 3% sodium alginate solution (Oancea et al., 2017b). The efficiency and reproducibility of the process was assured by using an equipment designed for beads formation, Encapsulator B-390 (Büchi, Flavil, Switzerland). The beads were dried at 35°C, in a vacuum oven (VD23, Binder, Tuttlingen).

Compost preparation

The resulting dried sawdust, depleted in polyphenols with potential anti-fungal activity, was normalized to 90% water activity with ammonium nitrate solution 0.2M - from 72.3 to 76.9 ml solution per 100 g of different batches of extracted sawdust. The water activity in the sawdust was determined by using a water activity measuring device, LabMaster-aw neo (Novasina, Lachen, Switzerland). The watered sawdust was supplemented with 10% eggshells powder, as a source of calcium and a pH buffering ingredient. The eggshell powder was obtained after washing abundantly with tape water, followed by rinsing with pure water, drying in the vacuum oven and grinding in a planetary ball mill (PM 200 Retsch, Verder Scientific, Haan, Germany), with agate jar and agate balls. The mixture watered sawdust - eggshell was inoculated with 1% alginates beads granules containing 10^8 spores *T. harzianum* Td50b per gram. The inoculated substrate for compost preparation was incubated at room temperature in aerobic condition (Haddadin et al., 2009), using 500 mL round bottom flask with large neck, containing ~250 g composting mixture and fitted with perforated caps and polyethylene tubes for aeration. The aeration with 0.25 L of air/min was done 15 min at every 4 h, using oil-

less air, from a screw compressor (OLS11-YD, Adicomp, Isola Vicentina, Italy). After 50 days of incubation at room temperature in aerobic condition, the compost was analysed for survival of *Trichoderma* and maintenance of the antagonistic and plant biostimulant characteristics. The control was represented by the same composting mixture, saw dust from sea buckthorn extracted for polyphenols, watered with ammonium nitrate solution and mixed with eggshell, without inoculation with alginate beads containing *Trichoderma*. Each experimental treatment, inoculated with alginate beds containing Td50b and control, included 5 jars, aerated at the same level by using an air distributors with mini valves and flowmeter. Each jar, with control pr the inoculated substrates, were weighted initially and after 50 days of incubation, by using an electronic scale.

***Trichoderma* (re)isolation from compost**

The selective medium for *Trichoderma*, containing chloramphenicol, streptomycin, quintozene, and propamocarb was used for re-isolation of *Trichoderma* (Williams et al., 2003). Briefly, a sample of around 10 g compost was mixed vigorously in aseptic condition with 500 ml sterile solution of phosphate buffer in a sterile plastic bags (Intervoid®, Coveris, Vienna, Austria). Serial dilution were performed, and 0.1 mL were platted on *Trichoderma* selective medium. From isolated colony developed after 5 days of incubation at 25°C, samples were taken and observed at microscope for typical Td50b characteristics (Oancea et al., 2017a).

Determination of antagonistic and plant biostimulant characteristic of (re)isolated *Trichoderma*.

The antagonistic activity was tested against *Fusarium graminearum* DSM 4527, by using the dual confrontation assay in a potato-dextrose-agar (PDA) medium (Whipps, 1987). The plant biostimulant assay was done by determining chlorophyll content of *A. thaliana* (cv. Columbia) seedlings exposed to volatiles produced by reisolated *Trichoderma* (Hung et al., 2013). Briefly, *A. thaliana* seeds were germinated and grown for 10 days on Murashike and Skoog (MS) medium, in a 9 cm Petri dish, at 22 ± 2°C with a light/dark photoperiod of 16:8 h (FitoClima 600, Aralab, Rio de Mouro, Portugal). The 10 day old seedling were exposed for 48 hours to volatiles produced by a 3 days old culture of re-isolate *Trichoderma* in a PDA medium distributed in a 9 cm Petri dish. The dish with *Trichoderma* was put together with the dish with *Arabidopsis* and separated by Breathe-Easy® sealing membrane. The incubation of the joint plated was done in the same conditions - at 22 ± 2°C with a light/dark photoperiod of 16:8 h. After 48 h the chlorophyll content was determined after extraction with dimethyl formamide (Zhang & Huang, 2013).

Statistical analysis

The experiments results were submitted to analysis of variance (ANOVA), using the SPSS 21 software package (IBM, Armonk, NY, USA) and Fisher's Least Significant Difference (LSD) test.

RESULTS AND DISCUSSIONS

The cascading process for the preparation from sea buckthorn branches sawdust of compost with the multipurpose *T. harzianum* Td50b stain that we developed in this work is illustrated in Figure 1.

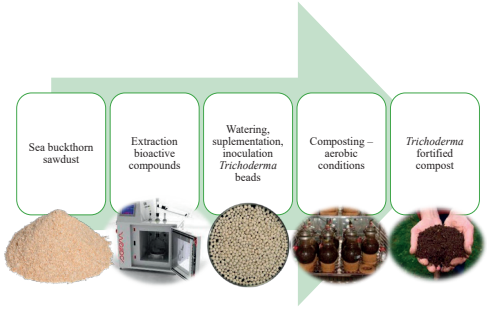

Figure 1. Illustration of the process for compost preparation from sea buckthorn branches by using a multipurpose *Trichoderma* strain

It involve grinding the harvested branches, bioactive extraction from the resulted branches sawdust, watering and supplementation of the extracted sawdust with nitrogen and calcium sources, inoculation with alginate beads containing *Trichoderma* strain and incubation for composting, 50 days in aerobic conditions.

The bioactive compounds were recovered from sea buckthorn branches according to pyramid-value principle, i.e., recovering of high-value components, mainly those useful for wellbeing industries, from biomass, prior others (bio)technological processes (Constantinescu-Aruxandei & Oancea, 2019). The total polyphenols and flavonoids contents is presented in Table 1.

Table 1. The total polyphenols and total flavonoids contents in the microwave assisted extract from sea buckthorn branches (mg/g d.w. extracted sample)

	Total polyphenols content (TPC)	Total flavonoids content (TFC)
Sea buckthorn branches - sawdust produced by cryo-milling with liquid nitrogen	56.8±9.9	48.7±8.2

The obtained values agree with the TPC and TFC contents determined by other authors in the sea buckthorn branches. The resulted extracts are useful for production of cosmetics and/or nutraceuticals, due to their high biological activity (Skalski et al., 2018; Żuchowski, 2023) – and correspond to the above mentioned principle of value pyramid. Also, the extraction of polyphenols with anti-microbial activities (Janceva et al., 2022; Tian et al., 2018) from branches promote the composting process.

The process used for production of Td50b spores and their formulation in alginate beads assure a survival of *Trichoderma* propagules and a good capacity to slowly release these propagules - Figure 2.

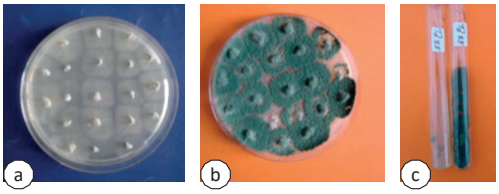

Figure 2. Release of the multipurpose *Trichoderma* Td50b strain from prepared alginate beads: a) immediately after alginate beads preparation; b) *Trichoderma* fungi released during a week of incubation on PDA medium from beads stored 1 month at room temperature; c) *Trichoderma* fungi released during a week of incubation on PDA medium from beads stored 3 month at room temperature

The medium and the cultivation conditions promote the formation of chlamydospores, fungal spreading forms with high resistance to harsh conditions (Zamfiropol-Cristea et al., 2017). Td50b is a strain with high saprophytic competence, able to survive and colonize composting substrate, plant rhizosphere and plant phyllosphere (Oancea et al., 2017a; Oancea et al., 2016; Şesan et al., 2020). The inoculation with *T. harzianum* Td50b alginate beads accelerate the decomposition of the extracted sea buckthorn sawdust by more than 23% - Figure 3.

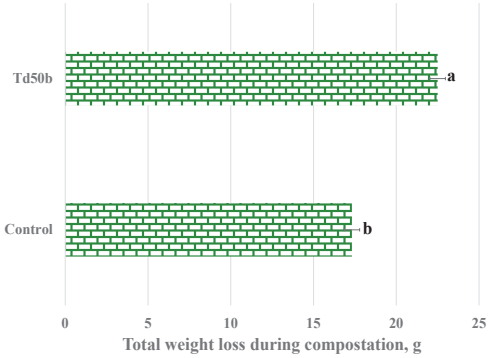

Figure 3. Total loss during the compostation process for the control (un-inoculated with lignocellulolytic microorganisms) and the treatment by inoculation with alginate beads releasing multipurpose *T. harzianum* Td50b strain. Different letters means statistically significant difference ($p<0.05$, $n = 5$)

Td50b strain produce significant amount of proteins acting on the lignocellulose matrix, enzymes - β-glucanases, xylanases, lytic polysaccharide mono-oxygenase (LPMO), laccases, peroxidases, and non-catalytic proteins (cerato-platanins) with expansin-like activities on lignocellulose (Oancea et al., 2016).

The cerato-platanins, non-catalytic proteins, loose the cell wall structure, due to breakage of the physical (hydrogen) bonds that stabilize cellulose and hemicellulose fibrils and promote the activity of enzymes acting on cellulose, hemicelluloses and lignin (Pennacchio et al., 2021). The enzymatic cocktails produced by Td50b strain release oligosaccharides and oxidize lignin, further enhancing composting process (Wu et al., 2022).

The rate of survival of the inoculated *Trichoderma* strain on the compost prepared according to the present work was high. We (re)isolated from the resulted compost fungi that have morphological characteristics and antagonistic properties similar to Td50b strain - Figure 4.

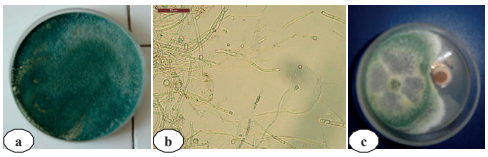

Figure 4. Different aspects of the re-isolated Trichoderma from Td50b inoculated compost: a) Morphological aspect of one isolate on PDA medium; b) Hyphae and spores on light microscopes; c) antagonism on PDA medium of *Trichoderma* isolate against *Fusarium graminearum* DSM 4527

The high rate of survival was promoted by the process that we developed. Our approach was to use a time released formulation of *Trichoderma* chlamydospores for the inoculation of the sawdust resulted from the extracted sea buckthorn branches. From such formulation *Trichoderma* propagules are continuous released, promoting the colonization of the substrate (Oancea et al., 2017b).

Production of volatiles regulates *Trichoderma* population by sporulation induction (Nemcovic et al., 2008) and maintains an additional reservoir of forms resistant to harsh conditions, able to recolonize the substrate in case of a drop of initial colonizing population.

The (re)isolated *Trichoderma* maintain its plant biostimulant characteristics, increasing the chlorophyll content of *A. thaliana* (cv. Columbia) - Table 2.

Table 2. The chlorophyll a and b content (mg per 100 g fresh weight) of *A. thaliana* seedlings (cv. Columbia) exposed for 48 h to the volatiles produced by the *Trichoderma* (re)isolate from compost inoculated with *T. harzianum* Td50b strain

	Chlorophyll a	Chlorophyll b
Control, not submitted to volatiles	12.47±1.38	3.24±0.37
Exposed for 48 h to the volatiles produced by *Trichoderma* (re)isolate from re-inoculated compost	18.62±1.92	3.94±0.28

Strain Td50b was demonstrated to enhance photosynthesis in the treated plant, by increasing chloroplast numbers and dimension and by higher contents of chlorophyll in the leaves (Şesan et al., 2020). Reisolated strains present similar effects on *A. thaliana* seedlings. Various type of composts were demonstrated to be suitable carriers for multipurpose *Trichoderma* strains. In the case of process, the Td50 strain, inoculated as alginate beads formulation, initially acts as compost accelerator, followed by a robust substrate colonization.

CONCLUSIONS

Treatment of the sea buckthorn branches sawdust, after the extraction of the polyphenols, with a time released alginate beads formulation of a multipurpose strain, *T. harzianum* Td50, accelerate the composting process. The resulted compost is a good substrate for further colonization. he proposed process recover the high-value ingredients from sea buckthorn branches and produce a compost that close the loop for mineral nutrients in sea buckthorn orchard in a sustainable manner, reduce the risk for proliferation of soil-borne pathogens producing sea buckthorn wilt/drying and increase the population of biostimulant *Trichoderma*.

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KERATIN EXTRACTION FROM CHICKEN FEATHERS IN AQUEOUS SOLUTIONS

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Abstract

Biowastes have emerged as a promising source for the production of value-added products, reducing the burden on landfills and promoting the concept of a circular economy. Chicken feathers, constituting a significant fraction of the poultry industry's waste stream, possess a robust protein structure composed mainly of keratin. Keratin is a biopolymer with unique properties, including high nitrogen content and slow degradation, making it an attractive candidate for various applications in agriculture and other fields. One of the main problems is the development of more eco-friendly methods for the efficient extraction of this biopolymer. The aim of this study was to compare the yield of keratin extraction from chicken feathers by three methods, alkaline, acidic and subcritical water and to characterize the keratin obtained by the most eco-friendly method, i.e., subcritical water. The subcritical water extraction is a promising alternative to the alkaline and acidic extractions, if proper optimization is carried out. We show that SDS-PAGE electrophoresis combined with FTIR analysis can offer valuable Information in this respect.

Key words*: poultry industry, feather keratin, subcritical water extraction, alkaline extraction, acidic extraction.*

INTRODUCTION

Keratin is one of the most abundant biopolymers, being produced by the epithelial cells of vertebrates (Meyers, Chen, Lin, & Seki, 2008). Together with collagen, keratin assures the structural rigidity of the different vertebrate coverages – skin, nails, hair, horns (McKittrick et al., 2012).

Keratin is a complex mixture of filament-forming proteins (Tinoco et al., 2020; Tomlinson, Mülling, & Fakler, 2004), it is characterised by a high content of sulfur-containing amino acids and is organized in two types of secondary structures, α-helix (e.g., wool) and β-sheet (e.g., feathers) (Calvaresi, Eckhart, & Alibardi, 2016; Chilakamarry et al., 2021).

The keratin chains are cross-linked by di-sulfuric bridges, which stabilize and rigidize the secondary and tertiary structures (Wang, Yang, McKittrick, & Meyers, 2016). Due to its complex and highly organized structures, keratin is insoluble in most of the known protein solvents and is resistant to the hydrolysis of the usual proteases, such as subtilisin, pepsin, papain or trypsin (Chaitanya Reddy et al., 2021; Jaouadi et al., 2013).

The bioeconomy, i.e., the economic sectors producing and using bioresources, generates large amounts of keratin- rich side streams – wool from sheep rearing or feathers, nails, hairs, skins from slaughterhouses, hair and wool from leather industries (Gaidau et al., 2019; Sharma & Gupta, 2016). Overall, such keratin-rich side streams are estimated to exceed yearly the

threshold of 40 million tons (Hussain, Memon, Khatri, & Memon, 2020; Suarato et al., 2020).
Disposal of keratin-rich material is difficult. Landfilling generates high epidemiological risks, due to the proliferation of the keratinolytic (human) pathogens (Călin et al., 2017; Duan et al., 2020). Incineration raises technical difficulties due to low flammability of keratin and produces hazardous substances like sulfur oxides (Ossai, Hamid, & Hassan, 2022; Tesfaye, Sithole, Ramjugernath, & Chunilall, 2017).
The ideal solution for keratin is to use the high protein content of keratin- rich side streams for various applications - in food and feed industries or as agricultural inputs (Alvarez-Castillo, Felix, Bengoechea, & Guerrero, 2021; Giteru et al., 2023; Perța-Crișan, Ursachi, Gavrilaș, Oancea, & Munteanu, 2021). Keratin protein is a source of plant biostimulants (Kaur, Bhari, & Singh, 2021; Popko et al., 2018), increasing plant tolerance to abiotic stress, improving crop quality traits and enhancing nutrients uptake and nutrients utilization. Due to their high resistance to biodegradation, keratin proteins are good candidates for sustainable fertilizer coating (Chen, Li, & Zhang, 2021; Choi & Nelson, 1996; Mihăilă et al., 2020; Yang, Tong, Geng, Li, & Zhang, 2013).
For such utilizations it is necessary to solubilize keratin from keratin-rich material. The aim of this work was to compare the yield of different methods for protein solubilization from chicken feathers and to characterize the protein extracted by the most eco-friendly method, subcritical water extraction.

MATERIALS AND METHODS

Materials

The chicken feathers were acquired from a local Romanian poultry farm. Cetrimonium bromide (CTAB) and Hydrochloric (HCl) acid 37% were from Sigma-Aldrich (Missouri, USA) and sodium hydroxide (NaOH) was from Chimreactiv (Bucharest, Romania). The chemicals for SDS-PAGE, Bradford and Biuret were: Precision Plus Protein™ Dual Xtra Standards, Mini-PROTEAN TGX Stain-Free Gels 4-20%, 2X Laemmli sample buffer, 2-mercaptoethanol, (Bio-Rad, California, USA), Bradford Reagent (Sigma-Aldrich, Missouri, USA), Brilliant Blue G ultrapure powder, Sodium n-dodecyl sulfate 99% (dry wt.) (Alfa Aesar, Massachusetts, USA), Glycine, Tris-(hydroxymethyl)-aminomethane, Sodium hydroxide, Hydrated copper(II) sulfate, Potassium sodium tartrate, Potassium iodide (Scharlau, Barcelona, Spain), Glacial acetic acid (Chimreactiv, Bucharest, Romania), Methanol (Honeywell, Indiana, USA), Albumin bovine fraction V, pH 7.0 (Janssen Chimica, Beerse, Belgium).
The feathers were initially washed with double distilled water (ddH_2O) + 1% CTAB in a ratio of 1/20 (w/v) at 50°C and magnetic stirring at 400 rpm for 2h, followed by rinsing with ddH_2O to remove CTAB. The rinsing process involved gently agitating the feathers in clean water until no more foam was observed. After rinsing, the feathers were air dried to remove excess moisture. They were spread out in a well-ventilated area and allowed to dry overnight at room temperature, ensuring complete drying. After the washing and drying steps, the feathers were further processed through milling to obtain a fine and homogeneous powder. Feather milling was conducted using a centrifugal ball mill (Retsch Type S 100, Haan, Germany) equipped with a ceramic grinding jar and ceramic balls. The centrifugal ball mill was operated at 380 rpm. The milling process lasted for a total of 4 hours to ensure thorough grinding and particle size reduction. To promote uniform milling and prevent sample aggregation, the direction of rotation was changed alternately between clockwise and counterclockwise every 2 minutes.

Alkaline hydrolysis of keratin from chicken feathers

For this extraction of keratin 6M solution of HCl and 1N solution of NaOH were used. The prewashed and grinded feathers were immersed in a mixture of NaOH 1N solution, with a ratio of 1:10 (w/v) for 1 hour at 90-95°C, under stirring (20 g feathers + 200 ml NaOH 1N). Samples were taken as to neutralize the hydrolysate with HCl 6M to a neutral pH (Mettler Toledo Seven Compact S210 pH meter) and further to acidify it down to the isoelectric point of feather keratin previously determined for the extraction in subcritical water, pH = 4.4 (Škerget et al., 2023). When the pH reached 4.4 the total sample was centrifuged at 15°C, 8500 rpm, 20 minutes (Hettich Universal Centrifuge

320 R, Tuttlingen, Germany). After that the precipitate was washed with ddH_2O and lyophilized (SCANVAC CoolSafe, Labogene, Lillerød, Denmark). The N content was determined in the supernatant after each reaction using the Kjeldahl method and reported to 100 g of feathers.

Acidic hydrolysis of keratin from feathers

For this extraction of keratin, 4 M solution of HCl and 1 M solution of NaOH were used. Pre-washed and grinded feathers (30 g) were mixed with 200 ml HCl 4 M. The mixture was boiled at 95°C until the feathers were dispersed. The resulted solution was filtered twice. The pH of filtrate was adjusted with NaOH 1M to reach the isoelectric point of keratin (pH = 4.4), at which the keratin precipitated. After that the precipitate was centrifuged (15°C, 8500 rpm, 20 minutes), washed with ddH_2O and lyophilized.

Keratin hydrolysis using super-heated (subcritical) water

The pre-washed and grinded feathers were mixed with ddH_2O at a substrate/water ratio of 1/20 (w/v), i.e., 6 g feathers with 120 mL ddH_2O. The experiments were done at two temperatures, 150°C and 200°C for 2 h, using a Parr Series 4523 Bench Top Reactor (Moline, USA), with 200 rpm agitation and pressure of 9 bar at 150°C and 11 bar at 200°C.

Characterization of keratin extracts

Infrared spectroscopy (FTIR) analyses were performed using a Shimadzu FTIR IRTracer-100 and FTIR automated microscope AIM-9000 spectrometer. A resolution of 4 cm^{-1} and a wavelength interval of 4000-400 cm^{-1} were used. For cryo - TEM analyses a Tecnai ™ G2 F20 TWIN Cryo-TEM (FEI Company ™) apparatus was used. The sample was prepared by embedding it in an epoxy resin (Agar 100 Resin Kit) from Agar Scientific (Stansted, UK). First, the fine powder was kept overnight at room temperature in a 1.5 mL volume from a mixture of epoxy resin (without accelerator) and acetone (1: 1 ratio by volume). The next day, the whole mixture was separated by centrifugation at 3000 g for 10 minutes at room temperature. The supernatant was discarded and the wet pellets containing the sample were left in a well-ventilated space at room temperature to remove all acetone by evaporation. The acetone-free wet pellets were then mixed with another 1.5 mL of epoxy resin with added accelerator and added to a silicon mold. After 48 h of crosslinking at 60°C, the embedded sample was cut to 80 nm slices using a Leica EM UC7 ultramicrotome (Wetzlar, Germany) at a cutting rate of 2 mm/s which were then collected on 200 mesh lacey carbon film/formvar grids from Ted Pella, Inc. (USA). The sample was analyzed at an accelerating voltage of 200 kV.

Biuret and Bradford assays

The protein concentration in the two keratin hydrolysate samples obtained by subcritical water was determined using Coomassie Brilliant Blue G-250 dye binding-assay – Bradford, and a cooper-based assay – Biuret. The Biuret assay was performed by mixing 40 µL of 1:4 diluted samples with 200 µL of Biuret reagent in 96-well plates. After 30 minutes of incubation at room temperature, the absorbance of the samples was read at 550 nm using a microplate reader (CLARIOstar Plus microplate reader, BMG LABTECH, Ortenberg, Germany) against a bovine serum albumin (BSA) standard curve prepared from a 10 mg/mL BSA stock solution and which underwent the same steps (Janairo, Linley, Yap, Llanos-Lazaro, & Robles, 2011). The Bradford assay was performed by mixing 200 µL of 1: 50 diluted samples with 50 µL of Bradford reagent in 96-well plates. After 5 minutes of incubation at room temperature, the absorbance of the samples was read at 595 nm. The BSA standard curve was prepared from a stock solution of 246 µg/mL BSA which was subjected to the same treatment (Bradford).

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Keratin hydrolysate samples obtained by subcritical water were analysed by SDS-PAGE using protein electrophoresis Mini-PROTEAN Tetra Cell (Bio-Rad, California, USA). After mixing 10 µL of sample with 5 µL of loading buffer, the samples were thermally denatured for 5 minutes at 95°C. 15 µL of sample and 5 µL of molecular mass marker were loaded into the electrophoresis gel well according to the manufacturer's instructions. The migration time up to the bottom of the gel was about 45 minutes at a constant voltage of 30 mA. Subsequently, the electrophoresis gel was washed 3 times with double distilled water and immersed in the staining solution based on Coomassie Blue R-250, where it was left for a few hours under stirring (ROCKER 3D digital, IKA, Staufenim

Breisgau, Germany). The gel was then washed with double distilled water, heated in the microwave and left under stirring for 24 hours, changing the water several times. A gel documentation system was used for the image acquisition (G:BOX Chemi XRQ, Syngene, Cambridge, United Kingdom).

RESULTS AND DISCUSSIONS

The alkaline and acidic hydrolysis gave the yield of 72.5% (14.5 g from 20 g feathers) and 64.6% (19.38 g from 30 g) extracted keratin, respectively, based on the total N measurements of extracted keratin. In fact, considering the previously reported keratin percent of 91% in feather, the yields are 79.7% and 71%, respectively. The precipitation of keratin at the isoelectric point resulted in approx. 50% precipitation of keratin in the case of the alkaline extraction and approx. 60% precipitation in the case of acidic extraction. This suggests higher molecular weight keratin fragments in the acidic extract than in the alkaline extract.

The yield in the case of the extractions with the subcritical water was 26.4% (29% of the total feather keratin) at 150°C (resulting approx. 13.75 mg/ml keratin concentration) and 90.5% (99.45% of the total feather keratin) at 200°C (resulting approx. 42 mg/ml keratin concentration). This is consistent with previous studies that reported, during the finalization of our studies, yield below 30% at 150°C and almost 80% yield at 200°C after 1h (Škerget et al., 2023). In the case of 200°C, although the yield was almost 100%, there were signs of compound degradation after the 2 h extraction, the extract color suffering a radical change from dark yellow to dark brownish (Figure 1).

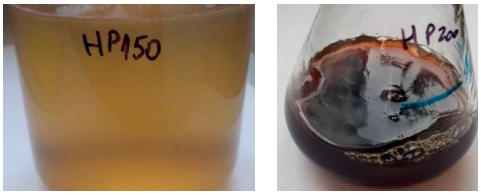

Figure 1. Reaction mixtures after hydrolysis with super-heated water: at 150°C (HP150) and at 200°C (HP200)

Signs of compounds degradation have been also reported previously at 250°C after 10 min of extraction, leading to a decrease in the yield probably caused by some volatile formation (Škerget et al., 2023). It is possible that in our case, after breaking down in small peptides and amino-acids at 200°C, some compounds suffered oxidation and possibly also Maillard-type reactions. Our study shows in addition that the extraction at 150°C does not improve after an additional hour compared to the previous study (Škerget et al., 2023), which indicates, together with the previous study, that temperatures up to 150°C are not efficient for extracting keratin in subcritical water even when using longer time periods.

Cryo-TEM analysis indicated the presence of high aggregates of colloidal-like structures formed from keratin proteins (Figure 2). Keratin was previously shown to have the tendency to form colloids. The aggregation is higher as the pH approaches the isoelectric point of the hydrolysate. The striations that can be observed at the 100 nm scale are an indication of the presence of keratin fragments obtained by the hydrolysis process.

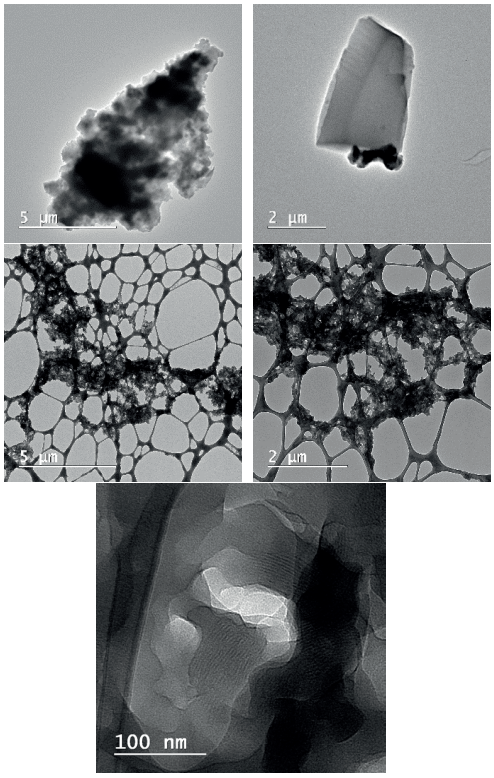

Figure 2. Cryo-TEM of keratin hydrolysate (HP200)

Bradford gave a protein concentration more than 50 times lower than Biuret i.e., 0.25 mg/mL compared to 14.81 mg/mL for HP150, and 0.45 mg/mL compared to 21.89 mg/mL for HP200 (Figure 3A). It is well known that the results obtained by the Bradford method depend on the amino acid composition, the Coomassie Blue R-250 i.e., dye binding readily to arginine and lysine residues (Compton & Jones, 1985). Lysine has not been found previously in significant amounts in keratin extracted by subcritical water, while arginine gave less clear results, one study reporting moderate to relatively high amounts and one study no mention about arginine (Di Domenico Ziero et al., 2022; Škerget et al., 2023). The difference between the two studies was that the first was performed in a semi-continuous flow. Moreover, the Bradford method is more suitable to pure proteins and other compounds could interfere with this method, including eventual CTAB traces remained after washing and rinsing (Aminian, Nabatchian, Vaisi-Raygani, & Torabi, 2013). The Biuret method does not depend on the amino-acid composition, but it is a direct evaluation of peptide bonds. Some differences between Bradford and Biuret have been reported in other cases as well, for example in the quantification of the protein from rapeseed meal (Kalaydzhiev, Ivanova, Uzunova, Manolov, & Chalova, 2018). Therefore, the differences between the two methods were not completely unexpected, although the differences are higher than estimated. The values obtained by Biuret are closer to the previously reported values (Di Domenico Ziero et al., 2022). Compared to the extracted keratin estimated based on the total N content, the keratin determined by Biuret was similar at 150°C (14.81 versus 13.74 mg/ml) but lower at 200°C (21.89 mg/ml versus 42 mg/ml). This indicates that at 200°C almost half of the peptide bonds got hydrolysed to amino-acids and other compounds that do not react with Biuret. The higher difference between the protein concentration of HP200 and HP150 measured with Bradford (ratio HP200/HP150 = 1.80) than with Biuret (HP200/HP150 = 1.5) show the differences between the two methods. The contribution to Bradford comes also from the free amino-acids and the results confirm that the amino-acids are probably more abundant in the HP200 sample than in the HP150 sample.

In the case of the SDS-PAGE the HP150 and HP200 samples were diluted 4-fold before mixing with loading buffer, and the 4-fold diluted HP200 sample (that had higher protein concentration than HP150) was additionally diluted to the concentration of the 4x diluted HP150 sample (HP200d).

In the case of the hydrolysate obtained at 150°C (HP150) the SDS gel shows a relatively narrow profile, with most protein fragments between 3 and 10 kDa (Figure 1B). This is in agreement with the previous study which reported molecular weights of extracted peptides in the range 4-12 kDa (Škerget et al., 2023).

Although the protein concentration shown in Figure 3A is higher in the case of sample HP200, apparently hydrolysis at a temperature of 200°C induces further hydrolysis of peptides the loss of high temperature sensitive amino acids (Bhavsar et al., 2016; Rajabinejad et al., 2017), as can be seen in Figure 1 B by the absence of bands for HP200 sample, and it also leads to an oxidation process, given by the brown colour of the hydrolysate, so these oxidation products may interfere with the methods used to determine protein content.

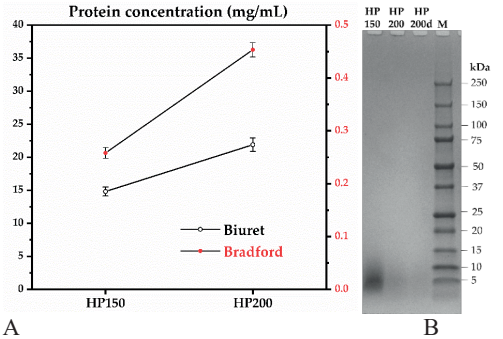

Figure 3. Protein analysis of keratin hydrolysates: (A) Protein concentration (mg/mL) determined by the Biuret and Bradford methods (error bars ± standard deviation (SD)); (B) Molecular weight analysis by SDS-PAGE; HP150=keratin hydrolysate obtained at 150°C, 4-fold dilution; HP200=keratin hydrolysate obtained at 200°C, 4-fold dilution; HP200d=keratin hydrolysate obtained at 200°C diluted to the protein concentration of sample H150; M=molecular weight marker

The FTIR spectra showed that the keratin extracted at 150°C maintained the structural features found in the feathers (Figure 4). The

main peaks the untreated feathers, known as the fingerprint for proteins, are present at approx. 1629 cm^{-1} (amide I), 1535 cm^{-1} (amide II), and 1240 cm^{-1} (amide III). Additionally, there is an additional band at 3285 cm^{-1}, characteristic for inter- and intra-molecular hydrogen (H)-bonds. The peaks characteristic for aliphatic C-H bonds around 2900 cm^{-1} are present as well. All these bands are present in the HP150 as well, with only small shifts in the case of certain peaks, indicating that the extraction at 150°C generates a significant amount of peptides. In the case of the extraction at 200°C, there are significant changes in the FTIR spectra. The amide and H-bond bands are almost completely reduced, and a large peak appears at 997 cm^{-1}. The peaks around 2900 cm^{-1} are maintained. This indicates that at this temperature the peptide structure is significantly hydrolyzed to amino acids/very small peptides together with the disruption of the H-bonds that stabilized the secondary and higher order structures. These data correlate with the observations from the SDS-PAGE, where the peptides are almost absent at 200°C.

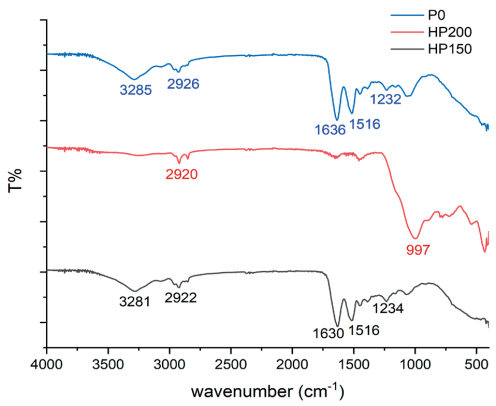

Figure 4. FTIR spectra of chicken feather (P0), keratin extraction by subcritical water at 200°C (HP200) and at 150°C (HP150)

CONCLUSIONS

In this study we compared three different methods of keratin extraction from chicken feathers in aqueous solutions: alkaline, acidic and subcritical water. Our data are in agreement with previous studies and show that the subcritical water extraction can be a viable alternative to the alkaline and acidic extractions, but that proper optimization, product characterization and selection of parameters need to be performed in order to meet the quality criteria depending on the application. SDS-PAGE electrophoresis combined with FTIR analysis can offer valuable Information in this respect.

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BLEACHING PRETREATMENT OF SULFITE PULP WITH COMMERCIAL MACERATION ENZYMES

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Abstract

The aim of our paper was to investigate the efficiency of some commercial products that contain blends of maceration enzymes on the bleaching of pulp obtained from soft wood, birch and polar, using a neutral sulfite semi-chemical pulping (NSSC) process. The enzyme biobleaching is a pretreatment process that releases from pulp the lignin-carbohydrate complexes and the hexenuronic acids and reduces the consumption of chemical bleaching agent. The biobleaching efficiency was evaluated by the reduction in the kappa number. The results demonstrate that the efficiency of the maceration enzymes depends on the degree of delignification and on the composition of the blends. The enzymes that contain a blend of pectinolytic enzymes, including polygalacturonase, have significant activity also on mechanically refined pulp. The maceration enzymes that include hemicellulases are highly actives on pulp resulted from chemical extraction and are less active on pulp that was further delignified by mechanical refining. The presence of pectin lyase tends to increase the kappa number due to the release of hexenuronic acids from pectin.

Key words*: biobleaching, sulfite pulp, maceration enzymes, pectinases.*

INTRODUCTION

The utilization of enzymes for pulp bleaching started in the last decades of the 20th century. This biotechnological process was a result of the significant concerns related to the toxic compounds formed during pulp bleaching with chlorine/chlorine derivatives - the adsorbable organic halogens, AOX (Crooks & Sikes 1990), e.g., polychlorinated compounds, with high environment impact due to their bio-accumulation potential (Freire et al., 2003; Solomon et al., 1996).

The brown-yellowish pulp color was related to the presence of the lignin-carbohydrate complexes precipitated on pulp fibers, including the xylan-lignin-glucomannan complex and glucomannan-lignin complex (Kantelinen et al., 1993; Lawoko et al., 2006). Therefore, the main enzymes initially used for enzymatic bleaching were hemicellulases/ xylanases (Viikari et al., 1994). Hemicellulases/xylanases increase the brightness of the pulp due to the solubilization of lignin-carbohydrate complexes and hydrolysis of xylan and mannan. Hemicellulose hydrolysis promotes pulp fibrillation and lamination and cellulose fibers accessibility to bleaching agents (Roncero et al., 2005).

In the last decades, it was demonstrated that the unsaturated 4-deoxy-*β*-l-*threo*-hex-4-enopyranosyluronic acid groups (hexenuronic acids - HexA), formed during the pulping process by partial degradation of the carbohydrate, and HexA complexes with lignin are also involved in the brown-yellowish color of the pulp (Vuorinen et al., 1999). Hexenuronic acids have other adverse effects on pulp bleaching. Due to the presence of unsaturated double bounds HexA determine an increased consumption of chemical bleaching agents / chlorine and leads to an enhanced AOX formation (Nie et al., 2015). Hexenuronic acids are the main compounds responsible for thermal and aging

induced yellowing of the bleached pulp (Kuwabara et al., 2012; Sevastyanova et al., 2006). Endo-xylanases remove hexenuronic acids (Shatalov & Pereira, 2009). Laccase activity is complementary to xylanase in removing hexenuronic acids and lignin (Valls & Roncero, 2009). An enzymatic preparation containing lipase A, obtained from *Aspergillus niger* was also proved to be effective in the degradation of the hexenuronic acid (Nguyen et al., 2008). Utilizing the enzymatic cocktails containing, besides xylanase, other enzymes, reduced the bleaching agent consumption and promoted lignin release from the pulp fibers (Immerzeel & Fiskari, 2023).

Recently, it was demonstrated that a combination between xylanases and pectinases has a synergic effect on pulp bleaching, mainly due to the hydrolytic activity of poly-galacturonase on hexenuronic acids (Agrawal et al., 2023; Nagpal et al., 2020; Nagpal et al., 2021). This leads us to the idea to evaluate the effect of commercial maceration enzymes on the bleaching of sulfite pulp.

Commercial maceration enzymes are used in winemaking and fruit juice industry, to improve juice color and aroma extraction (Danalache et al., 2018; Espejo, 2021). These enzyme mixtures includes several classes of hydrolase, such as hemicellulases and pectinolytic enzymes (Toushik et al., 2017; Uzuner & Cekmecelioglu, 2019). As we mentioned already, these hydrolases proved to be effective for pulp (pretreatment) enzymatic bleaching. Therefore, this paper aims to evaluate the effects of several commercial products containing macerating enzyme cocktails on the bleachability of sulfite pulp and on the mechanical resistance of paper resulted from the enzymatically treated pulp.

MATERIALS AND METHODS

Sulfite pulp

The used pulp samples were supplied by Cellulose and Paper Factory (CCH), Drobeta-Turnu Severin (Mehedinți, Romania), and was obtained from softwood, birch and poplar, by a neutral sulfite semi-chemical pulping (NSSC) process.

Commercial enzymes preparations

The following commercial macerating enzyme preparations were used: Safizyme Clean® (Fermentis, Marcq-en-Baroeul, France); VinoTaste® Pro (Novozyme, Bagsværd, Denmark), Zymovarietal Pro Ice (Sodinal, Bucharest, Romania); Enovin Pectinase (Agrovin, Alcázar de San Juan, Spain). Safizyme Clean® is a blend of pectinases, endo polygalacturonase, pectin methyl esterase and pectin lyases. VinoTaste® Pro includes, besides pectinases, β-glucanases, and hemicellulase / arabinanase. Zymovarietal Pro Ice is a mixture of pectinases, including pectin lyase, and β-glucuronidase and it targets especially the aroma and color extraction from grape. Enovin Pectinase is a mixture of pectinolytic enzymes.

Chemicals

The following chemical reagents, analytic purity, were purchased from Sigma-Aldrich (Merck Group, Darmstadt, Germany): Potassium permanganate $KMnO_4$ (used as solution 0.1 N); sodium thiosulfate, $Na_2S_2O_3 \cdot 5\ H_2O$ (used as solution 0.2 N); potassium iodide (KI, used as solution 1 N); sulfuric acid, H_2SO_4 (used as solution 4 N) and Starch (ACS reagent, used as solution 1%). Pure water was produced by reverse osmosis in a Centra 120 system (Elga Veolia Labwater, Celle, Germany).

Kappa number determination

Kappa number was determined in accordance with ISO 302:2015 standard. Kappa number is a measure of pulp bleachability and estimates the quantities of carbon oxidable bonds (COB) by titration with potassium permanganate, according to the following reactions:

$COB + MnO_4^- + H^+ \rightarrow COB_{ox} + H^+ + MnO_4^-$ (*excess*) (*1*)

$2MnO_4^- + 10I^- + 16H^+ \rightarrow 2Mn^{2+} + 5I_2 + 8H_2O$ (*2*)

$I_2 + 2S_2O_3^{2-} \rightarrow 2I^- + S_4O_6^{2-}$ (*3*)

Enzymatic treatments

The pulp samples (30 g) were dispersed in 270 mL of pure water and mixed for 5 min, followed by pH adjustment to 7. An equivalent of 5 units per ml pectinolytic activity was added from each commercial enzymes separately. The pectinolytic activity of each enzyme was determined by using dinitrosalicilic (DNS) reagent for reducing sugars (Miller 1959) and poligalacturonic acid as substrate, at 40°C and pH 6.5 (Joshi et al., 2013). One unit is the quantity of enzymes that releases the equivalent of 1 μM reducing sugar

per min at 40°C and pH 6.5. The treatment was done for 4 hours at 40°C and pH 6.5. The bio-bleaching efficiency was determined by measuring the reduction in kappa number. Each experiment was repeated three times.

Determination of mechanical characteristics

The tensile strength has been evaluated by the constant elongation gradient method, according to TAPPI T 494 standard, by using a ZwickyLine Z05 dynamometer (ZwickRoell, Ulm, Germany). The double bending resistance has been evaluated according to ISO 5626 TAPPI T423s standard, by using a Schopper type device (IDM Test, San Sebastian, Spain). The assay were done in paper board made on labor according to ISO 5264-2:2011 standard. The assay was done for the mechanical refined NSSC pulp, RAF3.

Statistical analysis

The results from the experiments were submitted to analysis of variance (ANOVA), using the SPSS 21 software package (IBM, Armonk, NY, USA). The statistically relevant differences were established by Fisher's Least Significant Difference (LSD) test.

RESULTS AND DISCUSSIONS

The aspect of semi-chemical pulp resulted from softwood, birch and polar, by using neutral sulfite semi-chemical pulping (NSSC) process, on Cellulose and Paper Factory (CCH), Drobeta-Turnu Severin (Mehedinți, Romania) is presented in Figure 1.

CW **RAF1** **RAF3**

Figure 1. Aspect of semi-chemical pulp resulted from softwood, birch and polar, by using neutral sulfite semi-chemical pulping (NSSC) process, at Cellulose and Paper Factory (CCH), Drobeta-Turnu Severin (Mehedinți, Romania). Left, the initial washed pulp obtained after the chemical treatment of softwood (CW). Middle, the pulp after delignification, the 1st mechanical refining step (RAF1). Right, after delignification, the 3rd mechanical refining step (RAF3)

The initial semi-chemical pulp (CW) is obtained after delignification of soft wood, birch and poplar. The wood chips are boiled with sulfite liquor at 170°C for 2 hours. The preparation of the sulfite liquor involves the following operations: preparation of sodium carbonate solution; melting and burning sulfur; absorption of sulfur dioxide in sodium carbonate solution. The spent (red) sulfite liquor is extracted, and the semi-chemical pulp is washed, resulting the washed chemical pulp - CW.

RAF 1 and RAF 3 are semi-chemical pulp samples, recovered from different mechanical refining steps, from the 1st and, respectively, from the 3rd step.

The mechanical refining step releases lignin-carbohydrate complexes from the pulp by application of the friction and shear forces generated by the pulp milling between steel plates / discs. Such mechanical refining process usually enhances the accessibility of enzymes, due to fibrillation and lamination of the pulp fibers (de Assis et al., 2018).

The initial Kappa number and the lignin equivalent for each sample, from different stages of the NSSC process are presented in Table 1.

Table 1. Initial Kappa number and the lignin equivalent for samples from different stage of the NSSC process

Specification	CW[1]	RAF1[2]	RAF3[3]
Kappa number	103.11±8.23	82.72±5.64	59.06±3.86
Lignin equivalent	15.46±1.24	12.40±0.86	8.85±0.58

[1]CW - the initial pulp obtained after chemical treatment of softwood
[2]RAF1 - after delignification, the 1st mechanical refining step.
[3]RAF3 - after delignification, the 3rd mechanical refining step

The NSSC process retains a large amount of lignin in the pulp. The pulp is further delignified by using refiner mechanical pulping process (Sandberg et al., 2020), in several steps. Sample from the 1st mechanical pulping stage has lower lignin content compared to initial sample, taken from chemical pulping process, and has a higher lignin content compared to 3rd mechanical pulping stage.

The efficacy of the enzymatic treatment on biobleaching the pulp sample from NSSC process is represented in Figure 2. The applied pectinolytic activity was similar. However, the difference comes from the additional enzymes existing in the process. Safizyme Clean® is a blend of pectinolytic enzymes and is highly efficient on the reduction of the kappa number including on the more mechanical refined pulp. Most probably the pectinolytic system, and especially polygalacturonase, is acting on the hexenuronic acids, that are similar with the

natural uronic acid substrate (Kumar 2021). Vino Taste® Pro, that includes xylanase, is more effective on the less delignified pulp – that contains more lignin-carbohydrate complexes, which are substrates for hemicellulase/xylanase (Lawoko et al., 2006). Enovin Pectinase and especially Zymovarietal Pro Ice, that contain also pectin lyase, an enzyme from the PL1 family polysaccharide lyases (PL1) class, which produce hexenuronic acids by the degradation of pectin (Lombard et al., 2010), are significantly less effective. On the highly refined mechanical pulp the use of enzyme maceration blends that contain pectin lyase tend to increase the kappa number, most probably due to the release of the hexenuronic acids from the pectin that is still included in the pulp.

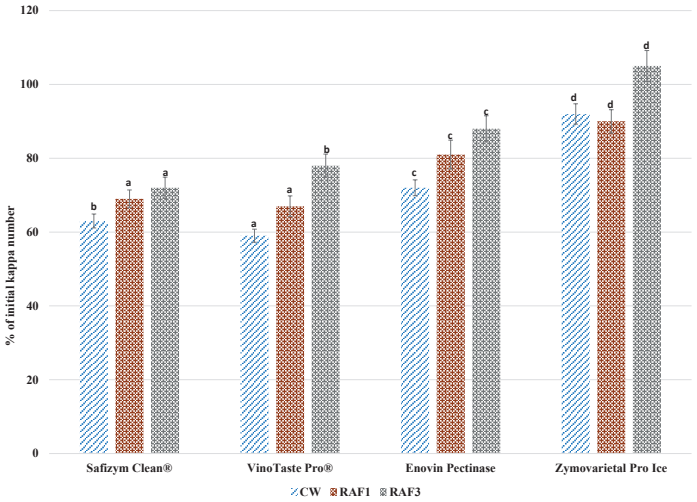

Figure 2. The biobleaching efficacy of treatment with commercial products containing different blends of maceration enzymes on the pulp sample from NSSC process, expressed as percentage of the initial kappa number. CW, the initial pulp obtained after chemical treatment of softwood. RAF1 - after delignification, the 1st mechanical refining step. RAF3 - after delignification, the 3rd mechanical refining step. The values with the same letter do not differ significantly at $p < 0.05$

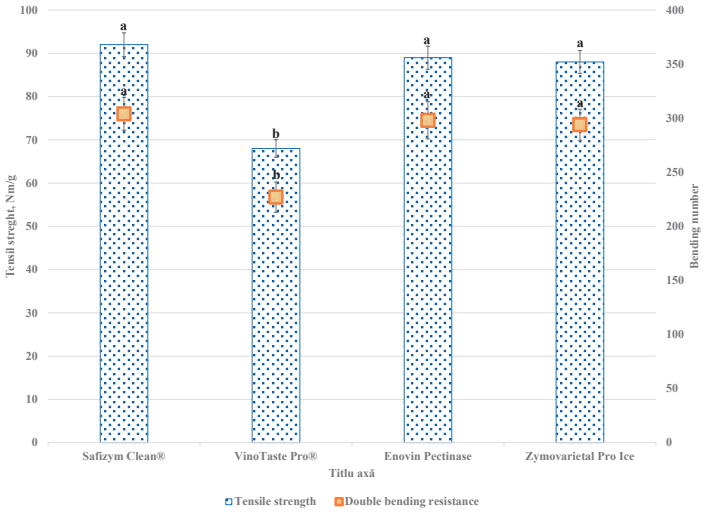

Figure 3. The effects of biobleaching with commercial products containing different blends of maceration enzymes on mechanical properties of the pulps sample from NSSC process, after delignification, the 3rd mechanical refining step (RAF3). The values with the same letter do not differ significantly at $p < 0.05$

In general, the enzymatic treatment does not significantly influence the mechanical characteristics of the pulp - Figure 3. However, VinoTaste® Pro, due to its β-glucanase activity, reduced the tensile strength of the paper produced from the pulp.

Our investigations demonstrate that it is possible to use commercial maceration enzymes for biobleaching of pulp resulted from the NSSC process. The decrease of the kappa number is comparable to that reported for a crude enzymatic preparation containing a xylanase, obtained from *Aureobasidium pullulans* (Christov & Prior, 1994).

The blend of the commercial maceration enzymes has been optimized to get the best results for their application in winemaking and juice production. Our results suggest that it is possible to introduce such blends of commercial maceration enzymes in the composition of the enzymatic cocktails used for the NSSC pulp bleaching. However, it is important to calibrate the enzymatic blends, to maximize bleaching effects and to reduce the negative effects. Better understanding of the effect of pectinolytic enzymes on hexenuronic acids will support a scientific design of the enzymatic cocktails used for pulp biobleaching.

CONCLUSIONS

Commercial products that include maceration enzymes, initially intended to be used in winemaking and juice production, are active also on pulp biobleaching. Most probably the polygalacturonases from the pectinolytic system hydrolyze the hexenuronic acids, releasing them from pulp.

The associated enzymes in commercial maceration blends, such as hemicellulases further support the biobleaching activity, releasing the lignin- carbohydrate complexes from pulp, especially from pulp that it is not mechanically refined. The blends that include polygalacturonase are effective also on the mechanically refined pulp. The enzymatic cocktail that includes pectin lyase tends to increase the kappa number due to the release of hexenuronic acid from pectin. The presence of β-glucanase reduces the mechanical strength of the treated pulp.

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EVALUATION METHODS OF MYCOTOXIGENIC CONTAMINANTS IN FEED RAW MATERIALS - A REVIEW

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Abstract

Mycotoxigenic contaminants in feed raw materials are a serious problem in animal production due to their negative impact on animal performance and health. The most hazardous genera of mycotoxigenic fungi that can contaminate feed raw materials are Aspergillus, Fusarium, and Penicillium. However, not all fungi of these genera are mycotoxin producers. Therefore, to differentiate contaminated from healthy feed it is important to apply fast detection methods. This review paper aims to present fast qualitative detection methods of mycotoxigenic contaminants. Studies are focused on molecular techniques such as PCR (polymerase chain reaction), as well as chromatographic method such as TLC (thin layer chromatography). Using the PCR method, there can be detected either the fungal species and genera, either the presence of certain genes or gene clusters encoding for mycotoxin synthesis. Mycotoxin detection through TLC is an affinity-based method, in which non-volatile compounds are detected based on their retention factor (Rf) depending on the stationary and mobile phase used. The mycotoxigenic contaminants reviewed in this paper are Aspergillus, Fusarium and Penicillium and their producers.

Key words*: feed, mycotoxins, PCR (polymerase chain reaction), TLC (thin layer chromatography).*

INTRODUCTION

Feed raw materials are exposed to various microbial contaminants, some responsible for toxic secondary metabolites called mycotoxins. Highly damaging are those fungi from *Aspergillus*, *Fusarium* and *Penicillium* genera. These pathogens are able to contaminate feed raw materials in a wide variety of their stages and conditions. Fungal infection can be installed either in the field, during harvest, while being stored in various settings, or by improper handling (Sîrbu et al., 2020; Twarużek et al., 2021).

Mycotoxin contamination levels are highly influenced by the mold type, ecologic factors and phytosanitary management practices. Once a substrate is contaminated, although the mycotoxins-producing molds are suppressed, the mycotoxins still persist, due to their stability over time and slow degradability (Cheli et al., 2013). Due to their toxicity, most hazardous mycotoxins have concentration limits that are governed by European Union regulations, which indicate their maximum levels in feeds and feeding stuffs (European Commission, 2016).

According to Food and Agricultural Organization (FAO) of the United Nations, mycotoxins are contaminating a quarter of world's crop production and compromising over one billion metric tons of food items and raw materials each year. Due to their damaging potential, in addition to food quality evaluation, timely assessment of these contaminants and identification of the most important toxigenic species are crucial for the improvement of management methods to ensure food and feed safety (Suanthie et al., 2009).

To maintain a high quality of the feed raw materials at any stage of their production (e.g., crop planting, culturing, harvesting, processing, storage, and transportation) requires periodic surveillance and monitoring of the mycotoxigenic contaminants, both fungi and toxic compounds. Molecular techniques based

on PCR are highly effective to detect mycotoxigenic fungi mostly by using species specific primers or by detecting the genes responsible for mycotoxins synthesis. As well, the chromatographic methods, such as TLC, are highly efficient in detecting various mycotoxins (Kim et al., 2011).

Considering the toxigenic potential of the samples, the PCR techniques are safer, and compared to the microbiologic methods, they are less time consuming. The PCR techniques not only that are quick, by they are also specific and sensitive if correct performed. Their versatility allows simultaneous amplification of species-specific genes, structural or regulatory genes involved in mycotoxin production pathways, by multiplex PCR. As well, by qPCR (quantitative PCR or Real Time PCR) the contamination level could be detected, while by RT-PCR (Reverse Transcription PCR) the expressed genes can be detected and even quantified through Quantitative Reverse Transcription PCR (RT-qPCR). All of these methods can be performed on various sample type including feed matrices (Rahman et al., 2020).

Chromatographic techniques have been used extensively to separate mycotoxins from other simultaneous produced non-toxic fungal metabolites (Betina, 1985). This is due to the fact that chromatographic methods, such as Liquid Chromatography (LC) and Gas Chromatography (GC), are able to isolate and reveal these compounds in their pure form. However, of all chromatographic methods used for studying mycotoxins, TLC is by far the most popular as it can detect toxins without complex or expensive equipment.

The present review aims to provide a comprehensive summary of the mycotoxigenic contaminants, relevant for public food and feed safety. Both fungi and related toxins are considered. A general description of mycotoxins detection through TLC is also presented, while PCR techniques are described for fungal identification and mycotoxin encoding genes detection.

Mycotoxigenic fungi in feed

The main toxigenic fungi found on feedstuffs belong to the *Aspergillus, Fusarium*, and *Penicillium* genera. Aside them there are also *Alternaria*, *Claviceps, Cephalosporium, Monascus, Myrothecium, Stachybotrys, Trichothecium, Verticimonosporium* etc. These fungi can be detected using traditional culture-based methods or by molecular techniques. To reduce the drawbacks of the culture-based methods, such as labor, time, costs, and for much more rapid and precise detection and identification, PCR methods are preferred. Therefore, most detection procedures are now DNA-based. A wide range of protocols allow the identification of a single species, multiple species belonging to the same genus or mixed populations of different genera (De Saeger et al., 2011). Moreover, molecular techniques based on PCR are able to provide valuable information on susceptible mycotoxin producing fungi (Stępień et al., 2012).

Molecular detection of fungi

There is a huge need for fungal contaminants detection in food and feed due to the safety concern they are raising, especially if they are mycotoxins producers. Classical microbiologic approaches for their detection, evaluation, identification and quantification have a number of drawbacks, as they are time-consuming, labour-intensive, dependent on the target, have poor result reliability, which can determine some difficulties in standardization (Pegels et al., 2012).

For early stage detection of fungal phytopathogens with high risk for human and animal health, scientists have increased the use of molecular techniques. Various PCR techniques can now provide relevant information regarding food and feed microbial contamination. Among these, beside the conventional PCR, there is the nested PCR, multiplex PCR, PCR restriction fragment length polymorphism (PCR-RFLP), amplified fragment length polymorphism PCR (AFLP-PCR), quantitative or real-time PCR (qPCR), droplet digital PCR (ddPCR), as well as some magnetic capture hybridization PCR (MCH-PCR), co-operational PCR (Deepa et al., 2021).

Food and feed microbiology are highly benefiting from the molecular technologies for partial and whole genome sequencing, which offer a wealth of data that are crucial in fungal identification and classification (Munaut et al., 2011).

PCR detection based on conserved DNA regions of taxonomic interest

The creation of species-specific PCR techniques based on conserved genes was mainly motivated by taxonomic and phylogenetic considerations. In the beginning, they were created to evaluate any probable sequence polymorphism between species belonging to the same genus and for further research on their evolutionary relationships. The level of conservation of some of the reported genes, however, was occasionally too high for complete resolution within a genus, so the conserved genes make the best templates for real-time PCR primers and probes when classifying because they enable the simultaneous detection, identification, and quantification of the target fungus species (Anantharajah et al., 2021).

The internal transcribed spacer region (ITS1-5.8S-ITS2) of the ribosomal RNA gene cluster (Figure 1) was selected as the official DNA barcoding area for fungi since it is the most frequently sequenced marker in fungi and has universally effective primers (Usyk et al., 2017).

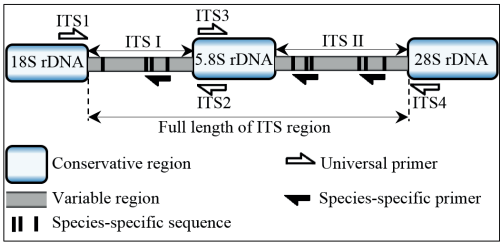

Figure 1. Primer sites within the ribosomal RNA genes of fungi (Horisawa et al., 2009; Srivastava et al., 2014)

The highly conserved 18S, 5.8S, and 28S subunits, on which universal primers are easily created, constitute the ribosomal genes. Two internal transcribed spacer regions (ITS1 and ITS2) divide them one from another, and repeated modules are connected by the intergenic spacer (IGS), these last three sequences were heavily used to distinguish different taxa because of their considerable diversity (Richard et al., 2008). In addition, compared to single gene copies, their multi-copy property is undeniably advantageous in the detection and measurement of extremely small quantities of DNA. Indeed, the sensitivity can be more crucial than a single gene copy for assays by more than 100 times (Edwards et al., 2002).

Moreover, based on the DNA sequence variety identified inside the internal transcribed spacer portions of the ribosomal DNA, several primers were created for *Aspergillus* species differentiation (Henry et al., 2000).

Several other coding regions are found interesting in fungal detection. They can help for taxonomic purposes, or molecular detection protocols of certain contaminants and pathogens (Table 1).

First of all, there is calmodulin, a calcium modulated protein present in all eukaryotic cells. Beside intracellular calcium signaling, it acts on several signaling metabolic pathways and gene expression regulation. Calmodulin encoding gene (*CaM*) is a good choice for taxonomic and phylogenetic studies due to its modest size and high degree of evolutionary conservation (Chin & Means, 2000).

Microtubules primary cytoskeletal elements present in all eukaryotic cells. The alfa and beta tubulin subunits play key roles in several vital cellular mechanisms. The α- and β-tubulin coding genes exhibit some evolutionary sequence variations between genera or species, which make them useful for molecular detection techniques (Munaut et al., 2011).

Translation elongation factors TEF1-α (or EF-Tu) and TEF2 (or EF-G) also exhibited remarkable evolutionary conservation, which makes their coding regions useful molecular differentiation of closely related species (Nouripour-sisakht et al., 2017).

The actin genes also facilitate deep-level phylogeny (Voigt & Wöstemeyer, 2000). Moreover, by analyzing various conserved regions within the genomes and combining the results, more reliable identification can be obtained.

For *Aspergillus* and *Penicillium* identification at species level, other target genes were also used. Phylogeny of *PB1* (RNA polymerase II largest subunit), *RPB2* (RNA polymerase II second largest subunit), *Tsr1* (putative ribosome biogenesis protein) and *Cct8* (putative chaperonin complex component TCP-1) genes were also employed (Hocking et al., 2006; Houbraken & Samson, 2011).

Table 1. Sequences of the most frequently used primers in fungal phylogeny studies

Locus	Gene/ region	Primer	Sequence (5'-3')	T (°C)	Fragment size (bp)	Target fungi	Reference
Internal transcribed spacer (ITS)	*ITS1-5,8S-ITS2*	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	55	565 to 613	*Aspergillus*	Henry et al., 2000; Als-huhaib et al., 2018
					~595	*Fusarium*	Zarrin et al., 2016
					~570	*Penicillium*	Demirel et al., 2013
		ITS1F ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC		574-576	*Fusarium*	Manter & Vivanco, 2007
Small Subunit (SSU, 18S) of the rRNA	*18S rRNA*	NS1 NS4	GTAGTCATATGCTTGTCTC CTTCCGTCAATTCCTTTAAG	52	~1200	all fungi	White et al., 1990
Large Subunit (LSU, 28S) of the rRNA	*28S rRNA*	LROR LR6	ACCCGCTGAACTTAAGC CGCCAGTTCTGCTTACC	52	~1200	all fungi	Vilgalys et al., 1990; Rehner & Samuels, 1995
β-tubulin	*β-tub*	βtub-F βtub-R	TGACGGGTGATTGGGATCTC CGTCCGCTTCTTCCTTGTTT	68	198	*A. fumigatus*	Serrano et al., 2011; Zarrin et al., 2017
		Tub-F Tub-R	CTCGAGCGTATGAACGTCTAC AAACCCTGGAGGCAGTCGC	60	340	*Fusarium* *Aspergillus*	Atoui et al., 2012
	Bt1 (benA)	Bt1a Bt1b	TTCCCCCGTCTCCACTTCT TCATG GACGAGATCGTTCATGTTGAACTC	55	450	*Aspergillus*	Al-Aayedi et al., 2020
					~ 540	various fungi	Glass & Donaldson, 1995
	Bt2 (tub2/BenA)	Bt2a Bt2b	GGTAACCAAATCGGTGCTGCTTTC ACCCTCAGTGTAGTGACCCTTGGC	58	~ 550	various fungi	Glass & Donaldson, 1995
					340	*F.oxysporum*	
					415-580	*Aspergillus*	Ćurčić et al., 2000
					~ 580	*Penicillium*	Yin et al., 2017
Calmodulin	*CaM*	CMD5 CMD6	CCGAGTACAAGGAGGCCTTC CCGATAGAGGTCATAACGTGG	55	475-595	*Aspergillus*	Hong et al, 2017
		CMD5 CMD6	CCGAGTACAAGGARGCCTTC CCGATRGAGGTCATRACGTGG		~ 580	all fungi	Hong et al, 2005; Yin et al., 2017
		CF1	GCCGACTCTTTGACYGARGAR	55	~ 750	*Penicillium*	Yin et al., 2017
		CF4	TTTYTGCATCATRAGYTGGAC				
Translation elongation factor 1-alpha	tef1	EF1-983F EF1-2218R	GCYCCYGGHCAYCGTGAYTTYAT ATGACACCRACRGCRACRGTYTG	66 >>56 touchdown (9 cycles) 56 (in the following 36 cycles)	~1000	all fungi	Rehner & Buckley, 2005
		EF1-1018F EF1-1620R	GAYTTCATCAAGAACATGAT GACGTTGAADCCRACRTTGTC		~600	all fungi	Stielow et al., 2015
		ef1 ef2	ATGGGTAAGGARGACAAGAC GGARGTACCAGTSATCATGTT	53	~700	*Fusarium*	Geiser et al., 2004; Maciá-Vicente et al., 2008.
RNA polymerase II subunit 1	RPB1	RPB1af RPB1cr	GARTGYCCDGGDCAYTTYGG CCNGCDATNTCRTTRTCCATRTA	52	~1200	various fungi	Stiller, & Hall, 1997; Matheny et al., 2002

Table 1. Sequences of the most frequently used primers in fungal phylogeny studies (continuation)

Locus	Gene/ region	Primer	Sequence (5'-3')	T (°C)	Fragment size (bp)	Target fungi	Reference
RNA polymerase II second largest subunit	RPB2-1	5F	GAYGAYMGWGATCAYTTYGG	61	~1000	*Penicillium*	Yin et al., 2017
		7CR	CCCATRGCTTGYTTRCCCAT				
RNA polymerase II second largest subunit	RPB2-2	5F eur	GAYGAYCGKGAYCAYTTCGG	61	~1000	*Penicillium*	Yin et al., 2017
		7CR eur	CCCATRGCYTGYTTRCCCAT				
Mini chromosome maintenance protein	MCM7	Mcm7-709for	ACIMGIGTITCVGAYGTHAARCC	56	~650	various fungi	Schmitt et al., 2009; Raja et al., 2011
		Mcm7-1348rev	GAYTTDGCIACICCIGGRTCWCCCAT				
T (°C) represents the primers' annealing temperature; R = A or G; Y = C or T; K = G or T; M = A or C; S = C or G; W = A or T; D = A, G or T; H = A, C or T; N = A, C, G or T; I=inosine.							

The ITS is considered suitable for fungal barcoding, as sequences databases are converting a wide spectrum of species, with a confident number of strains within taxon. However, for some fungal genera with a wide species range (including *Aspergillus, Fusarium,* and *Penicillium*), the ITS region is not always precise enough for species identification. Therefore, the ITS sequencing should be coupled with the analysis of other conserved coding regions, such as actin, β-tubulin, calmodulin, translation elongation factors (Raja et al., 2017).

Other research studies focused on fungal identification combine the PCR amplification of ITS region with RFLP, using different restriction enzymes to cleave the ITS amplification product.

For phylogenetic analysis, mainly for highlighting intraspecific variation, anonymous DNA sequences can also be detected through PCR. Such target sequence of the genomic DNA may or may not contain functional conserved genes (Gherbawy & Voigt, 2010). AFLP and RAPD are the main two methods for detecting these so-called anonymous DNA sequences (Vos et al., 1995; Carter & Vetrie, 2004). Most RAPD or AFLP markers were transformed into PCR markers for effective detection and identification, as they can find species-specific DNA fragments.

Identifying toxigenic fungal contaminants

Some pathogenic fungi not only they can cause considerable yield losses, but due their mycotoxin synthesis they can also compromise the quality of the harvest. The main mycotoxin-producing genera are primarily *Aspergillus, Fusarium*, and *Penicillium* (Marasas et al., 2008). Most of the toxigenic fungi can produce more than a single mycotoxin, while a given mycotoxin can also be produced by fungi belonging to different genera (Perrone & Gallo 2017).

The mycotoxigenic *Aspergillus* species are able to produce aflatoxins (B1, B2, G1, G2), ochratoxin A, and, less commonly fumonisins, gliotoxin and patulin (Munaut et al., 2011).

Fusaria are mainly producing particularly for trichothecenes and fumonisins. There are four types of trichothecenes classified from type A to D. The type A include diacetoxyscirpenol (DAS), HT-2, T-2, and neosolaniol. Type B trichothecenes are deoxynivalenol (DON), nivalenol, (NIV), and their acetylated derivatives. The type C and D trichothecenes are a minor group of toxins which are produced by other fungi than *Fusarium* species (Munkvold, 2017).

The most important mycotoxins produced by *Penicillium* species, are ochratoxin A and patulin, while at a less extent is cyclopiazonic acid (Perrone & Susca, 2017).

However, the most hazardous mycotoxins are by far the aflatoxins and DON, with highest negative impact for trade, and for the animal and human health (Munkvold, 2017).

In order to identify and characterize the aflatoxigenic molds, some genes encoding for the enzymes involved in aflatoxin production have been cloned and sequenced. Through these studies molecular markers have been

found, and primer pairs for PCR and qPCR were developed for faster identification and quantification of the contamination level (Buslyk et al., 2022). Due to the hazardous, teratogenic, mutagenic, and carcinogenic effects of aflatoxins (Sicuia et al., 2014), multiplex PCR methods were created to amplify simultaneously different genes involved in mycotoxin synthesis (Table 2).

Table 2. Specific primers for detecting mycotoxigenic fungi

Mycotoxin	Coding gene	Primer	Primer's sequence (5'to 3')	Annealing temperature (°C)	Fragment size (bp)	Reference
Aflatoxin	*AflR*	AflR660 AflR1249	CGCGCTCCCAGTCCCCTTCATT CTTGTTCCCCGAGATGACCA	59	630	Sweeney & Dobson, 1998
	AflR	APA-450 APA-1482	TATCTCCCCCCGGGCATCTCCCGG CCGTCAGACAGGCACTGGACACGG	65	1032	Schnerr et al., 2002
	*Ord*1	Ord1501 Ord2226	TTAAGGCAGCGGAATACAAG GACGCCCAAAGCCGAACACAAA	58	719	Sweeney & Dobson, 1998
	*nor*1	nor1 nor2	ACCGCTACGCCGGCACTCTCGGCAC GTTGGCCGCCAGCTTCGACACTCCG	65	400	Geisen, 1996
	*ver*1	ver1 ver2	GCCGCAGGCCGCGGAGAAAGTGGT GGGGATATACTCCCGCGACAAGCC	65	537	Geisen, 1996
	*ver*1	VER-496 VER-1391	ATGTCGGATAATCACCGTTTAGATGGC CGAAAAGCGCCACCATCCACCCCAATG	65	895	Schnerr et al., 2002
	*omt*A	Omt1 Omt2	GTGGACGGACCTAGTCCGACATCAC GTCGGCGCCACGCACTGGGTTGGGG	65	797	Geisen, 1996
	*omt*A	OMT-208 OMT-1232	GGCCCGGTTCCTTGGCTCCTAAGC CGCCCCAGTGAGACCCTTCCTCG	65	1024	Schnerr et al., 2002
Fumonisin	Fum5	Fum5F Fum5R	GTCGAGTTGTTGACCACTGCG CGTATCGTCAGCATGATGTAGC	62	845	Bluhm et al., 2002
Patulin	*IDH*	IDH-1 IDH-2	CAATGTGTCGTACTGTGCCC ACCTTCAGTCGCTGTTCCTC	52	600	Paterson et al., 2010
15-acetyl-deoxynivalenol 15A-DON	*tri3*	Tri3F971 Tri3R1679	CATCATACTCGCTCTGCTG TTRTAGTTTGCATCATTRTAG	53	708	Quarta et al., 2006
3-acetyl-deoxynivalenol (3A-DON)	*tri3*	Tri3F1325 Tri3R1679	GCATTGGCTAACACATGA TTRTAGTTTGCATCATTRTAG	53	354	Quarta et al., 2006
DON	*tri5*	3551H 4056H	ACTTTCCCACCGAGTATTTC ATCCCTCAAAAACTGCCGCT	55	525	Quarta et al., 2005
various trichothecene	*tri5*	Tr5F Tr5R	AGCGACTACAGGCTTCCCTC AAACCATCCAGTTCTCCATCG	60	544	Doohan et al., 1999
		Tox5-1 Tox5-2	GCTGCTCATCACTTTGCTCAG CTGATCTGGTCACGCTCATC	68	658	Niessen & Vogel, 1998
		HATri/F HATri/R	CAGATGGAGAACTGGATGGT GCACAAGTGCCACGTGAC	62	260	Edwards et al., 2001
	tri6	Tri6F Tri6R	CTCTTTGATCGTGTTGCGTC CTTGTGTATCCGCCTATAGTGATC	62	596	Bluhm et al., 2002
DON	*tri5-tri6*	N1-2 N2-2R	CTTGTTAAGCTAAGCGTTTT AACCCCTTTCCTATGTGTTA	55	200	Bakan et al., 2002
deoxynivalenol (DON)	*tri*7	GzTri7/f1 GzTri7/r1	GGCTTTACGACTCCTCAACAATGG AGAGCCCTGCGAAAG (C/T) ACTGGTGC	60	~161	Lee et al., 2001
nivalenol	*tri7*	Tri7F340 Tri7R965	ATCGTGTACAAGGTTTACG TTCAAGTAACGTTCGACAAT	53	625	Quarta et al., 2006
NIV	*tri*7	GzTri/fl GzTri/rl	GGCTTTACGACTCCTCAACAATGG AGAGCCCTGCGAAAG(C/T)ACTGGTGC	60	161	Lee et al., 2001

A problematic aspect regarding mycotoxin contamination is their chemical stability at high temperatures. Therefore, they are hardly destroyed by food and feed processing. But more important they are problematic due to their chronic effects. Some mycotoxins are now under regulation in several countries, while the risk related to emerging problems and/or new discovered mycotoxins requires urgent and wide investigations (Moretti et al., 2017).

Principles in mycotoxins extraction and analysis

To evaluate in desired feed stuff the mycotoxins spectrum and their level, several steps are required to be correctly performed. Sampling is the first step in collecting relevant results. Due to the uneven fungal and mycotoxin distribution within feed, proper sampling will influence the relevance of the results within the analyzed batch.

The collected samples within the same batch should be homogenized for proper accuracy, and sample division must be prepared according to the procedures to generate representative analytical samples. Grinding has an important impact on the extraction success, and it allows a better access of the solvents during the extraction of the desired compounds from the matrix (Köppen et al., 2010).

After sample preparation the desired compounds should be extracted from the matrix using appropriate solvents. Some protocols, especially designed to extract from large volume samples (kilogram) recommend to prepare the ground sample as a paste, by mixing with water, to improve the extraction when adding the solvents. Typically, organic solvents are used, sometimes diluted in acetic acid or water.

The most used solvents are chloroform, methanol (Al-Jaal et al., 2019), ethanol, benzene, acetonitrile, acetone, toluene, ethyl acetate, ethyl ether, dimethyl sulfoxide (Zhang et al., 2018), hexane, cyclohexane (Pereira et al., 2015), sometimes single or more commonly as mixtures (Agriopoulou et al., 2020).For higher sensitive detection methods, the crude extract should be cleaned-up before detection and quantitative determination (Köppen et al., 2010).

Mycotoxins can be analyzed using a variety of techniques, such as (i) spectrometry, (ii) TLC, (iii) enzyme linked immunosorbent assays (ELISA), (iv) high-performance liquid chromatography (HPLC) coupled with fluorescence detection (HPLC-FL), diode array detector (HPLC-DAD), ultraviolet and visible detector (HPLC UV-Vis), single mass spectrometry (HPLC-MS), tandem mass spectrometry (HPLC-MS/MS), thermo-spray MS (HPLC-TSP MS) (Valenta, 1998; Lawrence & Scott, 2000; Köppen et al., 2010).

Less recommended are gas chromatographic methods (GC) as mycotoxins are not volatile. Therefore, if GC is used, it must by coupled with electron capture (ECD), flame ionization (FI) or mass spectrometry (MS) (Turner et al., 2009). Moreover, by chemical derivatization of the samples, GC-MS could be performed for mycotoxin detection (Valenta, 1998).

The TLC method

The TLC is considered a popular analytical method due to its relative simplicity and affordability, high sensitivity, and fast separation time (Gololo et al., 2016). The procedure is carried out on a glass or aluminum plates, lightly covered with an adsorbent substance (e.g. silica gel or aluminum oxide), known as binding matrix. These thin coated plates are the stationary phase, while the mobile phase is the migration solution made of organic solvents. The samples should be loaded as spots on the bottom of the plate, which is then placed in to an enclosed tank loaded with the migration solution. During the TLC migration, the plates adsorb the mobile phase, through capillary action. The compounds from the migration solution are interacting with the analyte, making the substances form the samples to ascend the TLC plate at various rates (Figure 2). The compounds present in the sample are migrating at a different rate separating themselves one form each other while the migration front in ascending (De Saeger, 2011). Due to the migration variability of the extracted compounds, the mycotoxin can be detected based on their different retention factor. However, to identify the compounds, standard mycotoxins should be analyzed through the same protocol. An important factor in mycotoxin detection by TLC, is choosing the appropriate organic solvent for the samples subjected to analysis (Waksmundzka-Hajnos et al., 2008)

The Rf values are distinctive for each mycotoxin. However, they are influenced by the TLC characteristics. More precisely, the Rf value of a certain mycotoxin may differ with the extraction solvents used, type of the stationary phase, and mobile phase composition, respectively. Thus, the Rf values refer to the compounds' movement distance on the TLC plate. These values provide the critical information needed to detect the mycotoxins present in the samples, and represent the essence of TLC. The Rf is calculated as the ratio between the compounds' moving distance, and the solvent migration front. Based on this calculation algorithm the Rf values are unitless, and range from 0 to 1.

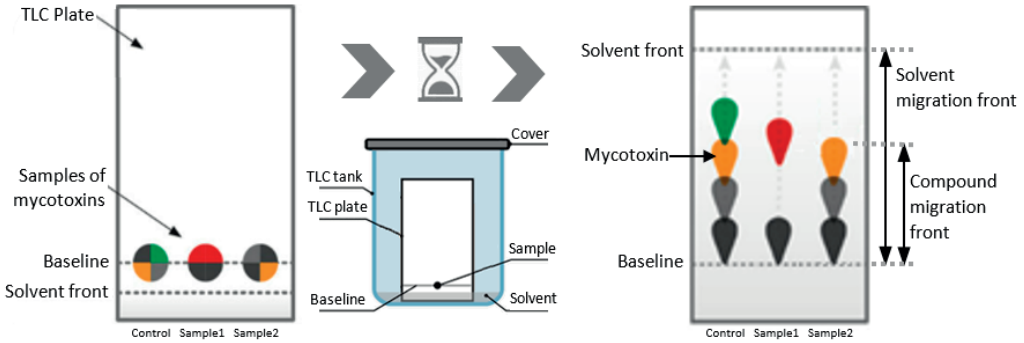

Figure 2. TLC performing steps in mycotoxin detection. The control containing standard mycotoxins and various samples are represented by the spots placed on the plate. Different colors are rebutted for each chemical component. Every component within a sample is separated from the others based on their ascent rates on the TLC plate during the solvent front migration. In the left side of the figure, the plate is represented after sample loading, while in the right side is represented after sample migration (https://www.mycotoxins.info/fileadmin/user_upload/MediaLibrary/Fig16_The-different-steps-in-the-TLC.jpg; https://theory.labster.com/tlc-procedure/)

In order to optimize a TLC protocol for mycotoxins detection it is recommended to use mycotoxin standards, which will provide the Rf values of the known compounds. Later on, the identification of the mycotoxins presence in the studied samples is more precise.

Well documented and reproducible TLC methods for *Aspergillus, Fusarium* and *Penicillium* mycotoxins are now available. The most important toxigenic compounds produced by these fungi can be separated and detected through TLC using the following solvent systems, toluene: ethyl acetate: 90% formic acid (6:6:1, v/v/v) or benzene: methanol: acetic acid (24:2:1, v/v/v) as described by Scott et al. (1970).

Aflatoxins, which are the most important *Aspergillus* mycotoxins, are well extracted in chloroform, at 4°C, overnight. An appropriate solvent mixture for TLC on silica gel plates is the 6:3:1 (v/v/v) mixture of chloroform: ethyl acetate/formic acid (Ciobotaru et al., 2014).

Similar to *Aspergillus*, *Fusarium* mycotoxin can also be extracted in chloroform (Vujanovic and Mansour, 2011). While for chromatographic elution on silica gel plates, an appropriate mobile phase is the mixture of toluene:ethyl acetate:formic acid (5:4:1, v/v/v) as described by (Ursan et al., 2018).

Mostly in mycotoxin detection, the last step in TLC evaluation of the extracted compounds, is the UV light exposure. Most compounds are better revealed under 254-365 nm UV light (Waksmundzka-Hajnos et al., 2008).

CONCLUSIONS

The unproper quality of animal feed due to mycotoxin exposure can cause acute or chronic illnesses, as well as occasionally fatalities. As a result, sensitive and precise analytical techniques and methods are required to acquire sufficient data on mycotoxin exposure levels, and to evaluate and determine the pertinent toxicological risk for both people and animals. Moreover, in order to support monitoring programs and the security of global trade, these analytical methods should also make it possible to measure such contaminants with high accuracy and precision at levels below the legal limits established by the European Union or other national or international legislation.

In order to meet performance criteria (accuracy and precision) and reliably approach the low detection limits required for risk assessment studies, a number of analytical techniques for the measurement of mycotoxins occurring in feeds have been developed and constantly refined.

The most effective methods for measuring the main known mycotoxins found in agricultural and feed commodities now is the classical methods for detecting based on chromatographic techniques such as HPLC and TLC. Furthermore, the most promising method for the simultaneous determination, evaluation and identification of many mycotoxins appears to be PCR technique.

Finally, a number of novel technologies, frequently combined with molecular methods, have been proposed for the quick analysis of mycotoxins in feed. However, more research is needed to validate these technologies and determine and evaluate whether they can be applied to real samples, particularly when mycotoxins are present at levels close to legal limits.

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TECHNOLOGIES FOR SOURDOUGH OBTAINING, FERMENTATION AND APPLICATIONS – A REVIEW

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Abstract

Recent trends are towards long-life, nutritious, preservative-free products that improve immunity, digestion and well-being and are tailored to nutritional needs and lifestyle of the consumer. In this perception, sourdough bread is part of this product category. The sourdough has a key role in obtaining a product with sensory properties such as: color, flavor, taste, texture even bread volume. The microflora is dominated by lactic acid bacteria and yeast, very important elements when we talk about the fermentation of bread dough. The factors influencing dough quality are: dough yield, temperature, microflora, environmental acidity and substrate. Dough can be classified into 3 types, of which the most used in the bakery industry is Type III. The beneficial contribution that this type of fermentation brings is not only to increase the volume of the bread, the flavor and the nutritional value, but also to extend the shelf life by inhibiting spoilage bacteria.

Key words*: lactic acid bacteria, yeast, sourdough technology, fermentation.*

INTRODUCTION

The sourdough dates back to antiquity (in the murals of the Egyptian civilization from 1500 BC). With the introduction of baker's yeast to the market (starting in the 19th century), consumer preferences shifted towards yeast bread, while in the recent years the trend has been towards sourdough bread. (https://bakerpedia.com/the-technology-of-sourdough-starters/)

The sourdough is a product obtained both in artisan bakeries and on a large scale, at the industrial level. Manufacturers supply the yeast either with starter for their own dough production or with fully fermented sourdough. Among these products are dry doughs, which are produced mainly from rye and wheat flour, but also from other plants containing starch, such as pseudocereals or legumes (Brandt, 2019).

Flour, water, salt, yeast and lactic acid bacteria cultures are needed to obtain the sourdough (Park et al., 2019). The microbiota of a certain dough can be influenced by its geographical origin, so the interpretation of research data depends on sampling, isolation and identification procedures (De Vuyst et al., 2017).

MATERIALS AND METHODS

The present paper is based on the research of the sourdough production and fermentation technologies, accessing the recently published articles on the e-nformation platform through keywords (lactic acid bacteria, yeast, sourdough technology, fermentation).

RESULTS AND DISCUSSIONS

From a microbiological perspective, the sourdough used in bakery production represents an ecosystem made up of yeasts and lactic acid bacteria. The last one has a role in the acidification of the dough, and in combination with the yeasts, help to form the aroma. Both yeasts and lactic acid bacteria of the heterofermentative type are needed for leavening.

Lactic acid bacteria (LAB) are part of the group of probiotics considered generally as safe elements (GRAS) for health with multiple benefits, such as: preventing the growth of

pathogens, immunomodulators, anticancer effects, improving the symptoms of lactose intolerance, reducing cholesterol levels, preventing and alleviating diarrheal symptoms (Kumar R. and Dhanda S., 2017; Nazir Y. et al., 2018).

During the sourdough-making process, a double-acting phenomenon occurs: the lactic acid bacteria multiply, and the pH drops with the subsequent hydrolysis of starch and proteins, favoring the growth of yeasts. At the same time, the yeast releases amino acids during autolysis, thus contributing to the growth of lactic bacteria. There is a synergistic growth of the lactic acid bacteria and yeasts used in the fermentation process, ultimately resulting in a product with a higher flavor concentration, a more elastic dough and a longer shelf life of the product (Siepmann et al, 2018).

De Vuyst et al. (2007) support the fact that the synergy between lactic acid bacteria and baker's yeast gives sourdough a more intense flavor, and other researchers, as Gerez C.L. et al. (2009) and Loponen J. et al. (2009), investigate forwards the action of lactic acid bacteria on the sourdough. According to some studies, Nionelli L. and Rizzello C.G. (2016) and Jonkuviene D. et al. (2016) support the fact that different species of lactic acid bacteria determine certain specific characteristics in bread, such as: some produce flavor, others are responsible for the production of antimicrobial compounds that prolong the aging time of bread during its storage. Moreover, lactic acid bacteria produce exopolysaccharides that cause an increase in bread volume, but also a decrease in firmness (Arendt E.K., Moroni A., Zannini E., 2011).

When we talk about dough, we can say that it has one of the ancient methods of grain fermentation (Chavan, R.S. and Chavan S.R., 2011). Getting a sourdough is within everyone's reach, as it requires a few steps: grind the grains, pseudo-grains or legumes and add the water. After that, spontaneous fermentation of the lactic bacteria present in the flour will take place. All these stages lead to the formation of the sourdough (Hammes, W.P. and Gänzle, M.G., 1998).

The dynamics and result of the dough fermentation processes (backslopped) are determined both by the flour, taking into account: its type, its quality state, etc., and by the process parameters, aiming at the fermentation temperature, pH evolution, dough yield, water activity and duration of fermentation.

Symbiotic colonies of lactic acid bacteria and yeasts present in our diverse ecosystem induce lactic acid fermentation of the dough, which eventually becomes a stable culture within a few hours (De Vuyst, L. et al., 2014).

Researchers such as Raimondi et al. (2017) performed a study in which the loss of competitiveness of a stable microbial community was described only after the addition of *S. cerevisiae.*

The variability of the number and type of microbiota in the dough depends on the native microbial flora of the environment, but also on other factors such as the choice of flour type, the time and frequency of starter's feeding, the hydration level, the leavening temperature and the fermentation time of the dough (Garofalo, C. et al., 2008; Lhomme, E. et al., 2014; Gobbetti, M. et al., 1994).

Microbial cultures give the product a unique flavor profile, thanks to a group of bacteria, such as lactic acid bacteria, that produces a similar flavor of yogurt. Apart from these, sourdough can also be fermented by acetic bacteria, which produce a similar flavor of vinegar (https://truesourdough.com/18-ways-to-make-sourdough-bread-more-or-less-sour/, accessed March 12, 2023), allowing the improvement of unsatisfactory sensory qualities, especially the aroma and taste of some gluten-free bakery products (Gobbetti et al., 2014; Moroni et al., 2009).

Due to the high temperature used during baking, products can significantly lose viable bacteria and thus become a significant challenge (Zhang et al., 2018).

Microorganisms in sourdough can affect the ecosystem and functions of the starter and by default the quality of the final product (Calvert et al., 2021).

Apart from the fact that the sourdough presents a significant potential for improving the organoleptic characteristics and nutritional quality of bakery products (especially bread), its uses have also extended to other food products, such as: biscuits, waffles, pancakes,

tortillas, muffins and noodles (Poutanen et al., 2009). Üçok and Sert (2020), studied the growth kinetics of a lactic acid bacteria species, namely *Lactobacillus plantarum* L14, naturally existing in sourdough to determine its biotechnological performance and the role of the effect on the fermentation process during dough preparation. Acidity, cell count and dough properties were analyzed. With the increase in acidity during fermentation, a coagulated and stickier dough was detected.

Belkova et al. (2021) provided comprehensive information in a study on the relationship between dough composition and the formation of processing contaminants in toast bread made from yeast wheat flour. The roasting process remarkably increased the formation of acrylamide. However, sulfur-containing compounds almost tripled the formation of acrylamide during roasting.

The year 2020 has had by far a significant impact on the trend of consumers to make sourdough bread at home, as millions of people have been restricted from leaving their homes during the Covid-19 pandemic, which has led sourdough bread to the third place in the globally top of recipes searched on the Google platform (https://trends.google.com/trends/yis/2020/GLOBAL/, accessed March 12, 2023).

One of the oldest methods of obtaining a dough turned out to be through the spontaneous fermentation of the sourdough. The technique consists in fermenting the sourdough to produce a gasier dough and, as such, an airier bread. Later, brewer's yeast was added to the sourdough fermentation (Decock and Cappelle, 2005; Spicher and Stephan, 1993), which appears to have been very successful.

Considering the technological configuration, it is possible to distinguish three methods, classified into sourdough types I, II and III.

Type I sourdough refers to traditionally made dough that requires backslopping by refreshing and adding fresh flour and water at regular intervals of time (Müller, M. et al., 2001) and the fermentation of yeasts and lactic bacteria present in the flour occurs spontaneously. The second type of sourdough is obtained by inoculating industrially adapted microbial cultures to acidify it. Type III sourdough is usually a dry form of type II, easy to store and use (De Vuyst, L.; 2017; Decock, P.; 2005; Meroth, C.B. et al., 2003). The sourdough obtaining methods, classified into sourdough types I, II and III can be observed below in the Figure 1.

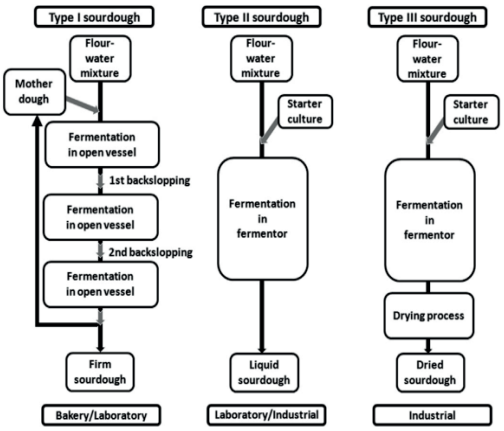

Figure 1. Types of sourdough fermentation processes according to the process technology applied (Source: https://doi.org/10.1016/bs.aambs.2017.02.003)

At the same time, some researchers considered the additional existence of another type of dough, called type 0 dough, used in the bakery industry as pre-dough or sponge dough, with the addition of baker's yeast (*Saccharomyces cerevisiae*) (De Vuyst, 2017) (Figure 2).

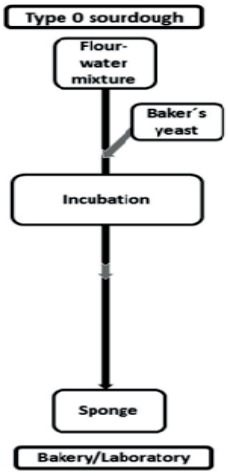

Figure 2. Type 0 dough, used in the bakery industry as pre-dough or sponge dough, with the addition of baker's yeast (Source: https://doi.org/10.1016/bs.aambs.2017.02.003)

Manufacturers in the bakery industry frequently develop type II and III dough, assuring the consumer of the quality of the product offered (Arena, M.P. et al., 2019). Type III dough brings bread an extension of the shelf life by inhibiting spoilage bacteria. Type I dough is one of the most commonly studied dough types due to its natural microbiome diversity (Plessas, 2021).

The high technology bakery industry has almost completely replaced small artisanal bakeries. Such as more efficient production technology was developed for both bread and bakery products. There has been a growing trend in demand for more flavorful bread. Thus, new businesses specialized in the production of bakery products with stabilized dough were born.

The dough fermentation produces a unique blend of flavors while improving the volume and texture of the bread. Apart from finally obtaining a product with special dough physical properties, fermentation brings bread an increased supply of nutrients, but also the extension of the shelf life (Park, D. M. et al., 2019; Katina, K. et al., 2005). The research trend is still directed towards obtaining a dough starter superior to that obtained from baker's yeast (Nionelli et al., 2018).

According to the research done by Park, D. M. et al., (2019), *Lactobacillus plantarum* SPC-SNU 72-2 proved net superior qualities in the sourdough fermentation process and was considered as a strain suitable for use in the bakery industry to obtain a bread with a slightly acidic taste.

Valerio et al. (2016) studied a fermentation product, *Lactobacillus plantarum* Bio21B. It was applied in the bread making process as a taste enhancer to obtain a yeast-leavened bread with reduced salt content (20% and 50%), compared to a reference bread (REF) that does not contain the fermentation product. Sensory analysis indicated that Bio21B bread with 50% reduced salt had a pleasant taste similar to bread containing salt (REF).

Zhou et al. (2017) had an incertitude regarding of the potential of several non-conventional yeasts and studied them as leavening agents and flavor producers under dough-like conditions in the presence of high sugar concentrations and stressful environments mimicking conditions found in the sourdough made from flour.

Hardness is an important parameter that affects the sensory quality of the bread and is a main indicator of staling during storage (Luo et al., 2018). Sensory evaluation is an important indicator, which mainly uses human senses such as: sight, smell and taste to evaluate the sensory characteristics of food, to understand people's preferences for products (Zhang, 2021).

Some researchers have come to the conclusion that the use of sourdough in the bread manufacturing process increases the feeling of satiety at the time of consumption (Zamaratskaia G., 2017). Moreover, the products obtained from cereals fermented with sourdough have a higher digestibility, compared to those fermented with yeast (Polese et al., 2018; Rizzello et al., 2019).

The composition and stability of yeast microbiota are at the basis of its applications (Table 1).

Table 1. Possible applications of sourdough lactic bacteria in bread preparation
(Source: https://doi.org/10.3390/ foods11030452)

Sourdough LAB Strains	Possible Applications
Pediococcus acidilactici LUHS29	For barley sourdough fermentation and the preparation of higher-value bread (Bartkiene, E, Vizbickiene, D. et al., 2017)
Combinations of LAB strains: *Pediococcus pentosaceus* LUHS183 and *Leuconostoc mesenteroides* LUHS242, *P. pentosaceus* LUHS183 and *Lactobacillus brevis* LUHS173, *P. pentosaceus* LUHS183 and *Enterococcus pseudoavium* LUHS234, *P. pentosaceus* LUHS183 and *Lactobacillus curvatus* LUHS51, *Lactobacillus plantarum* LUHS135 and *L. curvatus* LUHS51, *L. plantarum* LUHS135 and *P. pentosaceus* LUHS183	For wheat bread quality improving (higher porosity, better sensory properties, lower acrylamide concentration) (Bartkiene, E., Bartkevics, V. et al., 2017)
Lactobacillus coryniformis LUHS71, *L. curvatus* LUHS51, *L. farraginis* LUHS206, *Leuconostoc mesenteroides* LUHS225	For wheat bread quality improving (higher porosity, better sensory properties, lower acrylamide concentration); For surface treatment of bread to prolong the shelf life (Bartkiene, E. et al., 2019)
Pediococcus pentosaceus LUHS183, *P. acidilactici* LUHS29, *Lactobacillus paracasei* LUHS244, *Lactobacillus brevis* LUHS173, *Lactobacillus plantarum* LUHS135, *Leuconostoc mesenteroides* LUHS242	As antifungal agents against *Aspergillus nidulans*, *Penicillium funiculosum* and *Fusarium poae*; For bread safety improving (lower acrylamide concentration) (Bartkiene, E., Bartkevics, V.; Lele, V. et al., 2018)

Table 1. Possible applications of sourdough lactic bacteria in bread preparation (continuation) (Source: https://doi.org/10.3390/ foods11030452)

Sourdough LAB Strains	Possible Applications
Lactobacillus plantarum LUHS135 in combination with savory plants *Thymus vulgaris*, *Carum carvi*, *Origanum vulgare*, *Ocimum basilicum* and *Coriandrum sativum*	For bread safety improving (lower acrylamide concentration) (Bartkiene, E.; Bartkevics, V.; Krungleviciute, V. et al., 2018)
Lacticaseibacillus casei LUHS210	For almond, coconut and oat drinks by-products valorisation and added-value bread preparation (Bartkiene, E. et al., 2021)
Lactobacillus paracasei LUHS244	For okara (soybean residue) valorisation and added-value bread preparation (Juodeikiene, G. et al., 2021)

Different types of dough are known for both artisanal and industrial applications. The variety of bakery products is determined by the diversity of products obtained from sourdough.
In Europe, the use of sourdough in bakery products is quite widespread (30-50%), whereas in North America, the sourdough has less applicability. Small or medium-sized, specialized bakeries still use traditional sourdough as a leavening agent in the production of regional specialties such as: Panettone, Pumpernickel or San Francisco Sourdough Bread.
To obtain sourdough fermentation at an industrial level, semi-automatic (discontinuous) batches are carried out according to traditional fermentation procedures, incompatible with large-scale, continuous bread production (Böcker G., 2006). Therefore, sourdough fermentation is carried out by specialized suppliers in the bakery industry (Brandt M.J., 2007).
In contrast to the starter cultures used in meat and dairy fermentation, the freeze-dried ones fail to develop the metabolic activity necessary in the sourdough production process and thus require revitalization before use. When obtaining leaven, bakeries most often use stabilized, dry preparations that last a long time. They allow the new products obtained to suit the individual needs of the consumers. These preparations include ready-to-use active sourdough, dry sourdough products with added exopolysaccharides or flavor compounds derived from the Maillard reaction, and starter cultures selected to improve bread quality.
Industrial production of active dry sourdough can also be done with other types of bacteria. Bifidobacteria (Sanz-Penella J.M. et al., 2012), propionibacteria (Kariluoto S. et al., 2010), fungi and acetic acid bacteria (Haruta S. et al., 2006) grow in cereal substrates and have been used in experimental fermentations of cereals. Traditional fermentations of grains used in Africa, Asia and Latin America for the production of bread, beverages, vinegar or spices are carried out with fermentation bacteria adapted to grain substrates. The metabolic potential of these organisms differs greatly from lactic acid bacteria in yeast, and their use enables new functions for bakery products.

CONCLUSIONS

The sourdough can be obtained by three methods, classified into dough types I, II and III.
Type I dough is studied for its natural microbiome diversity, while in the bakery industry is developed frequently the type II and III dough.
Traditional sourdough is rich in microorganisms, and their composition have a great influence on the fermentation characteristics of the dough and on the appearance, taste and texture of the finished product. Lactic acid bacteria contribute to improving the dough rheology, increasing the volume and softening the texture of traditional fresh bread due to the action of the yeast to transform the soluble sugars in the dough into CO_2, alcohols, aldehydes and other substances. Lactic acid bacteria play a role in dough acidification, inhibit harmful microorganisms and react with yeast fermentation products to form aromatic substances.

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BIOETHANOL PRODUCTION THROUGH *Saccharomyces* AND Non-*Saccharomyces* YEAST STRAINS ISOLATED FROM COCOA MUCILAGE JUICE FERMENTED

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Abstract

Due to its significant contribution to lowering the use of crude oil and environmental pollution, bioethanol has been selected as the biofuel that is used the most frequently worldwide. The bioethanol was produced by fermentation carried out by microorganisms, particularly yeasts. Thus, yeasts resistance to ethanol remained a criterion important in bioethanol production. In this context, this study, the ability of Saccharomyces strain (Saccharomyces cerevisiae) and non-Saccharomyces strains (Debaryomyces hansenii, Rhodotorula mucilaginosa, and Pichia kudriavzevii) to produce bioethanol by sorghum wort fermentation following distillation was investigated. The results revealed that, globally, all yeast strains studied exhibited a similar fermentation behavior. The difference between yeast strains was observed in the alcoholic degrees of distillates. The alcoholic degree of distillates ranged between 7.01±0.007 and 7.38±0.063% (v/v) where the highest concentration has been observed with Rhodotorula mucilaginosa. More, statistical analysis showed a significant difference ($P<0.05$) between Rhodotorula mucilaginosa and other species and mixed culture. Among the strains studied, Rhodotorula mucilaginosa specie seemed the suitable strain to produce bioethanol.

Key words*: bioethanol, fermentation, non-Saccharomyces strains, Rhodotorula mucilaginosa, Saccharomyces strains*

INTRODUCTION

Environmental disasters (e.g., pollution, global warming) caused by the use of fossil fuels have resulted in the development of new, more environmentally friendly, and less energy-intensive sources (Hoekman, 2009; Demirbas, 2010; Kiran et al., 2014). Thus, bioethanol known as ethyl alcohol or chemically C_2H_5OH or EtOH remains one of the oldest environmentally friendly resources in use. Although its use and production are essentially non-existent in Africa, this is not the case everywhere else. The first commercial uses of bioethanol were initiated in Brazil in 1925. At the beginning of the 20th century, bioethanol was widely used in European countries and the United States. The growing interest in bioethanol and its adoption as an alternative by several countries was observed in the early 1980s (Azhar et al., 2017). Depending on the raw materials used, there are three generations of bioethanol. Feedstocks high in sucrose (such as sugar cane, sugar beet, sweet sorghum, and fruits) and starch (corn, wheat, rice, potato, cassava, sweet potato, and barley) were used in the production of first-generation bioethanol. Wood, straw, and grasses were examples of lignocellulosic biomass that is used to produce second-generation bioethanol. Algal biomass, which includes both microalgae and macroalgae, has been used to produce third-generation bioethanol (Nigam et al., 2011). Also food industrial waste has been reported such as fermentation medium to produce high concentration of biotehanol (Gropoşilă-Constantinescu et al., 2019). Whatever the bioethanol generation, it is produced through

the fermentation process by microorganisms. On other hand, some technical regarding microorganisms are required to optimize bioethanol production such as use of powder inoculum and microorganism encapsulation (Safitri et al., 2017).

S. cerevisiae has long been known for its fermentation performance and high resistance to ethanol and has been widely used for bioethanol production (Choi et al., 2010; Zhao et al., 2010; Mussato et al., 2012; Scordia et al., 2012; Kumari et al., 2013; Kim et al., 2014; Mossi et al., 2018). However, other authors reported the use of non-*Saccharomyces* yeast species in bioethanol production, such as *Debaryomyces hansenii* (Calahorra et al., 2009; Kurian et al., 2014); *Rhodotorula mucilaginosa* (Bura et al., 2012), and *Pichia kudriavzevii* (Ndubuisi et al., 2018; Akita et al., 2021; Pongcharoen, 2022). For bioethanol production, the microorganisms involved must require some skills namely resistance to ethanol. Although there are no universal methods for determining the resistance of microorganisms to ethanol, studies have relied on the relative values of cell growth, the specific rate of ethanol production, cell viability, and proton flux across the plasma membrane (Thomas et al., 1979; Beavan et al., 1982; Jiménez et al., 1985; Dombek et al., 1986; Birch et al., 2000). Also, bioethanol production is one most important biotechnological properties of yeasts. Finding yeast strains that can produce bioethanol is always a current concern. Thus, in this study, the ability of *Saccharomyces* and non-*Saccharomyces* yeast strains isolated and identified from cocoa mucilage juice fermented to produce bioethanol by using sorghum wort was investigated.

MATERIALS AND METHODS

Yeast strains

One *Saccharomyces cerevisiae* strain (YA5) and 3 three non-*Saccharomyces* strains (AK2; AK3; TIAS6) isolated from the cocoa juice in fermentation where the ethanol rate ranged between 7 and 10% have been taken into account in this study. All strains belonged to culture collection of the Food Technology Department (University Nangui Abrogoua, Abidjan, Côte d'Ivoire) and maintained in a 30% glycerol solution, at -20°C.

These yeast strains have been identified by Polymerase Chain Reaction Restriction Fragment Length polymorphism (PCR-RFLP) of the Internal Transcribed Spacer (ITS) region and sequencing of D1/D2 domains of rDNA sequence. The codes of strains are following : *Saccharomyces cerevisiae* (YA5), *Rhodotorula mucilaginosa* (AK2), *Debaromyces hansenii* (AK3), *Pichia kudriavzevii* (TIAS6). They were used for carrying out the alcoholic fermentation.

Fermentation conditions

Triplicate fermentations were performed with agitation in 1-L sterile Erlenmeyer flasks that contained 500 mL of pasteurised sorghum wort (10 min at 100°C) and were sealed with dense cotton plugs. A dense suspension of each specie from a YPD agar containing 10 g/L yeasts extract (Difco); 10 g/L Bacto Peptone (Becton Dickinson); 10 g/L D-glucose (Sordalab), and 10 g/L Agar (Oxford) plate was prepared in sorghum wort using a loop. The flasks containing 500 mL sterile wort were inoculated with each specie in mixed and monoculture [(*S. cerevisiae* (YA5), *R. mucilaginosa* (AK2), *D. hansenii* (AK3), *P. kudriavzevii* (TIAS6)] at O.D. 0.5 (10^7 CFU/mL) and shaken at 120 rpm for 120 h at 25 °C. Over time, samples were collected each 24 h for physicochemical and microbiology analyses. Three independent experiments were carried out.

Analytical determination

pH, titratable acidity, and total soluble solids (TSS)

After calibration with phosphate buffer, the pH of the yeast cultures during fermentation was measured using a pH meter (Hanna Instruments; HI 8010). Titration with 0.1 N NaOH was used to evaluate the titratable acidity which is expressed in lactic acid meq%. Using a hand refractometer, the total soluble solids (TSS) concentration, reported as °Brix, was determined in each sample. This latter parameter has been used to determine the sugar consumption rate. Three separate measurements were taken for every parameter.

Gas (CO_2) production during alcoholic fermentation

An experimental technique described by Lai (2010) was used in combination with fermentation tests to measure the volume of gas produced during fermentation. The fermentations performed through of were carried out in 500 mL flasks Erlenmeyer. Samples are taken regularly during alcoholic fermentation. The flasks Erlenmeyer are weighed before Weight (t-1) and after Weight (t) each sample to determine the kinetics of CO_2 production.

$$CO_2 = CO_2\,(t-1)\,\frac{\text{Weight}\,(t-1) - \text{Weight}\,(t)}{\text{Volume}(t)}$$

Volume (t) = V0-n*Vp,
where:
V0 - Volume at t = 0
N - Number of sampling
Vp - Volume of the collected sample

Microbial growth during fermentation

The method mentioned by Antunovics et al. (2005) was used to cultivate the yeast during fermentation. The increase in cell population was observed using the optical density at 600 nm. The experiments were replicated three times.

Distillation of fermented sorghum worts

The fermented worts are distilled to extract ethanol using the vigorous column distiller Quickfit/FC3/13, which measures 85 cm in length and 4.45 cm in diameter. Until all of the alcohol in the fermented must be used up in the heating flask, the temperature at the head of the column was kept at 79°C. The ethanol content was determined using an alcohol meter. Three independent experiments were carried out.

Statistical analysis

The collected data were processed using statistical analysis. XLStat software was used to do an analysis of variance (ANOVA) (version 2016). Duncan and Tukey's tests were used to assess the mean values of the physicochemical parameters of fermenting worts and fermented worts. Values of $P < 0.05$ were regarded as significant differences.

RESULTS AND DISCUSSIONS

The role of yeast involved in bioethanol and beverage production still one of the most studied biotechnological properties (Novidzro et al., 2013; Alexandre, 2014; Gbohaida et al., 2016) in contrast to their role in biofuel production (Hadiyanto et al., 2013; Tofighi et al., 2014; Mardawati et al., 2022; Saleh et al., 2022). Thus, pH and titratable acidity changes during alcoholic fermentation were shown in Figure 1.

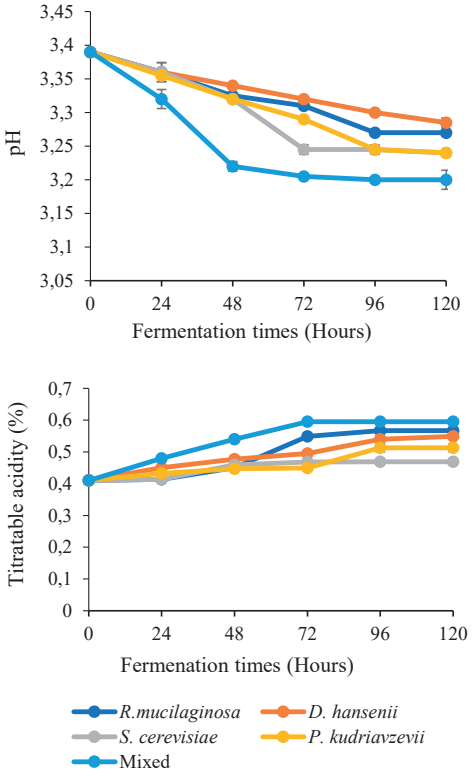

Figure 1. Changes of pH (a) and TA (b) during alcoholic fermentation of sorghum wort

During fermentation, it developed an inverse relationship between the sorghum worts fermenting pH and titratable acidity values (Figure 1). The change of the pH values was characterized by a decrease, while that of the titratable acidity increased. Thus, the mixed culture had more acidified the sorghum wort which expressed by more low value of pH 3.2 and more high value of 0.594% at the end of fermentation.

The growth performance of the yeast strains was assessed through the sugar consumption rate and the release of CO_2 during fermentation.

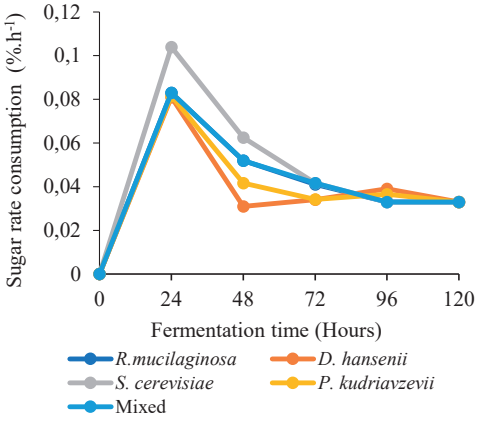

Figure 2. Sugar rate consumption during fermentation

Figure 2 showed the rate of sugar consumption by the yeast species used in this study during alcoholic fermentation. Regardless of the strain, an increase in the consumption rate was observed during the first 24 hours of fermentation, with the highest rate recorded for *S. cerevisiae* (0.104%.h^{-1}), while the rates for the other species were almost similar. After more than 24 hours, a decrease in the rate of sugar consumption was observed for the mixed culture and the *S. cerevisiae* and *R. mucilaginosa* species, from 0.083 to 0.033%.h^{-1} for the mixed culture and *R. mucilaginosa* species, respectively, after 96 hours, and from 0.104 to 0.033%.h^{-1} for the *S. cerevisiae* species after 96 hours of fermentation. From 96 h to the end of fermentation (120 h), the consumption rate remained constant at 0.033%.h^{-1}. The species *P. kurdiavzevii* was characterized by a decrease in the rate of sugar consumption from 24 h to 72 h, from 0.081 to 0.034%.h^{-1}, before a slight increase in the rate of consumption after 96 h, at 0.036%.h^{-1}. The *D. hansenii* species showed a decrease in the rate of sugar consumption from 24 to 48 h, with a slight increase in this rate between 48 and 96 h, from 0.031 to 0.039%.h^{-1}. However, at the end of fermentation (120 h), all species showed the same sugar consumption rate of 0.033%.h^{-1}. At the end of fermentation, no significant difference (P>0.05) was observed between the other samples.

CO_2 production during alcoholic fermentation by yeast species tested was presented in Figure 3.

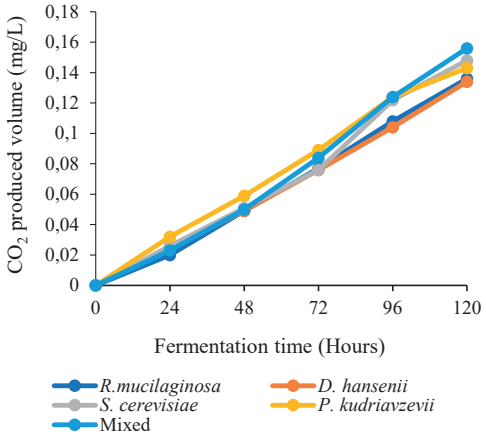

Figure 3. CO_2 produced volume (mg/L) during alcoholic fermentation

In general, a continuous increase in the volume of CO_2 released was recorded for all the yeast species tested, as well as the mixed culture. During the first 72 h of fermentation, the highest volume of CO_2 released was observed in the sorghum wort fermented by the *P. kurdiavzevii* species, increasing from 0 to 0.089 g/L. From 72 h to the end of fermentation, a more rapid increase in the volume of CO_2 released was obtained in the sorghum wort inoculated with *S. cerevisiae* species and the mixed culture. At the end of fermentation, the values for the volume of CO_2 released were 0.156 g/L for the mixed culture, 0.148 g/L for the *S. cerevisiae* species, and 0.143 g/L for the *P. kurdiavzevii* species. No significant difference (P>0.05) was observed between the other samples at the end of fermentation.

Yeast growth was determined by measuring its optical density during fermentation. Although this method is not precise enough, it does indicate the growth of the species tested. Thus, for all species tested, a continuous increase in OD values was recorded (Figure 4).

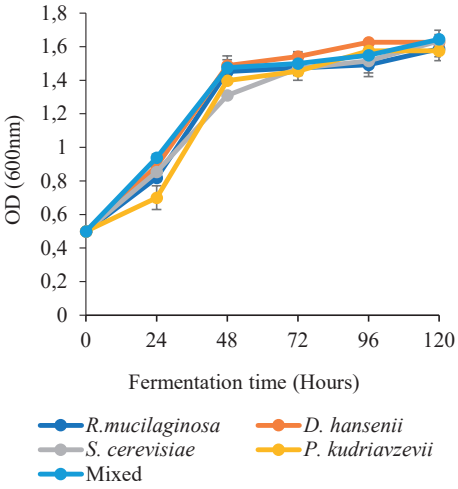

Figure 4. Yeast strains growth during alcoholic fermentation

For all the species tested, two growth phases were observed: a rapid growth phase during the first 48 hours with OD values increasing from 0.5 for all species and the mixed culture at the beginning of fermentation to 1.451; 1.487; 1.310; 1.470; 1.398; and 1.474, respectively, for the species *Rhodotorula mucilaginosa, Debaromyces hansenii, Saccharomyces cerevisiae, Pichia kurdiavzevii*, and the mixed culture. The second phase of yeast growth, which extends from 48 hours to the end of fermentation, was characterized by slower growth. The OD values evolved from 1.451 to 1.578 for *Rhodotorula mucilaginosa*; from 1.487 to 1.626 for *Debaromyces hansenii*; from 1.310 to 1.634 for *Saccharomyces cerevisiae* species; from 1.398 to 1.575 for *Pichia kurdiavzevii* cod; and from 1.474 to 1.644 for the mixed culture. Thus, this increase in OD correlated with the decrease in attenuation values and the increase in CO_2 release values. No significant difference ($P>0.05$) was observed between the other samples.

Overall, every strain exhibited remarkable fermentation performance, as seen by a continuous increase in CO_2 volume and optical density values as well as nearly comparable sugar consumption kinetics. The same characteristics have been reported by Mossi et al. (2018) and Coulibaly et al. (2021). This similarity between the different strains was also reflected in the alcohol content produced, which ranged from 7.01±0.007 to 7.12±0.084% (v/v) except *Rhodotorula mucilaginosa* where the alcohol content of distillate was 7.38±0.063% (v/v) (Figure 5).

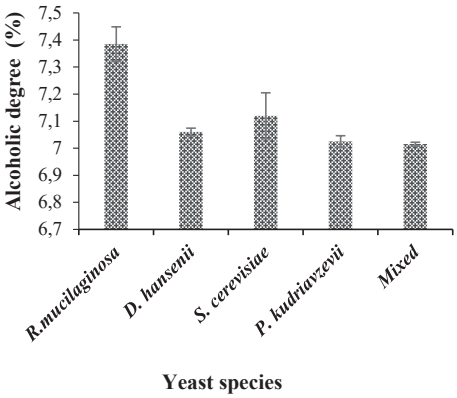

Figure 5. Alcoholic degree of distillates of yeasts isolates

Furthermore, the flasks containing the bioethanol produced are shown in Figure 6.

Figure 6. Flasks containing the bioethanol

Statistical analysis showed a significant difference between *Rhodotorula mucilaginosa* and the others strains. In our investigation, the ability of the yeast species to produce ethanol was used to assess ethanol resistance. Thus, the alcohol content of 7% v/v determined in our study was similar to that found by Yan et al. (2015), which was 7.34% (v/v), but far below the values found by Singh et al. (2013), which was 15.3% (v/v), by Ishola et al. (2015) with 37.1% (v/v), and by Moon et al. (2012) with 86.1% (v/v). This difference in alcohol content could depend on several factors, namely the ecological niche from which the yeast strain was isolated, the nature of the yeast (*Saccharomyces* and non-*Saccharomyces*), the fermentation conditions (temperature, stirring

speed, fermentation time, pH of the fermentation medium), and the raw materials (sorghum, maize, wheat, cassava, etc.) (Azhar et al., 2017). The strains used in this study were *Saccharomyces* species (*Saccharomyces cerevisiae*) and non-*Saccharomyces* species (*Debaryomyces hansenii, Rhodotorula mucilaginosa*, and *Pichia kudriavzevii*), with the fermentation conditions as follows: temperature: 25°C, stirring speed: 120 rpm, duration: 120 h and the raw material was sweet sorghum wort. Furthermore, the non-*Saccharomyces* yeast species showed the same capacity to produce ethanol as the *Saccharomyces* species and even more. Thus, the highest alcohol content was obtained with the non-*Saccharomyces* species, *Rhodotorula mucilaginosa*. For example, according to Mussato et al. (2012), the strains *Pichia stipitis* (NRRL-Y-7124) and *Kluyveromyces fagilis* (Kf1) were reported to be good ethanol producers from different types of sugars on par with the *Saccharomyces cerevisiae* species (RL-11).

Known to be an inhibitor of yeast growth and at the same time, a limiting factor for alcoholic fermentation, the mechanism of resistance to ethanol by yeast is the modification of their lipid composition to counteract these effects and avoid the permeabilization of their membranes (Stanley et al., 2010). According to You et al. (2003), adaptation to polar solvents such as ethanol results in an increase in unsaturated fatty acids and unsaturation levels. It is thought that the rise in unsaturated fatty acid content, especially C18:1 oleic acid, is a response to an adaptation to high ethanol concentrations. This allows for the stability of membrane integrity and the maintenance of metabolic stability and balance. This was also the same finding made by Coulibaly et al. (2018) when studying the resistance of yeast species to ethanol. Furthermore, other fat compounds such as ergosterol and phospholipids have also been cited as means of response to ethanolic stress (Swan et al., 1998; Inoue et al., 2000). Also, face to unable of yeast strains to produce ethanol at high concentrations, some yeast strains have been genetically modified (Alper et al., 2006). Location-specific mutagenesis has been used to create yeast capable of producing high levels of ethanol (Alper et al., 2006). The process involved changing specific words of the DNA code in a particular gene. However, the use of genetically modified microorganisms remains a controversial subject. Thus, certain non-*Saccharomyces* yeast strains were able to produce ethanol comparatively to *Saccharomyces cerevisiae* specie. *Debaryomyces hansenii* (Calahorra et al., 2009; Kurian et al., 2014); *Rhodotorula mucilaginosa* (Bura et al., 2012), and *Pichia kudriavzevii* (Ndubuisi et al., 2018; Akita et al., 2021; Pongcharoen, 2022) were used in bioethanol production.

CONCLUSIONS

Study of the ability of *Saccharomyces* yeast strain (*Saccharomyces cerevisiae*), non-*Saccharomyces* (*Debaryomyces hansenii, Rhodotorula mucilaginosa*, and *Pichia kudriavzevii*), and mixed culture (*Saccharomyces* and non-*Saccharomyces*) isolated form cocoa mucilage fermented juice, to produce bioethanol was investigated. All strains studied showed similar fermentation performances, but a difference was observed in alcohol content. Bioethanol content from different distillates ranged between 7.01±0.007 to 7.38±0.063% (v/v). The highest alcohol content was obtained with *Rhodotorula mucilaginosa* with 7.38±0.063% (v/v). The statistical analysis showed a significant difference ($P<0.05$) between *Rhodotorula mucilaginosa* and other strains and mixed culture. *Rhodotorula mucilaginosa* specie appeared as a suitable candidate to produce bioethanol. In further investigations, other raw materials from plants could be tested as substrates of fermentation.

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MOISTURIZERS MADE WITH NATURAL INGREDIENTS

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Abstract

The skin has remarkable properties of protection and defense against external aggressions, so it is very important that its health is permanently maintained. The main purpose of this research paper consisted in the formulation of two moisturizing creams based only on natural ingredients. The main ingredients were: fresh lavender flowers, aloe vera gel and coconut butter. The moisturisers were organoleptically, physicochemically and microbiologically analysed. The organoleptic tests showed that the moisturizing creams homogenized very well, did not show phase separations and the smell was very pleasant, specific to lavender. The pH values, between 5.0 and 5.3, were close to the optimal recommended, 5.5, which means that their use will have positive effects on the skin. Following the determination of the acidity index, values between 1.68 and 1.91 were obtained, which means that the products can be used safely, as they have not undergone alteration processes. Microbiological analyses showed low contamination with bacteria and fungi, as both lavender and aloe vera are known for their antibacterial and antifungal effects.

Key words: *aloe vera; lavender; moisturizing creams; skin.*

INTRODUCTION

Herbs have always been used as remedies, selected over millennia, excluding harmful ones and using beneficial ones to treat certain diseases or injuries (Maqbool et al., 2019).

Until today's chemical and biological analysis of active ingredients, plants have stood the test of time, so that now that drug abuse is one of civilisation's shortcomings, the return to natural products is a process that is increasingly encouraged (Grigore et al., 2020). In this respect, collaboration between doctors, pharmacists, chemists and biologists has led to the preparation of natural supplements and adjuvants used to prevent and treat various diseases and pathological conditions (Maqbool et al., 2019).

Since the beginning of time we enhanced our attractiveness and magnetism by primping, perfuming, and decorating our bodies. Although we no longer use crocodile manure for face masks and lead paint for whitening the skin, natural skin care has always been a priority for people (Oumeish, 2001.). Botanical extracts have remained the most important resource for healing and beautifying in the natural world, since the beginning civilization. The use of botanical extracts such as essential oils in the right amounts to handmade moisturizing creams and lotions allow for customization the products, contributing to both psychological and physiological well-being (Bijauliya et al., 2017). That's why blends that offer a combination of important botanicals in lotions, creams and ointments are gaining more and more popularity.

An important advantage of natural homemade moisturizing creams is that they can produced and customized using special ingredients for their specific properties. Homemade moisturizing creams made only with natural ingredients are full of skin-nourishing vitamins and minerals (Goldner, 1992). Their natural benefits include the ability to restore damaged skin cells, to hydrate and rejuvenate skin and to prevent future damage caused by harsh environmental factors by creating protective temporary barriers on skin (Morais, 2019). Because skin products can be labeled and stored in reusable containers, there is less waste from the disposal of packaging (Drobac et al., 2020).

There is a very large variety of moisturizers on the world market, the most popular being body lotions, face creams, face milk and body butters. They can be easily prepared at home with only

a few simple natural ingredients that basically need to be melted together and cooled before they are stored in their containers for later use. Creams and lotions are comprised of three main components: a) "base" or "carrier" oil, which will be a healing and nourishing oil of personal preference; b) water that is purified of toxins and pollutants, distilled water, pure floral waters or other water-based liquids may also be used; c) emulsifier (wax or a combination of other natural ingredients that provide emulsifying properties) (Ruiz et al., 2007).

Emulsifiers are binding agents that keep the oils and water joined together in a moisturizer, because these two components can not be combine naturaly; they are the key component for creating the fixed and lasting emulsion of oil and water (Mukherjee et al., 1999).

Lavender is one of the most popular herbs, especially for treating nervous, digestive or joint problems.

The versatility of lavender makes it the preferred ingredient for preparing topical solutions. Products that use lavender to moisturize the skin, control dryness, span all stages of a skincare routine, from cleansers, to toners and serums, and finally to body and face creams (Shahdadi et al., 2017).

The natural antimicrobial and antioxidant properties in lavender refresh and neutralize the skin, which helps eliminate bacteria and excess oil (Shahdadi et al., 2017).

Aloe vera is a plant with healing properties, used as a remedy since ancient times, with a multitude of beneficial effects for the health of the whole body. The leaves of the plant contain up to 240 active substances, with a role in the treatment of various ailments, and it is used both in the cosmetic industry and in phytotherapy (Dal'Belo et al., 2006). It has a strong moisturizing effect due to its high water content (about 95-99%) and is used as a beneficial ingredient in skin care products.

The gel extracted from this plant is a natural remedy for many ailments and has multiple beneficial properties for beauty and health, with countless uses in nutrition and care.

In the cosmetic industry it is used for skin care: moisturizing, stimulating collagen production, reducing wrinkles and acne, soothing psoriasis.

The nutrients and active substances give plants the therapeutic properties: anti-inflammatory, analgesic, antiseptic, antioxidant and antibacterial.

Coconut oil is a great natural remedy to keep skin looking younger for longer. It contains powerful antioxidants and ferulic acid that help to fade fine lines and blemishes, leaving skin feeling light and refreshed. Applied to the skin after sun exposure, coconut oil has moisturising and healing properties, reducing burns and soothing irritated skin (Nasir et al., 2018).

MATERIALS AND METHODS

The purpose of this research consisted in the formulation of two moisturizing creams based only on natural ingredients. The main ingredients were: fresh lavender flowers, aloe vera gel and coconut butter.

Both creams can be used as moisturizers, make-up removers, face or hair masks, to treat sunburns, minor wounds, eczema and psoriasis. They were prepared according to our own methods and formulas, using only natural ingredients (Tables 1 and 2):

Table 1. Ingredients used in the formulation of aloe vera moisturizing creams

Ingredient	Therapeutic role/therapeutic effect
Fresh aloe vera gel (extracted from leaves)	Antioxidant and antibacterial, prevents the formation of wrinkles.
Coconut oil	Emolient, nutritious, anti-aging
Grape seed oil	Emollient, antioxidant, nutritious
Olive oil	Emollient, nutritious

The moisturizing creams were prepared taking into account the physico-chemical properties of each ingredient.

Table 2. Ingredients used in the formulation of lavender moisturizing creams

Ingredient	Therapeutic role/therapeutic effect
Lavender flowers	Antibacterial, anti-inflammatory
Aloe vera gel	Antioxidant and antibacterial, prevents the formation of wrinkles
Coconut oil	Emolient, nutritious, anti-aging

Formulation of aloe vera moisturizing creams

Freshly harvested aloe vera, coconut butter, olive oil and grape-seed oil were used to make the aloe vera cream.

To prepare the fresh aloe vera moisturizing cream, first the aloe vera gel was obtained. Aloe vera leaves were cut into 3-5 cm pieces, then kept for 30 minutes in a bowl of water to remove the sap ("latex"). The leaves were peeled to harvest the fresh aloe gel, and the obtained pulp was blended until completely homogenized. Coconut oil, grape-seed oil and olive oil were added successively, mixing moderately after each ingredient.
The moisturizing creams with fresh aloe vera was distributed in a hermetically sealed container and stored in the refrigerator.

Formulation of lavender moisturizing creams
Freshly harvested lavender, fresh aloe vera gel and coconut butter were used to make the lavender cream.
The lavender inflorescence was first washed with water to remove impurities and soil. The flowers were dried and subjected to extraction of the active principles with coconut butter. The extraction was carried out at 90°C, for 2 hours. The fresh lavender oil was coarsely filtered, decanted, and then filtered again under heat. The aloe vera gel was added in portions over the cooled lavender oil, stirring continuously, until homogenised and a stable emulsion was obtained.
The moisturizing creams were distributed in hermetically sealed containers and stored in a cold refrigerator to avoid spoilage.
Several types of creams were prepared, varying the quantities of ingredients, as shown in Tables 3 and 4.

Table 3. Variants of aloe vera moisturizing creams

Ingredient	Quantity (g)		
	Sample 1 (AV_1)	Sample 2 (AV_2)	Sample 3 (AV_3)
Fresh aloe vera gel	70	60	80
Coconut oil	50	60	40
Grape-seed oil	10	10	10
Olive oil	10	10	10

Table 4. Variants of lavender moisturizing creams

Ingredient	Quantity (g)		
	Sample 1 (L_1)	Sample 2 (L_2)	Sample 3 (L_3)
Lavender flowers	20	20	20
Fresh aloe vera gel	70	60	80
Coconut oil	70	80	60

The quality of the moisturizing creams was assessed through a series of analyses (FDC legislation, 2011):
- Organoleptic tests (appearance, smell, colour);
- Physic-chemical analysis (pH and acidity index);
- Microbiological analyses;
- Stability analysis (physical, chemical and microbiological).

The organoleptic tests consists in the preliminary verification of the main characteristics of the moisturizing creams: appearance, color, smell, smoothness, adhesion.
They must have a homogeneous appearance, color and a pleasant smell, characteristic of the products from the composition, all these properties making the cream attractive and pleasant to administer on the skin. These characteristics must remain unchanged during storage.
The determination of pH is carried out to check whether or not the pharmaceutical preparation irritates the skin and mucous membranes. According to Romanian Pharmacopoeia 10th Edition, the pH of creams must be 4.5-8.5, as close as possible to that of the skin (Romanian Pharmacopoeia 10th Ed., 1998). The pH measurement was carried out with a pH-meter.
Determination of pH was done by the usual colorimetric or potentiometric methods in aqueous solution obtained by shaking the ointment with the required amount of water (Mukherjee et al., 1999).
5 g of product and 20 ml of distilled water are vigorously stirred for 1 minute, filtered and then the pH is determined in the filtrate.
The value of the acidity index allows to assess the quality of creams, by determining the free fatty acids that occur as a result of partial hydrolysis in the presence of microorganisms, as well as the degradative transformations of the rancidity process.
To determine the acidity index, 5 g of cream were dissolved in 50 ml of alcohol-ether mixture neutralized with phenolphthalein. The titration was done with a potassium hydroxide solution, until the pink coloration.
Microbial contamination control aims to determine the total number of aerobic microorganisms or the absence of pathogenic or conditionally pathogenic microorganisms, possibly present in pharmaceutical products,

from raw materials to finished forms. The microbiological determinations were carried out by direct seeding of the samples on culture media specific to each type of microorganism: aerobic bacteria, fungi. After incubation, plates were examined for colony counts.
The most important consideration with respect to pharmaceutical and cosmetic emulsions is the stability of the finished product. The chemical and physical characteristics, including the variation of pH and acidity index, observation of color and odor were determined (John, 1985).
Tests were performed for 4 consecutive weeks at room temperature. The measurements were made in triplicate and the average value was determined for each parameter.

RESULTS AND DISCUSSIONS

Organoleptic tests

The organoleptic tests showed that the moisturizing creams had a homogeneous appearance and a creamy consistency, adhering to the skin, pleasant color and smell, specific to the ingredients used. All these characteristics made the creams attractive and pleasant to apply on the skin.
The creams prepared from lavender were particularly noted, due to the specific smell of the plant, recognized for its special qualities.

pH tests

A balanced pH level plays a significant role in the way skin looks. A high pH level, above 7, damages the skin by drying it out, and a low pH level, below 4, irritates it.
Dry skin has a pH<5.5, with increased acidity, while oily skin is alkaline and has a pH>6. Bacteria and other micro-organisms prefer alkaline environments, which can lead to acne breakouts in oily skin, while acidic pH inhibits them but can trigger skin irritations and eczema (Hye-Yeon et al., 2018).
The pH values obtained from the testing of aloe vera moisturizing creams were between 5.2-5.4, while for lavender creams the values were slightly higher, between 5.3-5.6 (Figure 1). Values slightly lower than the optimal value of 5.5 mean that the preparations obtained will have beneficial effects on the skin and can be used effectively for moisturising or treating various conditions.

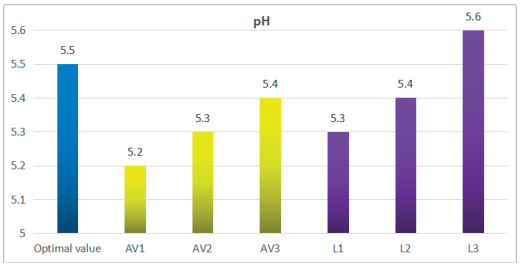

Figure 1. pH of aloe vera and lavander moisturizing creams

Acidity index

The acidity index represents the volume of potassium hydroxide required to neutralize the content of free fatty acids in the sample.
The acidity index values were between 1.71 and 1.97, for aloe vera creams, and 1.64 and 1.88, for lavender creams, which means a low content of free fatty acids (Figure 2).
Considering that these products are used for moisturizing and/or treating various skin conditions, a content as low as possible in free fatty acids is beneficial, meaning that the products can be used with confidence, since they have not undergone alteration processes.

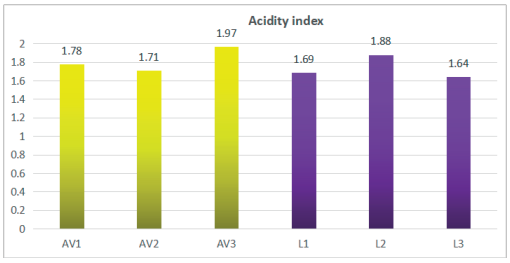

Figure 2. Acidity index of moisturizing creams

Total aerobic bacteria count

For the aloe vera moisturizing creams, a fairly high bacterial contamination was found, but within the limits of the FRX (Figure 3). This is due to the fact that the aloe vera leaves were only washed with cold water and then processed to extract the gel.
For lavender moisturizing creams, bacterial contamination was much lower compared to aloe vera moisturizing creams (Figure 3).
The reduced presence of bacteria in the prepared moisturizing creams is primarily due to the recognised antibacterial effect of lavender and aloe vera.

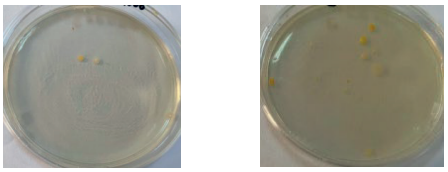

Figure 3. Contamination with aerobic bacteria of: Aloe vera moisturizing cream Lavender moisturizing cream

Total fungi count (yeasts and filamentous fungi)

Fungal contamination was extremely low, with colony growth only observed in plates inoculated with 1: 10 dilutions. For these plates a CFU/g value of 3 x 10^4 was calculated for the aloe vera cream and 2 x 10^4, for the lavender cream, as shown in Figure 4.

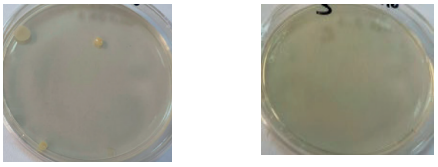

Figure 4. Contamination with fungi of: Aloe vera moisturizing cream Lavender moisturizing cream

Again, the ingredients used played an important role, as both lavender and aloe vera are well known for their antifungal effects.

Stability tests

Having a complex composition, with ingredients of varying degrees of unsaturation within wide limits and a predominantly organic structure, moisturizing creams are subject to the destructive processes of UV radiation and atmospheric oxygen and moisture.

During four weeks, the moisturizing creams were analysed four times, once a week, monitoring changes in organoleptic, physico-chemical and microbiological characteristics.

The organoleptic characteristics of the tested products did not change significantly during the test period. The organoleptic tests did not particularly highlight a special moisturizing cream, because all characteristics were maintained very well during the testing period.

The pH of samples AV_1, AV_2 and AV_3 decreased slightly over the four weeks, from 5.4 to a minimum of 5.0. The pH of sample L_1, L_2 and L_3 showed an excellent stability during the four weeks, as shown in Figures 5 and 6.

Of all six moisturizing creams, AV_3 and L_3 had the best evolutions as they maintained their pH during the entire testing period.

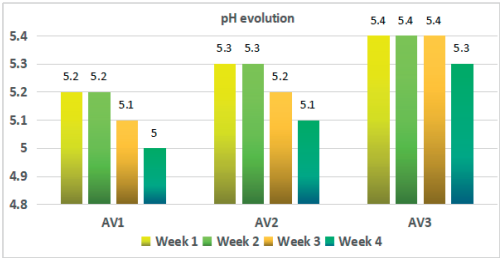

Figure 5. pH evolution of aloe vera moisturizing creams

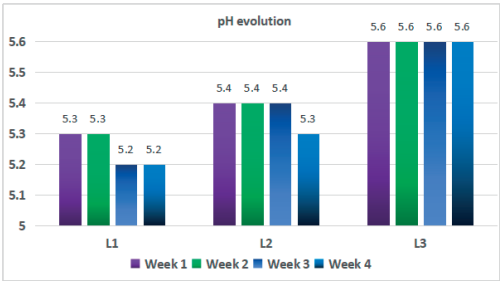

Figure 6. pH evolution of lavander moisturizing creams

The evolution of the acidity index was similar to that of the pH, it changed quite little during the stability test (Table 5). The results showed the importance of using as fresh and natural ingredients as possible.

Table 5. Variation of acidity index of moisturizing creams

Sample/ Acidity index	Week 1	Week 2	Week 3	Week 4
AV_1	1.78	1.88	1.86	2.05
AV_2	1.71	1.8	1.92	2.07
AV_3	1.97	1.93	1.95	1.98
L_1	1.69	1.74	1.79	1.82
L_2	1.88	1.89	1.86	1.90
L_3	1.64	1.65	1.68	1.71

From a microbiological point of view, the moisturizing creams proved to be valuable products, as the degree of contamination with aerobic bacteria and fungi did not increase, as expected. The main reason for inhibiting the growth of microorganisms is given by the main

ingredients, aloe vera and lavender, known for their antibacterial and antifungal effects.
Of the six moisturizing creams, AV_3 and L_3 stood out, haveing the highest content in aloe vera, proving once again the superior value of this plant.

CONCLUSIONS

All moisturizing creams have exhibited stable organoleptic characteristics.
The initial pH remained constant over time (between 5-5.6). These values, close to the skin's optimum, means that the moisturizing creams do not cause irritation when applied to the skin and have beneficial effects on the skin.
Following the analysis of the acidity index, the importance of using the freshest and most natural ingredients in obtaining these types of pharmaceutical products was highlighted.
The low contamination with aerobic bacteria and fungi further increases the value of the products, proving once again the benefits of using aloe vera and lavender as main ingredients.
The technological processes applied to obtain the creams led to the production of quality pharmaceutical products, proved by the results obtained in the verification of some physico-chemical parameters and stability tests.
Analyzing the results in this research, we selected AV_3 and L_3 creams as the most valuable and beneficial pharmaceutical products because they maintained their physical and chemical characteristics and had the least microbial contamination.

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STUDIES REGARDING ANTIOXIDANT PROPERTIES OF ANTIMICROBIAL BIOPRODUCTS FORMULATED WITH NATURAL POLYMERS

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Abstract

The antioxidant activity of antimicrobial bioproducts formulated with natural polymers most often depends on the activities of the active compounds from their composition. In the case of bioproducts with biopolymers, the antioxidant activities depend on the limonene source. In the present case, the antimicrobial activities of antimicrobial gels formulated with biopolymers were determined by the chemiluminescence method, using two sources of limonene, respectively the citrus essentials oils with 97% and respectively with 25% limonene. The results obtained from the chemiluminescence studies have shown that, if an essential oil (EO) with a high limonene content is used in the antimicrobial formulations, such as EO of Citrus sinensis (97% limonene), then the product will most often act as a prooxidant, due to the high limonene content. If the antimicrobial formulations are performed with less concentrated sources of limonene, such as the EO of bergamot (Citrus bergamia), then the resulting bioproducts, in addition to the maximized antimicrobial effect, will also act as antioxidants, the antioxidant activity obtained being 70%.

Key words*: antioxidant activities, antimicrobial gels.*

INTRODUCTION

The subproducts resulting from the food industry such as chitin, hides of cattle, and citrus peels can represent valuable raw materials, from which is possible to obtain products like hydrolyzed collagen, chitosan, or limonene, raw materials with huge applications in the pharmaceutical industry and not only. These raw materials can be functionalized with small quantities of antimicrobial reagents (quantities needed are less than 10 times in comparison with common products from the market) in order to obtain antimicrobial formulations such as gels (Mohammed et al., 2022). Formulations obviously exhibit antimicrobial activity, more or less specific, strongly influenced by the antimicrobial reagent type, respectively by synergies that can appear between the gel components. Considering that these formulations are intended for topical applications, it is important to know other properties of them. These properties, such as antioxidant/prooxidant activities, are important and obviously depend on the antioxidant/prooxidant activities of each component used in the gel formulations. According to Nurilmala et al., bioactive peptides with high antioxidant activity have a molecular weight of fewer than 10 kDa (Nurimala et al., 2020; Chen et al., 2020). Such a hydrolyzed collagen, obtained from the skin of yellowfin tuna, was investigated from the point of view of antioxidant activities with the ABTS method (2,20-azino-bis(3-ethylbenzothiazoline-6-sulphonic acids)) and DPPH (2,2 -diphenyl-1-picrylhydrazyl). The hydrolyzed collagen obtained, (with a content of 3.27% arginine, 1.78% lysine, 1.41% threonine, 0.89% phenylalanine, 0.44% leucine, 0.29% histidine, 8.85% glycine, 4.09%

proline, 3.64% alanine, 3.35% glutamate, 1.93% aspartate, 1.35% serine, and 0.17% tyrosine), exhibit antioxidant properties expressed towards ABTS (90%) and respectively towards DPPH (40%) (Nurimala et al., 2020). Chen and col. (Chen et al., 2020), in their bibliographic studies carried out, showed that only peptides with a certain sequence of amino acids show antioxidant activity (i.e: GETGPAGPAGPIGPVGARGPAGPQGPRGD KGETGEQ; DGAR; LEELEEELEGCE; GPLLGFLGPLGLS, FDSG-PAGVL; NGPLQAGQPGER; DPALATEPDPMPF).
The same authors found that the skin healing properties possess only the peptides obtained from the cattle's Achilles tendon, which contain the following sequence of amino acids: GETGPAGPAGPIGPVGARGPAGPQGPRGD KGETGEQ (Chen et al., 2020). Leon Lopez et al. (Leon-Lopez et al., 2019), found that human collagen contains 33% glycine, and 22% (proline+hydroxyproline). The human collagen has a structure of a triple helix, with three alpha chains, and 1014 amino acids in each alpha chain. This type of collagen has an average molecular weight of about 100 kD. According to the same authors, only the collagen obtained by enzymatic hydrolysis from bovine tendons and respectively from pig skins (the last one obtained by aqueous hydrolysis and ultrafiltration) have antioxidant properties and contain peptides with a molecular weight between 1-10 kD (Leon-Lopez et al., 2019).
Regarding the antioxidant properties of chitosan, Yen et al., in their research on DPPH and OH- scavenging capacity, found that the antioxidant activity of chitosan increases in direct proportion with an increasing deacetylation degree of this (Yen et al., 2008).
Gumgumjee and col. reported that if the chitin exoskeletons obtained from marine species are previously calcinated at 160^{o}C, colled, grinding, and dissolved in dimethyl sulphoxide (DMSO), then the scavenger properties for DPPH ions of these chitin solutions are higher than the chitosan solutions made with DMSO, with the chitosan obtained from the chitin of the same marine species, without calcination. These results can be due to the amino groups from the chitin macromolecules, which possess a pair of non-participating electrons, these acting as scavengers for hydrogen ions (Gumgumjee et al., 2018). The aim of this present study is to highlight the antioxidant/prooxidant properties of some raw materials or liquid formulations used in obtaining gels with antimicrobial activity.

MATERIALS AND METHODS

Antioxidant activity was studied by the chemiluminescence method after a methodology presented in detail by Zaharie et al. (Zaharie et al., 2022), using the same device and the same reagents. The raw materials used in the formulation of solid biopolymer composites used in regenerative medicine were previously obtained by Babeanu and col. (Babeanu et al., 2022) and the bioproducts type gell, with antimicrobial properties previously obtained (Mohammed et al., 2022) were subjected to analysis. It is important to highlight the fact that until now, this methodology has not been used yet for the evaluation of antioxidant/prooxidant activity of the natural biopolymers (collagen, chitosan) or for gels formulations, which include them.

RESULTS AND DISCUSSIONS

Results obtained by chemiluminescence studies reveal that in the case of collagen, antioxidant activities are 42% after 5 s, and 44%; after 60 when the collagen concentration in the chemiluminescence matrix is 50 µL/mL. At concentrations less than 25 µL colagen/mL the system become prooxidant (Figure 1).
The IC50 of collagen is 46.29 µL/mL (Table 1), and according to Nurilama and col. (Nurilama et al., 2020) this value represents a very strong antioxidant reagent when this biomaterial is used alone. The prooxidant value obtained at a concentration less than 25 µL/mL is probably due to the source of this biomaterial (hides of cattle) which does not contain a proper content of peptide with amino acids sequence favorable for antioxidant activity (Chen et al., 2020). In the case of chitosan (Figure 2), at all concentration studied biomaterials act as a pro-oxidant, at all concentrations studied. This behavior is probably due to the moderate deacetylation grade of this biomaterial and to average molecular mass, and to its structure of wrapped

coil, unfavorable for antioxidant activities (Babeanu et al., 2022). Results obtained in the case of the essential oil of *Citrus sinensis*, which contains 97% Limonene (Schroder et al, 2022), results obtained reveal antioxidant properties at 5 s, for all concentrations studied (Figure 3). After 60 s, all systems become pro-oxidant.This behavior can be due to high levels of limonene, which does not have antioxidant activities.

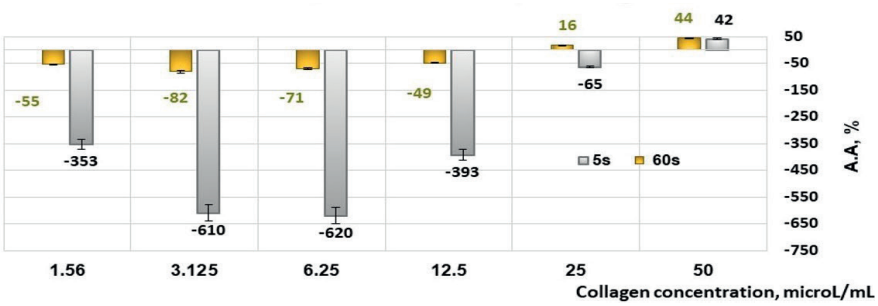

Figure 1. Antioxidant/prooxidant activity of the collagen obtained from beef hides, used as raw material for antimicrobial gels formulations

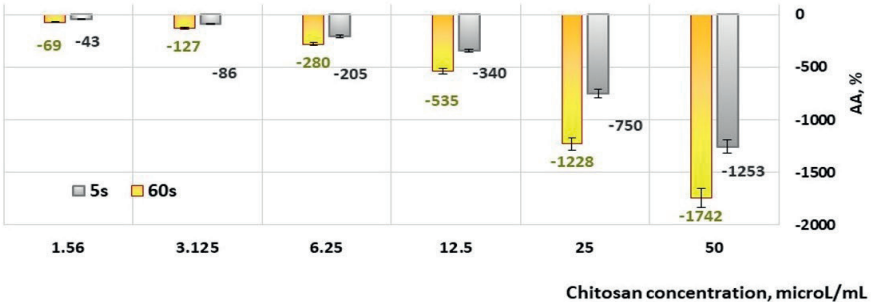

Figure 2. Prooxidant activity of the chitosan obtained from crab exoskeletons, used as raw material for antimicrobial gel formulations

The value obtained for IC5 (154.32 µL/mL) indicates that the essential oils of *Citrus sinensis* have average antioxidant activity (Nurilama et al., 2020). Lin et al. (Lin et al., 2021), in their studies with the DPPH technique, reported an IC50 ranging between (0.02-30) mg/mL, for more essential oils derived from different citrus varieties.Values obtained in the case of essential oils of *Citrus beragamia* (Figure 4, Table 1) indicate an antioxidant activity, probably due to the low content of limonene (30.41%) (Schroder et al., 2022). The value of IC50 obtained in this case (32.54 µL/mL) indicates it is a very strong antioxidant biomaterial. These observations are in agreement with the conclusions of other scientists (Sicari et al, 2016; Da Pazzo et al, 2018) which found for bergamot juice an IC50 between 8.77-17 mg GAE/g and respectively of (19.6-27.3) µL/mL, the last one measured by DPPH method. Analyses performed on the two gels with antimicrobial properties (Mohammed et al., 2022) and the chemiluminescence studies reveal the prooxidant activities for both of them (Figures 5 and 6). This behavior can be due to the fact that the properties of the final bioproducts are given by the raw materials which represent the majority, in this case, the chitosan and collagen. In addition, limonene, chitosan, and clotrimazole have prooxidant activities, and is much more probable that these raw materials imprint their properties on the end products. The antibiotic reagent used in antimicrobial formulations (an imidazole derivative named clotrimazole) does not exhibit antioxidant activities but the formulation made in the presence of plant extract or essential oil derived from plants (Elshaer et al., 2022; Carbone et al., 2019; Ouédraogo et al., 2012). Oh et al., in their studies of antioxidant

activities regarding DPPH free radical and hydroxyl free radical scavenging by a different type of chitosan, reported that the scavenger activities of chitosan increase with decreased average molecular mass of chitosan used. For example, chitosan with an average molecular mass of 3 kDa exhibits antioxidant activity greater than chitosan with an average molecular mass of 10 kDa, 20 kDa, 50 kD, or 100 kDa (Oh et al., 2019).

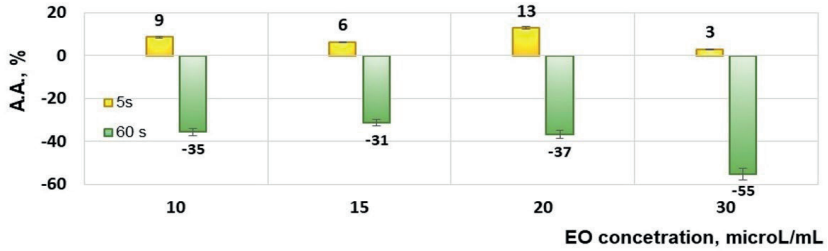

Figure 3. Antioxidant/prooxidant activity of the limonene obtained from *Citrus sinensis* (Life supplier), used as raw material for antimicrobial formulations

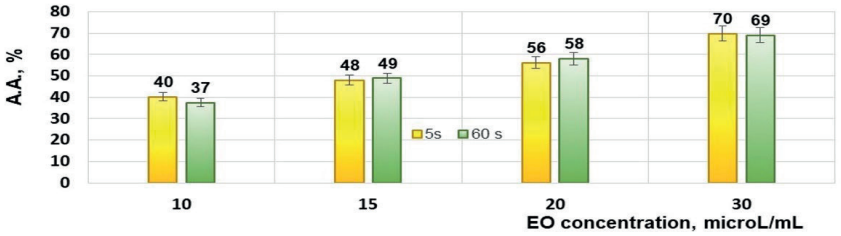

Figure 4. Antioxidant activity of the limonene obtained from *Citrus bergamia* (Life supplier), raw material in antimicrobial formulations

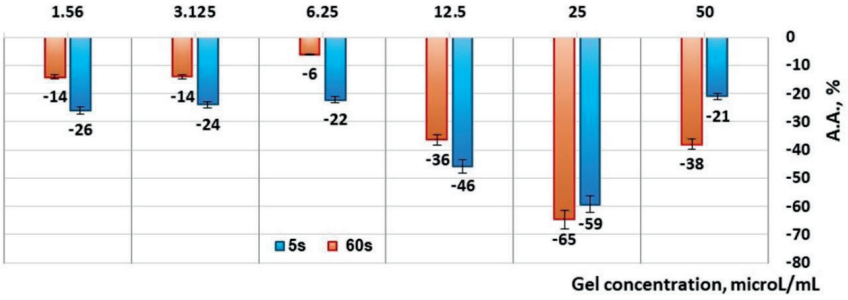

Figure 5. Prooxidant activity of the antimicrobial gel with a mass ratio of COL:CHIT:LIM:CT = 1:0:1:0.1

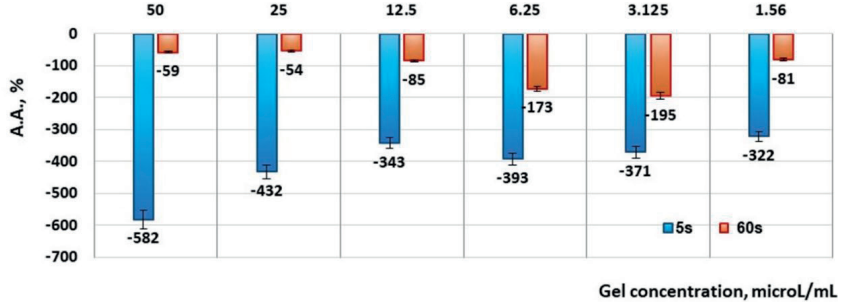

Figure 6. Prooxidant activity of the antimicrobial gel with a mass ratio of COL:CHIT:LIM:CT = 1:1:1:0.1

According to other scientists (Ivanova & Yaneva, 2020; Muthu et al., 2021) the presence of chitosan, increases the level of reactive oxygen species (ROS) inside the tumors cells, under the presence of these species the mitochondrial membrane is depolarized, is released the de cytochrome C, increase the expression of Caspase 3, the pro-inflammatories cytokines are inhibited and finally the tumor cell proliferation decrease (tumor cells have a mitochondrial redox metabolism changed).

In normal cells, (cells with normal mitochondrial redox metabolism) chitosan plays the role of ROS scavenging, increases the expression of antioxidant enzymes, preventing cell destruction due to oxidative stress (Ivanova & Yaneva, 2020; Muthu et al., 2021).

Table 1. Main characteristics of biomaterials tested by chemiluminescence for antioxidant/prooxidant activity

Product/Sources	**Biologic activity**	**IC 50** μL/mL	**Observation**	**Bibliographic sources**
Collagen (beef hides)	Decrease cytotoxicity in the magistral formula-tion; Improve tissue regeneration	46.29	Antioxidant activity at high concentra-tion. Low concen-trations act as prooxidants.	Babeanu et al., 2022; Ioan et al., 2020; Radu et al., 2016; Schroder et al., 2022; Nurilama et al., 2022
Chitosan (crabs exoskeleton)	Local antimicrobial activity for *E. coli, S. aureus*, *C. albicans*	-	Prooxidant acti-vities that increase over time	Babeanu et al., 2022; Schroder et al., 2022; Nurilama et al., 2022
Essential oil of *Citrus sinensis*	Antimicrobial activity against *E. coli* and *P. aeruginosa*	154.32	Low antioxidant activity; after 5s it becomes pro-oxidant;	Schroder et al., 2022; Nurilama et al., 2022
Essential oil of *Citrus bergamia*	High antimicrobial activity for *S. aureus; S. aureus* MRSA; *P. aeruginosa*	32.54	High antioxidant activity	Schroder et al., 2022
Bioproduct COL:CHIT:LIM:CT = 1:0:1:0.1	Local antimicrobial activity for *E. coli.* Moderate antimicrobial activity for *S. aureus.* Significant antimicro-bial activity for *S. aureus* MRSA; *C. albicans*	-	Prooxidant activities	Mohammed et al., 2022
Bioproduct COL:CHIT:LIM:CT = 1:1:1:0.1	Local antimicrobial activity for *E. coli.* Moderate antimicrobial activity for *S.aureus MRSA.* Significant anti-microbial activity for *S. aureus*; *C. albicans*	-	High prooxidant activities	Mohammed et al., 2022

IC50 = inhibition concentration of 50% of free radicals from the system. Quantification of antioxidant activities from IC 50 value, expressed in mg/mL (after Nurilama et al., 2020): IC50<0.05: very strong; 0.05≤IC50<0.1 strong; 0.1≤IC50<0.15 average; 0.15 ≤IC50<0.2 weak; IC50>0.2 wery weak

CONCLUSIONS

The antioxidant activity of some biomaterials such as collagen, chitosan, two essential oils obtained from citrus peels (*Citrus sinensis*; *Citrus bergamia*), and two gels with antimicrobial properties obtained with them have been highlighted for the first time, using the chemiluminescence method. The results obtained in the case of biomaterials used as raw materials, in gels formulations, confirmed the existing results in the specialized literature with the DPPH or ABTS method. Regarding the finished products (two gels with antimicrobial activities formulated with chitosan, collagen, clotrimazole, and limonene), the chemiluminescence studies carried out showed that they have pro-oxidant activities, properties most likely generated by major components from the system, which imprint their own properties in end products.

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LIPOSOMES WITH PLANT EXTRACTS IN THE TREATMENT OF CARDIOVASCULAR DISEASES

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Abstract

Worlwide, cardiovascular diseases (CVDs) is the leading cause of death, taking an estimated 17.8 million lives each year. CVDs include coronary heart disease, rheumatic heart disease, cerebrovascular disease and other conditions. In order to help the treatment of CVDs, this work aims to develop a food supplement based on liposomes. The market for food supplements is growing rapidly but many active ingredients have low bioavailability, especially when taken in high doses. Therefore, this paper is focused on obtaining and characterisation liposomes containing a combination of two plant extracts of Sambucus ebulus and Lycium barbarum. The preparation of liposomes was done by the technique of hydration of the lipid film. Reduction of particle size and degree of uniformity was ensured by the sonication process of the liposomal solutions. The formulations were characterised in terms of entrappment efficiency, particle size, polydispersity index, stability, and the evaluation of antioxidant activity was assessed by DPPH method.

Key words*: lipsomes, cardiovascular, Sambucus ebulus, Lycium barbarum, plant extracts.*

INTRODUCTION

Worldwide, cardiovascular diseases (CVDs) are a multifactorial condition responsible for approximately 17.5 million deaths each year. An estimated 17.9 million people died from CVDs in 2016, accounting for 31% of all deaths globally. Of these deaths, 85% occurred as a result of heart attack and stroke.The World Health Organization estimates that by 2030, this number of patients will increase to approximately 24 million (McMurray et al., 2012; W.H.O Report, 2019).

In Europe, CVDs rank first as a cause of mortality, with a higher prevalence in women (56 %) compared to men (44 %). Each year, approximately 4.40 million deaths in the member states of the World Health Organization - European Region, and approximately 2.0 million deaths in the European Union states were caused by CVDs (W.H.O Report, 2019).

According to the European Society of Cardiology, there is an increasing slope in the risk of death from CVDs in Europe, starting in western countries and reaching to the eastern countries. Therefore, Romania is included in the category of high-risk countries, immediately followed by Ukraine and Bulgaria. Thus, annually, approximately 60% of Romanians die due to a form of CVDs. Also, in terms of the mortality rate of the population due to CVDs, at the end of 2017, Romania ranked first in Europe and third in the world (approximately 109 deaths per 100,000 inhabitants, compared to the average offered by the European Union - about 44 per 100,000 inhabitants) (W.H.O Report, 2019).

Following these statistics, currently, the medical-pharmaceutical specialists places a special emphasis on the prophylaxis of CVDs, on early diagnosis, the correct treatment of these categories of patients, as well as on the long-term follow-up of patients by applying primary, secondary preventive measures and some cardiovascular recovery programs.

The market for dietary supplements for prevention of CVDs is expanding quickly, but many of the active ingredients - vitamins, minerals, and other substances - have low bioavailability, particularly when taken in high dosages.

Herbal therapeutic products are used worldwide, according to the WHO, with an estimated 80% of the world's population preferring natural treatments as the first alternative in preventing and combating various diseases. The advantages of products based on

natural compounds over conventional drugs are: a lower risk of side effects, wide availability, low cost, etc. (Sobhani et al., 2017). However, the use of natural extracts/natural compounds in biomedical applications is limited, due to the fact that they present various drawbacks, such as: toxicity, instability in very acidic pH environments, solubility and absorption problems; these inconveniences can lead to a low level of the concentration of the active principle(s) in the plasma, resulting in an ineffective therapeutic effect. To overcome these drawbacks, various innovative delivery systems (NDDS) are proposed: liposomes, micro/nano emulsions, microspheres, nanoparticles, etc. (Lacombe et al, 2017).

Particulate delivery technologies, such as liposomes, have gained attention because they can restructure a drug's *in vivo* behavior to lessen toxicity and solubilize water-insoluble active substances into nanoscale structures (Guo & Huang, 2014; Lee, 2020). Because liposomes are biocompatible enough to be allowed for parenteral administration, they have undergone the most testing of any nanoparticles (Allen & Cullis, 2013; Lee, 2020). Water-insoluble substances can be dissolved into the lipid domain of the liposomal membrane by liposomes, which are vesicles surrounded by phospholipid bilayers (Guo & Huang, 2014; Allen & Cullis, 2013; Has & Sunthar, 2019).

The structural and compositional similarity of liposomes with bio-membranes, in addition to their solubilizing ability and biocompatibility, has promoted their application for non-invasive oral delivery of poorly-permeable substances. The comparatively large size of liposomes has presented a number of challenges, including instability in the gastrointestinal tract and low permeability across the intestinal epithelia (He et al., 2019).There are numerous studies on the use of liposomes for the transport and release of natural phytoconstituents/extracts. Liposomes have been used to encapsulate plant extracts, such as: ginkgo biloba (*Ginkgo biloba*), grape seed (*Vitis vinifera*), milk thistle (*Silymarin marianum*), green tea (*Camelia sinensis*), ginseng (*Panax ginseng*), etc., or phytoconstituents, such as: vincristine, curcumin, quercitin, resveratrol (Alexander et al., 2016)

Formulation of natural extracts/natural compounds into liposomes is one of the strategies to improve bioavailability and thus potentiate the biological activity of natural extracts/compounds.

Moreover, there are numerous clinical studies for the evaluation of liposomes with natural compounds/plant extracts in various biomedical applications, such as:

a) evaluation of the therapeutic activity of liposomes with Ginkgo biloba (Agheron phytosome) - the study was conducted on patients affected by chronic venous disorders of the lower limbs (Montes et al., 2015)

b) evaluation of the effect of liposomes with Ginko biloba in the treatment of migraines (Montes et al., 2015)

c) evaluation of the release of silybin from liposomes to a target organ - study carried out on patients who previously underwent surgery to remove gallstones (Kidd & Head, 2005)

d) evaluation of the efficiency of liposomes with silybin - study carried out on patients with chronic hepatitis (Kidd and Head, 2005)

e) the effect of liposomes with green tea extract on obesity (Matsuda et al., 2016; Lin et al., 2020)

f) the effect of lipsomes with curcumin on diabetes symptoms (Antiga et al., 2015)

Therefore, this paper is focused on obtaining and characterisation liposomes containing a combination of two plant extracts of *Sambucus ebulus* and *Lycium barbarum*. The preparation of liposomes was done by the technique of hydration of the lipid film. Reduction of particle size and degree of uniformity was ensured by the sonication process of the liposomal solutions.

The formulation was characterised in terms of entrappment efficiency determined by UV-Vis spectrophotometry, particle size and polydispersity index assessed by the technique of dynamic light diffusion, and antioxidant activity evaluated by DPPH method. Also, all liposomes were characterised in terms of stability for three months.

MATERIALS AND METHODS

Materials

Phosphatidylcholine (from egg yolk), Folin-Ciocalteu reagent, 2,2-diphenyl-1-

picrylhydrazyl (DPPH), sodium carbonate, sodium cholate, Triton X-100, were purchased from Sigma-Aldrich Co (Germany).

Plant material and Lycium barbarum and Sambucus ebulus extract preparation

The mature leaves of *Lycium barbarum* (LB) and *Sambucus ebulus* (SE) were collected in Dambovita County, Romania (45°18'15.4" N; 25°23'28.4" E). The botanical team at the National Institute of Chemical-Pharmaceutical R&D (ICCF) in Bucharest, Romania, made the taxonomic identification. Based on their greater quantities of polyphenolic compounds that have a strong protection capacity against reactive oxygen species, LB and SE leaves were chosen as the vegetal material for the preparation of the extracts (Koehn & Carter, 2005; Vlachojannis et al, 2010; Zhou et al, 2017). LB and SE extracts were obtained by a technique described by Pavaloiu et al. (Pavaloiu et al, 2019; Pavaloiu et al, 2021). Therefore, an ethanolic extract of LB leaves with a polyphenol content of 18.30 ± 0.010 milligrams of gallic acid equivalents (mg GAE)/g of dry material and respectively an ethanolic extract from SE leaves with 25.50 ± 0.010 mg GAE/g dry material were combined and entrapped into liposomes.

Preparation of liposomes loaded with LB and SE extracts

The thin-film hydration method was used to produce liposomes that were loaded with SE and LB, fixed-dose (1:1 w/w) before being sonicated and extruded. In a brief, 10 mL of methanol was used to dissolve phosphatidylcholine, sodium cholate, and LB and SE extracts with various ratio (8:2:2.5; 9:1:2.5; 10:0:2.5 w/w).

The solutions were then maintained at room temperature for one night to facilitate the swelling of the phosphatidylcholine. The lipid solutions were added to a round-bottom flask and evaporated for two hours at 37°C under vacuum using a Laboranta 4000 Rotary evaporator from Heidolph Instruments GmbH & Co. KG. The resulting thin lipid layer was hydrated with distilled water at 37°C after the entire solvent had been removed. The resultant dispersions were stabilized for 2 hours at room temperature. Liposomes were sonicated, then extruded, to shrink their size. During 20 minutes, the sonication procedure was carried out in an ice-filled sonication bath (Sonorex Digital 10P, Bandelin Electronic GmbH & Co), with power delivery controlled by a 20% percentage amplitude. By utilizing 0.4 m and then 0.2 m pore size filters with 6 cycle extrusions for each, the extrusion was done sequentially. Centrifugation at 10,000 rpm and 5°C for 30 minutes was used to separate the loaded liposomes from the free extracts. The supernatant was then removed, and the sediment containing the loaded liposomes with LB and SE was re-dispersed in water. As controls, empty liposomes were prepared. Triplicates of each sample were prepared, and they were all kept at 4°C.

Table 1. Preparation of liposomes - parameters

Sample	PC: Sodium colat (w/w)	Plant Extracts (mg)	Parameters
P1	80:20	25	$t_{evaporation}$ = 37°C
P2	90:10	25	
P3	100:0	25	$t_{hydration}$ = 37°C
L1	80:20	-	
L2	90:10	-	Stirring rate = 200 rpm
L3	100:0	-	

Characterisation of liposomes loaded with LB and SE extracts

Particle size, polydispersity index (PDI), and entrappment efficiency (EE) of liposomes containing LB and SE extracts were assessed. The first step to evaluate the EE was the separation of liposomes loaded with SE and LB extracts from the free extracts combination by centrifugation (10,000 rpm at 5°C, 30 min), followed by redispersion of sediment in water. After two centrifugation cycles, the pellets were mixed with 0.5 ml Triton X-100 (0.5%) and vortexed to break up the lipid membrane. The final suspensions were diluted ten times with methanol. The Folin-Ciocalteu method, which has been described in multiple studies (Miere et al., 2019; Miere et al., 2021; Pavaloiu et al., 2019) was used to measure the EE. Samples without the Folin-Ciocalteu reagent were used as a control. Using a particle size analyzer and the Dynamic Light Scattering (DLS) method, the mean diameter and PDI of liposomes were determined (Beckman Coulter N4 PCS Submicron, Coulter Company, France). The stability of liposomes containing the combination of SE and LB extracts was

evaluated by EE assessment at different storage periods of time. The samples were kept in amber-coloured glass vials and stored at a temperature of 4°C for three months.

Radical Scavenging Activity Assessment

Using the Sanchez-Moreno et al. method, antioxidant activity was evaluated for the liposomes containing LB and SE extracts (Sanchez-Moreno et al., 1998). 2950 mL of the DPPH methanolic solution (0.025 g/L) were combined with 50 mL of each sample at various concentrations. For 30 minutes, the mixtures were maintained at 25°C in the dark. Using a UV/VIS spectrophotometer (Helios, Thermo Electron Corporation), the absorbance was determined at 517 nm. Five repetitions of the experiment for the antioxidant activity were run.

The following equation was used to calculate the percentage of DPPH inhibition:

$$\%inhibition = \frac{A_0 - A_{sample}}{A_0} \times 100$$

where A_0 is the absorbance of the control (without sample) and A_{sample} is the absorbance in the presence of the sample.

Statistical analysis

At least three replicate samples were used in each experiment, and the results were given as mean ±standard deviation (SD). At $p < 0.05$, differences were considered significant.

RESULTS AND DISCUSSIONS

Liposomes have attracted much attention as one of the most promising vehicles for the delivery of bioactive substances, owing to their remarkable properties: biocompatible, biodegradable, non-immunogenic, non-toxic, and the ability to incorporate hydrophilic and hydrophobic compounds. These unique properties therefore enable liposomes to enhance solubility, improve bioavailability, modulate pharmacokinetics, increase cellular uptake, and provide greater stability of bioactive compounds from plant extracts. Several papers showed that liposomes can enhance therapeutic activity for quercetin (Priprem et al., 2008), silymarin (El-Samaligy et al., 2006), curcumin (Sinjari et al., 2019), resveratrol (Joraholmen et al., 2015), rutin (Bonechi et al., 2018), chamomile (Das et al., 2019), etc.

Characterization of liposomes loaded with combination of SE and LB

The film hydration process, followed by sonication and extrusion, was used to produce the liposomes loaded with SE and LB extracts. The characteristics of liposomes influence their behavior *in vitro* and *in vivo*. Therefore, their characterization is an essential step in order to use them in the biomedical field. The size, PDI, EE and three-month stability of liposomes loaded with SE and LB extracts were determined. As a control, empty liposome were used. Table 2 lists the features of the liposomes.

Table 2. Features of liposomes loaded with combination of SE and LB extracts

Sample Code	Particle size (nm)	PDI
P1	196.1 ± 0.421	0.177 ± 0.001
P2	187.6 ± 0.560	0.114 ± 0.001
P3	167.6 ± 0.360	0.124 ± 0.001
L1	100.01± 0.141	0.300 ± 0.003
L2	96.76± 0.151	0.280 ± 0.003
L3	86.1± 0.211	0.298 ± 0.003

The empty liposomes had a smaller value (86.1± 0.211 - 100.01± 0.141 nm), while the encapsulation of combination of SE and LB extracts increased the particle size (167.6 ± 0.360 - 196.1 ± 0.421 nm).All liposomal formulations had particle sizes that were less than 200 nm.

A surfactant called sodium cholate may destabilize the phospholipids in an empty liposome, which would increase the size of the vesicle because of its intercalation in the phospholipid bilayer's structure (Gupta et al., 2012). The incorporation of compounds from plant extracts in the vesicle structure led to the increase of particle size following the extracts loading process. Differently polarized molecules are present in both the SE and LB extracts. Their distribution within liposomes is distinct: non-polar molecules are situated in the bilayer, and polar ones in the watery inner core, both of which lead to an increase in particle size (Castangia et al., 2015). Furthermore, the

addition of non-polar substances may result in a fluidization of the liposome membrane by disrupting the phospholipid bilayer by a deep insertion of polyphenols (Castangia et al., 2015). The EE of the combination of SE and LB extracts in liposomes was higher than 75% (reported in Figure 1). These results demonstrated that the preparation technique was effective and that there was minimal extract loss during preparation. Similar outcomes for the entrapment of *Polygonum aviculare* (EE = 83%), *Glycyrrhiza glabra* (EE = 84%), and *Artemisia arborescens* (EE = 74%) (Castangia et al., 2015; Sinico et al, 2005; Soon et al, 2015). Moreover, sodium cholate used as an "edge activator" in liposomes can improve the flexibility of the lipid layer, which leads to the incorporation of a greater amount of polyphenols (Gupta et al., 2012).

The PDI values show the homogeneity of the systems. In general, PDI values less than 0.1 indicate a homogeneous population, while values greater than 0.3 show high heterogeneity (Danaei et al., 2018). All liposomes showed a narrow size distribution (PDI < 0.3), demonstrating good homogeneity and low aggregation tendency.

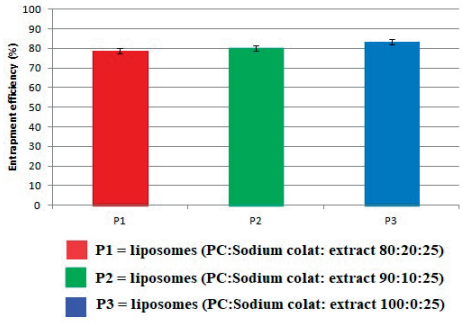

Figure 1. Values of EE of the combination of SE and LB extracts in liposomes

Antioxidant activity of liposomes loaded with SE and LB extracts

Serious health problems like cancer, cardiovascular disorders, cataracts, and diabetes are brought on by the oxidation of biomolecules (Berger, 2005). Utilizing antioxidant chemicals is one potential defense against oxidation. Due to the negative consequences of synthetic antioxidants, such as the potential for liver damage and carcinogenesis, more people are choosing to use natural antioxidants instead of synthetic ones. To guard diverse food products from oxidation, herbal extracts must be used as natural antioxidants (Komes et al., 2011). Polyphenolic chemicals, which can be utilized to stop oxidation and lengthen the shelf-life of delicate food material, are one of the most significant families of natural antioxidants (Rispail et al., 2005).

Numerous therapeutic plants contain antioxidants, primarily polyphenols, which are known as free radical scavengers and are recognised to reduce the degenerative consequences caused by oxidative stress (Forman et al., 2014; Halliwell, 2008; Vinson et al., 2001). As a by-product of aerobic respiration's mitochondrial electron transport or as a result of oxido-reductase biocatalysts and metal-catalyzed oxidation, ROS (Reactive Oxygen Species) are frequently created in a variety of reactions in living organisms (Hancock et al, 2001). Even at low doses, polyphenols can protect cells by inhibiting oxidative stress, which halts the oxidative stress-dependent molecular damage via a number of pathways (Galano et al., 2016). Due to the presence of several particular groups, specifically phenylhydroxyl groups, the major mechanisms of phenolic compounds as antioxidant agents have been documented in the literature (Galano et al., 2016; Pereira et al., 2009). Due to the way these groups behave as hydrogen donors, they have the ability to combine with reactive nitrogen species and ROS to produce a phenoxy radical (Pereira et al., 2009).

In this paper, the antioxidant activity of ethanolic extracts of LB and SE leaves was determined compared to the combination of SE and LB extracts. Also, as a control, empty liposome were used. The highest value of the antioxidant activity was presented by sample P3. Due to the lack of sodium colate in the structure of P3 liposome, there was not a competition between the plant extracts mixture and the sodium colate, therefore the EE and the ability of protection against reactive oxygen species were higher than other samples. Figure 2 shows the values of the antioxidant activity of the lipid vesicles loaded with extracts determined by the DPPH method. As controls

were used free liposomes and the combination of SE and LB extracts.

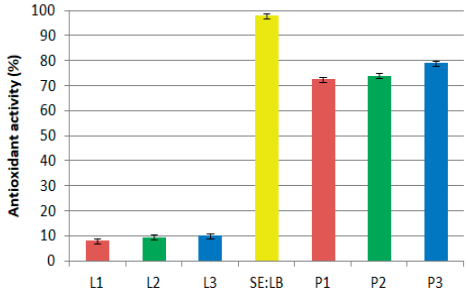

Figure 2. Antioxidant activity of liposomes loaded with SE and LB extracts (P1, P2, P3) compared to the mixture of SE and LB
L1,L2, L3 - liposomes without plant extract (PC:Sodium colat 80:20; 90:10; 100:0)
P1, P2, P3 - liposomes with plant extract (PC:Sodium colat:plant extract 80:20:25; 90:10:25;100:0:25)

Stability of liposomes loaded with combination of SE and LB

The stability of liposomes containing SE and LB extracts was investigated during a three-month period. The samples were kept in the dark at 4°C to prevent the oxidation and hydrolysis of lipids, processes caused by light. EE evaluation was used to determine the samples' stability across varied storage durations (up to three months). Liposomes containing SE and LB extracts were stable during three-month period, losing about the same amounts of plant compounds after one month (below 0.75%) and after three months (plant compunds loss below 4.50%) (Table 3). The liposomes loaded with SE and LB extracts were also visually stable because storage didn't result in any sedimentation. These findings could most likely be explained by higher Brownian motion and diffusion rates than those caused by gravitational-induced sedimentation (due to liposomes) (Gharib et al., 2017). Antioxidants with high sensitivity are polyphenols. In light of this, a number of variables, including oxygen content, an alkaline pH, and even their concentration, can weaken their antioxidant abilities (Zou et al., 2017). To prevent oxidation and degradation, increase the stability of the molecules during storage, and maintain their antioxidant action, these compounds can be encapsulated in a carrier system (Dehkharghanian et al., 2009). Lipid-based carrier systems have the most applications in the food and nutraceutical industries of all the entrapment methods now in use. These carrier systems are utilized to transfer materials to the desired place inside or outside the body while encapsulating and protecting compounds with varied solubilities.

The stability of the plant extracts against unfavorable environmental factors is increased when it is enclosed in a liposome. This effect might be brought on by high concentration of polyphenols and controlled oxygen levels, which considerably improve polyphenol stability (Bummer, 2004; Dehkharghanian et al., 2009). Because liposomes have membranes, the reaction between oxygen and polyphenols can only take place to a certain extent. Additionally, encapsulated polyphenols in liposomes degrade more slowly than free polyphenols because of their sluggish release (Bummer, 2004). The most widely used lipid-based entrapment technology, liposomes have a number of benefits, such as being made of natural materials, having the capacity to entrap bioactive materials with various solubilities, and preventing the oxidation of ingredients by free radicals, metal ions, and enzymes (Bummer, 2004).

Tabel 3. Entrapment efficiency of liposomes loaded with SE and LB for three-month period

Code	EE (%)	EE (%) 1 month	EE (%) 2 months	EE (%) 3 months
P1	78.60 ± 1.230	77.98 ± 1.030	76.50 ± 1.050	75.80 ± 1.130
P2	80.23 ± 1.761	79.89 ± 1.361	78.21 ± 1.091	77.23 ± 1.341
P3	83.43 ± 1.061	82.69 ± 1.071	81.33 ± 1.081	79.23 ± 1.231

CONCLUSIONS

Three formulations of liposomes loaded with a combination in a fixed-dose of *Sambucus ebulus* and *Lycium barbarum* with various ratio of PC:sodium colat:mixture of plant extracts (8:2:2.5; 9:1:2.5; 10:0:2.5 w/w) were obtained by the film hydration method combined with sonication and extrusion and characterized in terms of entrapment efficiency, size, polydispersity, and stability. Also, for all liposomal formulations it was investigated the antioxidant activity using the DPPH method.

Loaded liposomes presented small sizes (less than 200 nm), high entrapment efficiency (more than 75%), and a low polydispersity index. Also, all samples presented good stability over three months at 4 °C and a significant antioxidant activity. Our findings imply that liposome encapsulation might be a great method for delivering some antioxidant compounds, such as polyphenols, but more research is required.

Our results suggest that liposome encapsulation may be considered a great strategy for the delivery of plant extracts/polyphenols, but further research is needed to evaluate the cytotoxicity, the efficiency and the release profile of polyphenols from liposome formulations.

In conclusion, an innovative and alternative technology that can enhance health is liposomal technology.

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METHODS TO IMPROVE BIOAVAILABILITY OF NATURAL SENOLYTICS - A MINI REVIEW

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Abstract

Senolytics represent a group of substances that can eliminate senescent cells in several aging-related pathologies including Alzheimer`s disease, atherosclerosis, and type 2 diabetes, etc. Also, senolytic use has been proposed as a potential adjuvant approach to improve the response to senescence-inducing conventional and targeted cancer therapies. In various studies natural compounds (quercetin, fisetin, piperlongumine and curcumin) have been discovered to be effective senolytic agents. Despite the unequivocal promise of senolytics, some senolytics have low bioavailability. In this review, we summarize and discuss the latest methods to improve the bioavailability of immunomodulatory and/or immunostimulatory senolytic bioactive substances. Therefore, an in-depth discussion on diverse delivery strategies of senolytic agents and latest updates on a novel senotherapeutic research will be provided.

Key words*: bioavailability, natural compounds, senolytics.*

INTRODUCTION

Senolytics are drugs or compounds that selectively induce the death of senescent cells, which are aged or damaged cells that no longer function correctly and can contribute to aging and age-related diseases including Alzheimer`s disease, atherosclerosis, and type 2 diabetes, etc (Van Deursen, 2014). Some natural compounds have also been shown to exhibit senolytic properties, such as: quercetin, fisetin, resveratrol, piperlongumine, curcumin, etc. The biggest challenge with natural senolytics is their low bioavailability, which refers to the amount of the compound that is absorbed and available for use in the body (Boccardi & Mecocci, 2021).

Several methods to improve the bioavailability of natural senolytics were described in literature like: i) encapsulation of natural senolytics in innovative delivery systems; ii) co-administration with absorption enhancers; iii) combination with other senolytics and iv) dose optimization.

Researchers are exploring novel delivery systems for natural senolytics, like nanoparticles or conjugating the compounds with carrier molecules that can help enhance their delivery to specific tissues or organs. These approaches hold promise for improving the bioavailability and effectiveness of natural senolytics (Obeid et al., 2017; Squillaro et al, 2018). Another strategy to improve the bioavailability of natural senolytics is to co-administration the compound with absorption enhancers, which can help improve the absorption and bioavailability of the compound (Hosseini et al., 2022).

Combining natural senolytics with other senolytic compounds, such as dasatinib or navitoclax, can also help improve their bioavailability and effectiveness, as these drugs can enhance the elimination of senescent cells and increase the overall therapeutic effect (Sierra-Ramirez et al., 2020).

Optimizing the dosage and timing of natural senolytics can also improve their bioavailability. For example, taking the compound with a high-fat meal can improve its absorption, while taking it at a specific time of day or in divided doses can help maintain optimal blood levels of the compound (Rein et al., 2013).

This review presents senolytics from natural sources and highlights the methods to improve the bioavailability of senolytic substances from natural sources. Therefore, a discussion on diverse delivery strategies of natural senolytic agents and latest updates on a novel senotherapeutic research were provided.

MATERIALS AND METHODS

This review is based on secondary research sources. The search was carried out in various databases like ScienceDirect, Pubmed, Scopus, etc. in February 2023, using keywords like "senescence", "senescent cells", "senotherapy", "natural senolytics", "improve bioavaibility natural senolytics" and "senolytic delivery". The search was conducted taking into consideration various types of papers like reviews, books, research articles, online articles ahead of print.

RESULTS AND DISCUSSIONS

Senolytics from natural sources

Natural compounds that possess antioxidant and anti-inflammatory properties hold the greatest potential for promoting healthy aging and combating senescence (Gurau et al., 2018). Table 1 displays the most encouraging natural compounds for combating senescence, which have demonstrated significant reduction of senescent cells in animal models.

Table 1. Natural compounds for combating senescence

Compounds	Target	References
Tocotrienols	Multiple pathways (mTOR, NFκB, miRNAs)	Lagoumtzi & Chondrogianni, 2021; Meganathan et al., 2016;
Quercetin	Multiple pathways (mTOR, NFκB, miRNAs)	Lagoumtzi & Chondrogianni, 2021; von Kobbe, 2019; Zhang et al, 2022; Zoico et al., 2021
Curcumin	Multiple pathways (MAPK, NF-κβ, COX2, Bcl-2,cyclin B1)	Bielak-Zmijewska et al., 2019; Lagoumtzi & Chondrogianni, 2021; Li et al., 2019
Resveratrol	Multiple pathways (ROS, SASP, miRNAs)	Mamun et al., 2022; von Kobbe, 2019
Vitamin B3	mTOR	Kirkland & Tchkonia, 2020; Zhang et al., 2021; Zhou, 2021
Piperlongumine	Multiple pathways (ROS, apoptosis)	Lagoumtzi & Chondrogianni, 2021; Liu et al., 2018; Mamun et al., 2022
Fisetin	Multiple pathways (PI3K, mTOR, SASP)	Lagoumtzi & Chondrogianni, 2021; Yousefzadeh et al., 2018

BCL= B-cell lymphoma 2
COX-2 = cyclooxygenase-2
MAPK = mitogen-activated protein kinases
miRNA = micro RiboNucleic Acid
mTOR = mammalian target of rapamycin
NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells
PI3K = phosphatidylinositol 3-kinase
ROS = reactive oxygen species.
SASP = senescence associated secretory phenotype

Considerable research efforts are currently focused on identifying senolytics from natural sources that may aid in the prevention of pathological conditions, particularly age-related diseases (Barrera et al., 2021; Kirkland and Tchkonia, 2020; Vázquez et al., 2022). Tocotrienols are members of the vitamin E family, which are a group of fat-soluble antioxidants that help to protect cells from oxidative damage caused by free radicals. Unlike the more well-known vitamin E form, tocopherols, tocotrienols have an unsaturated side chain that allows them to more easily penetrate cell membranes and reach deep into fatty tissues, providing enhanced antioxidant protection. They have been shown to have a range of health benefits, including anti-inflammatory and anti-cancer effects, and are being studied for their potential to prevent or treat various age-related diseases (Mecocci et al., 2018). Quercetin can targets senescent cells and triggers their programmed cell death which can help to eliminate damaged cells that might otherwise contribute to age-related diseases. At the same time, it can delay senescence and/or promote the clearance of senescent cells in healthy tissues, which can help to maintain healthy cellular function and prevent the onset of age-related diseases. This dual and complementary action makes quercetin an attractive candidate for developing senolytic therapies aimed at preventing or treating age-related diseases (Dagher et al., 2021; Geng et al., 2018; Hwang et al., 2018; Zhu et al., 2015). Fisetin (3,3′,4′,7-tetrahydroxyflavone), a flavonoid is found in strawberries, apples, persimmons, and cucumbers, has been shown to selectively induce apoptosis in senescent cells (Zhang et al., 2018; Yousefzadeh et al., 2018). Curcumin, a compound is found in turmeric, a spice commonly used in cuisine, has been shown to have anti-inflammatory and antioxidant effects, and it may also have senolytic properties (Li et al., 2019). Resveratrol, a compound found in grapes, peanuts, and red wine, has been shown to have various anti-aging effects, including the ability to modulate cell senescence (Farhadnejad et al., 2019). B3 vitamins like nicotinamide (niacinamide), niacin (nicotinic acid), and nicotinamide riboside, found in white meat, peanuts, and mushrooms, are all precursors of nicotinamide adenine dinucleotide (NAD). NAD+ is the oxidized form of NAD and serves as an essential cofactor in various cellular pathways, including energy metabolism and

oxidative stress (Sauve, 2008). Piperlongumine is a natural compound found in Piper longum, known for its anticancer properties. Research has shown that piperlongumine has a selective effect on senescent cells, inducing their cell death (Bogan & Brenner, 2008; Wang et al., 2016; Zhang et al., 2018).

Methods to improve the bioavailability of natural senolytics

Encapsulation of senolytics in innovative delivery systems

The use of nanotechnology in natural senolytics delivery is an emerging field that shows great promise in improving the effectiveness and reducing the side effects of senolytic therapies (Squillaro et al., 2018). Nanotechnology offers several advantages in senolytic drug delivery, such as targeted delivery, controlled release, and increased cellular uptake. By designing nanocarriers with specific properties, such as size, shape, and surface charge, the delivery of senolytics can be targeted to senescent cells while minimizing damage to healthy cells. Moreover, nanocarriers can encapsulate senolytics, protecting them from degradation and enhancing their bioavailability (Obeid et al., 2017). Additionally, smart nanocarriers can be designed to release senolytics in response to specific signals or biomarkers, such as senescence-associated beta-galactosidase (SA-β-gal), which is highly expressed in senescent cells (Adamczyk-Grochala & Lewinska, 2020; Lee et al., 2006).

The initial innovative nanosystem that was developed to deliver cargo specifically to senescent cells is a mesoporous silica nanoparticle structure coated with galacto-oligosaccharides and loaded with rhodamine B, in which the nanoparticles are taken up by human senescent cells, they become activated by SA-β-gal (Agostini et al., 2012). Additionally, research has demonstrated that encapsulating a senolytic agent, navitoclax, with β (1,4)-galacto-oligosaccharides effectively removes senescent cells in models of senescence induced by damage or chemotherapy (Muñoz-Espín et al. 2018). Conjugating drugs with nanostructures can also potentially exhibit a senomorphic effect by blocking the SASP (Thapa et al., 2017; Lewinska et al., 2020). An *in vitro* study conducted recently found that nanoparticles modified with a monoclonal antibody against the CD9 receptor (which is overexpressed in aging cells) and loaded with rapamycin (a well-known mTOR inhibitor with anti-aging properties) can exhibit an anti-senescence effect (Thapa et al., 2017). Additionally, promising results have been documented for CD9-targeted PEGylated liposomes as a drug delivery system to target senescent cells. The uptake of these liposomes was found to be higher in premature senescent human dermal fibroblasts compared to young human dermal fibroblasts (Nguyen et al., 2017). Furthermore, there has been research conducted to explore the targeted delivery of rapamycin (known as a senolytic) to decrease senescence in cells that overexpress the CD9 receptor. Results indicated that rapamycin promoted cell proliferation and reduced the number of SA-β-gal-positive cells (Nguyen et al., 2017). Ke et al. (2018) showed that MoS2 NPs suppressed hydrogen-peroxide-induced senescence in endothelial cells.

All of these systems were proven effective for the delivery various senolitic substances, and could be explored for senolytics from natural sources. Furthermore, research has demonstrated that magnetite nanoparticles functionalized with quercetin (known as MNPQ) exhibit both senolytic and senostatic activity in prematurely-senescent human fibroblasts (via hydrogen peroxide treatment *in vitro*). *In vitro* experiments showed that MNPQ particles were able to eliminate senescent human fibroblast cells. MNPQ was also found to decrease the senescence-mediated proinflammatory response, as evidenced by reduced secretion of IL-8 and IFN-β, accompanied by the activation of AMP-activated protein kinase (Lewinska et al., 2020). Other innovative delivery systems include nanosuspensions, dendrimers, carbon nanotubes, polymeric micelles and lipid based nanoparticles such as liposomes, solid lipid nanoparticle, nanoemulsion and nanostructured lipid carriers are extensively reported to enhance the solubility, bioavailability of resveratrol, curcumin, quercetin, epigallocat echin gallate (EGCG), and fisetin, but were not yet explored for senotherapy (Nagesh et al.,

2019; Obeid et al., 2017; Squillaro et al., 2018). Overall, the use of nanotechnology in senolytic drug delivery holds tremendous potential in improving the efficacy and safety of senolytic therapies and could pave the way for new treatments for ageing and age-related diseases.

Co-administration with absorption enhancers

Co-administration of natural senolytics with absorption enhancers has been proposed as a potential strategy to improve the bioavailability and efficacy of these compounds. Absorption enhancers are compounds that increase the absorption of other substances across biological membranes. Studies have shown that absorption enhancers can increase the bioavailability and pharmacological activity of natural senolytics (Hosseini et al., 2022). For example, the natural senolytic fisetin has low bioavailability due to its limited absorption and rapid elimination from the body. However, co-administration of fisetin with quercitin has been shown to increase its bioavailability (Hosseini et al., 2022), that has the potential to improved efficacy in eliminating senescent cells. Similarly, another study showed that co-administration of the natural senolytic quercetin with the absorption enhancer piperine increased the bioavailability and therapeutic effects of quercetin in age-related diseases (Sharma et al., 2020). Therefore, co-administration of natural senolytics with absorption enhancers has the potential to be a promising approach to improve the efficacy of natural senolytics in eliminating senescent cells,

Combination with other senolytics

The combination of natural senolytics with other senolytics is an emerging strategy to increase the effectiveness of senotherapy in the treatment of age-related diseases.

Studies have shown that combining natural senolytics with other senolytics can increase their senolytic activity and improve their therapeutic effects. For example, the combination of the natural senolytic fisetin with the senolytic dasatinib has been shown to improve the clearance of senescent cells in vitro and in vivo, resulting in improved healthspan and lifespan in animal models of age-related diseases (Colman et al., 2020). Similarly, the combination of the natural senolytic quercetin with the senolytic navitoclax has been shown to have synergistic effects in eliminating senescent cells and improving the healthspan in mouse models of age-related diseases (Sierra-Ramirez et al., 2020). The combination of senolytic dasatinib and quercetin has been extensively studied and has been shown to eliminate senescent cells in both human and animal models (Xu et al, 2018; Zhu et al., 2015). While the combination of dasatinib and quercetin is not FDA-approved for this indication at the present, it has been tested/proposed in several clinical trials for the treatment of various age-related diseases, such as frailty, mild cognitive impairment and Alzheimer's disease (Boccardi & Mecocci, 2021). Overall, combining natural senolytics with other senolytics may represent a suitable approach for enhancing the effectiveness of senotherapics in the treatment of age-related diseases. However, more research is needed to optimize the dosages and combinations of natural senolytics and other senolytics, as well as to assess their safety and potential side effects.

Dose optimization

Optimizing the dosage and timing of natural senolytics is an important consideration for improving bioavailability and efficacy of natural senolytics. There are several factors to consider when determining the optimal dosage and timing of natural senolytics. One factor is absorption, which can be affected by factors such as food intake. For example, some natural senolytics have poor solubility and limited bioavailability, which can be improved by taking them with a high-fat meal. The presence of fat in the meal can increase the absorption of these compounds by enhancing their solubility and uptake in the intestines (Rein et al., 2013). Another factor is the timing of administration, which can influence the pharmacokinetics and bioavailability of the compound. For example, some natural senolytics have a short half-life and may require frequent dosing to maintain effective blood levels. Therefore, optimizing the dosage and timing of natural senolytics can improve their bioavailability and therapeutic effects, and may have important clinical implications for the treatment of age-related diseases. However, it is important to note that

the optimal dosages and timing of natural senolytics may vary depending on the specific compound, individual patient factors, and the disease being treated, and should be determined on a case-by-case basis (Rein et al., 2013).

CONCLUSIONS

This review presents senolytics from natural sources and highlights the methods to improve their bioavailability like encapsulation in innovative delivery systems, co-administration with absorption enhancers, combination with other senolytics and dose optimization. Overall, these methods have shown promise in improving the bioavailability of natural senolytics, which could lead to enhanced therapeutic efficacy and improved health outcomes.

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