University of Agronomic Sciences
and Veterinary Medicine of Bucharest
Faculty of Biotechnology

SCIENTIFIC BULLETIN

SERIES F. BIOTECHNOLOGIES

Volume XXVI, No. 1

2022
Bucharest

SCIENTIFIC BULLETIN
SERIES F. BIOTECHNOLOGIES
VOLUME XXVI, NO. 1, 2022

University of Agronomic Sciences
and Veterinary Medicine of Bucharest
Faculty of Biotechnology

SCIENTIFIC BULLETIN

SERIES F. BIOTECHNOLOGIES

Volume XXVI, No. 1

2022
BUCHAREST

PUBLISHERS:
University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania – Faculty of Biotechnology
Address: 59 Mărăști Blvd., District 1, Zip code 011464, Bucharest, Romania,
Phone: + 40 21 318 25 64, Fax: +40 21 318 28 88,
E-mail: florentina.matei@biotechnologii.usamv.ro, Webpage: http://biotechnologyjournal.usamv.ro

CERES Publishing House
Address: 29 Oastei Street, District 1, Bucharest, Romania
Phone: + 40 21 317 90 23, E-mail: edituraceres@yahoo.com, Webpage: www.editura-ceres.ro

To be cited: Scientific Bulletin. Series F. Biotechnologies, Volume XXVI, No. 1, 2022

ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

International Database Indexing: COPERNICUS, CABI, GOOGLE SCHOLAR, DOAJ, Scipio, PBN (Polish Scholarly Bibliography), OCLC, Research Bible, Cite Factor (Academic Scientific Journals), Universal Impact Factor

SUMMARY

SUBSECTION AGRICULTURAL BIOTECHNOLOGY

SUBSECTION FOOD BIOTECHNOLOGY

SUBSECTION MEDICAL AND PHARMACEUTICAL BIOTECHNOLOGY

SUBSECTION MISCELLANEOUS

AGRICULTURAL BIOTECHNOLOGY

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

ECO-EFFICIENCY AND TECHNO-ECONOMIC ANALYSIS OF *TRICHODERMA* BASED PLANT BIOSTIMULANT UTILISATION ON TOMATOES CULTIVATED IN A CONSERVATION FARMING SYSTEM

Valentin ZAMFIROPOL-CRISTEA[1], Iulia RĂUT[2], Florin OANCEA[2, 1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania
[2] National Institute for Research & Development in Chemistry and Petrochemistry - ICECHIM, 202 Splaiul Independenței, District 6, Bucharest, Romania

Corresponding author email: florin.oancea@icechim.ro

Abstract

Conservation agriculture is a farming system that includes no-tillage and coverage of the soil with plant residues. Despite many advantages, there are also drawbacks of such conservative systems. Plant residues promote the development of soil-borne pathogens and delay early crop development stages. Bioproducts based on microbial plant biostimulant strains were applied to compensate for the drawbacks of the conservation farming system. This paper evaluates the eco-efficiency and the economic benefits of using plant biostimulant Trichoderma strains as a plant residues treatment. To determine eco-efficiency, we used a Life-Cycle Analysis approach. We calculate the gross margin based on average yields and available statistical costs for outputs (tomatoes) and inputs for economic benefits estimation. The application of Trichoderma-based plant biostimulant bioproducts significantly increased the yield by 16.03%. The greenhouse gas (GHGs) production calculation reveals that the biostimulant application to plant residues reduced GHGs emissions per production unit. The yield increase compensates for the additional costs of the bioproducts. The gross margin is higher in the conservation farming system, which utilizes Trichoderma plant biostimulants.

Key words*: conservation farming, plant residues, Trichoderma-based plant biostimulants, eco-efficiency, gross-margin.*

INTRODUCTION

Industrial vegetables, produced in intensive field-grown systems, have a low phytonutrient content, a high risk of contamination with agrochemicals residues (pesticides, nitrates/ nitrites), and are less attractive for the consumers to their lower organoleptic characteristics (García-Mier *et al.*, 2013). Intensive monocultures reduce biodiversity due to weeding and high chemical inputs (Frison *et al.*, 2011).

The conditions for field-grown tomatoes in Romania are similar to those from semi-arid Mediterranean areas (Alexe *et al.*, 2015; Ronga *et al.*, 2017). From May till August, the growing season is characterized by low precipitation (well below 200 mm in the last decade) and high temperature - reaching 40°C (Paltineanu *et al.*, 2007). In such conditions, field-grown tomatoes are exposed to water and heat stress in the critical phenological phases (Voican *et al.*, 1995). The farming system is characterized by a massive application of agricultural inputs - water (up to 600 m^3ha^{-1}), fertilizers, and plant protection products (Dima *et al.*, 2020).

To compensate for these drawbacks, sustainable and low-input systems were proposed. Organic farming reduces the utilization of chemical inputs and increases the vegetables' edible yield (Tuomisto *et al.*, 2012). However, the cost of organic vegetable production is high, and the environmental impact per unit of product is sometimes higher than in intensive agriculture (Ronga *et al.*, 2019). A typical example is phosphorus eutrophication of the continental water bodies, promoted by the extensive use of organic fertilizers in organic farming. To ensure proper nitrogen fertilization, an excess of phosphorus is introduced into the soil, negatively impacting eutrophication (Möller *et al.*, 2018).

A low-input, sustainable production system for fresh-marked tomatoes and other vegetables was developed with cover crop mulches (Abdul-Baki *et al.*, 2002; Teasdale & Abdul-Baki, 1997). This system involves using winter annual legume hairy vetch (HV; *Vicia villosa* L. Roth) both as a cover crop and as a mulch source for

vegetable transplants cultivation (Campiglia *et al.*, 2010). It was shown to reduce soil losses, maintain high soil fertility, lower production costs, and maintain yield and product quality (Abdul-Baki *et al.*, 1996).

When is used as a cover crop, the winter hairy vetch simulates nitrogen fixation and nutrient recycling, reduces soil erosion and compaction, and supplements organic soil matter (Butler *et al.*, 2016; Muchanga *et al.*, 2020). When the cover crop is converted into mulch, plant residues covering the soil reduce weed seed germination, increases the nitrogen content in the soil, reduces water loss, and acts as a controlled-release growth-promoting pool - nutrients for cultivated plants and biologically active compounds that modulate agronomically valuable physiological processes (Massantini *et al.*, 2021).

It was demonstrated that when the tomatoes plants are cultivated in this HV farming plant, the expression of several genes related to nitrogen assimilation and ethylene signaling is up-regulated compared to those grown on black polyethylene (BP) mulch (Kumar *et al.*, 2004). HV farming system enables a metabolic system in tomatoes somewhat akin to higher polyamine-accumulating transgenic fruit with higher phytonutrient content (Neelam *et al.*, 2008). The positive responses of tomatoes to a hairy vetch cover crop observed in the field seem to be mediated by physiological cues other than the additional N provided by the vetch cover crop (Fatima *et al.*, 2016; Fatima *et al.*, 2012). Our hypothesis was that the polyamines released from the HV mulch modulate microbiome and plant physiology (Oancea, 2011)

The HV farming system has several drawbacks: (*i*) stimulation by plant residues of the soil-borne plant pathogens (Kerdraon *et al.*, 2019; Van Agtmaal *et al.*, 2017); (*ii*) lower soil temperature compared to bare soil, which could affect the development of the vegetables transplants during springs (Hobbs *et al.*, 2008); (*iii*) reduced bioavailability of nitrogen due to the increase of soil carbon pool (Ranaivoson *et al.*, 2017); (*iv*) low mechanical stability of vegetable mulch, which reduces the weeding ability (Sicuia *et al.*, 2011).

To compensate for these negative aspects of the high residues vegetable farming system, our group proposed using hydrogelified and film-forming formulation of microbial plant biostimulants based on *Trichoderma* (Oancea *et al.*, 2017). *Trichoderma* antagonize the development of the soil-borne plant pathogens (Hewedy *et al.*, 2020) and activate tomatoes plant defense against various foliar pathogens (Fernández *et al.*, 2014; Gomes *et al.*, 2017), aphids (Coppola *et al.*, 2019), and nematodes (Poveda *et al.*, 2020). Due to their production of bioactive compounds, *Trichoderma* increases nutrient uptake and nutrient use efficiency and stimulates plant growth (López-Bucio *et al.*, 2015). The tackifier and the film-forming adhesives increase mulch mechanical stability and weeding efficiency (Oancea *et al.*, 2016).

This paper aims to evaluate eco-efficiency through a life cycle impact assessment targeted on carbon footprint/climate change impact and the economic benefits of using plant biostimulant *Trichoderma* strains as a plant residues treatment.

MATERIALS AND METHODS

Study area, farming system, and yield. This study focuses on field-grown tomatoes in the Romanian plain, in the following farming system: bare-soil intensive system, HV- mulch system, *Trichoderma* plant biostimulant + HV mulch system. The yields used in this study are those reported already in our previous studies (Oancea *et al.*, 2017; Sicuia *et al.*, 2011), ranging from 67.5 to 82.3 tones.ha^{-1}.

System description. The system boundary of the Life Cycle Analysis (LCA) performed in this study is cradle-to-gate, respectively, during the farming phase. Such farming phase includes tomato transplant production, soil fertilization/ mulching, transplant replication, plant protection treatments, including weeding, irrigation, and harvesting. In the case of the HV system, the farming phase also included the hairy vetch establishment costs. HV mulch system is a low-input system. Fertilizer, weeding, and irrigation are reduced by around 25% on average comparing with the intensive system/ In the HV + *Trichoderma* biostimulant mulch system, the need for plant protection treatment is reduced by 40%, according to our previous studies (Sicuia *et al.*, 2011), due to activation of the plant innate immunity. Figure 1

illustrates the studied farming system and its boundary.

Life cycle inventory. Data used for the LCA study were collected from the data source (EcoInvent) and recently published inventory from peer-review articles (Pineda *et al.*, 2021). Trichoderma production and formulation data were collected from the peer-reviewed paper regarding cellulase production using selected *Trichoderma* strains. The data were adapted to the optimal biosynthesis conditions of the plant biostimulant strains used in our studies (Zamfiropol-Cristea *et al.*, 2017).

Figure 1. The cradle to gate system boundary was used for the Life Cycle Analysis performed in this paper

Life cycle impact assessment. The eco-efficiency can be evaluated as the environmental burden, spotlighted by the LCA specific indicators, such as carbon footprint/climate change impact, damage to continental water bodies, acidification potential, impact on human health. HV farming reduces erosion and nitrate leakage (Rice *et al.*, 2002). Therefore, damage to the continental water bodies and acidification potential were not calculated. Plant biostimulants and hairy vetch mulch cultivation enhance field-grown tomatoes' quality (Dima *et al.*, 2020; Hong *et al.*, 2000). The main LCA indicator which was considered in this paper was the carbon footprint. The carbon footprint was made by modeling bare-soil intensive system, HV- mulch system, *Trichoderma* plant biostimulant + HV mulch system in the GaBi software (Sfera Solutions, Leinfelden-Echterdingen, Germany). The chosen approach was the attributional LCA. The energy grid was considered the Romanian grid.

Techno-economic analysis. For the field-grown tomatoes in bare soil intensive farming system, the water consumption for irrigation was considered 600 m^3ha^{-1}. The fertilization was considered NPK 8:11:23, 500 $kg.ha^{-1}$ and one foliar treatment with 2 $liters.ha^{-1}$, with a 3:1:1 type NPK fertilizer, with microelement, including selenium (Dima *et al.*, 2020). The considered plant protection treatments were: bactericides against tomatoes bacterial disease (*Xanthomonas* spp., *Pseudomonas* spp.); fungicides against fungal foliar diseases (*Phytophthora infestans, Alternaria solani*, *Septoria lycopersici, Leveillula taurica*), against fungal vascular diseases (*Fusarium* spp., *Verticillium* spp.) and gray mold, *Botrytis cinerea* control; insecticides against *Tuta absoluta* and *Helicoverpa armigera*; pre-emergent herbicides for weed control. For the HV mulch system, the consumption of fertilizers, herbicides, and irrigations is lower by 25%. In the HV + *Trichoderma* biostimulant mulch system, the need for plant protection treatment was reduced by 40% (Sicuia et al., 2011). For both HV mulch systems, the winter cover crop's costs during the fall were considered. According to the cost, which is reimbursed for establishing the winter cover crop according to agro-environmental support, this cost was estimated to be 128 euro per ha.
To determine the costs for the production of *Trichoderma* hydrogelified and film-forming formulation, the model of *Trichoderma* cellulase production costs was used (Olofsson *et al.*, 2017). This model was combined with our data regarding optimal biosynthesis conditions for our plant biostimulant strain (Zamfiropol-Cristea *et al.*, 2017). Mass energy balance, capital costs/investment amortization, material consumption were estimated in Aspen Plus v8.0 software (Aspen Technology, Cambridge, MA, USA). All calculated costs and income were expressed in euros.

RESULTS AND DISCUSSIONS

The data used for calculation of carbon footprint of the three systems, bare-soil intensive system, HV- mulch system, *Trichoderma* plant

biostimulant + HV mulch system analyzed in this paper, are presented in Table 1. These data were validated by publication as peer-reviewed papers (Oancea *et al.*, 2016; Oancea *et al.*, 2017; Sicuia *et al.*, 2011).

Similar data were used for other LCA studies focused on the assessment of the carbon footprint/climate change impact of the tomatoes farming system, both in protected systems and open-field systems (Garofalo *et al.*, 2017; Pineda *et al.*, 2021; Ronga *et al.*, 2019; Zarei *et al.*, 2019). The resulted values related to carbon footprint for each type of farming system analyzed in this paper are presented in Figure 2.

As we already mentioned, one of the main differences in the HV-mulch system comparing to bare-soil intensive systems is tillage (Campiglia *et al.*, 2010). However, fuel consumption is not significantly reduced in the HV mulching system because of other mechanized works - establishment of the winter cover crops, termination of the hairy vetch, and conversion to mulch by roller-crimper. The reduced carbon footprint is due mainly to the reduced production of greenhouse gases (CO_2, CH_4, N_2O) due to soil coverage and nitrogen immobilization (Massantini *et al.*, 2021).

Table 1. The data used for the calculation of the carbon footprint of the three analyzed farming systems for field-grown tomatoes

	Unit	Bare soil	HV-mulch	HV-mulch + *Trichoderma*
Outputs to the Technosphere				
Tomato fruits yield	tones	82.324	67.538	79.696
Inputs from the environment				
Water	m^3	600	450	450
Inputs from Technosphere				
Transplants production				
Seeds	number	50,000	50,000	50,000
Peat based substrate	m^3	2.53	2.53	2.53
Fertilizer (Calcium nitrate, superphosphate, potassium sulphate)	kg	4.75	4.75	4.75
Amendment (calcium carbonate)	kg	2.25	2.25	2.15
Heating fuel	kg	264	264	264
Tomato fruit production – open field				
Mineral fertilizer NPK 8:11:2	kg	500	400	400
Foliar fertilization	kg	2	2	2
Trichoderma plant biostimulants	kg	0	0	4
Plant protection products		12,70	9.52	7.62
Electricity (irrigation)	kWh	14.10	10.57	10.57
Diesel (tillage, transplantation, harvesting)	kg	46.50	0	0
Diesel (hairy vetch establishment, hairy vetch termination, transplantation, harvesting)		0	52.5	52.5
Lubricant	kg	3.50	3.80	3.80
Outputs to the environment				
Emissions to air				
CO_2	kg	198,958.20	146,451.85	138,656.42
CH_4	kg	23.25	12.26	12.46
N_2O	kg	1.39	1.08	0.92

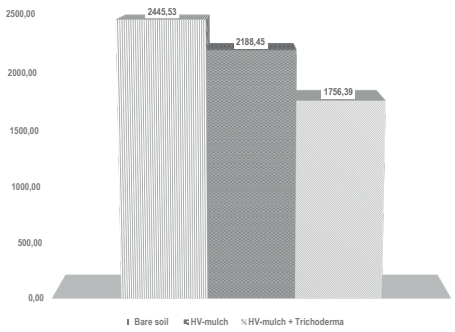


Figure 2. The carbon footprint, as equivalent kg CO_2 per tone of tomatoes fruits for the three farming systems studied - bare soil, HV-mulch, HV-mulch + *Trichoderma* plant biostimulants

Another difference that contributes significantly to the carbon footprint is the lower plant protection product consumption. Other studies also underlined this difference. Reduction of plant protection product utilization benefits also from other environmental indicators (Guo *et al.*, 2021; Ntinas *et al.*, 2017; Ronga *et al.*, 2019).

The techno-economic analysis for the three farming systems is presented in Table 2. The results demonstrated that the *Trichoderma* plant biostimulant application on mulch enhances the HV-system's profitability - mainly due to increased yield. Similar results related to the gross margin decreases in the hairy vetch low-inputs farming systems were also reported for

other semi-arid areas (Delate *et al.*, 2012; Leavitt *et al.*, 2011). It seems that the benefits of nitrogen and water storage are lower in semi-arid regions, especially during this period of climate change (Muchanga *et al.*, 2020)

Table 2. The techno-economic analysis for the three farming systems studied - bare soil, HV-mulch, HV-mulch + *Trichoderma* plant biostimulants

Incomes										
Tomatoes fruits yield per ha	kg	82324	0.11	9055.6	79696	0.11	8766.5	67538	0.11	7429.18
Subvention per ha	€	1.00		1410	1.00		1538.00	1.0		1538.00
Total				**10465.64**			**10304.56**			**8967.18**
										Direct costs
Seeds	Nr	50000.00	0.02	1000.00	50000.00	0.02	1000.00	50000.00	0.02	1000.00
Transplant production costs	Nr	50000.00	0.03	1500.00	50000.00	0.03	1500.00	50000.00	0.03	1500.00
Hairy vetch establishment and mulching	Nr	0.00	0.00	0.00	1.00	128.00	128.00	1.00	128.00	128.00
Fertilizers (soil)	kg	500.00	0.45	225.00	400.00	0.45	60.00	400.00	0.45	60.00
Fertilizer (foliar)	kg	2.00	16.50	33.00	2.00	16.50	33.00	2.00	16.50	33.00
Plant protection products	kg	12.70	30.50	387.35	9.52	30.50	290.36	7.62	30.50	M232,41
Trichoderma bioproduct	kg	0.00	0.00	0.00	4.00	12.75	51.00	0.00	0.00	0.00
Irrigation	m^3	600.00	0.75	450.00	450.00	0.75	337.50	450.00	0.75	337.50
Diesel	kg	46.50	1.05	48.83	52.50	8.00	8.00	52.50	1.01	8.00
Lubricant	kg	3.50	18.50	64.75	3.80	18.50	70.30	3.80	18.50	70.30
Direct working force	h	12.00	5.50	66.00	15.00	5.50	82.50	16.00	5.50	88.00
Others direct costs		1.00	48.00	48.00	1.00	48.00	48.00	1.00	28.00	28.00
Total				**3822.93**			**3608.66**			**3485.21**
Goss margin EUR per ha				**6642.72**			**6695.90**			**5481.97**

Low-input farming systems, such as organic farming or HV-farming, have a general environmental impact lower when expressed per production area unit, i.e., ha (Tuomisto *et al.*, 2012; Zarei *et al.*, 2019). However, due to lower production, the environmental impact is sometimes higher than in intensive agriculture (Ronga *et al.*, 2017). Therefore, yield increase was suggested for the low-inputs systems, especially in the semi-arid area (Delate *et al.*, 2012; Ronga *et al.*, 2019), to improve the eco-efficiency.

Our proposed systems, involving applying the *Trichoderma* plant biostimulant as a hairy vetch mulch treatment to compensate for the drawbacks of the traditional HV-mulch farming systems, increase the yield and the gross margin. At the same time, retain the benefits of the HV-system related to soil health and fertility (Butler *et al.*, 2016; Muchanga *et al.*, 2020), as our previous work demonstrated (Oancea *et al.*, 2017).

CONCLUSIONS

The low-input farming system of tomato cultivation into the hairy vetch mulch was improved by utilizing the treatment with a plant biostimulant *Trichoderma* strain. This strain was included in the hydrogelified and film-forming formulation.

Life cycle assessment demonstrates a significant reduction of the carbon footprint. This reduction was almost 30% compared to the bare-soil intensive farming system and more than 20% compared to the traditional HV farming system. The yield increase compensates for the additional costs of the bioproducts. The gross margin is higher in the conservation farming system, which utilizes *Trichoderma* plant biostimulants.

ACKNOWLEDGEMENTS

The research leading to these results has received funding from the NO Grants 2014-2021, under Project RO-NO-2019-540 "Integrated use of the next generation plant biostimulants for enhanced sustainability of field vegetable high residue farming systems STIM 4^{+}", contract no. 14/2020.

REFERENCES

Abdul-Baki, A. A., Teasdale, J., Korcak, R., Chitwood, D. J., & Huettel, R. (1996). Fresh-market tomato production in a low-input alternative system using cover-crop mulch. *HortScience, 31*(1), 65-69.

Abdul-Baki, A. A., Teasdale, J. R., Goth, R. W., & Haynes, K. G. (2002). Marketable yields of fresh-market tomatoes grown in plastic and hairy vetch mulches. *HortScience, 37*(6), 878-881.

Alexe, C., Vintilă, M., Popescu, S., Lămureanu, G., & Chira, L. (2015). The influence of culture technology upon the physical quality of some early tomatoes varieties. *Scientific Papers - Series B, Horticulture,* (59), 171-175.

Butler, D. M., Bates, G. E., & Inwood, S. E. E. (2016). Tillage System and Cover Crop Management Impacts on Soil Quality and Vegetable Crop Performance in Organically Managed Production in Tennessee. *HortScience, 51*(8), 1038-1044. doi:10.21273/hortsci.51.8.1038

Campiglia, E., Caporali, F., Radicetti, E., & Mancinelli, R. (2010). Hairy vetch (*Vicia villosa* Roth.) cover crop residue management for improving weed control and yield in no-tillage tomato (*Lycopersicon esculentum* Mill.) production. *European Journal of Agronomy, 33*(2), 94-102. doi:https://doi.org/10.1016/j.eja.2010.04.001

Coppola, M., Diretto, G., Digilio, M. C., Woo, S. L., Giuliano, G., Molisso, D., Pennacchio, F., Lorito, M., & Rao, R. (2019). Transcriptome and metabolome reprogramming in tomato plants by Trichoderma harzianum strain T22 primes and enhances defense responses against aphids. *Frontiers in physiology, 10*, 745.

Delate, K., Cwach, D., & Chase, C. (2012). Organic no-tillage system effects on soybean, corn and irrigated tomato production and economic performance in Iowa, USA. *Renewable Agriculture and Food Systems, 27*(1), 49-59. doi:10.1017/s1742170511000524

Dima, S. O., Neamtu, C., Desliu-Avram, M., Ghiurea, M., Capra, L., Radu, E., Stoica, R., Faraon, V. A., Zamfiropol-Cristea, V., Constantinescu-Aruxandei, D., & Oancea, F. (2020). Plant Biostimulant Effects of Baker's Yeast Vinasse and Selenium on Tomatoes through Foliar Fertilization. *Agronomy-Basel, 10*(1). doi:10.3390/agronomy10010133

Fatima, T., Sobolev, A. P., Teasdale, J. R., Kramer, M., Bunce, J., Handa, A. K., & Mattoo, A. K. (2016). Fruit metabolite networks in engineered and non-engineered tomato genotypes reveal fluidity in a hormone and agroecosystem specific manner. *Metabolomics, 12*(6). doi:10.1007/s11306-016-1037-2

Fatima, T., Teasdale, J. R., Bunce, J., & Mattoo, A. K. (2012). Tomato response to legume cover crop and nitrogen: differing enhancement patterns of fruit yield, photosynthesis and gene expression. *Functional Plant Biology, 39*(3), 246-254.

Fernández, E., Segarra, G., & Trillas, M. (2014). Physiological effects of the induction of resistance by compost or Trichoderma *asperellum* strain T34 against *Botrytis cinerea* in tomato. *Biological Control, 78*, 77-85.

Frison, E. A., Cherfas, J., & Hodgkin, T. (2011). Agricultural biodiversity is essential for a sustainable improvement in food and nutrition security. *Sustainability, 3*(1), 238-253.

García-Mier, L., Guevara-González, R. G., Mondragón-Olguín, V. M., del Rocío Verduzco-Cuellar, B., & Torres-Pacheco, I. (2013). Agriculture and bioactives: achieving both crop yield and phytochemicals. *International journal of molecular sciences, 14*(2), 4203-4222.

Garofalo, P., D'Andrea, L., Tomaiuolo, M., Venezia, A., & Castrignanò, A. (2017). Environmental sustainability of agri-food supply chains in Italy: The case of the whole-peeled tomato production under life cycle assessment methodology. *Journal of Food Engineering, 200*, 1-12.

Gomes, E. V., Ulhoa, C. J., Cardoza, R. E., Silva, R. N., & Gutiérrez, S. (2017). Involvement of Trichoderma harzianum Epl-1 protein in the regulation of Botrytis virulence-and tomato defense-related genes. *Frontiers in plant science, 8*, 880.

Guo, X.-X., Zhao, D., Zhuang, M.-H., Wang, C., & Zhang, F.-S. (2021). Fertilizer and pesticide reduction in cherry tomato production to achieve multiple environmental benefits in Guangxi, China. *Science of The Total Environment, 793*, 148527. doi:https://doi.org/10.1016/j.scitotenv.2021.148527

Hewedy, O. A., Abdel Lateif, K. S., Seleiman, M. F., Shami, A., Albarakaty, F. M., & M El-Meihy, R. (2020). Phylogenetic diversity of Trichoderma strains and their antagonistic potential against soil-borne pathogens under stress conditions. *Biology, 9*(8), 189.

Hobbs, P. R., Sayre, K., & Gupta, R. (2008). The role of conservation agriculture in sustainable agriculture. *Philosophical Transactions of the Royal Society B: Biological Sciences, 363*(1491), 543-555.

Hong, J. H., Mills, D. J., Coffman, C. B., Anderson, J. D., Camp, M. J., & Gross, K. C. (2000). Tomato Cultivation Systems Affect Subsequent Quality of Fresh-cut Fruit Slices. *Journal of the American Society for Horticultural Science jashs, 125*(6), 729. doi:10.21273/jashs.125.6.729

Kerdraon, L., Laval, V., & Suffert, F. (2019). Microbiomes and pathogen survival in crop residues, an ecotone between plant and soil. *Phytobiomes Journal, 3*(4), 246-255.

Kumar, V., Mills, D. J., Anderson, J. D., & Mattoo, A. K. (2004). An alternative agriculture system is defined by a distinct expression profile of select gene transcripts and proteins. *Proceedings of the National Academy of Sciences, 101*(29), 10535-10540.

Leavitt, M. J., Sheaffer, C. C., Wyse, D. L., & Allan, D. L. (2011). Rolled Winter Rye and Hairy Vetch Cover Crops Lower Weed Density but Reduce Vegetable Yields in No-tillage Organic Production. *Hort Science, 46*(3), 387-395. doi:10.21273/hortsci.46.3.387

López-Bucio, J., Pelagio-Flores, R., & Herrera-Estrella, A. (2015). *Trichoderma* as biostimulant: exploiting the multilevel properties of a plant beneficial fungus. *Scientia Horticulturae, 196*, 109-123.

Massantini, R., Radicetti, E., Frangipane, M. T., & Campiglia, E. (2021). Quality of Tomato (*Solanum lycopersicum* L.) Changes under Different Cover Crops, Soil Tillage and Nitrogen Fertilization Management. *Agriculture-Basel, 11*(2). doi:10.3390/agriculture11020106

Möller, K., Oberson, A., Bünemann, E. K., Cooper, J., Friedel, J. K., Glæsner, N., Hörtenhuber, S., Løes, A.-K., Mäder, P., Meyer, G., Müller, T., Symanczik, S., Weissengruber, L., Wollmann, I., & Magid, J. (2018). Improved Phosphorus Recycling in Organic Farming: Navigating Between Constraints. In D. L. Sparks (Ed.), *Advances in Agronomy* (Vol. 147, pp. 159-237): Academic Press.

Muchanga, R. A., Hirata, T., Uchida, Y., Hatano, R., & Araki, H. (2020). Soil carbon and nitrogen and tomato yield response to cover crop management. *Agronomy Journal, 112*(3), 1636-1648. doi:10.1002/agj2.20098

Neelam, A., Cassol, T., Mehta, R. A., Abdul-Baki, A. A., Sobolev, A. P., Goyal, R. K., Abbott, J., Segre, A. L., Handa, A. K., & Mattoo, A. K. (2008). A field-grown transgenic tomato line expressing higher levels of polyamines reveals legume cover crop mulch-specific perturbations in fruit phenotype at the levels of metabolite profiles, gene expression, and agronomic characteristics. *Journal of Experimental Botany, 59*(9), 2337-2346.

Ntinas, G. K., Neumair, M., Tsadilas, C. D., & Meyer, J. (2017). Carbon footprint and cumulative energy demand of greenhouse and open-field tomato cultivation systems under Southern and Central European climatic conditions. *Journal of Cleaner Production, 142*, 3617-3626.

Oancea, F. (2011). Conservation agriculture system based bioactive vegetative mulch consists of green crop. *Scrisul Romanesc, Craiova*, 67-87.

Oancea, F., Raut, I., Sesan, T. E., Cornea, P. C., Badea-Doni, M., Popescu, M., & Jecu, M. L. (2016). Hydro-gelified and film forming formulation of microbial plant biostimulants for crop residues treatment on conservation agriculture systems. *Studia Universitatis "Vasile Goldis" Arad. Seria Stiintele Vietii (Life Sciences Series), 26*(2), 251.

Oancea, F., Răut, I., & Zamfiropol-Cristea, V. (2017). Influence of soil treatment with microbial plant biostimulant on tomato yield and quality. *J. Int. Sci. Publ. Agric. Food, 5*, 156-165.

Olofsson, J., Barta, Z., Borjesson, P., & Wallberg, O. (2017). Integrating enzyme fermentation in lignocellulosic ethanol production: life-cycle assessment and techno-economic analysis. *Biotechnology for Biofuels, 10*. doi:10.1186/s13068-017-0733-0

Paltineanu, C., Tanasescu, N., Chitu, E., & Mihailescu, I. (2007). Relationships between the De Martonne aridity index and water requirements of some representative crops: A case study from Romania. *International agrophysics, 21*(1).

Pineda, I. T., Lee, Y. D., Kim, Y. S., Lee, S. M., & Park, K. S. (2021). Review of inventory data in life cycle assessment applied in production of fresh tomato in greenhouse. *Journal of Cleaner Production, 282*, 124395.

Poveda, J., Abril-Urias, P., & Escobar, C. (2020). Biological control of plant-parasitic nematodes by filamentous fungi inducers of resistance: Trichoderma, mycorrhizal and endophytic fungi. *Frontiers in Microbiology, 11*, 992.

Ranaivoson, L., Naudin, K., Ripoche, A., Affholder, F., Rabeharisoa, L., & Corbeels, M. (2017). Agro-ecological functions of crop residues under conservation agriculture. A review. *Agronomy for Sustainable Development, 37*(4), 1-17.

Rice, P. J., McConnell, L. L., Heighton, L. P., Sadeghi, A. M., Isensee, A. R., Teasdale, J. R., Abdul-Baki, A. A., Harman-Fetcho, J. A., & Hapeman, C. J. (2002). Comparison of copper levels in runoff from fresh-market vegetable production using polyethylene mulch or a vegetative mulch. *Environmental Toxicology and Chemistry, 21*(1), 24-30. doi:https://doi.org/10.1002/etc.5620210104

Ronga, D., Gallingani, T., Zaccardelli, M., Perrone, D., Francia, E., Milc, J., & Pecchioni, N. (2019). Carbon footprint and energetic analysis of tomato production in the organic vs the conventional cropping systems in Southern Italy. *Journal of Cleaner Production, 220*, 836-845. doi:10.1016/j.jclepro.2019.02.111

Ronga, D., Zaccardelli, M., Lovelli, S., Perrone, D., Francia, E., Milc, J., Ulrici, A., & Pecchioni, N. (2017). Biomass production and dry matter partitioning of processing tomato under organic vs conventional cropping systems in a Mediterranean environment. *Scientia Horticulturae, 224*, 163-170. doi:https://doi.org/10.1016/j.scienta.2017.05.037

Sicuia, O., Oancea, F., Dinu, S., Zamfiropol, R., Fătu, V., Fătu, C., Anton, L., & Voicu, E. (2011). Weed bio-control and tomato plants growth promotion by applying an alternative cultivation system into bio-composite mulch. *Romanian Journal of Plant Protection, 3*, 23-37.

Teasdale, J. R., & Abdul-Baki, A. A. (1997). Growth analysis of tomatoes in black polyethylene and hairy vetch production systems. *HortScience, 32*(4), 659-663.

Tuomisto, H. L., Hodge, I., Riordan, P., & Macdonald, D. W. (2012). Does organic farming reduce environmental impacts?–A meta-analysis of European research. *Journal of environmental management, 112*, 309-320.

Van Agtmaal, M., Straathof, A., Termorshuizen, A., Teurlincx, S., Hundscheid, M., Ruyters, S., Busschaert, P., Lievens, B., & de Boer, W. (2017). Exploring the reservoir of potential fungal plant pathogens in agricultural soil. *Applied Soil Ecology, 121*, 152-160.

Voican, V., Lacatus, V., & Tanasescu, M. (1995). Growth and development of tomato plants related to climatic conditions from some areas of Romania. *Acta Horticulturae, 412*, 355-365. doi:10.17660/ActaHortic.1995.412.42

Zamfiropol-Cristea, V., Răut, I., Șeșan, T. E., Trică, B., & Oancea, F. (2017). Surface response optimization of submerged biomass production for a plant biostimulant trichoderma strain. *Sci. Bull. Ser. F. Biotechnol, 21*, 56-65.

Zarei, M. J., Kazemi, N., & Marzban, A. (2019). Life cycle environmental impacts of cucumber and tomato production in open-field and greenhouse. *Journal of the Saudi Society of Agricultural Sciences, 18*(3), 249-255.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

ASSESSMENT OF GENETIC SIMILARITY AND PURITY DEGREE AMONG SEVERAL ROMANIAN MAIZE INBRED LINES USING SSR MARKERS

Valentina VASILE[1, 2], Matilda CIUCĂ[3], Elena NICOLAE[1, 2], Cătălina VOAIDEȘ[1], Călina Petruța CORNEA[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd., District 1, Bucharest, Romania
[2]Central Laboratory for Quality of Seeds and Planting Material, 10 Constantin Sandu Aldea Alley, District 1, Bucharest, Romania
[3]National Agricultural Research and Development Institute Fundulea, 1 Nicolae Titulescu Street, Fundulea, Călărași, Romania

Corresponding author email: valiv26@yahoo.com

Abstract

Maize (Zea mays ssp. mays) is today one of the most important cereal crops used not only for human consumption but although for feed or for industrial purposes, without the genetic evolution and the active intervention of breeders in the plant constant improvement, maize would not have today significance.
In this study, seeds from thirteen maize inbred lines (LC1-LC13) were analysed using eight SSRs markers recommended for seed varietal purity assessment. The seeds classes for maize inbred lines used in this study were pre-basic and basic seeds. Three of the maize inbred lines chosen for testing from both pre-basic and basic categories were analysed in order to verify that the varietal purity is preserved. High genetic similarity was between inbred line LC1 and LC2. SSR marker phi015 was the most polymorphic marker followed by umc1545, umc1448 and umc1117. The SSRs markers that showed low polymorphism were umc1061 and phi109275. The aim of this study was to select the most informative SSRs markers which fit to prove the varietal purity and to assess genetic diversity for maize seeds.

Key words*: SSR markers, maize, varietal purity, genetic diversity.*

INTRODUCTION

The history of maize (*Zea mays* ssp. *mays)* is thought to have begun 9,000 years ago, when the inhabitants of southern Mexico domesticated the plant called teosinte. Due to genetic evolution and human intervention in plant improvement maize is nowadays one of the main crops in the world, along with wheat and rice (Balter, 2007).
In Romania, maize is one of the main cereal crops. Statistical data on cultivated areas with cereals and their production from 2010 to 2019 showed an annual increase in cultivated areas but also in crops production. In 2020, according to data provided by INS (2021), in Romania, the area cultivated with cereals decreased by 2.4%, compared to 2019. Due to climatic changes especially the severe drought the production decreased by 37.6% with low yields for the vast majority of crops. Maize represents in Romania 55.8% of the total cereal production, wheat 35.2%, and barley 6.2%. The area cultivated with maize was the largest and the maize yields ranked second in the European Union (INS, 2021).
In recent years it has been observed in maize an extensive progress in breeding programs which led to a large number of new hybrids and different types of varieties selections (Bocianowski et al., 2021; Biţică, 2016).
Molecular markers and especially simple sequence repeats (SSRs) markers have wide applicability such as plant genetic diversity analysis, studies of germplasm conservation of maize genotypes (Vivodík et al., 2018), varietal purity or varietal identification (Chaudhary et al., 2018). In crop improvement strategies, SSRs markers are important tool for breeders in marker assisted selection (MAS) (Șuteu et al., 2014; Ahmad et al., 2017).
As a result of climate change and the growth of the world's population, it is becoming a general interest in selection and creating new maize hybrids showing valuable characters such as

drought or pests resistance (Sun et al, 2021; Li et al., 2016; Gaikpa et al., 2021), improved content in essential nutrients (Zawadi et al., 2021; Prasanna et al., 2020).
SSRs markers are preferred in research studies because they are codominant and have a high degree of polymorphism and reproducibility thus easy to identify by PCR technique (Raza et al., 2019; Vasile et al., 2020).
Selection of the most informative SSRs markers suitable for varietal purity assessment and genetic diversity of maize inbred lines chosen is the main purpose of this study.

MATERIALS AND METHODS

Plant material consisted of seeds from thirteen Romanian maize inbred lines (LC1-LC13) obtained from Central Laboratory for Quality of Seeds and Planting Material (LCCSMS), Romania. The seeds classes for these thirteen maize inbred lines used in this study were pre-basic second generation and basic seeds. In order to verify that the varietal purity is preserved for three of the maize inbred lines seeds from both pre-basic and basic categories were analysed.

DNA extraction

The DNA extraction was performed using the DNA extraction kit NucleoSpin Plant II (Macherey-Nagel) which contains two lysis buffers PL1 with CTAB and PL2 buffer with SDS. The DNA extraction protocol was adapted in order to meet the desired purity and concentration requirements for extracted DNA. The buffer chosen for DNA extraction in the present study was PL1 buffer based on CTAB. In order to highlight the fact that the choice of the extraction method is the correct one, an experimental plan was made which consisted in DNA extraction from dry maize seeds from all maize inbred lines and embryo and plant material resulting from maize seed germination for three of the maize inbred lines. Thus, after homogenizing the seeds samples chosen for testing a number of 3-5 seeds from each of the thirteen maize inbred lines were ground and about 40 mg of ground powder was transferred to a sterile microcentrifuge tube and PL1 buffer was added. The mixture was vortexed thoroughly and RNase A solution was added. The amount of buffer was also adjusted, thus increasing the amount recommended by the manufacturer for a better homogenization of the mixture. Incubation time at 65°C was increased from 10 to 30 mins. After centrifugation, the lysates were cleared by filtration using kit column and mixed with PC binding buffer. The mixture was loaded on a silica membrane column and the contaminants were removed by washing the column three times with kit wash buffers. The genomic DNA was eluted with kit elution buffer containing 5 mM Tris/HCl, pH 8.5 and frozen at -20 °C for longer storage.
DNA extraction from maize inbred line LC9, LC10 and LC11 was also made of 1-4 ground embryos. The seeds were placed in distilled water at room temperature for 2 hours after which the embryos were grounded.
In order to perform DNA extraction from germinated seeds 3-5 seeds from maize inbred lines LC9, LC10 and LC11 were covered with filter paper soaked in water. Germination was performed at a temperature of 26-28°C and the plant material for DNA extraction was taken after 48 hours and the germinated material was ground. In both cases, the DNA extraction followed the same steps mentioned above.
The concentration and quality of the extracted genomic DNA was assessed by spectrophotometry using the Biochrom Biowave DNA UV-Vis spectrophotometer. DNA amplification was verified by performing a PCR endogenous assay using primers to detect maize *hmg* (high mobility group) reference gene (Bonfini et al., 2012).

SSRs markers

Eight SSRs markers were chosen for this study in order to assess varietal purity and genetic diversity for the thirteen Romanian maize inbred lines. These SSRs markers are recommended as suitable for verification of maize varieties (ISTA, 2021). The SSRs markers, PCR primers sequence and approximate allele size range obtained for SSRs markers used in this study is presented in Table 1 (ISTA, 2021; Woodhouse et al., 2021).

Table 1. SSRs markers and PCR primers sequence

SSRs marker	Forward	Reverse	Approximate allele size range (bp)
umc1545	GAAAACTGCATCAACAACAAGCTG	ATTGGTTGGTTCTTGCTTCCATTA	70-96
umc1448	ATCCTCTCATCTTTAGGTCCACCG	CATATACAGTCTCTTCTGGCTGCTCA	160–190
umc1117	AATTCTAGTCCTGGGTCGGAACTC	CGTGGCCGTGGAGTCTACTACT	146–170
umc1061	AGCAGGAGTACCCATGAAAGTCC	TATCACAGCACGAAGCGATAGATG	100–120
phi109275	CGGTTCATGCTAGCTCTGC	GTTGTGGCTGTGGTGGTG	150-160
phi102228	ATTCCGACGCAATCAACA	TTCATCTCCTCCAGGAGCCTT	140-170
phi083	CAAACATCAGCCAGAGACAAGGAC	ATTCATCGACGCGTCACAGTCTACT	143-166
phi015	GCAACGTACCGTACCTTTCCGA	ACGCTGCATTCAATTACCGGGAAG	89-121

(ISTA, 2021; Woodhouse et al., 2021)

PCR conditions

In order to be able to choose the best PCR reaction conditions, optimizations of the PCR reaction were made. Thus, the final primer concentration was varied from 1 µM to 0.6 µM in the final mix also the final PCR reaction volume was adjusted from 20 µL to 15 µL for SSR marker umc1545. For all SSRs markers a temperature profile was created in order to choose the optimal annealing temperature. PCR products amplification was done in BIO- RAD T100™ Thermal Cycler system. The annealing temperatures chosen for testing were 61°C, 60°C, 59°C, 57°C and 56°C.

PCR reaction components and final concentration chosen for all SSRs markers was 1x Green GoTaq® Flexi Buffer, upstream and downstream primers 0.6 µM, 0.2 mM PCR Nucleotide Mix 10mM, 1.5 mM $MgCl_2$ solution 25mM, 0.5U GoTaq G_2 Hot Start DNA Polymerase (Promega). Additional reagents: nuclease-free water up to 15µL final volume and about 60-90 ng/µL template DNA.

The thermal cycling profile for PCR products amplification with SSRs markers were: initial denaturation 5 min at 95°C, denaturation 30 s at 95°C, annealing 30 s at 59°C for SSR marker umc1545 and 60°C for the remaining selected SSR markers, extension 30 s at 72°C, 35 cycles, final extension 5 min at 72°C.

To verify DNA amplification a different PCR reaction mix was performed containing PCR mix composed of 1x Fast Start PCR Master (Roche) a ready-to-use hot start PCR mix, 0.3 µM upstream and downstream primers final concentration, 5 µL template DNA and nuclease-free water up to 50µL final volume. The thermal cycling profile was initial denaturation 4 min at 95°C, denaturation 30 s at 95°C, annealing 30 s at 60°C, extension 1 min at 72°C, 37 cycles, final extension 7 min at 72°C. Fragments separation and highlighting of the resulting PCR amplification products was performed by agarose gel electrophoresis. The agarose gel concentration was between 2.4 and 2.8 % agarose (Agarose I™, VWR Life Science) in 1X TAE (TAE Buffer, 10X, Molecular Biology Grade/ Promega).

For nucleic acid visualization in agarose gel Red Safe™ Nucleic Acid Staining Solution (Intron) and ECO Safe Nucleic Acid Staining Solution was used. The power supply was provided by Consort EV243, the migration being performed at a voltage between 64-67 V. The migration time was between 1h 45 min and 2 hours. PCR products were visualized in UV light using Vilber Lourmat E-BOX VX2 imaging system.

RESULTS AND DISCUSSIONS

An important first step when using molecular biology methods is that the chosen DNA extraction method to result in a genomic DNA which meets the methods desired concentration and purity requirements. As mentioned in other studies the DNA requirements when using SSR markers are small amount of DNA. The genomic DNA does not require high purity ratio (Raza et al., 2019; Vasile et al., 2020).

As stated before the chosen buffer for DNA extraction in the present study was CTAB based lysis buffer and variations were only related to DNA extraction from dry maize seeds, embryos and plant material resulting from maize seed germination. These variations were applied to

only three of the maize inbred lines tested (LC9, LC10 and LC11).

Following the analysis of the spectrophotometric data resulting from the extracted DNA evaluation, it was found that the lowest values of concentration were obtained after DNA extraction from embryos, an average yield of 15 ng/µL. Given the fact that the concentration values are heterogeneous and there are insufficient data to exclude this type of approach an optimization of the extraction method from embryos is needed.

The highest concentration values were obtained after extraction from plant material resulting from seed germination, namely an average yield of 94 ng/µL. This type of approach was not considered appropriate for the present study because it requires a longer time for DNA extraction.

DNA extraction from dry maize seeds has proven to be the fastest and most effective method. The DNA yield for all thirteen maize inbred lines used in this study was between 28-57 ng/µL. Even if the DNA concentration is not very high compared to the DNA extraction after seeds germination it proved to be sufficient for PCR amplification.

Regarding the purity ratio for all measurements, it could be seen that the ratio was 1.8 for A_{260}/A_{280} ratio and 1.94 for A_{260}/A_{230} ratio.

Verification of the extracted DNA was performed in this study by amplifying the extracted DNA with specific primers for *Zea mays* reference gene (*hmg*).

All the resulting PCR products showed amplification regardless the extraction method. Figure 1 shows the 79bp amplification products for maize *hmg* (high mobility group) reference gene, for the PCR products obtained by amplifying the extracted DNA from dry maize seeds, embryos and plant material resulting from maize seed germination.

Although the data analysis related to DNA concentration showed that there are very heterogeneous values between the three extraction methods, no significant differences were observed for PCR products obtained by amplifying the extracted DNA with *Zea mays* reference gene (*hmg*).

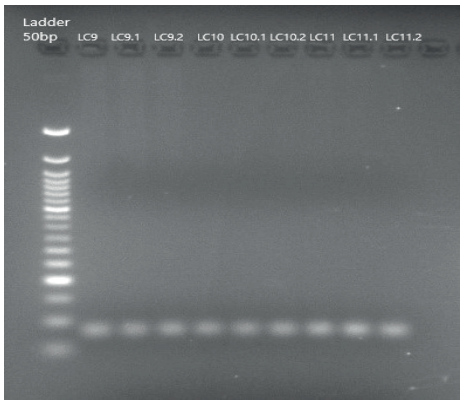


Figure 1. Agarose gel electrophoresis of PCR product obtained by amplifying the extracted DNA with *Zea mays* reference gene (*hmg*)
Legend: LC9-LC11 - PCR products obtained by amplifying the extracted DNA from dry seeds; LC9.1-LC11.1 - PCR products obtained by amplifying the extracted DNA from embryos; LC9.2-LC11.2 - PCR products obtained by amplifying the extracted DNA from seeds germination

Another important step when using methods based on SSRs markers is to optimize the working method in order to choose the right PCR conditions.

Choosing the most informative markers is also a priority for the success of a study (Raza et al., 2019; Vasile et al., 2020).

The eight SSRs markers chosen for this study are recommended as suitable for verification of maize varieties (ISTA, 2021).

In order to choose the best PCR conditions, a temperature gradient was created for all SSR markers.

No significant differences were observed in highlighting the amplification products at the chosen annealing temperatures. Figure 2 shows the results of PCR amplification products from two maize inbred line LC10 and LC11 with the umc1061 SSR marker at annealing temperatures of 61°C, 60°C, 59°C, 57°C and 56°C.

The final annealing temperature chosen for the umc1545 SSR marker was 59 °C and for the other SSRs markers 60°C.

Variations in primers concentration and final volume did not significantly influence product amplification, thus it was decided to reduce the concentration of primers and the final reaction volume for umc1545 SSR marker.

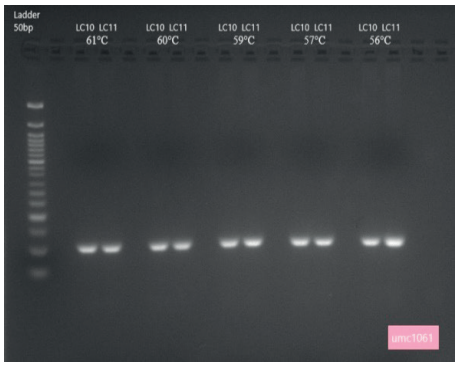


Figure 2. Agarose gel electrophoresis of PCR product obtained with umc1061 SSR marker at different annealing temperatures

In order to increase the specificity of the PCR reaction and to eliminate the risk of producing non-specific amplification products, it was decided to use a hot start enzyme in the PCR reaction.

Under the chosen conditions for testing all SSR markers presented good amplification products.

Another goal in this study was to characterize using SSRs markers the chosen thirteen maize inbred lines which will serve eventually as the seed parents to various maize hybrids.

The approximate allele size range (bp) obtained for all SSRs markers used in this study is presented in Table 1. The estimation of the values for the alleles obtained after PCR products electrophoresis running was made using the image analysis E-CAPT software.

After analysing the images, it was observed that all the selected markers showed some degree of polymorphism on the chosen maize inbred lines. Thus for SSRs markers umc1545, umc1448 and umc1117 four alleles were observed, for SSRs markers umc1061 and phi109275 two alleles were estimated, for SSRs markers phi102228 and phi083 three alleles were estimated and for SSR marker phi015 were estimated five allele. The highest number of estimated alleles was after using the SSR marker phi015. In Figure 3 we can observe the agarose gel electrophoresis of PCR product obtained with SSR marker phi015 for all thirteen maize inbred lines and the five alleles estimated (89, 112, 121,116, 97 bp) after image analysis with Vilber Lourmat, E-CAPT software.

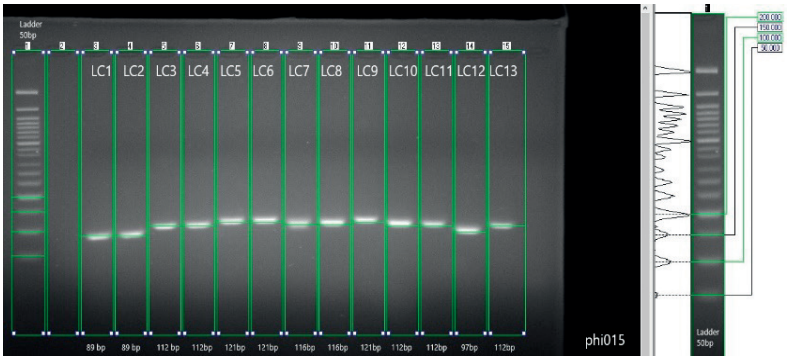


Figure 3. Agarose gel electrophoresis of PCR product obtained with phi015 SSR marker and approximate allele size range (bp) estimated after image analysis

As expected, the PCR products evaluation for maize inbred lines LC1 and LC2 (pre-basic second generation seeds and basic seeds) revealed high similarity with all SSRs markers used in this study. For maize inbred lines LC3, LC13 (pre-basic second generation seeds and basic seeds) and LC4, LC10 (basic and pre-basic second generation seeds) the PCR products evaluation of the eight SSRs markers, revealed a degree of similarity between 25-37% which may suggests some kind of contamination or an improvement of the initial inbred line desired by the breeder. After analysing the PCR products, high similarity was observed for inbred lines LC3 and LC13 with SSRs markers phi015, phi102228 and umc1061 and for LC4 and LC10 high similarity was observed with SSRs markers phi015 and phi102228.

For a better accuracy of determining the genetic purity of the seeds, it is recommended to analyse a larger number of seeds (Jhansi et al., 2015; ISTA, 2021).

Although maintaining genetic purity is an important factor in breeding process, genetic purity deterioration can occur due to various causes such as variations in plant adaptation to different environmental conditions or the influence of certain diseases, natural crossing but also due to precarious mechanical handling of seeds (Sendekie, 2020). It should be noted that the evaluation of the PCR products was performed in agarose gel and this method has its limitations (Sserumaga et al., 2014).

In order to assess genetic diversity for all thirteen maize inbred lines used in this study a dendrogram showing the relationship of maize inbred lines based on UPGMA (Unweighted Pair Group Method with Arithmetic Mean) cluster analysis was performed using Dice coefficient for comparison among sets of variables. For statistical data analysis a binary matrix was made, thus the amplified polymorphic bands were marked as present with "1" and absent with "0" and the dendrogram was generated using an online dendrogram construction utility (Garcia - Vallvé & Puigbo, 2009) retrieved from http: // genomes. urv. cat / UPGMA/. In Figure 4 is presented the relationship of maize inbred lines based on UPGMA cluster analysis.

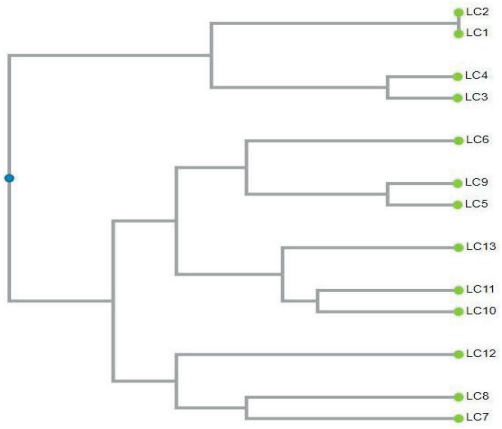


Figure 4. Dendrogram showing the relationship of maize inbred lines based on UPGMA cluster analysis

The dendrogram showed three clusters: cluster I consisting of LC7, LC8 and LC12, cluster II consisting from two sub-clusters (LC10, LC11, LC13 in sub-cluster II-1 and sub-cluster II-2 with LC5, LC9 and LC6) and cluster III consisting of LC1, LC2, LC3 and LC4.

Between inbred lines LC1 and LC2 can be seen 100 % similarity. High similarity it was also observed between maize inbred lines LC3 and LC4 and between inbred lines LC5 and LC9. Approximately 80% similarity was observed between maize inbred lines from LC1 to LC4 and between maize inbred lines LC5 to LC13. Evaluation of genetic relationships between different inbred lines can therefore be estimated using SSRs markers, being able to differentiate among closely related maize inbred lines (Sserumaga et al., 2014).

To better highlight the obtained allele it is possible to take into account to optimize the PCR reaction conditions as well as the use of much more SSRs markers.

Improvements can also be made in the way of highlighting the resulting products, namely the use of either a high resolution agarose instead of routine use agarose or highlighting the PCR products in polyacrylamide gel (Shiri, 2011; Adu et al., 2019; Zhang et al., 2021). Another effective way to analyse the data obtained after using the SSRs markers is capillary electrophoresis (Sserumaga et al., 2014; Tsonev et al., 2015; Bocianowski et al., 2021) which can reduce the possible risks related to results misinterpretation. However, it must be taken into account that using this method increases the final cost of the analysis.

The high genetic diversity of maize offers important opportunities for breeders in plant selection process where inbred lines are important in the development of hybrid varieties with high disease resistance, improved nutritional principles, higher yield or drought tolerance (Madobe et al., 2021; Oluwaranti et al., 2018; Zhang et al., 2021). That's why SSRs markers are important tools in assessing the varietal purity of maize inbred lines and the resulting hybrids in breeding process but also in the variety protection (Jhansi et al., 2015; Wani et al., 2017).

The availability of SSRs markers has played an important role in the development of the agricultural field, so for maize it was possible to observe an improvement in breeding programs as a result of the development of techniques that use molecular markers (Ahmad et al., 2017). The methods that use SSR markers have an advantage in terms of simplicity, efficiency, accessibility and reproducibility of the method,

so SSR markers are preferred in studies on testing the varietal purity and genetic diversity in maize and other crops (Sudharani, 2014; Jhansi et al., 2015; Wani et al., 2017; Adu et al., 2019).

In seeds quality control it is important to maintain or to confirm the genetic purity of an inbred line. Thus using methods based on SSRs markers or other techniques such as competitive allele specific PCR (KASP) or next generation sequencing (NGS) technology are very useful when phenotypic approach methods are far too laborious (Semagn et al., 2014; Raza et al., 2019; Vasile et al., 2020; Chen et al., 2021). The main problem is still the final cost of the implemented method and methods based on using SSRs markers are much more affordable than other modern technologies.

CONCLUSIONS

The chosen DNA extraction method was suitable and the extracted DNA met the desired criteria for the chosen PCR amplification method.

All SSRs markers chosen in this study gave good PCR amplification products under the tested reaction conditions and remained unaffected by variation made from initial conditions.

All the selected markers showed some degree of polymorphism on the chosen maize inbred lines. The highest number of estimated alleles was after using the SSR marker phi015 (five products were estimated) followed by markers umc1545, umc1448 and umc1117 (four alleles were estimated), markers phi102228 and phi083 (three alleles were estimated) and markers umc1061 and phi109275 (two alleles were estimated). Thus, the most polymorphic SSR marker of the eight SSRs markers used in this study was phi015 and the SSRs markers that showed low polymorphism were umc1061 and phi109275.

High genetic similarity was observed between lines LC1 and LC2 proving that that the varietal purity is preserved among seeds categories. Regarding varietal purity assessment among seeds categories for a better evaluation of the results much more seeds need to be analysed.

The SSRs markers turned out to be very useful tool for varietal purity and genetic diversity assessment being able to differentiate among closely related maize inbred lines and place the maize inbred lines into groupings based of genetic similarity.

Assessment of genetic diversity and varietal purity among different maize inbred lines can play an important role in hybrid maize breeding process.

This study can be a starting point for evaluating other maize inbred lines and selecting lines with desired traits in hybrid maize breeding program.

REFERENCES

Adu, G. B., Awuku, F. J., Amegbor, I. K., Haruna, A., Manigben, K. A., & Aboyadana, P. A. (2019). Genetic characterization and population structure of maize populations using SSR markers. *Annals of Agricultural Sciences, 64*(1), 47-54.

Ahmad, F., Akram, A., Farman, K., Abbas, T., Bibi, A., Khalid, S., & Waseem, M. (2017). Molecular markers and marker assisted plant breeding: current status and their applications in agricultural development. *Journal of Environmental and Agricultural Sciences, 11*, 35-50.

Balter, M. (2007). Seeking agriculture's ancient roots. *Science*, 316(5833), 1830-1835.

Bițică, A. R., Giura, A., & Cornea, P. (2016). The influence of the female parent on the inducing rate with five different inducer lines in maize DH technology. *Scientific Bulletin. Series F. Biotechnologies, 20*, 2285-1364.

Bocianowski, J., Nowosad, K., Wróbel, B., & Szulc, P. (2021). Identification of Associations between SSR Markers and Quantitative Traits of Maize (*Zea mays* L.). *Agronomy, 11*(1), 182.

Bonfini, L., Van den Bulcke, M. H., Mazzara, M., Ben, E., & Patak, A. (2012). GMOMETHODS: The European Union database of reference methods for GMO analysis. *Journal of AOAC International, 95*(6), 1713-1719.

Chen, Z., Tang, D., Ni, J., Li, P., Wang, L., Zhou, J., & Liu, J. (2021). Development of genic KASP SNP markers from RNA-Seq data for map-based cloning and marker-assisted selection in maize. *BMC Plant Biology, 21*(1), 1-11.

Gaikpa, D. S., Kessel, B., Presterl, T., Ouzunova, M., Galiano-Carneiro, A. L., Mayer, M., & Miedaner, T. (2021). Exploiting genetic diversity in two European maize landraces for improving Gibberella ear rot resistance using genomic tools. *Theoretical and Applied Genetics, 134*(3), 793-805.

Garcia-Vallvé, S., & Puigbo, P. E. R. E. (2009). DendroUPGMA: a dendrogram construction utility. *Universitat Rovira i Virgili*.

I.N.S. (2021). National Institute of Statistics. Productia vegetala la principalele culturi in anul 2020 (Plant production in the main crops in 2020). *Publishing house of the National Institute of Statistics* ISSN-L 1842-0575.

ISTA. (2021). International Seed Testing Association. International rules for seed testing. Rules 2021, Chapter 8, *Species and variety testing, i–8-28* (36).

Jhansi, G., Surender, M., Aparnna, S., & Sunitha, B. (2015). DNA finger printing of maize hybrids and development of molecular ID's using SSR markers. *Res. J. Agri. Sci, 6*(1), 43-47).

Li, Y., Bingxin, Y., Yiming, Y., Xiantao, H., Quanwei, L., Zhijie, L., & Dongxing, Z. (2016). Global overview of research progress and development of precision maize planters. International *Journal of Agricultural and Biological Engineering, 9*(1), 9-26.

Madobe, M. A., Raihan, M. S., Hasan, M., & Biswas, M. S. (2021). Morpho-molecular Characterization of Maize Inbred Lines Accelerating Parental Selection for Hybridization. *American Journal of Agricultural Science, Engineering and Technology, 5*(2), 431-452.

Oluwaranti, A., Edema, R., Ajayi, S. A., Atkinson, C. J., Asea, G., Makumbi, D., & Kwemoi, D. B. (2018). Characterization of elite maize inbred lines for drought tolerance using Simple Sequence Repeats markers. *International Journal of Agriculture and Environmental Research, 4*(2), 552-566.

Prasanna, B. M., Palacios-Rojas, N., Hossain, F., Muthusamy, V., Menkir, A., Dhliwayo, T., & Fan, X. (2020). Molecular breeding for nutritionally enriched maize: status and prospects. *Frontiers in genetics, 10*, 1392.

Raza, A., Mehmood, S. S., Shah, T., Zou, X., Yan, L., Zhang, X., & Khan, R. S. A. (2019). Applications of Molecular Markers to Develop Resistance Against Abiotic Stresses in Wheat. In M. Hasanuzzaman, K. Nahar, A. Hossain (Eds.), *Wheat Production in Changing Environments* (pp. 393-420). Springer, Singapore.

Semagn, K., Babu, R., Hearne, S., & Olsen, M. (2014). Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in crop improvement. *Molecular breeding*, *33*(1), 1-14.

Sendekie, Y.(2020). Review on; Seed Genetic Purity for Quality Seed Production. *International Journal of Scientific Engineering and Science*,*4*(10), 1-7

Chaudhary, S., Yashveer, S. Dinesh, D., Prajapati R., Kharb, P., & Kamboj, M.C. (2018). Utilization of SSR Markers for Seed Purity Testing in Popular Maize Hybrids. *Int.J.Curr. Microbiol. App. Sci*., *7*(4), 1117-1126.

Shiri, M. (2011). Identification of informative simple sequence repeat (SSR) markers for drought tolerance in maize. *African Journal of Biotechnology, 10*(73), 16414-16420.

Sserumaga, J. P., Makumbi, D., Ji, H., Njoroge, K., Muthomi, J. W., Chemining'wa, G. N. & Kim, H. (2014). Molecular characterization of tropical maize inbred lines using microsatellite DNA markers. *Maydica 59*(3), 267-274.

Sudharani, M., Rao, P. S., & SubbaRao, L. V. (2014). Identification of SSR markers for testing of hybridity and seed genetic purity in maize. *Int J Sci Res*, *3*(10), 92-95.

Sun, Q., Liu, J., Zhang, K., Huang, C., Li, L., Dong, J., & Ma, Z. (2021). De novo transcriptome assembly, polymorphic SSR markers development and population genetics analyses for southern corn rust (Puccinia polysora). *Scientific reports, 11*(1), 1-11.

Șuteu, D., Băcilă, I., Haș, V., Haș, I., & Miclăuș, M. (2014). Correction: Romanian Maize (*Zea mays*) inbred lines as a Source of genetic diversity in SE Europe, and Their Potential in Future Breeding efforts. *PLoSONE,9*(1),.https://dx.doi.org/10.1371/annotation/85f0b730-b723-4d59-a653d48b974c366c.

Tsonev, S., Todorovska, E. G., & Christov, N. K. (2015). A versatile fluorescence-based multiplexing assay for combined genotyping of SSR and insertion-deletion polymorphism (IDP) markers on capillary electrophoresis systems. *Bulgarian Journal of Agricultural Science, 21*(4), 843-850.

Vasile, V., Ciucă, M., Voaideș, C., & Cornea, C. P. (2020). DNA-based methods used for varietal purity detection in wheat cultivars. *AgroLife Scientific Journal, 9*(1), 342-354.

Vivodík, M., Balážová, Ž., Gálová, Z., & Petrovičová, L. (2018). Study of polymorphism of maize using DNA and protein markers. *Potravinarstvo, 12*(1), 157-165.

Wani, M. A., Khan, G. H., Gazal, A., & Lone, R. A. (2017). Genetic purity analysis in maize under temperate conditions. *Int. J. Curr. Microbiol. App. Sci, 6*(9), 2710-2722.

Woodhouse, M. R., Cannon, E. K., Portwood, J. L., Harper, L. C., Gardiner, J. M., Schaeffer, M. L., & Andorf, C. M. (2021). A pan-genomic approach to genome databases using maize as a model system. BMC *PlantBiol, 21*, 385.

Zawadi, M., Sibiya, J., Mashingaidze, K., Amelework, A. B., Kondwakwenda, A., & Musvosvi, C. (2021). Molecular characterization and diversity analysis of selected maize inbred lines using single-nucleotide polymorphism markers. *Canadian Journal of Plant Science, 101*(2), 240-248.

Zhang, W., Deng, S., Zhao, Y., Xu, W., Liu, Q., Zhang, Y., & Liu, B. (2021). qMrdd2, a novel quantitative resistance locus for maize rough dwarf disease. *BMC plant biology, 21*(1), 1-11.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

THE GREAT POTENTIAL OF ENTOMOPHTHORALEAN FUNGI FOR BIOLOGICAL CONTROL: A REVIEW

Mihaela Monica DINU[1, 2], Ana-Cristina FĂTU[2], Narcisa BĂBEANU[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd., District 1, Bucharest, Romania
[2]Research-Development Institute for Plant Protection, 8 Ion Ionescu de la Brad Blvd., District 1, Bucharest, Romania

Corresponding author email: mihaela.dinu@icdpp.ro

Abstract

Entomopathogenic fungi are well known for their role in the biological control of pests. The manipulation techniques of filamentous entomopathogenic fungi belonging to order Hypocreales (Beauveria bassiana, B. brongniartii, Metarhizium anisopliae, Lecanicillium longisporum, etc.) are already well developed in the biotechnological industry. At the moment, these types of fungi are the only ones authorized for inundative biological control of pest. However, numerous studies from recent years draw attention to some ecological attributes of order Entomophthorales as being more advantageous than order Hypocreales. In this review, we discuss the general characteristics of the Entomophthorales, the differences between Hypocreales and Entomophthorales, and the advances and challenges of using entomophthoralean fungi as myco-insecticides.

Key words*: entomopathogenic fungi, Entomophthorales, entomophthoralean fungi, biological control.*

INTRODUCTION

Entomophthoralean fungi

The order Entomophthorales is currently classified as part of the subphylum Entomophthoromycotina. The majority of extant species in this subphylum are soil living and saprotrophic, but most studied species are obligate or facultative parasites of insects (Möckel et al., 2022). In the present review, the entomopathogenic fungi we name entomophthoralean fungi to refer only to species of the ord. Entomophthorales.

Many species within Entomophthorales are pathogenic for insects and few species attack nematodes and mites (Jaronski, 2014). Entomophthoralean fungi are, from this point of view, considered important candidates for pest management with biological control agents (BCA). Even the name of ord. Entomophthorales is suggestive, the etymology of the word revealing that it comes from ancient Greek and it translates mot-a-mot "insect destroyer" (gr. entomo-= referring to insects - actually meaning “notched”, refers to the segmented body plan of the insect, and phthorá = “destruction”) (Britannica, 2019).

Biological control

The term “microbial control” was first used by Steinhaus in 1949: “that phase of biological control concerned with the employment by man of microorganisms for the control and reduction of the number of animals (or plants) in particular area or a given population” (Ravensberg, 2010). A comprehensive study of the history of biological control was conducted in the early twentieth century by Steinhaus (1956). However, this idea has its roots in entomology studies and cannot be associated with a single researcher (Stern et al., 1959; DeBach, 1964; van den Bosch, 1971).

Nowadays, biological control (or biocontrol) is defined in the plant protection discipline as an ecological alternative to chemical crop protection and a component of the integrated pest management (IPM) strategy. Biological control strategies use BCA as natural enemies (viruses, bacteria, fungi, arthropods, etc.) named beneficial organisms, to control crops, pests, and diseases. Eilenberg et al. (2001) suggested that the term “biological control should be restricted to the use of living organisms.” However, other authors consider botanical insecticides (plant extracts such as essential oils, alkaloids,

flavonoids, glycosides, esters, and fatty acids) are also biological control tools (Hikal et al., 2017; Tembo et al., 2018). For example, The International Biocontrol Manufacturers Association (IBMA) defines biological control as: “pest and disease control for plant protection based on living organisms and naturally-sourced compounds”. Also, IBMA considers three types of biocontrol: conservation biological control, augmentative biological control, and classical biological control.

According to Eilenberg et al. (2001), biological control can be carried out following four strategies: (1) Classical biological control (“the intentional introduction of an exotic, usually co-evolved, biological control agent for the permanent establishment and long-term pest control”); (2) Inoculation biological control (“the intentional release of a living organism as a biological control agent with the expectation that it will multiply and control the pest for an extended period, but not permanently”); (3) Inundation biological control (“the use of living organisms to control pests when control is achieved exclusively by the released organisms themselves”); (4) Conservation biological control (“modification of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests”). Inundation biological control use bioinsecticides based on entomopathogenic fungi (mycoinsecticides) and it is the most widely used microbial control strategy. The application of mycoinsecticides in pest management is similar to chemical pesticides.

The term "biological control" has had many definitions and approaches over time, but the four strategies are now generally accepted by most authors.

European legislation on BCA

European Union legislation on the sustainable use of pesticides, EU Directive 2009/128/EC, encourages the development and introduction of IPM and alternative approaches or techniques to reduce dependence on pesticides. However, the implementation of this directive at the Member State level has not been as successful, and “very little progress has been made in promoting the uptake of alternative techniques, which are the key to ensuring real pesticide dependency reductions” (Committee on the Environment, Public Health, and Food Safety, 2019). At present, the approval of pesticides based on microorganisms follows the exact requirements as for chemicals. Research on biological low-risk pest control products should be encouraged, and legislation on the authorization of micro-organisms for use in plant protection should be adapted to the specific properties and hazards of micro-organisms and not clone chemical pesticide regulations (Sundh & Eilenberg, 2021; Reinbacher et al., 2021). European legislation has approved for plant protection 449 active substances, safeners, and synergists, of which only 66 are microbials (strains of fungi, bacteria, and viruses). The approval expiration is set on 30.04.2022 for 29 of these strains (source EU Pesticides database, accessed 01.03.2022). Currently, no Entomophthorales-based mycoinsecticides are available (Litwin et al., 2020). Member States of the European Union endorsed four legal acts on February 10, 2022, which will shorten the authorization process of biological plant protection products based on micro-organisms. These acts reflect the latest scientific developments and could become applicable in Q4 2022 (European Commission, 2022). Relaxing the regulations governing the approval of microorganism-based plant protection products will reduce costs and speed up the development of new items, such as entomophthoralean-based mycoinsecticides. In recent years, progress has been made in the study of these fungi formulation. This review aims to provide an overview of entomophthoralean fungi and their potential as microbial control agents.

MATERIALS AND METHODS

We systematically searched several scientific databases such as ScienceDirect and Wiley Online Library, for literature from 1980 to 2022 using the following search terms: “entomophthoralean”, “Entomophthorales” and “Entomophthoromycotina”. We retrieved 1456 results, to which we added publications from Academia, Research Gate, and Google Scholar. Duplicates and publications that were not relevant to entomopathogens or biological control of pests were eliminated after a thorough screening. The exclusion criteria selected was the field of application. A comprehensive search

of the European Commission website and EUR-Lex database for legislation relating to Plant Protection Products based on microorganisms was also done, yielding three pertinent legal acts. Following the suitability evaluation, a total of 75 papers were selected for this review.

RESULTS AND DISCUSSIONS

Entomopathogenic fungi

Entomopathogenic microorganisms are currently used worldwide in microbial control (the use of micro-organisms in biological control) for controlling pests in agriculture, forestry, horticulture, etc., being a healthier alternative, environmentally friendly, and more sustainable to conventional chemical insecticides. Unlike other insect pathogenic microorganisms, most entomopathogenic fungi have the unique property of infecting through the cuticle, therefore they do not need to be ingested. (Roberts & Hajek, 1992). Only a few taxa (e.g. Culicinomyces) infect the host by invading through the alimentary canal (Inglis et al., 2001).

One of the earliest known accounts of insect diseases was found in the writings of Aristotle in Historia Animalium (probably written between 335 and 322 B.C.) (Steinhaus, 1956). The first known studies on entomopathogenic fungi were conducted in the 1800s to find a solution for managing silkworm disease, later called white muscardine, which severely affected the silk industry (Steinhaus, 1975). Following this discovery, studies of the interaction between arthropods and entomopathogenic fungi have been of particular interest due to their potential use of fungal entomopathogens for pest control (Keller, 1998; Hajek & Goettel, 2008.).

Not all arthropod species are necessarily closely associated with entomopathogenic fungi (Humber 2012a), but the number of insect-associated fungi is very high (Blackwell, 2011). Currently, arthropod species are estimated to be between 5-10 million (Ødegaard, 2000). The entomopathogenic property of about 700 fungal species belonging to 90 genera is already known, but only a few have been studied (Khachatourians & Qazi, 2008). Many researchers have studied entomopathogenic fungi (EPF) belonging to ord. Hypocreales (formerly Deuteromycetes) for use in the biological control of pests, especially fungi of the genera *Beauveria*, *Metarhizium*, *Isaria* (*Paecilomyces*), and *Lecanicillium* (*Verticillium*) (Inglis et al., 2001). For example, McCoy et al. (1988), Evans (1997), Ferron et al. (1991), Roberts & Hajek (1992), Tanada & Kaya (1993), Hajek & St. Leger (1994), Boucias & Pendland (1998), Wraight & Carruthers (1999), Zimmermann (2007), Butt et al. (2016), Islam et al. (2021), Rajula et al. (2021) to name a few, reviewed the main information about hyphomycetes and their use as microbial insecticides. Accordingly, *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin, *Isaria fumosorosea* Wize, *Metarhizium anisopliae* (Metchnikoff) Sorokin, and *Lecanicillium lecanii* (Zimmerman) Viegas were mainly studied (Bamisile et al., 2021). These species have a wide range of hosts and are easy to produce on an industrial scale. Epizootics usually occur only in insect populations in soil (Keller & Zimmerman, 1989).

On the other hand, entomophthoralean fungi have a high host specificity (Jaronski, 2014) and could be combined with useful arthropods in pest control which is one of the significant advantages of using these fungi in biological control programs. Furthermore, these entomopathogens occur in temperate, subtropical, and equatorial climates, and they are natural enemies of many harmful insects of agricultural interest such as thrips, aphids, lepidopterous adults, and larvae. They have great potential in triggering epizootics in foliar insect or mite populations (Evans, 1989) and could remain active for years as resistant spores. In an experiment performed in America, the fungus caused an epizootic five years after its artificial introduction, demonstrating the advantages of a specific trait of these fungi, that could generate resistant spores. The identification and confirmation of the strain have been made using enzyme and restriction fragment length polymorphism analyses (Hajek et al., 1990).

Entomophthoralean fungi have been less studied, mainly because their use in biological control has proved to be more difficult due to the limitations on mass production, which seems to be the most critical bottleneck (Ravensberg, 2010). However, their great biological control potential has long been known (Pell et al., 2001).

Augmentation strategies followed by conservation, i.e., use of irrigation, an increase of humidity, and providing banker plants with alternative hosts (reservoirs of entomopathogens) have been shown to have very good results (Wilding et al., 1986; Shah et al., 2004; Gonzalez et al., 2016; Dinu et al., 2017). But any microorganism used to control an insect must be registered with the appropriate regulatory body. The approval procedure is both costly and lengthy. A commercial motivation of inoculation biological control is insufficient (Jaronski, 2014). Formulation and mass production of entomophthoralean fungi for inoculation biological control has been and continues to be a major challenge.

Entomophthorales taxonomy

The subphylum Entomophthoromycotina has been the subject of discussion among taxonomists for decades (Möckel et al., 2022). It originated from the oldest known lineages of terrestrial fungi, most likely appearing in the Silurian, more than 400 mya (Humber, 2012b; Gryganskyi et al., 2013). It does not appear to have co-evolved with insects, which occurred 300 million years ago, yet they have high degree of specialization to their hosts. The appearance and the radiation of Pterygota (winged insects) have been shown to contribute to the dispersal of the entomopathogenic lines of this phylum. Currently, Pterygota constitute the most parasitized host group (Möckel et al., 2022).

One of the newest phylogenetic classifications is proposed by Spatafora et al. (2016) and includes two phyla, Mucoromycota and Zoopagomycota, with Entomophtoromycotina classified as a subphylum of Zoopagomycota. Also, in the most recent phylogenomic studies that reassessed the phylogeny of this group, respectively based on conserved genes encoding ribosomal RNA and RNA polymerase II subunits, the authors taxonomically classify these fungi in the subphylum Entomophthoromycotina (Li et al., 2021; Möckel et al., 2022), within three classes, three orders and 6 families. The study on entomophthoromycotan genome characteristics has lately grown and it will also reveal key evolutionary mechanisms behind selection adaptation (Hajek, 2004). A novel isolation unit of entomophthoralean fungi, has been developed lately (Hu et al., 2018). It is operational in the field, making it easier to collect conidia, preserve them, and identify new species and fungal strains.

Currently, order Entomophthorales follows the same classification as the one proposed by Humber (2012b):

Order Entomophthorales G. Winter, Rabenh. Krypt.-Fl.

Family Ancylistaceae J. Schröt.

Family Completoriaceae Humber

Family Entomophthoraceae Nowak.

Subfamily Entomophthoroideae S. Keller

Subfamily Erynioideae S. Keller

Family Meristacraceae Humber

High potential of entomophthoralean fungi as naturally occurring biological control agents

Entomophthoralean fungi have unique physiological characteristics which are important factors for biological control effectiveness.

Host specialization evolved genetically in response to the challenge of utilizing resources and dealing with the immune systems of different hosts. Genomic and transcriptome techniques have the potential to help researchers better understand the molecular processes of entomophthoralean pathogenesis (Licht et al., 2016).

The behavior of diseased insects inside the colony has an impact on pathogen transmission. Arthropods infected with entomophthoralean fungi have been observed to exhibit behavioral patterns that facilitate fungal dissemination. (Roy et al., 2006). For example, some entomophoralean diseases drive infected insects to migrate to the plant's top before dying. (summit disease syndrome). Consequently, the conidia ejected by the insect's cadaver have a higher chance of landing on possible hosts, nearby or on the same plant (Figure 1).

Figure 1. Epizooty in an aphids' colony ©MMD

This is probably the most successful evolutionary adaptation of these pathogens (Inglis et al., 2001). Insect behavior is even more altered: once at the top of the plant, they fix themselves to the plant with their mandible or feet, so that when they die, they remain immovable to the plant. This type of behavior generally comes across solitary insects. The gregarious insects (as aphids) stay in the colony and become reservoirs of infectious spores. Rhizoids, which are specific to some fungal species, grow from the insect's abdomen and anchor it to the substrate. (Bałazy, 1993).

On the other hand, entomophtoralean fungal spores are actively discharged (forcibly ejected), so they have greater chances to come in contact with another host (Figure 2).

Figure 2. Forcibly discharged conidia ("halo" of conidia) in a colony of aphids from the corpse of *Sitobion* sp. infected by an entomophthoralean fungus ©MMD

More than that, if the ejected conidia fall on a non-host surface, it will produce higher-order conidia, named secondary conidia. A secondary conidium may germinate to produce a tertiary conidium, also actively discharged (Pell et al., 2001). When the spores reach a potential host, the mechanism specific to entomopathogenic fungi is triggered, respectively invasion of the host by germ hyphae produced by conidia. It multiplies inside insect hosts as hyphae, hyphal bodies, or protoplasts.

The central hypothesis as entomophthoralean grows as protoplasts in the hemolymph of insects is to advantage the fungus in escaping host immune recognition (Boomsma et al., 2014). Because protoplasts lack a sugar-rich cell wall, they are not recognized as invaders by the hemocytes that normally protect insects. The

insect does not die immediately but slow down, feed less, stop laying eggs, or deposit eggs in unsuitable spots (Roy et al., 2016). For example, the average survival time of insects infected with the entomopathogen Pandora is 5-6 days. This feature encourages the rapid spread of the disease in pest populations (Görg et al., 2021).

Solitary insects seek cooler places, such as the top of the plant, during the last 1-2 days of infection. These locations are favorable for pathogen dissemination. Before the host dies, protoplasts acquire cell walls and are ready to resume their life cycle. Shortly after the insect dies, the fungus sporulates from its body. Conidia are produced externally on cadavers and are relatively short-lived (Licht et al., 2016).

Numerous authors have studied how dissemination is done by beneficial organisms used in biological control and have observed that they carry conidia in the foraging activity (Baverstock et al., 2008; Wells et al., 2011). It has also been observed that the attack of these entomopathogens stimulates transgenerational wing induction in some insects, thus contributing to the pathogen's spread (Hatano et al., 2012).

When the cold season approaches, the absence of the host insects triggers other physiological processes in entomophthoralean fungi. Winter survival is essential because the method for overwintering can be a key role in triggering epizootics during the seasons. Entomophthoralean fungi have four known winter survival strategies.: (1) as hyphal bodies in dead hosts (Keller, 1987); (2) as hyphal bodies in hibernating (living) hosts; (3) through a slow disease development and a slow disease transmission among hibernating hosts (Eilenberg et al., 2013); (4) as resting spores in soil (Hajek et al., 2018). Some *Conidiobolus* spp. and other less specialized entomophthoralean pathogens can survive and grow in the soil (Gryganskyi et al., 2017).

Production and formulation as biological control products

The inundation biological control requires large quantities of mycoinsecticide and mass production is the most critical bottleneck of entomophthoralean fungi (Ravensberg, 2010). Entomophthorales species have specific nutritional requirements for growth and sporulation *in vitro* (Latgé, 1981) They can be classified into four broad groups: (1) *Conidiobolus* spp. (family Ancylistaceae), which can be grown on standard media; (2) *Batkoa* (subfamily Entomophthoroideae), *Erynia,* and *Zoophthora* spp. (subfamily Erynioideae), which need supplements; (3) *Entomophthora* and *Entomophaga* spp. (subfamily Entomophthoroideae), which need special media; (4) *Strongwellsea* (subfamily Erynioideae) and *Neozygites* (order Neozygitales, family Neozygitaceae), which need tissue culture media (Keller, 1997; Pell et al., 2001). A synthesis of the formulation of fungi belonging to the order Entomophthorales was made by Pell et al. (2001). The author describes experiments in which various stages of the biological cycle of entomophthoralean fungi were exploited: production of the hyphal stage, formulation of hyphal material, and production of resting spores. By 2001, 46 species that produced resting spore *in vitro* had been described (Pell et al., 2001). The fragile nature of the mycelium and conidia makes these fungi more difficult to formulate than Hypocreales, which has led to their lack of commercial success.

Several other entomophthoralean formulations with fungal mycelium have been tested in recent years, some of them including broomcorn pellets (Hua & Feng, 2003), granules of broomcorn millet and polymer gel (Zhou & Feng, 2009), alginate pellets (Zhou & Feng, 2010), secondary conidia in inverted emulsion (water-in-oil formulation) (Batta et al., 2011), mycelium-encapsulated alginate pellets that float and sporulate continuously for utilization in watery fields (Zhou et al., 2015), encapsulation in calcium alginate beads (Muskat et al., 2022, a), to name a few. A complex nutrition source containing skimmed milk, yeast extract, and a low-cost fungal protein has increased biomass in a liquid shaking culture, according to the results of a recent experiment (Muskat et al., 2022, a). This is the first successful attempt to explore biomass production in a liquid media and it is a crucial step toward the fungus's potential for mass production.

CONCLUSIONS

Even though order Entomophthorales has some ecological advantages over order Hypocreales, there are no commercially available plant

entomophthoralean mycoinsecticides on the market. They were not developed because the alternative was more economically viable. Despite mass production challenges, significant progress has been made in determining the best formulation for entomophthoralean species. The recent submerged fermentation laboratory experiment success paves the way for large-scale fermentation and formulation processes. Given the recent legislation relaxation regarding the use of microorganisms in pest control and the European Parliament's recommendations to reduce pesticide dependency, it is critical to investigate and utilize all available natural resources. The physiology of these fungi and the multitrophic interactions in the environment are not yet fully understood, and future studies will need to focus on this. Research on the physiology of entomphthoralean fungi is essential for developing strategies for mass production, storage, and application.

In the context of regulatory relaxation and the newest results on the mass production process, this paper outlined the major characteristics of entomophthoralean fungi and their current development potential as plant protection products.

REFERENCES

Bałazy S. (1993). *Flora of Poland. Fungi (Mycota), vol. 24, Entomophthorales*. Polish Academy of Science, Warsaw.

Bamisile, B. S., Akutse, K. S., Siddiqui, J. A., & Xu, Y. (2021). Model application of entomopathogenic fungi as alternatives to chemical pesticides: prospects, challenges, and insights for next-generation sustainable agriculture. *Frontiers in Plant Science, 12*. 741–804.

Batta, Y., Rahman, M., Powis, K., Baker, G., & Schmidt, O. (2011). Formulation and application of the entomopathogenic fungus: *Zoophthora radicans* (Brefeld) Batko (Zygomycetes: Entomophthorales). *Journal of Applied Microbiology*, 110. 831–839

Baverstock, J., Baverstock, K.E., Clark, S.J., & Pell, J.K. (2008). Transmission of *Pandora neoaphidis* in the presence of cooccurring arthropods. *Journal of Invertebrate Pathology*, 98. 356–359.

Blackwell, M. (2011). The fungi: 1, 2, 3..5.1 million species? *American Journal of Botany, 98*(3). 426–438.

Boomsma, J. J., Jensen, A. B., Meyling, N. V., & Eilenberg, J. (2014). Evolutionary interaction networks of insect pathogenic fungi. *Annual Review of Entomology, 59*. 467–485.

Boucias, D. G., & Pendland, J. C. (1998). *Principles of Insect Pathology.* Kluwer Academic Publishers, Boston/Dordrecht/London.

Britannica (2019) The Editors of Encyclopaedia. "entomology". *Encyclopedia Britannica*. Retrieved March 1, 2022 from https://www.britannica.com/science/entomology.

Butt, T.M., Coates, C.J., Dubovskiy, I.M., & Ratcliffe, N.A. (2016). Entomopathogenic Fungi: New Insights into Host-Pathogen Interactions. *Advances in Genetics, 94*. 307–64.

Committee on the Environment, Public Health and Food Safety of European Parliament (2019). Report on the implementation of Directive 2009/128/EC on the sustainable use of pesticides. Retrieved March 1st, 2022 from https://www.europarl.europa.eu/doceo/document/A-8-2019-0045_EN.html .

DeBach, P. (1964). *Biological Control of Insect Pests and Weeds.* London: Chapman & Hall.

Dinu, M. M., Bloemhard, C. M. J., van Holstein-Saj, R. & Messelink, G. J. (2017). Exploring opportunities to induce epizootics in greenhouse aphid populations. *Acta Horticulturae, 1164.* 371–376.

Eilenberg, J., Hajek, A., & Lomer, C. (2001). Suggestions for unifying the terminology in biological control. *BioControl, 46.* 387–400.

Eilenberg, J., Thomsen, L., & Jensen A. (2013). A third way for entomophthoralean fungi to survive the winter: slow disease transmission between individuals of the hibernating host. *Insects,* 4. 392–403.

European Commission (2022). Questions and Answers: Farm to Fork: new rules for micro-organisms used in plant protection products. *Press material from the Commission Spokesperson's Service*. Retrieved March 1st, 2022 from https://ec.europa.eu/ commission/presscorner/detail/en/qanda_22_852 .

European Parliament and of the Council (2019). EU Directive 2009/128/EC establishing a framework for Community action to achieve the sustainable use of pesticides.

Evans, H.C. (1989). Mycopathogens of insects of epigeal and aerial habitats. In Wilding, N., Collins, N.M., Hammond, P.M. and Webber, J.F. (Ed.), *Insect-Fungus Interactions* (pp. 205–238). Academic Press, London.

Evans, H.F. (1997). Microbial insecticides: novelty or necessity? *BCPC Symposium Proceedings No. 68*. Coventry, BCPC, Farnham.

Ferron, P., Fargues, J., & Riba, G. (1991). Fungi as microbial insecticides. In Arora, D.K., Ajello, L. and Mukerji, K.G. (Ed.), *Handbook of Applied Mycology* (pp. 665–706). Marcel Dekker, New York.

Gonzalez, F., Tkaczuk, C., Dinu, M. M., Fiedler, Ż., Vidal, S., Zchori-Fein, E., & Messelink, G. J. (2016). New opportunities for the integration of microorganisms into biological pest control systems in greenhouse crops. *Journal of Pest Science*, 89. 295–311

Görg, L.M., Eilenberg, J., Jensen, A.B, Jensen, A. H., & Gross, J. (2021). Pathogenicity against hemipteran vector insects of a novel insect pathogenic fungus from Entomophthorales (*Pandora* sp. nov. inedit.) with potential for biological control. *Journal of Invertebrate Pathology*, *183:*107621.

Gryganskyi, A.P., Humber, R.A., Smith, M.E., Hodge, K., & Huang, B. (2013). Phylogenetic lineages in

Entomophthoromycota. *Molecular Phylogenetics and Evolution, 65.* 682–694.

Gryganskyi, A. P., Mullens, B. A., Gajdeczka, M. T., Rehner, S. A., Vilgalys, R., & Hajek, A. E. (2017). Hijacked: Co-option of host behavior by entomophthoralean fungi. *PLoS pathogens*, 13(5), e1006274.

Hajek, A. E., Humber, R. A., Elkinton, J. S., May, B., Walsh, S. R., & Silver, J. C. (1990). Allozyme and restriction fragment length polymorphism analyses confirm *Entomophaga maimaiga* responsible for 1989 epizootics in North American gypsy moth populations. *Proceedings of the National Academy of Sciences, 87*(18). 6979–6982.

Hajek, A. E., & St. Leger, R. J. (1994). Interactions between fungal pathogens and insect hosts. *Annual Review of Entomology, 39*. 293–322.

Hajek, A. (2004). Classical biological control. In *Natural Enemies: An Introduction to Biological Control* (pp. 39–61). Cambridge: Cambridge University Press.

Hajek, A.E., & Goettel, M.S. (2008). Guidelines for evaluating effects of entomopathogens on non-target organisms. In L.A. Lacey and H.K. Kaya (Ed.), *Field manual of techniques in invertebrate pathology, 2nd edition* (pp. 815–833). Springer, Dordrecht.

Hajek, A.E., Steinkraus, D.C.,& Castrillo, L.A. (2018). Sleeping Beauties: Horizontal Transmission via Resting Spores of Species in the Entomophthoromycotina. *Insects*, 9(3):102.

Hatano, E., Baverstock, J., Kunert, G., Pell, J.K., & Weisser, W.W. (2012). Entomopathogenic fungi stimulate transgenerational wing induction in pea aphids, *Acyrthosiphon pisum* (Hemiptera: Aphididae). *Ecological Entomology, 37*. 75–82.

Hikal, W. M, Baeshen, R. S., & Said-Al Ahl, H.A.H. (2017). Botanical insecticide as simple extractives for pest control. *Cogent Biology, 3:1.*

Hu, Y., Chen, C.,Ye, S., & Hu, H. (2018). Development of a novel isolation unit for entomophthoralean fungi. *Journal of Invertebrate Pathology*, 155. 1–4.

Hua, L., & Feng, M. G. (2003). New use of broomcorn millets for production of granular cultures of aphid-pathogenic fungus *Pandora neoaphidis* for high sporulation potential and infectivity to *Myzus persicae*. *FEMS Microbiology Letters, 227.* 311−317.

Humber, R. A. (2012a). Identification of entomopathogenic fungi. In Lacey L.A. (Ed), *Manual of Techniques in Invertebrate Pathology*, *2nd ed.* (151–187). Academic Press; San Diego, CA, USA.

Humber, R. A. (2012b). Entomophthoromycota: a new phylum and reclassification for entomophthoroid fungi. *Mycotaxon, 120*(1). 477–492.

Inglis, G. D., Goettel, M.S., Butt, T. M., & Strasser, H. (2001). Use of hyphomycetous fungi for managing insect pests. In T.M. Butt, C. Jackson and N. Magan (Ed.), *Fungi as biocontrol agents: progress, problems and potential* (pp. 23–69). CAB International, Wallingford.

Islam, W., Adnan, M., Shabbir, A., Naveed, H., Abubakar, Y. S., Qasim, M., Tayyab, M., Noman, A., Nisar, M. S., Khan, K.A., & Ali, H. (2021). Insect-fungal-interactions: A detailed review on entomopathogenic fungi pathogenicity to combat insect pests. *Microbial Pathogenesis, 159*. 105–122.

Jaronski, S.T. (2014). Chapter 11 - Mass Production of Entomopathogenic Fungi: State of the Art. In Morales-Ramos, J.A., Rojas, G. M., and Shapiro-Ilan, D. I (Ed.), *Mass Production of Beneficial Organisms* (pp. 357-413). Academic Press.

Keller, S. (1987). Observations on the overwintering of *Entomophthora planchoniana*. *Journal of Invertebrate Pathology*, 50. 333–335

Keller, S., & Zimmerman, G. (1989). Mycopathogens of soil insects. In Wilding, N., Collins, N.M., Hammond, P.M. and Webber, J.F. (Ed.), *Insect–Fungus Interactions* (pp. 240–247). Academic Press, London.

Keller, S. (1997). The genus Neozygites (Zygomycetes, Entomophthorales) with special refere-nce to species found in tropical regions. *Sydowia, 49.* 118-146.

Keller, S. (1998). Use of fungi for pest control in sustainable agriculture. *Phytoprotection, 79*(4). 56–60.

Khachatourians, G. G., & Qazi, S. S. (2008). Entomopathogenic fungi: biochemistry and molecular biology. In Brakhage, A. A. and Zipfel, P. F. (Ed.), *Human and Animal Relationships* (33–61). Berlin, Heidelberg: Springer.

Latgé, J. P. (1981). Comparaison des exigences nutritionelles des Entomophthorales. *Annals of Microbiology (Inst Pasteur) 132B.* 299–306.

Li, Y., Steenwyk, J. L., Chang, Y., Wang, Y., James, T. Y., Stajich, J.E., Spatafora, J. W., Groenewald, M., Dunn, C. W., Hittinger, C. T., Shen,X. X., & Rokas, A. (2021). A genome-scale phylogeny of the kingdom Fungi. *Current Biology*, 31-*8.* 1653–1665.

Licht, H. D. F., Hajek, A. E., Eilenberg, J., & Jensen, A. B. (2016). Utilizing genomics to study entomopathogenicity in the fungal phylum Entomophthoromycota: a review of current genetic resources. *Advances in genetics*, 94. 41–65.

Litwin, A., Nowak, M. & Różalska, S. (2020). Entomopathogenic fungi: unconventional applications. *Reviews in Environmental Science and Bio/Technology* 19, 23–42

McCoy, C. W., Samsom, R. A., &. Boucias, D. G. (1988). Entomogenous fungi. In Ignoffo, C.M. (Ed.), *Handbook of natural pesticides, Volume 5: Microbial insecticides, Part A, Entomogenous protozoa and fungi* (pp. 151–236). CRC Press, Boca Raton.

Möckel, L., Meusemann, K., Misof, B., Schwartze, V. U., De Fine Licht, H. H., Voigt, K., Stielow, B., de Hoog, S., Beutel, R. G., & Buellesbach, J. (2022). Phylogenetic Revision and Patterns of Host Specificity in the Fungal Subphylum Entomophthoromycotina. *Microorganisms 10*, *256.*

Muskat, L.C., Görg, L.M., Humbert, P., Eilenberg, J., & Patel, A.V. (2022, a). Encapsulation of the psyllid-pathogenic fungus *Pandora* sp. nov. inedit. and experimental infection of target insects. *Pest Management Science. 78(3).* 991–999.

Muskat, L.C., Przyklenk, M., Humbert, P., Eilenberg, J., & Patel, A.V. (2022, b). Fermentation of the psyllid-pathogenic fungus *Pandora* sp. nov. inedit. (Entomophthorales: Entomophthoraceae), *Biocontrol Science and Technology*, 0. 1–22.

Ødegaard, F. (2000) How many species of arthropods? Erwin's estimate revised. *Biological Journal of the Linnean Society 71.* 583–597.

Pell, J. K., Eilenberg, J., Hajek, A. E., & Steinkraus, D. C. (2001). Biology, ecology and pest management potential of entomophthorales. In Magan, N., Butt, T. M. and Jackson, C. (Ed.), *Fungi as biocontrol agents* (pp. 71-153). CABI International, Wallingford, Oxon.

Rajula, J., Karthi, S., Mumba, S., Pittarate, S., Thungrabeab, M., & Krutmuang, P. (2021). Chapter 4 - Current status and future prospects of entomopathogenic fungi: A potential source of biopesticides, Editor(s): Surajit De Mandal, Ajit Kumar Passari, *Recent Advancement in Microbial Biotechnology,* Academic Press. 71–98.

Ravensberg, W.J. (2010). The development of microbial pest control products for control of arthropods: a critical evaluation and a roadmap to success. *Ph.D. Thesis* Wageningen University, Wageningen, NL.

Reinbacher, L., Bacher, S., Praprotnik, E., & Grabenweger, G. (2021). Standard non-target tests for risk assessment of plant protection products are unsuitable for entomopathogenic fungi - a proposal for a new protocol. *Journal of Soils and Sediments,* 21, 2357–2368.

Roberts, D. W., & Hajek, A. E. (1992). Entomopathogenic Fungi as Bioinsecticides. In Leatham, G.F. (Ed.), *Frontiers in Industrial Mycology*. Springer, Boston, MA.

Roberts, D.W., Fuxa, J.R., Gaugler, R., Goettel, M., Jaques, R., & J. Maddox (1990). Use of insect pathogens. In Pimentel, D. and Hanson, A.A. (Ed.), *Handbook of pest management in agriculture, Vol.II.* (pp. 243–278). CRC Press, Boca Raton.

Roy, H. E., Steinkraus, D. C., Eilenberg, J., Hajek, A. E., & Pell, J. K. (2006). Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts. Annual Review of Entomology, 51.331–357.

Shah, P. A., Clark, S. J., & Pell, J. (2004). Assessment of aphid host range and isolate variability in *Pandora neoaphidis* (Zygomycetes: Entomophthorales). *Biological Control,* 29. 90–99.

Spatafora, J. W., Chang, Y., Benny, G. L., Lazarus, K., Smith, M. E., Berbee, M. L., Bonito, G., Corradi, N., Grigoriev, I., Gryganskyi, A., James, T. Y., O'Donnell, K., Roberson, R. W., Taylor, T. N., Uehling, J., Vilgalys, R., White, M. M., & Stajich, J. E. (2016). A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. *Mycologia*, *108(5).* 1028–1046.

Steinhaus E. A. (1949). *Principles of Insect Pathology*. New York, McGraw-Hill Book Co., Inc.

Steinhaus E. (1956). Microbial control - the emergence of an idea. A brief history of insect pathology through the nineteenth century. *Hilgardia 26(2).*107–160.

Steinhaus, E. A. (1975). *Disease in a Minor Chord.* Ohio State University Press, Columbus.

Stern, V. M, Smith, R. F., van den Bosch, R., & Hagen, K. S. (1959). The integrated control concept. *Hilgardia*, *29.* 81–101.

Sundh I., & Eilenberg, J. (2021). Why has the authorization of microbial biological control agents been slower in the EU than in comparable jurisdictions? *Pest Management Science.77(5).* 2170–2178.

Tanada, Y., & Kaya, H. K. (1993). *Insect Pathology.* San Diego, Academic Press.

Tembo, Y., Mkindi, A. G., Mkenda, P. A., Mpumi, N., Mwanauta, R., Stevenson, P. C., Ndakidemi, P. A., & Belmain, S. R. (2018). Pesticidal Plant Extracts Improve Yield and Reduce Insect Pests on Legume Crops Without Harming Beneficial Arthropods. *Frontiers in Plant Science, 9*. 1425.

Van den Bosch, R. (1971). Biological control of insects. *Annual Review of Ecology and Systematics,* 2:1. 45–66.

Wells, P.M., Baverstock, J., Majerus, M.E.N., Jiggins, F.M., Roy, H.E., & Pell, J.K. (2011). The effect of the coccinellid *Harmonia axyridis* (Coleoptera: Coccinellidae) on transmission of the fungal pathogen *Pandora neoaphidis* (Entomophthorales: Entomophthoraceae). *European Journal of Entomology, 108*. 87–90.

Wilding, N., Mardell, S.K., & Brobyn, P.J. (1986). Introducing *Erynia neoaphidis* into a field population of *Aphis fabae*: form of the inoculum and effect of irrigation. *Annals of Applied Biology, 108 (2)*. 373–385.

Wraight, S.P., & Carruthers, R.I. (1999). Production, delivery, and use of mycoinsecticides for control of insects' pests on field crops. In: Hall, F.R. and Menn, J.J. (Ed), *Biopesticides: use and delivery* (pp. 233–269). Humana Press, Totowa.

Zhou, X., & Feng, M. G. (2009). Sporulation, storage and infectivity of obligate aphid pathogen *Pandora nouryi* grown on novel granules of broomcorn millet and polymer gel. *Journal of Applied Microbiology, 107*: 1847−1856.

Zhou, X., & Feng, M. G. (2010). Improved sporulation of alginate pellets entrapping *Pandora nouryi* and millet powder and their potential to induce an aphid epizootic in field cages after release. *Biological Control 54.* 153−158.

Zhou, X., Su, X., & Liu, H. (2015). A floatable formulation and laboratory bioassay of *Pandora delphacis* (Entomophthoromycota: Entomophthorales) for the control of rice pest *Nilaparvata lugens* Stål (Hemiptera: Delphacidae). *Pest Management Science, 72(1).* 150–154.

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Science and Technology,17:9*. 879-920.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

ENTOMOPATHOGENIC BACTERIA VIRULENCE FACTORS AND TARGET PESTS

Daniel-Nicolae COJANU[1, 2], Oana-Alina BOIU-SICUIA[1, 2], Ana-Maria ANDREI[1], Călina Petruţa CORNEA[2]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd., District 1, Bucharest, Romania
[2]Research-Development Institute for Plant Protection, 8 Ion Ionescu de la Brad Blvd., District 1, Bucharest, Romania

Corresponding author email: sicuia_oana@yahoo.com

Abstract

Bacillus spp. gained worldwide recognition and continues to be both a benchmark in biological control and also an important source of biological material for future genetic approaches. Although predominant bioinsecticidal toxins are derived from Bacillus thuringiensis (Bt) varieties, there are several other virulence factors associated with different Gram-positive bacteria, as well as with Gram-negatives. Identifying the best strains with entomopathogenic activity ensures a high success of pests' biocontrol products. Moreover, detecting virulence factor genes in entomopathogenic bacteria can suggest general host pest spectrum. However, recently found toxins with entomopathogenic activity identified throughout the bacterial kingdom in other species than Bt, can broaden our knowledge regarding insect pest management. This review aims to analyse the status of bacterial based bioinsecticides focusing on Bt varieties accepted as active ingredients in EU commercial pesticides, listing other potential entomopathogenic bacteria, and describing the genetic virulence factors against arthropod and nematode pests.

Key words*: Bacillus, biocontrol, entomopathogens, virulence factor genes.*

INTRODUCTION

Plant protection against insect pests is traditionally managed with chemical insecticides (Hernández-Rosas et al., 2020). However, the continuous use of related pesticides in agriculture could be associated with various risks, such as acquired resistance, pests' recurrence, environmental pollution, residues accumulation in the food chain, as well as human and animal health risks. To counteract such problems, continuous research is made in plant protection field in order to improve present technologies and to design new control strategies. Various alternative approaches have emerged for pest management strategies (Kidanu & Hagos, 2020), some based on natural enemies, semiochemicals or bioinsecticides of microbial or plan origin. Therefore, the development and use of new pest control agents that are both safe and environmentally friendly becomes important (Karabörklü et al., 2017).

Although successfully presented as alternatives to chemical insecticides, the use of microbial based bioinsecticides is still limited, whether we are talking about a narrow spectrum of activity, specificity on a particular larval stage, low persistence in the environment, or even the implementation of application methods to ensure their efficiency. Therefore, the identification and constant characterization of the insecticidal activity of various microorganisms can ensure their successful introduction into organic and integrated pest management programs.

From the beginning of the 21st century, the opportunities and need for effective biological control are greater than ever, especially given the reluctance of consumers regarding the sustainability of genetically modified pest-resistant crops (Bale et al., 2008).

Although for 2022, the global market of synthetic and biologic pesticides was expected to grow with 5.3% CAGR (Compound Annual Growth Rate) starting from 2017 (Chen, 2018), due the pandemic situation and the repeated lockdowns the biopesticide production, especially, as well as trade movements, were seriously affected. However, for the next years, it is predicted an annual increase of biopesticides with 15.1% CAGR till 2027, with an increase of 5% CAGR only for

bioinsecticides (Mordor Intelligence, 2022 a,b). Such encouraging data confirms the European Union's initiative to reduce the use of chemical pesticides by 50% until 2030 (European Commission, 2020), and to increase the organic farming at 25% of the EU's agricultural land (European Commission, 2021). According to the EU Pesticide Database, (EU Pesticides database, 2022) we currently have 27 microbial-based active substances to be used in agriculture pest control against detrimental insects, mites and nematodes, of which 12 are based on various bacterial strains (Table 1).

Tabel 1. List of bacterial based active substances approved for use in pest control within the European Union and Romania (EU Pesticides database, 2022)

No.	Active Substance as microbial strains and consortia	Category	EU approval dates from-to	Romanian authorized
1	*Bacillus firmus* I-1582	Nematicide	01.10.2013 - 30.09.2023	Yes
2	*Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857	Insecticide	01.05.2009 - 30.04.2023	No
3	*Bacillus thuringiensis* subsp. *aizawai* strain GC-91	Insecticide	01.05.2009 - 30.04.2023	No
4	*Bacillus thuringiensis* subsp. *aizawai* strains ABTS-1857, GC-91	Insecticide	01.05.2009 - 30.04.2023	No
5	*Bacillus thuringiensis* subsp. *israeliensis* (serotype H-14) strain AM65-52	Insecticide	01.05.2009 - 30.04.2023	No
6	*Bacillus thuringiensis* subsp. kurstaki strain ABTS 351	Insecticide	01.05.2009 - 30.04.2023	Yes
7	*Bacillus thuringiensis* subsp. kurstaki strain EG 2348	Insecticide	01.05.2009 - 30.04.2023	No
8	*Bacillus thuringiensis* subsp. kurstaki strain PB 54	Insecticide	01.05.2009 - 30.04.2023	Yes
9	*Bacillus thuringiensis* subsp. kurstaki strain SA 11	Insecticide	01.05.2009 - 30.04.2023	No
10	*Bacillus thuringiensis* subsp. kurstaki strain SA 12	Insecticide	01.05.2009 - 30.04.2023	No
11	*Bacillus thuringiensis* subsp. kurstaki strains ABTS 351, PB 54, SA 11, SA12 and EG 2348	Insecticide	01.05.2009 - 30.04.2023	No
12	*Pasteuria nishizawae* Pn1	Nematicide	14.10.2018 - 14.10.2033	No

In Romania, the general use of plant protection products for pests and diseases is decreasing (https://www.fao.org/faostat/en/#data/RP), but herbicides seem not to be in the same trend (Table 2).

Table 2. Tons of pesticide used (according to FAO)

Year	Romania	EU	Worldwide
Insecticides			
2017	1001	63322	688145
2018	641	65018	690004
2019	583	69752	698168
Fungicides and Bactericides			
2017	2282	192006	951780
2018	1760	198020	975539
2019	1711	187935	969061
Herbicides			
2017	3576	194420	2234155
2018	2740	183247	2172865
2019	3052	186012	2222273
Pesticides			
2017	6859	490260	4185592
2018	5141	480270	4141023
2019	5346	478389	4168778

However, the pesticide use worldwide is showing various fluctuations depending on the year, region, and application purposes.

This review aims to analyse the status of bacterial based bioinsecticides, focusing on *Bacillus thuringiensis* varieties accepted as active ingredients in EU commercial pesticides, listing other potential entomopathogenic bacteria, and describing the genetic virulence factors against arthropod and nematode pests.

Bacillus thuringiensis HISTORY UPDATE

Bacillus thuringiensis is a Gram-positive, spore forming bacteria, capable of producing crystalline inclusions with entomopathogenic properties. It was first isolated in 1901, by the Japanese biologist Shigetane Ishiwatari, which called it *Bacillus sotto* due to the sotto disease (sudden-collapse disease) caused by this pathogen that killed large populations of silkworms. A decade later, Emile Berliner rediscovered this bacterium as it killed a Mediterranean flour moth in Thüringen region, Germany. He called this as *Bacillus*

thuringiensis (*Bt*), a name which is still valid (Knowles, 1994). In addition to Ishiwata's first important notations, that under-sporulation, cultures showed higher pathogenicity than active young cultures, Berliner further reveals that those sporulated cells contain inclusion crystals, yet he didn't attribute them to bacterial pathogenesis.

In 1920, farmers already started to use *Bt* as insecticide. But it was only in 1938, when the first *Bt* commercial product was released. The pesticide was named Sporeine, and the production was made in France (Milner, 1994).

Later on, in 1953, after purification process, Hannay C.L. confirmed that the insecticidal activity of *Bt* was given by protein crystals.

In 1958, *Bt* started to be used as commercial product also in the United States of America. Although in the 1970s chemical pesticides proved to be more efficient, the progress in biotechnology stimulated *Bt* research, and allowed the first cloning of the crystal toxin gene into another bacterial specie, as well as large-scale culture production at relatively low costs (Osman et al., 2015).

Nowadays, *Bt* based products are the most widely used microbial insecticides in the world (Ibrahim et al., 2010; Dinu et al., 2013), accounting almost 90% of the bioinsecticide market (Chattopadhyay et al., 2004), with a high rate of success in pests' control in both agriculture and environment (Jouzani et al., 2017). Studies on mosquito control using *Bacillus thuringiensis* subsp. *israelensis* showed that its larvicidal effects significantly decrease malaria transmission by reducing the population of the vector (Dambach et al., 2014, 2020).

With time, the specific cytotoxic activity of *Bt* was showed against different pests, such as insects (Gonzalez-Vazquez et al., 2021), nematodes (Baghaee Ravari & Mahdikhani Moghaddam, 2015; Huang et al., 2018), mites (Erban et al., 2009; Dunstand-Guzmán et al., 2015) gastropods (Abd El-Ghany & Abd El-Ghany, 2017), plathelmintes and protozoa (Feitelson, 1993). Although various laboratory studies have showed that *Bt* toxins could have various applications in agriculture and environmental pest control, and even strong cytocidal action against the human cancer cells (Palma et al., 2014a), the main activity is insecticidal, with high specificity on target pest. This host rage specificity allows the use of *Bt* proteins in environmentally friendly technologies for pest control. This way, *Bt* insecticides ensure good biocontrol efficacy, protecting the biodiversity, reducing environmental risks, and any detrimental effects on vertebrates and non-target insects (Jurat-Fuentes & Crickmore, 2017).

PREVALENCE AND GENERAL CHARACTERISTICS OF *Bt*

Bt is considered a ubiquitous soil bacterium, that could be also associated to plants, dead insects and water, however spread worldwide (Nair et al., 2018). Some studies reveal its presence in marine sediments (Maeda et al., 2000) and even Antarctica (Waschulin et al., 2022).

Phylogenetic studies attributed *Bt* to the *Bacillus cereus* group, based on 16S rRNA, 23S rRNA, as well as *gyrB* gene sequences, (Bavykin et al., 2004). The *B. cereus sensu lato* contains Gram-positive bacteria including *B. cereus*, *B. thuringiensis* (*Bt*), *B. mycoides* and *B. anthracis*. Although closely related, the main distinguishing differences are reported in their mobile genetic elements (Pacheco et al., 2021).

Considering that *Bt* is known as an insect pathogen, particular targeting certain insect orders, the identification is very important, not only for classification, but mainly to establish the pathogenicity (Chowdhury, 2020).

According to the List of Prokaryotic names with Standing in Nomenclature (LPSN), there are 23 *Bt* subspecies listed, although considered not validly published. However, the World Health Organization (1999) is mentioning 67 subspecies that had been described. Generally known *Bacillus thuringiensis* subspecies are *aizawai (Bta), entomocidus (Bte), galleriae (Btg), israelensis (Bti), kurstaki (Btk), thuringiensis (Btt),* and *tenebrionis (Btte)*.

Different serotypes are also listed, without being correlated to the toxic properties of the crystal proteins. Generally, there is a single type of crystals in each serologic group, although in *Btk* there is an exception (Xu et al., 2014).

The biopesticide properties of *Bt* against various pests' types is due to the toxic proteins produced during its vegetative and sporulation phases. During vegetative growth, *Bt* is able to produce secreted insecticidal protein (Sip), and

vegetative insecticidal proteins (Vir), while during sporulation it could produce parasporal crystalline δ-endotoxins, encoded by *Cyt* genes (responsible for the cytolytic toxin Cyt) and *Cry* genes (responsible for crystal toxin Cry) (Chattopadhyay & Banerjee, 2018).
Due to the negative connotations of the word *toxins*, especially outside of the academic context, it is advisable to avoid this term and refer to the insecticidal toxins, Cry and Cyt toxins, *Bt* toxins and so on, as insecticidal proteins, Cry and Cyt proteins etc (Crickmore et al., 2021).

REVISED NOMENCLATURE WITHIN INSECTICIDAL PROTEINS

One of the most important aspects of *Bt* is that it produces some parasporal crystals during sporulation, also known as δ-endotoxins. These trigger the toxicity to certain susceptible insect types, depending on their specificity. The genes encoding for such proteins are the *Cry* and *Cyt* genes. At first, the encoded toxic proteins were named based on their activity on target pests (Table 3).

Table 3. Outdated representation of *Cry* and *Cyt* genes based on the insecticidal activity expressed by the encoded δ-endotoxins (adapted from Khasdan, 2002)

Gene	Host specificity
CryI	Lepidoptera
CryIIA	Lepidoptera and Diptera
CryIIB	Lepidoptera alone
CryIII	Coleoptera
CryIV	Diptera larvae
CryV	Both Lepidoptera and Coleoptera larvae
CryVI	Nemathode
	Hymenoptera
Cyt	Diptera, Coleoptera, Lepidoptera, and *in vitro* cytolitic activity against mammalian cells

The nomenclature, however, had to be changed when the advanced analysis and continuous findings have showed new proteins, encoded by homologous DNA sequences of the *Cry* gene family, which showed different insecticidal specificity against new target pests (Crickmore et al., 1998). The high homology within amino-acids sequences of toxic proteins, as well as their different target pest categories, compared to the already known insecticidal activity, triggered the need for another nomenclature. Fatherly, these proteins were classified based on their amino acid similarity (www.lifesci.sussex.ac.uk /home/Neil_Crickmore/Bt/) and currently have four-level classifiers. The first and the fourth ranking classifiers are Arabic letters, the second and third are Latin scripts of a capital letter followed by a lowercase letter (Figure 1).

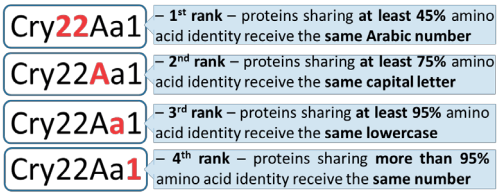


Figure 1. Nomenclature of bacterial pesticidal proteins

Although this four-ranking procedure started to put in order the pesticidal proteins, advanced research along with the improved techniques revealed some proteins incorrect correlated to the Cry, Cyt or Vip classes. Additionally, new proteins expressing enthomopathogenic activity were also found in other non *Bt* bacteria. Therefore, in order to maintain the very clear established rule of four-ranking, which have been widely spread and well accepted, mnemonics are currently used in order to connect this new classes of proteins (Crickmore et al., 2021). Such amendments were applied to the Cry6Aa, Cry34Ab, Cry35Ab and Cry51Aa protein groups found in *Bt*, which now are named App6Aa, Gpp34Aa, Tpp35Ab, and Mpp51Aa respectively (Tetreau et al., 2021). Or to the Cry75Aa proteins found in *Brevibacillus laterosporus* which are currently named Mpp75Aa insecticidal proteins (Bowen et al., 2021).
Along with Cry proteins, Cyt are also pore forming toxins with cytolytic activity within the insect midgut cells. They are able to express toxicity to different insect types, such as dipteran, coleopteran and lepidopteran pests. Moreover, they are able to increase the insecticidal potential of certain Cry toxins, which is a very important trait, able to overcome pest resistance to Cry toxins, already seen in mosquitoes (Soberón et al., 2013).
Beside *in vivo* insecticidal activity, Cyt toxins, except Cyt1Ca, also showed *in vitro* cytolytic activity against different mammalian cultured cells and erythrocytes hemolysis (Thomas & Ellar, 1983; Manasherob et al., 2006).

Another class of toxins, although non-proteinaceous, are β-exotoxins (Chattopadhyay & Banerjee, 2018). These show no target specificity, being able to affect not only insects but also mammals (Liu et al., 2014). As they are heat resistant, they are not removed by autoclaving. Therefore, *Bt* producing strains are forbidden to be used in pest control in many countries, over the UE and SUA (Obeidat et al., 2012).

Additionally, to the parasporal crystal endotoxins, during vegetative growth, *Bt* and other related species are able to produce vegetative insecticidal proteins, known as Vir (Estruch et al., 1996), secrete insecticidal proteins, named Sip (Donovan et al., 2006), and other pesticide important compounds.

A high number of *Vip* genes are currently known, almost 140, which have been classified into 4 groups (Jouzani et al., 2017). The Vip1 and Vip2 proteins are having binary insecticidal toxicity against various coleopteran and hemipteran pests (Sattar & Maiti, 2011), while Vip3 proteins affect a wide range of lepidopteran pests (Palma et al., 2014b; Palma, 2015). Meanwhile, for Vir4 proteins, no insecticidal activity was detected (Chattopadhyay & Banerjee, 2018). In the case of Sip proteins, they are mentioned to be insecticidal against coleopteran larvae (Chattopadhyay & Banerjee, 2018).

Although pesticide genes are plasmid-borne, they are known to be associated to mobile genetic elements (Fagundes et al., 2011). As Cry toxin genes express high mobility, they are important for the horizontal transfer, and for their potential to associate to other entomotoxin genetic determinants. This could increase the pesticidal activity and overcome the risks of insect resistance (Fayad et al., 2021). A recent study on the complete genome of *Bt* HER1410, revealed this strain to have a Cry-containing chromosome. The integration of the *Cry* genes within the chromosome, especially close to the replication origin, may influence the entomopathogenic activity of this strain, in a positive way for Lepidoptera control (Lechuga et al., 2020). Apart from the mentioned insecticidal proteins the entomopathogenic bacteria, both *B. thuringiensis* as well as non-*Bt* bacteria, can reduce pest populations by releasing chitinases, metalloproteases as well as some low-weight moieties. These compounds can act complementary to the insecticidal proteins or can be the only virulence factors responsible for insecticidal activity the in non-Bt bacteria (Malovichko et al., 2019).

Bt NEMATOCIDAL ACTIVITY

Plant parasitic nematodes are among most problematic pests in agriculture (Pulavarty et al., 2021). They are responsible of causing significant economic losses every year (Mesa-Valle et al., 2020). The negative impact of nematodes on the agricultural sector was estimated to 14% (Chitwood et al., 2003). The commonly used management approaches are soil fumigation with certain chemicals (White et al., 2016) and formaldehyde disinfection of seeds and planting materials (Dong & Zhang, 2006). These chemical methods are expensive and dangerous for the environment, animals or humans (Pulavarty et al., 2021). Based on these considerations, there is a worldwide interest for finding alternative methods that can ensure nematodes control with minimal impact on the environment and biodiversity. For such alternative methods, biocontrol microorganisms seem to be a promising solution.

Among *Bt* strains, many families of crystal proteins (i.e., *cry* 1, *cry* 5, *cry* 6, *cry* 14, *cry* 21 or *cry* 55) have been reported to have nematicidal activities (Huang et al., 2018, Meirizka et al., 2021, Li et al., 2008, Kahn et al., 2021). There are other biocontrol bacteria also mentioned, such as *Brevibacillus laterosporus*, (Carneiro et al., 1998), *Bacillus megaterium* (Mohamed, 2001) and *B. circulans* (El-Hadad et al., 2011).

DIVERSITY OF ENTOMOPATHOGENIC BACTERIA

Various studies confirmed the entomopathogenicity of different bacterial strains, some being currently approved as biopesticides, even in highly restrictive countries such as in EU (table 1).

Based on their target pests and proven efficiency, many strains of *Bt* and other *Bacillus* related species were listed as entomopathogenic, along with some other Gram-positive and Gram-negative bacteria (table 4).

Table 4. Biocontrol bacteria, non-*Bt*, listed to have entomopathogenic potential (adapted from Gouli et al., 2021)

Bacterial species	Pest categories	References
***Bacillus* related species**		
Bacillus circulans	Mosquitoes	Darriet & Hougard, 2002
	Nematodes	El-Hadad et al., 2011
Bacillus lentimorbus	Scarabaeidae	Rippere et al., 1998
Bacillus megaterium	Lepidoptera	Aksoy et al., 2018
	Nematodes	Mostafa et al., 2018
Bacillus moritai	Diptera	Berry et al., 2002
Brevibacillus brevis	Mosquitoes	Khyami-Horani et al., 1999
	Lepidoptera	Tozlu et al., 2021
Bacillus sphaericus	Mosquitoes	Medeiros et al., 2005
Brevibacillus laterosporus	Mosquitoes	Barbieri et al., 2021
	Coleoptera	Bowen et al., 2021
	Nematodes	Carneiro et al., 1998
Lysinibacillus sphaericus	Mosquitoes	Bernal & Dussán, 2020
Paenibacillus popilliae	Scarabaeidae	Chalivendra, 2021
Various Gram-positive bacteria		
Arthrobacter gandavensis	Coleoptera	Danismazoglu et al., 2012
Pasteuria nishizawae	Nematodes	Lund et al., 2018
Streptomyces griseoplanus	Lepidoptera	Vijayabharathi et al., 2014
S.bacillarys		
S. albolongus		
Various Gram-negative bacteria		
Burkholderia rinojensis	Lepidoptera	Cordova-Kreylos et al., 2013
	Mites	
Pseudomonas chlororaphis	Lepidoptera	Raio & Puopolo, 2021
Pseudomonas fluorescens	Lepidoptera	Redouan et al., 2019
Pseudomonas putida	Lepidoptera	Awad, 2012
Raoultella terrigena	Hemiptera	Ozsahin et al., 2014
Serratia marcescens	Lepidoptera	Sikorowski et al., 2001; Konecka et al., 2019
Photorhabdus luminescens	Lepidoptera	Adithya et al., 2020
Xenorhabdus nematophila		

Entomopathogenic specificity and variable virulence are highly influencing bacterial efficacy in pest control. Therefore, selecting the appropriate strains is not so easy and requires assiduous laboratory and field research. Moreover, bacterial fate in the environment can also influence the future success of plant protection products. If the bacteria are not having satisfying survival rates there is also the possibility of formulating only the toxic pesticide compounds, cells viability not being required.

CONCLUSIONS

Bt insecticides have gained worldwide recognition as one of the safest, most successful and most sustainable methods of pest management and control. With many advantages in terms of benefits, *Bt* continues to be a material with extraordinary potential for researchers, in the desire to obtain either biopesticides or to respond to problems such as pest resistance. These advantages do not stop only at the insecticidal properties manifested by *Bt*. Also, numerous studies analyse *Bt* as a potential biofertilizer, endophyte, or even as bioremediation agent in heavy metals and pollutions soils or as antagonist against plant and human pathogenic fungi.

Virulence factors related to Cry and Cyt families are also found in non-*Bt* bacteria from *Bacillus* genus and *Bacillus* related species, such as *Brevibacillus brevis*, *Paenibacillus popilliae* and *Lysinibacillus sphaericus*.

Entomopatogenic bacteria express their virulence against agricultural arthropod and nematode pests by various virulence factors and mechanisms such as insecticidal proteins, chitinases and metalloproteases enzymes, low-weight moieties or inducing systemic resistance in plants.

Although research results sustain entomopatogenic activity of various bacterial species it is quite difficult to integrate them as pesticide active ingredients. This is triggered by various aspects, such as the UE precautions on allowing the large-scale use of new species and strains inoculants without extensive evaluation, and the long process of pesticide active ingredients approval, which is non-differential between biologic and chemical pesticides.

REFERENCES

Abd El-Ghany, A.M. & Abd El-Ghany, N.M. (2017). Molluscicidal activity of *Bacillus thuringiensis* strains against *Biomphalaria alexandrina* snails. *Beni-Suef University Journal of Basic and Applied Sciences, 6*(4), 391-393.

Adithya, S., Shivaprakash, M.K., & Sowmya E. (2020). Evaluation of insecticidal activity of entomopathogenic bacteria *Photorhabdus* and *Xenorhabdus* against shoot and fruit borer *Earias vittella* (Lepidoptera: Noctuidae) of vegetable crops. *Journal of Entomology and Zoology Studies, 8*(4), 2343-2348.

Aksoy, H.M., Saruhan, I., Kaya, Y., & Ozturk, M (2018). Morphological Changes Caused by *Bacillus megaterium* on Adult Emergence of Fall Webworm's Pupa, *Hyphantria cunea* (Drury) (Lepidoptera: Erebidae). *Journal of Agricultural Sciences, 24*, 539-546.

Awad, H.H., 2012. Effect of *Bacillus thuringiensis* and farnesol on haemocytes response and lysozymal activity of the black cut worm *Agrotis ipsilon* larvae. *Asian J. Biol. Sci., 5*, 157-170.

Baghaee Ravari, S. & Mahdikhani Moghaddam, E. (2015). Efficacy of *Bacillus thuringiensis* Cry14 Toxin against Root Knot Nematode, *Meloidogyne javanica. Plant Protect. Sci., 51*, 46–51.

Bale, J. S., van Lenteren, J. C., & Bigler, F. (2008). Biological control and sustainable food production. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 363(1492), 761–776. doi: https://doi.org/10.1098/rstb.2007.2182.

Barbieri, G., Ferrari, C., Mamberti, S., Gabrieli, P., Castelli, M., Sassera, D., Ursino, E., Scoffone, V.C., Radaelli, G., Clementi, E., Sacchi, L., Ferrari, E., Gasperi, G., & Albertini, A.M. (2021). Identification of a Novel Brevibacillus laterosporus Strain With Insecticidal Activity Against Aedes albopictus Larvae. *Front. Microbiol., 12*, 624014.

Bavykin, S. G., Lysov, Y. P., Zakhariev, V., Kelly, J. J., Jackman, J., Stahl, D. A., & Cherni, A. (2004). Use of 16S rRNA, 23S rRNA, and gyrB gene sequence analysis to determine phylogenetic relationships of Bacillus cereus group microorganisms. *Journal of clinical microbiology*, *42*(8), 3711–3730.

Bernal, L., & Dussán, J. (2020). Synergistic effect of *Lysinibacillus sphaericus* and glyphosate on temephos-resistant larvae of *Aedes aegypti*. *Parasites Vectors, 13*, 68.

Berry, C., O'Neil, S., Ben-Dov, E., Jones, A. F., Murphy, L., Quail, M. A., Holden, M. T. G., Harris, D., Zaritsky, A. & Parkhill, J. (2002). Complete Sequence and Organization of pBtoxis, the Toxin-Coding Plasmid of *Bacillus thuringiensis* subsp. *israelensis*. *Applied and Environmental Microbiology, 68*(10), 5082–5095.

Bowen, D., Yin, Y., Flasinski, S., Chay, C., Bean, G., Milligan, J., Moar, W., Pan, A., Werner, B., Buckman, K., Howe, A., Ciche, T., Turner, K., Pleau, M., Zhang, J., Kouadio, J.L., Hibbard, B.E., Price, P., & Roberts, J. (2021). Cry75Aa (Mpp75Aa) insecticidal proteins for controlling the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), isolated from the insect-pathogenic bacterium *Brevibacillus laterosporus. Appl. Environ. Microbiol., 87*, e02507-20.

Carneiro, R.M.D.G., Desouza, I.S., & Belarmino, L.C. (1998). Nematicidal activity of *Bacillus* spp. strains on juveniles of *Meloidogyne javanica*. *Nematol. Brasileira, 22*, 12-21.

Chalivendra,S. (2021). Microbial Toxins in Insect and Nematode Pest Biocontrol. *Int. J. Mol. Sci., 22*, 7657.

Chattopadhyay, A., Bhatnagar, N. B., & Bhatnagar, R. (2004). Bacterial Insecticidal Toxins. *Critical Reviews in Microbiology*, 30, 33–54.

Chattopadhyay, P., & Banerjee, G. (2018). Recent advancement on chemical arsenal of *Bt* toxin and its application in pest management system in agricultural field. *3 Biotech*, *8*(4), 201.

Chen, J. (2018). Biopesticides: Global Markets to 2022. *BCC Research*; Wellesley, MA, USA: 2018. Report Code CHM029G.

Chitwood, D.J. (2003). Research on plant-parasitic nematode biology conducted by the United States Department of Agriculture-Agricultural Research Service. *Pest Management Science, 59*, 748–753.

Chowdhury, M.H. (2020). Isolation and characterization of bacteria of Amjhupi and Ishurdi soil series of Bangladesh. *International Journal of Applied Chemical and Biological Sciences, 1*(4), 33–39.

Cordova-Kreylos, A. L., Fernandez, L. E., Koivunen, M., Yang, A., Flor-Weiler, L., & Marrone, P. G. (2013). Isolation and characterization of *Burkholderia rinojensis* sp. *nov*., a non-*Burkholderia cepacia* complex soil bacterium with insecticidal and miticidal activities. *Applied and environmental microbiology*, *79*(24), 7669–7678.

Crickmore, N., Berry, C., Panneerselvam, S., Mishra, R., Connor, T.R., & Bonning, B.C. (2021). A structure-based nomenclature for *Bacillus thuringiensis* and other bacteria-derived pesticidal proteins. *The Journal of Invertebrate Pathology, 186*, 107438.

Crickmore, N., Zeigler, D. R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Dean, D. H. (1998). Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev. 62*, 807–813.

Dambach, P., Louis, V.R., Kaiser, A., Ouedraogo, S., Sié, A., Sauerborn, R., & Becker, N. (2014). Efficacy of *Bacillus thuringiensis* var. *israelensis* against malaria mosquitoes in northwestern Burkina Faso. *Parasites Vectors 7*, 371.

Dambach, P., Winkler, V., Bärnighausen, T., Traoré, I., Ouedraogo, S., Sié, A., Sauerborn, R., Becker, N., & Louis, V.R. (2020). Biological larviciding against malaria vector mosquitoes with *Bacillus thuringiensis israelensis* (Bti) - Long term observations and assessment of repeatability during an additional intervention year of a large-scale field trial in rural Burkina Faso. *Global Health Action, 13*:1, 1829828.

Danismazoglu, M., Demir, I. Sevim, A., Demirbag, Z., & Nalcacioglu, R. (2012). An investigation on the bacterial flora of Agriotes lineatus (Coleoptera:

Elateridae) and pathogenicity of the flora members. *Crop Protection, 40,* 1–7.

Darriet, F., & Hougard, J. M. (2002). An isolate of Bacillus circulans toxic to mosquito larvae. *Journal of the American Mosquito Control Association*, *18*(1), 65–67.

Dinu, M.M., Fătu, A.C., Ştefan, S., & Andrei, A.A. (2013). In vitro study on the interaction between *Bacillus thuringiensis* and chemical pesticides used for corn crop protection. *Scientific Bulletin. Series F. Biotechnologies, XVII*, 29–34.

Dong, L.Q., & Zhang, K.Q. (2006). Microbial control of plant-parasitic nematodes: A five-party interaction. *Plant Soil, 288*, 31–45.

Donovan, W. P., Engleman, J. T., Donovan, J. C., Baum, J. A., Bunkers, G. J., Chi, D. J., Clinton, W. P., English, L., Heck, G. R., Ilagan, O. M., Krasomil-Osterfeld, K. C., Pitkin, J. W., Roberts, J. K., & Walters, M. R. (2006). Discovery and characterization of Sip1A: A novel secreted protein from *Bacillus thuringiensis* with activity against Coleopteran larvae. *Applied Microbiology and Biotechnology*, *72*(4), 713–719.

Dunstand-Guzmán, E., Peña-Chora, G., Hallal-Calleros, C., Pérez-Martínez, M., Hernández-Velazquez, V.M., Morales-Montor, J., & Flores-Pérez, F.I. (2015). Acaricidal effect and histological damage induced by *Bacillus thuringiensis* protein extracts on the mite *Psoroptes cuniculi*. *Parasites Vectors, 8*, 285.

El-Hadad, M.E., Mustafa, M.I., Selim, Sh.M., El-Tayeb, T.S., Mahgoob, A.E.A., & Abdel Aziz, N.H. (2011). The nematicidal effect of some bacterial biofertilizers on meloidogyne incognita in sandy soil. *Brazilian Journal of Microbiology, 42*, 105–113.

Erban, T., Nesvorna, M., Erbanova, M., & Hubrt, J. (2009). *Bacillus thuringiensis* var. *tenebrionis* control of synanthropic mites (Acari: Acaridida) under laboratory conditions. *Exp. Appl. Acarol., 49*, 339–346.

Estruch, J. J., Warren, G. W., Mullins, M. A., Nye, G. J., Craig, J. A., & Koziel, M. G. (1996). Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(11), 5389–5394.

European Commission (2020, May 20). EU Biodiversity Strategy for 2030 - Bringing nature back into our lives. https://ec.europa.eu/commission/presscorner/detail/en/qanda_20_886.

European Commission (2021, March 25). Communication from the Commission to the European Parliament, the Council, the European economic and social committee and the Committee of the regions on an action plan for the development of organic production. https://ec.europa.eu/info/sites/default/files/food-farming-fisheries/farming/documents/com2021_141_act_organic-action-plan_en.pdf.

European Union Pesticides Database (2022). https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/active-substances/?event=search.as (Accessed on May 3rd, 2022).

Fagundes, R.B.S., Picoli, E.A.T., Lana, U.G.P., & Valicente, F.H. (2011). Plasmid patterns of efficient and inefficient strains of *Bacillus thuringiensis* against *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae). *Neotrop. Entomol. 40*(5), 600–606.

Fayad, N., Kambris, Z., El Chamy, L., Mahillon, J., & Kallassy Awad M. (2021). A Novel antidipteran *Bacillus thuringiensis* strain: Unusual cry toxin genes in a highly dynamic plasmid environment. *Applied and Environmental Microbiology, 87*(5), e02294-20

Feitelson, J.S. (1993) The *Bacillus thuringiensis* family tree. In L. Kim (Ed.) *Advanced Engineered Pesticides* (pp. 63–71). Marcel Dekker, New York.

Food and Agriculture Organisation of the United Nations (n.d.). https://www.fao.org/faostat/en/#data/RP (Accessed on February 28th, 2022)

Gonzalez-Vazquez, M. C., Vela-Sanchez, R. A., Rojas-Ruiz, N. E., & Carabarin-Lima, A. (2021). Importance of Cry Proteins in Biotechnology: Initially a Bioinsecticide, Now a Vaccine Adjuvant. *Life (Basel, Switzerland), 11*(10), 999.

Gouli, V.V., Marcelino, J.A.P., Gouli, S.Y. (2021). The basic biological resources for the production of microbial pesticides. *In*: Vladimir V. Gouli, Jose A.P. Marcelino, Svetlana Y. Gouli (Eds.). Microbial Pesticides (pp. 1-134) Academic Press. ISBN 9780128244517.

Hannay, C.L. (1953). Crystalline inclusions in aerobic sporeforming bacteria. *Nature, 172*, 1004.

Hernández-Rosas, F., Figueroa-Rodríguez, K.A., García-Pacheco, L.A., Velasco-Velasco, J., Sangerman-Jarquín, D.M. (2020). Microorganisms and Biological Pest Control: An Analysis Based on a Bibliometric Review. *Agronomy*, 10, 1808.

Höfte, H., & Whiteley, H.R. (1989). Insecticidal crystal proteins of Bacillus thuringiensis. Microbiological Reviews, 53, 242–255.

Huang, T. Lin, Q., Qian, X., Zheng, Y., Yao, J., Wu, H., Li, M., Jin, X., Pan, X., Zhang, L. & Guan, X. (2018). Nematicidal Activity of Cry1Ea11 from *Bacillus thuringiensis* BRC-XQ12 against the Pine Wood Nematode (*Bursaphelenchus xylophilus). Phytopathology, 108*, 44–51.

Ibrahim, M. A., Griko, N., Junker, M., & Bulla, L. A. (2010). *Bacillus thuringiensis*: a genomics and proteomics perspective. *Bioengineered Bugs, 1*(1), 31–50.

Jouzani, G.S., Valijanian, E. & Sharafi, R. (2017). *Bacillus thuringiensis*: a successful insecticide with new environmental features and tidings. *Applied Microbiology and Biotechnology, 101*, 2691–2711.

Jurat-Fuentes, J.L., & Crickmore, N. (2017). Specificity determinants for Cry insecticidal proteins: Insights from their mode of action. *The Journal of Invertebrate Pathology,* 142, 5–10.

Kahn, T. W., Duck, N. B., McCarville, M. T., Schouten, L. C., Schweri, K., Zaitseva, J., & Daum, J. (2021). A *Bacillus thuringiensis* Cry protein controls soybean cyst nematode in transgenic soybean plants. *Nature Communications*, *12*, 3380.

Karabörklü S., Azizoglu U., & Azizoglu Z.B. (2017). Recombinant entomopathogenic agents: a review of biotechnological approaches to pest insect control. *World Journal of Microbiology and Biotechnology*. doi: 10.1007/s11274-017-2397-0.

Khasdan, V. (2002) Cloning Combinations of Four Genes from *Bacillus thuringiensis* subsp. *israelensis* and *tmf* for expression in various bacteria to enhance mosquito biocontrol. PhD thesis. Ben-Gurion University of the Negev, Israel

Khyami-Horani, H., Katbeh-Bader, A., & Mohsen, Z.H. (1999). Isolation of endospore forming bacilli toxic to *Culiseta longiareolata* (Diptera: Culicidae) in Jordan. *Lett Appl Microbiol., 128*, 57–60.

Kidanu, S., & Hagos, L. (2020). Entomopathogenic Fungi as a Biological Pest Management Option: A Review. *International Journal of Research Studies in Agricultural Sciences (IJRSAS)*, Volume 6. doi: http://dx.doi.org/10.20431/2454-6224.0606001.

Knowles, B.H. (1994) Mechanism of Action of *Bacillus thuringiensis* Insecticidal δ-Endotoxins. *Advances in Insect Physiology, 24*, 275–308.

Konecka, E., Mokracka, J., Krzymińska, S., & Kaznowski, A. (2019). Evaluation of the pathogenic potential of insecticidal *Serratia marcescens* strains to humans. *Polish Journal of Microbiology*, *68*(2), 185–191.

Lechuga, A., Lood, C., Salas, M., van Noort, V., Lavigne, R., & Redrejo-Rodríguez, M. (2020). Completed Genomic Sequence of *Bacillus thuringiensis* HER1410 Reveals a *Cry*-Containing Chromosome, Two Megaplasmids, and an Integrative Plasmidial Prophage. *G3 (Bethesda, Md.)*, *10*(9), 2927–2939.

Li, X.Q., Tan, A., Voegtline, M.S., Bekele, S.T., Chen, C., & Aroian, R.V. (2008). Expression of Cry5B protein from *Bacillus thuringiensis* in plant roots confers resistance to root-knot nematode. *Biological Control*, *47*, 97–102.

Liu, X., Ruan, L., Peng D., Li, L., Sun, M., & Yu, Z. (2014). Thuringiensin: a thermostable secondary metabolite from *Bacillus thuringiensi*s with insecticidal activity against a wide range of insects. *Toxins, 6*, 2229–2238.

Lund, M.E., Mourtzinis, S., Conley, S.P. & Ané, J.M. (2018). Soybean cyst nematode control with *Pasteuria nishizawae* under different management practices. *Agronomy Journal, 110*(6), 2534–2540.

Maeda, M., Mizuki, E., Nakamura, Y., Hatano, T., & Ohba, M. (2000). Recovery of *Bacillus thuringiensis* from marine sediments of Japan. *Current microbiology*, *40*(6), 418–422.

Malovichko, Y.V., Nizhnikov, A.A., & Antonets K.S (2019). Repertoire of the *Bacillus thuringiensis* virulence factors unrelated to major classes of protein toxins and its role in specificity of host-pathogen interactions. *Toxins, 11*, 347.

Manasherob, R., Itsko, M., Sela-Baranes, N., Ben-Dov, E., Berry, C., Cohen, S., & Zaritsky, A. (2006). Cyt1Ca from *Bacillus thuringiensis* subsp. *israelensis*: production in *Escherichia coli* and comparison of its biological activities with those of other Cyt-like proteins. *Microbiology, 152*(9), 2651–2659.

Medeiros, F.P., Santos, M.A., Regis, L., Rios, E.M., & Rolim Neto, P.J. (2005). Development of a *Bacillus sphaericus* tablet formulation and its evaluation as a larvicide in the biological control of *Culex quinquefasciatus*. *Mem Inst Oswaldo Cruz., 100*, 431–434.

Meirizka, R., Budiharjo, A., & Pujiyanto, S. (2021). Screening of novel cry genes active against nematodes from *Bacillus thuringiensis* Thai isolates. *Bioma : Berkala Ilmiah Biologi, 22*(2), 155-160. https://doi.org/10.14710/bioma.22.2.155-160

Mesa-Valle, C. M., Garrido-Cardenas, J. A., Cebrian-Carmona, J., Talavera, M., & Manzano-Agugliaro, F. (2020). Global Research on Plant Nematodes. *Agronomy*, 10(8), 1148.

Milner, R.J. (1994). History of *Bacillus thuringiensis. Agriculture, Ecosystems and Environment, 49*, 9-13.

Mohamed, A.A. (2001). Studies on Some Asymbiotic N2 Fixing Bacteria. M.Sc. Thesis, Fac. Agric., Ain Shams Univ., Egypt.

Mordor Intelligence, 2022 a. www.mordorintelligence.com/industry-reports/ global- biopesticides-market-industry (Accessed on February 28th, 2022)

Mordor Intelligence, 2022 b. www.mordorintelligence.com/industry-reports/ global-insecticides-market-industry (Accessed on February 28th, 2022)

Mostafa, F.A.M., Khalil, A.E., Nour El-Deen, A.H. & Ibrahim D.S. (2018). The role of *Bacillus megaterium* and other bio-agents in controlling root-knot nematodes infecting sugar beet under field conditions. *Egypt J Biol Pest Control, 28*, 66.

Nair, K., Al-Thani, R., Al-Thani, D., Al-Yafei, F., Ahmed, T. & Jaoua, S. (2018). Diversity of *Bacillus thuringiensis* strains from Qatar as shown by crystal morphology, δ-endotoxins and Cry gene content. *Front. Microbiol., 9*, 708.

Obeidat, M., Khyami-Horani, H., & Al-Moman, F. (2012). δ-exotoxins and & β toxicity of *Bacillus thuringiensis* endotoxins to *Drosophila melanogaster*, *Ephestia kuhniella* and human erythrocytes. *Afr. J. Biotechnol., 11*(46), 10504–10512.

Osman, G.H., Already, R., Assaeedi, A., Organji, S.R., El-Ghareeb, D.K., Abulreesh, H.H., & Althubiani, A.S. (2015). Bioinsecticide *Bacillus thuringiensis* a comprehensive review. *Egyptian Journal of Biological Pest Control*, 25, 271–288.

Ozsahin, E., Sezen, K., Demir, I. & Demirbag, Z. (2014). Bacterial isolates from *Palomena prasina* (Hemiptera: Pentatomidae) include potential microbial control agents. *Biocontrol Science and Technology, 24*(9), 1039–1051

Pacheco, S., Gómez, I., Chiñas, M., Sánchez, J., Soberón, M., & Bravo, A. (2021). Whole Genome Sequencing Analysis of *Bacillus thuringiensis* GR007 Reveals Multiple Pesticidal Protein Genes. *Frontiers in microbiology, 12*, 758314.

Palma, L., Muñoz, D., Berry, C., Murillo, J., & Caballero, P. (2014a). *Bacillus thuringiensis* Toxins: An Overview of Their Biocidal Activity. *Toxins, 6*, 3296–3325.

Palma, L., Muñoz, D., Berry, C., Murillo, J., & Caballero, P. (2014b). Draft genome sequences of two *Bacillus thuringiensis* strains and characterization of a putative 41.9-kDa insecticidal toxin. *Toxins, 6*, 1490–1504.

Palma, L. (2015). Protocol for the fast isolation and identification of insecticidal *Bacillus thuringiensis* strains from soil. *Bt Res., 6*, 1–3.

Pulavarty, A., Egan, A., Karpinska, A., Horgan, K., & Kakouli-Duarte, T. (2021). Plant Parasitic Nematodes: A Review on Their Behaviour, Host Interaction, Management Approaches and Their Occurrence in Two Sites in the Republic of Ireland. *Plants (Basel, Switzerland), 10*(11), 2352.

Raio, A., & Puopolo, G. (2021). Pseudomonas chlororaphis metabolites as biocontrol promoters of plant health and improved crop yield. *World journal of microbiology & biotechnology*, *37*(6), 99.

Redouan, Q., Rachid, B., Abderahim, A., Hind, L., Abdelhadi, A., Naima, A.A., Abdelghani, T., El Hassan, M., & Bouchra C. (2019). Effect of *Pseudomonas* as a preventive and curative control of tomato leafminer *Tuta absoluta* (Lepidoptera: Gelechiidae). *J. Applied Sci., 19*, 473–479.

Rippere, K.E., Tran, M.T., Yousten, A.A., Hilu, K.H., & Klein, M.K. (1998). *Bacillus popilliae* and *Bacillus lentimorbus*, bacteria causing milky disease in Japanese beetles and related scarab larvae. *International Journal of Systematic and Evolutionary Microbiology, 42*(2), 395–402.

Sattar S., Maiti M. K. (2011). Molecular characterization of a novel vegetative insecticidal protein from *Bacillus thuringiensis* effective against sap-sucking insect pest. *J. Microbiol. Biotechnol., 21*, 937–946. 10.4014/jmb.1105.05030

Sikorowski, P.P., Lawrence, A.M., & Inglis, G.D. (2001). Effects of *Serratia marcescens* on rearing of the tobacco budworm (Lepidoptera: Noctuidae). *American Entomologist 47*(1), 51–60.

Soberón, M., López-Díaz, J.A., & Bravo, A. (2013). Cyt toxins produced by Bacillus thuringiensis: A protein fold conserved in several pathogenic microorganisms. *Peptides, 41*, 87–93.

Tetreau, G., Andreeva, E.A., Banneville, A.S., De Zitter, E., & Colletier, J.P. (2021). How does *Bacillus thuringiensis* crystallize such a large diversity of toxins? *Toxins, 13*, 443.

Thomas, W.E. & Ellar, D.J. (1983). *Bacillus thuringiensis* var. *israelensis* crystal - endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*. *J. Cell. Sci., 60*, 181–197.

Tozlu, E., Tozlu, G., Kotan, R., Çalmaşur, Ö., & Dadaşoğlu F. (2021). Investigation of some enthomopathogens as biocontrol agents of Tinocallis (Sappocallis) saltans (Nevsky, 1929) (Hemiptera: Aphididae). *Turkish Journal of Agriculture and Forestry, 45*, 124–132.

Vijayabharathi, R., Kumari, B.R., Sathya, A., Srinivas, V., Abhishek, R., Sharma, H.C., & Gopalakrishnan S. (2014). Biological activity of entomopathogenic actinomycetes against lepidopteran insects (Noctuidae: Lepidoptera). *Canadian Journal of Plant Science, 94*(4), 759–769.

Waschulin, V., Borsetto, C., James, R., Newsham, K. K., Donadio, S., Corre, C., & Wellington, E. (2022). Biosynthetic potential of uncultured Antarctic soil bacteria revealed through long-read metagenomic sequencing. *The ISME journal*, *16*(1), 101–111.

White, C.A., Holmes, H.F., Morris, N.L., & Stobart, R.M. (2016). *A review of the benefits, optimal crop management practices and knowledge gaps associated with different cover crop species*. Agriculture and Horticulture Development Board, *AHDB Cereals and Oilseeds,* Cambridge, UK.

World Health Organization & International Programme on Chemical Safety. (1999). Microbial pest control agent: *Bacillus thuringiensis*. World Health Organization. https://apps.who.int/iris/handle/10665/42242

www.lifesci.sussex.ac.uk /home/Neil_Crickmore/Bt/

Xu, C., Wang, B.C., Yu, Z., & Sun, M. (2014). Structural insights into Bacillus *thuringiensis* Cry, Cyt and parasporin toxins. *Toxins, 6*, 2732–2770.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

COMPARATIVE STUDY OF TWO VARIETIES OF PURPLE FLASH POTATO GROWN *IN VITRO*

Alexandra-Mihaela NAGY[1], Paula BOBOC (OROS)[3], Corina CĂTANĂ[3], Maria-Mihaela ANTOFIE[2], Camelia SAVA SAND[1, 2]

[1]University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Doctoral School of Agricultural Engineering, 3-5 Calea Mănăștur, Cluj-Napoca, Romania
[2]"Lucian Blaga" University of Sibiu, Faculty of Agricultural Science, Food Industry and Environmental Protection, 7 Dr. Ion Rațiu, Sibiu, Romania
[3]University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Centre for Biodiversity and Conservation, Department of Horticulture and Landscaping, 3-5 Calea Mănăștur, Cluj-Napoca, Romania

Corresponding author emails: corina.catana@usamvcluj.ro, alexandra.nagy@ulbsibiu.ro

Abstract

Solanum tuberosum L. is considered a major food/feed source since ancient times for both humans and animals. Potato cultivation is important at the global level to its extraordinary yield per unit area, being cultivated in over 120 countries worldwide. Over time, the consumption tendencies regarding potato have changed, and new varieties including purple flesh, are gradually growing in popularity. Purple flash potatoes are native to Peru but during the past decades became popular in Europe as well as in Romania. Among the main varieties of popular purple flash potatoes grown in Romania are 'Salad Blue' and 'Violet Negretin' both being rich in nutritional substances including anthocyanins. The scope of this study is to analyse the in vitro initiation and micropropagation of these cultivars. In this regard the best cultivation media is Murashige & Skoog (1962, MS62) for micropropagation. By adding Gamborg vitamins to MS62 minerals we also tested the effects of chitosan (2 mg/l) and/or active charcoal (2 g/l). Visible positive effects on micropropagation only on the culture medium supplemented with active charcoal were obtained.

Key words*: charcoal, chitosan, micropropagation, purple potato.*

INTRODUCTION

Purple flash potatoes are rich source of anthocyanins (Zhang *et al.,* 2018), which is why there has been a growing interest in introducing them in food consuming in recent years (Nemś *et al*., 2015). Purple pigmentation is due to the accumulation and storage of anthocyanins in the parenchymal tissue of potato tubers (Smeriglio *et al.,* 2016).

Anthocyanins are currently being studied extensively (Chen *et al.*, 2020; Oancea *et al.,* 2021), an essential role being attributed to their presence in the daily diet (Chen *et al.*, 2019). Potatoes rich in anthocyanin have proven antioxidant and anti-inflammatory properties (Henriques *et al*., 2020), while also possessing beneficial effects in preventing many human diseases (Mishra *et al*., 2020).

The colour of the potato flesh is a very clear indication of the presence of anthocyanins, therefor the ones with purple or red flesh have a high concentration of them (from 61.5 to 573.5 mg/kg cyanidin). Also, they have an antioxidant action of 4 to 5 times higher compared to yellow or white potato tubers (Hamouz *et al*., 2011).

In addition to cyanidin, other anthocyanins have been identified. They belong to the following groups of anthocyanidins: petunidin (Tang & Giusti, 2020), pelargonidin (Sampaio *et al*., 2021), delphinidin (Makori *et al*., 2022), peonidin (Sigurdson *et al.*, 2019) and malvidin (Pino *et al.*, 2021).

For this study, two varieties of purple potato are studied, `Salad Blue` and `Violet Negretin`, cultivated in Romania as well. Multiplication *in vitro* is a complex process material influenced by the mineral composition of culture media, vitamins formula, hormone balance as well as other chemical factors that can be supplemented to the culture media (Murashige & Skoog, 1962).

The purpose of this article is to test the effect of vitamins, chitosan, and active charcoal on the *in*

vitro development two purple potato varieties as following.

MATERIALS AND METHODS

Plant material

Two varieties of purple flash potatoes (*Solanum tuberosum* L.) as certified seed tubers were used in this study.

`Salad Blue` variety was provided by the National Research and Development Institute for Potato and Sugar Beet from Brașov, Romania. The second variety, `Violet Negretin` was provided by a nationally recognized chain of stores. This is cultivated, and distributed by Agro Brava Farm from Constanța, Romania.

The `Salad Blue` potato is originally from Scotland. Contrary to its name, due to its floury pulp, it is suitable for baking, frying, pureeing or even for making chips. It is an early variety, with flowers in shades of blue to purple. The shape of the tubers is oval, and their size is average. The epidermis has a dark blue shade, the flesh is purple to blue with white insertions (Pęksa *et al.*, 2013).

`Violet Negretin` variety, also called `Vitelotte Noire` or `Truffe de Chine` is a late potato variety of French origin. Potatoes of this variety have a dark blue peel, almost black, and the flesh is dark blue or dark purple. The shape of tubers is elongated, has a thick shell and sunken mesh (Ombra *et al*., 2015).

***In vitro* culture initiation and multiplication**

Starting material was represented by meristematic apexes taken from the sprouts of purple potato tubers in the sterile hood. The clean tubers were maintained in humidity chambers at the room temperature in jars, filter paper and soaked with distilled water.

Sterilization and initiation. The potatoes sprouts were sterilized in the sterile hood in 5% sodium hypochlorite solution for 15 minutes, followed by 3-5 times rinsing in sterile water and inoculated into test tubes with MS62 medium.

The multiplication. The first and second multiplication of the plant material was done by micro cuttings and transfer on MS62 medium without hormones (Table 1), 4 weeks after their initiation. 5 micro cuttings were transferred to each culture jar.

Micropropagation. On each culture medium variant (Table 1), micro cuttings were transferred in jars for each of the two potatoes cultivars (Figure 1) 4 weeks after their multiplication. Each jar contains 5 micro cuttings with two leaves.

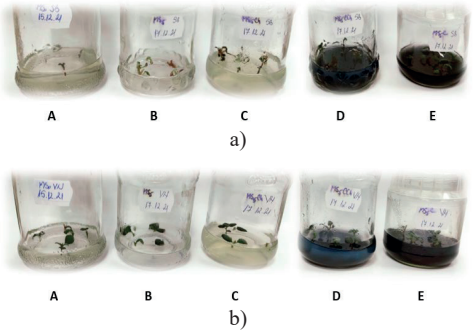


Figure 1. **a)** Transfer of the `Salad Blue` variety on culture medium variants; **b)** Transfer of the `Violet Negretin` variety on culture medium variants (**A** = MS62 medium; **B** = MS62 medium with B5 vitamins; **C** = MS62 medium with B5 vitamins and chitosan; **D** = MS62 medium with B5 vitamins, chitosan, and active charcoal; **E** = MS62 medium with B5 vitamins and active charcoal)

Culture and growth room conditions

For meristem initiation test tubes with a volume of 50 ml, height 14.5 cm, Ø of 2.5 cm, with 5 ml culture medium were used.

Jars used for multiplication and plant transfer of 200 ml, 8 cm in height, a diameter of 6 cm and filled with 50 ml of culture medium.

Culture medium was sterilized by autoclaving at 121°C for 20 minutes for test tubes and 30 minutes for the rest of the culture jars.

The culture jars were placed in a growth room with fluorescent tubes (2000 lx), at 23°C ± 2°C for night/day, with a photoperiod of 16 hours light and 8 hours darkness.

Culture media

The culture medium used for initiation was preparate according to the formula of Murashige & Skoog, 1962 medium (MS62), with 20 g sucrose and 3 g of Gelrite (Duchefa). pH before adding the Gelrite was 5.7. The pH was adjusted with NaOH 1 N and/or HCl 1 N.

The first and second multiplication were performed on MS62 without hormones.

The third multiplication was dedicated to the following experiments.

Table 1. Culture media used for studying multiplication of `Salad Blue` and `Violet Negretin` cultivars

	1	2	3	4	5
	MS62 minerals	MS62 vitamins	B5 vitamins	Chitosan	Active charcoal
Initiation and multiplication					
Initiation	X	x			
1st and 2nd multiplication	X	x			
1st experiment regarding the vitamins effect					
Control	X	x			
MS62 with B5	X		x		
2nd experiment regarding the chemicals effect					
Control	X		x		
Chitosan	X		x	x	
Active charcoal	X		x		x
Chitosan and active charcoal	X		x	x	x

1 - minerals in according to the original recipe of MS62; **2** - vitamins in according to the original recipe of MS62; **3** - B5 vitamins according to Gamborg B5 medium instead of regular vitamins; **4** - 2 mg/l chitosan; **5** - 2 g/l active charcoal.

Micro cuttings were transferred on two types of culture medium: MS62 (full formula of minerals and vitamins according to Murashige & Skoog, 1962) and a modified MS62 medium where the MS62 vitamins were replaced with B5 vitamins of Gamborg medium.

Second experiment was conducted by using the modified MS62 medium (vitamins replaced with B5 vitamins) and testing the effects of chitosan (2 mg/l) and/or active charcoal (2 g/l).

Chitosan is a biomaterial obtained more than 50 years ago through the extraction of polysaccharides substance from the exoskeleton of crustaceans (crabs, lobsters, and shrimps) (Periayah *et al.,* 2016). This biomaterial has been of interest to plant biotechnologies for over 20 years, as well as today (Hirano, 1995; Struszczyk *et al*., 2002; Li *et al*., 2020).

Active charcoal is commonly used in tissue culture media. The addition of it to the culture medium may promote or inhibit *in vitro* growth, depending on the species and tissues used (Pan & Staden, 1998).

The active charcoal used was produced by Duchefa and solubilized in the culture medium.

The chitosan was produced by Sigma-Aldrich and was solubilized with 3-4 drops of glacial acetic acid, then added to the composition of the culture medium before boiling.

All these types of media were solidified by Gelrite (Duchefa) after checking the pH and bringing it to 5.7.

Data analysing

Morphometry. The number of shoots obtained per explant as well as the heigh of plantlets were monitored.

Data were collected after 4 weeks and analysed by applying the Polifact statistical program, performing bifactorial analyses, using the Duncan test, where the first factor was the variety, and the second factor was the type of culture medium. Graphs were obtained using Excel from the Microsoft Office suite.

RESULTS AND DISCUSSIONS

Initiation and multiplication

As the plant material used for initiation comes from the natural environment conditions, it was sterilized to minimize the risk of infection and to achieve the highest rate of success of the crop.

The success rate of the initiation was 60-70%. Plantlets resulting after initiation and first multiplication were used as stock plant material for the experiments discussed in this paper.

The effect of vitamins

Each purple potato variety was grown on two types of culture medium (MS62 and MS62 with addition of B5 vitamins) after the second multiplication for studying the influence of vitamins on shooting.

The results represent an average of the values from 10 plantlets of each variety.

The analysis of the results of this experiment shows that the vitamins from the composition of the culture media doesn't have a significative influence at the shooting process for either of `Salad Blue` and `Violet Negretin` potato varieties.

The highest average shoots` number was 2.03 for the `Violet Negretin` variety grown on the MS62. This was followed by the `Salad Blue` variety grown on the same type of medium, with an average up to 1.97 shoots/explant. For the culture medium supplemented by B5 vitamins, in case of `Salad Blue` variety was obtained a higher average value (1.67) compared to the `Violet Negretin` variety (1.50) of shoots number.

However, even if different values were recorded (Figure 2), there are no statistical difference.

It is possible that multiple shoots will be generated by the stress of injury of the explants (Levshin *et al*., 2019).

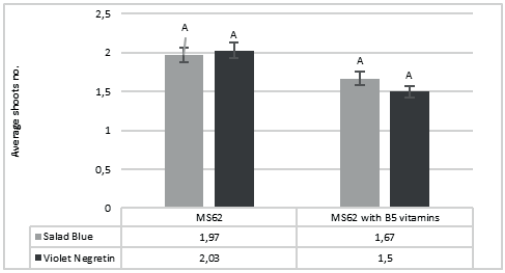


Figure 2. The effect of vitamins on shooting
(A-A - no statistical difference according Duncan test)

Another indicator followed in this experiment was the shoots heigh induced on the two types of culture medium. MS, and MS with addition of B5 vitamins. The results represent the average of the 10 length shoots per variant. The obtained results as well as the statistical approach show that vitamins influence the growth of plantlets (Figure 3).

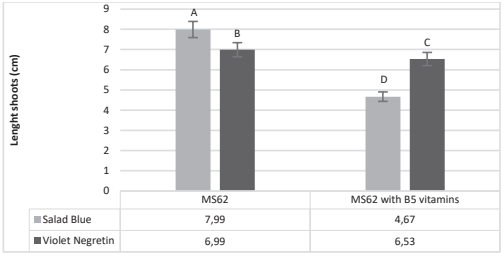


Figure 3. The effect of vitamins in the growth shoots
(A-D - significant statistical difference
according Duncan test)

The plantlets grown on the MS62 medium have a uniform growth, the average length of the shoots being insignificant between the two varieties of potato.
On the MS62 medium supplemented by B5 vitamins, the `Violet Negretin` variety (6.53 cm) registered an approximately equal increase compared to the control sample (6.99 cm), while for the `Salad Blue` variety there is a significant reduction of the growth in length of the shoots. On the MS62 medium the average of length shoots was 7.99 cm compared to that cultivated on the medium supplemented by B5 vitamins, where the average of length shoots was 4.67 cm.

The effect of the active charcoal and chitosan
Due to a noticed reduction in the shoots` height, we continued to study the effect of active charcoal and chitosan on shoots development.

The growth of the shoots for `Salad Blue` and `Violet Negretin` purple potato varieties were studied in four types of culture media (Table 1). The results represent the average of the 10 length shoots of each variant.
From the analysis of the data presented in Figure 4, it is observed that the type of culture medium influences the elongation of the shoots.

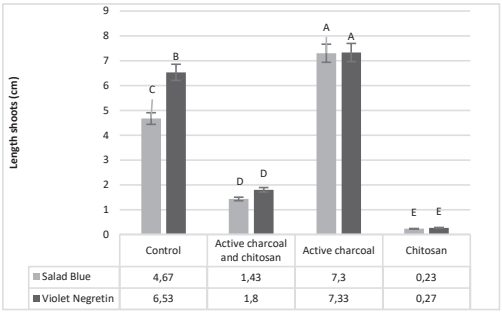


Figure 4. The effect of culture medium on the length shoots (A-E - significant statistical difference according Duncan test)

Significant differences have been observed between potato plants grown on the two-culture media regardless of the variety.
Considering the results of the second experiment, we can appraise that the effect of adding active charcoal together with chitosan addition in the culture medium could decrease the inhibitory effects of chitosan.
Compared to the control, the best results were observed for the culture medium supplemented by active charcoal. Regarding the culture medium that contains only chitosan, the plantlets stopped growing, remaining at the stage they were transferred on the culture medium.
Asghari-Zakaria *et al.* (2009) showed that chitosan, as a growth promoter and stimulator of plant protection mechanism, could alleviate the stress caused by *in vitro* culture conditions and acclimatization. The addition of chitosan into the culture medium at a concentration of 500 mg/l mainly influenced the increase in the production of greenhouse and minitubers. The increase in the yield and the number of minitubers after the application of 500 mg/l of chitosan was 12.6% and 36.3%. The results of that study indicate that chitosan can also be successfully incorporated into artificial seeds (Asghari-Zakaria *et al*., 2009).

In some species, active charcoal promotes tissue growth for apex and anthers cultures, as it has been found that charcoal powder inactivates toxic compounds produced and secreted into the culture medium (Ngomuo *et al*., 2014).

CONCLUSIONS

The initiation and multiplication of the plant material consisting of the two varieties of purple flash potato (`Salad Blue` and `Violet Negretin`) was successful.

The general formulas of the culture media used, MS62 and MS62 supplemented by B5 vitamin do not greatly influence the development processed in potato.

The culture medium that favoured a uniform and constant growth for purple potato plantlets, regardless of variety, was MS62.

Regarding the MS supplemented by B5 vitamins, the growth was negatively influenced. From the analysis of the results of these experiments it can be considered that the addition of chitosan to the MS62, also supplemented by B5 vitamins culture medium completely inhibits the growth.

On the other hand, supplementing the culture medium with active charcoal stimulates the growth compared to the control variant in both potatoes' cultivars.

In a future experiment it is desirable to monitor the effect of the chitosan on the micro-tuberization process.

ACKNOWLEDGEMENTS

This study was realized in the Centre of Biodiversity and Conservation of the University of Agricultural Sciences and Veterinary Medicine Cluj Napoca, Romania during the PhD training program.

REFERENCES

Asghari-Zakaria, R., Maleki-Zanjani, B., & Sedghi, E. (2009). Effect of in vitro chitosan application on growth and minituber yield of Solanum tuberosum L. *Plant soil environ*, *55*(6), 252-256.

Chen, C. C., Lin, C., Chen, M. H., & Chiang, P. Y. (2019). Stability and quality of anthocyanin in purple sweet potato extracts. *Foods*, *8*(9), 393.

Chen, S., Wu, M., Lu, P., Gao, L., Yan, S., & Wang, S. (2020). Development of pH indicator and antimicrobial cellulose nanofibre packaging film based on purple sweet potato anthocyanin and oregano essential oil. *International Journal of Biological Macromolecules*, *149*, 271-280.

Hamouz, K., Lachman, J., Pazderů, K., Tomášek, J., Hejtmánková, K., & Pivec, V. (2011). Differences in anthocyanin content and antioxidant activity of potato tubers with different flesh colour. *Plant, Soil and Environment*, *57*(10), 478-485.

Henriques, J. F., Serra, D., Dinis, T. C., & Almeida, L. M. (2020). The anti-neuroinflammatory role of anthocyanins and their metabolites for the prevention and treatment of brain disorders. *International Journal of Molecular Sciences*, *21*(22), 8653.

Hirano, S. (1995). Some ecologically friendly applications of chitin and chitosan in biotechnology. *Industrial Biotechnology Polymers*, 189-203.

Levshin, A. G., Gasparyan, I., Bitsoev, B., & Shchigolev, S. (2019). Constructive features of the device to remove the apical shoots of potatoes. In *18th International Scientific Conference``Engineering for Rural Development,* 532-537.

Li, Q., Dunn, E. T., Grandmaison, E. W., & Goosen, M. F. (2020). Applications and properties of chitosan. In *Applications of chitin and chitosan* (pp. 3-29). CRC Press.

Makori, S. I., Mu, T. H., & Sun, H. N. (2022). Profiling of Polyphenols, Flavonoids and Anthocyanins in Potato Peel and Flesh from Four Potato Varieties. *Potato Research*, *65*(1), 193-208.

Mishra, T., Raigond, P., Thakur, N., Dutt, S., & Singh, B. (2020). Recent updates on healthy phytoconstituents in potato: a nutritional depository. *Potato Research*, *63*(3), 323-343.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia plantarum*, *15*(3), 473-497.

Nemś, A., Pęksa, A., Kucharska, A. Z., Sokół-Łętowska, A., Kita, A., Drożdż, W., & Hamouz, K. (2015). Anthocyanin and antioxidant activity of snacks with coloured potato. *Food Chemistry*, *172*, 175-182.

Ngomuo, M., Mneney, E., & Ndakidemi, P. A. (2014). The in vitro propagation techniques for producing banana using shoot tip cultures. *American Journal of Plant Sciences*, *2014*.

Oancea, S., Perju, M., Coman, D., & Olosutean, H. (2021). Optimization of conventional and ultrasound-assisted extraction of *Paeonia officinalis* anthocyanins, as natural alternative for a green technology of cotton dyeing. *Romanian Biotechnological Letters*, *26*(2), 2527-2534.

Ombra, M. N., Fratianni, F., Granese, T., Cardinale, F., Cozzolino, A., & Nazzaro, F. (2015). In vitro antioxidant, antimicrobial and anti-proliferative activities of purple potato extracts (*Solanum tuberosum* cv Vitelotte noire) following simulated gastro-intestinal digestion. *Natural product research*, *29*(11), 1087-1091.

Pan, M. J., & Staden, J. V. (1998). The use of charcoal in in vitro culture–A review. *Plant growth regulation*, *26*(3), 155-163.

Pęksa, A., Kita, A., Kułakowska, K., Aniołowska, M., Hamouz, K., & Nemś, A. (2013). The quality of

protein of coloured fleshed potatoes. *Food Chemistry*, *141*(3), 2960-2966.

Periayah, M. H., Halim, A. S., & Saad, A. Z. M. (2016). Chitosan: A promising marine polysaccharide for biomedical research. *Pharmacognosy reviews*, *10*(19), 39.

Pino, M. T., & Vergara, C. (2021). Red and Purple Flesh Potatoes a Healthy and Attractive Alternative Associated with New Market Trends. In *Solanum tuberosum-a Promising Crop for Starvation Problem*. IntechOpen.

Sampaio, S. L., Lonchamp, J., Dias, M. I., Liddle, C., Petropoulos, S. A., Glamočlija, J., ... & Barros, L. (2021). Anthocyanin-rich extracts from purple and red potatoes as natural colourants: Bioactive properties, application in a soft drink formulation and sensory analysis. *Food Chemistry*, *342*, 128526.

Sigurdson, G. T., Robbins, R. J., Collins, T. M., & Giusti, M. M. (2019). Molar absorptivities (ε) and spectral and colorimetric characteristics of purple sweet potato anthocyanins. *Food chemistry*, *271*, 497-504.

Smeriglio, A., Barreca, D., Bellocco, E., & Trombetta, D. (2016). Chemistry, pharmacology, and health benefits of Anthocyanins. *Phytotherapy Research*. 30, 1265-1286.

Struszczyk, M. H., Pośpieszny, H., & Gamzazade, A. (2002). Chitin and chitosan. *Polimery*, *47*(5), 316-325.

Tang, P., & Giusti, M. M. (2020). Metal chelates of petunidin derivatives exhibit enhanced color and stability. *Foods*, *9*(10), 1426.

Zhang, L., Zhao, L., Bian, X., Guo, K., Zhou, L., & Wei, C. (2018). Characterization and comparative study of starches from seven purple sweet potatoes. *Food hydrocolloids*, *80*, 168-176.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

PLANT BIOSTIMULANTS BASED ON NANOFORMULATED BIOSILICA RECOVERED FROM SILICA-RICH BIOMASS

Liliana-Adriana PAIRAULT[1, 2], Naomi TRITEAN[3, 4], Diana CONSTANTINESCU-ARUXANDEI[3], Florin OANCEA[3, 1*]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd., District 1, Bucharest, Romania
[2]Amia International, 7 Gorunului Street, Otopeni, Romania
[3]National Institute for Research & Development in Chemistry and Petrochemistry - INCDCP-ICECHIM, 202 Spl. Independentei, District 6, Bucharest, Romania
[4]Faculty of Biology, University of Bucharest, 91-95 Spl. Independentei, Bucharest, Romania

[*]Corresponding author email: florin.oancea@icechim.ro

Abstract

This review evaluates the technological solutions used to produce nanoformulated plant biostimulants made from biosilica recovered from silicon-rich biomass. Silicon improves root nutrient uptake/nutrient use efficiency, increases plant tolerance to stress, and promotes phytonutrients in edible crop yield. The exact mechanism of silicon action is not yet known. However, it is generally accepted that a flow of soluble silicon species through plant simplast is essential to produce the aforementioned effects. The main difficulties in applying soluble silicon species, silicic acid, and its di- and trimers to plants are related to the polycondensation features at very low concentrations. One of the solutions to this technical problem is to use amorphous silica, which constantly releases small quantities of soluble silicon species. For example, phytoliths formed in several plant species that concentrate the simplast flow of soluble silicon in their simplast are an excellent source of soluble silicon. Nanoformulation increases the surface/volume ratio and further improves the release of soluble silicon species. Our review focuses on the techniques used to extract and nanoformulate the biosilica from silicon-rich biomass.

Key words*: biosilica, nanoformulation, plant biostimulant, soluble silicon.*

INTRODUCTION

Bionanoparticles and biomass-derived nanoformulations are spreading in agriculture and plant-related applications due to their remarkable physical-chemical particularities such as size, shape, specific surface area, multi-functionality, biochemical activity, and stability, aiming to enhance yield and crop quality in a sustainable, circular paradigm.

Silicon (Si) is not considered a nutrient for plants but mainly a microelement with beneficial effects (Constantinescu-Aruxandei *et al.*, 2020). It is known that Si has two main functions in plants: a structural one, associated mainly with Si translocated by apoplast, and a physiological one, usually associated with soluble Si translocated by simplest (Casey *et al.*, 2003).

Soluble silicon was classified as an inorganic plant biostimulant (Savvas & Ntatsi, 2015). Plant biostimulants represent a new category of agricultural inputs, and their production from plant extracts is a sustainable approach to valorize the side streams (du Jardin, 2015). Recent studies and debates have led to the conclusion that "*A plant biostimulant shall be an EU fertilizing product, the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: i) nutrient use efficiency, ii) tolerance to abiotic stress, iii) quality traits, or iv) availability of confined nutrients in the soil or rhizosphere*", as stated in the European Regulation 2019/1009 (Regulation, 2019).

Although plants have the ability to regulate their functions under stress, there is a reduction in crop and yield productivity below an optimal level of the parameters that play a vital role in plant development (Fahad *et al.*, 2017). Silicon-free plants are structurally weaker and highly prone to developmental, reproductive, and

growth changes because Si acts as a trigger for plant protection mechanisms against biotic and abiotic stress. Therefore, in terms of plant resistance to abiotic stress, two major factors are thought to be involved (a) the mechanical and/or physical protection provided by deposited amorphous silica, respectively (b) the biochemical response that triggers different metabolic pathways (Etesami & Jeong, 2018).

SILICON IN PLANTS

Even if all terrestrial plants have silicon (Si) in their tissues, the concentration varies according to species, ranging from 0.1 to 10% Si in dry weight (Epstein, 1994; Ma *et al.*, 2007). This difference in silicon accumulation between plant species has been attributed to the specificity of roots to take up silicon. Thus, three ways in which Si can be accumulated by higher plants in relation to water uptake have been proposed: active uptake (the Si uptake rate is much higher than the water uptake), passive uptake (soluble silicon and water are taken up similarly in terms of uptake rate) and partial rejection (water uptake rate is higher than that of soluble silicon) (Carey & Fulweiler, 2014; Ma *et al.*, 2007). Plants that accumulate a high amount of silicon include horsetail (*Equisetaceae* family) and rice, corn, or wheat (*Poaceae* family) species. Other plant species, such as nettle (*Urticaceae* family), accumulate a relatively moderate amount (Luyckx *et al.*, 2017).

The available soluble in soil largely depends on biosilica recycling (Schaller *et al.*, 2021). The available silicon in soil refers to the amount of silicon that the growing plants can take up during the growing season. The liquid phase (soil solution) contains soluble silicon, i.e., orthosilicic acid (H_4SiO_4) and its di- and trimers, the only silicon molecular species that plants take up. Orthosilicic acid, H_4SiO_4, is taken up from the soil solution at concentrations between 0.2 and 0.6 mM (Epstein, 1994). Orthosilicic acid is a very weak acid with four acid functions and with the lowest pKa value of 9.8. This means that at pH 9.8, orthosilicic acid is present in the undissociated state in a proportion of 50% and 50% of it in the dissociated state. Between pH 2 and 8, orthosilicic acid is a neutral, completely non-dissociated molecule. At concentrations higher than 2 mM, it starts to polymerize through polycondensation reactions with the release of water (Spinde *et al.*, 2011). The main factors that influence the bioavailability of silicon in soil are the soil type, pH, texture, temperature, organic matter, or the ions present in the soil (Kawaguchi & Kyūma, 1977). In the soil, silicon is an element with reduced mobility (Cornelis & Delvaux, 2016). Soluble silicon species, formed by weathering of the silicate rocks, are precipitated by the co-released aluminum ions and generate clays (de Tombeur *et al.*, 2021).

In the last decade, different silicon transporters have been discovered in the plant tissues of several species. The flux of soluble silica, i.e., H_4SiO_4, and the species resulting from its dimerization/trimerization, taken up from the soil by the roots and passing through the symplasts, causes balanced priming of the different defense pathways in the plants (Van Bockhaven *et al.*, 2013). Once in the symplast via the *Lsi1* transporter, Si is transferred to the apoplast via the *Lsi2* transporter and further via the transpiration process. Si reaches the xylem as monosilicic acid (Casey *et al.*, 2003). Subsequently, in the metabolic active plant tissues, by the concentration of silicic acid due to water loss by evaporation, silicic acid polymerizes to amorphous silica [$(SiO_2)_n$ *x* nH_2O], known as opal or phytolith. The resulted amorphous biosilica is deposited in specific cells. In some species, removal of Si from the xylem is also an active, transporter-mediated process. *Lsi6* is thought to be involved in the translocation of Si from the xylem, a transporter identified in the *Poaceae* family (Ma & Yamaji, 2015; Mitani-Ueno & Ma, 2021).

In plants, silicon is stored in a solid form defined by phytoliths which are micrometer-sized amorphous silica structures that protect the plant cell wall against abiotic stress. The cell wall polysaccharides mediate silica deposition and phytolith formation (Nawaz *et al.*, 2019). Silica from the plant cell wall is coupled with lignin polymerization and cell wall oxidative level (Zexer & Elbaum, 2020).

It is known that abiotic stress can take many forms that hinder plant growth and development by slowing down physiological processes, and that can even lead to plant tissue senescence. One of the ways in which silica delays cell senescence and thus strengthens the plant cell

wall is by amplifying the synthesis pathway of suberin monomers and monolignols (Fleck *et al.*, 2011). The silicic acid-mediated suberification process slows down the water loss through evapotranspiration and even protects plants against pathogenic microorganisms (Harman-Ware *et al.*, 2021).

Plant exposure to various abiotic and biotic stress conditions also leads to the release of reactive oxygen species (ROS), activating plant defense responses. ROS accumulation determines a cascade of biochemical reactions at the cellular level (Hasanuzzaman *et al.*, 2020). Silicon has the ability to alleviate ROS induced stress in plant cells and tissues in several ways, such as attenuating the lipid peroxidation process of cell membranes, increasing or decreasing the level of endogenous phytohormones (Kim *et al.*, 2014), or activating the antioxidant defense system of plants (Ali & Hassan, 2017; Verma *et al.*, 2021). We summarized in Table 1 some of the effects reported in silicon-treated plants as a response to stress.

Table 1. Response of silicon-treated plants to abiotic and biotic stress

Plant type	Stress	Effect in Si-treated plants compared to untreated plants	References
Rice (*Oryza sativa*)	Heavy metals	Decrease in SA* and JA*; increase in ABA* Decrease in lipid peroxides Increase in plant growth Increase in chlorophyll content	(Kim *et al.*, 2014)
Strawberry (*Fragaria* sp.)	Salt	Increase in chlorophyll content Increase in carotenoid content Increase in plant growth	(Avestan *et al.*, 2019)
Roselle (*Hibiscus sabdariffa* L.)	Drought (water deficit)	Increase in SOD*, CAT*, POD* Increase in plant growth	(Ali *et al.*, 2017)
Sugarcane (*Saccharum officinarum* L.)	Water excess	Increase in SOD, CAT, APx* activity Increase in chlorophyll content Increase in photosynthesis activity	(Verma *et al.*, 2021)
Wheat seedlings (*Triticum aestivum* L.)	UV-B	Increase in antioxidant enzyme activity Increase in chlorophyll, flavonoid, and anthocyanins content Increase in plant growth	(Yao *et al.*, 2011)
Maize (*Zea mays* L.)	Low temperature	Constant level of IAA*, GA*, CKs* Increase in antioxidant enzyme activity	(Moradtalab *et al.*, 2018)
Tomato seeds (*Solanum lycopersicum* L.)	Heat	Activating plant defense mechanisms (increase in antioxidant enzyme activity)	(Khan *et al.*, 2020)
Yellow melon	*Acidovorax citrulli* (bacterial infection)	Protection against bacterial fruit blotch (BFB) Decrease in ABA and SA phytohormones	(Ferreira *et al.*, 2015)
Arabidopsis thaliana	*Erysiphe cichoracearum* (fungal disease)	Stress alleviation	(Fauteux *et al.*, 2006)

*Abbreviations: SA (salicilyc acid), JA (jasmonic acid), SOD (superoxide dismutase), CAT (catalase), POD (peroxidase), APx (ascorbate peroxidase), IAA (auxins), GA (gibberellins)

Soluble silicon modulates the level of various types of plant hormones. According to the effect of phytohormones on plant growth, they are divided into stimulants - auxins (IAA), gibberellins (GA), cytokinins (CKs), salicylic acid (SA), and inhibitors: abscisic acid (ABA), ethylene (ET), jasmonic acid (JA) (El Sabagh *et al.*, 2022). Depending on the stress type, silicon cross-talks with each of these hormones, balancing the stimulation and the inhibition of the tissular and cellular processes (Lesharadevi *et al.*, 2021).

NANOBIOSILICA EXTRACTED FROM SILICON-RICH BIOMASS

As we mentioned, silicates mineral weathering determines the formation of clays (de Tombeur *et al.*, 2021). Hydrated silica nanoparticles, SiO_2 x nH_2O, represent the only form with known

efficiency in the released quantities and bioavailability of silicon to the root and further to the plant tissues (Epstein, 1994). The phytoliths accumulated in plants, especially in the silicon-accumulator plants, act as silicon sinks and silicon sources(Cornelis *et al.*, 2016). This section will focus on the recovery of silica nanoparticles from phytoliths, namely chemical extraction and maceration of silica-rich plants, widely used in organic agriculture.

Particular types of biomass wastes were found to be rich in biosilica, such as rice and oat husks, wheat and rice straws, horsetail, corn cobs, sugarcane bagasse, or bamboo and reed (*Phragmites australis*) leaves, with a very high level of biosilica (Athinarayanan *et al.*, 2017; Kow *et al.*, 2016; Schaller *et al.*, 2013; D. Schneider *et al.*, 2020; Vaibhav *et al.*, 2015). From such biosilica-rich biomass, silicon is recovered mainly in the form of nanosilica. The silicon extraction methods can generally be divided into alkaline or acid techniques. Some relevant studies in various extraction conditions are presented in Table 2.

Table 2. Extraction conditions of biosilica from different types of biomass wastes

Biomass type	Extraction conditions	Silica yields and properties	References
Rice husk	Ball milling, 0.2M NaOH, KOH, 80°C, 3 h, 6% w/v, washing, calcination 6 h at 900°C	98.5% Silica, 1.97 m^2/g, SSA, 0.004 cm^3/g, pore-volume, NaOH leads to a higher yield than KOH	(Park *et al.*, 2021)
Rice husk Sugarcane bagasse Groundnut shell Bamboo leaves	Drying, calcination 7 h at 900°C, 1M NaOH to dissolve SiO_2, precipitation 24 h with 6M H_2SO_4	98% SiO_2	(Vaibhav *et al.*, 2015)
Sorghum husk	Drying, 1:5 S/L, 0.1N HCl, 120°C, 15 lbs., drying, calcination 20 min at 600°C	95% Silica, 50-300 µm particle size	(Periasamy *et al.*, 2018)
Horsetail	Drying, 1:10 S/L, 2% H_2S0_4, 140°C, calcination 2h at 650°C	30-50 nm particle size	(Mattos *et al.*, 2018)
Rice Oat Spelt husk Rice husk Horsetail	Drying, 1:13 S/L, water, 24 h, filtration, 1:13 S/L, 3.25M citric acid, 323K, 24 h, filtration, washing, calcination 30-210 min at 583-783K	>90% Silica, 185-303m^2/g SSA, 0.35-0.46 cm^3/g pore volume, 2-30 nm pore size	(Denise Schneider *et al.*, 2020)
Sugarcane bagasse	Crushing, sieving to a size of 0.5-1 cm, 1:6 S/L, 1N HCl, 120°C, 2 h, 15 lbs., washing, drying, calcination at 550°C, 650°C, 750°C	251, 210, 191 average particle size for the nanobiosilica prepared at three different calcination temperature	(Athinarayanan *et al.*, 2017)
Rice husk	1:5 S/L, 1N HCl, 120°C, 2 h, 15 lbs., washing, drying, calcination at 500°C, 600°C, 700°C	10-30 nm particle size, spherical shape,	(Athinarayanan *et al.*, 2015)
Horsetail	Drying, sieving, 1:50 S/L, HCl 4M, 2 h boiling, washing, drying, calcination 2h at 500°C	Mesoporous silica network, 450m^2/g SSA, about 15 nm particle size	(Hosseini Mohtasham & Gholizadeh, 2020)
Sugarcane bagasse Corn cob	Drying, sieving, calcination 400-1000°C, 1:6 S/L, 1N NaOH, 80°C, cooling, 1N HCl to reach neutral pH, desiccation 12 h, 80°C	17.23 nm average crystallite size, nano-agglomerated, irregular shape	(Goswami & Mathur, 2022)

*Abbreviations: S/L = solid/liquid ratio

The extraction processes generally start with a grinding step and end with a calcination step. Mechanical grinding using ball milling or chopping blender is necessary to reduce particle size and increase the surface area for the next leaching step. Alkaline or acidic treatments aim to destruct the lignocellulose matrix by dissolving the soluble fibers, mainly hemicelluloses, and further decrease the particle size for the carbonization/calcination step. By calcination at 400-900°C, the remaining organic phase consisting of cellulose and lignin is decomposed in volatiles, CO_2, and water, leaving behind the inorganic phase predominant in silica. The resulting nano(bio)silica from phytoliths was used to promote plant growth and enhance stress tolerance in cultivated plants (Mathur & Roy, 2020).

Maceration is a process used since ancient times through which bioactive compounds are

released from plant material into the extraction solvent. This process has become widespread as a recovery method for compounds with productivity and crop quality enhancing properties. As we already mentioned, there are silica-rich plants due to their capacity to uptake silicon faster than water (Richmond & Sussman, 2003). Such plants were used to manufacture fermented macerates used in organic/biodynamic agriculture (Proctor, 2012). Horsetail is the common name that has been assigned to plant species that belong to the genus *Equisetum*. These plants contain a significant amount of silicon deposited in plant tissues in the form of phytoliths. There are theories that the rich silicon content, i.e., 25% of the dry mass of horsetail species, has replaced the lignin content, which is about 12%. Therefore, silicon plays one of its functions in strengthening the cell wall structure (Yamanaka *et al.*, 2012). Moreover, the potential of *Equisetum arvense* L. in crop protection has been recognized, as stated in the European Regulation No 462/2014 (Regulation, 2014), especially for the ability of silicon to alleviate the stress induced by fungal diseases (García-Gaytán *et al.*, 2019).

Nettle is the common name for plant species belonging to the genus *Urtica* (Đurić *et al.*, 2019; Kregiel *et al.*, 2018), and they have been used for centuries to treat various medical conditions due to their rich content in compounds with antimicrobial (Kregiel *et al.*, 2018), anti-inflammatory (Johnson *et al.*, 2013), antioxidant (Telo *et al.*, 2017) activity. In nettles, the rate of silicon uptake from the soil is similar to that of water uptake, and Si gets deposited even in the stinging nettle (Sowers & Thurston, 1979). Thus, they are considered moderate accumulators of silicon (Luyckx *et al.*, 2017; Sowers *et al.*, 1979). Several studies have been reported in which horsetail or nettle macerates have been used as plant biostimulants or control agents against plant pathogens. We presented some of them in Table 3.

Table 3. Response of macerate-treated plants to different conditions

Plant type	S:L ratio	Maceration time	Final concentration applied	Environment condition	Effect in horse-tail macerate-treated plants compared to untreated plants	References
Horsetail	200 g: dry plant:10L water	Soaking 30 min, boiling 45 min	2 mg/mL	Grapevine trunk diseases (GTDs)	25% inhibition of fungal growth	(Langa-Lomba *et al.*, 2021)
Horsetail	600 g dry leafs:10L water	7 days for Si release	12 kg/hL	Fungal pathogens	Si slowed down the water excess that could lead to fungal growth Increase in crop yield with 30%	(Trebbi *et al.*, 2021)
Nettle	15 g dry leafs:1L water	3-4 days	2 mg/mL	Grapevine trunk diseases (GTDs)	25% inhibition of fungal growth	(Langa-Lomba *et al.*, 2021)
Nettle	183 g dry plant:10L water	24 h / 14 days	Dilution 1:3 ratio	-	Increase in leaf area of green beans Increase in height and stem diameter	(Maričić *et al.*, 2021)

NANOFORMULATED PLANT BIOSTIMULANTS MADE FROM BIOSILICA EXTRACTED FROM SILICON-RICH BIOMASS

Nanoparticles (NPs) are defined as particles with a size between 1 and 100 nm (Khan *et al.*, 2019). The abundance of nanoparticles in plants depends on various factors such as plant age, growth environment, or species (Luyckx *et al.*, 2017; Rajput *et al.*, 2020; D. Tripathi *et al.*, 2016). The uptake rate, translocation, or degree of storage of nanoparticles also depend on the nanoparticles' physicochemical properties, such as size, shape, chemical composition, surface/volume ratio, or nanoparticles stability in solution (Ferdous & Nemmar, 2020). The chemical structure of most silica forms consist of one silicon atom bonded to four oxygen atoms in a tetrahedral unit. Crystalline forms of silica

have a regular structure. In contrast, amorphous forms are composed of highly disordered tetrahedral units of silicon and oxygen, bonded randomly, without a defined pattern (Perry, 2009). The polymeric structure of silica nanoparticles consists of siloxane (-Si-O-Si-) groups formed by covalently bonded oxygen and silicon atoms and silanol (Si-OH), highly concentrated at the surface (Zhuravlev, 2000). In addition, depending on the use, silica nanoparticles (SiNPs) can be functionalized with various compounds resulting in multifunctional nanoconjugates.

Nanoparticles uptake into the plant structure depends on the pore diameter of the cell wall (2-20 nm) (Kurczyńska *et al.*, 2021). Thus, the size of the synthesized nanoparticles or nanoparticles aggregate must be smaller than the pore diameter of the cell so they can easily pass through the cell wall and reach the cell membrane (Augustine *et al.*, 2020). As a pathway into plant tissues, silica nanoparticles can form complexes with specific transporters or root exudates (Bhat *et al.*, 2021; de Moraes & Lacava, 2022; Wang *et al.*, 2022). Translocation of nanoparticles through plasmodesmata has also been reported (Kurczyńska *et al.*, 2021).

Plenty of studies support the role of silicon nanoparticles in agriculture due to their remarkable properties - their nanometric size and high mobility through the plant tissues. Some relevant studies are presented in Table 4..

Table 4. Response of SiNPs-treated plants to different conditions

Plant type	SiNPs size	Stress	Effect in SiNPs-treated plants compared to untreated plants	References
Hawthorn berry (*Crataegus* sp.)	10-30 nm	Drought	Increase in plant growth Constant electrolyte leakage index Decrease of MDA Increase in chlorophyll content	(Ashkavand *et al.*, 2015)
E. sativa	17.23 nm	-	Increase plant growth Increase in protein content Increase in chlorophyll level Increase in polyphenol content	(Goswami *et al.*, 2022)
Maize (*Zea mays* L.)	20-40 nm	-	Increase in phenolic compounds	(Suriyaprabha *et al.*, 2014)
Maize (*Zea mays* L.)	20-40 nm	-	Enhance soil nutrient content	(Rangaraj *et al.*)
Lentil (*Lens culinaris* Medik.)	20-30 nm	Salt	Increase in seed germination Stress alleviation Increase in plant weight	(Sabaghnia & Janmohammadi, 2015)
Maize	150-200 nm	Heavy metals	Stress alleviation	(D. K. Tripathi *et al.*, 2016)

*Abbreviations: MDA (malondialdehyde)

Despite many studies demonstrating the silicon plant biostimulant activities, soluble silicon and nanobiosilica produced from plant phytoliths are still not-used routinely in crop production (Zellner *et al.*, 2021). The main reason for such underutilization is presented in Figure 1. Future studies, including those that will reinforce the link between (nano)biosilica and the beneficial effects of macerated plant extracts, will promote silicon utilization by the farmers.

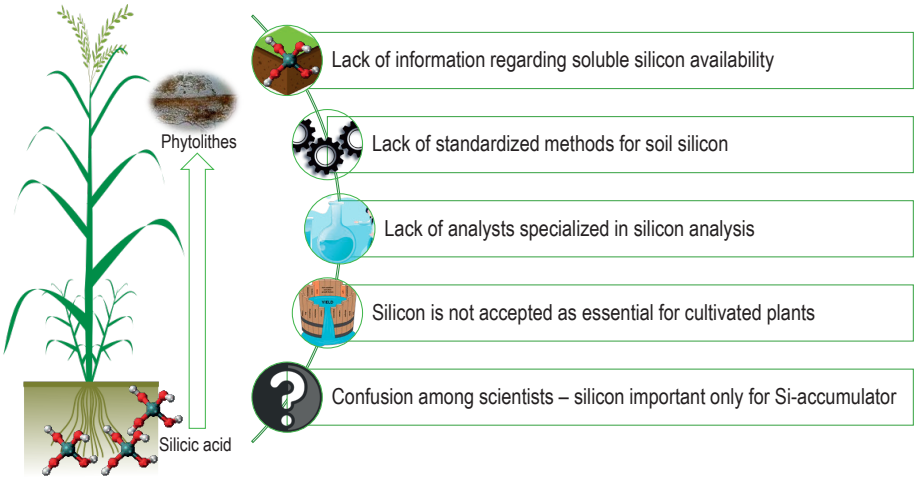


Figure 1. The main reason for the underutilization of silicon in crop management. Information adapted from Zelnner *et al*., 2021

CONCLUSIONS

Soluble silicon is beneficial for cultivated plants and the proper function of ecosystems. The silicon mode of action is not fully understood. However, the studies reported till now reveal silicon potential in mitigating abiotic stress amplified by climatic changes.

Soluble silicon is still not routinely used in crop management. One direction to increase silicon utilization in agriculture is to accelerate the studies demonstrating (nano)biosilica as one of the main ingredients of macerated plant extracts. Such macerated plant extracts are largely used in practice. The academic community's interest in studying such macerated plant extract is limited due to a lack of active ingredient characterization. Such bi-directional knowledge transfer should promote silicon farming.

ACKNOWLEDGEMENTS

This research work was carried out with the support of the Ministry of Research, Innovation, and Digitalization, project 1PS Development of innovative technologies using artificial intelligibility to valorize the biomass from wet areas as a support for sustainable green energy and Project Nucleu P.N.19.23.01.01 Integrated platform for the "smart" utilization of biomass – Smart-Bi.

REFERENCES

Ali, E., & Hassan, F. (2017). Water Stress Alleviation of Roselle Plant by Silicon Treatment Through Some Physiological and Biochemical Responses. *Annual Research & Review in Biology, 21*, 1-17.

Ashkavand, P., Tabari, M., Zarafshar, M., Tomášková, I., & Struve, D. (2015). Effect of SiO2 nanoparticles on drought resistance in hawthorn seedlings. *Forest Research Papers, 76*(4), 350-359.

Athinarayanan, J., Periasamy, V. S., Alhazmi, M., Alatiah, K. A., & Alshatwi, A. A. (2015). Synthesis of biogenic silica nanoparticles from rice husks for biomedical applications. *Ceramics International, 41*(1, Part A), 275-281.

Athinarayanan, J., Periasamy, V. S., Alhazmi, M., & Alshatwi, A. A. (2017). Synthesis and biocompatibility assessment of sugarcane bagasse-derived biogenic silica nanoparticles for biomedical applications. *Journal of Biomedical Materials Research Part B-Applied Biomaterials, 105*(2), 340-349.

Augustine, R., Hasan, A., Primavera, R., Wilson, R. J., Thakor, A. S., & Kevadiya, B. D. (2020). Cellular uptake and retention of nanoparticles: Insights on particle properties and interaction with cellular components. *Materials Today Communications, 25*, 101692.

Avestan, S., Ghasemnezhad, M., Esfahani, M., & Byrt, C. S. (2019). Application of Nano-Silicon Dioxide Improves Salt Stress Tolerance in Strawberry Plants. *Agronomy, 9*(5).

Bhat, J. A., Rajora, N., Raturi, G., Sharma, S., Dhiman, P., Sanand, S., Shivaraj, S. M., Sonah, H., & Deshmukh, R. (2021). Silicon nanoparticles (SiNPs) in sustainable agriculture: major emphasis on the practicality, efficacy and concerns. *Nanoscale Advances, 3*(14), 4019-4028.

Carey, J. C., & Fulweiler, R. W. (2014). Silica uptake by Spartina - evidence of multiple modes of accumulation

from salt marshes around the world. *Frontiers in Plant Science, 5*.

Casey, W., Kinrade, S., Knight, C., Rains, D., & Epstein, E. (2003). Aqueous silicate complexes in wheat, *Triticum aestivum* L. *Plant, Cell & Environment, 27*, 51-54.

Constantinescu-Aruxandei, D., Lupu, C., & Oancea, F. (2020). Siliceous Natural Nanomaterials as Biorationals - Plant Protectants and Plant Health Strengtheners. *Agronomy, 10*(11).

Cornelis, J. T., & Delvaux, B. (2016). Soil processes drive the biological silicon feedback loop. *Functional Ecology, 30*(8), 1298-1310.

de Moraes, A. C. P., & Lacava, P. T. (2022). Chapter 5 - Use of silicon and nano-silicon in agro-biotechnologies. In H. Etesami, A. H. Al Saeedi, H. El-Ramady, M. Fujita, M. Pessarakli, & M. Anwar Hossain (Eds.), *Silicon and Nano-silicon in Environmental Stress Management and Crop Quality Improvement* (pp. 55-65): Academic Press.

de Tombeur, F., Roux, P., & Cornelis, J. T. (2021). Silicon dynamics through the lens of soil-plant-animal interactions: perspectives for agricultural practices. *Plant and Soil, 467*(1-2), 1-28.

du Jardin, P. (2015). Plant biostimulants: Definition, concept, main categories and regulation. *Scientia Horticulturae, 196*, 3-14.

Đurić, M., Mladenović, J., Bošković-Rakočević, L., Šekularac, G., Brković, D., & Pavlović, N. (2019). Use of different types of extracts as biostimulators in organic agriculture. *Acta agriculturae Serbica, 24*, 27-39.

El Sabagh, A., Islam, M. S., Hossain, A., Iqbal, M. A., Mubeen, M., Waleed, M., Reginato, M., Battaglia, M., Ahmed, S., Rehman, A., Arif, M., Athar, H., Ratnasekera, D., Danish, S., Raza, M., Rajendran, K., Mushtaq, M., Skalicky, M., Brestic, M., & Abdelhamid, M. (2022). Phytohormones as Growth Regulators During Abiotic Stress Tolerance in Plants. *2022*, 765068.

Epstein, E. (1994). The anomaly of silicon in plant biology. *Proceedings of the National Academy of Sciences, 91*(1), 11-17.

Etesami, H., & Jeong, B. R. (2018). Silicon (Si): Review and future prospects on the action mechanisms in alleviating biotic and abiotic stresses in plants. *Ecotoxicology and Environmental Safety, 147*, 881-896.

Fahad, S., Bajwa, A. A., Nazir, U., Anjum, S. A., Farooq, A., Zohaib, A., Sadia, S., Nasim, W., Adkins, S., Saud, S., Ihsan, M. Z., Alharby, H., Wu, C., Wang, D., & Huang, J. (2017). Crop Production under Drought and Heat Stress: Plant Responses and Management Options. *Frontiers in Plant Science, 8*.

Fauteux, F., Chain, F., Belzile, F., Menzies James, G., & Bélanger Richard, R. (2006). The protective role of silicon in the Arabidopsis–powdery mildew pathosystem. *Proceedings of the National Academy of Sciences, 103*(46), 17554-17559.

Ferdous, Z., & Nemmar, A. (2020). Health Impact of Silver Nanoparticles: A Review of the Biodistribution and Toxicity Following Various Routes of Exposure. *International journal of molecular sciences, 21*(7), 2375.

Ferreira, H. A., Nascimento, C. W. A. d., Datnoff, L. E., Nunes, G. H. d. S., Preston, W., Souza, E. B. d., & Mariano, R. d. L. R. (2015). Effects of silicon on resistance to bacterial fruit blotch and growth of melon. *Crop Protection, 78*, 277-283.

Fleck, A. T., Nye, T., Repenning, C., Stahl, F., Zahn, M., & Schenk, M. K. (2011). Silicon enhances suberization and lignification in roots of rice (*Oryza sativa*). *Journal of experimental botany, 62*(6), 2001-2011.

García-Gaytán, V., Bojórquez-Quintal, E., Hernández-Mendoza, F., Tiwari, D. K., Corona-Morales, N., & Moradi-Shakoorian, Z. (2019). Polymerized silicon (SiO2· nH2O) in equisetum arvense: potential nanoparticle in crops. *Journal of the Chilean Chemical Society, 64*(1), 4298-4302.

Goswami, P., & Mathur, J. (2022). Application of agro-waste-mediated silica nanoparticles to sustainable agriculture. *Bioresources and Bioprocessing, 9*(1), 9.

Harman-Ware, A. E., Sparks, S., Addison, B., & Kalluri, U. C. (2021). Importance of suberin biopolymer in plant function, contributions to soil organic carbon and in the production of bio-derived energy and materials. *Biotechnology for Biofuels, 14*(1), 75.

Hasanuzzaman, M., Bhuyan, M., Zulfiqar, F., Raza, A., Mohsin, S. M., Al Mahmud, J., Fujita, M., & Fotopoulos, V. (2020). Reactive Oxygen Species and Antioxidant Defense in Plants under Abiotic Stress: Revisiting the Crucial Role of a Universal Defense Regulator. *Antioxidants, 9*(8).

Hosseini Mohtasham, N., & Gholizadeh, M. (2020). Nano silica extracted from horsetail plant as a natural silica support for the synthesis of H3PW12O40 immobilized on aminated magnetic nanoparticles (Fe3O4@SiO2-EP-NH-HPA): a novel and efficient heterogeneous nanocatalyst for the green one-pot synthesis of pyrano[2,3-c]pyrazole derivatives. *Research on Chemical Intermediates, 46*(6), 3037-3066.

Johnson, T. A., Sohn, J., Inman, W. D., Bjeldanes, L. F., & Rayburn, K. (2013). Lipophilic stinging nettle extracts possess potent anti-inflammatory activity, are not cytotoxic and may be superior to traditional tinctures for treating inflammatory disorders. *Phytomedicine : international journal of phytotherapy and phytopharmacology, 20*(2), 143-147.

Kawaguchi, K., & Kyūma, K. (1977). *Paddy soils in tropical Asia, their material nature and fertility / Keizaburo Kawaguchi, Kazutake Kyuma*. Honolulu: University of Hawaii Press.

Khan, A., Khan, A. L., Imran, M., Asaf, S., Kim, Y.-H., Bilal, S., Numan, M., Al-Harrasi, A., Al-Rawahi, A., & Lee, I.-J. (2020). Silicon-induced thermotolerance in *Solanum lycopersicum* L. via activation of antioxidant system, heat shock proteins, and endogenous phytohormones. *BMC Plant Biology, 20*(1), 248.

Khan, I., Saeed, K., & Khan, I. (2019). Nanoparticles: Properties, applications and toxicities. *Arabian Journal of Chemistry, 12*(7), 908-931.

Kim, Y.-H., Khan, A. L., Kim, D.-H., Lee, S.-Y., Kim, K.-M., Waqas, M., Jung, H.-Y., Shin, J.-H., Kim, J.-G., &

Lee, I.-J. (2014). Silicon mitigates heavy metal stress by regulating P-type heavy metal ATPases, Oryza sativalow silicon genes, and endogenous phytohormones. *BMC Plant Biology, 14*(1), 13.

Kow, K. W., Yusoff, R., Aziz, A. R. A., & Abdullah, E. C. (2016). Determination of kinetic parameters for thermal decomposition of bamboo leaf to extract bio-silica. *Energy Sources Part a-Recovery Utilization and Environmental Effects, 38*(21), 3249-3254.

Kregiel, D., Pawlikowska, E., & Antolak, H. (2018). Urtica spp.: Ordinary Plants with Extraordinary Properties. *Molecules (Basel, Switzerland), 23*(7), 1664.

Kurczyńska, E., Godel-Jędrychowska, K., Sala, K., & Milewska-Hendel, A. (2021). Nanoparticles - Plant Interaction: What We Know, Where We Are? *Applied Sciences, 11*(12).

Langa-Lomba, N., Buzón-Durán, L., Martín-Ramos, P., Casanova-Gascón, J., Martín-Gil, J., Sánchez-Hernández, E., & González-García, V. (2021). Assessment of Conjugate Complexes of Chitosan and Urtica dioica or Equisetum arvense Extracts for the Control of Grapevine Trunk Pathogens. *Agronomy, 11*(5).

Lesharadevi, K., Parthasarathi, T., & Muneer, S. (2021). Silicon biology in crops under abiotic stress: A paradigm shift and cross-talk between genomics and proteomics. *Journal of Biotechnology, 333*, 21-38.

Luyckx, M., Hausman, J.-F., Lutts, S., & Guerriero, G. (2017). Silicon and Plants: Current Knowledge and Technological Perspectives. *Frontiers in Plant Science, 8*.

Ma, J. F., & Yamaji, N. (2015). A cooperative system of silicon transport in plants. *Trends in Plant Science, 20*(7), 435-442.

Ma, J. F., Yamaji, N., Tamai, K., & Mitani, N. (2007). Genotypic difference in silicon uptake and expression of silicon transporter genes in rice. *Plant physiology, 145*(3), 919-924.

Maričić, B., Radman, S., Romić, M., Perković, J., Major, N., Urlić, B., Palcic, I., Zorić, Z., & Goreta Ban, S. (2021). Stinging Nettle (*Urtica dioica* L.) as an Aqueous Plant-Based Extract Fertilizer in Green Bean (*Phaseolus vulgaris* L.) Sustainable Agriculture. *Sustainability, 13*.

Mathur, P., & Roy, S. (2020). Nanosilica facilitates silica uptake, growth and stress tolerance in plants. *Plant Physiology and Biochemistry, 157*, 114-127.

Mattos, B. D., Gomes, G. R., de Matos, M., Ramos, L. P., & Magalhães, W. L. E. (2018). Consecutive Production of Hydroalcoholic Extracts, Carbohydrates Derivatives and Silica Nanoparticles from *Equisetum arvense*. *Waste and Biomass Valorization, 9*(11), 1993-2002.

Mitani-Ueno, N., & Ma, J. F. (2021). Linking transport system of silicon with its accumulation in different plant species. *Soil Science and Plant Nutrition, 67*(1), 10-17.

Moradtalab, N., Weinmann, M., Walker, F., Höglinger, B., Ludewig, U., & Neumann, G. (2018). Silicon Improves Chilling Tolerance During Early Growth of Maize by Effects on Micronutrient Homeostasis and Hormonal Balances. *Frontiers in Plant Science, 9*.

Nawaz, M. A., Zakharenko, A. M., Zemchenko, I. V., Haider, M. S., Ali, M. A., Imtiaz, M., Chung, G., Tsatsakis, A., Sun, S., & Golokhvast, K. S. (2019). Phytolith Formation in Plants: From Soil to Cell. *Plants, 8*(8), 249.

Park, J. Y., Gu, Y. M., Park, S. Y., Hwang, E. T., Sang, B. I., Chun, J., & Lee, J. H. (2021). Two-Stage Continuous Process for the Extraction of Silica from Rice Husk Using Attrition Ball Milling and Alkaline Leaching Methods. *Sustainability, 13*(13), 11.

Periasamy, V. S., Athinarayanan, J., & Alshatwi, A. A. (2018). Extraction and biocompatibility analysis of silica phytoliths from sorghum husk for three-dimensional cell culture. *Process Biochemistry, 70*, 153-159.

Perry, C. C. (2009). An Overview of Silica in Biology: Its Chemistry and Recent Technological Advances. In W. E. G. Müller & M. A. Grachev (Eds.), *Biosilica in Evolution, Morphogenesis, and Nanobiotechnology: Case Study Lake Baikal* (pp. 295-313). Berlin, Heidelberg: Springer Berlin Heidelberg.

Proctor, P. (2012). *Grasp the Nettle: Making Biodynamic Farming & Gardening Work*: Penguin Random House New Zealand Limited.

Rajput, V., Minkina, T., Mazarji, M., Shende, S., Sushkova, S., Mandzhieva, S., Burachevskaya, M., Chapligin, V., Singh, A., & Jatav, H. (2020). Accumulation of nanoparticles in the soil-plant systems and their effects on human health. *Annals of Agricultural Sciences, 65*, 137-143.

Rangaraj, S., Gopalu K Fau - Rathinam, Y., Rathinam Y Fau - Periasamy, P., Periasamy P Fau - Venkatachalam, R., Venkatachalam R Fau - Narayanasamy, K., & Narayanasamy, K. Effect of silica nanoparticles on microbial biomass and silica availability in maize rhizosphere. (1470-8744 Electronic).

Regulation, E. U. (2019). 1009 of the European Parliament and of the Council of 5 June 2019 Laying Down Rules on the Making Available on the Market of EU Fertilising Products and Amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and Repealing Regulation (EC) No 2003/200. *J. Eur. Union*, L170.

Richmond, K. E., & Sussman, M. (2003). Got silicon? The non-essential beneficial plant nutrient. *Current opinion in plant biology, 6*(3), 268-272.

Sabaghnia, N., & Janmohammadi, M. (2015). Effect of nano-silicon particles application on salinity tolerance in early growth of some lentil genotypes / Wpływ nanocząstek krzemionki na tolerancję zasolenia we wczesnym rozwoju niektórych genotypów soczewicy. *Annales UMCS, Biologia, 69*.

Savvas, D., & Ntatsi, G. (2015). Biostimulant activity of silicon in horticulture. *Scientia Horticulturae, 196*, 66-81.

Schaller, J., Brackhage, C., Paasch, S., Brunner, E., Bäucker, E., & Dudel, E. G. (2013). Silica uptake from nanoparticles and silica condensation state in different tissues of Phragmites australis. *Science of The Total Environment, 442*, 6-9.

Schaller, J., Puppe, D., Kaczorek, D., Ellerbrock, R., & Sommer, M. (2021). Silicon Cycling in Soils Revisited. *Plants-Basel, 10*(2).

Schneider, D., Wassersleben, S., Weiß, M., Denecke, R., Stark, A., & Enke, D. (2020). A Generalized Procedure for the Production of High-Grade, Porous Biogenic Silica. *Waste and Biomass Valorization, 11*(1), 1-15.

Schneider, D., Wassersleben, S., Weiss, M., Denecke, R., Stark, A., & Enke, D. (2020). A Generalized Procedure for the Production of High-Grade, Porous Biogenic Silica. *Waste and Biomass Valorization, 11*(1), 1-15.

Sowers, A. E., & Thurston, E. L. (1979). Ultrastructural evidence for uptake of silicon-containing silicic acid analogs by*Urtica pilulifera* and incorporation into cell wall silica. *Protoplasma, 101*(1), 11-22.

Spinde, K., Pachis, K., Antonakaki, I., Paasch, S., Brunner, E., & Demadis, K. D. (2011). Influence of Polyamines and Related Macromolecules on Silicic Acid Polycondensation: Relevance to "Soluble Silicon Pools"? *Chemistry of Materials, 23*(21), 4676-4687.

Suriyaprabha, R., Karunakaran, G., Yuvakkumar, R., Rajendran, V., & Kannan, N. (2014). Foliar Application of Silica Nanoparticles on the Phytochemical Responses of Maize (*Zea mays* L.) and Its Toxicological Behavior. *Synthesis and Reactivity in Inorganic, Metal-Organic, and Nano-Metal Chemistry, 44*(8), 1128-1131.

Telo, S., Halifeoglu, I., & Ozercan, I. H. (2017). Effects of Stinging Nettle (Urtica Dioica L.,) on Antioxidant Enzyme Activities in Rat Model of Mammary Gland Cancer. *Iranian journal of pharmaceutical research : IJPR, 16*(Suppl), 164-170.

Trebbi, G., Negri, L., Bosi, S., Dinelli, G., Cozzo, R., & Marotti, I. (2021). Evaluation of *Equisetum arvense* (Horsetail Macerate) as a Copper Substitute for Pathogen Management in Field-Grown Organic Tomato and Durum Wheat Cultivations. *Agriculture, 11*(1).

Tripathi, D., Gaur, S., Singh, S., Singh, S., Pandey, R., Singh, D. V., Sharma, N., Prasad, S., Dubey, N., & Chauhan, D. (2016). An overview on manufactured nanoparticles in plants: Uptake, translocation, accumulation and phytotoxicity. *Plant Physiology and Biochemistry, 110*.

Tripathi, D. K., Singh, S., Singh, V. P., Prasad, S. M., Chauhan, D. K., & Dubey, N. K. (2016). Silicon Nanoparticles More Efficiently Alleviate Arsenate Toxicity than Silicon in Maize Cultiver and Hybrid Differing in Arsenate Tolerance. *Frontiers in Environmental Science, 4*.

Vaibhav, V., Vijayalakshmi, U., & Roopan, S. M. (2015). Agricultural waste as a source for the production of silica nanoparticles. *Spectrochimica Acta Part a-Molecular and Biomolecular Spectroscopy, 139*, 515-520.

Van Bockhaven, J., De Vleesschauwer, D., & Höfte, M. (2013). Towards establishing broad-spectrum disease resistance in plants: silicon leads the way. *Journal of Experimental Botany, 64*(5), 1281-1293.

Verma, K. K., Song, X.-P., Tian, D.-D., Singh, M., Verma, C. L., Rajput, V. D., Singh, R. K., Sharma, A., Singh, P., Malviya, M. K., & Li, Y.-R. (2021). Investigation of Defensive Role of Silicon during Drought Stress Induced by Irrigation Capacity in Sugarcane: Physiological and Biochemical Characteristics. *ACS Omega, 6*(30), 19811-19821.

Wang, L., Ning, C., Pan, T., & Cai, K. (2022). Role of Silica Nanoparticles in Abiotic and Biotic Stress Tolerance in Plants: A Review. *International Journal of Molecular Sciences, 23*(4).

Yamanaka, S., Sato, K., Ito, F., Komatsubara, S., Ohata, H., & Yoshino, K. (2012). Roles of silica and lignin in horsetail (*Equisetum hyemale*), with special reference to mechanical properties. *Journal of Applied Physics, 111*(4), 044703.

Yao, X., Chu, J., Cai, K., Liu, L., Shi, J., & Geng, W. (2011). Silicon Improves the Tolerance of Wheat Seedlings to Ultraviolet-B Stress. *Biological Trace Element Research, 143*(1), 507-517.

Zexer, N., & Elbaum, R. (2020). Unique lignin modifications pattern the nucleation of silica in sorghum endodermis. *Journal of Experimental Botany, 71*(21), 6818-6829.

Zhuravlev, L. T. (2000). The surface chemistry of amorphous silica. Zhuravlev model. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 173*(1), 1-38.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

IN VITRO INFLUENCE OF CULTIVARS AND DIFFERENT CULTURE MEDIA ON VITRIFICATION AND DARKNESS ON PEACH (*Prunus persica* L. Batsch) SHOOTS MULTIPLICATION

Nazar AL GHASHEEM[1, 2], Florin STĂNICĂ[1], Adrian George PETICILĂ[1], Oana VENAT[1], Ana BUTCARU[1], Imad Al-SUWAID[1], Basim K. HASAN[2], Ihasn J. AZEB[2], Kawther ALGHASHEEM[2]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd., District 1, Bucharest, Romania
[2]College of Agricultural and Marshlands, University of Thi- Qar, Iraq

Corresponding author email: nizar.shihan.post.am@utq.edu.iq

Abstract

Three peach varieties (Florin, Filip and Mimi), two explants (shoots-tip and nodes) were tested. The explants were cultivated on 3 medium MS, B5 and QL adding of 30 g/l sucrose and 7 g/l agar without any hormone supplements during initiation stage. Cultures tubes were kept in the dark (0, 1, 2, 3, 4) days to get rid of the process of oxidation of phenolic materials resulting from cutting plant tissues. It was found during this study that the placement of the tubes of culture in the dark has a positive effect in the disposal of phenolic materials. Results presented that placing the explants in dark for 2-3 days was the best result to explants phenolic-free, in 2 days/dark (Florin 89% explant growing, Filip 83% explant growing and Mimi 85% explant growing), in 3 days/dark (Florin 89% explant growing, Filip 84% explant growing and Mimi 86% explant growing). Also QL medium give yellowish green shoots with a high resistance 95% to vitrification.

Key words*: B5, culture media, explants, MS, QL.*

INTRODUCTION

Peach (*Prunus persica* L. Batsch) was cultivation in China before 4000 years ago (Faust and Timon, 1995). Micropropagation peaches method has been used in recent years to produce seedlings with resistant and desirable qualities in large numbers (Al Ghasheem, 2018a). Micropropagation technique depends on several factors including: genetic structure; success of the sterilization process; components of the nutrient medium and added hormones; sources Carbon; blackening phenomenon and appearance of phenolic substances; vitrification phenomenon; etc. (Hammerschlag, 1986; Kubota, 2001; Kozai and Kubota, 2005). Browning was important factors for the successful plant tissue culture, phenolic leaching and contamination is one of the most common problems micropropagation. This process begins by changing and transforming the surface of the cutting plant tissues due to the oxidation of phenolic compounds to brown and thus the formation of quinine, which is considered a highly reactive substance and a toxic substance for plant tissues. (Ko et al., 2009; Taji and Williams, 1996; Xu et al., 2011a; Dayarani et al., 2013). In addition to the use of darkness and subculture, there are many methods that are used to get rid of the browning phenomenon in plant tissue culture, such as the use of activated charcoal (Weatherhead, 1979; Madhusudhanan, and Rahiman, 2000; Thomas, 2008); Antioxidants (Yari Khosroushahi, 2011; Ndakidemi, 2014); Polyphenol inhibitors (Jones and Saxena, 2013; Erland and Mahmoud, 2014; Jones et al., 2012; 2015). Vitrification or Hyperhydricity is a big problem in plants tissue culture technique which can effect on plants multiplication and culture developing (Hammerschlag, 1986). Its affects the morphological characteristics of the plant such as formed leaves and shoots (Pasqualetto, 1990). Anatomically and chemically, xylem with sclerenchyma tissues is less differentiated and lignified accompanied by a hypertrophy in the cortical and pith parenchyma. (Vieitez et al., 1986). Some researcher's divided plants into plants that are excessive in water or not, depending the vitrifaction phenomenon on the

shape of the formed leaves and shoots (Dewir et al., 2006; Tsay et al., 2006, Gribble, 1999; Casanova et al., 2008; Rojas-Martinez and Klerk, 2010) confirmed that the phenomenon of vitrification is a feature of the qualitative characteristics possessed by plants. While others confirmed that the excess water in the culture medium is the cause of the vitrification (Debergh et al., 1992; Dewir et al., 2006). While others mentioned the effect of hormones and Silicon (Si) elements added to the culture medium (Badr-Eldin et al., 2012; Sivanesan, 2014; Soundararajan, 2017) or effect of ventilation and Agar (Majada et al., 1997; Tsay et al., 2006; Badr-Eldin et al., 2012). The study aims to know the effect of the genetic factor and the period of darkness on the phenomena of browning and vitrification on peach micropropagation.

MATERIALS AND METHODS

Three peach varieties were included in the experiment: Florin, Filip and Mimi. Two types of explants (shoots and nodes) were taken at a length of 0.5-1 cm. Explants were collected from healthy trees grown at the Agricultural Research Station of the University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania in 2019. For clean and sterilization of explants was carried out to get rid of dust and pathogens via washed with tap water for 30 min. Under Laminar flow cabinet, explants were treated with alcohol (70% ethanol) for several minutes (2-3), after which the alcohol was removed by washing with distilled water 3 times. The explants were treated with NaOCl (10% v/v) for 15-20 minutes for surface sterilized with continuous stirring and shaking by magnetic stirrer to increase the effectiveness of the sterile material and its spread on the surface of the explants, and then rinsed with distilled water at least three times(Alghasheem, 2018b). The explants were cultured on 3 cultures media MS (Murashige and Skoog, 1962), B5 (Gamborg, 1968) and QL (Quoirin and Lepoivre, 1977) with 30 g/l sucrose and 7 g/l agar without any hormone. The pH was adjusted to 5.7. The test tubes were kept in the dark (0, 1, 2, 3, 4) days to get rid of the oxidation process of phenolic substances resulting from the cutting of plant tissues. The experiment was repeated twice, each treatment containing 30 replicates (one explant). Measurements and results were taken every day in terms of the interaction of phenolic substances and the appearance of the phenomenon of vitrification.

RESULTS AND DISCUSSIONS

Effect of variety and cultures media on tissue browning

During the study, placing the tubes in the dark had a positive effect in removing phenolic substances that cause the explants to discolour to black or brown and cause the toxicity of the cultured tissue and the low success rate of the culture. In Table 1 we were found that placing the explants in the dark for 2-3 days gave the best results, obtaining explants without phenols. Thus, they were recorded for a period of 2 days/dark (Florin 89% healthy explants, Filip 83% healthy explants and Mimi 85% healthy explants), respectively of 3 days/dark (Florin 89%, Filip 84% explants and Mimi 86% healthy explants).(Figures 1, 2 and 3)

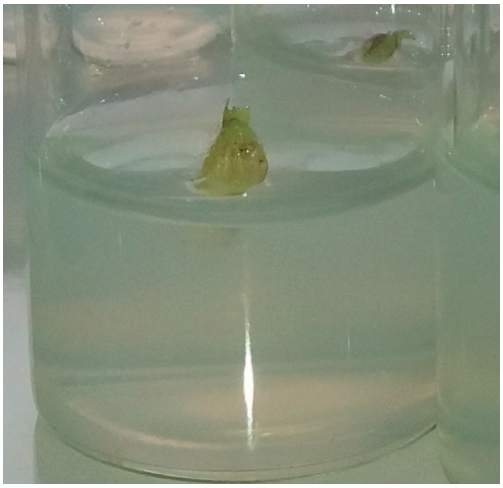


Figure 1. The appearance of phenolic substances after put the explants in culture media (Filip variety)

Explants incubation in the dark decreases browning of the culture medium caused by exudation of phenolic by explants (Nehra et al., 1989; Rugini, 1992; Bhatia and Ashwath, 2005; Xu et al., 2011b). When the plant is cut many enzymes such as polyphenol oxidase,

Table 1. Effect of darkness on phenolic compounds that cause the oxidation of tissue of plant cultured in 3 culture media

Variety	Number of explants	Days														
		0			1			2			3			4		
		% Dead	% growth	Notiţe	% Dead	% growth	Notes	% Dead	% growth	Notes	% Dead	% growth	Notes	% Dead	% growth	Notes
Florin	30	26	74	green to brown green	14	86	Green to yellowish green	11	89	Gree n	11	89	Green	10	90	Green to yellowish green
Filip	30	34	66	green to brown green	20	80	Green to yellowish green	17	83	Gree n	16	84	Green	16	84	Green to yellowish green
Mimi	30	31	69	green to brown green	19	81	Green to yellowish green	15	85	Gree n	14	86	Green	15	85	Green to yellowish green

superoxide dismutase and peroxidase are released into the affected part of the plant. These enzymes work to maintain plant tissues by catalysing various reactions for the purpose of eliminating reactive oxygen and thus healing the plant (Titov et al., 2006).

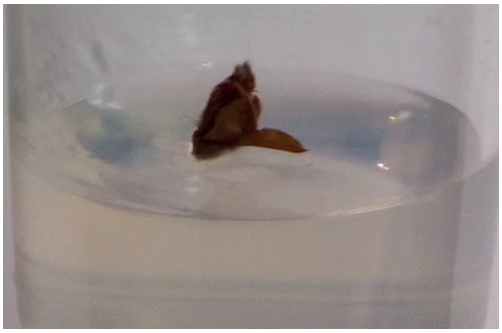


Figure 2. Loss of explant after one week from culture due to reactions of phenolic substances (Florin variety)

In this process, many compounds are produced, including "melanin", which is a pigment characterized by a dark colour and causes the colour of the media as well as the explants to turn brown, and an increase in this substance leads to stopping or impeding the growth of the plant and may lead to its death (Banerjee et al., 1996; Murata et al., 2001; Wu and Lin, 2002; Aquino-Bolaños and Mercado-Silva, 2004; Yari Khosroushahi, 2011).

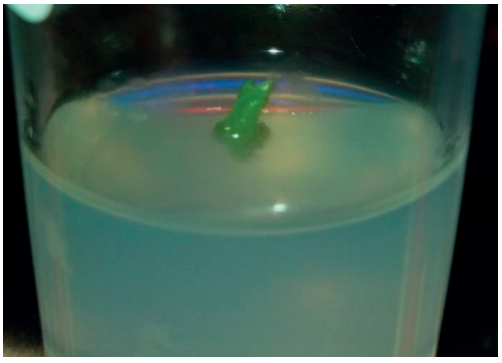


Figure 3. Shoot Mimi variety in QL medium (vitrification resistance of 95%)

Effect of variety and cultures media on vitrification

The study showed that the variety and culture medium affect the resistance to vitrification. The QL medium gives the shoots a high vitrification resistance of 95% (Mimi variety) compared to the MS and B5 media **(**Table 2**).** The study results was similar. Vieitez et al. (1986) studies when using Heller's macronutrient formula with MS medium, Heller's macronutrient recorded the highest vitrification resistance. Morphological abnormalities may be associated with decreased chlorophyll and lignin and increased tissue moisture**.** Vitrification can lead to leaf deformation and bud necrosis or loss the dominance in apical shoots (Cassells and Curry, 2001; Machado et al., 2014). Plants are characterized by abnormal growth and buds, stems and leaves are easy to break; their leaves are also characterized by shrunken or thin and shiny and slow growth and may eventually die(Figure 4) Which leads to a decrease in the vital processes inside the plant from carbon building and the formation of enzymes necessary for the plant and the lack of ionic and phenolic content (Phan and Letouze, 1983; Kevers and Gaspar, 1986; Bottcher et al., 1988; Perry et al., 1999; Frank et al., 2004).

Table 2. Effect of varieties and culture media on resistance of shoots to vitrification

V / M	Florin %	Filip %	Mimi %
MS	45	65	85
B5	70	50	80
QL	85	80	95

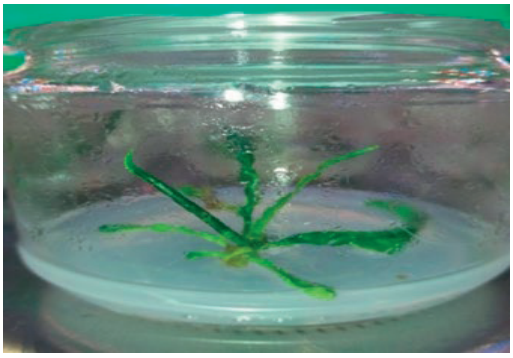


Figure 4. Shoots in MS medium, Filip variety with vitrification state

CONCLUSIONS

In our study we were found that the placement of the cultures tubes in the dark has a positive effect in the disposal of phenolic materials. Results presented that placing the explants in

dark for 2-3 days was the best result to explants phenolic-free, Also QL culture medium gave a high resistance 95% to vitrification.

ACKNOWLEDGEMENTS

The authors want to thanks all their colleagues, students, researchers, management and staff in micropropagation laboratory, Centre for Studies of Food and Agricultural Products Quality, USAMV of Bucharest, for their assistance in this papers.

REFERENCES

Alghasheem, N., Stănică, F., Peticilă, A.G. & Venat, O. (2018a). *In vitro* effect of various sterilization techniques on peach (*Prunus persica* L. Batsch) explants, Scientific Papers. Series B, Horticulture. Vol. LXII, 2018, Pp:227-234.

Alghasheem, N., Stănică, F., Peticilă, A.G. & Venat O. (2018b). *In vitro* effect of genotype, growth season and cytokinines on peach varieties (*Prunus persica* L. Batsch) propagation, Scientific Papers. Series B, Horticulture. Vol. LXII, 2018, Pp: 217-226.

Aquino-Bolaños, E.N. & Mercado-Silva, E. (2004). Effects of polyphenol oxidase and peroxidase activity, phenolics and lignin content on the browning of cut jicama. Post. Harvest. Biol. Tec. 33(3), 275–283.

Badr-Elden, A.M., Nower, A.A., Ibrahim, I.A., Ebrahim, M. K.H. & Abd Elaziem, T.M. (2012). Minimizing the hyperhydricity associated with *in vitro* growth and development of watermelon by modifying the culture conditions .January. AFRICAN JOURNAL OF BIOTECHNOLOGY, 11:8705-8717.

Banerjee, S., Upadhyay, N., Kukreja, A.K., Ahuja, P.S., Kumar, S., Saha, G.C., Sharma, R.P. & Chattopadhyay, S.K. (1996). Taxanes from *in vitro* cultures of the *Himalayan yew*, *Taxus wallichiana*. Planta Medica, 62(4), 329-331.

Bhatia, P. & Ashwath, N. (2005). Effect of Duration of Light: Dark Cycles on *in vitro* Shoot Regeneration of Tomato. Asian Journal of Plant Sciences, 4: 255-260.

Bottcher, I., Zoglauer K. & Goring, H. (1988). Induction and reversion of vitrification of plants cultured *in vitro*. Physiol Plant 72: 560–564.

Casanova, E., Moysset, L. & Trillas, M.I. (2008). Effects of agar concentration and vessel closure on the organogenesis and hyperhydricity of adventitious carnation shoots. Biol Plant, 52, 1-8.

Cassells, A.C. & Curry R. (2001). Oxidative stress and physiological epigenetic and genetic variability in plant tissue culture: implications for micropropagators and genetic engineers. Plant Cell Tiss. Org. Cult. 64: 145–167.

Dayarani, M., Dhanarajan, M.S., Uma, S. & Durai P. (2013). Macropropagation for regeneration of wild bananas (*Musa spp*.). Advanced BioTech 12:16–18.

Debergh, P., Aitken-Christie, J., Cohen, D., Grout, B., von Arnold, S., Zimmerman, R. & Ziv, M. (1992). Reconsideration of the term 'vitrification' as used in micropropagation. Plant Cell Tiss Org Cult, 30, 135-140.

Dewir, Y.H., Chakrabarty, D., Ali, M.B., Hahna, E.J & Paek, K.Y. (2006). Lipid peroxidation and antioxidant enzyme activities of *Euphorbia millii* hyperhydric shoots. Environ Exp Bot 58:93–99

Erland, L.A.E. & Mahmoud, S.S. (2014). An efficient method for regeneration of lavandin (*Lavandula x intermedia* cv. "Grosso"). *In vitro* Cell Dev Biol - Pl 50:646–654.
doi: 10.1007/s11627-014-9614-4.

Faust, M. & Timon, B. (1995). Origin and dissemination of peach. Hortic. Rev. 17, 331–379.

Franck, T., Kevers, C., Gaspar, T., Dommes, J., Deby, C., Greimers, R., Serteyn, D. & Deby-Dupont, G. (2004). Hyperhydricity of *Prunus avium* shoots cultured on gelrite: A controlled stress response. Plant Physiology and Biochemistry 42(6): 519-527.

Gamborg, O.L., Miller, R.A. & Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Research 50: 151–158.

Gribble, K. (1999). The influence of relative humidity on vitrification, growth and morphology of *Gypsophila paniculata* L. Plant Growth Regul, 27, 181-190.

Hammerschlag, F. A. (1986). Temperate fruits and nuts. In: "Tissue culture as a plant production system for horticultural crops". 221–236, Zimmerman, R. H., Griesbach, R. J., Hammerschlag, F. A., & Lawson, R. H. (eds). Martinus Nijhoff Publishers, Dordrecht.

Jones, A.M.P., Chattopadhyay, A., Shukla, M., Zon, J. & Saxena, P.K. (2012). Inhibition of phenylpropanoid biosynthesis increases cell wall digestibility, protoplast isolation, and facilitates sustained cell division in American elm (*Ulmus americana*). BMC Plant Biol, 12:75.
doi: 10.1186/1471-2229-12-75.

Jones, A.M.P. & Saxena, P.K. (2013). Inhibition of phenylpropanoid biosynthesis in *Artemisia annua* L.: A Novel Approach to Reduce Oxidative Browning in Plant Tissue Culture. PLOS ONE 8:e76802.
doi: 10.1371/journal.pone.0076802.

Jones, A.M.P., Shukla, M.R., Biswas, G.C.G. & Saxena, P.K. (2015). Protoplast-to-plant regeneration of American elm (*Ulmus americana*). Protoplasma, 252:925–931.
doi: 10.1007/s00709-014- 0724-y.

Kevers, C. & Gaspar, T.H. (1986). Vitrification of carnation *in vitro*: changes in water content, extracellular space, air volume, and ion levels. Physiol Vég 24: 647–653.

Ko, W.H., Su, C.C., Chen, C.L. & Chao, C.P. (2009). Control of lethal browning of tissue culture plantlets of Cavendish banana cv. Formosana with ascorbic acid. Plant Cell Tissue Organ Cult 96:137–141.

Kozai, T. & Kubota, C. (2005). *In vitro* root zone environments and their effects on growth and development of plants. In Photoautotrophic (sugar-free medium). Micropropagation as a New Micropropagation and Transplant Production System

Pp.53-60. Springer Netherlands. https: //doi.org/10.1007/1-4020-3126-2_5.

Kubota, C. (2001). Concepts and Background of Photoautotrophic Micropropagation. Molecular Breeding of Woody Plants, Proceedings of the International Wood Biotechnology Symposium (IWBS), 325–334. doi: 10.1016/s0921-0423(01) 80089-7.

Machado, M.P., da Silva, A.L.L., Biasi, L.A., Deschamps, C., Filho, J.C.B. & Zanette, F. (2014). Influence of calcium content of tissue on hyperhydricity and shoot-tip necrosis of *in vitro* regenerated shoots of *Lavandula angustifolia* Mill. Braz. Arch. Biol. Technol. 57, 636-643.

Madhusudhanan, K. & Rahiman, B.A. (2000). The effect of activated charcoal supplemented media to browning of *In vitro* cultures of piper species. Biologia plantarum, 43(2), 297-299. doi: 10.1023/A:1002729032527.

Majada, J.P., Fal, M.A. & Sánchez-Tamés, R. (1997). The effect of ventilation rate on proliferation and hyperhydricity of *Dianthus caryophyllus* L. *In Vitro* Cell. Dev. Biol.-Plant 33, 62. https://doi.org/10.1007/s11627-997-0042-6.

Murata, M., Nishimura, M., Murai, N., Haruta, M., Homma, S. & Itoh, Y. (2001). A transgenic apple callus showing reduced polyphenol oxidase activity and lower browning potential. Bioscience Biotechnology and Biochemistry, 65(2), 383-388.

Murashige, T. & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Ndakidemi, C., Mneney, E. & Ndakidemi, P. (2014). Effects of ascorbic acid in controlling lethal browning in *in vitro* culture of *Brahylaena huillensis* using nodal segments, American Journal of Plant Sciences, Vol. 5 No. 1, 2014, pp. 187-191. doi: 10.4236/ajps.2014.51024.

Nehra, N.S., Stushnoff, C. & Kartha, K. (1989). Direct shoot regeneration from strawberry leaf disks. Journal of the American Society for Horticultural Science, 114, 1014–1018.

Pasqualetto, P.L. (1990). Vitrification in plant tissue culture. In: Rodríguez, R., Tamés, R.S., Durzan, D.J. (eds) Plant Aging. NATO ASI Series (Series A: Life Sciences), vol 186. Springer, Boston, MA. https://doi.org/10.1007/978-1-4684-5760-5_16.

Perry, P.L., Ueno, K. & Shetty, K. (1999). Reversion to hyperhydration by addition of antibiotics to remove Pseudomonas in unhyperhydrated oregano tissue cultures Process Biochem. 34, 717-723.

Phan, C.T., & Letouze, R. (1983). A comparative study of chlorophyll, phenolic and protein contents and of hydroxycinnamate: CoA ligase activity of normal and vitreous plants (*Prunus avium* L.) obtained *in vitro*. Plant Sci. Lett., 31:323–327.

Quoirin, M. & Lepoivre, P. (1977). Etude de milieux adaptes aux cultures *in vitro* de Prunus. Acta Hortic, 78:437-442.

Rojas-martinez, L. & Klerk, G. (2010). Hyperhydricity in plant tissue culture. Drowing from within. In Prophyta Annual 2010; Agro Business Solutions: Grootebroek, The Netherlands, pp. 22-25.

Rugini, E. (1992). High efficiency shoots regeneration from calluses of strawberry (*Fragaria* × *ananassa Duch*) stipules of *in vitro* shoot cultures. Journal of Horticultural Science, 67, 577-582.

Sivanesan, I. & Park, S.W. (2014). The role of silicon in plant tissue culture. Front. Plant Sci., 5:571. doi: 10.3389/fpls.2014.00571.

Soundararajan, P., Manivannan, A., Cho, Y.S. & Jeong, B.R. (2017). Exogenous Supplementation of Silicon Improved the Recovery of Hyperhydric Shoots in *Dianthus caryophyllus* L. by Stabilizing the Physiology and Protein Expression. Front. Plant Sci. 8:738. doi: 10.3389/fpls.2017.00738.

Thomas, T.D. (2008). The role of activated charcoal in plant tissue culture. Biotechnology Advances Volume 26, Issue 6, Pages 618-631.

Titov, S., Bhowmik, S.K., Mandal, A., Alam, M.S. & Uddin, S.N. (2006). Control of phenolic compound secretion and effect of growth regulators for organ formation from *Musa spp*. cv. Kanthali floral bud explants. Am J Biochem Biotechnol, 2:97-104.

Taji, A. & Williams, R. (1996). Tissue culture of Australian plants. UNE, Armidale, pp 1–15.

Tsay, H.S. Lee, C.Y., Agrawal, D.C. & Basker, S. (2006). Influence of ventilation closure, gelling agent and explant type on shoot bud proliferation and hyperhydricity in *Scrophularia yoshimurae*: A Medicinal Plant. *In Vitro* Cellular & Developmental Biology. Plant Vol. 42, No. 5 (Sep. - Oct., 2006), pp. 445-449.

Vieitez, A., Ballester, A., San José, M.C. & Vieitez, E. (1986). Anatomical and chemical studies of vitrified shoots of chestnut regenerated *in vitro*. Physiologia Plantarum 65(2):177 – 184. DOI: 10.1111/j.1399-3054.1985.tb02379.x.

Weatherhead, M.A., Burdona, J. & Henshaw, G.G. (1979). Effects of activated charcoal as an additive plant tissue culture media: Part 2. Volume 94, Issue 5, Pages 399-405.

Wu, J. & Lin, L. (2002). Ultrasound-induced stress responses of *Panax ginseng* cells: enzymatic browning and phenolics production. Biotechnology Progress, 18(4), 862-866.

Xu, Y.W., Zou, Y.T., Husaini, A.M., Zeng, J.W., Guan, L.L., Liu, Q. & Wu, W. (2011a). Combined effect of dark and wounding on regeneration potential of *Houttuynia cordata* Thunb. leaves. Indian Journal of Experimental Biology 49, 540–546.

Xu, Y.W., Zou, Y.T., Husaini, A.M., Zeng, J.W., Guan, L.L., Liu, Q. & Wu, W. (2011b). Optimization of potassium for proper growth and physiological response of *Houttuynia cordata* Thunb. Environmental and Experimental Botany 71, 292–297.

Yari Khosroushahi, A., Naderi-Manesh, H., & Toft Simonsen, H. (2011). Effect of antioxidants and carbohydrates in callus cultures of *Taxus brevifolia*: evaluation of browning, callus growth, Total Phenolics and Paclitaxel Production. BioImpacts: BI, 1(1), 37–45. https://doi.org/10.5681/bi.2011.006.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

DEVELOPMENT STATUS AND TREND OF PLANT FACTORY INTELLIGENCE IN CHINA

Xinfa WANG[1, 2], Vladislav ZUBKO[1], Viktor ONYCHKO[1], Mingfu ZHAO[2]

[1]Sumy National Agrarian University, 160 Herasyma Kondratieva Street, Sumy, 40000, Ukraine
[2]Henan Institute of Science and Technology, East Section of Hualan Avenue, Xinxiang City, 453003, Henan Province, China

Corresponding author emails: wangxf2006@qq.com, zubkovladislav@ukr.net

Abstract

This paper analyzes the development status and trend of intelligent plant factories in China, hoping to give enlightenment to the development of plant factories in Ukraine or other countries. With the development of modern agricultural science and technology, plant industrialized production has become the preferred development mode of modern agriculture. At present, the plant factory is not highly intelligent and is in the stage of semi artificial intelligence. Most of the production operations and management are completed manually, which is difficult to manage, high cost and low efficiency. Process and analyze the real-time monitoring data, dynamically generate the management decision-making model, intelligently control the integrated irrigation equipment of water and fertilizer, the automatic recycling system of nutrient solution and other equipment and systems, and comprehensively intelligently regulate the LED artificial light, temperature and humidity, CO_2 concentration and air flow. These require highly intelligent facilities and equipment to jointly provide the most suitable growth environment for crops. Therefore, the effective integration of agricultural machinery and agronomy is the key to realize the intelligent management of plant factories. The deep integration of agricultural equipment with artificial intelligence and information technology to improve its automation and intelligence level will help to solve the problems existing in the industrialization of plant factories and the scale of crop planting.

Key words*: intelligent agriculture, plant factory, sustainable agriculture, urban agriculture, vertical farm.*

INTRODUCTION

Due to the rapid growth of population, large-scale expansion of cities, decreasing cultivated land, shortage of land resources, global spread of epidemic diseases, frequent extreme climate, pesticide abuse and serious biological pollution, organic food crop production, food supply, fruit and vegetable raw eating security are facing great threats and challenges. On the other hand, with the continuous improvement of people's living standards, the requirements for food hygiene, nutrition, health and greens are higher and higher. At present, a plant factory is one of the effective methods to solve the above contradiction. Plant factory refers to an efficient agricultural system that realizes the annual planned production of crops in vertical three-dimensional space through high-precision environmental control under completely closed or semi closed conditions (Yang, 2019). Artificial light plant factory refers to a plant industrial production facility with artificial light, heat insulation and almost closed warehouse structure (Kozai, 2013). Compared with open-air agriculture, the annual crop productivity of protected planting with the same floor area has increased by one order of magnitude (Mitchell, 2004), and the productivity of multi-layer indoor crop production has increased by two orders of magnitude (Kozai et al., 2015). This environmentally controlled agriculture is called indoor agriculture, urban agriculture, vertical agriculture or plant factories all over the world and they have great potential to provide fresh and healthy agricultural products all year round without long-distance transportation and can be built in any place and climate conditions (Kozai et al., 2019). However, the plant factory has large initial investment, high power consumption, high operation cost, and the management is mostly completed manually, so the management is difficult and the management cost is also high. Therefore, how to improve the automation of production

process is the research and development trend of the plant factory.

In recent years, the plant factory has gradually realized the semi automation of the production process from sowing, seedling raising, transplanting, harvesting, handling and logistics. Models of some industrial production automation technologies have been taken over, the automatic logistics system is developed to realize the automatic handling of the seedbed in the process of transplanting and harvesting, which is conducive to reducing the work intensity of labor personnel, improving productivity and reducing production costs. However, the plant factory with weak artificial intelligence still can not meet the unmanned planting. In order to reduce labor intensity, human resource cost and damage to plant growth environment caused by frequent access of operators, intellectualization and unmanned will become an important direction for the intelligent development of plant factories.

In addition, the light, temperature, humidity, carbon dioxide and nutritional conditions required for the growth of different crops vary greatly, even if the crops of the same variety have different requirements for the environment at each growth stage. Therefore, it is impossible to control the growth process of all plants with a unified growth model, but different comprehensive regulation is required according to different crops. However, up to now, there is still a lack of fine production guidance and necessary data support in plant production practice, resulting in poor crop growth, low comprehensive utilization rate of resources and low production efficiency. Therefore, the accuracy of production management indicators and production modes is an important guarantee to improve the work productivity of plant factories.

DEVELOPMENT STATUS

At present, in the field of plant factories, many countries such as Japan, the Netherlands, the United States, Sweden, the United Kingdom, Israel, etc., are very advanced in implementing a high level of mechanization systems, automation and intelligent leadership, without a lot of manual participation and coordination. Moreover, plant factories in the form of miniaturization, household, containerization, hotel and vertical agriculture are developing rapidly, and are also developing in a more intelligent direction. In recent years, China's plant factory research progress and technological development have been very fast. By the end of 2020, more than 200 commercial plant factories, more than 600 laboratories in artificial light plants and more than 1,200 air-conditioned room laboratories have been built, which has made progress gradually, on the road to the industrialization of plant production (China Zhiyan Data Research Center, 2021). However, compared with the technical characteristics and intelligent requirements of the plant factory itself, there is still a big gap (Zhang et al., 2021). Most of the existing equipment and systems come from solar greenhouses and other technologies in the field of facility agriculture. There is still a huge room for improvement in the hardware and software technologies required for the application environment of the plant factory. Researchers predict that in the not-too-distant future there will be creative breakthroughs in scientific and technical research to improve the automation of the efficient operation of plant cultivation (Zhang et al., 2019). In terms of hardware, due to the complexity of crop growth process, plant factory automation equipment needs to be further integrated with facility agronomy. In terms of software, due to the lack of a large amount of experimental data support, most plant factories in China mainly carry out single factor independent regulation by computer program based on empirical parameters or expert system. Its rationality and accuracy need to be further improved (Fang et al., 2021).

EXISTING PROBLEMS

Passive perception of environmental information

The acquisition of growth environment and biological characteristics information is the basis of the digital, intelligent and modern plant factory (Wang et al., 2021). Traditional monitoring and information acquisition mainly rely on the deployment of various sensors or detection equipment in plant factories, which are transmitted through various wired or

wireless buses or protocols, and collected centrally by computers. This method not only increases the initial investment cost of the plant factory, but also makes the communication wiring between systems complex and cumbersome, and the movement of automatic mechanical equipment in the plant factory is greatly restricted (Xu, 2020). In addition, there are many metal material frames in the planting plant of the plant factory, which cause strong electromagnetic interference, poor stability of wireless communication, low transmission rate of monitoring information and frequent information loss (Zhang et al., 2019).

Low positioning accuracy of indoor intelligent mobile equipment

Plant factories have high requirements for air tightness, walls and insulation materials may shield radio waves, and many indoor intelligent mobile devices that rely on global positioning system (GPS) or BeiDou Navigation Satellite System (BDS) and other satellite positioning systems cannot meet precise movement control due to reduced positioning accuracy (Liu & Huang, 2021). At present, indoor positioning mainly uses infrared, ultrasonic, Bluetooth, ultra-wideband, wireless LAN, RRFID and other wireless positioning technologies, but these technologies have electromagnetic interference, low accuracy, complex construction, limited scale, high equipment cost and many other problems (Zhang, 2021; Li et al., 2021; Cao et al., 2020).

Low automation of planting management

In the plant factory, in addition to the planting management of sowing, germination, seedling raising, transplanting, field planting, patrol, replanting, pruning and harvesting, the planting tray, nutrient solution transmission tank, reservoir, filter, pipeline and other equipment need to be cleaned for crop cultivation. At present, these tasks do not realize automatic operation, and the degree of intelligence is also very low, which is mainly completed manually (Liu et al., 2021; Ren et al., 2020). In addition, in order to make full use of space and expand the planting area, the three-dimensional vertical cultivation mode of multi-layer hydroponics is generally adopted, which has heavy planting equipment, high labor intensity and climbing operation, and has great potential safety hazards (Zhang et al., 2019). Moreover, due to the low degree of overall automation, a large number of personnel and equipment are required to enter and leave the cultivation workshop repeatedly, which is easy to bring pathogens and cause environmental pollution (Liu, 2020; Yu & Liu, 2014).

Inaccurate nutrient solution regulation and circulation

Plant growth cannot be separated from adequate nutrients, lack of nutrients will reduce plant yield and quality, and excessive supply will cause huge waste (Shao et al., 2021). In the existing plant factories in China, the preparation of nutrient solutions is mostly based on the experience of experts to determine the mixing ratio of water and fertilizer. After mixing and stirring, it is directly transported to the root of the plant through pipes, and then recycled. The mixing and supplement of nutrients lack scientific experimental data and crop growth model support, and precise regulation has not yet been achieved (Yang et al., 2021; Zhang, 2021; Sun et al., 2018). Moreover, the dissolution and dilution of solid nutrients, the supplement of nutrient solution required for growth, and the control of waste liquid recovery and discharge require a lot of human labor (Guo et al., 2020; Xia, 2020).

DEVELOPMENT SUGGESTIONS

Research and development of information active perception and acquisition system

An agricultural ecological environment detection sensor, an image sensor and the like are installed on the unmanned aerial vehicle mobile device. This was made to construct target self-searching and active mobile unmanned monitoring system equipment, so as to realize full-automatic and all-weather nondestructive inspection of the planting environment and the growth of plants in a plant factory and active perception of comprehensive information. Such a detection system is flexible and intelligent, and has strong adaptability to different crops. They form a network and interconnect through the Internet of things technology without installing communication devices and additional wiring, which reduces system

expense and makes communication more efficient. In addition, multi-sensor fusion technology is used to comprehensively process multi-sensor or multi-source information and data (Yang & Han, 2019), so as to obtain more abundant and operative information, enhance the effectiveness and robustness of the sensor system and avoid the limitations of a single sensor.

Development of unmanned intelligent equipment for indoor high-precision positioning

Indoor high-precision positioning technology is one of the key technologies for intelligent equipment in unmanned plant factories. It is found that the visible light emitted by LED lamps for plant growth illumination can be used not only for plant photosynthesis, but also for fast and high-precision positioning and navigation of intelligent equipment in plant factories (Wei et al., 2021). The dual functions of lighting and positioning can be realized without the installation of extra special positioning and navigation devices, and it also overcomes the difficulties of no satellite positioning signal indoors, high complexity and low positioning accuracy of rf positioning technology, and has the advantages of strong anti-interference and high positioning accuracy.

Research on precise regulation of environmental multi-factors

Each environmental factor of canopy and root zone has different effects on plant growth and development, mainly including temperature and humidity, light, moisture, CO_2 in air, dissolved oxygen in root zone, canopy air circulation, nutrients and minerals, etc. Plants are affected by the comprehensive action of many factors at the same time, and a small change of one factor may cause large changes of other factors, and while acting together on plant growth, it will have a greater impact on plant development. Therefore, it is challenging to not only promote the rapid development of plants but also enhance the comprehensive utilization rate of resources. At present, the precise and coupling regulation of environmental multi-factors has become one of the important contents of research of intelligent plant factories.

Crop growth process model and agricultural expert system are the basis for the intelligent plant factory to realize multi factor coupling and accurate regulation (Xu et al., 2021). The crop growth model can quantitatively describe the dynamic processes of crop growth, development, fruit formation and yield according to meteorological conditions, soil conditions and crop cultivation and management measures.

The agricultural expert system can be applied to various fields of agriculture, such as crop cultivation, plant protection, formula fertilization, agricultural economic benefit analysis, marketing management and so on.

In China, the research on these aspects mainly focuses on four aspects: Multi-source environmental information fusion monitoring (Yang et al., 2021), non-destructive monitoring of plant growth based on computer vision (Liu, 2020), construction of crop growth model based on in-depth learning (Cen et al., 2020), and environmental multi factor coupling and accurate regulation based on crop growth model (Zhu et al., 2020), mainly focusing on the comprehensive intelligent regulation of plant canopy and root zone.

The smart plant factory oriented crop growth model and expert decision-making system, including expert decision-making model library, is mainly used for accurate collaborative management of crop growth, environmental change and intelligent facilities and equipment, prediction of crop growth trend, comprehensive analysis of a variety of real-time monitoring information, and formulation of nutrient solution management, LED light modulation, comprehensive dynamic management decision-making scheme for environmental factor regulation. At the same time, the system also has the functions of agricultural materials management, technical database and personnel management, which helps to improve the planting production efficiency and reduce the management cost.

Automatic precision logistics equipment research and development

A low-cost autonomous mobile seedbed, three-dimensional multi-layer cultivation rack and corresponding logistics control system are designed and developed by comprehensively using sensor, automatic control, model driving, visible light communication and other technologies (Tang, 2017).

The system will automatically transport the mobile seedbed or planting shelves that need irrigation and planting to the designated position of the planting area, and also automatically transport the mobile seedbed or planting shelves that need harvesting or treatment to the operation workshop, so as to facilitate workers to concentrate on efficient operation or other mechanical equipment for automatic processing.

CONCLUSIONS

In recent years, with the rapid development of science and technology and national economy, China's facility agriculture has developed rapidly. Plant factory is an important part of facility agriculture, and its basic scientific research, engineering construction and production management technology development are also improving steadily. The intelligent development of plant factory equipment is promoting the plant factory to become a new industrial form of modern agriculture. In the future, it is possible to build plant factories directly in urban areas as a sustainable form of urban productive agriculture.

ACKNOWLEDGEMENTS

This research work was carried out with the support of Henan provincial government, Henan Provincial Department of science and technology, Henan Provincial Department of Education and also was financed from project of science and technology of the Henan province for tackling key problems, Project No. 212102110234.

REFERENCES

Cao, Z.T., Guo, Y., Zhao Z.X., & Liu, M.Y. (2020). Indoor positioning technology research review. *Computer technology and development*, (06), 202-206.

Cen, H.Y., Zhu, Y.M., Sun, D.W., & He, Y. (2020). Application status and Prospect of deep learning in plant phenotype research. *Journal of Agricultural Engineering,* (09), 1-16.

China Zhiyan Data Research Center (2021). 2021-2027 China Plant Factory Market Analysis and Development Trend Research Report. Beijing, China: China Institute of Economics and Industry, 156-182. http://www.abaogao.com/b/qita/11438247MM.html

Fang, H., Cheng, R.F., Wu, G., & Liu, Q.X. (2021). The effect of greenhouse multi-layer stereoscopic cultivation combined with supplementary light on the yield and quality of lettuce. *Journal of China Agricultural University,* (07), 147-154.

Guo, X.Y., Xue, X.Y., Lu, J.L., & Sun, S.H. (2020). Research status and prospect of intelligent equipment in plant factories in China. *Chinese Journal of Agricultural Machinery Chemistry*, (09), 162-169.

Kozai, T. (2013). Plant factory in Japan: current situation and perspectives. *Chron Hortic,*53(2), 8-11.

Kozai, T., Niu, G.H., & Takagaki, M. (2015 Eds.). *Plant Factory: An Indoor Vertical Farming System for Efficient Quality Food Production* (pp.423). London, Academic Press.

Kozai, T., Niu, G.H., & Takagaki, M. (2019). *Plant Factory: An Indoor Vertical Farming System for Efficient Quality Food Production* (Second Edition) (pp.3-5). Academic Press: London, United Kingdom.

Li, G., Yu, X., & Wang H.C. (2021). Simulation of indoor pedestrian positioning method based on WiFi fingerprint filtering method. *Computer simulation,* (04), 366-370.

Liu, H.F., Gao, X., Li, S.Z., & Wen, X.Y. (2021). Design of automatic logistics system in plant factories. *Modern agricultural equipment,* (03), 22-26 + 48.

Liu, T. (2020). Countermeasures for pest control of pollution-free vegetables in solar greenhouse. *Modern agriculture research,* (01), 126-127.

Liu, Z.H., & Huang, J.J. (2021). Multi-source fusion indoor positioning method based on image calibration. *Surveying and mapping geographic information,* (S1), 272-275.

Liu, W.Q. (2020). Using Computer Vision Technology to Realize Non-Destructive Monitoring of Greenhouse Plant Growth. *The Frontiers of Society, Science and Technology,* 2(1), 131-138.

Mitchell, C.A. (2004). Controlled environments in plant-science research and commercial agriculture. *IJOB,* 33, 1-12.

Ren, R.B., Nie, R.X., Chen, X.W., & Zhang, T.Z. (2020). Development status and prospect of intelligent plant factories. *Modern horticulture,* (07), 69-70.

Shao, M.J., Liu, W.K., Zhou, C.B., & Li, B.S. (2021). The effects of duration and frequency of strong light irradiation on the growth and nutrient absorption of lettuce under LED red and blue combined spectrum. Spectroscopy and spectral analysis, (09), 2853-2858.

Sun, M.Y., Song, J., Hu, Q.X., & Yang, S.J. (2018). Key technology development of plant factories. *Agricultural engineering,* (06), 62-66.

Tang, C.S. (2017). Research on the composite process of ADSS model based on model-driven and workflow. doctoral dissertation, Chinese Academy of Agricultural Sciences (pp. 32-56). https://kns.cnki.net/KCMS/detail/detail.aspx?dbname=CDFDLAST2018&filename=1017269542.nh.

Wang, N., Jiao, Z.W., Li, D.Y., & Wang, N.P. (2021). Research progress on key technologies of green facility agriculture cultivation in China. *Jiangsu Agricultural Sciences,* (18), 18-24.

Wei, Z.S., Xue, Q., Sun, X.H., & Wang, Z. (2021). High-precision visible light positioning method based on

adaptive filter. *Optical communication technology,* (09), 5-10. doi: 10.13921/j.cnki.issn1002-5561.2021.09.002.

Xia, B. (2020). The intelligent sprinkler irrigation system in plant factories. *Fujian Agricultural Machinery,* (02), 39-41.

Xu, L.H., Zhang, D.S., Li, X., & Wei, R.H. (2021). Construction and validation of second-scale greenhouse tomato crop-environment interaction model. *Journal of Agricultural Engineering,* (08), 212-222.

Xu, D. (2020). Research on double closed-loop optimal control of greenhouse based on online feedback of crop state. Doctoral dissertation, Chinese Academy of Agricultural Sciences. https://kns.cnki.net/KCMS/detail/detail.aspx?dbname=CDFDLAST2021&filename=1021040879.nh.

Yang, J., Zhang, D.S., Li, X., & She, G.X. (2021). High-efficiency grape-cucumber interplanting cultivation mode under precise control of digital agriculture. *Yangtze vegetable,* (17), 34-35.

Yang, P., Zhao, Y.Y., Li, Y.M., & Li, Z.B. (2021). Summary of agricultural open space integration based on multi-source information fusion. *Journal of agricultural machinery*, (S1), 185-196.

Yang, Q.C. (2019). Plant Factory. *Chapter I, Summary* (pp. 2-5). Beijing: Tsinghua University Press.

Yang, Q.J., & Han, L. (2019). Research on multi-sensor data intelligent fusion method in agricultural Internet of Things. *Modern electronic technology,* (20), 29-32.

Yu, X.S., & Liu, Y.P. (2014). Factors affecting plant quality in plant factories and their control. *Agricultural outlook,* (05), 52-55.

Zhang, C., Chen, X.B., Cao, P.B., & Yang, Z.M. (2019). Based on multi-spectral continuous adjustable plant Internet of Things cultivation system. *Industrial control computer,* (01), 33-35+38.

Zhang, L., Zhou, J.Q., Ju, W.J., & Yang, W.J. (2021). Research and application of ranging system based on ultrasonic and infrared technology. *Electrical automation,* (03), 99-101.

Zhang, M.Q. (2021). Temperature and humidity environment control and experimental study of artificial light source plant factory. PhD thesis, Heilongjiang Bayi Agricultural University .https://kns.cnki.net/KCMS/detail/detail.aspx?dbname=CDFDTEMP&filename=1021064369.nh.

Zhang, Z.W., Zhao, X., & Gao, J.L. (2021). Research and implementation of plant factory environment monitoring system based on LoRa and configuration software. *Agricultural equipment technology,* (04), 14-17.

Zhang, C. Huang, X., Liu, L., & Wei, F. (2019). Orbital efficient cultivation and benefit analysis of green leaf vegetables in solar plant factories. *Chinese vegetables,* (09), 89-92.

Zhu, Y., Tang, L., Liu, L.L., & Cao, W.X. (2020). Research progress of crop growth model. *Chinese Agricultural Sciences,* (16), 3235-3256.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

IN VITRO EFFECT OF CULTURE MEDIA AND GROWTH HORMONES ON THREE PEACH (*Prunus persica* L. Batsch) CULTIVARS

Nazar AL GHASHEEM[1, 2], Florin STĂNICĂ[1], Adrian George PETICILĂ[1], Oana VENAT[1], Ana BUTCARU[1], Imad Al-SUWAID, Basim K. HASAN[2], Ihasn J. AZEB[2], Kawther ALGHASHEEM[2]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd., District 1, Bucharest, Romania
[2]College of Agricultural and Marshlands, University of Thi- Qar, Iraq

Corresponding author email: nizar.shihan.post.am@utq.edu.iq

Abstract

In vitro three peach cultivars (Florin, Filip and Mimi) included in this experiment, two explants (shoots-tip and nodes) were taken at 0.5-1cm length. The explants were cultivated on 3 medium MS, B5 and QL adding of 30 g/l sucrose and 7 g/l with tested different concentrations of plant hormones BAP and NAA in 12 variants. The aim of this study was to test the effect of culture medium and growth hormones on some peach cultivars. Cultures media MS and QL showed the highest mean number of shoots at 5 mg/l BAP in (MS) medium (Florin 4.20 shoots/explant, Filip 3.60 shoots/explant and Mimi 4.00 shoots/explant respectively), and (QL) medium (Florin 4.40 shoots/explant, Filip 2.40 shoots/explant and Mimi 4.00 shoots/explant) respectively, while the (B5) medium gave the lowest value. Moreover, we were found that there is a relationship between BAP and NAA, since the addition of both hormones has led to the emergence of callus tissue, while adding just BAP to all media in the experiment gave shoots.

Key words: *BAP, culture media, explants, NAA, shoots.*

INTRODUCTION

In recent years, the technique of plant tissue culture has been widely used in the production of plants in large quantities, good characteristics, pathogens free and in regeneration, propagation and *in vitro* preservation of rare and economically important plants (Altman and Hasegawa, 2012a; Bhatia, 2015). *In vitro* technique propagation is growthing and maintaining and developing any plant parts (cells, tissues or organs) in a culture medium in suitable containers under controlled environmental conditions (Debergh and Read, 1991; Sharma et al., 2015). Cultures media should generally contain: Macronutrients, micronutrients components, vitamins, carbon sources, unspecified organic supplements, growth hormones and antioxidants. The quantities of nutrients supported in cultures media must be compatible to promote growth during culture period (Altman and Hasegawa, 2012b; Datta, 2019). Currently, there are many culture media used in tissue culture technique depending on the purpose of propagation, such as Gautheret (1939); White (1943); Murashige and Skoog MS (1962); Linsmaier and Skoog LS (1965); Gamborg B5 (1968); Nitsch and Nitsch NN (1969); Quoirin and Lepoivre QL (1977). Most experiments used the Murashige and Skoog (1962) medium without modification, while the experiments focused on the effect of plant growth regulators. There are many studies in effect of cultures medium such as: Shoot- tips and axillary buds in MS and B5 on Penta Rootstock (Balapour et al., 2020); shoot tips and axillary buds in MS, WPM and DKW on GF677 rootstock (Hamidi and Rezagholy, 2016); axillary buds in MS, LS, B5, N6 and QL on *Salvia guaranitica* Benth (Echeverrigaray et al., 2010); callus induction in MS, B5 and WPM on *Crataeva tapia* L. (Sharma et al., 2017). Despite extensive studies in other species, *in vitro* multiplication of peach (*Prunus persica* L. Batsch) is still finite. Peach has been successfully regenerated *in vitro* from immature cotyledons (Mante et al., 1989; Pooler and Scorza, 1995); cell suspension cultures (Schiavone and Wisniewski, 1990; Bhansali et al.,1991); callus induction (Hammerschlag et al., 1985; Declerck and Korban, 1996; Pérez-Jiménez et al,. 2012); shoot-tip and leaf (Cordts et al., 1987; Al ghasheem et al., 2018a). The aim

of this study was to test the effect of culture medium and growth hormones on some peach cultivars of Romania via micropropagation technique.

MATERIALS AND METHODS

Peach varieties (Florin, Filip and Mimi) were included in the experiment. Shoots and nodes explants (0.5-1 cm) were collected from healthy trees grown in the agricultural research station of Faculty of Horticulture / University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania in 2019. All explants were washed with washing liqued to remove plankton and dust under tap water to 30 min. For primary sterilization, the explants were immersing in 70% ethanol with constant stirring to 2-3 minutes, after which the alcohol was removed by washing with distilled water 3 times under the laminar flow cabinet. The explants surface were sterilized with NaOCl (10% v/v) with constant stirring by magnetic stirrer for 15-20 minutes, and then rinsed with distilled water at least three times (Al ghasheem et al., 2018b). The explants were cultured on 3 cultures media MS (Murashige and Skoog, 1962), B5 (Gamborg, 1968) and QL (Quoirin and Lepoivre, 1977) with 30 g/l sucrose and 7 g/l agar without hormones supplements during the initiation stage. In the multiplication stage, 12 variants were tested (Table 1).

Table 1. Scheme of treatments for concentration of hormones added to three culture media

Treatments	Media	BAP mg/l	NAA mg/l
T1	1/2MS	1.00	0.50
T2	1/2MS	5.00	0.50
T3	1/2B5	1.00	0.50
T4	1/2B5	5.00	0.50
T5	1/2QL	1.00	0.50
T6	1/2QL	5.00	0.50
T7	1/2MS	1.00	0.00
T8	1/2MS	5.00	0.00
T9	1/2B5	1.00	0.00
T10	1/2B5	5.00	0.00
T11	1/2QL	1.00	0.00
T12	1/2QL	5.00	0.00

The pH was adjusted to 5.7 by using drops from solution of NaOH and HCl. All cultures media were placed in tubes culture and sterilized by autoclaving at 121°C for 20 min. Growth chamber were controled at 22°C, 2000-2500 lux with relative humidity of 80-85%. The test tubes were kept in the dark (2-3 days) to get rid of the oxidation process of phenolic substances resulting from the cutting of plant tissues. The experiment was repeated twice, each treatment containing 30 replicates (one explant) in the initiation stage and 5 replicates (three explants) tested in multiplication. All experiments were arranged in a completely randomized design. The duration of the culture varied between four and eight weeks, depending on the individual experiment. The data were recorded on number shoots formed, shoots length (cm) and leaves number/shoot. The significance of the differences between the results was estimated by analysis of variance (ANOVA) on the SPSS (2005) program compared to the standard error means of the mean difference and lowest difference (LSD) test at probability level 0.05.

RESULTS AND DISCUSSIONS

In the initiation stage, the results showed that there were responses of all varieties to the tissue culture technique, the results showed that there was growth in all varieties on all cultures media used in the experiment.

MS culture medium gave highest average of shoots for all the varieties used (Florin 3.33 cm, Filip 2.61 cm and Mimi 2.59 cm), while B5 culture medium gave lower value (Florin 2.15 cm, Filip 1.84 cm and Mimi 2.01 cm). (Tables 2, 3 and Figures 1, 2). MS has a higher concentration of nitrogen and phosphorus compared to B5 and QL.

Nitrogen plays an important role in the construction of the plant through enzymes that stimulate the absorption of nitrates which is used in the manufacture of plant tissues (Mashayekhi, 2000). Our results are similar to what the researchers Bell and Reed (2002) reached when they planted 20 pear cultivars in (MS, LP and DKW) media. In the multiplication stage, the results showed that there were significant differences at the level of 0.05.

All varieties shoots formed depending on the amount of phosphorus in the culture medium and the concentration of plant hormones were added. The results showed that there is an effect of genotypes and cultures media. Culture media

MS and QL showed the highest average shoots number formed in 5 mg/l BAP: medium MS (Florin 4.20 shoots / explant, Filip 3.60 shoots / explant and Mimi 4.00 shoots / explant) and QL culture medium (Florin 4.40 shoots / explant, Filip 2.40 shoots / explant and Mimi 4.00 shoots / explant), while B5 culture medium gave the lowest value (Tables 4 and 5).

The study also showed that the shoots growth on QL culture medium have a yellowish-green colour may be due to the concentration of the element nitrogen compared to the MS and B5 media (Figure 3).

Figure 1. Shoots of Florin variety growing in the MS media after 8 weeks of multiplication stage

Table 2. Effect of variety and culture medium on average shoots length formed (cm) at initiation stage on three peach varieties. Data were taken after 4 weeks of culture

V / M	Florin	Filip	Mimi	Total
MS	**3.33 ± 0.10**	**2.61 ± 0.25**	**2.59 ±0.13**	2.71 ± 0.12
B5	2.15 ± 0.19	1.84 ± 0.06	2.01 ± 0.16	2.15 ± 0.10
QL	2.59 ± 0.18	2.01 ± 0.16	2.25 ± 0.16	2.28 ± 0.09
Total	2.84 ± 0.10	2.00 ± 0.09	2.30 ± 0.09	

Table 3. Effect of variety and culture medium on average leaves number formed/explants in initiation stage on three peach varieties. Data were taken after 4 weeks of culture

V / M	Florin	Filip	Mimi	Total
MS	4.50 ± 0.23	5.30 ± 0.27	4.10 ± 0.18	4.63 ± 0.20
B5	**5.80 ± 0.29**	5.00 ± 0.28	**5.10 ± 0.26**	5.30 ± 0.23
QL	4.70 ± 0.25	**5.80 ± 0.29**	4.80 ± 0.25	5.10 ± 0.20
Total	5.00 ± 0.28	5.36 ± 0.27	4.66 ± 0.19	

Table 4. Effect of variety, BAP and culture medium on average shoots grown number at multiplication stage on three peach varieties. Data were taken after 8 weeks of culture

Varieties	Treatments	Mean shoots number formed		
		MS	B5	QL
Florin	T7	2.16 ± 0.092	1.10 ± 0.089	2.95 ± 0.120
	T8	**4.20 ± 0.181**	**1.80 ± 0.136**	**4.40 ± 0.158**
Filip	T9	1.74 ± 0.082	1.35 ± 0.078	1.81 ± 0.086
	T10	**3.60 ± 0.128**	1.40 ± 0.118	**2.40 ± 0.136**
Mimi	T11	2.01 ± 0.081	1.79 ± 0.092	2.09 ± 0.084
	T12	**4.00 ± 0.136**	2.20 ± 0.128	**4.00 ± 0.137**

Table 5. Effect of variety, BAP and culture medium on average shoots length formed (cm) in multiplication stage on three peach varieties. Data were taken after 8 weeks of culture

Varieties	Treatments	Mean shoots length formed		
		MS	B5	QL
Florin	T7	2.19 ± 0.04	1.12 ± 0.04	**2.59 ± 0.04**
	T8	1.98 ± 0.03	1.37 ± 0.03	1.84 ± 0.02
Filip	T9	**2.63 ± 0.04**	1.55 ± 0.04	2.56 ± 0.04
	T10	1.12 ± 0.04	1.15 ± 0.04	1.09 ± 0.04
Mimi	T11	2.15 ± 0.04	**1.63 ± 0.04**	2.18 ± 0.04
	T12	1.89 ± 0.03	1.10 ± 0.05	1.91 ± 0.03

Figure 2. Shoots of Filip variety growing in the B5 media after 8 weeks at multiplication stage

These results are similar to those studied (Nowakowska et al., 2019) on two groups of culture medium (MS and WPM) where they were used on explants of *Daphne mezereum* L. and the superiority of MS medium over WPM medium was found. Also, our results are similar to those studied by Aviles et al., (2009) on 4 groups of culture media (MS, BTM, DKW and WPM) on the callus formed in the common walnut (*Juglans regia* L.) where the BTM medium was superior on all cultures media. According to Garton et al., (1984), there is effect of clonal origin or genotype of the plant material on organogenesis while Thakur et al., (2011) suggested that the growth of the plant depends on the components of the nutrient medium used in the plant tissue culture technique.

Figure 3. Shoots of Mimi variety (yellowish-green color) growing in the QL media after 8 weeks at multiplication stage

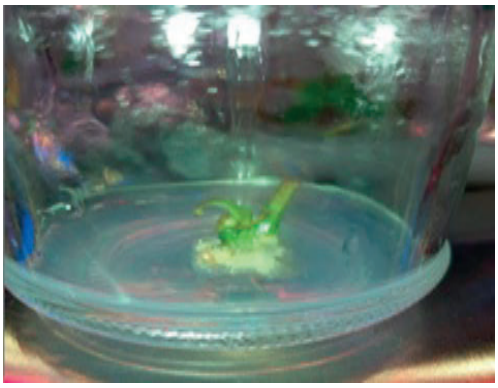


Figure 4. Effect of interaction between BAP and NAA in callus production from shoots growing in B5 culture media after 8 weeks of culture, Filip T1 (1/2 B5 + 1.00 mg/l BAP + 0.50 mg/l NAA)

Many studies reported that the amount of shoots formed was related to the phosphorus amounts absorbed by the explants (Pierik, 1990) confirmed that the amounts of nutrients in the culture medium have an important impact on the morphogenic response, also even possible to exclude the effect of hormones in plants by changing the compositional concentration of the culture medium (Ramage and Williams, 2002). In our study, we were found that there is a relationship between BAP and NAA, when were addition of both hormones led to the appearance of callus tissue (the highest amount of callus Florin 44% callus / explant in T1 (1/2 MS + 1.00 BAP mg / l + 0.50 NAA mg / l), while were adding only BAP to all media in the experiment give shoots formed (Table 6 and Figure 4). We were found that the BAP concentration of 5 mg / l led to the formation of the highest amount of shoots number formed compared to the concentration of 1 mg / l BAP. Auxin and cytokinins are plant hormones that play a very important role in controlling the plant growth process. These hormones have been used extensively in plant tissue culture technology. There are relationship between the concentration of auxins and cytokinins in the culture medium on growth nature and specialization of explants grown in culture media. We were found that increasing the ratio of auxin to cytokinins makes this medium a catalyst for the formation of root mass of plant parts, while increasing the ratio of cytokinin to auxin makes the culture medium ready to stimulate plant shoots to grow and form new

shoots or callus. The results of our study are similar to previous studies that were confirmed by Gentile et al., (2002) on *Prunus species* when different concentrations of BAP were used, which led to good results in peach genotypes used in experiments.

Table 6. Effect of auxin and cytokinin interaction (BAP and NAA) on shoots growth and callus formed in 3 culture media

V / T	Florin			Filip			Mimi		
	Callus %	**Shoots %**	**Dead %**	**Callus %**	**Shoots %**	**Dead %**	**Callus %**	**Shoots %**	**Dead %**
T1	**44**	18	38	22	37	41	**38**	34	28
T2	25	34	41	13	32	55	29	42	29
T3	42	27	31	**27**	43	30	37	22	41
T4	37	48	15	19	25	56	23	37	40
T5	21	36	43	11	27	62	19	32	49
T6	16	28	**56**	06	34	60	12	26	**62**
T7	00	78	22	00	65	35	00	32	24
T8	00	77	23	00	**82**	18	00	26	39
T9	00	**89**	11	00	49	51	00	67	33
T10	00	69	31	00	67	33	00	**70**	30
T11	00	76	33	00	44	**66**	00	55	45
T12	00	66	34	00	70	30	00	68	32

CONCLUSIONS

The study confirmed that MS culture media showed the highest average shoots number formed in 5 mg / l BAP: (MS) medium (Florin 4.20 shoots / explant, Filip 3.60 shoots / explant and Mimi 4.00 shoots / explant) respectively, while (B5) medium gave the lowest value (Florin 1.80 shoots/ explant, Filip 1.40 shoots/ explant and Mimi 2.20 shoots / explant). Also found that there is a relationship between BAP and NAA, when addition of both hormones led to the appearance of callus tissue (the highest amount of callus in Florin 44% callus / explants in T1 (1/2 MS + 1.00 mg / l BAP + 0.50 mg /l NAA)), while adding only BAP to all cultures media were got shoots formed . Also, we were found that the BAP concentration of 5 mg / l led to the formation of the highest number of shoots compared to the concentration of 1 mg / l BAP.

ACKNOWLEDGEMENTS

The authors would like to thank all students, researchers, management and staff of micropropagation laboratory, Centre for Studies of Food and Agricultural Products Quality, USAMV, to help complete these research papers.

REFERENCES

Alghasheem, N., Stănică, F., Peticilă, A.G. & Venat, O. (2018a). *In vitro* effect of various sterilization techniques on peach (*Prunus persica* L. Batsch) explants. *Scientific Papers. Series B, Horticulture*. Vol. LXII, 2018, Pp:227-234.

Alghasheem, N., Stănică, F., Peticilă, A.G. & Venat O. (2018b). *In vitro* effect of genotype, growth season and cytokinines on peach varieties (*Prunus persica* L. Batsch) propagation. *Scientific Papers. Series B, Horticulture*. Vol. LXII, 2018, Pp:217-226.

Altman, A. & Hasegawa, P.M. (2012a). Plant Biotechnology and Agriculture: Prospects for the 21st Century. Elsevier/Academic Press, London.

Altman, A. & Hasegawa, P.M. (2012b). Introduction to Plant Biotechnology: Basic Aspects and Agricultural Implications. In Altman, A., & Hasegawa, P. M. (eds.), Plant Biotechnology and Agriculture: Prospects for the 21st Century. Elsevier/Academic Press, London.

Aviles, F., Rios, D., Gonzalez, R. & Sanchez-Olate, M. (2009). Effect of culture medium in callogenesis from adult walunt leaves (*Juglans regia* L.). *Chilean journal of agricultural research*, 69(3):460-467.

Balapour, Z., Moghaddam, H.H., Zarei, M. & Mollashahi, M. (2020). Micropropagation of Penta rootstock (*Prunus domestica*) in the two culture media (MS and B5). *Plant Prod*., 42(4): 441-454. DOI: 10.22055/ppd.2019.24937.1570.

Bell, R.L. & Reed, B.M. (2002). *In vitro* tissue culture of pear: Advances in techniques for micropropagation and germplasm preservation. *Acta. Hortic*. 596: 412-418.

Bhansali, R.R., Driver, J.A. & Durzan, D.J. (1991). Somatic embryogenesis in cell suspension cultures of *Prunus persica* (L.). *J Hortic Sci*, 66:601-605

Bhatia, S. (2015). Plant Tissue Culture, in Modern Applications of Plant Biotechnology in Pharmaceutical Sciences. Editors S. Bhatia, K. Sharma, R. Dahiya, & Bera, T. (Boston: Academic Press), 31-107. b978-0-12-802221-4.00002-9.

Cordts, J.M., Scorza, R. & Bell, R.L. (1987). Effects of carbohydrates and nitrogen on the development of anthocyanins of a red leaf peach (*Prunus persica* L. Batsch) *in vitro*. *Plant Cell Tiss Organ Cult*, 9, 103–110. https://doi.org/10.1007/BF00044245

Datta, S.K. (2019). Need based tissue culture in floriculture: a success story. *Journal of Plant Science and Research*, Vol. 35, No.2, pp. 245–254.

Debergh, P.C. & Read, P.E. (1991). Micropropagation. In: Debergh, P.C. and Zimmerman, R.H., Eds., *Micropropagation. Technology and Application*, Kluwer Academic Publishers, Dordrecht, 1-14.

Declerck, V. & Korban, S.S. (1996). Influence of growth regulators and carbon sources on callus induction, growth and morphogenesis from leaf tissues of peach (*Prunus persica* L. Batsch). *J Hortic Sci*, 71:49–55.

Echeverrigaray, S., Carrer R. P. & Andrade L. B. (2010). Micropropagation of *Salvia guaranitica* Benth. through axillary shoot proliferation. *Biological and Applied Sciences, Braz. arch. biol. technol*., 53(4). https://doi.org/10.1590/S1516-89132010000400018

Gamborg, O.L., Miller, R.A. & Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Research*, 50: 151-158.

Garton, D.W., Koehn, R.K., & Scott, T.M. (1984). Multiple-locus heterozygosity and the physiological energetics of growth in the coot clam, *Mulinia lateralis*, from a natural population. *Genetics*, 108:445–455.

Gautheret, R.J. (1939). Sur la possibilite de realiser la culture indéfinie des tissus de tubercules de carotte. *C.R. Acad. Sci*., Paris, 208:118-120.

Gentile, A., Monticelli, S. & Damiano, C. (2002). Adventitious shoot regeneration in peach (*Prunus persica* L. Batsch). *Plant Cell Reports*, vol. 20, no. 11, pp. 1011–1016.

Hamidi, H. & Rezagholy, A.A. (2016). Effects of culture media and growth regulators on propagation of rootstock GF677 in tissue culture conditions. *Journal of Plant Research (Iranian Journal of Biology)*. Volume 29, Issue 1 , Pages 1-14.

Hammerschlag, F.A., Bauchan, G. & Scorza, R. (1985). Regeneration of peach plants from callus derived from immature embryos. *Theor Appl Genet*, 70:248-251.

Linsmaier, E.M. & Skoog, F. (1965). Physiol. Plant. 18: 100-127.

Mante, S., Scorza, R. & Cordts, J.M. (1989). Plant regeneration from cotyledons of *Prunus persica* L., *Prunus domestica* L. and *Prunus cerasus* L. *Plant Cell Tissue Organ Cult*., 19:1-11.

Mashayekhi, N.K. (2000). The protein synthesis spectrum during the induction phase of somatic embryogenesis in carrot (*Daucus carota* L.) cultures and the role of nitrogen forms for embryo development. Dr. Sci. Thesis. Justus Liebig University, Giessen, Germany.

Murashige, T. & Skoog F. (1962). A revised medium for rapid growth and bioassay with Tobacco tissue cultures. *Physiologia Plantrum*, 15:460-497.

Nitsch J.P. & Nitsch C., (1969). Haploid plants from pollen grains. *Science*, 163:85-87.

Nowakowska, K., Pacholczak, A. & Tepper, W. (2019). The effect of selected growth regulators and culture media on regeneration of *Daphne mezereum* L. 'Alba'. *Rend. Fis. Acc. Lincei*, 30, 197-205. https://doi.org/10.1007/s12210-019-00777-w

Pérez-Jiménez, M., Carrillo-Navarro, A. and Cos-Terrer, J. (2012). Regeneration of peach (*Prunus persica* L. Batsch) cultivars and *Prunus persica* × *Prunus dulcis* rootstocks via organogenesis. *Plant Cell Tiss Organ Cult*, 108:55-62.

Pierik, R. (1990). Cultivo *in vitro* de las plantas superiores. 326 p. Ediciones Mundi-Prensa, Madrid, España.

Pooler, M.R. & Scorza, R. (1995). Regeneration of peach [*Prunus persica* (L.) Batsch] rootstock cultivars from cotyledons of mature stored seed. *HortScience*, 30, 355–356.

Quoirin, M. & Lepoivre, P. (1977). Etude de milieux adaptes aux cultures *in vitro* de Prunus. *Acta Hortic*, 78:437-442.

Ramage, C.M. & Williams, R.R. (2002). Mineral nutrition and plant morphogenesis. *In Vitro Cell Dev Pl*, 38: 116-124.

Schiavone, F.M. & Wisniewski, M.E. (1990). Callus and Cell Suspension Cultures from Dormant Stems of Peach. *Hortscience*, Vol. 25(4), 483.

Sharma, G.K., Jagetiya, S. & Dashora, R. (2015). General techniques of plant tissue culture. Lulu Press Inc, Raleigh, North Carolina, United States.

Sharma, P., Patil, A. & Patil, D. (2017). Effect of culture media and growth hormones on callus induction in *Crataeva tapia* L. *International Journal of Pharmaceutical Research*, 9(2):70-76.

SPSS, Inc. (2005). SPSS Base 14.0 for Windows User's Guide. SPSS Inc., Chicago, IL.

Thakur, T., Dadsena, R., Nandeshwar, S.B. & Kumar, A. (2011). *In vitro* study of callus induction in *Wedelia trilobata* using different growth regulators, *Plant Tissue Cult. And Biotech*., 21(1): 95-99.

White, P.R. (1943). *A handbook of plant tlssue culture*. Ronald Press Co, New York.

FOOD BIOTECHNOLOGY

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

CONVENTIONAL *VERSUS* MODERN TECHNIQUES USED FOR THE DETECTION OF PATHOGENS IN FOOD MATRICES: A REVIEW

Quthama AL-ZAIDI, Camelia Filofteia DIGUȚĂ, Getuța DOPCEA, Florentina MATEI

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Biotechnology, 59 Marasti Blvd., District 1, Bucharest, Romania

Corresponding author email: alzaidy2004.n3@gmail.com

Abstract

Microbial contamination is one of the most important obstacles in the food industry. In order to control microbial contamination, many methods have been developed over the years to reveal the behaviour and characteristics of microorganisms in order to control them and in order to understand the impact of microorganisms on foods. Increasing concerns about outbreaks of foodborne diseases require rapid on-site and sensitive methods for the detection of microorganisms in various food matrices. In the current review, a brief discussion is presented about the methods used for the detection of pathogenic microorganisms present in food matrices, especially the tools based on nucleic acids extraction.

Key words: *microbial contamination, food industry, food matrices, detection.*

INTRODUCTION

Foodborne pathogens are considered a major public health risk, and diseases cause a significant burden on food workers, consumers, and governments.

Despite significant advances in diagnosis and awareness of food safety worldwide, many foodborne germs may be detected to the consumer, and many foods, such as meat and other animal products including dairy, are contaminated with potentially harmful microorganisms (Minarovikova et al., 2020). Also the impact of microorganisms such as bacteria, viruses and fungi on human life is significant. *Salmonella*, followed by *Escherichia coli*, are the two most common types of microorganisms responsible for outbreaks of food-borne illness and disease (Jayan et al., 2020).

Microorganisms can adapt to different environments and perform a variety of functions in diverse commodities. Therefore, in order to detect microorganisms, appropriate tools and techniques are needed.

In the recent years, the conventional detecting methods include, aside microscopy, cell culture, biochemical tests, tools which benefit of immunology (serotype, Elisa) or even molecular biology tools (classical PCR or DNA-DNA hybridisation).

In general, cell culture, colony counting, microscopic analysis, polymerase chain reaction (PCR), and immunoassay are often used to identify and quantify microbes. Sometimes the role of 'culture methods and colony counting for results' can be inappropriate. Especially in samples that contain different types and high concentrations of microorganisms.

Although PCR is a specific molecular method, this technique is time consuming, while the real-time PCR method can be more specific and faster (Persson et al., 2018). However, it is considered one of the most important modern methods for detecting microorganisms (Paniel & Noguer, 2019).

There are many methods for isolating bacteria from different food matrices. The traditional methods for isolating bacteria depend on a fresh medium, which requires a differential agar medium and colony counting (Paniel & Noguer, 2019) (Figure 1).

Some other disadvantages of conventional culture methods can be pointed out, like their low sensitivity, risk of bacterial contamination leading to inhibition in the growth of bacteria of interest, and presence of viable but non-culturable bacteria (VBNC).

Consequences of having VBNC in a food matrices include an underestimation of the number of viable cells or the impossibility of isolating pathogens from the sample.

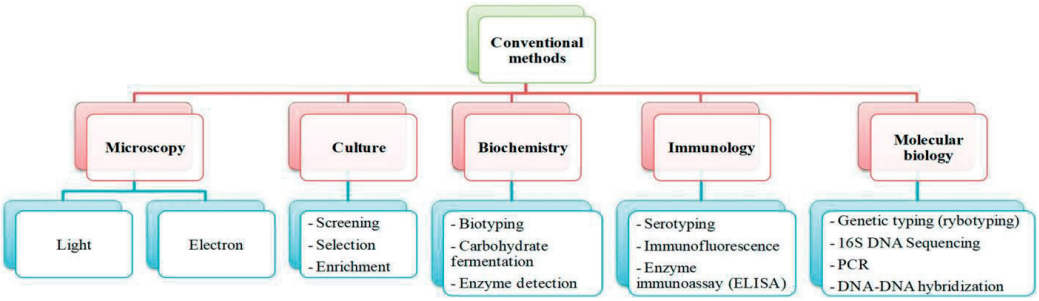


Figure 1. Conventional methods used for food borne pathogenic bacteria detection (Paniel & Noguer, 2019)

The VBNC condition is commonly found in environmental and food samples due to bacterial starvation and a large variety of stressful conditions, including growth inhibition temperature, hypoxia, suboptimal pH and salinity. In food, VBNC has been reported to occur, in some cases, while cannot be detected by bacterial culture, but can be detected with modern techniques. Therefore, these bacteria pose a growing threat in the food industry (Yang et al., 2021).

Some authors showed that VBNC cells can be found in the case of *Salmonella typhi* (*S. typhi*), as well as *Escherichia coli* (*E. coli*) and *Legionella pneumophila*. Therefore, in order to reach more accurate results, researchers often combine standard microbiological counting methods with other automated or semi-automated detection techniques that include DNA, antibody or biochemical methods (Cao et al., 2019). However, there are still many drawbacks to these traditional methods, and there is still a need to develop more rapid, sensitive and specific techniques for pathogen detection and quantification.

The development of new technologies with faster response time, better sensitivity and selectivity is very important to ensure the safety of consumers.

PCR-based methods have been applied to detect and identify bacteria in a large variety of samples. Compared with the old traditional methods, modern methods based on advanced qPCR techniques showed better specificity, higher sensitivity, shorter analysis time and better accuracy (Durand et al., 2020).

The PCR and qPCR methods can be applied to in situ and real-time monitoring for many applications, including the detection of many microorganisms.

These techniques are distinguished in the detection of bacterial populations even in the absence of selective culture medium and in the presence of other dominant groups.

To increase the accuracy of the analysis and reduce the time, some PCR methods have been developed that allow the identification of several pathogens in a single sample within a single reaction.

In the following, the classical pathogen detection methods will be described against the modern developed detection tools. This review is based mainly on the last five years scientific publications in the field of pathogen detection in different types of food. The study was structured based on different food matrices, respectively animal (Table 1), plant (Table 2) and beverages (Table 3), and for each were revealed the main pathogens to be detected and associated detection methods. Through this study it was emphasized that there is a difference between the old traditional methods and modern methods in detecting the existing germs in terms of work speed and results accuracy. Among the modern methods that are characterized by accuracy and speed in work, the most important are classical PCR and Real Time PCR, which is one of the most important modern methods for detecting microorganisms.

Table 1. Different tools used for the pathogen detection in animal food matrices

Food matrices	Pathogen	Tools	Reference
Beef Burgers	*Escherichia coli*	Real-Time PCR	Rey et al., 2021
Cheese	*Salmonella typhimurium, S. aureus, L. monocytogenes*	Real-Time PCR	Mendonça et al., 2019; Jana et al., 2020
Chicken breast, turkey, Beef, Raw Pork, Sausage	*L. monocytogenes, E. coli, S. enterica, Campylobacter* spp., *C. coli, C. lari, C. upsaliensis, Salmonella*	PCR, Real-Time PCR	Kim et al., 2021; Vizzini et al., 2021; Hyeon et al., 2019
Chicken carcasses	*Salmonella* spp., *Salmonella enteritidis*	Multiplex PCR	Ferone et al., 2020
Dairy products	*Salmonella* spp., *Listeria monocytogenes*, *Cronobacter sakazakii*	Real-Time PCR, PMAxx-ddPCR	Ferone et al., 2020; Lv, 2021
Fish	*Aeromonas* spp., *Streptococcus* spp.,	Multiplex PCR, LAMP, Biosensors	Pires et al., 2021
Fresh pork	*Staphylococcus aureus, Salmonella* and *Shigella*	Real-Time PCR	Ferone et al., 2020
Meat	*Pseudomonas*, *Enterobacteriaceae*, *Brochothrix thermosphacta*, *Staphylococcus*	Multiplex Qpcr	Bahlinger et al., 2021
Meat Products	*Listeria monocytogenes*	Real-Time PCR	Labrador et al., 2021
Milk	*Salmonella, Listeria monocytogenes,* Pseudomonas, *Bacillus cereus, Staphylococcus aureus*, *Hafnia alvei, Serratia marcescens, Citrobacter freundii, E. coli*	Real-Time PCR, Multiplex Real Time PCR, qPCR, Biosensors	Wei et al., 2019; Zhou et al., 2019; Ferone et al., 2020; Jayan et al., 2020; Du, 2021; Huang et al., 2021; Lonczynski, and Cowin 2021; Maier et al., 2021
Minced meat	*Listeria monocytogenes, Staphylococcus aureus, Bacteriophage*	Real-Time PCR, PCR	Ferone et al., 2020; Spilsberg et al., 2021
Minced pork meat, Egg white, Egg yolk, Whole egg	*Salmonella enterica*	Direct PCR	Vinayaka et al., 2019
Pork meat	*S. typhimurium*	Biosensors	Jayan et al., 2020
Poultry meat, Red meat, Beef meat, Liver samples.	*Salmonella* spp.	Real-Time PCR	Siala et al., 2017
Raw Poultry	*Salmonella* spp.	Real-Time PCR	O'Bryan et al., 2021
Raw Seafood	*Vibrio vulnificus*	Real-Time PCR	Yang et al., 2021
Sausages	*Latilactobacillus sakei*	ePCR	Iacumin, et al., 2020
Shrimp, Mussels, Seafood	*Toxoplasma gondii, Vibrio parahaemolyticus, Vibrio cholerae, V. parahaemolyticus, V. vulnificus*	Real-Time PCR, LAMP Methods	Bonnin-Jusserand et al., 2019; Cao, 2019; Durand, 2020; Yang, 2020
sliced turkey, raw cheese, chicken salad, shrimp	*Listeria* species *monocytogenes*	Multiplex Real Time PCR	Lonczynski et al., 2021
Yogurt	*S. enterica*, *L. monocytogenes*	PCR	Kim et al., 2021

Food born pathogen detection by cell culture

The culture-based detection method has the advantages of being simple to use, detecting only live cells, and not requiring expensive experimental equipment. Sensitivity and selectivity are crucial features in choosing a selective/differential media, although certain culture-based methods lack the sensitivity and/or selectivity needed to isolate the target pathogen. When the media is insensitive, the chance of a food borne outbreak rises because the danger goes unrecognized. Target pathogens, on the other hand, cannot be separated from other microorganisms when selectivity is poor. Traditional media are constantly being improved, and new media are being developed to successfully isolate target microorganisms.

Table 2. Different tools used for the pathogen detection in vegetable matrices

Food matrices	Pathogen	Tools	Reference
Cantaloupes, Watermelons, Pineapples, Radishes	*Salmonella enterica*, *Listeria monocytogenes*	Bacterial strains-Luria-Bertani (LB) media	Huang et al., 2019
Chamomile, Mint	*Salmonella*	Multiplex Real-Time PCR	Koprinarova, 2021
Cherry tomato	*Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus*	Multiplex qPCR	Wei et al., 2019
Chocolate bar	*Salmonella* spp., *Listeria monocytogenes*	Real-Time PCR	Ferone et al., 2020
Fruits, Vegetables,	*Salmonella* spp. *and Listeria monocytogenes, Fungi*	Real-Time PCR	Ferone et al., 2020; Roumani et al., 2021
Lettuce, Cabbage	*E. coli O157:H7, S. typhimurium, Salmonella*	Real-Time PCR	Kim et al., 2019; Kim et al., 2020; Huang et al., 2021
olive fruit	*Colletotrichum acutatum*	Real-Time PCR	Azevedo-Nogueira, 2021
Pineapple	*Aspergillu, Rhizopus, Geotrich , Neurospora, Candida, Fungi*	PCR, culture-based detection	Koffi et al., 2019; Koffi et al., 2021
Soybean sprouts	*Listeria monocytogenes*	Real-Time PCR	Wei et al., 2019

Table 3. Different tools used for the pathogen detection in beverages

Food matrice	Pathogen	Tools	Reference
Apple juice, grape juice.	*Salmonella paratyphi, S. typhimurium, Penicillium expansum, Paenibacillus* spp., *Alicyclobacillus* spp.	LAMP, PCR, qPCR, Biosensors	Frisch, L. M. et al., 2021; Li, H. et al., 2021; Elie, S. C. 2020; Jayan et al., 2020
Beer, grape wine, other fruit wine, refined rice wine, traditional Korean turbid rice wines.	*Bacillus cereus*	Soy polymyxin broth	Kim et al., 2020
Boza	*Enterococcus faecium* YT52	PCR	Gök Charyyev, M. et al., 2019
Coconut water	*Escherichia coli*	PCR	Wang et al., 2021
Kombucha	*Starmerella davenportii Do18*	Potato dextrose agar (PDA)	Tu et al., 2020
Orange juice	*E. coli, Salmonella typhimurium, Yersinia enterocolitis, Shigella boydii*	FTIR measurements, Biosensors	Paniel and Noguer 2019; Jayan et al., 2020
Rice beer (makgeolli)	*Cronobacter, Enterobacter, Klebsiella*	qPCR	Jung et al., 2012

A variety of food samples were used, including leafy greens, seafood, beef, and pork items (Baek et al., 2021).

The researchers found many bacterial species in a viable but non-culturable state known as VBNC. These cells are characterized by their loss of ability to be cultured on routine agar, which impairs their detection by conventional techniques. Hence, failure to detect them poses a threat to public health. Therefore, the researchers had to find a way to detect all the germs present in the sample to be examined (Cao et al., 2019), and the methods take advantage of different molecular tools.

Polymerase chain reaction (PCR)

Is a relatively newly used and widely method to quickly make millions of copies of a given DNA sample (complete copies or partial copies), in a series of cycles of temperature changes. It allows researchers to take very small samples of DNA and amplify them as much as possible

enough to study in detail. PCR was invented in 1983 by American scientist Cary Mullis at Cetus Corporation. It is fundamental to many procedures used in genetic testing and research, including analysis of ancient DNA samples and identification of infectious agents and diseases.
PCR is nowadays a common and often indispensable technique used in many medical laboratory research as well as for a variety of applications including biomedical research and forensics.
Applications of this technology include DNA cloning for sequencing, gene transcription and manipulation, and mutagenesis; DNA-based phylogenetic construction, or functional analysis of genes; diagnostics and monitoring of genetic disorders; ancient DNA amplification; Analysis of genetic fingerprints to determine DNA traits (for example, in pedigree and forensic testing); the detection of pathogens in DNA tests to diagnose infectious diseases. In food matrices, PCR was used for the detection of *Salmonella* or *E. coli* in different food products, as seen in Table 1.

Real-time polymerase chain reaction (real-time PCR)

It is one of the laboratory technique of molecular biology based on the PCR. It monitors the amplification of a targeted DNA molecule during the PCR, as in conventional PCR. Real-time PCR can be used quantitatively (quantitative real-time PCR) and semi-quantitatively (i.e., above/below a certain amount of DNA molecules) (semi-quantitative real-time PCR) (Minarovičová et al., 2020). Real-time PCR, sometimes referred to as quantitative or qPCR, determines the amount of PCR product present during a particular cycle. This approach allows you to quantify DNA creation in the qPCR experiment by employing a fluorescent report in the PCR reaction. SYBR Green and probe-based qPCR are the two types of qPCR. In a SYBR Green qPCR process, you start with a template that contains the target sequence you want to analyze. You'll also need primers, dNTPs, and your preferred DNA polymerase. The SYBR Green I dye is commonly included in the reaction mix that contains the DNA polymerase. In a probe-based qPCR process, you start with a template that includes the target sequence you want to analyze. You'll also need primers, dNTPs, and your preferred DNA polymerase. You'll also need a probe that's tagged with both a reporter and a quencher molecule. Probes are usually sold separately as custom items because they are unique to the target sequence. The first step in the PCR process is denaturation. The double-stranded DNA helix melts open into two single-stranded DNA templates as the thermocycler heats up to around 95 degrees Celsius. The temperature cools to 45-65 degrees Celsius during the annealing step, and the single-stranded primers connect to the appropriate ends of the target sequence. DNA polymerase connects to the prepared template and begins incorporating complimentary nucleotides during the cycle. Finally, the temperature rises to 65-75 degrees Celsius during extension. The sequence-specific primer is extended by DNA polymerase by adding complementary nucleotides to the DNA template.
Probe-based differs from SYBR Green in this stage because: in probe-based The fluorescence is caused when the DNA polymerase displaces the reporter molecule from the probe. The fluorescence builds up as the PCR cycle progresses, and it is measured at the end of each cycle. The number of freshly formed double-stranded DNA strands is quantified by measuring the intensity of the fluorescence created by the reporter molecule above background level (the Cq value). SYBR Green binds and fluoresces all newly produced double-stranded DNA complexes. The fluorescence builds up as the PCR cycle progresses, and it is measured at the end of each cycle. The amount of freshly produced double-stranded DNA is quantified by measuring the intensity of fluorescence emitted by SYBR Green I above background level (the Cq value). You're ready to start analyzing after repeating the denaturation, annealing, and extension processes 35-40 times. The Cq values can be used to quantify beginning DNA amounts, create a standard curve for gene expression research, and perform other analyses. Different examples of pathogens detection are provided in Table 1, like the detection of *Campylobacter* spp. in chicken samples by Real-Time PCR (Vizzini et al., 2021), or *Salmonella* in milk samples also by Real-Time PCR (Huang et al., 2021).

End-point polymerase chain reaction (ePCR methods)

The analysis performed after all PCR cycles have been finished is known as end point PCR. End point analysis is predicated on the plateau phase of amplification, unlike qPCR, which permits quantification as the template doubles (exponential phase).

Northern blot or P32 radioisotope (for RNA). Your RNA is run on an agarose gel, then transferred to a nucleic acid binding membrane, which is subsequently hybridized with a P32 labelled DNA probe that identifies your transcript. After exposure to an Xray film, the quantification is done using densitometry.

End-point polymerase chain reaction has been used in DNA amplification techniques since 1985, real-time polymerase chain reaction (RT-PCR) has been used since 1993, and reverse transcription real-time polymerase chain reaction (RT-qPCR) has been used in many research applications including cloning, analysis, gene expression genotyping, mutagenesis, sequencing, and many others. The cyclic amplification of a single DNA molecule into billions of copies of DNA molecules in a short period of time using DNA polymerase is the core premise of DNA amplification technologies (Figure 2). The DNA polymerases, on the other hand, stay with the result (DNA copies) in one system at the end of the enzymatic reaction and are normally discarded after usage. Separating the enzyme from the products, which is a difficult, expensive, and time-consuming process, is required to reuse DNA polymerases in a repeating reaction.

Furthermore, DNA polymerases are still quite expensive; therefore, to prevent wasting time and other resources, DNA-modifying enzyme immobilization method for nucleic acid detection is advised.

Research on the electrochemical real-time amplification technology, which is based on the solid-phase PCR methodology, have sparked a lot of curiosity. The electrochemical polymerase chain reaction (PCR), which is based on the hybridization of a target sequence and an oligonucleotide probe and immobilization on an electrode: graphite oxide, has been the focus of research for the past years.

The sample surface was passivated to prevent adsorption of additional PCR components, and the electrochemical response was accomplished in the requisite thermal conditions. The plots of the current signal against the number of ePCR cycles were recorded, and after 25-40 reaction cycles, a plateau was reached. However, this invention does not solve the problem because DNA polymerases remain with the product in one system and are typically discarded after usage. As a result, the use of electrochemical techniques as well as the immobilization of some extra enzymes makes a lot of sense (Dronina et al., 2021). Example for ePCR - detection of *Latilactobacillus sakei* in sausages samples (Iacumin et al., 2020).

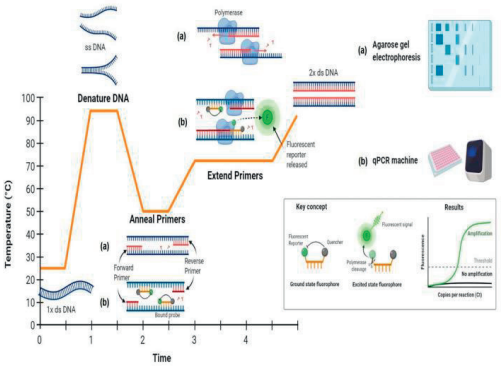


Figure 2. Schematic illustration of two DNA amplification techniques: (a) PCR; (b) real-time PCR (Dronina et al., 2021)

Biosensor

A biosensor is a three-part analytical instrument that includes: a bio-receptor or recognition element, a transducer, and a signal reading device (an enzyme, a receptor, an antibody fragment, a nucleic acid, a full microbial cell, plant or animal tissues, polysaccharides, and other biosensor recognition elements).

A transducer is a device that transfers a signal with high sensitivity from one form (physical, chemical, or biological) to another (electrical). Many transducer systems have been developed and are constantly being developed (Figure 3, after Rupak et al., 2021). An example is the detection of *Salmonella paratyphi, S. typhimurium* in Apple juice samples (Jayan et al., 2020).

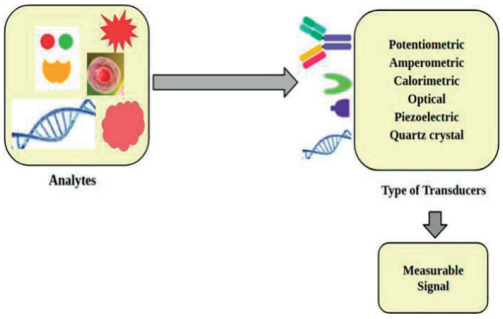


Figure 3. Detection of analytes by biosensor based methods (Rupak et al., 2021)

FTIR Analysis

FTIR Analysis, often known as FTIR Spectroscopy, is a technique for identifying organic, polymeric, and inorganic materials. Infrared light is used to scan test materials and examine chemical characteristics using the FTIR analysis method. The FTIR instrument passes infrared light in the range of 10,000 to 100 cm^{-1} through a material, with part of it being absorbed and some passing through. The sample molecules transform the absorbed radiation into rotational and/or vibrational energy. The resulting signal at the detector appears as a spectrum, ranging from 4000 cm^{-1} to 400 cm^{-1}, and represents the sample's molecular fingerprint. Because each molecule or chemical structure has its own spectral fingerprint, FTIR analysis is an excellent technique for chemical identification. When assessing industrially created material, FTIR spectroscopy is a well-established technique for quality control, and it is frequently used as the initial stage in the material analysis process. A change in the absorption bands' distinctive pattern suggests a change in the material's composition or the presence of contamination. If visual inspection reveals a problem with the product, FTIR micro-analysis is usually used to discover the source. This method is excellent for determining the chemical composition of tiny particles (10-50 microns) as well as larger areas on the surface.

FTIR analysis is used to: identify and characterize unknown materials (e.g., films, solids, powders, or liquids); identify contamination on or in a material (e.g., particles, fibers, powders, or liquids); identify additives after extraction from a polymer matrix; identify oxidation, decomposition, or uncured monomers in failure analysis investigations. An example is the detection of *E. coli, Salmonella typhimurium, Yersinia enterocolitis, Shigella boydii* in orange juice samples (Paniel and Noguer 2019).

LAMP (loop-mediated isothermal amplification)

Is a single-tube DNA amplification technology that offers a low-cost alternative for detecting certain diseases. To detect RNA, reverse transcription loop-mediated isothermal amplification (RT-LAMP) combines LAMP with a reverse transcription step.

LAMP stands for "isothermal nucleic acid amplification". In contrast to polymerase chain reaction (PCR) technology, which uses a number of alternating temperature steps or cycles to complete the reaction, isothermal amplification uses a constant temperature and does not require the use of a thermal cycler. An example is the detection of *Aeromonas* spp., *Streptococcus* spp. in fish samples (Pires et al., 2021).

CONCLUSIONS

The purpose of this review is to point out the methods used recently in the food borne pathogens transmitted in different food matrices. Through the modern described methods (biochemical or molecular), it can be identified the best, most accurate and fastest the food borne pathogens. During the current period that the world is going through in the development in the production and transportation of food products in the same country or from one country to another.

As reported in the scientific publications, there are many methods used recently to detect bacterial contamination in different food matrices. Through PCR technology, we can know the characteristics and behaviour of the microorganisms present in the samples examined, even if they are few, simply by taking a small sample and multiplying it by copying it into millions of copies by PCR technology.

Different modern tools, like Real-Time PCR, or biosensors, were employed to identify the most common pathogenic bacteria, like *Salmonella, E. coli,* O157:H7, *L. monocytogenes*, *S. aureus*, as well as new emerging pathogens, like *Cronobacter* spp., *Hafnia* spp., *Shigella* spp. Such tests can be performed on a few samples by doubling the DNA to millions of copies, thus

obtaining more accurate results which cannot obtain it by the old traditional methods, nor by most modern methods**.**

It was found that one of the most employed of these methods is qPCR, which is one of the very successful and accurate modern methods, comparing to the old traditional methods, especially bacterial culture methods, which do not allow the detection of viable but non-cultivable bacteria (VBNC).

The ELISA approach has a benefit in that it may be used on-site, but it can only be used on a limited number of food samples, for example (ELISA) used for detection of foreign proteins in milk and other foods (Nagraik et al., 2021).

Few bacteriophage amplification-based detection techniques are now marginal, but they have a greater specificity than other methods.

The approaches based on gold nanoparticle aggregation are quick, and they can be used on-site, but they are challenging to employ in a liquid–solid matrix. Consumers and food companies may soon be able to conduct their own microbiological tests before purchasing or consuming food based on these efforts.

REFERENCES

Azevedo-Nogueira, F., Gomes, S., Lino, A., Carvalho, T., & Martins-Lopes, P. (2021). Real-time PCR assay for Colletotrichum acutatum sensu stricto quantification in olive fruit samples. *Food Chemistry, 339*, 127858.

Baek, H.-W., Kim, S.-A., Min, W.-K., Dal Kang, S., Shim, S., Han, N. S., & Seo, J.-H. (2021). A species-specific qPCR method for enumeration of *Lactobacillus sanfranciscensis*, *Lactobacillus brevis*, and *Lactobacillus curvatus* during cocultivation in sourdough. *Food Analytical Methods, 14*(4), 750-760.

Bahlinger, E., Dorn-In, S., Beindorf, P.-M., Mang, S., Kaltner, F., Gottschalk, C., . . . Schwaiger, K. (2021). Development of two specific multiplex qPCRs to determine amounts of *Pseudomonas, Enterobacteriaceae, Brochothrix thermosphacta* and *Staphylococcus* in meat and heat-treated meat products. *International Journal of Food Microbiology, 337*, 108932.

Bonnin-Jusserand, M., Copin, S., Le Bris, C., Brauge, T., Gay, M., Brisabois, A., ... & Midelet-Bourdin, G. (2019). *Vibrio* species involved in seafood-borne outbreaks (*Vibrio cholerae, V. parahaemolyticus* and *V. vulnificus*): review of microbiological versus recent molecular detection methods in seafood products. *Critical reviews in food science and nutrition*, *59*(4), 597-610.

Cao, X., Zhao, L., Zhang, J., Chen, X., Shi, L., Fang, X., . . . Wang, L. (2019). Detection of viable but nonculturable *Vibrio parahaemolyticus* in shrimp samples using improved real-time PCR and real-time LAMP methods. *Food Control, 103*, 145-152.

Dronina, J., Bubniene, U. S., & Ramanavicius, A. (2021). The application of DNA polymerases and Cas9 as representative of DNA-modifying enzymes group in DNA sensor design. *Biosensors and Bioelectronics*, *175*, 112867.

Du, M., Li, J., Liu, Q., Wang, Y., Chen, E., Kang, F., & Tu, C. (2021). Rapid detection of trace *Salmonella* in milk using an effective pretreatment combined with droplet digital polymerase chain reaction. *Microbiological Research, 251*, 126838.

Durand, L., La Carbona, S., Geffard, A., Possenti, A., Dubey, J. P., & Lalle, M. (2020). Comparative evaluation of loop-mediated isothermal amplification (LAMP) vs qPCR for detection of *Toxoplasma gondii* oocysts DNA in mussels. *Experimental parasitology, 208*, 107809.

Elie, S. C. (2020). Heat resistance of *Paenibacillus* sp. spores isolated from carbonated juice beverage.

Ferone, M., Gowen, A., Fanning, S., & Scannell, A. G. (2020). Microbial detection and identification methods: bench top assays to omics approaches. *Comprehensive Reviews in Food Science and Food Safety, 19*(6), 3106-3129.

Frisch, L. M., Mann, M. A., Marek, D. N., & Niessen, L. (2021). Development and optimization of a loop-mediated isothermal amplification (LAMP) assay for the species-specific detection of *Penicillium expansum*. *Food Microbiology*, *95*, 103681.

Gök Charyyev, M., Özden Tuncer, B., Akpınar Kankaya, D., & Tuncer, Y. (2019). Bacteriocinogenic properties and safety evaluation of *Enterococcus faecium* YT52 isolated from boza, a traditional cereal based fermented beverage. *Journal of Consumer Protection and Food Safety*, *14*(1), 41-53.

Huang, C., Mahboubat, B. Y., Ding, Y., Yang, Q., Wang, J., Zhou, M., & Wang, X. (2021). Development of a rapid Salmonella detection method via phage-conjugated magnetic bead separation coupled with real-time PCR quantification. *LWT, 142*, 111075.

Huang, J., Luo, Y., Zhou, B., Zheng, J., & Nou, X. (2019). Growth and survival of Salmonella enterica and *Listeria monocytogenes* on fresh-cut produce and their juice extracts: Impacts and interactions of food matrices and temperature abuse conditions. *Food Control, 100*, 300-304.

Hyeon, J.-Y., Mann, D. A., Wang, J., Kim, W. K., & Deng, X. (2019). Rapid detection of Salmonella in poultry environmental samples using real-time PCR coupled with immunomagnetic separation and whole genome amplification. *Poultry science, 98*(12), 6973-6979.

Iacumin, L., Cecchini, F., Vendrame, M., & Comi, G. (2020). Emulsion pcr (Epcr) as a tool to improve the power of dgge analysis for microbial population studies. *Microorganisms, 8*(8), 1099.

Jayan, H., Pu, H., & Sun, D.-W. (2020). Recent development in rapid detection techniques for microorganism activities in food matrices using bio-recognition: A review. *Trends in Food Science & Technology, 95*, 233-246.

Jung, M.-J., Nam, Y.-D., Roh, S. W., & Bae, J.-W. (2012). Unexpected convergence of fungal and bacterial communities during fermentation of traditional Korean alcoholic beverages inoculated with various natural starters. *Food microbiology, 30*(1), 112-123.

Kim, E., Yang, S.-M., & Kim, H.-Y. (2021). Differentiation of lacticaseibacillus zeae using pan-genome analysis and real-time pcr method targeting a unique gene. *Foods, 10*(9), 2112.

Kim,[a] J. H., & Oh, S. W. (2019). Optimization of bacterial concentration by filtration for rapid detection of foodborne *Escherichia coli* O157: H7 using real-time PCR without microbial culture enrichment. *Journal of food science, 84*(11), 3241-3245.

Kim,[b] J.-H., & Oh, S.-W. (2020). Rapid and sensitive detection of E. coli O157: H7 and S. *Typhimurium* in iceberg lettuce and cabbage using filtration, DNA concentration, and qPCR without enrichment. *Food Chemistry, 327*, 127036.

Kim,[c] J.-H., & Oh, S.-W. (2021). Pretreatment methods for nucleic acid-based rapid detection of pathogens in food: a review. *Food Control, 121*, 107575.

Kim, S. A., Park, H. J., Cho, T. J., & Rhee, M. S. (2020). Toxic Potential of Bacillus cereus isolated from fermented alcoholic beverages. *Food Research International, 137*, 109361.

Koffi F. Y., Moumouny, T., Eric, Y. Y., Koffi, L. B., Diguta, C., Alloue-Boraud, M. W., & MATEI, F. (2021). Biocontrol of Post-harvest Fungal Diseases of Pineapple (*Ananas comosus* L.) Using Bacterial Biopesticides. *American Journal of Microbiological Research, 9*(2), 34-43.

Koffi, Y. F., Diguta, C., Alloue-Boraud, M., Koffi, L. B., Dje, M., Gherghina, E., & Matei, F. (2019). PCR-ITS-RFLP identification of pineapple spoilage fungi [J]. *Rom Biotechnol Lett, 24*, 418-424.

Koprinarova, M. (2021). Methods To Improve Molecular Detection of *Salmonella* in Complex Herbal Matrices Containing Inhibitors. *Journal of Food Protection, 84*(8), 1309-1314.

Labrador, M., Giménez-Rota, C., & Rota, C. (2021). Real-Time PCR Method Combined with a Matrix Lysis Procedure for the Quantification of Listeria monocytogenes in Meat Products. *Foods, 10*(4), 735.

Li, H., Chen, H., Liu, B., Cai, R., Jiang, N., Yue, T., & Wang, Z. (2021). Establishment of quantitative PCR assays for the rapid detection of *Alicyclobacillus* spp. that can produce guaiacol in apple juice. *International Journal of Food Microbiology*, *360*, 109329.

Lonczynski, T., & Cowin, L. (2021). Validation of the Applied Food Diagnostics, Inc. Simultaneous Multiplex Real Time PCR (SIMUL-qPCR) *Listeria* species and *L. monocytogenes* Assay in Selected Foods and Environmental Surfaces: AOAC Performance Tested MethodSM 062001. *Journal of AOAC International*.

Lv, X., Wang, L., Zhang, J., He, X., Shi, L., & Zhao, L. (2021). Quantitative detection of trace VBNC *Cronobacter sakazakii* by immunomagnetic separation in combination with PMAxx-ddPCR in dairy products. *Food Microbiology, 99*, 103831.

Maier, C., Hofmann, K., Huptas, C., Scherer, S., Wenning, M., & Lücking, G. (2021). Simultaneous quantification of the most common and proteolytic *Pseudomonas* species in raw milk by multiplex qPCR. *Applied microbiology and biotechnology, 105*(4), 1693-1708.

Mendonça, J. F. M. d., Vieira, F. d. O., Fonseca, I., Ribeiro, J. B., Arcuri, E. F., Borges, M. d. F., Martins, M. F. (2019). Detection of viable *Salmonella typhimurium* and *Staphylococcus aureus* in Coalho cheese by Real-Time PCR. *Food Science and Technology, 39*, 690-696.

Minarovičová J., Véghová A. and Kaclíková E. (2020). Evaluation of DNA extraction methods for culture-independent real-time PCR-based detection of *Listeria monocytogenes* in cheese. *Food Analytical Methods, 13*(3), 667-677.

Nagraik, R., Sharma, A., Kumar, D., Chawla, P., & Kumar, A. P. (2021). Milk adulterant detection: Conventional and biosensor based approaches: A review. Sensing and Bio-Sensing Research, 33, 100433.

O'Bryan, C. A., Ricke, S. C., & Marcy, J. A. (2022). Public health impact of *Salmonella* spp. on raw poultry: Current concepts and future prospects in the United States. *Food Control, 132*, 108539.

Paniel, N., & Noguer, T. (2019). Detection of *Salmonella* in food matrices, from conventional methods to recent aptamer-sensing technologies. *Foods, 8*(9), 371.

Persson, S., Eriksson, R., Lowther, J., Ellström, P., & Simonsson, M. (2018). Comparison between RT droplet digital PCR and RT real-time PCR for quantification of noroviruses in oysters. *International journal of food microbiology*, *284*, 73-83.

Pires, N. M., Dong, T., Yang, Z., & da Silva, L. F. (2021). Recent methods and biosensors for foodborne pathogen detection in fish: progress and future prospects to sustainable aquaculture systems. *Critical Reviews in Food Science and Nutrition, 61*(11), 1852-1876.

Rey, M. D. L. A., Cap, M., Favre, L. C., Rodríguez Racca, A., Dus Santos, M. J., Vaudagna, S. R., & Mozgovoj, M. (2021). Evaluation of PMA-qPCR methodology to detect and quantify viable Shiga toxin-producing *Escherichia coli* in beef burgers. *Journal of Food Processing and Preservation, 45*(4), e15338.

Roumani, F., Azinheiro, S., Rodrigues, C., Barros-Velázquez, J., Garrido-Maestu, A., & Prado, M. (2022). Development of a real-time PCR assay with an internal amplification control for the detection of spoilage fungi in fruit preparations. *Food Control*, *135*, 108783.

Siala, M., Barbana, A., Smaoui, S., Hachicha, S., Marouane, C., Kammoun, S., ... & Messadi-Akrout, F. (2017). Screening and detecting *Salmonella* in different food matrices in Southern Tunisia using a combined enrichment/real-time PCR method: correlation with conventional culture method. *Frontiers in microbiology*, *8*, 2416.

Spilsberg, B., Sekse, C., Urdahl, A. M., Nesse, L. L., & Johannessen, G. S. (2021). Persistence of a stx-encoding bacteriophage in minced meat investigated by Application of an improved DNA extraction Method and digital droplet PCR. *Frontiers in microbiology, 11*, 3305.

Tu, C., Hu, W., Tang, S., Meng, L., Huang, Z., Xu, X., Dong, M. (2020). Isolation and identification of *Starmerella davenportii* strain Do18 and its application in black tea beverage fermentation. *Food Science and Human Wellness, 9*(4), 355-362.

Vinayaka, A. C., Ngo, T. A., Kant, K., Engelsmann, P., Dave, V. P., Shahbazi, M.-A., Bang, D. D. (2019). Rapid detection of *Salmonella enterica* in food samples by a novel approach with combination of sample concentration and direct PCR. *Biosensors and Bioelectronics, 129*, 224-230.

Vizzini,[a] P., Manzano, M., Farre, C., Meylheuc, T., Chaix, , Ramarao, N., & Vidic, J. (2021). Highly sensitive detection of *Campylobacter* spp. In chicken meat using a silica nanoparticle enhanced dot blot DNA biosensor. *Biosensors and Bioelectronics, 171*, 112689.

Vizzini,[b] P., Vidic, J., & Manzano, M. (2021). Enrichment Free qPCR for Rapid Identification and Quantification of *Campylobacter jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* in Chicken Meat Samples by a New Couple of Primers. *Foods, 10*(10), 2341.

Wang, J., Kanach, A., Han, R., & Applegate, B. (2021). Application of bacteriophage in rapid detection of *Escherichia coli* in foods. *Current Opinion in Food Science, 39*, 43-50.

Wei,[a] S., Daliri, E. B. M., Chelliah, R., Park, B. J., Lim, J. S., Baek, M. A., . . . Oh, D. H. (2019). Development of a multiplex real-time PCR for simultaneous detection of *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus* in food samples. *Journal of Food Safety, 39*(1), e12558.

Wei,[b] S., Park, B.-J., Kim, S.-H., Seo, K.-H., Jin, Y.-G., & Oh, D.-H. (2019). Detection of *Listeria monocytogenes* using Dynabeads® anti-Listeria combined with real-time PCR in soybean sprouts. *LWT, 99*, 533-539.

Yang, X., Zhang, X., Wang, Y., Shen, H., Jiang, G., Dong, J., Gao, S. (2020). A real-time recombinase polymerase amplification method for rapid detection of *Vibrio vulnificus* in seafood. *Frontiers in microbiology, 11*, 2751.

Yang, X., Zhao, P., Dong, Y., Chen, S., Shen, H., Jiang, G., Gao, S. (2021). An isothermal recombinase polymerase amplification and lateral flow strip combined method for rapid on-site detection of Vibrio vulnificus in raw seafood. *Food Microbiology, 98*, 103664.

Zhou, P., Xie, G., Liang, T., Yu, B., Aguilar, Z., & Xu, H. (2019). Rapid and quantitative detection of viable emetic *Bacillus cereus* by PMA-qPCR assay in milk. *Molecular and cellular probes, 47*, 101437.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

HIGHLIGHTING THE INFLUENCE OF PEA SPROUTS (*Pisum sativum* L.) ADDED TO MANGO (*Mangifera indica* L.) OR KIWI (*Actinidia deliciosa*) SORBET ON THE FINAL CONSUMER'S PURCHASING PREFERENCES

Oana LIVADARIU*, Ioana-Alexandra DICU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Biotechnology, 59 Marasti Blvd., District 1, Bucharest, Romania

*Corresponding author email: ombioteh@gmail.com

Abstract

This paper contains theoretical and practical information that has allowed the experimental setup of an effective working protocol for obtaining food sprouts in peas (Pisum sativum L.), as well as obtaining mango (Mangifera indica L.) or kiwi (Actinidia deliciosa) sorbet. In order to create a new product, mango or kiwi sorbet were combined with pea sprouts. The experimental results obtained after performing a sensory analysis of the final products, as well as an online questionnaire filled by the panelists are presented in the paper based on charts, which are demonstrating the influence of pea sprouts on consumers preferences and its purchasing behaviour.

Key words: *kiwi, mango, pea, sorbet, sprouts.*

INTRODUCTION

The pea (*Pisum sativum* L.) belongs to the family *Papilionaceae* (*Fabaceae*) (www.britannica.com/topic/list-of-plants-in-the-family-Fabaceae-2021803). This is an annual herbaceous plant with a strong but poorly developed root system (Popovici et al., 2007). Due to the short growing season and the low heat requirements, the pea crop has spread rapidly in all countries. Thus, in 1906, Denaiffe described about 200 varieties of peas (Indrea et al., 2007). Peas (*Pisum sativum* L.) are mainly used for green beans, which contain a lot of protein, vitamins and minerals. In order to have a balanced diet, an adult should consume about 11 kg of peas *per* year, of which 8 kg should be in canned form (Ciofu et al., 2003).

Light has a strong influence on the germination and growth processes of pea plants (*Pisum sativum* L.), due to its photosensitive pigments. It influences plant growth by duration, intensity and spectral composition (Dobrescu, 2003).

Pea crops are the most common in their own households. The soil used for growing peas must be rich in nutrients, loose, permeable and also have a high water retention capacity. The pH of the soil must be neutral to alkaline, with values between 6.5 and 7.5 (Ciofu et al., 2003). Soil moisture in the field should be maintained at 65-70% during the growing season (Indrea et al., 2007).

Sorbet is an extremely popular dessert with a long history. It is considered to be the vegetarian alternative of ice cream or smoothies usually eaten, but also a quick and unique snack (https://sanovita.ro/blog/sorbet-de-citrice/).

Sorbets tipically include very few ingredients and do not include any dairy. Also, they are simply to produce (Whetzel, 2012).

In order to obtain the sorbet, all the ingredients (fruit, water and, if wanted, sugar), should be mix in a blender and place in the freezer. One hundred grams of mango fruit (fresh or frozen), contain 60 calories. Likewise, one hundred grams of kiwi fruit contain 58-68 calories (https://www.nutritionvalue.org/nutritioncalculator.php).

These may be reasons for which mango or kiwi sorbet can be consumed daily. Furthermore, if fresh fruits are used to obtain the sorbet, than the final product will have nutriational properties, such as antioxidant capacity which is well maintained in this product by freezing technology.

Mango (*Mangifera indica* L.) has become a well-known fruit and it has been considered the "king of fruits" in Asia (Purseglove, 1972).

The mango fruit is a large fleshy drupe, with an edible mezocarp. His flavor can vary from sweet to turpentine. Mango fruit is rich in amino acids, carbohydrates, organic acids, fatty acids, proteins, vitamins and minerals. Ripe mango fruits contain moderate levels of vitamin C, but also a semnificative quantity of provitamin A and vitamins B_1 and B_2 (Litz, 2009).

Kiwi (*Actinidia deliciosa*), also called kiwifruit or Chinese gooseberry is an edible fruit, which has a slightly acid taste and belongs to the *Actinidiaceae* family.

The kiwi plant is a woody vine native to Asia mainland, respectively from China and Taiwan. (https://www.britannica.com/plant/kiwi-fruit).

Kiwi fruits contain high quantities of bioactive compounds, such as: ascorbic acid (vitamin C), total phenols, anthocyanins, chlorophylls, carotenoids, tannins and flavonoids. All these compounds are important for nutritional values preservation during the food products preparation (Park et al., 2016).

It is well known that kiwi fruits have strong antioxidant effects and may prevent the development and deterioration of diseases caused by oxidative stress (Iwasawa et al., 2011).

The aim of this work was to create a new product from mango or kiwi sorbet and pea (*Pisum sativum* L.) sprouts, as well as highlighting the consumer's purchasing preferences regarding the final product.

The objectives set were as follows:

1. Obtaining mango (*Mangifera indica* L.) and kiwi (*Actinidia deliciosa*) puree;
2. Obtaining pea (*Pisum sativum* L.) sprouts;
3. Obtaining the new product from mango or kiwi sorbet and pea sprouts;
4. Highlighting consumer preferences through sensory analysis and online questionnaire that was filled by the sensory analysis participants.

MATERIALS AND METHODS

Obtaining pea (*Pisum sativum* L.) sprouts

For this work, pea (*Pisum sativum* L.) seeds belonging to the *Carouby de Moussane* variety, purchased online from the Tulipshop store, were used. Approximately 150 pea seeds were used for the experiment.

Pea sprouts were obtained on a substrate from a commercial source - *Universal flower soil*: organic substrate dry product at least 70%, pH value 6.5-7, humidity 60-70%, N 1.78%, P 0.21%, K 0.82% and organic carbon 13.96%.

The temperature during the germination and growth steps was a 19°C ± 1°C. The sprouts were collected for use after 10 days from planting in January 2022. After collecting, the pea sprouts were cleaned with sterile distilled water to separate them from the soil substrate.

Obtaining mango (*Mangifera indica* L.) and kiwi (*Actinidia deliciosa*) puree

The mango and kiwi fruits used to prepare the puree were bought from the Carrefour hypermarket chain.

The fruit puree was obtained by blending mango and kiwi fruits with a blender purchased from a commercial source - *Tefal Blendforce*, with a total capacity of 2 liters and 600W maximum power. This, 400 g from each fruit were blended at maximum capacity for 3 minutes.

Obtaining the new product from (*Mangifera indica* L.) and kiwi (*Actinidia deliciosa*) sorbet and pea (*Pisum sativum* L.) sprouts

After obtaining pea (*Pisum sativum* L.) sprouts and mango (*Mangifera indica* L.) and kiwi (*Actinidia deliciosa*) puree, the next step was to create the new product. For this purpose, pea sprouts were added whole to the fruits puree. In order to obtain the sorbet, the final product was put in the freezer.

Eight experimental sorbet variants were made, 4 for each fruit - mango/kiwi, as follows:

- + 10% pea sprouts
- + 20% pea sprouts
- + 30% pea sprouts
- control - no pea sprouts added

Highlighting consumer preferences through sensory analysis and online questionnaire that was filled by the participants in the sensory analysis

In order to highlight the influence of pea sprouts on consumer purchasing preferences, a survey in the form of an online questionnaire was made. The platform used in the questionnaire design was “Google Forms”, which has been distributed through social media platforms to be completed by the people.

Data collection through an online survey appears to have the potential to collect large amounts of data efficiently, economically and

within relatively short time frames (Regmi et al., 2016).

The information about the influence of pea sprouts on consumers preferences and its purchasing behaviour were achieved by answers to the questions *via* the online questionnaire. The first questions were used in obtaining socio-demographic information about the consumers, such as age, the environment they come from, monthly income, etc. The next part of the questionnaire included 16 questions about flavour, texture, quality of the new intended product and the decision of marketing this product.

RESULTS AND DISCUSSIONS

After obtaining the novel product, the last step in highlighting the influence of pea (*Pisum sativum* L.) sprouts added in mango (*Mangifera indica* L.) or kiwi (*Actinidia deliciosa*) sorbet on the final consumer's purchasing preferences was to perform the sensory analysis and complete an online questionnaire based on it.

The questionnaire was completed by 59 people (whose socio-demographic profile is presented in Table 1).

Table 1. The socio-demographic profile of the respondents

Age group	66.10% - 18-25 years 18.60% - 26-35 years 6.80% - 36-45 years 6.80% - 46-60 years 1.70% - over 60 years
Gender	59.30% - feminine 40.70% - masculine 0.00% - other
Highest level of education completed	33.90% - High school 55.90% - University 10.20% - Postgraduate
Professional status	54.90% - student 38.00% - employed 4.90% - unemployed 1.60% - entrepreneur 0.50% - pensioner
Household's net monthly income	11.90% - under 2.500 RON 59.30% - 2.501-5.000 RON 28.80% - over 5.000 RON
Where do you live (living area)?	67.80% - urban area 32.20% - rural area

In the first part of the questionnaire, the participants were asked if they consume sorbet products (Figure 1).

The results obtained indicate that a majority of 61.00% of people are consuming sorbet products very rare and 35.60% of them are consuming it occasional. Only one of 59 respondents are consuming fruit sorbet once a month or once a week. This highlights the lack of consumers knowledge about the meaning of fruit sorbet, as well as the low interest in consuming a natural and healthy dessert.

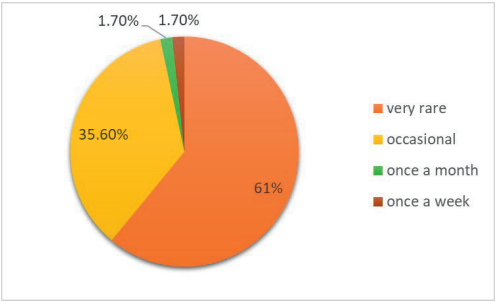


Figure 1. Q1 - How often do you consume sorbet?

In the next question (Figure 2), a 54.20% majority chose mango sorbet as a preference when buying such a product. With a percentage of 39.00%, it is followed in the preferences of consumers by kiwi sorbet. Other preferences mentioned by consumers were lemon, rose, quince and strawberry sorbet.

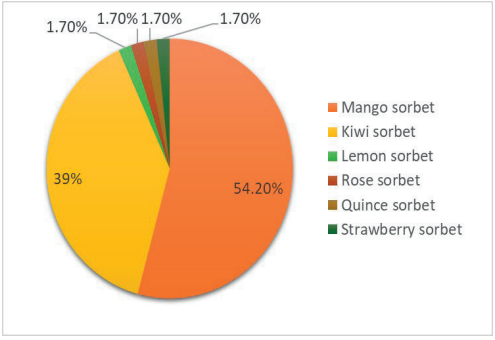


Figure 2. Q2 - What type of sorbet would you prefer to buy?

From Figure 3, it can be seen that most people (50.80), are influenced by many factors (brand, quality, price and packaging), in choosing to purchase a food product. Only 10.20% of those surveyed consider that these factors have a minor influence on the purchase decision of a food product.

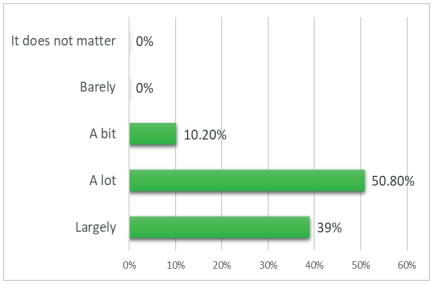


Figure 3. Q3 - How much are you influenced in the purchase of a food product by the following factors: brand, quality, price, packaging?

According to the illustrated data (Figure 4), 81.40% of people consider that the most important sense in choosing sorbet is the taste, while 13.60% of them consider the color or texture the most important factors and only 5.10% take into account the aroma of the product.

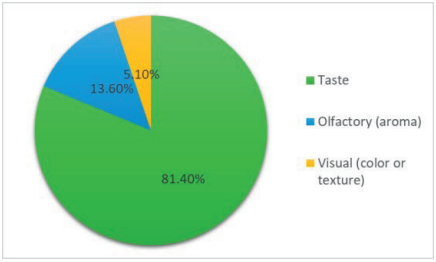


Figure 4. Q4 - From your point of view, what is the most important sense in choosing sorbet?

In terms of taste quality (Figure 5), the mango product with the addition of pea sprouts obtained majority results with a pleasant taste quality, and it was voted very satisfactory and satisfactory in proportion of 23.70% and 32.20% respectively. Only, 3 out of 59 people found the taste quality unsatisfactory.

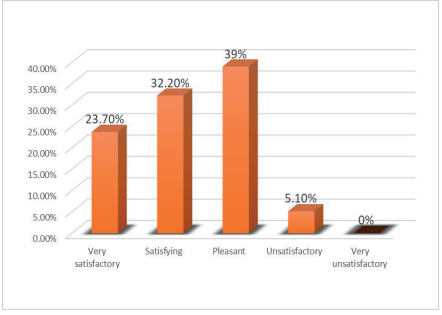


Figure 5. Q5 - What is the taste quality of the mango sorbet preparation with the addition of pea sprouts (*Pisum sativum* L.)?

Regarding the quality of the kiwi product taste (Figure 6), it was pleasant for a majority of 44.00% of people, satisfactory for 30.50%, very satisfactory only for 16.90%.
Four persons participating in the questionnaire declared the quality to be unsatisfactory, and only one person stated that they were not at all satisfied with the taste quality of this product.

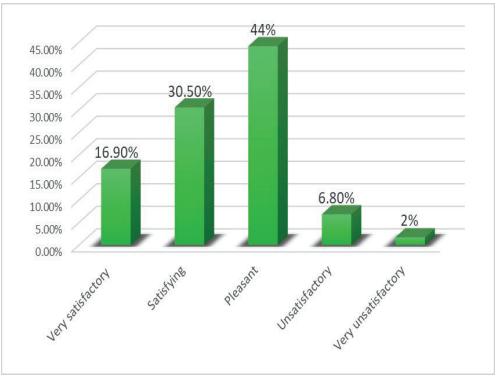


Figure 6. Q6 - What is the taste quality of the kiwi sorbet preparation with the addition of pea sprouts (*Pisum sativum* L.)?

In terms of the texture of the product obtained, the results were similar for both mango and kiwi sorbet (Figure 7 and Figure 8).
A majority of 42.40% answered that the change in texture by adding pea sprouts was insignificant in obtaining the final product.
Nearly a quarter of respondents thought that the texture was significantly altered by the presence of pea sprouts.
One person considered the texture changed a lot due to the addition of sprouts in both cases.

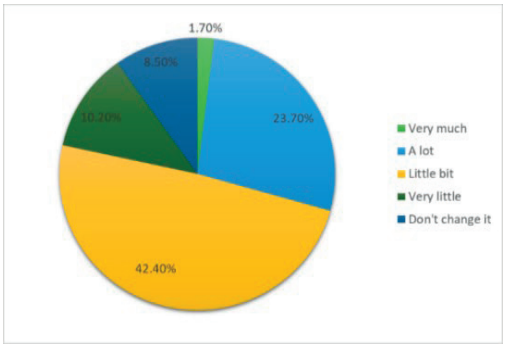


Figure 7. Q7 - Do you think that the pea sprouts (*Pisum sativum* L.) added to mango sorbet change its texture?

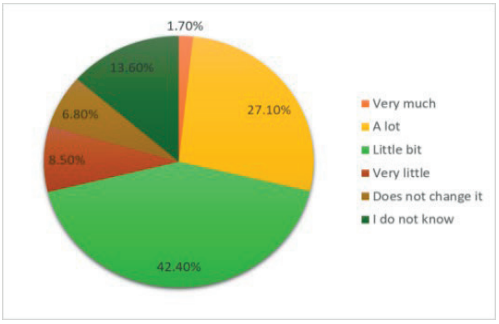


Figure 8. Q8 – Do you think that the pea sprouts (*Pisum sativum* L.) added to kiwi sorbet change its texture ?

In proportion to 40.70% (mango - Figure 9), respectively 50.80% (kiwi - Figure 10), of respondents considered that aroma of the product was less influenced by the presence of pea sprouts. Thereby, 33.90% respondents considered that the aroma changed after the addition of sprouts in mango sorbet, while 27.10% respondents for kiwi sorbet. Also, only 1.70% respondents considered that the aroma was very much changed by the presence of pea sprouts.

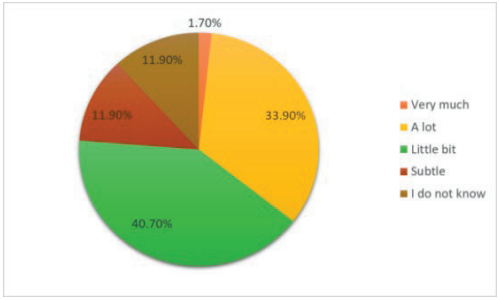


Figure 9. Q9 - Do you think that pea sprouts (*Pisum sativum* L.) added to mango sorbet change its flavor?

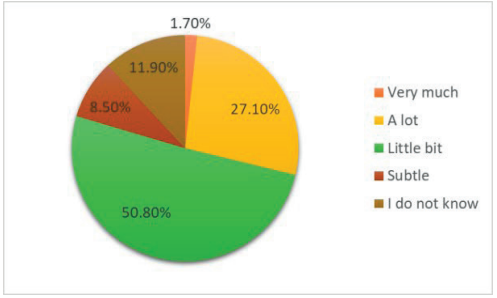


Figure 10. Q10 – Do you think that pea sprouts (*Pisum sativum* L.) added to kiwi sorbet change its flavor ?

About quantity of pea sprouts (*Pisum sativum* L.) from sorbet (Figure 11), 57.60% of the respondents considered that the new fruit sorbet product with the pea sprouts should contain a moderate amount of sprouts (20% of the final product), while 37.30% of them would prefer a small amount of sprouts (10% of the final product), and 5.10% would like pea sprouts to be found in an large of 30% of the total amount of the product.

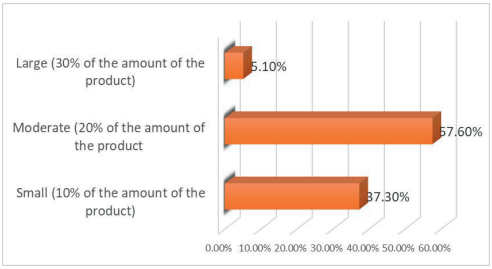


Figure 11. Q11 - Which quantity of pea sprouts (*Pisum sativum* L.) do you consider the sorbet must contain?

Compared to other marketed sorbets, the product with mango sorbet and pea sprouts has obtained positive results (67.80 good and 28.80 very good), in terms of quality (Figure 12), and 94.90% of people want this product to be marketed (Figure 13).

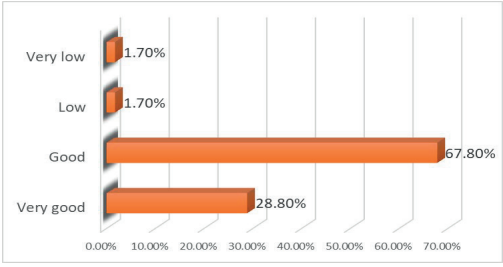


Figure 12. Q12 - What quality would you give to the mango sorbet with pea sprouts (*Pisum sativum* L.), compared to other marketed sorbets?

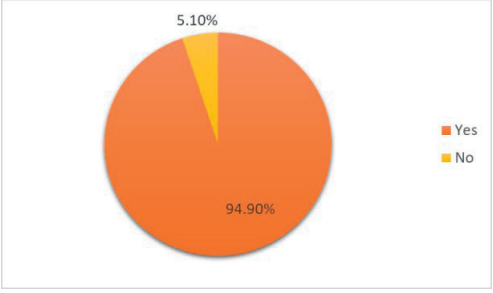


Figure 13. Q13 - Would you like this product to be marketed?

Like the mango sorbet with pea sprouts, the kiwi product enjoyed the appreciation of quality, a majority of 67.80% considering it of good quality (Figure 14), and 91.50% of the participants in the questionnaire want this product to be marketed (Figure 15).

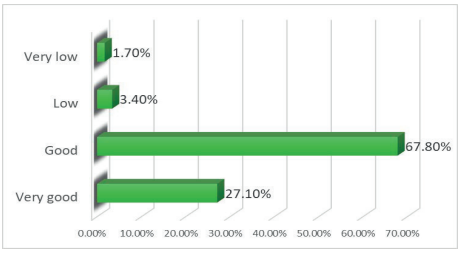


Figure 14. Q14 - What quality would you give to the kiwi sorbet with pea sprouts (*Pisum sativum* L.), compared to other marketed sorbets?

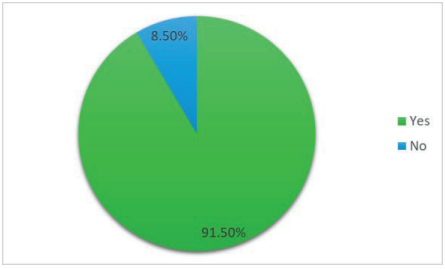


Figure 15. Q15 - Would you like this product to be marketed?

Because it is desired to market these new products from mango or kiwi and pea sprouts, the participants chose, in proportion of 44.10%, the amount of 9-10 RON to be the most suitable price for 100 grams of product. 35.60% of respondents would pay the amount of 8-9 RON, and 20.30% would prefer the price of 7-8 RON *per* 100 grams of product (Figure 16).

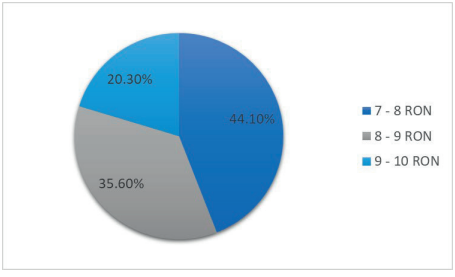


Figure 16. Q16 - What would be the maximum price you would be willing to pay for 100 g of mango or kiwi sorbet with the addition of pea sprouts (*Pisum sativum* L.)?

Following the questionnaire, the resulting data were obtained:

- 61.00% of people consume sorbet very rarely, this highlighting the limited knowledge about this healthy alternative dessert;
- half of consumers are greatly influenced by brand, quality, price and packaging in purchasing a product;
- 81.40% of the taste is considered the most important sense in choosing sorbet;
- sorbet texture is very little modified by the addition of pea sprouts (*Pisum sativum* L.) in mango sorbet in the case of 42.40% of consumers. Approximately the same preferences are observed in the case of kiwi sorbet with the addition of pea sprouts (*Pisum sativum* L.);
- 57.60% of consumers prefer a moderate addition of pea sprouts (*Pisum sativum* L.) to mango or kiwi sorbet, and
- in the case of both types of sorbet with the addition of pea sprouts *(Pisum sativum* L.), over 90.00% of consumers would like to purchase them.

CONCLUSIONS

In order to formulate the novel food product, pea sprouts obtained from soil substrate and mango or kiwi sorbet obtained by blending the fruit were used. The novel food product preparation was followed by the sensory analysis of the product and an online questionnaire completed by 59 people.

The sensory analysis results presented in this paper showed the influence of pea sprouts (*Pisum sativum* L.) added to mango (*Mangifera indica* L.) or kiwi (*Actinidia deliciosa*) sorbet on the consumer preferences and the questionnaire results established the consumer's purchasing preferences. Thus, more than half of consumers considered the addition of pea sprouts (*Pisum sativum* L.) to mango or kiwi sorbet to be pleasant and satisfying in terms of taste quality and according to consumer preferences, a corresponding price *per* 100 grams of product would be between 9 and 10 RON.

REFERENCES

Ciofu, R., Stan N., Popescu, V., Chilom, P., Apahidean, S., Horgoş, A., Berar, V., Lauer, K. F., Atanasiu, N.,

(2003). *Tratat de legumicultură.* Editura Ceres, București, RO.
Dobrescu, A. (2003). *Botanica și fiziologia plantelor*, Universitatea de Științe Agronomice și Medicină Veterinară, București, pp. 201.
https://sanovita.ro/blog/sorbet-de-citrice/
https://www.britannica.com/plant/kiwi-fruit
https://www.britannica.com/topic/list-of-plants-in-the-family-Fabaceae-2021803
https://www.nutritionvalue.org/nutritioncalculator.php
Indrea, D., Apahidean, S. Al., Apahidean, M., Mănuțiu, D. N., Sima, R. (2007). *Cultura legumelor.* Editura Ceres, București, RO.
Iwasawa, H., Morita, E., Yui, S., & Yamazaki, M. (2011). Anti-oxidant effects of kiwi fruit in vitro and in vivo, *Biological & Pharmaceutical Bulletin*, 128-134.
Litz, R. E. (2009). *The Mango: Botany, production and uses*, Tropical Research and Education Center and Center for Tropical Agriculture, 2nd Edition, Edited by: Richard Litz, University of Florida, pp. 3.
Park, Y. S., Ham, K.-S., Park, Y.-K., Leontowicz, H., Leontowicz, M., Namieśnik, J., Katrich, E., Gorinstein, S. (2016). The effects of treatment on quality parameters of smoothie-type 'Hayward' kiwi fruit beverages, *Food Control, 70,* 221-228. (http://www.bashanfoundation.org/inmemoriam/Trakhtenberg-S/2016.-Gorinstein-FC.pdf)
Popovici, L., Moruzi, C., Toma, I., (2007). *Atlas botanic.* Editura Didactică și Pedagogică, București, RO.
Purseglove, J.W. (1972). Mangoes west from India. *Acta Horticulturae*, *24.* 107-174.
Regmi, P. R., Waithaka, E., Paudyal, A., Simkhada, P., & van Teijlingen, E. (2016). Guide to the design and application of online questionnaire surveys. *Nepal journal of epidemiology*, *6*(4), 640-644. https://doi.org/10.3126/nje.v6i4.17258 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5506389/).
Whetzel, S. (2012). *The Everything Ice Cream, Gelato, and Frozen Desserts Cookbook* (pp. 6).

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

THE USE OF ACID DRIED SOURDOUGH STARTER TO IMPROVE SENSORY PROPERTIES AND BREAD`S SHELF LIFE - A REVIEW

Sabina-Andreea BOBEA[1, 2], Nastasia BELC[1, 2], Călina Petruța CORNEA[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd., District 1, Bucharest, Romania
[2]National Research & Development Institute for Food Bioresources - IBA Bucharest, 6 Dinu Vintilă Street, District 2, Bucharest, Romania

Corresponding author email: sabina.bobea@bioresurse.ro

Abstract

Bread is one of the main products of Romanian food industry that is always present in the human daily diet. Current trends are to return to traditional bread making methods using biotechnological processes based on the use of certain bacterial cultures and yeasts. Thus, different types of dough fermentation are used in the bakery industry, such as: acidic substances, pure microbial cultures and microbial cultures developed on a nutritious support (acid sourdough). Lactic acid bacteria (LAB) represent an important group of GRAS (Generally recognized as safe) microorganisms, as they are used industrially mainly in the production of fermented foods and beverages. They have a major advantage, being recognized as safe elements in the food industry. Lactic acid bacteria are also used in starter cultures, thus contributing to the sensory characteristics of finished products. The present study reviews about the use of acid dried sourdough starter and how it can improved the sensory properties and bread`s shelf life.

Key words*: microbial cultures, lactic acid bacteria, biotechnological processes, dough fermentation.*

INTRODUCTION

The current challenges of food industry research are to find alternatives to developing probiotic products other than dairy products, as the latter can cause allergies and lactose intolerance (Lu Zhang et al., 2018; De Prisco and Mauriello, 2016).
In the recent years many probiotic fortified foods have appeared on the market (De Prisco and Mauriello, 2016; Rivera-Espinoza and Gallardo Navarro, 2010) due to the fact that they offer several health benefits, helping to maintain a balance of the intestinal flora and increasing resistance to pathogens (Tripathi M.K. and Giri S.K., 2014).
Of these, bakery products are a very important category, attracting continued interest in research (Pinto et al., 2014; Reid et al., 2007; Soukoulis et al., 2014; Vitaglione et al., 2015; Zhang et al., 2014).
The population is aware of the impact that functional probiotic foods have on health and therefore, there is a fairly high demand for this. Many factors can affect the viability of probiotic organisms during processing and storage. On the other hand, there may be undesirable effects, such as affecting the quality and sensory properties of the finished products. In this sense, by different methods such as: encapsulation, or by adding different protectors and by modifying the processing and storage conditions, it was desired to protect microorganisms (Tripathi M.K. and Giri S.K., 2014).
From ancient times, dough production is considered a biotechnological process, used in the manufacture of bread (Albagli et al., 2021).
Dough is a leavening agent with an important role in baking having as raw material wheat flour, yeast *Saccharomyces cerevisiae*, water (Arendt et al., 2007; Yu et al., 2018) and lactic acid bacteria (Siepmann et al., 2018).
Due to the fermentation of lactic acid bacteria and yeast, the sensory characteristics of the dough, the nutritional value of the bread, but also its taste and aroma change (Chavan et al., 2011; Katina et al., 2006; Tafti et al., 2013). Some researchers (Oshiro et al., 2021; Ganzle et al., 2016) believe that it can also contribute to the shelf life of bakery products.

MATERIALS AND METHODS

This paper is based on recently published articles, accessing Science Direct on the e-nformation platform. The keywords were based on research on lactic fermentation in sourdough, to improve sensory characteristics, such as: taste, smell, texture, shape (appearance) of bread.

RESULTS AND DISCUSSIONS

The introduction of probiotic fortified foods on the market in recent years has been a real success (De Prisco and Mauriello, 2016; Rivera-Espinoza and Gallardo Navarro, 2010).
Bakery products are an area of interest for researchers because they are an important category in the segment of probiotic foods (Pinto et al., 2014; Reid et al., 2007; Soukoulis et al., 2014; Vitaglione et al., 2015; Zhang. et al., 2014).
As an acidifying agent, lactic acid bacteria have an important advantage because they are generally recognized as safe (GRAS) for use in the food industry. LAB contributes to the extension of the shelf life of fermented bakery products, making an important contribution to their organoleptic and nutritional properties (Cucu S.E. and Popa M.E., 2020). They are used in the food industry as initial inoculum in food and beverage production (Zamfir et al., 2014).
In order to improve the quality of the final product in terms of texture, shelf life and flavor, an important step is the fermentation of the dough, largely attributed to the metabolic interaction of microorganisms (Gobbetti et al., 2019). Lactic acid bacteria, along with yeast, are the predominant microflora. Most of those that have been isolated from dough are the genus *Lactobacillus*, and among the yeast species, *Candida* and *Saccharomyces* being the most common (De Vuyst and Neysens, 2005). *Lactobacillus sanfranciscensis* is mainly used in bakery production in the USA and Italy, while *Lactobacillus plantarum* and *Lactobacillus brevis* in Spain (De Vuyst et al., 2014).
The addition of live bacteria should be limited to a minimum number that should be retained in the baked product at the time of consumption (> 6-7 log CFU/g) (Tripathi M.K. and Giri S.K., 2014). Due to the high temperature used during baking, the products can significantly lose viable bacteria and thus become a significant challenge (Zhang et al., 2018). Therefore, it is important to study the bacteria during the baking process, in order to facilitate their development.
According to Chavan and Chavan (2011), most LAB species isolated from dough or used as a starter inoculum, belong to the genera: *Lactobacillus, Pediococcus, Leuconostoc* and *Weisella,* with few exceptions. Among the yeasts, the most common species are: *Candida milleri, Candida holmii, Kazachstania exigua* and *Saccharomyces cerevisiae*. Researching the microbiology of yeast from 1970 to 2013, Huys, Daniel and De Vuyst (2013) reviewed 40 publications revealing six species of yeast often observed in stable dough: *Saccharomyces cerevisiae, Kazachstania exigua, Candida humilis, Pichia kudriavzevii, Torulaspora delbrueckii, Wickerhamomyces anomalus*. They observed the presence of over 60 species of LAB, and most are heterofermentative (*Levilactobacillus brevis - Lactobacillus brevis, Fructilactobacillus sanfranciscensis - Lactobacillus sanfranciscensis, Lactobacillus citreum, Limosilactobacillus reuteri - Lactobacillus reuteri*). There are some optional heterofermentative (*Companilactobacillus alimentarius - Lactobacillus alimentarius, Lacticaseibacillus casei - Lactobacillus casei and Lactiplantibacillus plantarum - Bacillus plantarum*) and homofermentative (*Lactobacillus acidophilus, Lactobacillus delbrueckii* subsp. *delbrueckii and Lactobacillus lactis* subsp. *lactis*).
Lactobacillus plantarum is a Gram-positive bacteria which grows at 10-15°C. *L. plantarum* cells are rod-shaped with rounded ends, usually 3-8 μm long and 0.9-1.2 μm wide. It can be observed microscopically in individual cells, cell pairs or short chains (Corsetti et al., 2016; Hammes and Vogel, 1995; Landete et al., 2010). *L. plantarum* has a relatively large 3.3 Mb genome compared to other *Lactobacillus* spp. species (Darby and Jones, 2017). The length of the genome is influenced by the variety of environmental niches in which *L. plantarum* is found (Landete et al., 2010). *L. plantarum* contributes to the fermentation of

fruits and vegetables. It has a higher tolerance to acid than other lactic acid bacteria (Fleming, 1984; Lu et al., 2003) and represent a potential probiotic (Georgieva, et al., 2009; Janković, 2012; Park and Lim, 2015; Yoon et al., 2006; Zago et al., 2011). The most commonly microbial cultures that are found in doughs belong to *Lactobacilus* sp.*: L. plantarum, L. sanfranciscensis, L. brevis* subsp. *lindneri and L. brevis* (Gobbetti, 1998), but also: *Weisella cibaria* and *Pediococcus pentosaceus* (Iacumin et al., 2009). In the production of bakery, a "starter" strain is used to improve their quality, but also to obtain a variety of standard and quality products (Pepe et al., 2004). *L. plantarum* has many beneficial effects on health due to its probiotic characteristics for fermented products.

Fast fermentation using traditional baking yeast (*Saccharomyces cerevisiae*) is now frequently used. Dough-based fermentation is used worldwide for the production of typical bread dough, pizza, biscuits and more sweet pastries (Reale et al., 2019; Ashaolu and Reale, 2020). Compared to sourdough, the yeast has several advantages, such as: higher productivity, more uniform products, a smaller amount of baking yeast used for a considerable production and lower costs. Comparing the yeast and the sourdough fermentation, the last is more expensive and takes longer; it lasts between 12-24 hours. Maintaining a dough starter consumes time and resources. It has advantages such as: sensory characteristics, digestibility and nutritional attributes compared to bread made from traditional baking yeast dough (Siepmann et al., 2018).

Globally, consumers prefer the products of artisanal bakeries (Albagli et al., 2021) although there are many challenges of drying the dough starter, regarding its microbiological composition. The microbiology of the dough starter is composed of lactic acid bacteria (LAB) and yeast in a ratio of 100: 1 in the usual way (Reale et al., 2019).

The scientific literature on the drying process of the microorganism starter culture is limited, but in many studies, it is found in dry form (Abagli et al., 2021). Drying, either by freeze-drying or by spraying, influences both the aroma and the shelf life of the product. Freeze-drying is done by reducing the activity of water without preheating, but it takes longer than conventional dehydration. Samples need to be frozen quickly, followed by vacuum water removal (Morgan et al., 2006). A freeze-dried starter can be a good replacement for the fresh dough starter used in the production process without pre-fermentation. A reduction in the concentration of flavor components was observed after the lyophilization process of a starter (Kirchhoff, 2000). During the freezing process, water drains from the cells. This causes the formation of ice crystals and an increase in the concentration of intracellular salt, influencing the viability of the cells. A cryoprotectant can be used to prevent cell damage (Stefanello et al., 2019). The cells obtained from the drying process can be recovered by rehydration (Morgan et al., 2006). Cryopreservation increases cell viability using high osmotic pressure, demonstrates Ray et al. (1971). Morgan et al. (2006) demonstrated that rehydration media can play an important role in reconditioning damaged cells by providing essential nutrients and components to damaged cells. Also, the rehydration temperature influences the cells recovery. Thus, a higher number of cells can be obtained by applying a temperature in the range of 15-25°C, than in the range of 35-45°C (Ray et al., 1971). *Saccharomyces cerevisiae* cells rehydrated for 7-16 days under controlled conditions showed greater viability compared to immediate rehydration cells (Poirier et al., 1999).

Meuser, Barber, and Fischer (1995) have shown that lyophilization is a good technique for ensuring cell viability by comparing dry dough with the initial lyophilized dough without cryoprotectant. Thus, they obtained a number of LAB and yeast cells approximately 100 times higher in the case of dry dough compared to the initial lyophilized dough without cryoprotectant, in the first 24 hours of rehydration. Stefanello et al. (2018) used trehalose (as cryoprotectant) for lyophilization of the dough and observed a microbial survival rate 81% more efficient for LAB species than for yeasts, but in terms of cost, trehalose is not the best option.

Stefanello et al., 2019 tested different cryoprotectants, such as: 0.1% peptone water solution, 10% sucrose solution, 5% trehalose solution, 10% skim milk solution and a mixture

of 5% glutamate monohydrate and 10% skimmed milk powder. These were added to the lyophilized species, previously isolated from the dough, of *Lactobacillus fermentum* IAL 4541 and *Wickerhamomyces anomalus* IAL 4533 before freezing, at -80°C (Stefanello et al., 2018). Of the above-mentioned variants, the least effective was sucrose. Caglar et al. (2021) observed a significant difference between the two methods: the powder produced by lyophilization performed a higher number of LAB and yeast than in the case of spray drying. Bread made from spray-dried powder had a larger and a specific volume. At the same time, the increase in the level of dry dough has led to an increase in the hardness of the bread. Dried sourdough acted as a chemical acidifier for the dough. The authors concluded that dry sourdough must be pre-treated, such as rehydrating the powder and refreshing the cells to activate the starter.

Spray drying is a method by which water is removed from a fluid material on contact with the hot air of a drying chamber (Tafti et al., 2013). The process takes place in three phases: atomization, conversion of droplets into particles and collection of particles (separated from the drying medium by a gravitational force and collected) in a tank (Santos et al., 2018).

One of the spray drying applications is mentioned by Tafti et al (2013) regarding the isolation of *L. paralimentarius* from traditional Iranian wheat dough and inoculated (10^8 CFU/g) into a mixture of wheat flour and tap water to make a dough starter. The starter was then spray dried using inlet and outlet air temperatures of 180°C and 90°C, respectively, and the dust was collected from the bottom of the chamber and from the cyclone. The microbial analysis was performed after rehydration with 90 ml of sterile 0.1% peptone water, resulting in a decrease of 10^4 CFU/g of cells, probably due to the high outlet temperature used to obtain a low moisture content (less than 5%, necessary for storage stability) (Tafti et al., 2013).

Using several methods, such as spray drying, freeze drying and oven drying, Ertop et al. (2018) evaluated the physico-chemical properties of bread produced by spontaneous fermentation and the addition of LAB as a starter. Thus, they observed the highest microbial viability, using the method of spray drying, which together with spontaneous fermentation improved the shelf life of bread by slowing down the growth of mold up to 9 days. Bread made with dry starters showed better water retention in the structure, improving moisture, texture and volume compared to the control bread. In addition, according to the authors, the antioxidant activity of the samples of bread baked with dry dough starter was higher compared to the control bread. Due to the metabolism of LAB in the dough, oxidation of lipids or strong antioxidant activity may occur during fermentation, as well as the release of bioactive peptides that are known for their antioxidant effect.

Dry sourdough either by spraying or by freeze-drying method, gives the bread a higher volume, lower elasticity, and a special aroma (Ertop et al., 2018). Other advantages that sourdough has compared to baking yeast dough: the hydrolysis of starch, the higher acidity of the dough and the degradation of gluten. All of that increase the concentration of volatile compounds (the flavor), help to enrich the nutritional value, improve the texture and the digestibility, prolong the shelf life of bread (Siepmann et al., 2018). Volatile compounds depends on the added water content and the microbial species presented in the sourdough (Damiani et al., 1996).

Adding different proportions of dry starter and baking yeast, Tafti, Peighambardoust, Hesari, Bahrami and Bonab (2013) studied the physical and sensory characteristics of bread. They obtained better sensory results than the control when using 9% spray-dried starter.

CONCLUSIONS

Although rapid fermentation with baking yeast is currently used in the manufacture of dough, worldwide trends are shifting to making bread using dough preparation technology, especially using sourdough. Bread made from sourdough fermentation has the advantage of better general sensory characteristics along with digestibility and nutritional attributes compared to bread made from dough with traditional baking yeast.

ACKNOWLEDGEMENTS

This paper was published under the frame of PhD USAMVB fellowship grant.

REFERENCES

Albagli G., do Monte Schwartz I., Amaral F.F.P., Ferreira T.F., Finotelli P.V. (2021). How dried sourdough starter can enable and spread the use of sourdough bread. *LWT - Food Science and Technology*, 149,111888.

Arendt, E. K., Liam, A. M. R. and Bello, F. D. (2007). Impact of sourdough on the texture of bread. *Food.Microbiology*, 24, 165–174. https://doi.org/10.1016/j.fm.2006.07.011.

Ashaolu, T. J. and Reale, A. (2020). A holistic review on euro-asian lactic acid bacteria fermented cereals and vegetables. *Microorganisms*, 8(8), 1–24. https://doi.org/10.3390/microorganisms8081176. MDPI AG.

Caglar, N., Ermis, E. and Durak, M. Z. (2021). Spray-dried and freeze-dried sourdough powders: Properties and evaluation of their use in breadmaking. *Journal of Food Engineering*, 292, 110355. https://doi.org/10.1016/j.jfoodeng.2020.110355

Chavan, R. and Chavan, R. (2011). Sourdough technology - A traditional way for wholesome foods: A review. *Comprehensive Reviews in Food Science and Food Safety*, 10, 170–183. https://doi.org/10.1111/j.1541-4337.2011.00148.x.

Corsetti, A., Ciarrocchi, A. and Prete, R. (2016). Lactic acid bacteria: *Lactobacillus* spp.: *Lactobacillus plantarum*. *Reference module in food science*. Elsevier.

Cucu, Ş.-E., Popa M.E. (2020). Packaging and technological solutions for bread shelf life improving. *Scientific Bulletin*. *Series F. Biotechnologies*, Vol. XXIV, No. 2, 2020, ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

Damiani, P., Gobbetti, M., Cossignani, L., Corsetti, A., Simonetti, M. S. and Rossi, J. (1996). The sourdough microflora. Characterization of hetero- and homofermentative lactic acid bacteria, yeasts and their interactions on the basis of the volatile compounds produced. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology*, 29(1-2), 63–70. https://doi.org/10.1006/fstl.1996.0009

Darby, T. M. and Jones, R. M. (2017). Chapter 10 - beneficial influences of *Lactobacillus plantarum* on human health and disease. In M. H. Floch, Y. Ringel and W. Allan Walker (Eds.). *The microbiota in gastrointestinal pathophysiology* (pp. 109–117). Boston: Academic Press.

De Prisco, A. and Mauriello, G. (2016). Probiotication of foods: A focus on microencapsulation tool. *Trends in Food Science & Technology*, 48, 27–39. http://doi.org/10.1016/j.tifs.2015.11.009.

De Vuyst, L., Van Kerrebroeck, S., Harth, H., Huys, G., Daniel, H. M. and Weckx, S. (2014). Microbial ecology of sourdough fermentations: Diverse or uniform? *Food Microbiology*, 37, 11–29. https://doi.org/10.1016/j.fm.2013.06.002.

De Vuyst, L. and Neysens, P. (2005). The sourdough microflora: Biodiversity and metabolic interactions. *Trends in Food Science & Technology*, 16(1-3), 43–56. https://doi.org/10.1016/j.tifs.2004.02.012.

Ertop, H. M., Ilter, S. M., YilMaz, F., BaltaCi, C. and Gündogdu, A. (2018). Quality properties of wheat breads incorporated with dried sourdoughs produced with different fermentation and drying methods. *Food Science and Technology Research*, 24(6), 971–980. https://doi.org/10.3136/fstr.24.971.

Fleming, H. P. (1984). Developments in cucumber fermentation. *Journal of Chemical Technology and Biotechnology*, 34(4), 241–252. https://doi.org/10.1002/jctb.280340404.

Ganzle, M. and Ripari, V. (2016). Composition and function of sourdough microbiota: from ecological theory to bread quality, *Int. J. Food Microbiol.*, 239, 19e25.

Georgieva, R., Iliev, I., Haertlé, T., Chobert, J. M., Ivanova, I. and Danova, S. (2009). Technological properties of candidate probiotic *Lactobacillus plantarum* strains. *International Dairy Journal*, 19(11), 696–702. https://doi.org/10.1016/j.idairyj.2009.06.006.

Gobbetti, M. (1998). The sourdough microflora: Interactions of lactic acid bacteria and yeasts. *Trends in Food Science & Technology*, 9(7), 267–274. https://doi.org/10.1016/S0924-2244(98)00053-3.

Gobbetti, M., De Angelis, M., Di Cagno, R., Calasso, M., Archetti, G. and Rizzello, C. G. (2019). Novel insights on the functional/nutritional features of the sourdough fermentation. *International Journal of Food Microbiology*, 302, 103–113. https://doi.org/10.1016/j.ijfoodmicro.2018.05.018.

Hammes, W. P. and Vogel, R. F. (1995). The genus *Lactobacillus*. In B. J. B. Wood & W. H. Holzapfel (Eds.), *The Genera of Lactic Acid Bacteria* (pp. 19–54). Boston, MA: Springer US.

Huys, G., Daniel, H. M. and De Vuyst, L. (2013). Taxonomy and biodiversity of sourdough yeasts and lactic acid bacteria. *In Handbook on sourdough biotechnology*. https://doi. org/10.1007/978-1-4614-5425-0_5

Iacumin, L., Cecchini, F., Manzano, M., Osualdini, M., Boscolo, D., Orlic, S., et al. (2009). Description of the microflora of sourdoughs by culture-dependent and culture-independent methods. *Food Microbiology*, 26(2), 128–135. https://doi.org/10.1016/j.fm.2008.10.010.

Janković, T., Frece, J., Abram, M. and Gobin, I. (2012). Aggregation ability of potential probiotic *Lactobacillus plantarum* strains. *Int J Sanit Eng Res*, 6(1), 19–24.

Katina, K., Heiniö, R., Autio, K. and Poutanen, K. (2006). Optimization of sourdough process for improved sensory profile and texture of wheat bread. *LWT-Food Science and Technology*, 39(10), 1189–1202. https://doi.org/10.1016/j.lwt.2005.08.001.

Kirchhoff, E. K. (2000). Charakterisierung von Schlüsselaromastoffen in Roggenbrot

Untersuchungen zum Einfluß des Herstellungsverfahrens und der Teigezeptur. Doctoral Thesis. Munich: Faculty of Chemistry, Technical University.

Landete, J. M., Rodríguez, H., Curiel, J. A., de las Rivas, B., de Felipe, F. L. and Muñoz, R. (2010). Chapter 43 - Degradation of Phenolic Compounds Found in Olive Products by Lactobacillus plantarum Strains. In V. R. Preedy & R. R. Watson (Eds.), *Olives and Olive Oil in Health and Disease Prevention* (pp. 387–396). San Diego: Academic Press.

Lu, Z., Breidt, F., Jr., Fleming, H. P., Altermann, E. and Klaenhammer, T. R. (2003). Isolation and characterization of a *Lactobacillus plantarum* bacteriophage, phiJL-1, from a cucumber fermentation. *Int J Food Microbiol*, 84(2), 225-235. https://doi:10.1016/s0168-1605(03)00111-9

Lu Z., Marieke A. Taal, Remko M. Boom, Xiao Dong Chen, Maarten A.I. Schutyser, (2018), „Effect of baking conditions and storage on the viability of *Lactobacillus plantarum* supplemented to bread", *LWT - Food Science and Technology*, 87, 318-325. http://dx.doi.org/10.1016/j.lwt.2017.09.005.

Meuser, F., Barber, B. and Fischer, G. (1995). Determination of the microbial activity of dried sourdoughs by revitalization of their lactic acid bacteria and yeasts. *Food Control*, 6(3), 147–154. https://doi.org/10.1016/0956-7135(94)00009-3

Morgan, C. A., Herman, N., White, P. A. and Vesey, G. (2006). Preservation of microorganisms by drying; A review. *Journal of Microbiological Methods*, 66(2), 183–193. https://doi.org/10.1016/j.mimet.2006.02.017

Oshiro M., Zendo T., and Nakayama J. (2021). Diversity and dynamics of sourdough lactic acid bacteriota created by a slow food fermentation system. *Journal of Bioscience and Bioengineering*. VOL. 131 No. 4, 333-340, https://doi.org/10.1016/j.jbiosc.2020.11.007

Park, S. Y. and Lim, S. D. (2015). Probiotic Characteristics of *Lactobacillus plantarum* FH185 541 Isolated from Human Feces. *Korean J Food Sci Anim Resour*, 35(5), 615-621. https://doi:10.5851/kosfa.2015.35.5.615

Pepe, O., Blajotta, G., Anastasio, M., Moschetti, G., Ercolini, D. and Villani, F. (2004). Technological and molecular diversity of *Lactobacillus plantarum* strains isolated from naturally fermented sourdoughs. *Systematic & Applied Microbiology*, 27(4), 443–453. https://doi.org/10.1078/0723202041438446.

Pinto, D., Castro, I., Vicente, A., Bourbon, A. I. and Cerqueira, M. A. (2014). Chapter 25: Functional bakery Products: An overview and future perspectives. In W. Zhou, Y. H. Hui, I. De Leyn, M. A. Pagani, C. M. Rosell, & J. D. Selman, (Eds.). *Bakery products science and Technology*, (pp. 431–452). (Second Edition). http://doi.org/10.1002/9781118792001.ch25.

Poirier, I., Maréchal, P. A., Richard, S. and Gervais, P. (1999). *Saccharomyces cerevisiae* viability is strongly dependant on rehydration kinetics and the temperature of dried cells. *Journal of Applied Microbiology*. https://doi.org/10.1046/j.1365-2672.1999.00638.x

Ray, B., Jezeski, J. J. and Busta, F. F. (1971). Effect of rehydration on recovery, repair, and growth of injured freeze-dried *Salmonella anatum*. *Applied Microbiology*. https://doi.org/10.1128/aem.22.2.184-189.1971

Reale, A., Di Renzo, T., Boscaino, F., Nazzaro, F., Fratianni, F. and Aponte, M. (2019a). Lactic acid bacteria biota and aroma profile of Italian traditional sourdoughs from the irpinian area in Italy. *Frontiers in Microbiology*, 10, 1621. https://doi.org/10.3389/fmicb.2019.01621

Reid, A. A., Champagne, C. P., Gardner, N., Fustier, P. and Vuillemard, J. C. (2007). Survival in food systems of Lactobacillus rhamnosus R011 microentrapped in whey protein gel particles. *Journal of Food Science*, 72(1), 31–37. http://doi.org/10.1111/j.1750-3841.2006.00222.x

Rivera-Espinoza, Y. and Gallardo-Navarro, Y. (2010). Non-dairy probiotic products. *Food Microbiology*, 27(1), 1–11. http://doi.org/10.1016/j.fm.2008.06.008.

Santos, D., Maurício, A. C., Sencadas, V., Santos, J. D., Fernandes, M. H. and Gomes, P. S. (2018). Spray drying: An overview. In Biomaterials - physics and chemistry (New Edition). *InTech*. https://doi.org/10.5772/intechopen.72247.

Siepmann, F. B., Ripari, V., Waszczynskyj, N. and Spier, M. R. (2018). Overview of sourdough technology: From production to marketing. *Food and Bioprocess Technology*, 11(2), 242–270. https://doi.org/10.1007/s11947-017-1968-2

Soukoulis, C., Yonekura, L., Gan, H. H., Behboudi-Jobbehdar, S., Parmenter, C. and Fisk, I. (2014). Probiotic edible films as a new strategy for developing functional bakery products: *The case of pan bread. Food Hydrocolloids*, 39, 231–242. http://doi.org/10.1016/j.foodhyd.2014.01.023.

Stefanello, R. F., Machado, A. A. R., Pasqualin Cavalheiro, C., Bartholomei Santos, M. L., Nabeshima, E. H., Copetti, M. V., et al. (2018). Trehalose as a cryoprotectant in freeze-dried wheat sourdough production. *LebensmittelWissenschaft und -Technologie- Food Science and Technology*, 89(May 2017), 510–517. https://doi.org/10.1016/j.lwt.2017.11.011

Stefanello, R. F., Nabeshima, E. H., Iamanaka, B. T., Ludwig, A., Fries, L. L. M., Bernardi, A. O., et al. (2019). Survival and stability of *Lactobacillus fermentum* and *Wickerhamomyces anomalus* strains upon lyophilisation with different cryoprotectant agents. *Food Research International*, 115(April 2018), 90–94. https://doi.org/10.1016/j.foodres.2018.07.044

Tafti, A. G., Peighambardoust, S. H., Behnam, F., Bahrami, A., Aghagholizadeh, R., Ghamari, M., et al. (2013). Effects of spray-dried sourdough on flour characteristics and rheological properties of dough. Czech Journal of Food Sciences, 31(4), 361–367. https://doi.org/10.17221/183/2012-CJFS.

Tafti, A. G., Peighambardoust, S. H., Hesari, J., Bahrami, A. and Bonab, E. S. (2013). Physico-chemical and functional properties of spray-dried sourdough in breadmaking. *Food Science and*

Technology International, 19(3), 271–278. https://doi. org/10.1177/1082013212452415

Tripathi, M. K. and Giri, S. K. (2014). Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of Functional Foods*, 9(1), 225–241. http://doi.org/10.1016/j.jff.2014.04.030.

Üçok, G., Sert, Durmuş.(2020), Growth kinetics and biomass characteristics of *Lactobacillus plantarum* L14 isolated from sourdough: Effect of fermentation time on dough machinability, *LWT - Food Science and Technology*, doi: https://doi.org/10.1016/j.lwt.2020.109516.

Vitaglione, P., Troise, A. D., De. Prisco, A. C., Mauriello, G. L., Gokmen, V., et al. (2015). Chapter 15: Use of microencapsulated ingredients in bakery Products: Technological and nutritional aspects. *Microencapsulation and microspheres for food applications*. Elsevier Inch http://doi.org/https://doi.org/10.1016/B978-0-12-800350-3.00020-0.

Yoon, K. Y., Woodams, E. E., & Hang, Y. D. (2006). Production of probiotic cabbage juice by lactic acid bacteria. *Bioresour Technol*, 97(12), 1427-1430. https://doi:10.1016/j.biortech.2005.06.018

Yu, Y., Wang, L., Qian, H., Zhang, H. and Qi, X. (2018). Contribution of spontaneously fermented sourdoughs with pear and navel orange for the breadmaking. *LWT-Food Science and Technology*, 89, 336– 343. https://doi.org/10.1016/j.lwt.2017.11.001.

Zago, M., Fornasari, M. E., Carminati, D., Burns, P., Suarez, V., Vinderola, G., Giraffa, G. (2011). Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. *Food Microbiol*, 28(5), 1033-1040. https://doi:10.1016/j.fm.2011.02.009

Zamfir M., Cornea C. P., De Vuyst L., Grosu T. S. S. (2014). Biodiversity and biotechnological potential of lactic acid bacteria. *AgroLife Scientific Journal*, Volume 3, Number 1, ISSN 2285-5718, 169-176.

Zhang, L., Huang, S., Ananingsih, V. K., Zhou, W. and Chen, X. D. (2014). A study on *Bifidobacterium lactis* Bb12 viability in bread during baking. *Journal of Food Engineering*, 122(1), 33-37. http://doi.org/10.1016/j.jfoodeng.2013.08.029

Zhang, L., Taal, M. A., Boom, R. M., Chen, X. D. and Schutyser, M. A. I. (2018). Effect of baking conditions and storage on the viability of *Lactobacillus plantarum* supplemented to bread. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology*, 87, 318–325. http://doi.org/10.1016/j.lwt.2017.09.005.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

BUCKWHEAT *VS*. SORGHUM FLOUR IN GLUTEN-FREE RICE COOKIES ENHANCED WITH PEA PROTEIN POWDER

Iulia-Elena SUSMAN[1, 2], Mihaela MULȚESCU[2], Gabriela STAMATIE[2], Alina CULEȚU[2], Mona Elena POPA[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd., District 1, Bucharest, Romania
[2]National Institute of Research and Development for Food Bioresources - IBA Bucharest, 6 Dinu Vintilă Street, District 2, Bucharest, Romania

Corresponding author email: iuliaelenasusman@yahoo.com

Abstract

Buckwheat (BF), sorghum (SF) and coconut (CF) flours as well as pea protein (PP) are considered alternative raw materials for gluten-free baked products. A cookie formulation based on 100% rice flour was control. Rice flour was substituted with different percentages of BF or SF (20%, 30% and 40%) and 20% PP and 10% CF were added to increase cookies nutritional values. This study showed how the addition of buckwheat and sorghum flour influenced the physico-chemical, texture and colour properties as well as the sensory attributes. Samples with 20% and 30% SF or BF had better scores than control, the highest acceptance scores were 6.44 for cookies with 20% SF and 6.11 for cookies with 20% BF. The colour measurement showed that the samples with the addition of SF were lighter than BF. Also, the samples with BF had similar colour to the control. Moreover, by adding different raw materials to rice flour, the level of protein and fiber increased.

Key words*: buckwheat, gluten-free cookies, pea protein powder, sorghum.*

INTRODUCTION

Nowadays food allergies are growing (Sicherer, 2011). According to Sicherer & Sampson (2010) about 5% of young children and 3- 4% of adults have food allergies. The main allergens are milk and eggs (about 2.5% of young children and 0.3% of adults are allergic to milk and 1.5% of young children and 0.2% of adults are allergic to eggs) followed by wheat and soy. The protein from wheat, gluten affects people which are suffering from celiac disease. This disease is a common autoimmune systemic disorder which affects approximately 1% of the global population.

Considering the fact that the population is growing and at the same time the number of people suffering from celiac disease is increasing, the gluten-free products are a growing demand, thus, the baking industry should expand and diversify this type of products (Rosell & Garzon, 2015). However, the development of gluten-free products remains a technological, sensory and nutritional challenge (da Silva & Conti-Silva, 2016). There are a lot of challenges that make gluten-containing products hard to replace. Gluten is the protein from wheat that plays a key role in dough developing determining the unique baking quality. Gluten provides water absorption ability, cohesiveness, viscosity and elasticity to wheat dough. The lack of gluten leads to the lack of dough cohesiveness, elasticity and baking quality. This fact negatively influences the way of dough handling (Bendera & Schonlechnera, 2019). Moreover, celiac people have various nutrient deficiencies such as significantly lower weight, body mass index, fat and lean body mass than control subjects therefore gluten-free diet should meet several requirements (Hallert et al., 2002). For a proper diet of people who suffer from celiac disease gluten-free products should be a source of nutrients such as: fiber, protein, vitamins and minerals (calcium, iron and zinc) (Martínez-Villaluenga et al., 2020).

Cookies represent a product range with a wide variety of diversity as texture, formulation, taste, flavour, colour, influenced by the addition of different ingredients. Therefore, we can find sweet and salty cookies, with a harder or softer texture, with or without cream or with various additions of fruit, seeds or powders or with

different colours. The global market for cookies was valued at USD 30.6 billion in 2018 and tends to grow with 5.3% each year (Grand View Research, Cookies Market Size, Share & Trends Analysis Report, 2019).

Rice and corn flour are one of the most used ingredients in the world for gluten-free cookies (GFC) development. However, for diversification and for nutritionally enhancement, a long list of ingredients such as pseudo-cereals, seeds, legumes and nuts (e.g. amaranth, quinoa, millet, sorghum, flax and chickpeas) could be integrated (Kupper, 2005).

Sorghum is a gluten-free cereal with a high level of phenolic compounds (phenolic acids, flavonoids and condensed tannins) and high antioxidant capacity which helps in chronic diseases prevention such as cardiovascular disease, obesity, non-fatty liver disease, 2 diabetes mellitus and cancer (Arbex et al., 2018; Lopes et al., 2018)

Buckwheat is a pseudo-cereal, also without gluten, rich in fiber, essential amino acids, vitamins, minerals and polyphenols. Moreover, buckwheat is one of the most researched pseudo-cereals for GF cookies formulation. During thermal treatments, buckwheat flour can maintain its antioxidant capacity (Sakac et al., 2011). The level of starch in buckwheat is similar to many cereal grains, also buckwheat is known for its high levels resistant starch (Xu et al., 2020; Zhang et al., 2020; Skrabanja et al., 2001). Therefore, this study aimed to establish differences between gluten-free cookies with buckwheat addition and cookies with sorghum addition.

MATERIALS AND METHODS

Rice flour was obtained from the National Institute of Research and Development for Food Bioresources - IBA Bucharest. Pea protein powder, coconut flour and sorghum flour (SF) were bought from Paradisul Verde (Romania) while buckwheat flour (BF) was purchased from Eurokalis.

First of all, the control (C) was made of 100% rice flour, then another 6 samples were developed. Three of them were based on buckwheat flour where the rice flour was substituted with 40%, 30% and 20% BF (B40, B30, B20) and the others three based on SF (S40, S30, S20) instead of buckwheat flour. In addition, BF and SF formulations contained pea protein powder (60 g) and coconut flour (30 g). Cookies were developed as follows: control with rice flour 100%, B40, B30, B20 with rice flour (150, 120, 90 g) and buckwheat flour (60, 90, 120 g) and S40, S30, S20 with rice flour (150, 120, 90 g) and sorghum flour (60, 90, 120 g). The rest of the ingredients which never change in all the cookies formulation were: sugar (40 g) coconut sugar flower (40 g), coconut milk (100 g), butter (100 g), egg (≈ 45 g), lemon juice (20 g), baking powder (3 g) and salt (2 g). Figure 1 shows the process of the gluten-free cookies manufacturing.

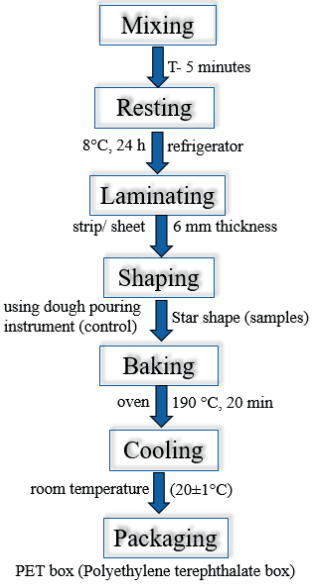


Figure 1. The process of the gluten-free cookies manufacturing

Chemical composition of gluten-free cookies

Chemical composition of gluten-free cookies was determined according to the Association Official of Analytical Chemists (AOAC, 2005) methods for ash (furnace Nabertherm, Lilienthal, Germany), fat (fat extraction system SOXTEC 2055, FOSS, Hillerød, Denmark), protein (Kjeldahl block digestion unit, Behr, Düsseldorf, Germany) and total dietary fibre (Fibertec system, FOSS, Hillerød, Denmark) content. Also, the carbohydrate values were calculated according to Regulation (EU) no. 1169/2011 of the European Parliament and of

the Council of 25 October 2011 using the following equation: 100 - (moisture + ash + proteins + lipids + fibres). These analysis were performed to calculate the energy value which was calculated using the following conversion factors: 9 for fat, 4 for carbohydrates, 4 for protein and 2 for fibre.

Moisture content

Moisture content was determined using Moisture analyzer METTLER TOLEDO, model HE73 at 130°C, sprinkling 5 grams of sample on the entire surface of the tray without pressing.

Colour measurement

Colour was performed using CM-5 Konica Minolta colorimeter on GF cookies sample. determining three parameters: parameter L* which measures the brightness of the sample on a scale from 0 to 100, where value 0 represents black and value 100 represents white; parameter a* which represents the colour of the sample on the scale from pure green to pure red, where the negative values are green, the positive values are red and 0 is neutral and parameter b* which represents the position of the sample on a scale from pure blue to pure yellow, where the negative values are blue, the positive values are yellow and 0 is neutral. Each value was an average of 10 measurements made on different points of the sample.

Texture measurement

Texture analysis was done using an Instron Texture Analyzer (5944, Illinois Tool Works Inc., SUA). The method of analysis included a cycle of compressions in the middle of each cookie up to a distance of 50% from the height of the cookie. The experimental conditions were: compression speed: 3 mm/min; load cell: 50 N. The texture parameter firmness (or hardness) was defined as the maximum force (expressed in N) which a cookie can bear before breaking. This parameter was calculated using the Bluehill 3.13 program.

Consumer acceptance

Consumer acceptance of the 7 types of cookies was performed by 18 people (12 females and 6 males from National Institute of Research and Development for Food Bioresources - IBA Bucharest using a 9-point hedonic scale. Scores were given based on the scale from 1 “I dislike it extremely” to 9 “I like it extremely”. Between each sample water was provided to people for mouth cleaning. 20-60 years old was the age group of the sensory panelists who performed sensory analysis of gluten-free cookies.

RESULTS AND DISCUSSIONS

Chemical composition of gluten-free cookies

Analyzing the protein content of each sample, the samples with pea protein as well as BF and SF addition, had a remarkable increase of the protein content up to 3.3 times more compared to the control (Table 1).

Moreover, differences of protein content can be seen between the samples with the buckwheat addition (B40, B30 and B20), which had a higher protein content when higher BF addition percentages were used.

Table 1. Chemical composition (%) of gluten- free cookies

	P	**F**	**Ch** of which sugar		**CF**	**A**	**M**	**EV**
C	3.73	13.99	49.38	12.35	1.1	0.80	12.16	350/1429
B40	12.51	15.20	38.11	13.53	3.21	1.76	10.83	355/1449
B30	12.20	15.17	38.79	13.62	3.03	1.70	9.22	356/1452
B20	11.95	15.07	39.46	13.40	2.84	1.65	11.29	357/1454
S40	11.89	15.05	40.25	13,57	2.21	1.43	15.76	358/1461
S30	11.77	15.04	40.38	13.39	2.28	1.37	16.3	358/1461
S20	11.66	14.99	40.80	13.36	2.35	1.36	13.93	359/1465

***S** - name of the sample; **P** - protein content; **F** - fat content; **Ch** - Carbohydrates of which sugar; **CF** - Crude fiber; **A** - Ash; **M** - cookie moisture. All of them are expressed as percentage (%) ***EV** - Energy Value expressed as kcal/kJ

The same trend was noticed for the sorghum cookies. The fat content increased from ≈ 14% for the control cookie to ≈ 15% for the enriched samples. Control had lower values of fat content because it was made from 100% rice, the fat content in the other formulations was influenced by the addition of coconut flour. Coconut dishes are rich in fats, proteins and some vitamins, they counterbalance some of the deficiencies (Palaniappan & Subramaniam, 2010). Coconut flour also has a high fiber content. The fiber content was higher in buckwheat and sorghum cookies compared to control. Moreover, the buckwheat cookies had a higher content of 3.21% compared to 2.35% for sorghum cookies due to the fact that buckwheat is richer in fiber than sorghum. Alvarez-Jubete, 2009 analyzed buckwheat seeds and showed the following results 12.5 ± 0.3% protein, 2.1 ± 0.1% fat, and 29.5 ± 1.2% dietary fiber.

Moisture content

The moisture content varies between samples. The lowest value was recorded for B30- 9.22% and the highest for S30- 16.3%. The other values were: 10.83% for B40, 11.29% for B20, 12.16% for C, 13.93% for S20, 15.76% for S40. The different values for the moisture content may be because of the cookies position in the oven.

Colour measurement

The colour analysis results showed similar values between the control and buckwheat samples. On the other side, the gluten-free cookies with the addition of sorghum showed a lighter colour than those with the addition of buckwheat. Although the percentages of buckwheat and sorghum varied, there were no significant changes in the colour parameters (Table 2). Some differences can be seen but there is no tendency. These differences may be due to the cookies position in the oven.

Table 2. Colour of gluten-free cookies

Sample	L*(D65)	a*(D65)	b*(D65)
C	72.82±0.12	7.06±0.06	25.07±0.03
B40	72.21±0.02	7.16±0.02	23.61±0.03
B30	69.18±0.05	9.19±0.03	24.96±0.04
B20	71.90±0.05	7.69±0.01	24.67±0.05
S40	74.49±0.07	5.95±0.02	22.53±0.06
S30	74.02±0.07	6.15±0.02	23.67±0.06
S20	74.29±0.03	6.39±0.02	23.46±0.05

Texture measurement

For consumers besides taste and appearance, the product texture is an important factor when they choose to buy a product.

The textural analysis showed that the values of sample hardness are similar (Table 3). The softest cookies were S40 and B20 with the addition of 40% sorghum and 20% buckwheat, respectively. The hardest sample is the one with 30% buckwheat (B30) and 20% sorghum (S20). It can be seen that the cookie hardness increased with the decrease of the percentage addition of sorghum. According to Naseer et al. (2021) who developed gluten-free cookies with 100% rice flour hardness registered values from 31.00 to 48.90 N. Hadnađev et al. (2013) claimed that cookie dough based on rice had lower water content, was strong and elastic which led to a harder cookie (36 N).

Table 3. Hardness of developed gluten-free cookies

Sample	Hardness (N)
C	12.30±1.24
B40	13.54± 3.18
B30	15.19±2.55
B20	10.94±2.57
S40	10.73±1.71
S30	12.83±0.86
S20	15.05±1.45

Consumer acceptance

The consumer acceptance test was conducted using eighteen untrained panellists. The most popular cookies were S20 (20% sorghum) followed by B20 (20% buckwheat) and B30 (30% buckwheat). The most disliked samples were those with 40% buckwheat and 40% sorghum, respectively (Table 4).

However, looking on the average values, almost all the sample obtained were higher than 5, so these were considered acceptable according to Lazaridou et al. (2007). Only one sample recorded a score below 5.

Table 4. Hedonic scale by consumer acceptance

Sample	Average score
C	5.67±2.28
B40	4.39±2.09
B30	6.00±1.41
B20	6.11±1.57
S40	5.28±2.08
S30	5.56±1.89
S20	6.44±1.25

CONCLUSIONS

The addition of pea protein powder had a major impact on increasing the protein content of cookies. In addition, it was observed that buckwheat GFC were richer in protein content than those with sorghum.

Comparing the fiber content of the 2 types of GFC, it was observed that by adding buckwheat flour in cookies formulation, higher values for the fiber content were obtained in buckwheat-based cookies than in those with sorghum flour.

The formulation of gluten-free cookies with addition of sorghum showed a lighter colour than those with the addition of buckwheat.

As expected, based on the ingredients used in the cookies formulations, the energy values for buckwheat and sorghum cookies was higher than the control.

ACKNOWLEDGEMENTS

This research was achieved through Core Program, with the support of the Ministry of Research, Innovation and Digitization (MCID), contract 22N/2019, project PN 19 02 02 02 and USAMVB PhD fellowship programme.

REFERENCES

Alvarez-Jubete, L., Arendt, E.K. Gallagher E. (2009). Nutritive value and chemical composition of pseudocereals as gluten-free ingredients. *International Journal of Food Science and Nutrition*, 60(1), 240-257.

Arbex, P. M., de Castro Moreira, M. E., Toledo, R. C. L., de Morais Cardoso, Pinheiro-Sant'ana, H. M., dos Anjos Benjamin, L., Martino, H. S. D., Licursi, L., Carvalho, C.W.P., Queiroz, V.A.V., & Martino, H.S.D. (2018). Extruded sorghum flour (Sorghum bicolor L.) modulate adiposity and inflammation in high fat diet-induced obese rats. *Journal of Functional Foods*, *42*, 346–355. https://doi.org/10.1016/j.jff.2018.01.010.

Barak, S., Mudgil, D., & Khatkar, B.S. (2015). Biochemical and functional properties of wheat gliadins: a review. *Critical Reviews in Food Science and Nutrition* 55, 357-368, https://doi.org/10.1080/10408398.2012.654863.

Bendera, D., & Schonlechnera, R. (2019). Innovative approaches towards improved gluten-free bread properties. *Journal of Cereal Science*, DOI: 10.1016/j.jcs.2019.102904.

Cookies Market Size, Share & Trends Analysis Report By Product (Bar, Molded, Rolled, Drop), By Distribution Channel (Offline, Online), By Region (North America, APAC, MEA, Europe, CSA), And Segment Forecasts, 2019-2025 (2019).

da Silva, T.F., Conti-Silva, A.C. (2016). Preference mappings for gluten-free chocolate cookies: Sensory and physical characteristics. *Nutrition & Food Science*, 46(3):374-387 DOI: 10.1108/NFS-11-2015-0139.

Hadnađev, T. R. D., Torbica, A. M., & Hadnađev, M. S. (2013). Influence of buckwheat flour and carboxymethyl cellulose on rheological behaviour and baking performance of gluten-free cookie dough. *Food and Bioprocess Technology*, 6(7), 1770–1781.

Hallert, C., Grant, C., Grehn, S., Granno, C., Hulten, S., & Midhagen, G. (2002). Evidence of poor vitamin status in coeliac patients on a gluten-free diet for 10 years. *Alimentary Pharmacology & Therapeutics*, 16, 1333–1339.

Kupper, C. (2005). Dietary guidelines and implementation for celiac disease. *Gastroenterology*, 128, 121–127.

Lazaridou, A., Duta, D., Papageorgiou, M., Belc, N., & Biliaderis, C.G. (2007). Effects of hydrocolloids on dough rheology and bread quality parameters in gluten-free formulations. *Journal of Food Engineering*, Volume 79, Issue 3, April, Pages 1033–1047.

Lopes, R. de C. S. O., de Lima, S. L. S., da Silva, B. P., Toledo, R. C. L., Moreira, M. E. de C., Anunciação, P. C., & Martino, H. S. D. (2018). Evaluation of the health benefits of consumption of extruded tannin sorghum with unfermented probiotic milk in individuals with chronic kidney disease. *Food Research International*, 107, 629–638. https://doi.org/10.1016/j.foodres. 2018.03.004.

Martínez-Villaluenga, C., Peñas, E., & Hernández-Ledesma, B. (2020). Pseudocereal grains: Nutritional value, health benefits and current applications for the development of gluten-free foods. *Food and Chemical Toxicology*, 137, 111–178.

Naseer, B., Naik, H. R., Hussain, S. Z., Beenish, Z. I., Bhat, T. A., & Nazir, N. (2021). Effect of carboxymethyl cellulose and baking conditions on in-vitro starch digestibility and physico-textural characteristics of low glycemic index gluten-free rice cookies. *Food Science and Technology*, 141 110885.

Palaniappan, G., & Subramaniam, S. (2010). The coconut revival. market survey, lecturers in commerce at Anbu Arts and Science College, Komarapalayam, Namakkal District, Tamilnadu.

Rosell, C. M. & Garzon, R. (2015). Chemical composition of bakery products. *Handbook of Food Chemistry*, 191–224.

Sakac, M., Torbica, A., Sedej, I., & Hadnadev, M. (2011). Influence of breadmaking on antioxidant capacity of gluten free breads based on rice and buckwheat flours. *Food Research International*, 44(9), 2806–2813.

Sakač, M., Pestorić, M., Mišan, A., Nedeljković, N.,

Jambrec, D., Jovanov, P., Banjac, V., Torbica, A., Hadnadev, M., & Mandic, A. (2015). Antioxidant capacity, mineral content and sensory properties of gluten-free rice and buckwheat cookies. *Food Technology and Biotechnology,* 53(1), 38–47.

Sicherer, S. H. (2011). Food allergy. The Mount Sinai. *Journal of Medicine*, 78(5), 683–696.

Sicherer, S. H., & Sampson, H. A. (2010). Food allergy. *The Journal of Allergy and Clinical Immunology*, 125 (Supplement 2), S116–S125 (2).

Skrabanja, V., Liljeberg, E.H.G.M., Kreft, I., & Bjorck, I.M.E. (2001). Nutritional properties of starch in buckwheat products: studies *in vitro* and *in vivo*. *J. Agric. Food Chemistry*. 49, 490–496.

Xu, J., Zhang, Y., Wang, W., & Li, Y., Advanced Properties of Gluten-Free Cookies, Cakes, 2 and Crackers: A Review *Trends in Food Science & Technology*, https://doi.org/10.1016/j.tifs.2020.07.017.

Zhang, Z.-L., Zhou, M.-L., Tang, Y., Li, F.-L., Tang, Y.-X., Shao, J.-R., Xue, W.-T., & Wu, Y.-M. (2012). Bioactive compounds in functional buckwheat food. *Food Research International* 49(1), 389–395.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

STABILIZATION OF SEA BUCKTHORN (*Elaeagnus rhamnoides*) TURBID JUICES

Mădălina GIURESCU[1, 2], Luminița DIMITRIU[3, 1], Florin OANCEA[3, 1*]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd., District 1, Bucharest, Romania
[2]BioCătina, 88 Calea Mosilor, sc. B, et.2, ap.28, District 3, Bucharest, Romania
[3]National Institute for Research and Development in Chemistry and Petrochemistry - ICECHIM Bucharest, 202 Spl. Independentei, District 6, Bucharest, Romania

*Corresponding author email: florin.oancea@icechim.ro

Abstract

This paper reviews the technological solutions used to stabilize the sea buckthorn (Elaeagnus rhamnoides, syn. Hippophae rhamnoides) juice. Sea buckthorn is a nutraceutical crop of growing interest due to the health benefits of its berries active ingredients - essential (unsaturated) fatty acids, vitamins, antioxidants, and minerals. The turbid juice produced from the whole berries without enzymatic clearance has a higher biological value due to the extraction of the main active ingredients. However, the turbid juice made from sea buckthorn berries has poor physical and chemical stability. The lipids from berry pulp, rich in monounsaturated palmitoleic acid (16: 1n-7), separate from juices and are prone to rapid oxidation. Syneresis also occurs due to the low water-holding ability of the pulp polysaccharides. Oxidation reduces the content of the antioxidant (pro) vitamins. Stabilization is done by physical homogenization and/or utilization of additives with emulsification and/or antioxidant characteristics. The paper analyzes and discusses the advantages and drawbacks of the existing solutions.

Key words*: sea buckthorn, turbid juice, syneresis, oxidation, stabilization, homogenization, additives.*

INTRODUCTION

Sea buckthorn, *Elaeagnus* (synonym *Hippophae*) *rhamnoides* (L.) A. Nelson is a thorny deciduous shrub recently domesticated (Kalia et al., 2011). Long-period sea buckthorn berries were harvested from nature (Yang et al., 2009). Domestication and establishment of orchards with this robust shrub tree were driven by its ecologic (Constandache et al., 2016) and economic importance (Gatlan & Gutt, 2021).

The ecologic importance of sea buckthorn results from its ability to colonize and stabilize marginal lands, including those from cold and dry areas or reclaimed spoil mines (Baig et al., 2021; Li & Schroeder, 1996; Zhao et al., 2013). The ability of sea buckthorn to grow well in poor soil is directly related to this robust shrub being both an actinorhizal plant and an endomycorrhizal plant (Zhou et al., 2017). As an actinorhizal plant, sea buckthorn has the ability to form symbiotic N2-fixing nodules with actinomycetes from *Frankia* genera (Diagne et al., 2013). The formation of symbiosis with mycorrhizae fungi supports phosphorus acquisition from immobile soil sources and the development of N2-fixing nodules and other beneficial rhizobacteria (Yang et al., 2018).

The economic importance of the sea buckthorn results from the high content of its fruits and leaves in bioactive ingredients - (pro)vitamins, essential (poly)unsaturated fatty acids, and antioxidant phytonutrients (Ren et al., 2020).

Due to its high content in healthy related compounds, sea buckthorn has been utilized for millennia as a nutraceutical/medicinal plant in Europe, China, and India (Suryakumar & Gupta, 2011; Wani et al., 2016).

Sea buckthorn leaves are occasionally used to prepare healthy infusions. The sea buckthorn fruits (berries) are the main edible parts used for nutraceutical purposes. Because of their acidic taste and short shelf life, sea buckthorn berries are not consumed in a fresh state (Alexandrakis et al., 2014). Usually the berries are converted into turbid juice prepared from the flashy pulp/pericarp and in a seed oil preparation, obtained by the mechanical extraction of the edible oil from the seeds (Beveridge et al., 1999). The pomace is further used as a source

for tocopherols and carotenoids, extracted with supercritical CO_2 (Kitrytė et al., 2017; Mihalcea et al., 2021), for the production of soluble dietary fiber (Hussain et al., 2021) or for recovery of natural lipophilic antioxidants (Patra et al., 2022).

Turbid juice needs stabilization because of its heterogeneous composition, including suspended microparticles and hydrophobic and hydrophilic soluble fractions. The turbid juice separated during storage in three phases, with the oily one on the upper surface and sedimented particles on the bottom (Beveridge et al., 1999). This work reviews the technological solutions used to stabilize the sea buckthorn turbid juice. Initially, we detail the technical problems associated with the composition of the turbid juice and its peculiar rheology. Further, we describe the physicomechanical processes used for turbid juice stabilization. We address the innovative solutions at the end of the paper, including those described in the published patents.

Active ingredients in the sea buckthorn turbid juice

The diverse agro-pedo-climatic conditions promote the accumulation of different bioactive compounds in sea buckthorn berries and leaves. Romanian cultivars, differentiated in a separate subspecies, *E*. (*H*.) *rhamnoides* subsp. *carpatica* accumulated in the berries five carotenoids, β-carotene, cis-β-carotene, β-cryptoxanthin, lutein, and zeaxanthin. The antioxidant activity of extract from these Romanian cultivars correlated well (r = 0.96) with the total flavonoids. The antibacterial activity against *Bacillus cereus* and *Pseudomonas aeruginosa* was higher in the sea buckthorn leaves (Criste et al., 2020).

The pulp oils from sea buckthorn cultivated in the Indian Himalayas had a high content of unsaturated fatty acid (65-75%), palmitoleic acid (16:1n-7), being the most abundant (32-53%) (Ranjith et al., 2006). The cultivars from *E*. (*H*.) *rhamnoides* subsp. *mongolica* that has been grown in Poland (51.59° N, 20.139° E) accumulated more monounsaturated fatty (palmitoleic, 16:1n-7; cis-vaccenic, C18:1 n-7; oleic C18:1 n-9) compared to polyunsaturated fatty acids (linoleic C18:2 n-6) (Teleszko et al., 2015). The berries from *E*. (*H*.) *rhamnoides* cultivated in Turkey accumulated a higher proportion of palmitoleic acid (47.3%) in the pulp (Cakir, 2004).

Despite variations due to subspecies - cultivars and the agro-pedoclimatic conditions, the presence in the pulp of a large amount of palmitoleic acid, in combination with different types of antioxidants, hydrophobic (carotenoids, flavonoids), and hydrophilic (hydroxycinnamic acids, ascorbic acid) is reported in all published studies - Table 1.

Table 1. Composition of the sea buckthorn fruit pulp

Active ingredients	Content ($mg.g^{-1}$ fresh weight)	Reference
Palmitoleic acid (16:1n-7)	2.54-15.92	(Yang & Kallio, 2001)
	4.27-16.7	(H. Kallio et al., 2002)
	2.64	(Cakir, 2004)
	4.95-23.76	(Dulf, 2012)
Total carotenoids	0.01 -0.18	(Andersson et al., 2009)
	0.02 -0.40	(Bal et al., 2011)
	0.17	(Pop et al., 2015)
Tocopherols	0.03-0.21	(Jiménez-Escrig & Sánchez-Muniz, 2000)
	0.06-0.14	(Heikki Kallio et al., 2002)
Flavonoids	3.54-10	(Bal et al., 2011)
	1.68-8.59	(Heinaaho & Julkunen-Tiitto, 2011)
Ascorbic acid	1.4-30	(Ranjith et al., 2006)
	0.25-25	(Bal et al., 2011)
Hydroxycinnamic acid	0.151-0.402	(Hajazimi et al., 2016)
Caffeic acid	0.03.6-0.087	
Ferulic acid	0.015-0.15	
p-Coumaric acid	0.10-0.265	

From 65 to 85% of the sea buckthorn berry flesh pulp is converted into turbid juice by (mechanical) squeezing the fruits. The turbid juice proved to have significant health benefits due to its high content in lipophilic and hydrophilic antioxidants and ω-7 palmitoleic acid (Ciesarová et al., 2020). For decades, sea buckthorn has been recognized for its effect on cardiovascular diseases and associated metabolic disorders (Olas, 2016). The immunomodulatory and anti-inflammatory effects were confirmed by several studies (Ren et al., 2020). Also, *in vitro* studies on transformed cells and *in vivo* studies on laboratory animals confirmed the anticancer activity of sea buckthorn (Olas et al., 2018).

Palmitoleic acid reduces the risk of type II diabetes by increasing insulin sensitivity (Hu et al., 2019). Serum palmitoleate acts as a lipokinin (Merino et al., 2016) and prevents harmful effects of metabolic syndrome (Talbot et al., 2014; Tricò et al., 2020). Sea buckthorn pulp oil enhanced the immunity of immunosuppressed

mice due to a significant prebiotic effect on probiotic bacteria producing post-biotics, i.e., short-chain fatty acids (Zhang et al., 2021).
The carotenoids present in high quantity in turbid sea buckthorn juice synergize the palmitoleic effect on type II diabetes and metabolic syndrome. Sea buckthorn is an accessible source of carotenoids, especially zeaxanthin (Tudor et al., 2020). Carotenoids levels in serum were inversely correlated with type II diabetes (Marcelino et al., 2020) and metabolic syndrome (Beydoun et al., 2018; Matsumoto et al., 2021). This inverse correlation is higher for zeaxanthin (Sugiura et al., 2015). The hydrophilic antioxidants present in the sea buckthorn turbid juice are also active against type II diabetes (J.-S. Zheng et al., 2020) and metabolic syndrome (Liu et al., 2019). The anticancer activity of the sea buckthorn extracts was directly correlated with the level of ursolic acids (Grey et al., 2010), flavonol glycosides (Enkhtaivan et al., 2017) and flavonoid aglycones, including quercetin, isorhamnetin and kaempferol (Guo et al., 2017).
Recently, the low polarity fraction from sea buckhorn juice was reported to have low cytotoxicity for the normal cell and induce DNA damage apoptosis in cancer cells (Marciniak et al., 2021). This low polarity fraction from sea buckthorn juice contains triterpenoids, acylated triterpenoids, phospholipids and their derivatives, and saponins (Marciniak et al., 2021).
The lipophilic and hydrophilic antioxidants from sea buckthorn were reported to affect amyloid-beta (Aβ) levels directly. Therefore sea buckthorn could be efficient in Alzheimer's disease (Dong et al., 2020). The neuroprotective effect of sea buckthorn was also demonstrated against oxidative stress damage on neural tissue (Shivapriya et al., 2015), including the oxidative stress resulting from reperfusion following ischemia (Godeanu et al., 2020) and for iron-induced epilepsy (Ladol & Sharma, 2021).

Instability factors of the sea buckthorn turbid juice

This unique combination of active ingredients with high biological value also represents the source of an intrinsic instability - Figure 1.
The sea buckthorn turbid juice has a low thermal stability. This thermal instability is related to the instability of several components with high biological value, such as flavonoids, terpenoids, and carotenoids, and the dehydration reaction of soluble sugars in the presence of high-level ascorbic acids (Li et al., 2014). Polyphenols/ hydroxycinnamic acids from the sea buckthorn juice tend to be more thermostable than carotenoids, flavonoids, and antioxidants (Ursache et al., 2017).

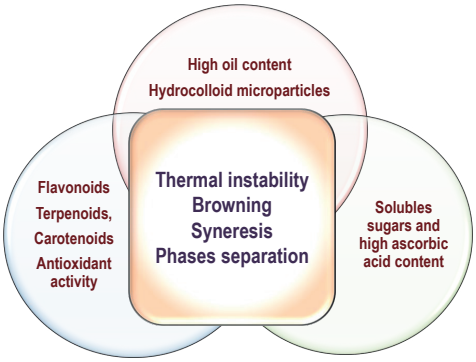


Figure 1. The instability factors of sea buckthorn turbid juice related to its specific composition

The formation of the 5-hydroxymethylfurfural (5-HMF) is associated with a non-enzymatic browning and a decrease in the level of ascorbic acid (Xu et al., 2015). HMF formation by dehydration of the soluble carbohydrates starts at a temperature lower than 60°C (Constantin et al., 2019). In sea buckthorn juice, HMF does not contribute to forming the Maillard type aroma/taste compounds and is considered a contaminant (Constantin et al., 2019; Rannou et al., 2016). The maximum allowed level of HMF in juice is 5 $mg.L^{-1}$ (Damasceno et al., 2008).
The formation of HMF could be associated with forming a more harmful contaminant, acrylamide (Constantin et al., 2019). However, the temperature promoting acrylamide formation is higher than 130°C (Constantin et al., 2019). The high oil content of the turbid juice, combined with the low water-holding ability of hydrocolloid microparticles, determine syneresis and phase separation (Beveridge et al., 1999).

Sea buckthorn turbid juice stabilization treatment

Several different mechanical treatments were proposed to stabilize the sea buckthorn turbid juice. The majority of these are inspired by the stabilization of the milk, a food system that is

similar in terms of the presence of hydrophilic and lipophilic phases. Such treatments target the formation of a more stable suspension by generating smaller lipid droplets and reducing the particle dimension. However, sea buckthorn turbid juice is a much more complex system. It is not a simple emulsion; it is a suspoemulsion that includes both hydrophobic fractions and suspended particles. Each of the proposed treatments has some drawbacks.

Homogenization at 100 MPa in a piston homogenizer Panda PLUS 2000 (GEA Niro Soavi S.p.A., Parma, Italy) stabilizes the emulsion. Still, it generates a lighter and yellowish color that influences customers' acceptance (Aaby et al., 2020).

Better stabilization was obtained by using dynamic high-pressure microfluidization (DHPM) by using a Microfluidizer® (model M-110EH, Microfluidics Inc., Newton, MA, USA) (Abliz et al., 2021). The difference between a piston homogenizer and a microfluidization equipment is related to the dimensions of the space wherein the high shearing force occurs. The space is limited to a homogenizing valve that separates the high-pressure chamber from the atmospheric pressure in a piston homogenizer. In a Microfluidizer® processor, the space is a whole interaction microfluidics chamber, with walls treated with high hardness materials (i.e., zirconia or diamond). Microfluidizer® is expensive equipment with low capacity that is not yet suitable for the food industry (Guo et al., 2020). Pretreatment is necessary to avoid clogging

Various emulsifiers were combined with a piston homogenizer to enhance the stabilization of the sea buckthorn turbid juice suspoemulsion. The most effective stabilization agents were sodium caseinate- and sugar esters. However, the best stabilization agents also influenced the antioxidant activity (H. X. Zheng et al., 2020). This decrease in the antioxidant activity is because the carotenoids from sea buckthorn juice bind to the proteins (Aprodu et al., 2017; Dumitraşcu et al., 2016).

Table 2 summarizes the homogenization treatments (including the combined homogenization and emulsifiers) intended to stabilize the turbid sea buckthorn juice. The effects and drawbacks are presented.

Table 2. Homogenization treatment proposed for the stabilization of turbid sea buckthorn juice

Treatment	Effect	Drawbacks	Reference
Homogenization in a piston homogenizer, 100 MPa	The reduction of the oily droplets and the polysaccharides particles increased the stability of the suspoemulsion	Lighter and yellowish color – consumer perception	(Aaby et al., 2020)
Microfludization, up to 250 MPa, through a Z-shaped interaction chamber	Increased stability of the suspoemulsion, carotenoids released from droplets, higher antioxidant activity	Expensive equipment. technology is not yet ready for the food industry	(Abliz et al., 2021)
Emulsified agent + homogenization in a piston homogenizer	Increased stability of the suspoemulsions	Decreased the antioxidant activity due to conjugation	(H. X. Zheng et al., 2020)

Innovative approaches were proposed for the stabilization of the sea buckthorn turbid juice. The purified additives were replaced with different plant extracts – for example, the infusion from *Hibiscus* fruits (Heilscher, 1995). Other innovative approaches involved stabilization with extracts from sea buckthorn by-products - Figure 2.

Sea buckthorn berries juicing produce two types of by-products, leaves, and pomace. The fruits are very tightly bunched to the brunches, and the harvesting techniques generate significant amounts of leaves (Fu et al., 2014). The leaves have a higher antioxidant content than fruits (Olas, 2016).

The pomace is rich in pectin and antioxidants (Patra et al., 2022). Several patents claim utilization of the extracts from sea buckthorn by-products to stabilize the juice (Shi et al., 2019; Tian et al., 2018).

There are two main drawbacks of these solutions based on plant extracts. One is related to the

taste - the plant extract enhances the astringency and the bitterness of the turbid juice. The other one is related to the low reproducibility of the stabilization process. This low reproducibility results from combining two highly variable products - the turbid juice and the plant extract (including the extract from the by-products of the sea buckthorn processing).

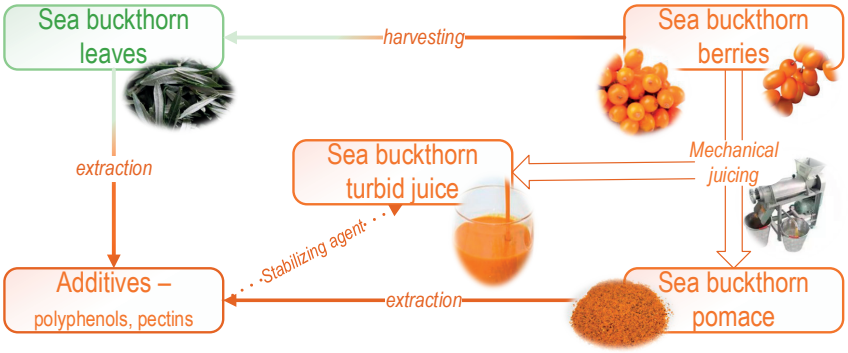


Figure 2. Illustration of the innovative approaches for stabilizing the sea buckthorn turbid juice by using extracts rich in polyphenols and/or pectin from sea buckthorn processing by-products

CONCLUSIONS

The sea buckthorn turbid juice is unstable and prone to browning and phase separation. Stabilization of the sea buckthorn turbid juice is done by physical homogenization and/or utilization of emulsifiers.

Innovative solutions involve the use as additives for stabilization of the extracts from the by-products of the sea buckthorn processing.

Complexity of the turbid juice suspoemulsion determine low reproducibility of the stabilization process.

Because of the drawbacks of the existing solutions, new approaches are needed for sea buckthorn turbid juice stabilization.

ACKNOWLEDGEMENTS

The research leading to these results has received funding from European Regional Development Fund (ERDF), the Competitiveness Operational Programme (POC), Axis 1, project POC-A1-A1.2.3-G-2015-P_40_352, My_SMIS 105684, "Sequential processes of closing the side streams from bioeconomy and innovative (bio)products resulting from it - SECVENT", subsidiary project 2609/2020 - Nutri-GUT.

REFERENCES

Aaby, K., Martinsen, B. K., Borge, G. I. A., & Røen, D. (2020). Bioactive compounds and color of sea buckthorn (*Hippophae rhamnoides* L.) purees as affected by heat treatment and high-pressure homogenization. *International Journal of Food Properties, 23*(1), 651-664.

Abliz, A., Liu, J. F., Mao, L. K., Yuan, F., & Gao, Y. X. (2021). Effect of dynamic high pressure microfluidization treatment on physical stability, microstructure and carotenoids release of sea buckthorn juice. *Lwt-Food Science and Technology, 135*.

Alexandrakis, Z., Kyriakopoulou, K., Katsaros, G., Krokida, M., & Taoukis, P. (2014). Selection of Process Conditions for High Pressure Pasteurization of Sea Buckthorn Juice Retaining High Antioxidant Activity. *Food and Bioprocess Technology, 7*(11), 3226-3234.

Andersson, S. C., Olsson, M. E., Johansson, E., & Rumpunen, K. (2009). Carotenoids in sea buckthorn (*Hippophae rhamnoides* L.) berries during ripening and use of pheophytin a as a maturity marker. *Journal of Agricultural and Food Chemistry, 57*(1), 250-258.

Aprodu, I., Ursache, F.-M., Turturică, M., Râpeanu, G., & Stănciuc, N. (2017). Thermal stability of the complex formed between carotenoids from sea buckthorn (*Hippophae rhamnoides* L.) and bovine β-lactoglobulin. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 173*, 562-571.

Baig, S. M., Khan, A. A., Ali, A., Khan, M. Z., Ahmed, S., Shah, G. M., & Ali, G. (2021). Enhancing socioeconomic resilience and climate adaptation through value chain development of mountain products in Hindu Kush Himalayas. *Environment, Development and Sustainability, 23*(6), 8451-8473.

Bal, L. M., Meda, V., Naik, S. N., & Satya, S. (2011). Sea buckthorn berries: A potential source of valuable nutrients for nutraceuticals and cosmoceuticals. *Food Research International, 44*(7), 1718-1727.

Beveridge, T., Li, T. S. C., Oomah, B. D., & Smith, A. (1999). Sea Buckthorn Products: Manufacture and Composition. *Journal of Agricultural and Food Chemistry, 47*(9), 3480-3488.

Beydoun, M. A., Chen, X., Jha, K., Beydoun, H. A., Zonderman, A. B., & Canas, J. A. (2018). Carotenoids, vitamin A, and their association with the metabolic syndrome: a systematic review and meta-analysis. *Nutrition Reviews, 77*(1), 32-45.

Cakir, A. (2004). Essential oil and fatty acid composition of the fruits of *Hippophae rhamnoides* L. (Sea Buckthorn) and Myrtus communis L. from Turkey. *Biochemical Systematics and Ecology, 32*(9), 809-816.

Ciesarová, Z., Murkovic, M., Cejpek, K., Kreps, F., Tobolková, B., Koplík, R., Belajová, E., Kukurová, K., Daško, Ľ., Panovská, Z., Revenco, D., & Burčová, Z. (2020). Why is sea buckthorn (Hippophae rhamnoides L.) so exceptional? A review. *Food Research International, 133*, 109170.

Constandache, C., Peticila, A., Dinca, L., & Vasile, D. (2016). The usage of Sea Buckthorn (Hippophae Rhamnoides L.) for improving Romania's degraded lands. *AgroLife Scientific Journal, 5*(2), 50-58.

Constantin, O. E., Kukurová, K., Daško, Ľ., Stănciuc, N., Ciesarová, Z., Croitoru, C., & Râpeanu, G. (2019). Modelling Contaminant Formation during Thermal Processing of Sea Buckthorn Purée. *Molecules, 24*(8), 1571.

Criste, A., Urcan, A. C., Bunea, A., Pripon Furtuna, F. R., Olah, N. K., Madden, R. H., & Corcionivoschi, N. (2020). Phytochemical Composition and Biological Activity of Berries and Leaves from Four Romanian Sea Buckthorn (*Hippophae rhamnoides* L.) Varieties. *Molecules, 25*(5), 1170.

Damasceno, L. F., Fernandes, F. A. N., Magalhães, M. M. A., & Brito, E. S. (2008). Non-enzymatic browning in clarified cashew apple juice during thermal treatment: Kinetics and process control. *Food Chemistry, 106*(1), 172-179.

Diagne, N., Arumugam, K., Ngom, M., Nambiar-Veetil, M., Franche, C., Narayanan, K. K., & Laplaze, L. (2013). Use of Frankia and Actinorhizal Plants for Degraded Lands Reclamation. *BioMed Research International, 2013*, 948258.

Dong, K., Fernando, W. M. A. D. B., Durham, R., Stockmann, R., W. Jayatunga, D. P., & Jayasena, V. (2020). A role of sea buckthorn on Alzheimer's disease. *International Journal of Food Science & Technology, 55*(9), 3073-3081.

Dulf, F. V. (2012). Fatty acids in berry lipids of six sea buckthorn (Hippophae rhamnoides L., subspecies carpatica) cultivars grown in Romania. *Chemistry Central Journal, 6*(1), 106.

Dumitraşcu, L., Ursache, F. M., Stănciuc, N., & Aprodu, I. (2016). Studies on binding mechanism between carotenoids from sea buckthorn and thermally treated α-lactalbumin. *Journal of Molecular Structure, 1125*, 721-729.

Enkhtaivan, G., John, K. M., Pandurangan, M., Hur, J. H., Leutou, A. S., & Kim, D. H. (2017). Extreme effects of Seabuckthorn extracts on influenza viruses and human cancer cells and correlation between flavonol glycosides and biological activities of extracts. *Saudi Journal of Biological Sciences, 24*(7), 1646-1656.

Fu, L., Su, H., Li, R., & Cui, Y. (2014). Harvesting technologies for sea buckthorn fruit. *Engineering in Agriculture, Environment and Food, 7*(2), 64-69.

Gatlan, A. M., & Gutt, G. (2021). Sea Buckthorn in Plant Based Diets. An Analytical Approach of Sea Buckthorn Fruits Composition: Nutritional Value, Applications, and Health Benefits. *International Journal of Environmental Research and Public Health, 18*(17).

Godeanu, C. S., Costea, T., Ghica, M., Lupuliasa, D., & Gird, C. E. (2020). Evidence-based use of sea buckthorn fresh juice for patients with traumatic brain injury. A pilot study. *FARMACIA, 68*(3), 541-546.

Grey, C., Widén, C., Adlercreutz, P., Rumpunen, K., & Duan, R.-D. (2010). Antiproliferative effects of sea buckthorn (Hippophae rhamnoides L.) extracts on human colon and liver cancer cell lines. *Food Chemistry, 120*(4), 1004-1010.

Guo, R., Guo, X., Li, T., Fu, X., & Liu, R. H. (2017). Comparative assessment of phytochemical profiles, antioxidant and antiproliferative activities of Sea buckthorn (Hippophaë rhamnoides L.) berries. *Food Chemistry, 221*, 997-1003.

Guo, X., Chen, M., Li, Y., Dai, T., Shuai, X., Chen, J., & Liu, C. (2020). Modification of food macromolecules using dynamic high pressure microfluidization: A review. *Trends in food science & technology, 100*, 223-234.

Hajazimi, E., Landberg, R., & Zamaratskaia, G. (2016). Simultaneous determination of flavonols and phenolic acids by HPLC-CoulArray in berries common in the Nordic diet. *LWT, 74*, 128-134.

Heilscher, K. (1995). Germany Patent No. DE19549289C1.

Heinaaho, M., & Julkunen-Tiitto, R. (2011). Efficient extraction of flavonoids of sea buckthorn berries. *BioChemistry: An Indian Journal, 5*, 83-87.

Hu, W., Fitzgerald, M., Topp, B., Alam, M., & O'Hare, T. J. (2019). A review of biological functions, health benefits, and possible de novo biosynthetic pathway of palmitoleic acid in macadamia nuts. *Journal of Functional Foods, 62*, 103520.

Hussain, S., Sharma, M., & Bhat, R. (2021). Valorisation of sea buckthorn pomace by optimization of ultrasonic-assisted extraction of soluble dietary fibre using response surface methodology. *Foods, 10*(6), 1330.

Jiménez-Escrig, A., & Sánchez-Muniz, F. (2000). Dietary fibre from edible seaweeds: Chemical structure, physicochemical properties and effects on cholesterol metabolism. *Nutrition research, 20*(4), 585-598.

Kalia, R. K., Singh, R., Rai, M. K., Mishra, G. P., Singh, S. R., & Dhawan, A. K. (2011). Biotechnological interventions in sea buckthorn (Hippophae L.): current status and future prospects. *Trees, 25*(4), 559-575.

Kallio, H., Yang, B., Peippo, P., Tahvonen, R., & Pan, R. (2002). Triacylglycerols, Glycerophospholipids,

Tocopherols, and Tocotrienols in Berries and Seeds of Two Subspecies (ssp. sinensis and mongolica) of Sea Buckthorn (Hippopha> ë rhamnoides). *Journal of Agricultural and Food Chemistry, 50*(10), 3004-3009.

Kallio, H., Yang, B. R., Peippo, P., Tahvonen, R., & Pan, R. L. (2002). Triacylglycerols, glycerophospholipids, tocopherols, and tocotrienols in berries and seeds of two subspecies (ssp sinensis and mongolica) of sea buckthorn (Hippophae rhamnoides). *Journal of Agricultural and Food Chemistry, 50*(10), 3004-3009.

Kitrytė, V., Povilaitis, D., Kraujalienė, V., Šulniūtė, V., Pukalskas, A., & Venskutonis, P. R. (2017). Fractionation of sea buckthorn pomace and seeds into valuable components by using high pressure and enzyme-assisted extraction methods. *LWT - Food Science and Technology, 85*, 534-538.

Ladol, S., & Sharma, D. (2021). The effects of Hippophae rhamnoides in neuroprotection and behavioral alterations against iron-induced epilepsy. *Epilepsy Research, 175*, 106695.

Li, H., Zhang, Q., S Bhadury, P., & Yang, S. (2014). Furan-type compounds from carbohydrates via heterogeneous catalysis. *Current Organic Chemistry, 18*(5), 547-597.

Li, T. S., & Schroeder, W. R. (1996). Sea buckthorn (Hippophae rhamnoides L.): a multipurpose plant. *HortTechnology, 6*(4), 370-380.

Liu, K., Luo, M., & Wei, S. (2019). The bioprotective effects of polyphenols on metabolic syndrome against oxidative stress: Evidences and perspectives. *Oxidative Medicine and Cellular Longevity, 2019*.

Marcelino, G., Machate, D. J., Freitas, K. d. C., Hiane, P. A., Maldonade, I. R., Pott, A., Asato, M. A., Candido, C. J., & Guimarães, R. d. C. A. (2020). β-Carotene: Preventive role for type 2 diabetes mellitus and obesity: A review. *Molecules, 25*(24), 5803.

Marciniak, B., Kontek, R., Żuchowski, J., & Stochmal, A. (2021). Novel bioactive properties of low-polarity fractions from sea-buckthorn extracts (Elaeagnus rhamnoides (L.) A. Nelson) – (in vitro). *Biomedicine & Pharmacotherapy, 135*, 111141.

Matsumoto, M., Suganuma, H., Ozato, N., Shimizu, S., Katashima, M., Katsuragi, Y., Mikami, T., Itoh, K., & Nakaji, S. (2021). Association between serum concentration of carotenoid and visceral fat. *Nutrients, 13*(3), 912.

Mihalcea, L., Turturică, M., Cucolea, E. I., Dănilă, G.-M., Dumitrașcu, L., Coman, G., Constantin, O. E., Grigore-Gurgu, L., & Stănciuc, N. (2021). CO2 Supercritical Fluid Extraction of Oleoresins from Sea Buckthorn Pomace: Evidence of Advanced Bioactive Profile and Selected Functionality. *Antioxidants, 10*(11), 1681.

Olas, B. (2016). Sea buckthorn as a source of important bioactive compounds in cardiovascular diseases. *Food and Chemical Toxicology, 97*, 199-204.

Olas, B., Skalski, B., & Ulanowska, K. (2018). The Anticancer Activity of Sea Buckthorn [Elaeagnus rhamnoides (L.) A. Nelson]. *Frontiers in Pharmacology, 9*.

Patra, B. R., Borugadda, V. B., & Dalai, A. K. (2022). Microwave-assisted extraction of sea buckthorn pomace and seed extracts as a proactive antioxidant to stabilize edible oils. *Bioresource Technology Reports, 17*, 100970.

Pop, E. A., Diaconeasa, Z. M., Fetea, F., Bunea, A., Dulf, F., Pintea, A., & Socaciu, C. (2015). Carotenoids, tocopherols and antioxidant activity of lipophilic extracts from sea buckthorn berries (*Hippophae rhamnoides*), apricot pulp and apricot kernel (*Prunus armeniaca*). *Bulletin UASVM Food Science and Technology, 72*(2), 169-176.

Ranjith, A., Kumar, K. S., Venugopalan, V. V., Arumughan, C., Sawhney, R. C., & Singh, V. (2006). Fatty acids, tocols, and carotenoids in pulp oil of three sea buckthorn species (*Hippophae rhamnoides, H. salicifolia*, and *H. tibetana*) grown in the Indian Himalayas. *Journal of the American Oil Chemists' Society, 83*(4), 359-364.

Rannou, C., Laroque, D., Renault, E., Prost, C., & Sérot, T. (2016). Mitigation strategies of acrylamide, furans, heterocyclic amines and browning during the Maillard reaction in foods. *Food Research International, 90*, 154-176.

Ren, R., Li, N., Su, C., Wang, Y., Zhao, X., Yang, L., Li, Y., Zhang, B., Chen, J., & Ma, X. (2020). The bioactive components as well as the nutritional and health effects of sea buckthorn. *RSC Advances, 10*(73), 44654-44671.

Shi, J., Xu, Z., Qian, J., Li, H., Zhou, T., & Zhou, L. (2019). CN108651786-A.

Shivapriya, S., Ilango, K., & Dubey, G. (2015). Evaluation of antioxidant and neuroprotective effect of *Hippophae rhamnoides* (L.) on oxidative stress induced cytotoxicity in human neural cell line IMR32. *Saudi Journal of Biological Sciences, 22*(5), 645-650.

Sugiura, M., Nakamura, M., Ogawa, K., Ikoma, Y., & Yano, M. (2015). High-serum carotenoids associated with lower risk for developing type 2 diabetes among Japanese subjects: Mikkabi cohort study. *BMJ Open Diabetes Research and Care, 3*(1), e000147.

Suryakumar, G., & Gupta, A. (2011). Medicinal and therapeutic potential of Sea buckthorn (*Hippophae rhamnoides* L.). *Journal of Ethnopharmacology, 138*(2), 268-278.

Talbot, N. A., Wheeler-Jones, C. P., & Cleasby, M. E. (2014). Palmitoleic acid prevents palmitic acid-induced macrophage activation and consequent p38 MAPK-mediated skeletal muscle insulin resistance. *Molecular and Cellular Endocrinology, 393*(1), 129-142.

Teleszko, M., Wojdyło, A., Rudzińska, M., Oszmiański, J., & Golis, T. (2015). Analysis of Lipophilic and Hydrophilic Bioactive Compounds Content in Sea Buckthorn (*Hippophaë rhamnoides* L.) Berries. *Journal of Agricultural and Food Chemistry, 63*(16), 4120-4129.

Tian, Z., Li, P., & Yu, H. (2018). CN111671783-A.

Tricò, D., Mengozzi, A., Nesti, L., Hatunic, M., Gabriel Sanchez, R., Konrad, T., Lalić, K., Lalić, N. M., Mari, A., & Natali, A. (2020). Circulating palmitoleic acid is an independent determinant of insulin sensitivity, beta cell function and glucose tolerance in non-diabetic individuals: A longitudinal analysis. *Diabetologia, 63*(1), 206-218.

Tudor, C., Bohn, T., Iddir, M., Dulf, F. V., Focşan, M., Rugină, D. O., & Pintea, A. (2020). Sea Buckthorn Oil as a Valuable Source of Bioaccessible Xanthophylls. *Nutrients, 12*(1), 76.

Ursache, F.-M., Ghinea, I. O., Turturică, M., Aprodu, I., Râpeanu, G., & Stănciuc, N. (2017). Phytochemicals content and antioxidant properties of sea buckthorn (*Hippophae rhamnoides* L.) as affected by heat treatment - Quantitative spectroscopic and kinetic approaches. *Food Chemistry, 233*, 442-449.

Wani, T. A., Wani, S., Ahmad, M., Ahmad, M., Gani, A., & Masoodi, F. (2016). Bioactive profile, health benefits and safety evaluation of sea buckthorn (Hippophae rhamnoides L.): A review. *Cogent Food & Agriculture, 2*(1), 1128519.

Xu, H., Hao, Q., Yuan, F., & Gao, Y. (2015). Non-enzymatic Browning Criteria to Sea Buckthorn Juice during Thermal Processing. *Journal of Food Process Engineering, 38*(1), 67-75.

Yang, B., Halttunen, T., Raimo, O., Price, K., & Kallio, H. (2009). Flavonol glycosides in wild and cultivated berries of three major subspecies of *Hippophaë rhamnoides* and changes during harvesting period. *Food Chemistry, 115*(2), 657-664.

Yang, B. R., & Kallio, H. P. (2001). Fatty acid composition of lipids in sea buckthorn (*Hippophae rhamnoides* L.) berries of different origins. *Journal of Agricultural and Food Chemistry, 49*(4), 1939-1947.

Yang, M., Yang, D., & Yu, X. (2018). Soil microbial communities and enzyme activities in sea-buckthorn (*Hippophae rhamnoides*) plantation at different ages. *PLOS ONE, 13*(1), e0190959.

Zhang, J., Zhou, H.-C., He, S.-B., Zhang, X.-F., Ling, Y.-H., Li, X.-Y., Zhang, H., & Hou, D.-D. (2021). The immunoenhancement effects of sea buckthorn pulp oil in cyclophosphamide-induced immunosuppressed mice. *Food & Function, 12*(17), 7954-7963.

Zhao, Z., Shahrour, I., Bai, Z., Fan, W., Feng, L., & Li, H. (2013). Soils development in opencast coal mine spoils reclaimed for 1–13 years in the West-Northern Loess Plateau of China. *European Journal of Soil Biology, 55*, 40-46.

Zheng, H. X., Mao, L. K., Yang, J. Y., Zhang, C. Y., Miao, S., & Gao, Y. X. (2020). Effect of Oil Content and Emulsifier Type on the Properties and Antioxidant Activity of Sea Buckthorn Oil-in-Water Emulsions. *Journal of Food Quality, 2020*.

Zheng, J.-S., Sharp, S. J., Imamura, F., Chowdhury, R., Gundersen, T. E., Steur, M., Sluijs, I., van der Schouw, Y. T., Agudo, A., & Aune, D. (2020). Association of plasma biomarkers of fruit and vegetable intake with incident type 2 diabetes: EPIC-InterAct case-cohort study in eight European countries. *bmj, 370*.

Zhou, X., Tian, L., Zhang, J., Ma, L., Li, X., & Tian, C. (2017). Rhizospheric fungi and their link with the nitrogen-fixing Frankia harbored in host plant Hippophae rhamnoides L. *Journal of Basic Microbiology, 57*(12), 1055-1064.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

THE USE OF LACTIC ACID BACTERIA AND THEIR METABOLITES TO IMPROVE THE SHELF LIFE OF PERISHABLE FRUITS AND VEGETABLES

Florentina BADEA, Camelia Filofteia DIGUȚĂ, Florentina MATEI

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Biotechnology, 59 Marasti Blvd., District 1, Bucharest, Romania

Corresponding author email: badea_florentina2003@yahoo.com

Abstract

As a result of biochemical transformation and microbiological contamination that can occur during storing and commercialization, freshly picked fruits and vegetables tend to be very perishable products, thus having a shortened shelf life. Lactic acid bacteria (LAB) are microorganisms that play a crucial role in a wide variety of fermentation processes. Using microorganisms Generally recognized as safe (GRAS), LAB, including their metabolites, is proven to be a promising alternative of food preservation by respecting food safety measures in a natural way. Such metabolites produced and released by probiotic lactic bacteria strains are known as postbiotics. Vegetables and fruits can benefit from the antagonistic activity of postbiotics due to their content of bioactive products, such as short-chain fatty acids, antimicrobial peptides, organic acids, carbohydrates, vitamins and enzymes. This review is focused on the advances of the sustainable biopreservation products to extend the shelf life of perishable fruits and leafy vegetables, by the use of synergic postbiotics from LAB strains.

Key words*: contamination, fruits, vegetables, lactic acid bacteria, postbiotics, shelf-life.*

INTRODUCTION

Consumers concern regarding safe and healthy food has driven the food industry to research new technologies and alternatives to enhance the quality of and reliability on food by using few chemical substances (Alegre et al., 2013). Using GRAS (Generally Recognized As Safe) microorganisms, like lactic acid bacteria (LAB) and/or their naturally occurring metabolites, is proven to be a promising alternative for maintaining food safety and is also perceived by costumers as a natural preservation method. Fruits and vegetables are a good source of vitamins and minerals, without which the human body could not maintain an adequate health state and could not fight diseases. Fruits, as a food source, are extremely delectable, have a low-calorie intake and a low quantity of fats, are facile to prepare, and also from the point of view of their impact on human health, they play a crucial role by preventing a plethora of diseases by consumption.

Fruits and vegetables contain pectin, cellulose, oils, healthy fats and proteins. As a result of biochemical transformation and microbiological contamination that can occur during storage and commercialization, freshly picked fruits and vegetables tend to be very perishable products thus having a shortened shelf life. Using preservation means to almost eliminate food losses and to enhance the quality and safety of food has been always a staple in food industry; however, contemporary consumers usually develop antipathy against food additives, seeing them as unhealthy, although they do not understand the action mechanism and how they affect health (Bearth & Hartmann, 2017). All these alterations can lead to a significant degradation of organoleptic characteristics, such as color, aroma and taste, which leads to economic losses (De Corato, 2020).

Because of these reasons, recent researches are focused on developing products with reduced contents of additives or using natural ingredients to assure the quality and safety and also meet the consumer requirements (Guimarães et al., 2020). In this regard, natural antimicrobial agents (AA) gained important attention from researchers, allowing manufacturers to replace synthetic additives whilst following food safety measures and help develop healthier food.

According to the scientific literature, as shown in Table 1, fruits and vegetables are classified into four categories based on their perishability.

Table 1. Fresh fruits and vegetables categorized by perishability (source: Ahmad et al., 2017)

Group	Vegetables	Fruits
Extremely perishable	Endives, early potatoes, spring onion and garlic, mushrooms, orache spinach, lettuce, parsley, garden	Blueberries, strawberries, gooseberries, blackcurrant, blackberries, raspberries, mulberry, dates, wild strawberry
Very perishable	Okra, cucumbers, cauliflower, leeks zucchini, green beans, green peas, cabbage, asparagus,	Apricots, cherries, quince, peaches, plum, grapes,sour cherries
Perishable	Potatoes, aubergines, bell peppers, artichoke, carrots, tomatoes	Apples, pears, cantaloupe, watermelon, bananas
Less perishable	Potatoes, onions, garlic, kohlrabi, horseradish, leek, root vegetables, red cabbage, white cabbage, beetroot	Apples, pears, chestnuts, oranges, lemons

LACTIC ACID BACTERIA

LAB are Gram-positive, nonrespiring and non-motile microorganisms with a variable optimum pH, between 5.5 and 5.8, that helps extend the shelf life of products by inhibiting the growth of the alteration microorganisms and improving their organoleptic properties (Jiang et al., 2021). Generally, LAB are referred to as probiotics due to their capacity to resist in the gastro-intestinal tract, or to produce exopolysaccharides, biological preservatives and bacteriocins.

As shown in Figure 1, LABs are a key component in various parts of the food industry (bread manufacturing, cheese industry, beverages, other fermented food, etc.) Fermentation is an efficient process that conserves energy and increases the shelf life of food products (Capozzi et al., 2017).

The LABs are microorganisms that perform various fermentation processes, and are used as primary or secondary starter cultures. They have antimicrobial properties and can extend the shelf life of fruits and vegetables (Ranadheera et al., 2019). The bioactive agents produced by these microorganisms are known as postbiotics, having, for instance, antimicrobial properties (Barros et al., 2020).

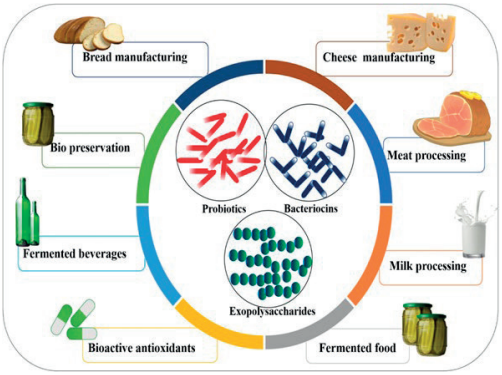


Figure 1. Lactic acid bacteria and their application (source: Raj et al., 2021)

Using LABs in food biopreservation is considered a promising technique to prevent pathogenic microorganisms' development, like *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli, Salmonella enterica* Serovar *Typhimurium, Proteus vulgaris Bacillus cereus*, but also the spoilage molds, such as *Aspergillus* spp., *Penicillium* spp., *Botrytis cinerea.* The antimicrobial capabilities of LABs are linked to various actions by the production of antimicrobial compounds such as bacteriocins, hydrogen peroxide, organic acids, etc. (Ramos et al., 2020).

LAB as PROBIOTIC

The innovations made in the last years in functional foods generated a wide variety of bioactive compounds that positively influence health, like probiotics, postbiotics, prebiotics or natural antioxidants, peptides, etc. (Fernandes et al., 2019).

In 2002, the United Nations' Food and Agriculture Organization and the World Health Organization defined probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host". Studies in this area showed that multiple LAB strains can be considered probiotics, and their postbiotic compounds frequently equally present health benefits to the consumer (Aguilar-Toalá et al., 2018). Some advantages of adding probiotics to food products are the interaction with pathogenic bacteria, due to the fact that probiotics can inhibit the growth of pathogenic agents by producing antimicrobial substances (Mousavi et al., 2020). The mix of

probiotics with fruits and vegetables can deliver both prebiotics and alimentary fibers needed by the human body, proving to be an important way of developing the probiotic industry in the future. The aforementioned combination has diverse forms and can be easily incorporated into the products of vegetables and fruits. The most used probiotic bacteria employed in the food industry belong to the genera *Lactoabcillus* spp. and *Bifidobacterium* spp. (Table 2), but much more other species were subject of researches (Table 3). For instance, the effect of washing fresh-cut lettuce with *Lactococcus lactis* solutions has been assessed to analyse the survival of *Listeria monocytogenes*.

Table 2. The most used probiotics species in the food industry (source: Žuntar et al., 2020)

Lactobacillus spp.	*Bifidobacterium* spp.	Other spp.
L. lactis	*B. lactis*	*Enterococcs faecalis*
L. fermentum	*B. infantis*	*Escherichia coli*
L. acidophilus	*B. breve*	*Bacilus cereus*
L. brevis	*B. longum*	*Enterococcus faecium*
L. delbrueckii	*B. bifidum*	*Pediococcus acidilactici*
L. paracasei	*B. animalis*	*Leuconostoc mesenteroides*
L. helveticus		*Bacilus coagulans*
L. bulgaricus		
L. plantarum		
L. casei		
L. rhamnosus		

The viability of this pathogen decreased by 1.2-1.6 log units instantly after washing. After washing the vegetables (green asparagus and beans) with *Enterococcus faecium*, cells of *Bacillus cereus* decreased by instantly 1.0-1.5 log unit (Borges et al., 2018). Alegre et al., 2013 discovered that some probiotics inactivated by heat can maintain important cellular structures and can exercise biologic activity in the host body. Direct use of probiotics and selected LABs in food packaging systems was researched and now it is known usually as food probiotic packaging (Espitia et al., 2016; Odila et al., 2016). Incorporation of bacterial cells in the packaging material can improve the antimicrobial potential of the whole package by developing and by freeing antimicrobial substances or even by the competition between the probiotics with spoilage microorganisms and pathogenic agents from the food surface (Motalebi et al., 2020).

POSTBIOTIC

The postbiotic term is relatively new and there is no generally recognized definition yet. Langella and Martin in 2019 defined postbiotics as viable bacterias products or metabolic products from microorganisms that exercise biologic activity inside the host. Consequently, this definition can be extended to soluble food grade bioactive factors produced by microorganisms while growing and fermenting in complex microbiological culture media, cellular compounds and substances produced by the action of the microorganism on food ingredients.
The word “postbiotic” is used now to refer to bioactive metabolites, such as organic acids, short chain fatty acids, carbohydrates, antimicrobial peptides, enzymes, cofactors, immune signalling compounds, and complex agents (Moradi et al., 2019; Rajakovich & Balskus, 2019).
The efficiency of postbiotics in food systems depends on the LAB strain from which the postbiotic is produced, the targeted microorganism/contaminant, concentration, the application type, as well as the food type. Fruits and vegetables can benefit from the antagonistic activity of postbiotics. They can be used as washing solutions, as disinfection agents for fresh products industry. The direct addition of bacteriocin can be used in vegetable and fruit wash pre-treatment in order to inhibit the growth of microbial pathogens.
Postbiotics can also restrict the growth of foodborne pathogenic agents from fruits and vegetables (Tenea & Barrigas, 2018). Alvarez et al. (2021), demonstrated the effectiveness of a *Lactobacillus plantarum* strain incorporated into an edible coating based on exopolysaccharide from *Weissella confusa* on the physicochemical and microbiological quality of cherry tomato; the coating managed to inhibit the griwth of *Fusarium* spp. and *Rhizopus stolonifera.* In a study by De Simone et al., 2021, cell-free supernatants of *L. plantarum*, managed to inhibit the growth of

B. cinerea by 14 days compared to the control samples.. Duran et al. (2016) incorporated natamycin (antifungal medication) and commercially nisin (a bacteriocin produce by *Lactococcus lactis*) in a chitosan edible coating which extended the shelf life of strawberries, decreasing the aerobic mesophilic bacteria, yeast and mold levels. In this regard, combining postbiotics with different natural antimicrobial agents (AA) can increase the overall biological activity.

The outcome of a recent study by Pique et al. from 2019, underlines that postbiotics have many pharmacodynamic characteristics on living bacteria. It was proved that bacteriocins can interact with many compounds present in the food matrix, such as enzymes, proteins and carbohydrates (Silva et al., 2018), which may lead to the inhibition of their active postbiotic potential.

Further, will be described the most important characteristics of some of the LABs' postbiotics: bacteriocins, organic acids, exopolysaccharides and oxygen peroxide. To the present day, the majority of reports focused on the potential use of postbiotic metabolites, as bacteriocins in biopreservation of different food products and EPS.

Table 3. Lactic acid bacteria used for the inhibition of different pathogens in agro-food products (source: Mostafidi et al., 2020)

Product	Probiotic	Pathogen
asparagus, broccoli, cauliflower, celery, lettuce, spinach	*L. mezenteroid*	*Aeromonas* spp.
alfalfa sprouts, carrot, lettuce, onions sprouts, parsley, radish	*Enterococcus mundtii*	*Staphylococcus aureus*
potatoes, green onions, spinach, lettuce, peppers, parsley, mushrooms	*Leuconostoc,* *L. mezenteroides*	*Campylobacter jejuni*
cabbage, coriander, apples	*Enterococcus mundtii*	*E. coli*
bean sprouts, cabbage, chicory cucumber, eggplant, lettuce, potatoes, radish, salad, vegetables, tomato	*E. faecium* *Pediococcus pentosaceus* *L. plantarum*	*Listeria monocytogenes* *Bacillus cereus*
salad, cauliflower, eggplant, endive, parsley, ananas, peppern, strawberries, tomatoes, melons	*Enterococcus mundtii* *L. mesenteroides* *L. plantarum*	*Salmonella enterica* Serovar *Typhimurium*

BACTERIOCINS

Bacteriocins are synthetized mainly by LABs, which possess a wide range of inhibitory activity and are molecules with low mass. They are thermoresistant antibacterial peptides and are synthetized in the ribosome. An important fact is, that bacteriocins are well known to not alter food products sensorial and nutritional properties, being postbiotics with great potential in food industry applications (O'Bryan et al., 2018, Rohani et al., 2011). Together with the aforementioned substances, some extracellular biologically active agents with antibacterial activity, namely exopolysaccharides (EPS),
gained more and more ground in food related processes (Mbye et al., 2020).

Antimicrobial metabolites are narrow range compounds and pathogenic agents treated with some of them, including bacteriocins, can develop immunity despite the fact that the costs of isolation and purification of bacteriocins is high (Kumariya et al., 2019). Regarding the case of the postbiotic mix, food can take advantage of the wide range antimicrobial activity, as well as of the synergic activities between the organic acids and other metabolites, besides the heightened thermostability of the postbiotic mix. LAB produce a series of extracellular antimicrobial substances which not only restricts pathogenic microorganisms, but also the ones that cause food alteration. *In situ* production of antimicrobial agents by protective LAB made their exploitation in means of food preservation more accessible and compatible (Hussein et al., 2018). Keeping in mind that many microorganisms produce bacteriocins, the ones produced by LABs attracted the attention most because of their extended uses in food processing and food fermentation as natural biopreservatives. For instance, these were widely used in food conservation, such as in cheese, meat, vegetable and fruit preservation (Drider et al., 2016). Using bacteriocins in the

food sector can lower the need of chemical preservatives or thermic treatments. Bacteriocins can be used conjunctively with other traditional treatments as part of the technology chain (Favaro et al., 2015). Using bacteriocins as an alternative to traditional preservation of fruits and vegetables is still under study, even though they have known preservation properties. Until the present day, only nisin (sold under the name Nisaplin) and pediocin (sold under the name Micocin) were approved the for use as food additives. Bacteriocin, enterocin, and nisin were commonly used in food factories to prevent product spoilage (Johnson et al., 2018).

Bacteriocins' classification was under revision many times. Recent classification organizes bacteriocins in three major groups based on their physiochemical and structural properties (Zacharof et al., 2012).

In the first class, bacteriocins, or lantibiotics, are small peptides, thermostable, which are largely modified during the post-translational phase and are made up of characteristic polycyclic amino acids (Kaur et al., 2015). In the second class the bacteriocins are also small thermostable peptides, but without lantinoin, such as pediocin and sakacin. They are mainly active against *Listeria monocytogenes* (Kaur et al., 2015).

Bacteriocins from the third class are thermolabile proteins with heavy molecular mass. Table 4 describes bacteriocins which have been tested for preservation of fruit products.

Nisin is the most researched and characterized bacteriocin for the preservation of fruit products. It is a first class bacteriocin produced by *Lactococcus lactis* strains. Nisin has inhibitory properties against foodborne enteropathogens, including *Clostridium difficile* (Martinez et al., 2016, Le Lay et al., 2015). The use of nisin in certain food is authorized as preservative additive (E-234) in EU (Allendeat et al., 2007). Nisin is effective against *Staphylococcus aureus*, *Clostridium difficile, Bacillus subtilis, Listeria monocytogenes*, *Alicyclobacillus acidoterrestris*, and even against some gram-negative bacteria (Barbosa et al., 2017). This bacteriocin inhibited the vegetative growth and sporulation of *Bacillus acidoterrestris* in apples, grapes and orange juice (Barbosa et al., 2017). Komitopoulou et al., 1999 in their research showed that by adding nisin in fruit juices inhibited *Alicyclobacillus acidoterrestris* development at all temperatures, storage conditions and storage time.

Pediocin is produced by *Pediococcus* spp., maintains stability in a wide pH range (3-9) and during high thermic processing techniques (65-121ºC). It has antibacterial activity against many foodborne pathogens, such as *C. perifringens* and *L. monocytogenes*, also against gram-positive bacteria that infects food (Barbosa et al., 2017).

This kind of bacteriocin was widely studied for its use in food preservation, mainly for animal food products, but recent reports reveal that pediocin can also be used in fruit products (Narsaiah et al., 2015).

Enterocin is a cyclic peptide produced by *Enterococcus faecalis*. It has a broad spectrum concerning both gram-positive and Gram-negative bacteria; it is thermostable and resists in high pH conditions. Enterocin is stable at heating temperature od 90ºC for 120 minutes and storage at 4ºC for 6 months (Barbosa et al., 2017). Molinos et al., 2008 showed that this bacteriocin has the potential to be used in fruit and vegetable conservation against *Listeria monocytogenes*.

Table 4. Bacteriocins that have been tested in the preservation of fruit products (source: Barbosa et al., 2017)

Bacteriocin	**Class**	**Producer strain**	**Antimicrobial activity**	**Applied product**
Nisin	I	*Lactococcus lactis sup lactis*	*A. acidoterrestris*	Fruit juices, mango pulp,
Bificin	I	*Bifidobacterium animalis*	*Alicyclobacillus acidoterrestris*	Fruit juices
Bovicin	I	*Streptococcus bovis*	*Clostridium tyrobutyricum, Bacillus cereus, A. acidoterrestris*	Mango pulp
Pediocin	II	*Pediococcus pentosaceus*	*Mesophilic bacteria* and *fungi*	Minimally processed papaya
Enterocin	II	*Enterococcus casseliflavus*	*Listeria monocytogenes*	Ready to eat fruits

Bificin is produced by *Bifidobacterium* spp. and thrives in lower pH ranges and higher temperatures. This characteristics are crucial for a bacteriocin to be used in fruit product preservation (Pei et al., 2013). Bificin presented antimicrobial activity against many strains of *S. aureus* and *E. faecium* (Pei et al., 2014). It could also be used in the edible coatings or in the plastic foil matrix both used in packaging minimal processed fruits.

EXOPOLYSACCHARIDE (EPS)

EPS produced by LAB are a diverse group of polysaccharides produced by many species, and are another useful characteristic of LAB. They can improve the stability and organoleptic characteristics of food (Michalak., 2018). The most prominent EPS producing lactic acid bacteria are *Weissella, Lactococcus, Pediococcus, Lactobacillus, Streptococcus* and *Bifidobacterium* sp.

EPS are classified in heteropolysaccharides produced intracellularly from several monosaccharides and homopolysaccharides which are produced in the extracellular medium. Homopolysaccharides can be characterized depending on the type of monosaccharide present in their structure. For instance, dextran is the only component that contains glucose. These are produced by *Streptococcus*, *Lactobacillus, Oenococcus, Weissella* and *Leuconostoc* (Farinazzo et al., 2020).

Heteropolysaccharides consist of repeated units with different degrees of polymerization and are composed of two to eight different monosaccharides such as fructose, rhamnose, glucose or galactose. Heteropolysaccharides are produced by members of the genera *Bifidobacterium, Lactococcus* and *Lactobacillus* (Ripari et al., 2019).

The technofunctional role of EPS in food is related to their ability to act like hydrocolloids and retain moisture in the product (Mende et al.,2016).

ORGANIC ACIDS

The goal of the LAB is to accelerate the production of organic acids in food by introducing them during the cell cycle. In fermented food, the souring effect is caused by the fermentation of carbohydrates to lactic and acetic acids. Treating fruits with organic acids can reduce the number of microorganisms in them. This method is usually used for treating fruits and vegetables perishable like strawberries, raspberries, blueberries and cantaloupes (Linares et al., 2018). Organic acids such as acid ascorbic and citric, malic, tartaric are commonly acids found in fruits and vegetables and can be used as preservatives.

Due to the decrease in environmental pH, organic acids reduce microbial loads and interfere with the cell's internal pH. They also affect the metabolic enzymes and protein synthesis (Corbo et al., 2015).

The effects of malic and citric acids on metabolism are minimal in peaches. In contrast, decreased malic acid contents have no significant effects on grapes (Famiani et al., 2016 a,b).

HYDROGEN PEROXIDE (H_2O_2)

When oxygen is present, LAB can produce hydrogen peroxide. This chemical is produced by the action of flavoproteinases or nicotinamide adenine dinucleotide peroxidase (NADH). Hydrogen peroxide is a precursor to the production of free radicals, which can be bactericidal by damaging DNA. It has been found that LAB that producing H_2O_2 can inhibit the growth of pathogenic *Clostridium botulinum, Listeria monocytogenes* bacteria and interfere with the development of food preservatives (Ben at al., 2019).

PEPTIDES

LAB can also produce antimicrobial peptides in addition to the well-known hydrogen peroxide and organic acids. (Muhialdin et al., 2016). Peptides are heat resistant and have low acidity, so they can also be used as preservatives in food. Peptides have multiple actions, such as degradation of the microbial membrane and inhibition of macromolecule synthesis (Waghu et al., 2020). A high antimicrobial activity has been attributed to LAB derived peptides against many pathogens *Escherichia coli*, *Salmonella enteritidis* and *Listeria monocytogenes* (Siroli et al., 2015).

The peptides obtained from the fermentation of vegetable performed by *Lactobacillus*

plantarum showed advantageous properties s (Jakubczyket al., 2017)

Brittany Forkus et al. (2017) used antimicrobial peptides produced by *Escherichia coli* and it was found that the inhibit the growth of *Salmonella enterica* by damaging the cell wall structure.

CONCLUSIONS

Aside from enhancing the functional features of fermented food, the selection of new autochthonus LAB is also important to improve the sensory quality of the finished product.

The composition and quality of the exopolysaccharides and other postbiotic compounds produced by these microorganisms can help improve the taste and texture of the perishable fruits and vegetables.

This paper highlights potential alternative methods, detrimental to the traditional ones of fruit and vegetable products preservation. New research is requested to find optimal mixture of such postbiotic products, of different LAB origins, to be used for new minimal processing technology for fruits and vegetables with short shelf-life.

REFERENCES

Allende, A., Martínez, B., Selma, V., Gil, M.I., Suárez, J.E., Rodríguez, A. (2007) Growth and bacteriocin production by lactic acid bacteria in vegetable broth and their effectiveness at reducing *Listeria monocytogenes in vitro* and in fresh-cut lettuce. *Food Microbiol.*, *24*, 759–766.

Alegre, I., Viñas, I., Usall, J., Anguera, M., Altisent, R., Abadias, M. (2013). Antagonistic effect of Pseudomonas graminis CPA-7 against foodborne pathogens in fresh-cut apples under simulated commercial conditions. *Food Microbiol*. 33, 139–148.

Ahmad, V., Khan, M. S., Jamal, Q. M. S., Alzohairy, M. A., Al Karaawi, M. A., & Siddiqui, M. U. (2017). Antimicrobial potential of bacteriocins: In therapy, agriculture and food preservation. *International Journal of Antimicrobial Agents*, *49*(1), 1–11. https://doi.org/10.1016/j.ijantimicag.2016.08.016.

Aguilar-Toalá, J. E., Garcia-Varela, R., Garcia, H. S., Mata-Haro, V., González-Córdova, A. F., Vallejo Cordoba, B., & Hernández Mendoza, A. (2018). Postbiotics: An evolving term within the functional foods field. *Trends in Food Science & Technology*, *75*,105–114. https://doi.org/10.1016/j.tifs.2018.03.009

Alvarez A., Manjarres J.J., Ramirez C., Bolivar G. (2021). Use of an exopolysaccharide-based edible coating and lactic acid bacteria with antifungal activity to preserve the postharvest quality of cherry tomato. *LWT - Food Science and Technology*, 151, 112225.

Bearth, A., & Hartmann, C. (2017). Consumers perception and acceptance of food additives. In *Reference module in food science*. Amsterdam, the Netherlands:Elsevier. https://doi.org/10.1016/B978-0-08-100596-5.21250-X

Ben Said, L., Gaudreau, H., Dallaire, L., Tessier, M., & Fliss, I. (2019). Bioprotective culture: A new generation of food additives for the preservation of food quality and safety. *Industrial biotechnology*, *15*(3), 138-147.

Barbosa, A. A. T., Mantovani, H. C., & Jain, S. (2017). Bacteriocins from lactic acid bacteria and their potential in the preservation of fruit products. *Critical Reviews in Biotechnology*, *37*(7), 852-864.

Barros, C. P., Guimarães, J. T., Esmerino, E. A., Duarte, M. C. K. H., Silva, M. C., Silva, R., ... Cruz, A. G. (2020). Paraprobiotics and postbiotics: Concepts and potential applications in dairy products. *Current Opinion in Food Science*, *32*, 1–8. https://doi.org/10.1016/j. cofs.2019.12.003

Borges, S., & Teixeira, P. (2018). Application of bacteriocins in food and health care. *Bacteriocins: Production, Applications and Safety*.

Capozzi, V., Fragasso, M., Romaniello, R., Berbegal, C., Russo, P. & Spano, G. (2017). Spontaneous Food Fermentations and Potential Risks for Human Health. *Fermentation*, *3*(4), 49.

Corbo, M. R., Campaniello, D., Speranza, B., Bevilacqua, A., & Sinigaglia, M. (2015). Non-conventional tools to preserve and prolong the quality of minimally-processed fruits and vegetables. *Coatings* 5, 931–961. doi:10.3390/ coatings 5040931.

De Corato U. (2020). Improving the shelf life and quality of fresh and minimally-processed fruits and vegetables for a modern food industry: A comprehensive critical review from the traditional technologies into the most promising advancements. *Critical Reviews in Food Science and Nutrition*, *60*(6), 940-975.

De Simone, N., Capozzi, V., de Chiara, M.L.V., Amodio, M.L., Brahimi, S., Colelli, G., Drider, D., Spano, G., Russo, P. (2021). Screening of Lactic Acid Bacteria for the Bio-Control of *Botrytis cinerea* and the Potential of *Lactiplantibacillus plantarum* for Eco-Friendly Preservation of Fresh-Cut Kiwifruit. *Microorganisms*.; *9*(4):773.

Drider, D., Bendali, F., Naghmouchi, K., Chikindas, M.L. (2016). Bacteriocins: Not only antibacterial agents. Probiotics Antimicrob Proteins, 8, 177–82.

Duran, M., Aday, M. S., Zorba, N. N. D., Temizkan, R., Büyükcan, M. B., & Caner, C. (2016). Potential of antimicrobial active packaging 'containing natamycin, nisin, pomegranate and grape seed extract in chitosan coating'to extend shelf life of fresh strawberry. *Food and Bioproducts Processing*, 98, 354-363.

Espitia, P. J. P., Batista, R. A., Azeredo, H. M. C., & Otoni, C. G. (2016).Probiotics and their potential applications in active edible films and coatings. *Food Research International*, *90*, 42–52. https://doi.org/10.1016/ j.foodres.2016.10.026

FAO/WHO. *FAO*/*WHO* Joint Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in

Food (30 April 2002 and 1 May 2002); Scientific Research Publishing: London, ON, Canada, 2002

Favaro, L., Penna, A. L. B. and Todorov, S. D. (2015) Bacteriocinogenic LAB from cheeses - Application in biopreservation? *Trends in Food Science and Technology*, 41, 37-48 https://doi.org/ 10.1016/J.TIFS.2014.09.001

Fernandes, S.S., Coelho, M.S., de las Mellado, M.M. (2019) Bioactive compounds as ingredients of functional foods: polyphenols, carotenoids, peptides from animal and plant sources new. In: Maira RSC, editor. Bioactive compound. Cambridge: Woodhead Publishing-Elsevier, 129–142.

Farinazzo, F. S., Valente, L. J., Almeida, M. B., Simionato, A. S., Fernandes, M. T. C., Mauro, C. S. I., ... & Garcia, S. (2020). Characterization and antioxidant activity of an exopolysaccharide produced by *Leuconostoc pseudomesenteroides* JF17 from juçara fruits (*Euterpe edulis* Martius). *Process Biochemistry*, 91, 141-148.

Forkus, B., Ritter, S., Vlysidis, M., Geldart, K., & Kaznessis, Y. N. (2017). Antimicrobial probiotics reduce *Salmonella enterica* in turkey gastrointestinal tracts. *Scientific reports*, *7*(1), 1-9.

Famiani, F., Farinelli, D., Frioni, T., Palliotti, A., Battistelli, A., Moscatello, S., et al. (2016a). Malate as substrate for catabolism and gluconeogenesis during ripening in the pericarp of different grape cultivars. *Biol. Plant*, 60, 155–162. doi: 10.1007/s10535-015-0574-2

Famiani, F., Farinelli, D., Moscatello, S., Battistelli, A., Leegood, R. C., and Walker, R. P. (2016b). The contribution of stored malate and citrate to the substrate requirements of metabolism of ripening peach (*Prunus persica* L. Batsch) flesh is negligible. Implications for the occurrence of phosphoenolpyruvate carboxykinase and gluconeogenesis. *Plant Physiol. Biochem*. 101, 33–42. doi: 10.1016/j.plaphy.2016.01.007

Guimarães, J. T., Balthazar, C. F., Silva, R., Rocha, R. S., Graça, J. S., Esmerino, E. A., ... Cruz, A. G. (2020). Impact of probiotics and prebiotics on food texture. *Current Opinion in Food Science*, *33*, 38–44. https://doi.org/10.1016/j.cofs.2019.12.002

Hussein, A. R., Khalaf, Z. Z., Samir, Z., & Samir, R. (2018). Antibacterial activity of crud Bacteriocin-like substance against food pathogens. *Iraqi Journal of Science*, *59*(1A), 16-24.

Jakubczyk, A., Karaś, M., Złotek, U., Szymanowska, U. (2017). Identification of potential inhibitory peptides of enzymes involved in the metabolic syndrome obtained by simulated gastrointestinal digestion of fermented bean (*Phaseolus vulgaris* L.) seeds. *Food Research International*, *100*, 489–496. https://doi.org/10.1016/j.foodres.2017.07.046

Jiang, X., Liu, X., Xu, H., Sun, Y., Zhang, Y., Wang, Y. (2021) Improvement of the nutritional, antioxidant and bioavailability properties of corn gluten-wheat bran mixture fermented with lactic acid bacteria and acid protease. *LWT*. 144, 111161. https://doi.org/ 10.1016/j.lwt.2021.111161

Johnson, E. M., Jung, D. Y. G., Jin, D. Y. Y., Jayabalan, D. R., Yang, D. S. H., & Suh, J. W. (2018). Bacteriocins as food preservatives: challenges and emerging horizons. *Critical reviews in food science and nutrition*, *58*(16), 2743-2767.

Kaur, S., & Kaur, S. (2015). Bacteriocins as potential anticancer agents. *Frontiers in pharmacology*, *6*, 272.

Kumariya, R., Garsa, A. K., Rajput, Y. S., Sood, S. K., Akhtar, N., & Patel, S. (2019). Bacteriocins: Classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria. *Microbial Pathogenesis*, *128*, 171–177. https://doi.org/10.1016/ j.micpath.2019.01.002

Komitopoulou, E., Boziaris, I. S., Davies, E. A., Delves-Broughton, J., & Adams, M. R. (1999). Alicyclobacillus acidoterrestris in fruit juices and its control by nisin. International journal of food science & technology, *34*(1), 81-85.

Le Lay, C., Fernandez, B., Hammami, R., Ouellette, M., Fliss I. On (2015) *Lactococcus lactis* UL719 competitivity and nisin (Nisaplin®) capacity to inhibit *Clostridium difficile* in a model of human colon. *Front Microbiol*. 6:1020.

Linares-Morales, J. R., Gutiérrez-Méndez, N., Rivera-Chavira, B. E., Pérez-Vega, S. B., & Nevárez-Moorillón, G. V. (2018). Biocontrol processes in fruits and fresh produce, the use of lactic acid bacteria as a sustainable option. *Frontiers in Sustainable Food Systems*, 50.

Langella, P, Martín, R. (2019) Emerging health concepts in the probiotics field: streamlining the definitions. *Front Microbiol*., 10, 1047.

Martinez, R. C. R., Alvarenga, V. O., Thomazini, M., Fávaro-Trindade, C. S., & de Souza Sant'Ana, A. (2016). Assessment of the inhibitory effect of free and encapsulated commercial nisin (Nisaplin®), tested alone and in combination, on Listeria monocytogenes and Bacillus cereus in refrigerated milk. *LWT-Food Science and Technology*, *68*, 67-75.

Mende, S., Rohm, H., Jaros, D. (2016). Influence of exopolysaccharides on the structure, texture, stability and sensory properties of yoghurt and related products. *International Dairy Journal*, 52, 57-71.

Michalak, M.; Gustaw, K.; Wa´sko, A.; Polak-Berecka, M. (2018), Composition of lactic acid bacteria during spontaneous curly kale (Brassica oleracea var. sabellica) fermentation. *Microbiol. Res.* 206, 121–130

Molinos, A. C., Abriouel, H., Ben Omar, N., Lucas, R., Valdivia, E., & Galvez, A. (2008). Inactivation of Listeria monocytogenes in raw fruits by enterocin AS-48. *Journal of food protection*, *71*(12), 2460-2467.

Moradi, M., Tajik, H., Mardani, K., & Ezati, P. (2019). Efficacy of lyophilized cell-free supernatant of *Lactobacillus salivarius* (LsBU2) on *Escherichia coli* and shelf life of ground beef. *Veterinary Research Forum*, *10*(3), 193–198.

Mousavi Khaneghah, A., Abhari, K., Eş, I., Soares, M. B., Oliveira, R. B. A., Hosseini, H., ... Sant'Ana, A. S. (2020). Interactions between probiotics and pathogenic microorganisms in hosts and foods: A review. *Trends in Food Science & Technology*, *95*, 205–218. https://doi.org/10.1016/j.tifs.2019.11.022

Moghanjougi, Z. M., Bari, M. R., Khaledabad, M. A., Almasi, H., & Amiri, S. (2020). Bio-preservation of white brined cheese (Feta) by using probiotic bacteria

immobilized in bacterial cellulose: Optimization by response surface method and characterization. *LWT*, 117, 108603.

Mostafidi, M., Sanjabi, M. R., Shirkhan, F., & Zahedi, M. T. (2020). A review of recent trends in the development of the microbial safety of fruits and vegetables. *Trends in Food Science & Technology*, *103*, 321-332.

Mbye, M., Baig, M. A., AbuQamar, S. F., El-Tarabily, K. A., Obaid, R. S., Osaili, T. M., ... Ayyash, M. M. (2020). Updates on understanding of probiotic lactic acid bacteria responses to environmental stresses and highlights on proteomic analyses. *Comprehensive Reviews in Food Science and Food Safety*, *19*(3), 1110-1124. https://doi.org/10.1111/1541-4337.12554

Muhialdin, B. J., Hassan, Z., Abu Bakar, F., and Saari, N. (2016). Identification of antifungal peptides produced by *Lactobacillus plantarum* IS10 grown in the MRS broth. *Food Control* 59:27–30. doi: 10.1016/j.foodcont.2015.05.022

Narsaiah, K., Wilson, R.A., Gokul, K., Mandge, H.M., Bhadwal, S., Anurag, R.H., ... Vij, S. (2015) Effect of bacteriocin-incorporated alginate coating on shelf-life of minimally processed papaya (*Carica papaya* L.). *Postharvest Biol Technol*.,100, 212–218

Odila Pereira, J., Soares, J., Sousa, S., Madureira, A. R., Gomes, A., & Pintado, M**.** (2016). Edible films as carrier for lactic acid bacteria. *LWT-Food Science and Technology*, *73*, 543–550. https://doi.org/10.1016/j.lwt.2016.06.060

O'Bryan, C. A., Koo, O. K., Sostrin, M. L., Ricke, S. C., Crandall, P. G., & Johnson, M. G. (2018). Characteristics of bacteriocins and use as food antimicrobials in the United States. In S. C. Ricke, G. G. Atungulu, C. E. Rainwater, & S. H. Park (Eds.), *Food and feed safety systems and analysis* (pp. 273–286). Cambridge, MA: Academic Press. https://doi.org/10.1016/B978-0-12-811835-1.00015-4

Pei, J., Yuan, Y., & Yue, T. (2013). Characterization of bacteriocin bificin C6165: a novel bacteriocin. *Journal of applied microbiology*, *114*(5), 1273-1284.

Pei, J., Yue, T., & Yuan, Y. (2014). Control of Alicyclobacillus acidoterrestris in fruit juices by a newly discovered bacteriocin. *World Journal of Microbiology and Biotechnology*, *30*(3), 855-863

Piqué, N., Berlanga, M., Miñana-Galbis, D. (2019) Health benefits of heat-killed (Tyndallized) probiotics: an overview, *Int J Mol Sci.*, 20, 2534.

Rajakovich, L. J., & Balskus, E. P. (2019). Metabolic functions of the human gut microbiota: The role of metalloenzymes. *National Product Reports*, *36*(4), 593–625. https://doi.org/10.1039/ C8NP00074C

Ranadheera, C. S., Evans, C. A., Baines, S. K., Balthazar, C. F., Cruz, A. G., Esmerino, E. A., ... Vasiljevic, T. (2019). Probiotics in goat milk products: Delivery capacity and ability to improve sensory attributes. *Comprehensive Reviews in Food Science and Food Safety*, *18*(4), 867–882. https://doi.org/10.1111/1541-4337.12447

Ramos, B., Brandão, T.R.S., Teixeira, P. Silva, C.L.M. (2020). Biopreservation approaches to reduce *Listeria monocytogenes* in fresh vegetables. *Food Microbiology*, 85, 103282.

Raj, T., Chandrasekhar, K., Kumar, A. N., & Kim, S. H. (2021). Recent biotechnological trends in lactic acid bacterial fermentation for food processing industries. *Systems Microbiology and Biomanufacturing*, 1-27.

Ripari, V. Techno-functional role of exopolysaccharides in cereal-based, yogurt-like beverages. *Beverages* 2019, *5*, 16

Rodrıguez, J.M., Martınez, M.I., Kok, J. (2002). Pediocin PA-1, a wide-spectrum bacteriocin from lactic acid bacteria. *Rev Food Sci Nutr*., 42,91–121.

Rohani, Razavi, M., S., Moradi, M., Mehdizadeh, T., Saei-Dehkordi, S.S., & Griffiths, M. W. (2011). The effect of nisin and garlic (*Allium sativum* L.) essential oil separately and in combination on the growth of Listeria monocytogenes. *LWT-Food Science and Technology*, *44*(10), 2260–2265. https://doi.org/10.1016/j.lwt.2011.07.020

Silva, C. C. G., Silva, S. P. M., & Ribeiro, S. C. (2018). Application of bacteriocins and protective cultures in dairy food preservation. *Frontiers in Microbiology*, *9*, 594

Siroli, L., Patrignani, F., Serrazanetti, D., Tabanelli, G., Mantanari, C., Gardini, F., et al. (2015). Lactic acid bacteria and natural antimicrobials to improve the safety and shelf-life of minimally processed sliced apples and lamb's lettuce. *Food Microbiol.*, 47, 74–84. doi: 10.1016/j.fm.2014.11.008

Tenea, G. N., & Barrigas, A. (2018). The efficacy of bacteriocin containing cell-free supernatant from *Lactobacillus plantarum* Cys5-4 to control pathogenic bacteria growth in artisanal beverages. *International Food Research Journal*, *25*(5), 2131–2137.

Zacharof, M.P.; Lovitt, R.W. Bacteriocins produced by lactic acid bacteria: A review article. *APCBEE Procedia* 2012, *2*, 50–56.

Žuntar, I., Petric, Z., Bursać Kovačević, D., & Putnik, P. (2020). Safety of probiotics: functional fruit beverages and nutraceuticals. *Foods*, *9*(7), 947.

Waghu, F.H., Idicula-Thomas, S. Collection of antimicrobial peptides database and its derivatives: Applications and beyond. *Protein Science* 2020, *29*, 36-42, https://doi.org/10.1002/pro.3714

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

IN SILICO APPROACH FOR THE IDENTIFICATION AND CHARACTERISATION OF BIOACTIVE PEPTIDES FROM SILVER CARP COLLAGEN

Grigore-Mihăiță STAN[1,2], Diana CONSTANTINESCU-ARUXANDEI[1], Dan Florin MIHĂILESCU[2], Florin OANCEA[1]

[1]National Institute for Research & Development in Chemistry and Petrochemistry – ICECHIM, District 6, Bucharest, Romania
[2]Department DAFAB, Faculty of Biology, University of Bucharest, 91-95 Splaiul Independenței, District 5, Bucharest, Romania

Corresponding author email: florin.oancea@icechim.ro

Abstract

The paper aimed to identify in silico bioactive peptides with antioxidant and antihypertensive effects from silver carp collagen. This approach involved the use of a wide range of specialized online databases and tools to identify bioactive peptides from various protein sources. In this case, the collagen type-I alpha-1 protein sequence was extracted from UniProtKB with the identification code A0A077B3P8. The digestion was simulated using the BIOPEP database with the following enzymes: subtilisin, papain and pepsin. ExPASy ProtParam, Peptide Ranker, PepCalc, and ToxinPred showed that silver carp collagen is a significant source of biologically active peptides, health promoters with potential antihypertensive and antioxidant effects. The computational approach used in this study offered useful initial insights for more extensive studies.

Key words*: bioactive peptides, collagen, health effects, in silico, silver carp,*

INTRODUCTION

By-products, generated from industrial fish processing represent over 50% of the total weight of the source, and their market value is low. Without processing, such by-products transform into dangerous wastes due to intrinsic microbiological instability. These residues sourced from skin, bones, viscera, scales, heads or fins are rich in collagen, which is a potential source of bioactive compounds applicable in the pharmaceutical, biomedical, cosmetic, and food industries (Pati et al., 2010; Pal & Suresh, 2016).

Until recently, collagen from various animals, such as bovine or porcine components, was preferred as a source of potential compounds with health-promoting effects. Due to various consumer perception issues, such as potential prion contamination of animal collagen, or ethical considerations, alternative sources of collagen have begun to be sought (Karim & Bhat, 2009).

Collagen derivatives from various sea sources are possible health promoters through various antihypertensive, antioxidant, neuroprotective, and regulatory effects. These effects result from bioactive collagen compounds such as bioactive peptides released when collagen interacts with proteolytic enzymes (Pal & Suresh, 2016). Due to the high content of amino acids such as glycine and proline in the structure of carp collagen, its potential for the generation of bioactive peptides is high (Pal & Suresh, 2017).

To the best of our knowledge, data on the biological activities of the bioactive peptides that could be generated from silver carp collagen are limited in the literature. This study explores the use of possible health-promoting agents of type I alpha-1 collagen in silver carp (*Hypophthalmichthys molitrix*) released at the interaction with various proteolytic enzymes. Using *in silico* approach, we attempted to predict the possible bioactive peptides with antihypertensive (angiotensin-converting enzyme/ACE inhibitors) and antioxidant activities when collagen is subjected to enzymatic hydrolysis. Also, we focused on the release from fish carp collagen of the peptides

with sensory characteristics, such as umami taste (Zhao *et al*., 2019). Collagen is an appropriate substrate to release umami peptides due to its high content in glutamic and aspartic acids (Gan et al., 2022).

MATERIALS AND METHODS

We used the alpha-1 type I collagen protein sequence from the silver carp (*H. molitrix*) that we acquired from the UniProt database (http://www.uniprot.org; UniProt entry: A0A077B3P8_HYPMO), accessed on 10.09.2021. To check the amino acid content of type I alpha 1 collagen in silver carp, for the entry mentioned above from UniProt, we used the Expasy ProtParam online tool (https://web.expasy.org/protparam/) (Gasteiger et al., 2005).

We chose the option "Profiles of potential biological activity" in the "Analysis" section of the BIOPEP instrument (http://www.uwm.edu.pl/biochemia/index.php/en/biopep; accessed on 10.09.2021), to predict the different profiles of the silver carp collagen protein potential biological activity. This option generated a list with various health-promoting effects, depending on the appearance of bioactive fragments in alpha-1 type I collagen chains in proteolysis simulation. The results obtained here included information such as ID, name, activity, number, sequence, and location of the peptide in the protein sequence. To check the potential of type I alpha-1 collagen protein as a possible substrate generating peptides with antihypertensive and antioxidant activities, the "Calculations" option was selected from the "Bioactive peptides" menu of the BIOPEP instrument. The result generated here is based on the equation: A=a/N, where A=frequency of occurrence of bioactive peptides, a=number of bioactive peptides, and N=total number of amino acid residues in the chosen protein sequence (Minkiewicz et al., 2019).

For the *in silico* prediction of the theoretical peptide sequences generated when collagen interacts with various proteolytic enzymes, the program "Enzyme (s) action", available in the BIOPEP instrument, was used. The enzymes used for protein proteolysis in this study were: subtilisin (EC 3.4.21.62), papain (EC 3.4.22.2), and pepsin (pH>2) (EC 3.4.23.1). The protein of interest, i.e., type I collagen from the silver carp (*H. molitrix*) was subjected to the simulation of enzymatic hydrolysis individually with each enzyme and a mix of two enzymes: pepsin+papain. The peptides obtained at this step were subjected to the "Search for active fragments" option, and the antihypertensive and antioxidant peptides were selected and extracted for further analysis.

The bioactive potential of ACE inhibitory and antioxidant peptides selected at the previous step was predicted using the online tool PeptideRanker. (http://bioware.ucd.ie/~compass/biowareweb/). This server can predict if a peptide is bioactive, generating peptide scores between 0-1, where a score close to 1 means high bioactivity (Pooja et al., 2017).

All the theoretical peptides obtained at the *in silico* proteolysis step, were subjected to the bioactivity test, after which they were manually included in the top 5 of the most bioactive peptide sequences.

To predict *in silico* theoretical parameters of peptides such as molecular weight, isoelectric point, or solubility of peptides in water, the peptides obtained in the previous step were subjected to prediction tests using PepCalc (https://pepcalc.com/) and ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/index.html) programs, the latter predicting possible toxicity of peptides (Pooja et al., 2017).

The prediction of the sensory characteristics of bioactive peptides obtained from type I alpha-1 collagen in silver carp was performed using the BIOPEP database. The number of bitter and umami-taste peptides resulting from the collagen protein, was calculated using the "Enzyme(s) action" option in the "Sensory peptide and amino acids" menu of the BIOPEP instrument (Iwaniak et al., 2016).

RESULTS AND DISCUSSIONS

Regardless of the origin, type I collagen contains about 20 different amino acids, which are found in different proportions and achieve the triple helix conformation due to their organization in three α chains, which wrap around each other (Shoulders & Raines, 2009).

Of these amino acids, glycine represents the highest percentage, about 27%, and

hydroxyproline and proline together about 25% of the total amino acids, which explains the repetitive Gly-X-Y sequence, where glycine is found at every third residue (Gauza-Włodarczyk et al., 2017; Coppola et al., 2020). Such repetitive sequence is directly related to the triple helix highly packed secondary structure. The high content of amino acids such as alanine, proline, and hydroxyproline is characteristic of type I collagen (Jafari et al., 2020)

Using the Expasy ProtParam program, we found varying amounts of amino acids in the silver carp protein sequence. Table 1 shows the amounts of amino acids according to the COL1A1 sequence extracted from the UniProtKB database.

Table 1. The amino acid content of type I alpha 1 collagen in silver carp (*H. molitrix*) according to Expasy ProtParam (https://web.expasy.org/protparam/)

Aminoacid	Acronym 3- letter*	Acronym 1- letter*	Amount (%)
Alanine	Ala	A	10.10%
Arginine	Arg	R	4.70%
Asparagine	Asn	N	2.10%
Aspartate	Asp	D	4.40%
Cysteine	Cys	B	1.20%
Glutamine	Gln	C	2.80%
Glutamate	Glu	E	5.50%
Glycine	Gly	Q	26.80%
Histidine	His	Z	0.60%
Isoleucine	Ile	G	2.30%
Leucine	Leu	H	2.60%
Lysine	Lys	I	4.10%
Methionine	Met	L	1.70%
Phenylalanine	Phe	K	2.00%
Proline	Pro	M	17.20%
Serine	Ser	F	3.90%
Threonine	Thr	P	4.30%
Tryptophan	Try	S	0.40%
Tyrosine	Tyr	T	0.70%
Valine	Val	W	2.60%

*IUPAC-IUB Commission on Biochemical Nomenclature - Rules, 1968

Table 1 shows glycine as the most abundant amino acid, with about 26%, followed by proline at 17%, alanine at 10%, glutamic acid at 5%, aspartic acid, and arginine threonine at 4%, serine at 3%, and methionine about 1.7%. These data are in agreement with the findings of other researchers in the analysis of type I collagen from various sources (Shoulders & Raines, 2009; Gauza-Włodarczyk et al., 2017; Song et al., 2017; Czerniecka-Kubicka et al., 2020).

The BIOPEP database contains a wide range of online tools that can be used in the predictive analysis of bioactive peptides in various protein chains. At the time of accessing the BIOPEP database, it contained a range of 740 proteins and 4199 sequences of bioactive peptides. It is important to note that in similar attempts to generate bioactive peptides from the same type of silver carp collagen protein used in this study, the results may be different, as the database may undergo changes in sequence content (Minkiewicz et al., 2019).

Table 2. Profile of the potential biological activity of type I alpha-1 collagen in silver carp (*H. molitrix*)

	Activity	Frequency (A)
1	dipeptidyl peptidase IV inhibitor	0.8273
2	ACE inhibitor	0.8087
3	antithrombotic	0.2079
4	regulating	0.1906
5	antiamnestic	0.1899
6	dipeptidyl peptidase III inhibitor	0.0725
7	antioxidative	0.0566
8	inhibitor	0.0456
9	alpha-glucosidase inhibitor	0.0407
10	chemotactic	0.0325
11	stimulating	0.0090
12	renin inhibitor	0.0076
13	neuropeptide	0.0055
14	activating ubiquitin-mediated proteolysis	0.0041
15	bacterial permease ligand	0.0028
16	CaMPDE inhibitor	0.0028
17	embryotoxic	0.0021
18	immunostimulating	0.0014
19	HMG-CoA reductase inhibitor	0.0007
20	hypolipidemic	0.0007
21	immunomodulating	0.0007

For alpha-1 type I collagen protein, our analyses showed the existence of several profiles of potential biological activity. The predicted significant potential biological

activity was dipeptidyl peptidase IV inhibitory activity with an activity frequency of A=0.8273, followed by inhibitory ACE activity with A=0.8087, antithrombotic activity A=0.2079, regulatory activity A=0.1906, anti-amnestic (Alzheimer preventive) activity A=0.1899 and the inhibitory activity of peptidyl peptidase III (protease involved in the modulation of peptide hormones signaling) A=0.0725. The antioxidant activity was ranked seventh with an activity frequency of A=0.0566 (Table 2).

Pal and Sureh (2016) researched an ACE inhibitory activity frequency of A=0.7011 and A=0.7993 for dipeptidyl peptidase IV inhibitory activity at *in silico* simulation of type 1 alpha-1 collagen from *Ctenopharyngodon idella*. The antioxidant activity found by researchers for this type of collagen was A = 0.0546 (Pal & Suresh, 2017).

Our study is in agreement with the data obtained by other researchers, type I alpha-1 collagen showing potential release effects of peptides with different bioactive roles, which places this protein as a potential candidate for the development of new products with antihypertensive, antioxidant, antidiabetic, or antithrombotic effects.

The number of bioactive peptides with ACE inhibitory and antioxidant activities obtained at the *in silico* simulations can be observed in Figure 1.

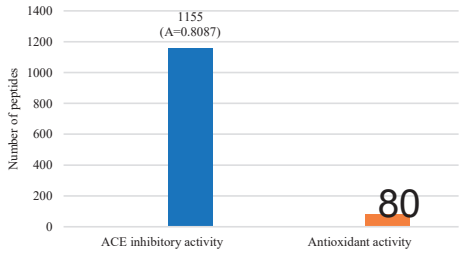


Figure 1. Prediction of the number of potential bioactive peptides in silver carp collagen showing the value of parameter A

The potentially significant biological activity was observed in the case of inhibitory ACE activity, with a number of 1155 potential bioactive peptides and a value of the quantitative parameter A=0.8087, compared to the antioxidant activity with 80 peptides obtained and A=0.0566. The value of the quantitative parameter A shows the ability of the protein to release bioactive peptides. Therefore, a higher value of this parameter means a higher possibility that a specific activity will be predominant (Minkiewicz et al., 2019).

Data on potential bioactive peptides resulting from type I alpha-1 collagen in silver carp are limited. The present work is among the first studies to demonstrate that this type of low-cost protein, with high accessibility, is a possible generator of peptides with antihypertensive and antioxidant activities, using an *in silico* approach.

Figure 2 shows how the silver carp protein sequence can give rise to a variety of peptide fragments when subjected to the simulation of enzymatic hydrolysis with various proteolytic enzymes.

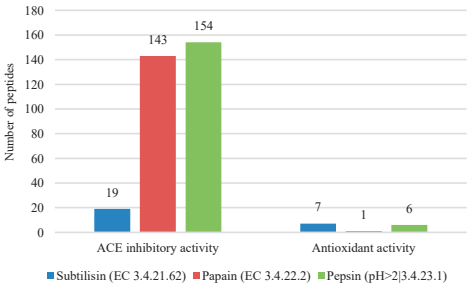


Figure 2. Prediction of the number of potential bioactive peptides in the *in silico* interaction of type I alpha-1 collagen with proteolytic enzymes

Of the three proteases selected for hydrolysis simulation, pepsin generated the highest number of bioactive peptides in the case of inhibitory ACE activity, namely 154. Papain showed 143 bioactive peptides, followed by subtilisin with 19 peptides with antihypertensive activity, generated from the chosen collagen protein.

The results for ACE inhibitory activity are in agreement with the data obtained by Zhang and co-workers (Zhang et al., 2019). They found pepsin to be able to hydrolyze a large number of peptide bonds from collagen substrate, generating ACE inhibitory activity. It is speculated that neutrase, acting at neutral pH, instead of pepsin, acting at acidic pH, could be a better choice for the generation of peptides

with ACE inhibitory and dipeptidyl peptidase IV inhibitory activities (Zhang et al., 2019).
The antioxidant activity was low for all three proteolytic enzymes. Subtilisin showed the highest activity, with a number of 7 peptides released, followed by pepsin with 6 peptides and papain with 1 peptide released.
Pal and Sureh (2017) have reported a small number of antioxidant peptide sequences in the *in silico* simulation of type I alpha-1 collagen proteolysis from *Ctenopharyngodon idella*. The researchers found a number of five antioxidant peptides released when papain was used as a hydrolysis protease and three peptides when digestion was simulated with pepsin (Pal & Suresh, 2017).
It is important to note that the ACE inhibitory activity was predominant when using these three enzymes in our study.
When we simulated the hydrolysis of collagen with a mix of two enzymes, pepsin + papain, a number of 121 peptides released with antihypertensive activity was observed, compared to 8 peptides in the case of antioxidant activity (Figure 3).

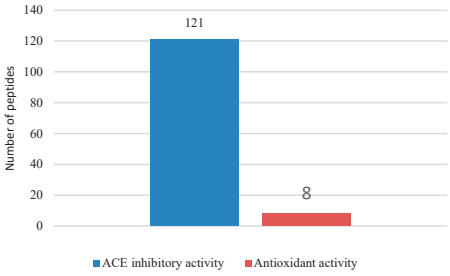


Figure 3. Prediction of the number of potential bioactive peptides in the *in silico* interaction of type I alpha-1 collagen with a mix of two enzymes (pepsin+papain)

In order to observe the potential of the peptides obtained from previous simulations, their profiles were subjected to bioactivity tests. For this purpose, the online tool PeptideRanker was used, which can classify peptides according to their bioactivity, based on structure-function analysis. Scores higher than 0.5 show a potential bioactivity (Pooja et al., 2017; Garg et al., 2018).
In the case of subtilisin, the most bioactive peptides with ACE inhibitory activity that are generated from type I alpha-1 collagen from silver carp (*H. molitrix*) were the MF (methionine – phenylalanine) peptide with a score of 0.99 and RF (arginine - phenylalanine) sequence with a score of 0.98 – detailed in Table 3.

Table 3. Bioactivity score of peptides* obtained from type I alpha-I collagen in the simulation with subtilisin

		Subtilisin		
	Seq	ACE inhibitor score	Seq	Antioxidant score
1	MF	0.996643	RHF	0.876772
2	RF	0.986556	VW	0.802223
3	PGL	0.855192	TY	0.113932
4	TF	0.826678	VY	0.0989681
5	GL	0.808777	-	-

*one letter amino acids abbreviation is used

The amino acid phenylalanine (F) was observed in several sequences. This amino acid was reported to generate bioactivity in short peptides (Mooney et al., 2012).
For the antioxidant activity, the RHF (arginine – histidine – phenylalanine) tripeptide showed a high score of 0.87, followed by the VW sequence with a score of 0.80. The TY and VY peptide sequences showed a bioactivity score below 0.5
The amino acid phenylalanine was also observed in the peptides obtained from papain (Table 4).

Table 4. Bioactivity score of peptides* obtained from type I alpha-I collagen in the simulation with papain

		Papain		
	Seq	ACE inhibitor score	Seq	Antioxidant score
1	SF	0.948796	IR	0.332363
2	NF	0.941145	-	-
3	PG	0.877086	-	-
4	MKG	0.559174	-	-
5	AG	0.546994	-	-

*one letter amino acids abbreviation is used

The SF (serine-phenylalanine) sequence was ranked 1st, with a bioactivity score of 0.948, followed by the NF (asparagine-phenylalanine) peptide with a score of 0.941 and PG peptide with a score of 0.87. The tripeptide MKG (methionine-lysine-glycine) was observed in the 4th place, with a score of 0.55. Significant antioxidant activity was not observed at the

hydrolysis simulation of collagen type I alpha-1 from silver carp (*H. molitrix*) with papain.
Pepsin showed high scores for WG, tryptophan-glycine (0.99), and SF (serine-phenylalanine) (0.94) sequences at ACE inhibitory activity (Table 5).

Table 5. Bioactivity score of peptides obtained from type I alpha-I collagen in the simulation with pepsin

		Pepsin		
	Seq	ACE inhibitor score	Seq	Antioxidant score
1	WG	0.992384	WG	0.992384
2	SF	0.948796	WY	0.974885
3	PG	0.877086	RHF	0.876772
4	RG	0.738353	VY	0.0989681
5	RL	0.626352	-	-

The WG (tryptophan - glycine) sequence was also observed in the case of antioxidant activity, followed by the WY (tryptophan - tyrosine) peptide with a score of 0.97 and the RHF (arginine- histidine – phenylalanine) tripeptide with a score of 0.87.
Table 6 shows the score of the peptides obtained from the combination of two enzymes: pepsin and papain.

Table 6. Bioactivity score of peptides obtained from type I alpha-I collagen in the simulation with a mix of two enzymes

		Pepsin+Papain		
	Seq	ACE inhibitor score	Seq	Antioxidant score
1	WG	0.992384	WG	0.992384
2	SF	0.948796	WY	0.974885
3	PG	0.877086	IR	0.332363
4	PR	0.787626	VY	0.0989681
5	IG	0.501816	-	-

In the case of ACE inhibitory activity, the first three peptides are similar to those obtained from pepsin. Additional 2 sequences, PR (proline-arginine) and IG (isoleucine-glycine), with scores of 0.78 and 0.50, respectively, were generated. The latter two peptides have also been reported by Ningrum and Munawaroh (2019). They used an *in silico* approach to observe the peptides released with antihypertensive activity when pepsin and papain together interact with type I collagen in the tuna fish. In the study of these researchers, PeptideRanker showed a score of 0.99 for the PR peptide and 0.54 for the IG peptide (Ningrum & Munawaroh, 2019). The different score in the case of PR peptide obtained in our study concerning that observed by Ningrum and Munawaroh is probably due to the update of the PeptideRanker program in the last years.
In the case of antioxidant activity, the WG (tryptophan – glycine) and WY (tryptophan – phenylalanine) sequences are the only peptides that showed significant bioactivity and are similar to those obtained from pepsin.
The most bioactive peptides obtained from the interaction of collagen with the three enzymes were subjected to the prediction of physico-chemical characteristics and the prediction of toxicity (Table 7).

Table 7. Prediction of toxicity and physicochemical characteristics of the most bioactive peptides obtained from silver carp collagen

Peptide	Molecular weight (g/mol)	Isoelectric point	Water solubility	Toxicity prediction	Taste
MF	296.39	3.45	low solubility	non-toxic	-
RF	321.37	10.55	good solubility	non-toxic	bitter
PGL	285.34	4.08	low solubility	non-toxic	-
TF	299.29	3.39	low solubility	non-toxic	-
GL	188.22	3.63	low solubility	non-toxic	bitter
RHF	458.51	10.55	good solubility	non-toxic	-
VW	303.63	3.57	low solubility	non-toxic	-
SF	252.27	3.43	low solubility	non-toxic	-
NF	279.29	3.28	low solubility	non-toxic	-
PG	172.18	4.06	low solubility	non-toxic	bitter
MKG	334.44	9.88	good solubility	non-toxic	-
AG	146.14	3.69	low solubility	non-toxic	-
WG	261.28	3.5	low solubility	non-toxic	-
RG	231.25	10.55	good solubility	non-toxic	bitter/ salt enhancer
RL	287.36	10.55	good solubility	non-toxic	bitter
WY	367.4	3.51	low solubility	non-toxic	-
PR	271.32	11.29	good solubility	non-toxic	bitter
IG	188.22	3.63	low solubility	non-toxic	bitter

Most of the peptides generated have a low molecular weight of 0.1-0.4 kDa. The peptide with the highest molecular weight (0.458 kDa) is RHF, followed by the peptides: WY (0.367

kDa), MKG (0.334 kDa), and RF (0.321 kDa). Most peptides showed an isoelectric point at acidic pH around 3 and were predicted to have low solubility in water. The peptides RF, RHF, MKG, RG, RL, and PR, showed the isoelectric point at alkaline pH, between 9 and 11, and were predicted to be soluble in water. The peptides analyzed do not contain amino acid residues considered potentially toxic, ToxinPred classifies all peptide sequences as non-toxic. It has been demonstrated that amino acids such as valine, threonine, arginine, glutamine, methionine, leucine, lysine, isoleucine, phenylalanine, or alanine are non-toxic, alone or in peptide sequences. Amino acids such as proline, histidine, cysteine, and asparagine have been reported to induce toxicity when they are included in some specific biotoxic peptide sequences (Ningrum & Munawaroh, 2019).

In conclusion, no toxic peptides were found for the threshold value set to 0 of the support vector machine, which makes these peptides good targets for further study.

Moreover, most of the peptides analyzed with ACE inhibitory activity showed bitter sensory characteristics. In addition to the bitter taste, the RG (arginine-glycine) peptide also showed salty sensory characteristics. Antioxidant peptides did not show taste characteristics (Table 7).

The last objective of this study was to predict the number of peptides with sensory characteristics resulting from the interaction of the three enzymes with type I alpha-1 collagen from silver carp (*H. molitrix*). Taste is an important indicator in evaluating food, which can help identify possible toxic substances. Peptides generated from various protein structures can affect food taste due to the presence of specific amino acids (Maehashi & Huang, 2009; Ningrum & Munawaroh, 2019).

Using the BIOPEP-UWM database, we found most of the peptides having bitter and umami tastes. We observed that pepsin generated a number of 198 bitter peptides, followed by papain with 87 peptides and subtilisin with 10 bitter peptides. The number of umami peptides was significantly smaller. Papain generated 18 umami peptides, pepsin 17 peptide sequence with umami taste and subtilisin one umami peptide (Figure 4).

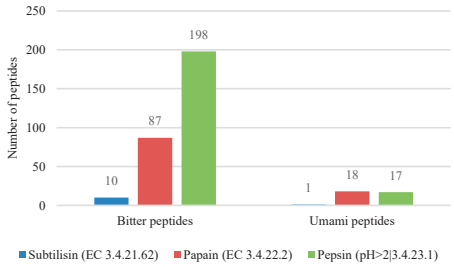


Figure 4. Prediction of the number of bitter and umami peptides in the *in silico* interaction of type I alpha-1 collagen from silver carp (*H. molitrix*) with different enzymes

CONCLUSIONS

This study is the first to show the advantages of using an *in silico* approach to obtain bioactive peptides from type I alpha-1 collagen in silver carp (*Hypophthalmichthys molitrix*).

The analysis of the profile of the potential biological activity of collagen showed several types of possible bioactivities, including antihypertensive and antioxidant activity.

Several non-toxic peptide sequences with possible beneficial bioactivity and appropriate physicochemical properties have been identified and can be utilized for more in-depth studies.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Ministry of Research, Innovation and Digitization, CCCDI – UEFISCDI, project number PN-III-P2-2.1-PTE-2019-018, contract 3PTE-COL-STIM, within PNCDI III. We also thank the Ministry of Research, Innovation and Digitization, Program 1 - Development of the national research and development system, Subprogram 1.2 - Institutional performance - Projects to finance excellence in RDI, Contract no. 31PFE / 2018 and Contract no 15PFE / 2021, for financial support.

REFERENCES

Coppola, D., Oliviero, M., Vitale, G. A., Lauritano, C., D'Ambra, I., Iannace, S., & de Pascale, D. (2020). Marine Collagen from Alternative and Sustainable Sources: Extraction, Processing and Applications. *Marine drugs, 18*(4), 214

Czerniecka-Kubicka, A., Neilsen, G., Dickson, M. S., Woodfield, B. F., Janus-Kubiak, M., Kubisz, L., . . . Pyda, M. (2020). Vibrational heat capacity of silver carp collagen. *International Journal of Biological Macromolecules, 163*, 833-841

Gan, R., He, Y., & Li, Y. (2022). Structural characteristics of taste active peptides in protein hydrolysates from tilapia by-products. *Journal of Food Measurement and Characterization*, *16*, 1674–1687.

Garg, S., Apostolopoulos, V., Nurgali, K., & Mishra, V. K. (2018). Evaluation of in silico approach for prediction of presence of opioid peptides in wheat. *Journal of Functional Foods, 41*, 34-40

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D., & Bairoch, A. (2005). Protein Identification and Analysis Tools on the ExPASy Server. In J. M. Walker (Ed.), *The Proteomics Protocols Handbook* (pp. 571-607): Humana Press.

Gauza-Włodarczyk, M., Kubisz, L., & Włodarczyk, D. (2017). Amino acid composition in determination of collagen origin and assessment of physical factors effects. *International Journal of Biological Macromolecules, 104*, 987-991

Iwaniak, A., Minkiewicz, P., Darewicz, M., Sieniawski, K., & Starowicz, P. (2016). BIOPEP database of sensory peptides and amino acids. *Food Research International, 85*, 155-161

Jafari, H., Lista, A., Siekapen, M. M., Ghaffari-Bohlouli, P., Nie, L., Alimoradi, H., & Shavandi, A. (2020). Fish Collagen: Extraction, Characterization, and Applications for Biomaterials Engineering. *Polymers, 12*(10), 2230

Karim, A. A., & Bhat, R. (2009). Fish gelatin: properties, challenges, and prospects as an alternative to mammalian gelatins. *Food Hydrocolloids, 23*(3), 563-576

Maehashi, K., & Huang, L. (2009). Bitter peptides and bitter taste receptors. *Cellular and Molecular Life Sciences, 66*(10), 1661-1671

Minkiewicz, P., Iwaniak, A., & Darewicz, M. (2019). BIOPEP-UWM Database of Bioactive Peptides: Current Opportunities. *International Journal of Molecular Sciences, 20*(23), 5978

Mooney, C., Haslam, N. J., Pollastri, G., & Shields, D. C. (2012). Towards the improved discovery and design of functional peptides: common features of diverse classes permit generalized prediction of bioactivity. *PloS one, 7*(10), e45012

Ningrum, A., & Munawaroh, H. S. (2019). In Silico approach of collagen from tuna fish by-product as angiotensin-converting enzyme inhibitor. *Asian Journal of Pharmaceutical and Clinical Research, 12*(10), 113-117

Pal, G. K., & Suresh, P. V. (2016). Sustainable valorisation of seafood by-products: Recovery of collagen and development of collagen-based novel functional food ingredients. *Innovative Food Science & Emerging Technologies, 37*, 201-215

Pal, G. K., & Suresh, P. V. (2017). Physico-chemical characteristics and fibril-forming capacity of carp swim bladder collagens and exploration of their potential bioactive peptides by in silico approaches. *International Journal of Biological Macromolecules, 101*, 304-313

Pati, F., Adhikari, B., & Dhara, S. (2010). Isolation and characterization of fish scale collagen of higher thermal stability. *Bioresource Technology, 101*(10), 3737-3742

Pooja, K., Rani, S., & Prakash, B. (2017). In silico approaches towards the exploration of rice bran proteins-derived angiotensin-I-converting enzyme inhibitory peptides. *International Journal of Food Properties, 20*(sup2), 2178-2191

Rules, T. (1968). IUPAC-IUB Commission on Biochemical Nomenclature A One-Letter Notation for Amino Acid Sequences 1–3. *Journal of Biological Chemistry*, 243(13), 3557-3559.

Shoulders, M. D., & Raines, R. T. (2009). Collagen structure and stability. *Annual review of biochemistry, 78*, 929-958

Song, J. J., Wang, Q., Du, M., Ji, X. M., & Mao, X. Y. (2017). Identification of dipeptidyl peptidase-IV inhibitory peptides from mare whey protein hydrolysates. *Journal of Dairy Science, 100*(9), 6885-6894

Zhang, Y., Liu, H., Hong, H., & Luo, Y. (2019). Purification and identification of dipeptidyl peptidase IV and angiotensin-converting enzyme inhibitory peptides from silver carp (Hypophthalmichthys molitrix) muscle hydrolysate. *European Food Research and Technology, 245*, 243-255

Zhao, Y., Zhang, M., Devahastin, S., & Liu, Y. (2019). Progresses on processing methods of umami substances: A review. *Trends in Food Science & Technology*, *93*, 125-135.

MEDICAL AND PHARMACEUTICAL BIOTECHNOLOGY

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

BACTERIAL NANOCELLULOSE AND ITS APPLICATION FOR MUCOADHESIVE FORMULATIONS

Angela MORARU[1, 2], Florin OANCEA[3, 1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd., District 1, Bucharest, Romania
[2]Laboratoarele Medica Srl, 11, Frasinului, Otopeni, Romania
[3]National Institute for Research & Development in Chemistry and Petrochemistry - ICECHIM, 202 Splaiul Independenței, District 6, Bucharest, Romania

Corresponding author email: florin.oancea@icechim.ro

Abstract

Bacterial nanocellulose (BNC) is a fibrillar nanomaterial composed of β-(1 → 4) glucan chains, with <100 nm widths. Usually, the BNC is produced by mechanical disintegration of the cellulose fibrils network biosynthesized by several bacterial species, both gram-negative bacteria such as acetic acid bacteria, agrobacteria, rhizobia, and gram-positive bacteria from Sarcina and Bacillus genera. One dimension of BC is still micrometric; therefore, it is considered a 1D nanomaterial. BNC presents suitable mucoadhesive formulation features – biocompatibility and biodegradability, water retention, shear-thinning, good interaction with mucin. In this review, we focus mainly on the non-Newtonian behavior / shear-thinning characteristic of the BNC hydrogel. Due to this characteristic, BNC could be used as an in-situ thickener for the mucoadhesive formulations, which generate low viscosity gel and droplets.

Key words*: bacterial nanocellulose, mucoadhesive formulations, in situ thickener, shear-thinning.*

INTRODUCTION

Nanocellulose is a fibrillar (cylindrical) nanomaterial composed of β-(1 → 4) glucan chains (Thomas *et al.*, 2020). Nanocellulose is considered a 1D-nanomaterial because one of its dimensions, the length, is usually outside of the upper limit of the nano-range, i.e., 200 nm (Fang *et al.*, 2019).

Nanocellulose is usually prepared from two approaches. The top-down process involves: (i) separation of cellulose fibrils from the other biopolymers associated with the plant lignocellulose matrix, majors (hemicelluloses, lignin) and minor (pectin / pectic fractions and glycoproteins such as arabinogalactan), and (ii) conversion of the separate cellulose fibers into nanocellulose (Pirich *et al.*, 2020). The nanocellulose produced from the top-down process is classified as cellulose-nanofibrils (CNF) and cellulose nanocrystals (CNC) (Gupta & Shukla, 2020). CNF diameter is from 5 to 60 nm, and its length is in the micron range (Oberlintner *et al.*, 2021). The average dimension of CNC depends on the preparation methods, from 3 to 35 nm in diameter and from 20 nm to 1000 nm in length (Clemons, 2016; Nechyporchuk *et al.*, 2016).

The bottom-up process is a biosynthetic one. The resulting nanomaterial is called bacterial nanocellulose because it is produced by converting the cellulose fibrils produced by bacteria (de Amorim *et al.*, 2020). Various bacterial species, both gram-negative bacteria such as acetic acid bacteria, agrobacteria, rhizobia, and gram-positive bacteria from *Sarcina* and *Bacillus* genera, produce cellulose (Jozala *et al.*, 2016; Romling & Galperin, 2015). Bacterial cellulose is free of lignin, hemicellulose, pectin, glycoproteins. Usually, bacterial cellulose is produced as a membrane/ pellicle, including a network of β-(1 → 4) glucan chains (Sharma & Bhardwaj, 2019). The conversion of bacterial cellulose ribbon to bacterial nanocellulose (fibrils) is usually done by applying high-shear forces intended to de-fibrillate the fibrils network. Such high-shear forces are typically provided by microfluidization equipment (Dima *et al.*, 2017; Salimi *et al.*, 2019), ball milling (Piras *et al.*, 2019), or colloidal milling (Panaitescu *et al.*, 2016).

Bacterial nanocellulose (BNC), prepared by a bottom-up approach, has several advantages comparing to plant nanocellulose. These advantages result from the absence of other biopolymers and the more porous structure and larger surface area (de Amorim *et al.*, 2020).
The absence of other associated biopolymers (and/or their degradation products formed during the preparation process) reduces the risk for adverse biological reactions. The porous structure promotes hydrogel formation and enhances the capacity of loading with bioactive substances. Due to these characteristics, bacterial nanocellulose gathered more interest in the biomedical field than plant nanocellulose (Jozala *et al.*, 2016; Sharma & Bhardwaj, 2019). Various biomedical applications were reported for nanocellulose: formulation agent for drug/ bioactive compounds delivery ((Pötzinger *et al.*, 2017); support and enhancer for wound-dressing formulation (Liyaskina *et al.*, 2017); scaffold for cell and tissue culture (Sämfors *et al.*, 2019), carriers for 3D bio-ink formulations used in 3D bioprinters (Apelgren *et al.*, 2019).
In this review, we focus on the BNC utilization for mucoadhesive formulations. We will describe several features essential for mucoadhesive formulations, such as non-Newtonian behavior/shear-thinning and mucin interaction of the BNC hydrogel.

NANOBACTERIAL CELLULOSE PREPARATION

As we already mentioned, bacterial cellulose (BC) is produced by bacterial biosynthesis. Several bacterial strains produce cellulose as the main component of a biofilm with specific functions. Such BC biofilms facilitate bacteria interactions with other microorganisms (in the mixed biofilms consortia) or with their metazoans hosts and promote survival in extreme environments.
In the case of Proteobacteria, BC facilitates the establishment of beneficial (i.e., symbiotic) or deleterious (i.e., pathogenic) relationships with plant, fungal or animal hosts (Augimeri *et al.*, 2015). Rhizobia produce BC during their early legume root colonization (Poole *et al.*, 2018). *Pseudomonadaceae* use their BC to promote phyllosphere colonization (Arrebola *et al.*, 2015). Enterobacteriaceae produce BC to facilitate their adherence to fresh vegetables and further intestinal colonization (Yu & Shi, 2021). In other bacteria, BC biofilm promotes their development in an environment with extreme conditions. Acetic acid bacteria produce BC pellicle, which allows them to survive at a pH lower than 1 (Qiu *et al.*, 2021). Thermophilic *Bacillus licheniformis* strain ZBT2 produces bacterial cellulose biofilm at 50°C temperature (Bagewadi *et al.*, 2020). Cellulose is the main component of the sulfur-turf bacterial mat developed in Yellowstone hot springs (Ogawa & Maki, 2003; Romling & Galperin, 2015).
The scheme of the BC production and conversion to BNC is presented in Figure 1.

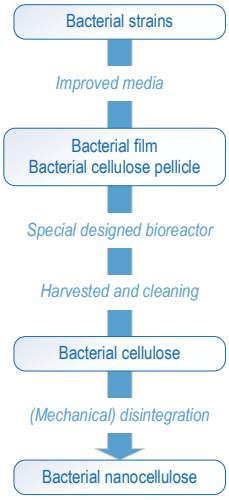


Figure 1. Scheme of bacterial nanocellulose (BC) production and its conversion to bacterial nanocelllulose (BNC)

Because BC is associated with biofilm formation, BC biosynthesis is challenging. The formation of the large pellicle, including strong cellulose chains/fibrils, require static conditions. However, BC producers are aerobes needing large O_2 amounts, challenging to be supplied in static conditions. BC production in submerged cultivation was also tested. In submerged fermentation, BC in the form of small pellets was produced, instead of BC membrane/pellicle BC prepared from pellets present worst mechanical properties, due to a lower degree of polymerization and reduced crystallinity, an, compared to BC prepared from pellicle produced by the same strains, in the same cultivation media, in static conditions. These

worst mechanical conditions, associated with a less-organized form of BC, can result from the shear stress resulting during the agitation for aeration of the submerged biosynthesis.

Various bioreactor designs were explored to increase BC production, improving the ratio between the oxygen-rich surface and total bioreactor volume. Such design included: cylindrical silicon vessel, aerated from bellow (Yoshino *et al.*, 1996); rotating disk reactors (Serafica *et al.*, 2002); rotary drum bioreactor (G. Chen *et al.*, 2019); moving bed biofilm reactor (Cheng *et al.*, 2009), static, intermittent fed-batch (Sharma & Bhardwaj, 2019).

Another challenge to producing bacterial cellulose/nanocellulose is related to the composition of the media used to make cellulose by cultivating the acetic acid bacteria, including the most known producer of bacterial cellulose - *Komagataeibacter xylinus*. The initial growing media was a complex media, including glucose, yeast extract, and peptone (Hestrin & Schramm, 1954). Other complex media were developed, replacing the peptone with corn steep liquor (Zhou *et al.*, 2007) or with ammonium sulfate (Yamanaka *et al.*, 1989). Various by-products from the bioeconomy were used as ingredients of such complex media to produce bacterial cellulose: raw glycerol, a by-product from the production of the biodiesel from soybean oil (Jung *et al.*, 2010; Tsouko *et al.*, 2015); the wastewater from acetone - butanol - ethanol fermentation (Huang *et al.*, 2015); spent beer yeast ((D. Lin *et al.*, 2014); vinasse from the production of (bio)ethanol from molasses (Barshan *et al.*, 2019); cheese whey and sugar beet molasses (Salari *et al.*, 2019); kitchen waste (M. Wu *et al.*, 2019); sugar cane molasses (Abol-Fotouh *et al.*, 2020).

Chemically defined media were also developed almost twenty years ago (Heo & Son, 2002), replacing bacterial growth factors from yeast extract with inositol and nicotinamide. Further developments lead to delivering a minimal defined media (de Souza *et al.*, 2019). Table 1 summarizes the composition of several complex media and the chemically defined and minimal defined media developed in the last years.

Table 1. Composition of the various media used to produce bacterial nanocellulose

Components	Complex Media (g/L) (Hestrin & Schramm, 1954)	Complex Media (g/L) (Yamanaka *et al.*, 1989)	Complex Media (g/L) (Zhou *et al.*, 2007)	Chemically defined medium (g/L) (Heo & Son, 2002)	Minimal defined medium (g/L) (de Souza *et al.*, 2019)
Glucose	20	50	40	40	4.5
Ethanol	-	-	-	11.05	
Corn steep liquor	–	–	20	-	-
Yeast extract	5	5	–	-	-
Peptone	5	–	–	-	-
Na_2HPO_4	2.7	–	–	3.0	2.32
Citric acid. H_2O	1.15	–	–	-	-
Inositol				0.0025	-
Nicotinamide				0.0006	-
$(NH_4)_2Cl$				-	0.5448
$(NH_4)_2SO_4$	–	5	4	2.0	-
KH_2PO_4	–	3	2	2.5	1.2
$MgSO_4.7H_2O$	–	0.05	0.4	0.05	0.1971
$CaCl_2x_2H_2O$	-	-	-	-	0.0294
H_3BO_3	-			0.0025	-
$FeSO_4x7H_20$	-	-	-	0.002	-

Bacterial nanocellulose was used for several biomedical applications. Several of these applications are listed in Table 2.

Essential BNC characteristics for biomedical applications are, besides those already mentionned, higher surface area and higher porosity, are water retention/thickener, and shear-thinning. Our group developed a process for preparing bacterial nanocellulose from the Kombucha pellicle, a side-stream of the production of a healthy beverage - tea fermented with a SCOBY consortium (Dima *et al.*, 2017).

Table 2. Biomedical applications of bacterial nanocellulose

Application	Other ingredients	References
Wound dressing	silver nanoparticles	(J. Wu *et al.*, 2014)
	chitosan	(W.-C. Lin *et al.*, 2013)
	montmorillonite	(Ul-Islam *et al.*, 2013)
Drug delivery	tizanidine, famotidine	(Badshah *et al.*, 2018)
	tetracycline	(Shao *et al.*, 2016)
Scaffold for cell culture	bone morphogenetic protein-2	(Shi *et al.*, 2012)
Bio-ink for the 3D bioprinter	alginate	(Jessop *et al.*, 2019)
Facial mask	essential oil	(Indrianingsih *et al.*, 2020)
Mucoadhesive formulation	curcumin, gelation	(Chiaoprakobkij *et al.*, 2020)

We should mention here that the bacterial pellicle protects the SCOBY consortium from extreme conditions similar to Mars (Orlovska *et al.*, 2021).

We develop the process involving an alkaline cleaning process (intended to remove both biomass and melanoidins formed during SCOBY fermentation) and a two-step defibrillation process - Figure 2. The resulting BNC exhibit higher swelling/higher water retention characteristics than the BNC produced by the pure culture of acetic acid bacteria. This higher porosity is also because the pellicle should accommodate a consortium that includes yeast, larger than acetic acid bacteria.

The nanocellulose produced from Kombucha has better water retention and shear thinning characteristics compared to nanocellulose made by pure acetic acid bacteria.

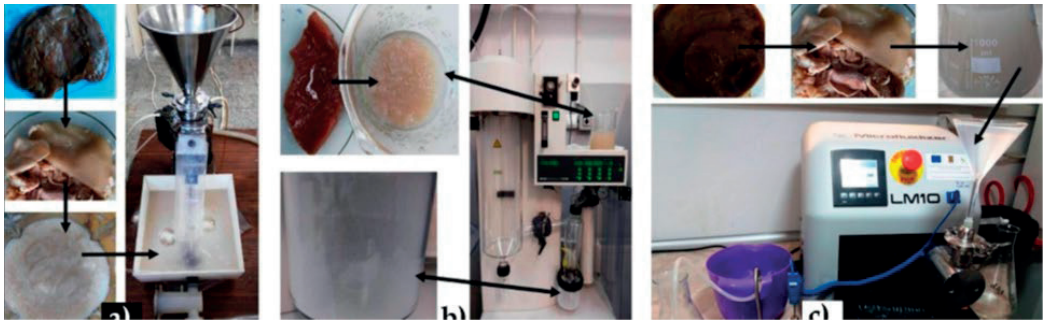


Figure 2. Purification and preparation of bacterial nanocellulose (BNC) from Kombucha pellicles: (a) purification, grinding with a blender, and deep grinding with a colloidal mill; (b) purification by alkaline treatment and drying by using a spray-dryer; (c) purification by alkaline treatment and defibrillation by microfluidization at pressures higher than 1300 bar. From Dima *et al.* (2017).

Never dried bacterial nanocellulose

For mucoadhesive formulations, one of the most efficient forms of BNC is the never-dried BNC. Never-dried BNC (NDBNC) has specific properties that result from its biosynthesis peculiarities. Clusters of the water molecules adsorbed by the nascent β-(1 → 4) glucan chains are kept in their initial, native stages. During the drying process, the structure of BNC collapse (S.-Q. Chen *et al.*, 2020), and the loading capacity for the bioactive ingredients significantly decreases.

BNC has a wide palette of properties, like biocompatibility, high hydrophilicity, flexibility, transparency, high mechanical strength, chemical stability, high surface area, and rich surface chemistry. NDBNC also has a higher purity compared to plant cellulose and a high degree of polymerization (up to 8000), presents a 3D network of nanofibrils of 40-60 nm in diameter and has a crystallinity up to 90% and a water content up to 99% (Muller *et al.*, 2013). Water vapor permeability of NDBNC varies from 1 x 10^{-13} g m^{-1} s^{-1} Pa^{-1} at 31% humidity to 1.1 x 10^{-12} g m^{-1} s^{-1} Pa^{-1} at 82% humidity. Moreover, NDBNC showed biocompatibility and favorable adhesion and growth of tissue cells such as chondrocytes on the BNC matrix (Ahrem *et al.*, 2014). Unmodified NDBNC showed a three-layer structure with a heterogeneous pore distribution and a relatively low diffusion of bovine chondrocytes through the outer layers, of a maximum 70 μm depth. From the rheological point of view, NDBNC showed

an elastic behavior, but not a visco-elastic one, and also presents a strain-independent elastic modulus, without meaningful changes after 26% cellulose loss by laser perforation. NDBNC has a superior mechanically resistance and a higher adsorption capacity for proteins, like cellobiose dehydrogenase, for peptides and chimeric proteins. (Muller *et al.*, 2013). Compared to freeze-dried BNC who presented a 7.2% uptake capacity and 12.5-128.3% bovine serum albumin loading, never-dried BNC showed a 9.8% uptake capacity and 15.5-132.2% protein loading. Also, NDBNC has a lower susceptibility towards esterification with organic acids than freeze-dried BNC. NDBC has a lower susceptibility to chemical functionalization in general, maybe because of the residual, intermolecular water molecules that block a part of the -OH groups (Lee & Bismarck, 2012).

NDBNC low susceptibility to chemical functionalization makes it more attractive to physical modification and blending. Nanocellulose blending is largely used to obtain nanocomposites, aerogels, hydrogels, and other nanomaterials. Still, the majority of the studies are using nanocellulosic materials (cellulose nanocrystals – CNC, cellulose nano-fibers – CNF) derived from wooden plants (Abitbol *et al.*, 2016; De France *et al.*, 2017; Dumanli, 2017; Zubik *et al.*, 2017). NDBNC can be physically functionalized in micro-channels creation using 3D laser perforation and cutting by pulsed CO_2 (Ahrem *et al.*, 2014). The 300 µm laser beam performed uniform 200 µm channels inside the BNC matrix. Channels proved to increase the porous hydrogel's homogeneity without changes in the elastic modulus. This finding means that, without losing biomechanical resistance, although an estimated 26% BNC is lost by laser perforation. Moreover, FT-IR and XPS analyses evidenced no chemical changes in the hydrogel's structure after laser contact and no undesirable effect on the vitality of bovine chondrocytes. Instead, a stimulated growth of vital cells inside the laser-perforated hydrogel, making the BNC hydrogels more appealing in tissue engineering as an implant material for *in situ* cellularizations (Ahrem *et al.*, 2014). The porosity of NDBNC can also be modified during the biosynthesis process using water-soluble and insoluble additives like β-cyclodextrin, poly(ethylene glycol), or carboxymethylcellulose (Muller *et al.*, 2013). Further physical modification can be performed by coating BNC with polymers, metals, metal oxides (Muller *et al.*, 2013). Chemical functionalization of NC includes phosphorylation, amidoximation, carboxy-methylation (Muller *et al.*, 2013), functionalization with carboxylic acids, acrylamide, xyloglucan, alkyl chains (Mondal, 2017), or with trimethylsilane (Grunert and Winter, 2002). NDBNC was less studied as nanofiller or structural nanomaterial. Some blends of NDBNC are reported to be performed using poly(vinyl alcohol) (Tan *et al.*, 2015) or chitosan (Cabañas-Romero *et al.*, 2020).

MUCOADHESIVE FORMULATIONS

Biocompatible drug-delivery systems are of high interest for the medical and pharmaceutical fields, particularly in developing new alternative products for adjusting vaginal microflora imbalances. Some contain probiotic lactic strains (Liu et al., 2012), but in the case of lactobacilli presence in the treated vagina, the products have limited efficacy due to intra-specific competition. Moreover, they can induce adverse effects, such as urinary tract infections (Czaja et al., 2007).

Various mucoadhesive compositions are known to be desirable for re-balancing vaginal microflora. The normal vaginal microflora in women at the age of reproduction is characterized by the dominance of lactic acid-producing bacteria from the *Lactobacillus* group. They maintain an acidic pH of the vaginal fluids and represent a biomarker of the health status (Palmeira-de-Oliveira et al., 2015).

More than 70% of adult women had vaginal problems and used vaginal products to treat infections. Recent studies have demonstrated the antimicrobial, antifungal, and immunomodulatory properties of lactic acid produced by lactobacilli and have evaluated the use of lactic acid or probiotic lactobacilli in the prevention or treatment of bacterial vaginitis (Tachedjian et al., 2017).

An alternative to probiotics is represented by the new generation of prebiotics such as polyphenols from plant extracts, which specifically stimulate the growth of microbial populations from healthy human microbiocenosis. In some

compositions, prebiotics compounds are the only active ingredients. In others, they are associated with estrogenic plant extracts that favor the trophism of vaginal mucosa (Bou A.S., 2010) or with anti-Candida thiazole antibiotics (Dikovskiy et al., 2015).

Essential oils have a significant capacity to remove biofilms formed by pathogenic microorganisms in the vagina (Bogavac et al., 2015) and are considered an important component of new strategies for local treatment of intestinal microflora imbalances. However, essential oils may inhibit the development of lactobacilli (Dunn et al., 2016). To increase the selectivity of essential oils towards beneficial vaginal microflora, new technical solutions are required to protect lactobacilli and stimulate their development.

Another essential characteristic of the (ND)BNC for the mucoadhesive formulation is related to the non-Newtonian behavior, i.e., shear-thinning. Such behavior means that when a force is applied to the (ND)BNC hydrogel, the viscosity of the solution is significantly decreesing. Such a feature allows the development of a formulation that is a robust and stable gel (keeping uniformly distributed low soluble ingredients), easy generate low-viscosity droplets, which reconstitute stable, adhesive droplets on the target organs - Figure 3.

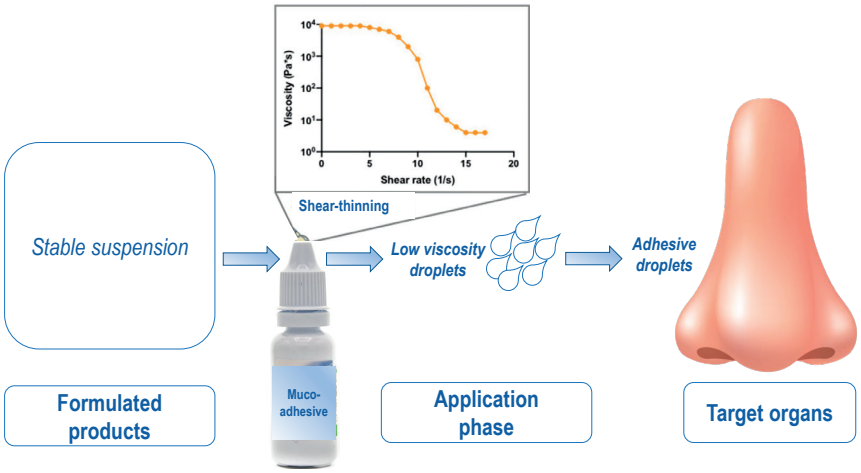


Figure 3. Illustration of the importance of the shear-thinning feature for the formulation of stable, easy to apply and efficient mucoadhesive product

Such a shear-thinning characteristic is essential also for the development of the bio-ink used for 3D-bioprinting. Stable suspension should be converted in low viscosity droplets, which produce stable and adhesive additive layers (Wilson *et al.*, 2017).

Another characteristic which is necessary for the mucoadhesive formulation is thermogelling. Thermogelling systems have been developed to improve vaginal drug delivery due to their liquid form at room temperature and the sol-gel transition at physiological temperature. Additionally, after gelling, these systems may exhibit mucoadhesion improving retention in the vaginal cavity. The gelling temperature (T_{gel}) specific to each termogelling system is crucial for their performance and ranges between 25-37°C. Termogelling properties of the vaginal formulations rely on the use of some specific polymers, among which poloxamers were the most studied. At physiological temperature in suitable interactions, poloxamers solutions change from micellar properties and hydrophobic interactions, leading to a reversible sol-gel transition. (Palmeira-de-Oliveira et al., 2015).

To develop such formulation, additional polymers are needed. Pharmaceutical poloxamers are available under the trade name Lutrol® (Europa) and Pluronic® (BASF), Pluronic® F127 (poloxamer 407, P407) and Pluronic® F68 (poloxamer 188, P188) (SUA) (Palmeira-de-Oliveira et al., 2015). The role of mucoadhesive polymers like poloxamer, chitosan, gelatin

cellulose derivatives, and the combination thereof is essential in obtaining various efficient product for the treatment of urogenital infections, especially vaginal infections.

CONCLUSIONS

Bacterial nanocellulose (BC) and never dried cellulose (NDBNC) presents essential features for mucoadhesive formulation features – biocompatibility and biodegradability, water retention, shear-thinning, good interaction with mucin. Shear-thinning is essential for a formulation that is a robust and stable gel (keeping uniformly distributed low soluble ingredients), easy generate low-viscosity droplets, which reconstitute stable, adhesive droplets on the target organs.
Additional biopolymers and active ingredients are necessary to optimise others characteristic, such as thermogelling, important for formulation of the efficient mucoadhesive products.

ACKNOWLEDGEMENTS

This work was supported by European Regional Development Fund (ERDF), the Competitiveness Operational Programme (POC), Axis 1, project POC-A1-A1.2.3-G-2015-P_40_352, My_SMIS 105684 SECVENT.

REFERENCES

Abitbol, T., Rivkin, A., Cao, Y., Nevo, Y., Abraham, E., Ben-Shalom, T., Lapidot, S., & Shoseyov, O. (2016). Nanocellulose, a tiny fiber with huge applications. *Current Opinion in Biotechnology, 39*, 76-88. doi:10.1016/j.copbio.2016.01.002

Abol-Fotouh, D., Hassan, M. A., Shokry, H., Roig, A., Azab, M. S., & Kashyout, A. E.-H. B. (2020). Bacterial nanocellulose from agro-industrial wastes: low-cost and enhanced production by Komagataeibacter saccharivorans MD1. *Scientific reports, 10*(1), 1-14.

Ahrem, H., Pretzel, D., Endres, M., Conrad, D., Courseau, J., Muller, H., Jaeger, R., Kaps, C., Klemm, D. O., & Kinne, R. W. (2014). Laser-structured bacterial nanocellulose hydrogels support ingrowth and differentiation of chondrocytes and show potential as cartilage implants. *Acta Biomaterialia, 10*(3), 1341-1353. doi:10.1016/j.actbio.2013.12.004

Apelgren, P., Karabulut, E., Amoroso, M., Mantas, A., Martínez Ávila, H. c., Kölby, L., Kondo, T., Toriz, G., & Gatenholm, P. (2019). In vivo human cartilage formation in three-dimensional bioprinted constructs with a novel bacterial nanocellulose bioink. *ACS Biomaterials Science & Engineering, 5*(5), 2482-2490.

Arrebola, E., Carrión, V. J., Gutiérrez-Barranquero, J. A., Pérez-García, A., Rodríguez-Palenzuela, P., Cazorla, F. M., & de Vicente, A. (2015). Cellulose production in Pseudomonas syringae pv. syringae: a compromise between epiphytic and pathogenic lifestyles. *FEMS microbiology ecology, 91*(7), fiv071.

Augimeri, R. V., Varley, A. J., & Strap, J. L. (2015). Establishing a Role for Bacterial Cellulose in Environmental Interactions: Lessons Learned from Diverse Biofilm-Producing Proteobacteria. *Frontiers in Microbiology, 6*(1282). doi:10.3389/fmicb.2015.01282

Badshah, M., Ullah, H., Khan, A. R., Khan, S., Park, J. K., & Khan, T. (2018). Surface modification and evaluation of bacterial cellulose for drug delivery. *International journal of biological macromolecules, 113*, 526-533. doi:https://doi.org/10.1016/j.ijbiomac.2018.02.135

Bagewadi, Z. K., Bhavikatti, J. S., Muddapur, U. M., Yaraguppi, D. A., & Mulla, S. I. (2020). Statistical optimization and characterization of bacterial cellulose produced by isolated thermophilic Bacillus licheniformis strain ZBT2. *Carbohydrate research, 491*, 107979.

Barshan, S., Rezazadeh-Bari, M., Almasi, H., & Amiri, S. (2019). Optimization and characterization of bacterial cellulose produced by Komagatacibacter xylinus PTCC 1734 using vinasse as a cheap cultivation medium. *International journal of biological macromolecules, 136*, 1188-1195.

Cabañas-Romero, L. V., Valls, C., Valenzuela, S. V., Roncero, M. B., Pastor, F. J., Diaz, P., & Martínez, J. (2020). Bacterial cellulose–chitosan paper with antimicrobial and antioxidant activities. *Biomacromolecules, 21*(4), 1568-1577.

Chen, G., Chen, L., Wang, W., Hong, F. F., & Zhu, M. (2019). Manufacture of a novel anisotropic bacterial nanocellulose hydrogel membrane by using a rotary drum bioreactor. *Carbohydrate Polymers, 211*, 281-288.

Chen, S.-Q., Cao, X., Li, Z., Zhu, J., & Li, L. (2020). Effect of lyophilization on the bacterial cellulose produced by different Komagataeibacter strains to adsorb epicatechin. *Carbohydrate Polymers, 246*, 116632.

Cheng, K.-C., Catchmark, J. M., & Demirci, A. (2009). Enhanced production of bacterial cellulose by using a biofilm reactor and its material property analysis. *Journal of biological engineering, 3*(1), 1-10.

Chiaoprakobkij, N., Suwanmajo, T., Sanchavanakit, N., & Phisalaphong, M. (2020). Curcumin-Loaded Bacterial Cellulose/Alginate/Gelatin as A Multifunctional Biopolymer Composite Film. *Molecules, 25*(17), 3800. Retrieved from https://www.mdpi.com/1420-3049/25/17/3800

Clemons, C. (2016). Nanocellulose in spun continuous fibers: a review and future outlook. *Journal of Renewable Materials, 4*(5), 327-339.

de Amorim, J. D. P., de Souza, K. C., Duarte, C. R., da Silva Duarte, I., de Assis Sales Ribeiro, F., Silva, G. S., de Farias, P. M. A., Stingl, A., Costa, A. F. S.,

Vinhas, G. M., & Sarubbo, L. A. (2020). Plant and bacterial nanocellulose: production, properties and applications in medicine, food, cosmetics, electronics and engineering. A review. *Environmental Chemistry Letters, 18*(3), 851-869. doi:10.1007/s10311-020-00989-9

De France, K. J., Hoare, T., & Cranston, E. D. (2017). Review of Hydrogels and Aerogels Containing Nanocellulose. *Chemistry of Materials, 29*(11), 4609-4631. doi:10.1021/acs.chemmater.7b00531

de Souza, S. S., Berti, F. V., de Oliveira, K. P., Pittella, C. Q., de Castro, J. V., Pelissari, C., Rambo, C. R., & Porto, L. M. (2019). Nanocellulose biosynthesis by Komagataeibacter hansenii in a defined minimal culture medium. *Cellulose, 26*(3), 1641-1655.

Dima, S.-O., Panaitescu, D.-M., Orban, C., Ghiurea, M., Doncea, S.-M., Fierascu, R. C., Nistor, C. L., Alexandrescu, E., Nicolae, C.-A., & Trică, B. (2017). Bacterial nanocellulose from side-streams of kombucha beverages production: Preparation and physical-chemical properties. *Polymers, 9*(8), 374.

Dumanli, A. G. (2017). Nanocellulose and its Composites for Biomedical Applications. *Current Medicinal Chemistry, 24*(5), 512-528. doi:10.2174/0929867323666161014124008

Fang, Z. Q., Hou, G. Y., Chen, C. J., & Hu, L. B. (2019). Nanocellulose-based films and their emerging applications. *Current Opinion in Solid State & Materials Science, 23*(4), 15. doi:10.1016/j.cossms.2019.07.003

Gupta, G. K., & Shukla, P. (2020). Lignocellulosic Biomass for the Synthesis of Nanocellulose and Its Eco-Friendly Advanced Applications. *Frontiers in Chemistry, 8*(1203). doi:10.3389/fchem.2020.601256

Heo, M. S., & Son, H. J. (2002). Development of an optimized, simple chemically defined medium for bacterial cellulose production by Acetobacter sp. A9 in shaking cultures. *Biotechnology and applied biochemistry, 36*(1), 41-45.

Hestrin, S., & Schramm, M. (1954). Synthesis of cellulose by Acetobacter xylinum. 2. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose. *Biochemical Journal, 58*(2), 345.

Huang, C., Yang, X. Y., Xiong, L., Guo, H. J., Luo, J., Wang, B., Zhang, H. R., Lin, X. Q., & Chen, X. D. (2015). Evaluating the possibility of using acetone-butanol-ethanol (ABE) fermentation wastewater for bacterial cellulose production by G luconacetobacter xylinus. *Letters in applied microbiology, 60*(5), 491-496.

Indrianingsih, A. W., Rosyida, V. T., Apriyana, W., Hayati, S. N., Darsih, C., Nisa, K., & Ratih, D. (2020). Antioxidant and antibacterial properties of bacterial cellulose—Indonesian plant extract composites for mask sheet. *Journal of Applied Pharmaceutical Science, 10*(07), 037-042.

Jessop, Z. M., Al-Sabah, A., Gao, N., Kyle, S., Thomas, B., Badiei, N., Hawkins, K., & Whitaker, I. S. (2019). Printability of pulp derived crystal, fibril and blend nanocellulose-alginate bioinks for extrusion 3D bioprinting. *Biofabrication, 11*(4), 045006.

Jozala, A. F., de Lencastre-Novaes, L. C., Lopes, A. M., de Carvalho Santos-Ebinuma, V., Mazzola, P. G., Pessoa-Jr, A., Grotto, D., Gerenutti, M., & Chaud, M. V. (2016). Bacterial nanocellulose production and application: a 10-year overview. *Applied microbiology and biotechnology, 100*(5), 2063-2072.

Jung, H.-I., Jeong, J.-H., Lee, O.-M., Park, G.-T., Kim, K.-K., Park, H.-C., Lee, S.-M., Kim, Y.-G., & Son, H.-J. (2010). Influence of glycerol on production and structural–physical properties of cellulose from Acetobacter sp. V6 cultured in shake flasks. *Bioresource technology, 101*(10), 3602-3608.

Lee, K. Y., & Bismarck, A. (2012). Susceptibility of never-dried and freeze-dried bacterial cellulose towards esterification with organic acid. *Cellulose, 19*(3), 891-900. doi:10.1007/s10570-012-9680-x

Lin, D., Lopez-Sanchez, P., Li, R., & Li, Z. (2014). Production of bacterial cellulose by Gluconacetobacter hansenii CGMCC 3917 using only waste beer yeast as nutrient source. *Bioresource technology, 151*, 113-119.

Lin, W.-C., Lien, C.-C., Yeh, H.-J., Yu, C.-M., & Hsu, S.-h. (2013). Bacterial cellulose and bacterial cellulose–chitosan membranes for wound dressing applications. *Carbohydrate Polymers, 94*(1), 603-611.

Liyaskina, E., Revin, V., Paramonova, E., Nazarkina, M., Pestov, N., Revina, N., & Kolesnikova, S. (2017). *Nanomaterials from bacterial cellulose for antimicrobial wound dressing.* Paper presented at the Journal of Physics: Conference Series.

Mondal, S. (2017). Preparation, properties and applications of nanocellulosic materials. *Carbohydrate Polymers, 163*, 301-316. doi:10.1016/j.carbpol.2016.12.050

Muller, A., Ni, Z., Hessler, N., Wesarg, F., Muller, F., Kralisch, D., & Fischer, D. (2013). The biopolymer bacterial nanocellulose as drug delivery system: Investigation of drug loading and release using the model protein albumin. *Journal of Pharmaceutical Sciences, 102*(2), 579-592. doi:10.1002/jps.23385

Nechyporchuk, O., Belgacem, M. N., & Bras, J. (2016). Production of cellulose nanofibrils: A review of recent advances. *Industrial Crops and Products, 93*, 2-25. doi:https://doi.org/10.1016/j.indcrop.2016.02.016

Oberlintner, A., Likozar, B., & Novak, U. (2021). Hydrophobic functionalization reactions of structured cellulose nanomaterials: Mechanisms, kinetics and in silico multi-scale models. *Carbohydrate Polymers*, 117742.

Ogawa, K., & Maki, Y. (2003). Cellulose as extracellular polysaccharide of hot spring sulfur-turf bacterial mat. *Bioscience, biotechnology, and biochemistry, 67*(12), 2652-2654.

Orlovska, I., Podolich, O. K., Olga, Zaets, I., Reva, O. K., Ludmila, Zmejkoski, D. R., Sergiy Barh, Debmalya Tiwari,Sandeep Kumavath, Ranjith Góes-Neto, Aristóteles , Azevedo, V., Brenig, B., Ghosh, P., & de Vera, J.-P. K., Natalia (2021). Bacterial Cellulose Retains Robustness but Its Synthesis Declines After Exposure to a Mars-Like Environment Simulated Outside the International Space Station. *Astrobiology, 21*(0), null. doi:10.1089/ast.2020.2332

Panaitescu, D. M., Frone, A. N., Chiulan, I., Casarica, A., Nicolae, C. A., Ghiurea, M., Trusca, R., & Damian, C. M. (2016). Structural and morphological

characterization of bacterial cellulose nano-reinforcements prepared by mechanical route. *Materials & Design, 110*, 790-801. doi:https://doi.org/10.1016/j.matdes.2016.08.052

Piras, C. C., Fernández-Prieto, S., & De Borggraeve, W. M. (2019). Ball milling: a green technology for the preparation and functionalisation of nanocellulose derivatives. *Nanoscale Advances, 1*(3), 937-947.

Pirich, C. L., Picheth, G. F., Fontes, A. M., Delgado-Aguilar, M., & Ramos, L. P. (2020). Disruptive enzyme-based strategies to isolate nanocelluloses: a review. *Cellulose, 27*(10), 5457-5475. doi:10.1007/s10570-020-03185-8

Poole, P., Ramachandran, V., & Terpolilli, J. (2018). Rhizobia: from saprophytes to endosymbionts. *Nature Reviews Microbiology, 16*(5), 291.

Pötzinger, Y., Kralisch, D., & Fischer, D. (2017). Bacterial nanocellulose: the future of controlled drug delivery? *Therapeutic delivery, 8*(9), 753-761.

Qiu, X., Zhang, Y., & Hong, H. (2021). Classification of acetic acid bacteria and their acid resistant mechanism. *AMB Express, 11*(1), 1-15.

Romling, U., & Galperin, M. Y. (2015). Bacterial cellulose biosynthesis: diversity of operons, subunits, products, and functions. *Trends in Microbiology, 23*(9), 545-557. doi:10.1016/j.tim.2015.05.005

Salari, M., Khiabani, M. S., Mokarram, R. R., Ghanbarzadeh, B., & Kafil, H. S. (2019). Preparation and characterization of cellulose nanocrystals from bacterial cellulose produced in sugar beet molasses and cheese whey media. *International journal of biological macromolecules, 122*, 280-288.

Salimi, S., Sotudeh-Gharebagh, R., Zarghami, R., Chan, S. Y., & Yuen, K. H. (2019). Production of nanocellulose and its applications in drug delivery: A critical review. *ACS Sustainable Chemistry & Engineering, 7*(19), 15800-15827.

Sämfors, S., Karlsson, K., Sundberg, J., Markstedt, K., & Gatenholm, P. (2019). Biofabrication of bacterial nanocellulose scaffolds with complex vascular structure. *Biofabrication, 11*(4), 045010.

Serafica, G., Mormino, R., & Bungay, H. (2002). Inclusion of solid particles in bacterial cellulose. *Applied microbiology and biotechnology, 58*(6), 756-760.

Shao, W., Liu, H., Wang, S., Wu, J., Huang, M., Min, H., & Liu, X. (2016). Controlled release and antibacterial activity of tetracycline hydrochloride-loaded bacterial cellulose composite membranes. *Carbohydrate Polymers, 145*, 114-120. doi:https://doi.org/10.1016/j.carbpol.2016.02.065

Sharma, C., & Bhardwaj, N. K. (2019). Bacterial nanocellulose: Present status, biomedical applications and future perspectives. *Materials Science and Engineering: C, 104*, 109963. doi:https://doi.org/10.1016/j.msec.2019.109963

Shi, Q., Li, Y., Sun, J., Zhang, H., Chen, L., Chen, B., Yang, H., & Wang, Z. (2012). The osteogenesis of bacterial cellulose scaffold loaded with bone morphogenetic protein-2. *Biomaterials, 33*(28), 6644-6649. doi:https://doi.org/10.1016/j.biomaterials.2012.05.071

Tan, B. K., Ching, Y. C., Poh, S. C., Abdullah, L. C., & Gan, S. N. (2015). A Review of Natural Fiber Reinforced Poly(Vinyl Alcohol) Based Composites: Application and Opportunity. *Polymers, 7*(11), 2205-2222. doi:10.3390/polym7111509

Thomas, P., Duolikun, T., Rumjit, N. P., Moosavi, S., Lai, C. W., Johan, M. R. B., & Fen, L. B. (2020). Comprehensive review on nanocellulose: Recent developments, challenges and future prospects. *Journal of the mechanical behavior of biomedical materials*, 103884.

Tsouko, E., Kourmentza, C., Ladakis, D., Kopsahelis, N., Mandala, I., Papanikolaou, S., Paloukis, F., Alves, V., & Koutinas, A. (2015). Bacterial cellulose production from industrial waste and by-product streams. *International journal of molecular sciences, 16*(7), 14832-14849.

Ul-Islam, M., Khan, T., Khattak, W. A., & Park, J. K. (2013). Bacterial cellulose-MMTs nanoreinforced composite films: novel wound dressing material with antibacterial properties. *Cellulose, 20*(2), 589-596.

Wilson, S. A., Cross, L. M., Peak, C. W., & Gaharwar, A. K. (2017). Shear-thinning and thermo-reversible nanoengineered inks for 3D bioprinting. *ACS applied materials & interfaces, 9*(50), 43449-43458.

Wu, J., Zheng, Y., Song, W., Luan, J., Wen, X., Wu, Z., Chen, X., Wang, Q., & Guo, S. (2014). In situ synthesis of silver-nanoparticles/bacterial cellulose composites for slow-released antimicrobial wound dressing. *Carbohydrate Polymers, 102*, 762-771.

Wu, M., Chen, W., Hu, J., Tian, D., Shen, F., Zeng, Y., Yang, G., Zhang, Y., & Deng, S. (2019). Valorizing kitchen waste through bacterial cellulose production towards a more sustainable biorefinery. *Science of the Total Environment, 695*, 133898.

Yamanaka, S., Watanabe, K., Kitamura, N., Iguchi, M., Mitsuhashi, S., Nishi, Y., & Uryu, M. (1989). The structure and mechanical properties of sheets prepared from bacterial cellulose. *Journal of materials science, 24*(9), 3141-3145.

Yoshino, T., Asakura, T., & Toda, K. (1996). Cellulose production by Acetobacter pasteurianus on silicone membrane. *Journal of fermentation and Bioengineering, 81*(1), 32-36.

Yu, L., & Shi, H. (2021). Recent advances in anti-adhesion mechanism of natural antimicrobial agents on fresh produce. *Current Opinion in Food Science, 42*, 8-14. doi:https://doi.org/10.1016/j.cofs.2021.01.009

Zhou, L., Sun, D., Hu, L., Li, Y., & Yang, J. (2007). Effect of addition of sodium alginate on bacterial cellulose production by Acetobacter xylinum. *Journal of industrial Microbiology and Biotechnology, 34*(7), 483.

Zubik, K., Singhsa, P., Wang, Y. A., Manuspiya, H., & Narain, R. (2017). Thermo-Responsive Poly(N-Isopropylacrylamide)-Cellulose Nanocrystals Hybrid Hydrogels for Wound Dressing. *Polymers, 9*(4). doi:10.3390/polym9040119

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

PREPARATION AND CHARACTERIZATION OF ANTIBACTERIAL CREAMS CONTAINING PLANT EXTRACTS FOR TOPICAL APPLICATION

Ramona-Daniela PAVALOIU[1], Fawzia SHAAT[1], Cristina HLEVCA[1], Georgeta NEAGU[2], Adela STARAS[2], Lucia PIRVU[3]

[1]Department of Synthesis Bioactive Substances and Pharmaceutical Technologies, National Institute for Chemical-Pharmaceutical Research and Development - ICCF Bucharest, 112 Vitan Avenue, District 3, Bucharest, Romania
[2]Department of Pharmacology, National Institute for Chemical-Pharmaceutical Research and Development - ICCF Bucharest, 112 Vitan Avenue, District 3, Bucharest, Romania
[3]Department of Pharmaceutical Biotechnologies, National Institute for Chemical-Pharmaceutical Research and Development - ICCF Bucharest, 112 Vitan Avenue, District 3, Bucharest, Romania

Corresponding author email: fawzya.shaat@gmail.com

Abstract

The use of herbal cosmetics is increasing in the world market, due to the good activity with no side effects of the active ingredients from plant extracts as compared to synthetic compounds. The aim of the present study was to formulate and characterize herbal creams containing several plant extracts (Arctium lappa, Magnolia virginiana, Tiliae flos) in different combinations. The creams were prepared with different ratio of plant extracts and essential oils, while the composition of the cream base was kept the same. The characterization of the formulated creams was carried out by standard methods, for evaluating the organoleptic characteristics, physicochemical properties, microbiological contamination level and in vitro antibacterial activity against Staphylococcus aureus. Also, the plant extracts were evaluated in terms of cytotoxicity using MTS/MTT assay on L-929 fibroblasts. The creams were homogeneous, non-irritant and easily removable. The pH of creams was in the range of 5.0-5.5 which is safe for human skin. The samples were found to be populated with aerobic bacteria, yeast and fungi up to 10 CFU/g and showed moderate antibacterial activity.

Key words*: plant extracts, antibacterial creams, Arctium lappa, Magnolia virginiana, Tiliae flos.*

INTRODUCTION

Arthritis is a term used to describe more than 100 different pathological conditions of the skin and joints, being a chronic inflammatory systemic disease characterized by persistent swelling and stiffness of the joints and destruction of the synovial joints, leading to severe disability with early mortality (Sayah & English, 2005; McInnes, & Schett, 2011). Among the most common causes are trauma, metabolic disorders, genetic factors (HLA class II -DR1 and DR4), infections, immune factors (Harth & Nielson, 2019). It is also one of the most common chronic diseases and one of the most common causes of disability in the world's population. The incidence of the disease is estimated at 95-150 new cases per 100,000 inhabitants annually (Harth & Nielson, 2019). The prevalence of the disease is about 1% in Caucasians, and ranges from 0.1% in rural Africans to 5% in Indians (Smolen et al., 2016; Coates & Helliwell, 2017). It is well known that topically applied medication products are preferred over their systemic counterparts for treating topical illnesses due to the numerous benefits they provide (Benson et al., 2019; Tang, 2019). Because there is no systemic absorption, topical therapies have no systemic negative effects. They are also simple to make, administer, and have a higher level of patient compliance (Chang et al., 2012; Benson et al., 2019). Currently, on the dermatocosmetics market, there is a wide range of topical products with an adjuvant role in the treatment of inflammatory diseases of the skin and joints (e.g. arthritis), such as creams, lotions, gels, and ointments, mainly based on plant extracts (Panda & Ghosh, 2010; Oltean et al., 2014; Ahuja et al., 2021). Most of these products contain highly aggressive synthetic chemicals for the skin (e.g. salicylic acid, urea), synthetic

surfactants (tween, borax, cetyl alcohol), preservatives and/or stabilizers (Singh Malik et al., 2016; Rodriguez-Merchan, 2018). Also, plant extracts used in most skincare products are not standardized. The aim of this study was to formulate and characterize herbal creams containing several standardized plant extracts (*Arctium lappa, Magnolia virginiana,* and *Tiliae flos*) in different combinations.

MATERIALS AND METHODS

Materials. The materials used include: white wax purchased from MAYAM Pure Cosmetics, Romania, cocoa butter (*Theobroma cacao*), shea butter (*Buthyrospermum parkii*) procured from Herbavit, Romania, vitamin E (α-tocopherol) acquired from Sigma-Aldrich, Germany, grapeseed oil (*Vitis vinifera*), sweet almond oil (*Prunus amygdalus dulcis*) and cinnamon essential oil (*Cinnamomum zeylanicum*) purchased from Sabio Cosmetics, Romania.
The lanolin used for cream base was purified by Pharmaceutical Technologies's team of the National Institute of Chemical-Pharmaceutical R & D (ICCF Bucharest), Romania. The plant extracts used for cream formulations were obtained and characterized by the botanist's team of the National Institute of Chemical-Pharmaceutical R & D (ICCF Bucharest), Romania. Each plant extract of burdock (*Arctium lappa)* leaf and linden inflorescence (*Tiliae flos*) had a concentration of 5 mg gallic acid equivalents [GAE] *per* 1 mL, and magnolia (*Magnolia virginiana*) petals extract had a concentration of 2.5 mg gallic [GAE] acid equivalents *per* 1 mL. All plant extracts were standardized in 50% glycerin. The composition of herbal cream formulations is shown in Table 1.

Formulation. The required quantities of the cream base constituents, namely, lanolin (12 g), white wax (6 g), cocoa butter (2.2 g), shea butter (3.8 g) were accurately weighed, heated in a water bath up to 60-70^{0}C and stirred continuously. Grapeseed oil (8.1 g), sweet almond oil (8.1 g) were also weighed accurately and were added to the base cream constantly until homogenous product was attained. The mixture of plant extracts (*Arctium lappa* leaves: *Tiliae flos* 1:1 w/w for F1 and *Arctium lappa* leaves: *Magnolia virginiana* petals 1:1 w/w for F2) was then incorporated into the cream base under continuous stirring. A homogeneous mixture was obtained, to which essential oil (1.5 g of cinnamon oil) and 1.5 g of vitamin E were added. The composition of the two different herbal cream formulations is given in Table 1.

Table 1. Composition of herbal cream formulations (F1 and F2)

No.	Constituent	F1 % w/w	F2 % w/w
1	Lanolin	28	28
2	White wax	14	14
3	Cocoa butter	5	5
4	Shea butter	9	9
5	Mixture of burdock leaf extract : linden inflorescence extract (1:1 w/w)	3	-
6	Mixture of burdock leaf extract : magnolia petals extract (1:1 w/w)	-	3
7	Grapeseed oil	19	19
8	Sweet almond oil	19	19
9	Cinnamon essential oil	1.5	1.5
10	Vitamin E	1.5	1.5

Organoleptic characterization. The formulations were examined for colour, odour, texture and homogeneity. A small amount of each formulation was split into two glass slides and examined visually.
Results for texture and homogeneity were presented as follows: (+++) = excellent, (++) = very good, (+) = good and (-) = poor.

pH determination. pH has been measured using a digital pH meter (MP 220, Mettler Toledo). 1 g of each formulation was mixed in 100 ml distilled water (1% w/v), then warmed, vigorously stirred and stored for two hours. The electrode was inserted three times into the sample for pH recording.

Stability study. The formulations were packed in foldable aluminium tubes and placed in an accelerated stability chamber at 40°C and 75% relative humidity for three months according to the international conference on harmonization (ICH) guidelines (ICH Harmonized Tripartite Guidelines, 2003). Samples were removed after 1, 2, and 3 months storage to evaluate the following parameters: texture, colour, odour, and pH.

Microbiological evaluation. Microbial counts and detection of microorganisms were performed according to the European Pharmacopoeia

(Ph.Eur. 9th edition). The test sample was prepared by suspending 1 g of the formulation in 9 mL of buffered sodium chloride peptone solution and shaking it for 15 minutes in a vortex blender.

Total Aerobic Bacteria Count

1 mL of test liquid was aseptically removed from the supernatant layer and spread over solid plates of soybeancasein digest agar medium (Sigma-Aldrich, Germany) containing amphotericin B (2.5 µg/mL) (2 ml/L of medium) to prevent the fungal growth, and incubated at 37 °C for 24 hours. The colony-forming unit (CFU) of each plate was counted using the colony meter and the CFU/g of the formulation was recorded.

Total number of yeasts and fungi

1 mL of test liquid was spread on solid Sabouraud dextrose agar medium (Sigma-Aldrich, Germany) containing a chloramphenicol (50 mg/L of medium) to prevent bacterial growth, and incubated at 25°C for 72 hours. The colony-forming unit (CFU) of each plate was counted using the colony meter and the CFU/g of the formulation was noted.

In vitro evaluation of antibacterial activity

In vitro antibacterial activity was evaluated against *Staphylococcus aureus* ATCC 6538 using the agar well diffusion technique in accordance with the requirements of Ph. Eur. 9th edition.

Determination of cell viability and cytotoxicity of cream formulations components (plant extracts). The experiments were performed on the L-929 cell line (ATCC CRL-6364), the murine fibroblast line. Cell cultures were performed in Eagle's Minimum Essential Medium (EMEM) adjusted with 10% equine fetal serum, 1% fetal bovine serum, penicillin-streptomycin-neomycin mixture in 0.9% NaCl solution (10.000 µg/mL/10.000 U/mL). At 75% confluence, cell cultures were harvested by trypsin-EDTA treatment to remove the cell monolayer, after which trypsin was neutralized with fetal bovine serum, and the cells were homogenized. The cell suspension was then harvested in 15 mL centrifuge tubes and the cells were centrifuged at 1200 rpm for 10 minutes. The cells were then resuspended in culture medium and adjusted to 10^6 cells/mL. 96-well plates were inoculated at a density of 8 x 10^3 cells/well. After 24 hours, the culture medium was replaced with fresh medium (180 µL/well). The cells were then incubated in the presence of the above samples for 24 hours at 37°C in an atmosphere with 5% CO_2, at concentrations of 100 µg/mL, 50 µg/mL, 25 µg/mL, 10 µg/mL and 5 µg/mL medium, after which cell viability was determined by a colorimetric method using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega, USA). After 24 hours of exposure to the above concentrations, the medium was replaced with 100 µL of MTS reagent, diluted 1:10 with fresh medium. The cells were incubated for 3 hours in an incubator with 5% CO_2, then the optical densities were measured at 490 nm using a Microplate Reader (Chameleon V Plate Reader, LKB Instruments).

Optical densities were recorded and related to the values of the control samples, considered to be the maximum values of cell viability.

All tests were performed in triplicate.

RESULTS AND DISCUSSIONS

This study was geared to obtain cream formulations based on natural ingredients:

(1) *Lanolin* is a waxy, anhydrous substance obtained by extraction after processing sheep's wool, used in a wide range of cosmetics, especially for skincare, and pharmaceuticals. It was used in these formulations because it is easily absorbed into the skin, reduces transepidermal water loss and has an emollient, protective and repairing role on the skin (Chang et al., 2012; Rodriguez-Merchan, 2018; Singh Malik et al., 2016).

(2) *White wax* is a natural ingredient widely used in the preparation of cosmetics and dermatocosmetics because it gives consistency and stability to the compositions, has an emollient, soothing role and helps maintain an optimal level of skin hydration (Chang et al., 2012; Singh Malik et al., 2016).

(3) *Cocoa butter* (*Theobroma cacao*) has a moisturizing, calming effect, stimulates the production of collagen, which leads to the fading of wrinkles, as well as their prevention and helps to treat and fade scars (Rodriguez-Merchan, 2018; Singh Malik et al., 2016).

(4) *Shea butter* (*Buthyrospermum parkii*) is anti-inflammatory, antibacterial, skin regenerating,

epithelializing, facilitates healing and soothes irritations, protects the skin from external factors, has a nourishing role and maintains the optimal level of skin hydration (Chang et al., 2012).

(5) *Vitamin E* (α-tocopherol) has an antioxidant effect, leading to a high stability of topical products. It is included in many cosmetics products, because it increases the hydration and elasticity of the skin (Chang et al., 2012; Singh Malik et al., 2016).

(6) *Burdock leaf extract* (*Arctium lappa*, family Asteraceae) has a high content of scaffeol-quinic acids, derivatives of quercetin and luteolin, and bitter sesquiterpene principles with germacranic structure (arctiopicrin); the natural complex provides antioxidant, antibacterial and anti-inflammatory properties. It is also strongly emollient, regenerating and moisturizing (Chan et al., 2011; Pirvu et al., 2017).

(7) *Magnolia petals extract* (*Magnolia virginiana*, family Magnoliaceae) contains a series of chemical compounds with a complex structure, including lignans, neolignans, terpenoids, alkaloids, polyphenolic acids (syringic acid), the most studied being neolignans, such as magnolol, honokiol and obovatol, with anti-inflammatory and antinociceptive effect (Ding et al., 2018).

(8) *Linden inflorescence extract* (*Tiliae flos*, family Malvaceae) presents polyphenols and flavonoids derived from cvercetol and kaempferol, gallic and catechin tannins, small amounts of fraxoside and esculoside, a non-hemolytic saponin, called tiliadin. Due to the hydroxyl groups presence in the structures of the compounds that neutralize reactive oxygen species, involved in inflammatory oxidative processes, linden inflorescence extract has antioxidant properties (Barreiro Arcos et al., 2006).

(9) *Grapeseed oil* (*Vitis vinifera*, family Vitaceae), *sweet almond oil (Prunus amygdalus dulcis*, family Rosaceae) and *cinnamon oil* (*Cinnamomum zeylanicum*, family Lauraceae) have antioxidant, invigorating, tonic, anti-inflammatory and moisturizing effect on the skin, maintaining the elasticity of epithelial cells in optimal parameters (Ahuja et al., 2021; Panda & Ghosh, 2010).

Also, the obtained herbal cream formulations were evaluated for organoleptic parameters, pH, microbial contamination level, antimicrobial activity, and for plant extracts was assessed the cytotoxicity effect.

Organoleptic characterization. The herbal creams (F1 and F2) showed similar organoleptic characteristics (Table 2). Generally, the results obtained for all characteristics were acceptable as all have light yellow consistent color, agreeable odour, highly homogenous and an excellent semi-solid texture. The odour was aromatic plant for both formulations.

Tabel 2. Organoleptic and physical characteristics of herbal cream formulations F1 and F2

No.	Characteristics	Formulation code	
		F1	F2
1	Texture	+++	+++
2	Homogeneity	+++	+++
3	Colour	Light yellow	Light yellow
4	Odour	Aromatic plant, pleasant	Aromatic plant, pleasant
5	*p*H	5.2 ± 0.2	5.4 ± 0.1
6	Stability	Stable	Stable

pH evaluation. F1 and F2 formulations had pH values ranged between 5.0 and 5.5 as displayed in Table 2. Such values of pH would guarantee physiological compatibility with human skin since pH of the human skin is ranged between 4.0 and 7.0 and underneath or over this range will unfavourably influence the human skin.

Stability study. Both herbal cream formulations (F1 and F2) showed stability at a temperature of 40^0C and 75% relative humidity for three months. It was not observed any differences in texture, colour, odour, or pH.

Microbiological evaluation. All herbal cream formulations exhibited Total Aerobic Bacteria Count < 10 CFU/g, Total Number of Yeasts and Fungi < 10 CFU/g (Table 3), and showed moderate *in vitro* antibacterial activity against *Staphylococcus aureus* (mean diameter of 18.44 ± 1.43 mm for F1 and 18.05 ± 1.09 mm for F2; Figure 1). The obtained results are in accordance to the Ph.Eur. requirements and indicated the good hygienic preparation, good microbial limit of raw materials and efficient preservation.

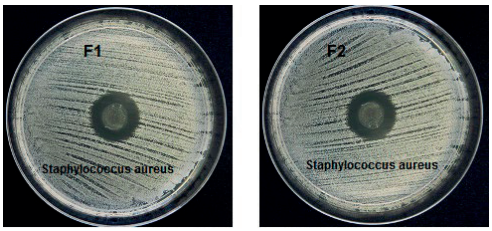


Figure 1. *In vitro* antibacterial activity against *Staphylococcus aureus*

Tabel 3. Microbiological contamination level and *in vitro* antibacterial activity of herbal cream formulations F1 and F2

Formulation Code	Characteristics	Results (CFU/g)
F1	Total Aerobic Bacteria Count	< 10
	Total number of yeasts and fungi	< 10
F2	Total Aerobic Bacteria Count	< 10
	Total number of yeasts and fungi	< 10

Evaluation of cytotoxicity effect of plant extracts. Following the experiments performed on the L-929 murine fibroblast line, given the dose levels used, the plant extracts (*Tiliae flos* and *Arctium lappa*) are practically free of cytotoxicity. Cytotoxic effects were observed only in the case of the plant extract of *Magnolia virginiana*, at concentrations higher than 25 µg ± 0.20 GAE/mL.

The IC_{50} value for *Magnolia virginiana* plant extract of 61.26 ± 0.30 µg GAE/mL, a difficult dose to achieve *in vivo*, indicates that therapeutic doses can be used safely.

The results lead to the conclusion that all the extracts tested are either non-toxic or have a very low cytotoxicity and can be used safely as ingredients for dermatocosmetics (Figures 2-4). The results are in accordance to the literature (Barreiro Arcos et al., 2006; Chan et al., 2011; Ifeoma & Oluwakanyinsola, 2013; Ding et al., 2018).

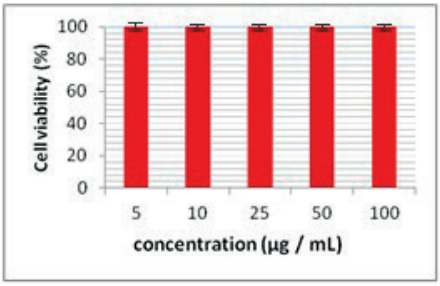


Figure 2. Effect of *Tiliae flos* extract on L-929 fibroblasts

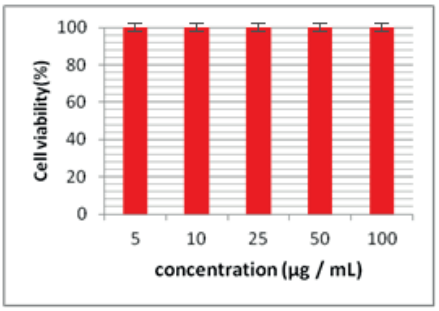


Figure 3. Effect of *Arctium lappa* extract on L-929 fibroblasts

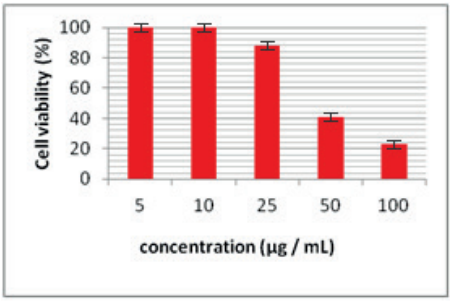


Figure 4. Effect of *Magnolia virginiana* extract on L-929 fibroblasts

CONCLUSIONS

Out of the numerous topical drug delivery systems, herbal cream formulations have shown favourable properties over other semisolid dosage forms, such as easily spreadable, greaseless, easily removable, non-staining, bio-friendly and have long shelf life. In this study two formulations were obtained containing several plant extracts (*Arctium lappa, Magnolia virginiana, Tiliae flos*) in different combinations, while the composition of the cream base was kept the same. The herbal cream formulations were homogeneous, non-irritant and easily removable. The pH of creams was in the range of 5.0–5.5 which is safe for human skin. The samples were found to be populated with aerobic bacteria, yeast and fungi up to 10 CFU/g and showed moderate antibacterial activity.

ACKNOWLEDGEMENTS

This research work has been financed by the Romanian National Authority for Scientific Research ANCS, Competitiveness Operational

Programme COP-A1-A1.2.3-G-2015, Project title “Innovative technologies for new, natural health products”, ID P_40_406, SMIS 105542.

REFERENCES

Ahuja, A., Gupta, J., & Gupta, R. (2021). Miracles of herbal phytomedicines in treatment of skin disorders: natural healthcare perspective. *Infectious disorders drug targets*, *21*(3), 328–338.

Barreiro Arcos, M. L., Cremaschi, G., Werner, S., Coussio, J., Ferraro, G., & Anesini, C. (2006). *Tilia cordata* Mill. Extracts and scopoletin (isolated compound): differential cell growth effects on lymphocytes. *Phytotherapy research : PTR*, *20*(1), 34–40.

Benson, H., Grice, J. E., Mohammed, Y., Namjoshi, S., & Roberts, M. S. (2019). Topical and transdermal drug delivery: from simple potions to smart technologies. *Current drug delivery*, 16(5), 444–460.

Chan, Y. S., Cheng, L. N., Wu, J. H., Chan, E., Kwan, Y. W., Lee, S. M., Leung, G. P., Yu, P. H., & Chan, S. W. (2011). A review of the pharmacological effects of *Arctium lappa* (burdock). *Inflammopharmacology*, *19*(5), 245–254.

Chang, R.K., Raw, A., Lionberger, R., Yu, L. (2012). Generic development of topical dermatologic products: formulation development, process development, and Testing of Topical Dermatologic Products. *The AAPS Journal*, 15(1), 41-52. 27.

Coates, L. C., & Helliwell, P. S. (2017). Psoriatic arthritis: state of the art review. *Clinical medicine (London, England)*, *17*(1), 65–70.

Ding, L. F., Su, J., Pan, Z. H., Zhang, Z. J., Li, X. N., Song, L. D., Wu, X. D., & Zhao, Q. S. (2018). Cytotoxic sesquiterpenoids from the leaves of *Magnolia grandiflora*. *Phytochemistry*, *155*, 182–190.

European Pharmacopoeia, 9th Edn. Council of Europe, Strasbourg, 2016

Harth, M., & Nielson, W. R. (2019). Pain and affective distress in arthritis: relationship to immunity and inflammation. *Expert review of clinical immunology*, *15*(5), 541–552.

ICH Harmonized Tripartite Guidelines, Stability Testing of New Drug Substances and Products. *ICH Committee*, 2003.

Ifeoma, O., & Oluwakanyinsola, S. (2013). Screening of herbal medicines for potential toxicities. In (Ed.), New Insights into Toxicity and Drug Testing. IntechOpen.

McInnes, I. B., & Schett, G. (2011). The pathogenesis of rheumatoid arthritis. *The New England journal of medicine*, *365*(23), 2205–2219.

Oltean, H., Robbins, C., van Tulder, M. W., Berman, B. M., Bombardier, C., & Gagnier, J. J. (2014). Herbal medicine for low-back pain. *The Cochrane database of systematic reviews*, *2014*(12), CD004504.

Panda, P., Ghosh, A. (2010). Formulation and evaluation of topical dosage form of *Eupatorium odoratum* Linn. and their wound healing activity. *International Journal of Pharma and Bio Sciences*, India, 1(2), 1–13.

Pirvu, L., Nicorescu, I., Hlevca, C., Albu, B. & Nicorescu, V. (2017). Burdock *(Arctium lappa)* Leaf extracts increase the *in vitro* antimicrobial efficacy of common antibiotics on Gram-positive and Gram-negative bacteria. *Open Chemistry,* 15(1), 92–102.

Rodriguez-Merchan E. C. (2018). Topical therapies for knee osteoarthritis. *Postgraduate medicine*, *130*(7), 607–612.

Sayah, A., & English, J. C. (2005). Rheumatoid arthritis: a review of the cutaneous manifestations. *Journal of the American Academy of Dermatology*, *53*(2), 191–212.

Singh Malik, D., Mital, N., & Kaur, G. (2016). Topical drug delivery systems: a patent review. *Expert opinion on therapeutic patents*, *26*(2), 213–228.

Smolen, J. S., Aletaha, D., & McInnes, I. B. (2016). Rheumatoid arthritis. *Lancet (London, England)*, *388*(10055), 2023–2038.

Tang C. H. (2019). Research of pathogenesis and novel therapeutics in arthritis. *International journal of molecular sciences*, *20*(7), 1646.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

HAND SANITIZERS MADE WITH NATURAL INGREDIENTS

Diana GROPOŞILĂ-CONSTANTINESCU, Andreea-Antonia MILITARU, Gabriela POPA, Luminiţa VIŞAN, Gabriela MĂRGĂRIT, Dana BARBA, Radu TOMA

University of Agronomic Sciences and Veterinary Medicine of Bucharest,
Faculty of Biotechnology, 59 Marasti Blvd., District 1, Bucharest, Romania

Corresponding author email: bth.diana@yahoo.com

Abstract

Hand hygiene is one of the most important and healthy habits that each of us should practice. The ingredients used in the formulation of hand sanitizers, as well as their concentration, must be chosen carefully not to affect the hand skin. Four sanitizers have been formulated, containing natural products that give a pleasant and moisturizing consistency, a pleasant smell, and an application as easy as possible. Two disinfectants, 96% ethyl alcohol and 99.9% isopropyl alcohol, glycerin, Aloe-Vera pulp and four flavoring substances, tea tree essential oil, lemon essential oil, eucalyptus essential oil, essential oil of lavender, were used. The obtained sanitizers were analyzed from an organoleptic and physico-chemical point of view. The products had a pleasant appearance, a fluid consistency without signs of phase separation, a very pleasant, aromatic odor, a pH around five and, most importantly, an antibacterial effect, along with an effective moisturizing of the skin. The sanitizer solutions were applied to the hands by spraying.

Key words: *alcohol, disinfectant, sanitizer, skin.*

INTRODUCTION

Hand sanitizers are a combination of different types of alcohol (ethyl alcohol, isopropyl alcohol, and/or n-propyl) mixed with moisturizers (glycerin or aloe vera), water and other ingredients such as dyes or perfumes. (Dhifi et al., 2016)

Although alcohol has long been used as an antiseptic, hand sanitizer is a discovery, dating back only a few decades (Gold et al., 2021).

The World Health Organization (WHO) defines the antibacterial product as "a product containning alcohol (liquid, gel or foam) designed to be applied to the hands to inactivate microorganisms and/or temporarily suppress their growth. Such products may contain one or more types of alcohol, other active ingredients with excipients and humectants" (Gold et al., 2021; Jing et al., 2020).

Alcohol-based hand antiseptics contain isopropyl alcohol, ethyl alcohol, n-propanol, or a mixture thereof as the active ingredients (Jing et al., 2020; Lachenmeier et al., 2008). The antimicrobial activity of alcohols is attributed to their ability to denature and coagulate proteins. This causes the microorganisms to lose their protective coatings and inhibit them.

The Centers for Disease Control and Prevention recommends formulations containing 80% (v/v) ethyl alcohol or 75% isopropyl alcohol, however, generally speaking, products containing 60-95% alcohol are accepted (Thaddeus et al., 2018; Jing et al., 2020). The recommended concentrations of ethyl alcohol and isopropyl alcohol are maintained between 80% and 75% (Lachenmeier et al., 2008). Concentrations higher than the recommended ones are, paradoxically, less effective, because the proteins do not denature quickly in the absence of water.

Alcohol concentrations in antiseptics are often expressed as a percentage by volume and rarely as a percentage by weight. A study of 85% (w/w) ethyl alcohol showed that a contact time of 15 seconds is enough to reduce gram-positive and gram-negative bacteria by more than 5 log10 steps (Golin et al., 2020). Research suggests that alcohols are fast germinators when applied to the skin but have no persistent residual activity (CDC, 2002).

The addition of chlorhexidine, octenidine, or triclosan to alcohol-based products may also lead to longer-term protection; 4% chlorhexidine demonstrated persistent bactericidal activity against methicillin-resistant *Staphylococcus aureus* for up to 4 hours after application (Grayson et al., 2008; CDC, 2002).

Ethyl alcohol seems to be the most effective alcohol against viruses, while propanol is

considered a better bactericidal alcohol (Cartner et al., 2017; Meyers et al., 2021). The combination of alcohols can also have a synergistic effect. Studies have shown that rubbing hands with an 85% ethanol solution significantly reduced bacterial populations compared to 60%-62% solutions (Edmonds et al., 2010). Disinfectants may also contain emollients and/or moisturizers, such as glycerin and aloe vera, which help prevent dry skin (Hamman, 2008; Javed et al., 2014).

None of the alcohols mentioned above have shown any potential for bacterial resistance and are therefore considered to be highly effective for medical use (Gold et al., 2021).

Hand hygiene products affect the skin by causing corneal layer proteins to distort, changes in intercellular lipids (depletion or reorganization of lipid fragments), decreased cohesion of corneocytes and decreased ability to bind water to the stratum corneum. Of these, the main concern is the depletion of the lipid barrier, which may be the consequence of contact with lipid emulsifying detergents and lipid dissolving alcohols (Plessis et al., 2013; Pendlington et al., 2001).

Disinfectants affect the skin's balance and cause burns and injuries, and contact dermatitis (García-Gavín et al., 2011).

Dermatologists warn that the number of dermatitis cases has increased alarmingly since the onset of the Covid 19 pandemic. The symptoms of patients who already had various dermatological conditions have worsened due to these products (Azelee et al., 2020; Rundle et al., 2020).

In addition to disinfectants, hand sanitizers also contain ingredients for skin protection and / or fragrances (Fallica et al., 2021; Javed et al., 2014).

The hand sanitizer should be used in urgent conditions where there is no availability of soap and water, which can offer a short-term solution. It can be considered only as a temporary standby solution (Singh et al., 2020; Edmonds et al., 2013).

MATERIALS AND METHODS

Four hand sanitizers were formulated, two based on 96% ethanol and the other two based on 99.9% isopropyl alcohol. The obtained products were analyzed organoleptically and physico-chemically.

Formulation of hand sanitizers

The solutions were made according to our own recipe, respecting the European rules on ingredients and concentrations allowed (EU Parliament, 2012).

The raw materials used were taken from the Guide to Local Production (2010), World Health Organization - recommended Handrub Formulation (WHO/IER/PSP/2010.5; Golin et al., 2020).

Four solutions were formulated, two of them based on 96% ethyl alcohol, labeled AE_{T+L} and AE_{Eu+Lv}, and the other two, based on 99.9% isopropyl alcohol, labeled AIz_{T+L} and AIz_{Eu+Lv}. The ingredients used to prepare the hand sanitizers were divided into three categories (A, B, C), depending on their role and importance (Table 1).

Table 1. Ingredients used to formulate the hand sanitizers

Phase	Ingredients
A	Ethanol 96%
	Isopropyl alcohol 99.9%
	Hydrogen peroxide 3%
	Glycerol 99.5%
B	Distilled water 99.98%
	Aloe Vera pulp
C	Tea tree essential oil
	Lemon essential oil
	Eucalyptus essential oil
	Lavender essential oil

The first solution, marked AE_{T+L}, consists of ethyl alcohol, hydrogen peroxide, glycerin, distilled water, aloe vera pulp, tea tree and lemon essential oils. The concentrations and quantities of all ingredients are presented in Table 2.

Table 2. Composition of AE_{T+L} hand sanitizer

Cat.	Ingredient	Concentration (% w/v or v/v)	Quantity/ UM for 100 ml
A	Ethanol 96%	83.33	83.33
A	Hydrogen peroxide 3%	4.17	4.17
A	Glycerol 99.5%	1.45	1.45
B	Distilled water 99.98%	5.05	5.05
B	Aloe Vera pulp	4	4
C	Tea tree essential oil	1	1
C	Lemon essential oil	1	1

The second solution, AE_{Eu+Lv}, consists of ethyl alcohol, hydrogen peroxide, glycerin, distilled water, aloe vera pulp, eucalyptus and lavender essential oils.
The concentrations and quantities of all ingredients are shown in Table 3.

Table 3. Composition of AE_{+Eu+Lv} hand sanitizer

Cat.	Ingredient	Concentration (% g/v or v/v)	Quantity/ UM for 100 ml
A	Ethanol 96%	83.33	83.33
A	Hydrogen peroxide 3%	4.17	4.17
A	Glycerol 99.5%	1.45	1.45
B	Distilled water 99.98%	5.05	5.05
B	Aloe Vera pulp	4	4
C	Eucalyptus essential oil	1	1
C	Lavender essential oil	1	1

AIz_{T+L}, the third hand sanitizer, contains isopropyl alcohol, hydrogen peroxide, glycerin, distilled water, aloe vera pulp, and tea tree and lemon essential oils.
Also, the concentrations and quantities of all ingredients of this product are shown in Table 4.

Table 4. Composition of AIz_{T+L} hand sanitizer

Cat.	Ingredient	Concentration (% g/v or v/v)	Quantity/ UM for 100 ml
A	Isopropyl alcohol 99.9%	75.15	75.15
A	Hydrogen peroxide 3%	4.17	4.17
A	Glycerol 99.5%	1.45	1.45
B	Distilled water 99.98%	13.23	13.23
B	Aloe Vera pulp	4	4
C	Tea tree essential oil	1	1
C	Lemon essential oil	1	1

AIz_{+Eu+Lv} hand sanitizer contains isopropyl alcohol, hydrogen peroxide, glycerin, distilled water, aloe vera pulp and eucalyptus and lavender essential oils.
Concentrations and quantities of all ingredients are shown in Table 5.

Table 5. Composition of AIz_{+Eu+Lv} hand sanitizer

Cat.	Ingredient	Concentration (% g/v or v/v)	Quantity/ UM for 100 ml
A	Isopropyl alcohol 99.9%	75.15	75.15
A	Hydrogen peroxide 3%	4.17	4.17
A	Glycerol 99.5%	1.45	1.45
B	Distilled water 99.98%	13.23	13.23
B	Aloe Vera pulp	4	4
C	Eucalyptus essential oil	1	1
C	Lavender essential oil	1	1

Obtaining alcohol-based hand sanitizers

Hand sanitizers were obtained considering the properties of all the ingredients and the general methods of obtaining these solutions.

- The ingredients of phase A, representing the active substances for all 4 product variants, are weighed, and placed in a graduated container, after which they are homogenized on a magnetic stirrer.
- The components of phase B, which play a role in hydrating the skin, are weighed, and added to the mixture obtained later.
- The ingredients of phase C with a flavoring role, represented by the essential oils, are incorporated in the obtained solution.

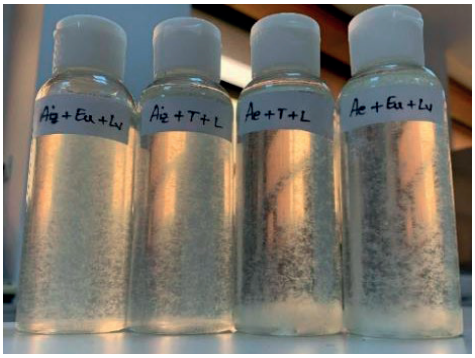


Figure 1. Hand sanitizers before maceration

The samples were soaked in the refrigerator at 4ºC for 14 days.
The solutions were then filtered on filter paper to remove aloe vera pulp.

Evaluation of hand sanitizers - Performing qualitative analyzes

Quality analyzes for disinfectants in the form of gels or solutions are divided into two categories:
a) Organoleptic analysis (appearance, smell, color);
b) Physico-chemical analysis (pH).

All these physico-chemical analyzes were performed according to the requirements of the European Pharmacopoeia (EDQM, 2017).

To determine the appearance of the solutions, 10 mL of each sample were taken and placed in glass tubes to check that they were clear (by comparison with water), transparent, opalescent or cloudy. They must not have solid particles in suspension.

To determine the odor, a piece of filter paper was soaked with 1 ml of samples and smelled from a distance of about 2-4 cm. Samples must not have a musty or rancid odor, must have a specific odor of ethanol/isopropyl alcohol or essential oil. Among the organoleptic characteristics, the determination of the smell is the one that allows the differentiation of the products.

The pH was determined for the final product, after maceration and filtration. The determination was made using pH-indicator paper.

RESULTS AND DISCUSSIONS

Qualitative evaluation of hand sanitizers was performed by physico-chemical and organoleptic (appearance, odor, color) analyzes, 14 days after their formulation (Figure 2).

Figure 2. The four hand sanitizers

Homogeneity, lack of crystalline solid particles, but also lack of phase separation are important indicators that reflect the good homogenization of the solution.

Due to the high quality of the active ingredients and the essential oils used, valuable products were obtained both in terms of antimicrobial efficacy, the degree of skin softening, but also the specific smell, which had a positive effect after use (Dhifi et al., 2016; Javed et al., 2014).

Following the organoleptic analyzes, it was found that the products obtained based on ethyl alcohol were very well homogenized, did not show phase separations, and the smell was much more pleasant than the other two solutions, based on isopropyl alcohol. The specific smell of alcohol has been faded by essential oils, resulting in two moisturizers with antimicrobial action and a fresh smell.

Both ethyl alcohol solutions had a slightly yellowish color, due to the aloe vera pulp, but also to the essential oils.

The smell was very pleasant, specific to the essential oils used in the preparation. Ethanol played a very important role, so that the solutions AE_{T+L} and AE_{Eu+Lv} did not present a pronounced alcoholic odor.

Following the organoleptic analyzes performed on the solutions based on isopropyl alcohol, it was found that they homogenized very well, showed no signs of phase separation, and the smell was much stronger, specific to isopropyl alcohol. The specific smell of alcohol could not be completely blurred by the essential oils used, which slightly reduced the quality of the product. The latter two solutions also showed a slightly yellowish color, due to the aloe vera pulp and essential oils.

The disinfectant solutions were preliminarily tested for five days. Our tests have confirmed the scientific research on the effect of aloe vera on human skin (Vogler et al., 1999; Dal'Belo et al., 2006). Due to their aloe vera content, hand sanitizers protected the hands from the aggressive effect of alcohol and had a moisturizing effect, so the skin did not suffer any injury.

CONCLUSIONS

Following the research carried out in this study, the following main conclusions could be drawn:

- The natural ingredients used to make hand sanitizers are accessible to all and the formulation was easy;

- Organoleptic determinations confirmed the obtaining of high quality products, with a pleasant appearance, a fluid consistency, easy to apply, without signs of phase separation and a very pleasant smell;
- The smell of alcohol-based products is much more pleasant than that of isopropyl alcohol, due to the alcohol used. The pungent odor of isopropyl alcohol persisted in hand sanitizers, which could lead to a lower degree of acceptance by users;
- Due to their aloe vera content, hand sanitizers protected the hands from the aggressive effect of alcohol and had a moisturizing effect.

REFERENCES

Azelee, N. (2020). Antimicrobial disinfectants and sanitizers: An effective tool for breaking the circle of pandemic disease. *Asian J. Agric. Biol.*, *8*, 348-367.

Cartner, T., Brand, N., Tian, K., Saud, A., Carr, T., Stapleton, P., & Rawlings, A. V. (2017). Effect of different alcohols on stratum corneum kallikrein 5 and phospholipase A2 together with epidermal keratinocytes and skin irritation. *International journal of cosmetic science*, *39*(2), 188-196.

CDC, 2002. Antimicrobial spectrum and characteristics of hand-hygiene antiseptic agents. MMWR Morb Mortal Wkly Rep. 51(RR16), 45.

Dal'Belo, S. E., Rigo Gaspar, L., & Berardo Gonçalves Maia Campos, P. M. (2006). Moisturizing effect of cosmetic formulations containing Aloe vera extract in different concentrations assessed by skin bioengineering techniques. *Skin Research and Technology*, *12*(4), 241-246.

Dhifi, W., Bellili, S., Jazi, S., Bahloul, N., & Mnif, W., (2016). Essential oils' chemical characterization and investigation of some biological activities: A critical review. *Medicines, 3*(4), 25.

Edmonds, S. L., Mann, J., McCormack, R. R., Macinga, D. R., Fricker, C. M., Arbogast, J. W., & Dolan, M. J. (2010). SaniTwice: a novel approach to hand hygiene for reducing bacterial contamination on hands when soap and water are unavailable. *J Food Prot., 73*(12), 2296-300.

Edmonds, S. L., Macinga, D. R., Mays-Suko, P., Duley, C., Rutter, J., Jarvis, W. R., & Arbogast, J. W. (2012). Comparative Efficacy of Commercially Available Alcohol-Based Hand Rubs and World Health Organization-Recommended Hand Rubs: Formulation Matters. *Am. J. Infect. Control*, *40*, 521-525.

EDQM, 2017 EDQM, European Pharmacopoeia 9th Edition, (Strasbourg, France).

Fallica, F., Leonardi, C., Toscano, V., Santonocito, D., Leonardi, P., & Puglia, C. (2021). Assessment of alcohol-based hand sanitizers for long-term use, formulated with addition of natural ingredients in comparison to WHO formulation 1. *Pharmaceutics*, *13*(4), 571.

García-Gavín, J., Lissens, R., Timmermans, A., & Goossens, A. (2011). Allergic contact dermatitis caused by isopropyl alcohol: a missed allergen? *Contact dermatitis, 65*(2), 101-106.

Gold N. A., Mirza T. M., & Avva U. (2021). Alcohol Sanitizer. Nov 4. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan. PMID: 30020626.

Golin, A. P., Choi, D., & Ghahary, A. (2020). Hand sanitizers: A review of ingredients, mechanisms of action, modes of delivery, and efficacy against coronaviruses. *American journal of infection control*, *48*(9), 1062-1067.

Grayson, M. L., Melvani, S., Druce, J., Barr, I. G., Ballard, S. A., Johnson, P.D., Mastorakos, T., & Birch, C. (2009). Efficacy of soap and water and alcohol-based hand-rub preparations against live H1N1 influenza virus on the hands of human volunteers. *Clin Infect Dis., 48*(3), 285-91.

Hamman, J. H. (2008). Composition and applications of Aloe vera leaf gel. *Molecules, 13*(8), 1599-1616.

Houben, E., De Paepe, K., & Rogiers, V. (2006). Skin Condition Associated with Intensive Use of Alcoholic Gels for Hand Disinfection: A Combination of Biophysical and Sensorial Data. *Contact Dermat.*, *54*, 261-267.

Javed, S., & Rahman, A. (2014). Aloe Vera Gel in Food, Health Products, and Cosmetics Industry. *Stud. Nat. Prod. Chem.*, *41*, 261-285.

Jing, J. L. J., Yi, T. P., Bose, R. J. C., McCarthy, J. R., Tharmalingam, N., & Madheswaran, T. (2020). Hand sanitizers: a review on formulation aspects, adverse effects, and regulations. *International Journal of Environmaental Research and Public Health, 14*, 3326.

Lachenmeier, D. W. (2008). Safety evaluation of topical applications of ethanol on the skin and inside the oral cavity. *Journal of Occupational Medicine and Toxicology, 3*(1), 1-16.

Meyers, C., Kass, R., Goldenberg, D., Milici, J., Alam, S., & Robison, R. (2021). Ethanol and isopropanol inactivation of human coronavirus on hard surfaces. *Journal of Hospital Infection*, *107*, 45-49.

Pendlington, R. U., Robinson, J. A., & Whittle, E. (2001). Howes D Fate of ethanol topically applied to skin. *Food Chem Toxicol., 39*(2), 169-74.

Plessis, J., Stefaniak, A., Eloff, F., John, S., Agner, T., Chou, T., Nixon, R., Steiner, M., Franken, A., & Kudla, I. (2013). International Guidelines for the in Vivo Assessment of Skin Properties in Non-Clinical Settings: Part 2. Transepidermal Water Loss and Skin Hydration. *Skin Res. Technol.*, *19*, 265-278.

Rundle, C. W., Presley, C. L., Militello, M., Barber, C., Powell, D.L., Jacob, S.E., & Dunnick, C.A. (2020). Hand hygiene during COVID-19: recommendations from the American Contact Dermatitis Society. *Journal of the American Academy of Dermatology*, *83*(6), 1730-1737.

Singh, P., Potlia, I., Malhotra, S., Dubey, H., & Chauhan, H. (2020). Hand Sanitizer an Alternative to Hand Washing-A Review of Literature. *Journal of Advanced Oral Research*, *11*(2), 137-142.

Thaddeus, N. I., Francis, E. C., Jane, O. O., Obumneme, A. C., & Okechukwu, E. C. (2018). Effects of some common additives on the antimicrobial activities of alcohol-based hand sanitizers. *Asian Pacific Journal of Tropical Medicine*, *11*(3), 222.

The EU Parliament And The Council Of The EU, Regulation (EU) No 528/2012 Of The European Parliament And Of The Council, Official Journal of the European Union

Vogler, B. K., & Ernst, E. (1999). Aloe vera: a systematic review of its clinical effectiveness. *British journal of general practice*, *49*(447), 823-828.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

Arctium lappa - A POTENTIAL SOURCE OF BIOACTIVE COMPOUNDS WITH PHARMACEUTICAL APPLICATIONS

Ioana TABREA[1], Lucia PIRVU [2], Narcisa BĂBEANU[1], Călina Petruța CORNEA[1], Nicoleta RADU[1, 3]*

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd , District 1, Bucharest, Romania
[2]National Institute for Chemical-Pharmaceutical Research and Development - ICCF Bucharest, 112 Vitan Avenue, District 3, Bucharest, Romania
[3]National Institute of Chemistry and Petrochemistry Research and Development of Bucharest, 202 Splaiul Independetei, District 6, Bucharest, Romania

*Corresponding author email: nicoleta.radu@biotehnologii.usamv.ro

Abstract

The study provides an overview of the bioactive molecules present in Arctium lappa. The main bioactive compounds in this plant, their pharmacological activities, and the main methods of obtaining bioproducts concentrated in these bioactive compounds are summarized. Due to the diversity of pharmaceutical activities of the bioactive components found in Arctium lappa (biomolecules with anti-inflammatory, antimicrobial, antioxidant, antitumor properties) this species can be considered a potential source of compounds with therapeutic properties of interest.

Key words*: Arctium lappa, pharmacological activities, bioproducts.*

INTRODUCTION

Arctium lappa (*Asteraceae*) known as burdock (Jiang et al., 2019), is a biennial plant that grows to about one meter in height, blooms from July to October, and is widespread and cultivated in East Asia and Europe (Skowronska et al., 2020). *Arctium lappa* can also be grown at temperatures between 10 and 25°C, but shows better development between 16 and 22°C, on moist and sandy soils (Skowronska et al., 2021; Tita et al., 2009).

In terms of chemical composition, the major active chemical compound, distinctive for *Arctium lappa*, is arctigenin (AR) and its glucosylated form arctiin, a phenolic compound of the lignan class (so it is part of lignin); arctigenin and arctiin are in the category of plant estrogens or phytoestrogens, due to their dibenzylbutanic backbone (Gao et al., 2018). Arctigenin and arctiin were identified in the fruits of *Arctium lappa*, from which is derived the plant name "arctii", and is widely used in China and other countries due to its biological activity (Chen et al., 2016).

The major secondary metabolites of the species are tannin and iron complexes, polyacetylenes, sulfuric acetylenes, essential oils, guainolides, bitter compounds, lignans (arctigenin, arcthin), and sterols (sitosterol, stigmasterol) (Maghsoumi et al., 2016). Studies on the metabolic profile of the compounds present in various parts of *Arctium lappa* have also shown the presence of polyphenols (4-o-glucoside caffeic acid, chlorogenic acid, quercitrin, quercetin, quercetin-3-O-glucuronide, nobiletin, p-coumaric acid, biachanin A, and tangeretin), tannins, and terpenoids (lupeol, ursolic acid, oleanolic acids) (Al-Snafi, 2014), gallic acid (Nema et al., 2019) but also some polysaccharides as inulin and pectin (Watanabe et al., 2020). Thus, according to the literature, burdock root, *Bardanae radix*, is used as a medicine, but also as a vegetable (similar to potatoes) in East Asian regions due to its respective nutritional value of high carbohydrate content (69%), inulin (27-50%), mucilages, to which fats, vitamins (B1-B6, C; E; K) and minerals (Ca; Fe; Mg; P; Zn) is added (Gentil et al. 2006; Awale et al. 2006).

Regarding secondary metabolites of burdock, studies have shown that the pharmacological activities are due to the rich content of caffeoylquinic derivatives, and flavonoids such as rutin, hyperoside, isoquercitrin, and quercitrin (Liu et al. 1997). According to literature, when traditional medicine is concerned, burdock root is used in skin conditions, kidney and liver disease, cancer, and diabetes (Maghsoumi et al., 2019). The leaves of this species have a high content of polyphenols, (Kim et al., 2020) which are used as treatments for burns, ulcers, and wounds (Carlotto et al., 2015). The seeds of Burdock (called niubangzi in China) are used in Chinese medicine and have anti-inflammatory, detoxifying, diuretic, sedative, and anti-inflammatory properties (Qina et al., 2019). Moreover, *Arctium lappa* is effective as a treatment for inflammatory diseases, raised tension, or viral hepatitis (Leea et al., 2019).
In Romanian popular medicine, the extract obtained in hot water (decoction) is used to prevent or relieve cough, being thus recommended for various lung, digestive, renal, and skin diseases (Maghsoumi et al., 2016; Pereira et al., 2005). Therapeutic applications were attributed to different parts of the plant, roots, leaves, seeds, and fruits. All of these are used to obtain bioproducts used in the treatment of intoxications, throat infections, rashes, and skin infections (Carlotto et. al., 2015).

METHODS FOR OBTAINING ENRICHED FRACTIONS IN BIOMOLECULES OF INTEREST

Regarding the location of the active compounds in the parts of the plant, lignans were identified in all parts of the plant. Arctigenin is present in higher concentrations in leaves, fruits, seeds, and roots (Table 1); actiin is found in leaves, fruits, and roots; diarctigenin is found mainly in fruits, roots, and seeds (Al-Snafi et al., 2014). Trachelogenin is mainly extracted from fruits; lappaol F is extracted from fruits and seeds; terpenoids such as beta-eudesmol and 3α-hydroxylanosta-5 have been isolated mainly from fruit. Regarding polyphenols, caffeic acid is present in large quantities in stems, leaves, and roots. Chlorogenic acid is dominant in leaves and roots and the tannins, inulin, and sterols are found in large quantities in the roots. Burdock root is considered an important source of fiber (prebiotic), cynarin, lignans, and quercetin (Al-Snafi, et al., 2014). The extraction methods used depend on the solubility of the compounds from the part of the plant used. For example, in the case of *A. lappa* root (Agha et al., 2020), lignans, fructans (polysaccharide fraction), polyphenols, amino acids, and phytosterols are compounds of maximum interest. The method of extraction of the secondary metabolites from the root is carried out with alcohol of different concentrations, more specifically with ethanol or methanol solutions (Machado et al., 2012). Regarding the proof of pharmacological activity of root extracts, it was reported that both ethanolic (EtOH) and ethyl acetate (EtOAc) extract inhibits the CaCo-2 cell lines proliferation. Antiproliferative activity was progressively improved by subsequent extraction in n-hexane (EHX). The efficacy of EHX extract was superior in comparison with bioproducts obtained in other solvents, for MCF-7 and EAhy926 cell lines. For these cell lines, the IC50 values obtained were 14.08±3.64 and 27.25±3.45 μg/ml, respectively (Machado et al., 2012). The EHX fraction also significantly regulated TGF β cytokine values in MCF-7. The efficacy of EHX against the MCF-7 adenocarcinoma cell line consisted in the regulation of the transcription factor NF-κB, which has a role in blocking cellular apoptosis (Machado et al. 2012). In a study performed on the leaves of *A. lappa*, other researchers showed that the extracts obtained in ethanol show high antioxidant activity in vitro. In a recent study, Jiang et al. evaluated the antioxidant capacity of the mono-, di- and tri-CQA (one to three units of cofeyl-bound quinidine, respectively), isolated in the alcoholic extract made from burdock root, using DPPH and FRAP methods (Jiang et al., 2016). Inulin, present in large quantities in burdock root (17%), belongs to the category of low molecular weight fructans (Liu et al., 2014). Studies performed on three fractions, named ALP40-1, ALP60-1, and ALP80-1, with an average molecular weight of 218 kDa 178 kDa, and 60 kDa, respectively, proven that these have a higher glucose content than fructose. Taleb and coworkers revealed that the fraction ALP60-1 has the best

antioxidant activity (Agha et al., 2020). Experimental studies made to elucidate this behavior have shown that the pronounced antioxidant activity of a polysaccharide obtained from *P. tricuspidata* (named PTP-4) is possible to be due to its structure's mannose content (Liang et al., 2018).

Studies performed on a fructan (named ALP1) with a molecular weight of about 4600 Da obtained from the roots of *A. lappa* using extraction with hot water revealed a significant antioxidant activity both *in vitro* and *in vivo* (Lou et al., 2009).

Studies performed to evaluate the IC50 for the antioxidant activities of ALP60-1 for two strong radicals (superoxide and hydroxyl) indicated values of 0.79 mg/ml and 1.38 mg/ml, respectively.

An optimized ultrasonic extraction method (83 min) was used to obtain ALP-60-1 (Liang et al. 2018). The AA activities of ALP60-1 could therefore be attributed either to the high mannose content (5.72%) or to the ratios of the various monosaccharides present in its structure (Liang et al., 2018).

Another process of obtaining fructans (APP) consists of the extraction of dry powder obtained from the roots of *A. lappa* L. with petroleum ether and ethanol for 3 hours. After the removal of the solvents, the solid residues were dried in a vacuum and used for ultrasonic extraction of crude ALP (fructans) (Jiang et al., 2019). The Sevag method was used to eliminate the proteins; the deproteinized solution was dialyzed at 8000 Da; after that, it was concentrated by evaporation. In a concentrated solution, anhydrous ethanol is added so that in the final solution the concentration of C_2H_5OH is 40% (ALP40), 60% (ALP60), and 80% (ALP80). The solutions thus prepared were left overnight at 4°C and then centrifuged at 4500 rpm for 15 min. After centrifugation, each precipitate was washed more times with C_2H_5OH (99%) and acetone and then was lyophilized to obtain a crude extract of ALP40, ALP60, or ALP80 (Jiang et al., 2019).

Concerning the extraction of polyphenols from the *Bardanae radix*, the highest concentrations of phenolic compounds were reported in the extraction of plant material in Soxhlet with solvent CH_2Cl_2 (79.45 mg GAE/g product) and EtOH (77.26 mg GAE/g product) (Predes et al., 2011).

Other scientists found that while the hydroethanolic extract (HE) contains 72.61 mg GAE/g product, the extraction with a mixture of CH_3Cl-C_2H_5OH (1:1), conduce to the content of 85.15±0.55 mg GAE/g dry brut extract.

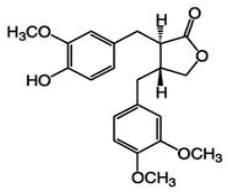
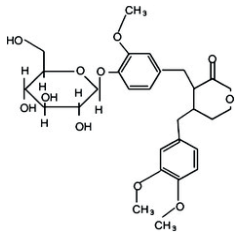
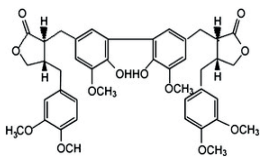
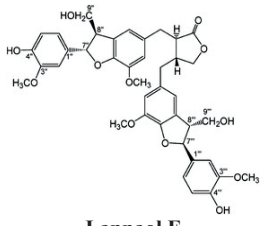
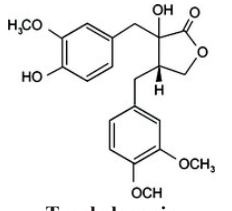
The products obtained with solvent mixtures contain high amounts of flavonoids 12.57±0.05 mg Quercetin/g product (Gilioli et al., 2007).

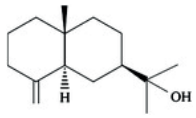
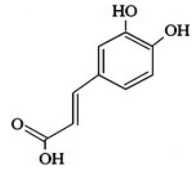
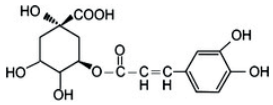
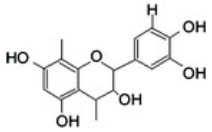
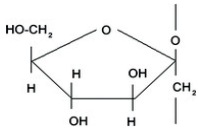
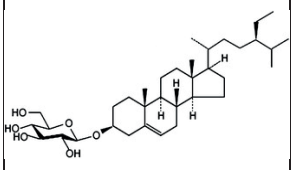
Predes and collab. have reported that in the product obtained by extraction with CH_3Cl the polyphenols content can attain 65.92±0.36 mg GAE/g (Predes et al., 2011).

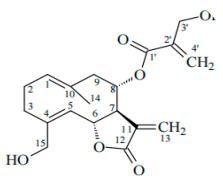
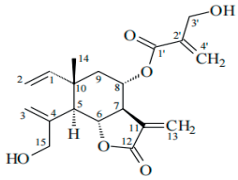
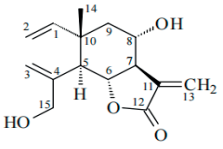
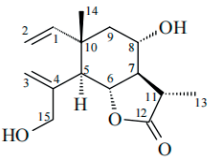
In this context, it has been determined that phenolic compounds such as anthocyanin, carotenoids, and flavonoids are responsible for the strong AA of burdock root extracts (Jianga et al., 2016). Other studies performed on polyphenolic content (Folin-Ciocalteu method) from *Bardanae radix* extracts found the following: the alcoholic extract (70% C_2H_5OH) contains 216.75 mg GAE/g (dry biomass); the C_6H_{14} extract contains 41.58 mg GAE/g; the $CHCl_3$ extract have 69.92 mg GAE/g; the aqueous extract has 96.92 mg GAE/g (Lea et al., 2019). The results obtained from these measurements show the fact that in the alcoholic media (ethanol) the polyphenolic compounds are best extracted (Jianga et al., 2016). The content of polyphenolic compounds from the roots is much lower in comparison with the polyphenols levels from the seed or leaves of the burdock plant (Thaísa et al., 2020).

Regarding the extraction of sesquiterpenes, studies performed on *A. lappa leaves* (Savina et al., 2006) have shown that the best extraction solvent is EtOAc. These extracts contain onopordopicrin and other sesquiterpenes, and their concentration in EtOAc attain 0.035-0.005% and 0.01% respectively (sesquiterpenes).

Table 1. Biomolecules of interest contained in *Arctium lappa*

Compound name/ Chemical structure	Methods of obtaining	Methods of identification	Biological activity	References
 **Arctigenin**	Extraction in 35% C_2H_5OH with reflux; after concentration, selective extractions are performed with petroleum ether, CH_2Cl_2, and C_4H_9OH	HPLC (Ultra performance liquid chromatograph) coupled with mass spectrometry; HR-ESI-MS (high-resolution mass spectrometry) with electrospray ionization); IR; UV-VIS; NMR	Antitumor activity; Antiviral activity; Anti-inflammatory activity. Anti-influenza virus.	Jianga et al., 2016; Zeng et al., 2018; Predes et al. 2011
 **Arctiin**	1.5 ml + 1 ml of 80% CH_3OH (23°C) for 8 hours	LC−MS, MALDI-QIT-TOF MS	Antiprolife-rative activity against B cell hybridoma cell, MH60; Antitumor activity; Antiviral activity	Liu et al., 2014
 **Diarctigenin**	Extraction with CH_2Cl_2, C_2H_5OH (95%) and water (plant solvent ratio: 2: 1) for 6 hours for each solvent	Folin-Ciocalteau method	Antioxidant activity; Anti-inflammatory activity; Inhibiting NO production	Predes et al., 2011
 **Lappaol F**	Extraction by maceration with a 96% C_2H_5OH	UPLC-ESI-QTOF-MS qualitative analysis; GC-MS	Antiviral activity; Antitumor activity	Dias et al., 2017
 **Trachelogenin**	Accelerated solvent extraction (extraction with liquid under PLE pressure) 200 g of dry vegetable material is subjected to PLE extraction for 1.45 h using 550 ml of 70% C_2H_5OH		Antioxidant activity; Antidiabetic activity	Petkova et al., 2020

Compound name/ Chemical structure	Methods of obtaining	Methods of identification	Biological activity	References
 **Beta-eudesmol**	Extraction with supercritical fluids (solvent: CO2; cosolvent: C_2H_5OH) Extract 1 was obtained below 15 MP, in 50 minutes, and extract 2 under 15 MP in 75 min	GC-MS	Anti-inflammatory activity; Antibacterial activity	Chan et al., 2011
 **Caffeic acid**	Extraction by maceration at 45°C for 48 hours with petroleum ether, C_2H_5OH and water (MTT tests). The extract obtained in C_2H_5OH had the highest biologic activity		Antioxidant activity; Antitumoral; Immunomodula -tory activity	Agha et al., 2020
 **Clorogenic acid**	Soxhlet extraction for 18-12 hours. *Arctium lappa* leaves were extracted in 70% C_2H_5OH		Neuroprotecto; antioxidant; anti-HIV	Agha et al., 2020; Chan et al., 2011
 **Tannin**	The alcoholic extract (made in 70% C_2H_5OH) is obtained by refluxing (3 times), at 100°C for 1 hour each, using 200 mL C_2H_5OH and 10 g of plant material		Antioxidant activity; Antitumoral; Immuno-modulatory activity	Chan et al., 2011
 **Inulin**	The dried burdock roots (20 kg) were extracted with 300 L C_2H_5OH at 80°C (3 times), at reflux, for 2 hours. Partition with petroleum ether (V_{water}:$V_{petroleum\ ether}$ = 1:13); dichloromethane (V_{water}: $V_{dichloromethane}$ = 1:1); 3 times and ethyl acetate **(**EtOAc) (V_{water}: V_{EtOAc} = 1:1); 6 times	NMR spectra IR spectra	Prebiotic activity; antihypertensiv; antidiabetic	Gaoa et al., 2020; Chan et al., 2011; Skawronsk a et al., 2021; Jiang et al., 2019. Zhang et al., 2019
 **Sitosterol-beta-D-glucopyranoside**	Maceration with methanol (2 L) at 25°C for 3 days. The extract was concentrated, and fractionated with open column of silica gel (210 g) using gradual elution with $CHCl_3$ and MeOH in various ratios (10:0, 50:1, 10:1, 5:1, 1:1, 1:5, and 0:10) with a volume of 840 ml for each ratio, to give 14 fractions (420 mL/fraction).	Mass spectra NMR analysis performed in $CHCl_3$	Antidiabetic activity.	Skawronsk a et al., 2021; Jiang et al., 2019.

Compound name / Chemical structure	Methods of obtaining	Methods of identification	Biological activity	References
 **Onopordopicrin**	The crude extract obtained in 50% C_2H_5OH was partitioned with C_6H_{14}, EtOAc and n-C_4H_9OH.		Antiproliferative activity	Machado et al., 2012
 **Dehidromeltesina**	Extraction with 70% C_2H_5OH at 40° C for 2 hours (at reflux). The filtered extract is concentrated and fractionated consecutively with n-C_6H_{14}, $CHCl_3$, EtOAc and water	UPLC-Q-TOF MS	Antioxidant activity	Leea et al., 2019
 **Dehydromelitesin**	Treatment of 30 g of ground material with 400 ml of diffrent solvents, choosed in order of increasing polarity: C_6H_{14}, EtOAc and 100% C_2H_5OH	LC-ESI-MS	Antioxidant activity	Ayoddhia et al., 2019
 **Melitesin**	Acetone extraction (plant solvent ratio: 1:20), for 1 h	LC-ESI-MS	Antioxidant activity	Olennikov & Tankhaev et al., 2011

METHODS FOR CHARACTERIZING BIOPRODUCTS CONTAINING MOLECULES OF INTEREST

Studies performed by Lin and Harnly on caffeoyl quinic derivatives in burdock root propose the LC-DAD-ESI/MS as analyzing method. Comparative analytical results on cultured burdock root indicate chlorogenic acid and cinnarine levels are 50% higher than the levels found in the same species from spontaneous flora (Lin. et al. 2008) techniques. In addition to the above-mentioned compounds, this method made it possible to identify 18 other hydroxycinnamoylquinic acids in the form of mono-, di- or tricaffeoylquinic compounds (Lin and Harnly, 2008). Ferracane and coworkers characterized polyphenolic compounds from seeds, root leaves using the LC/MS technique (Ferracane et al., 2010). A UPLC/MS/MS method was developed to identify benzoic acid, p-coumaric acid, and flavonoids from burdock leaves (Table 1) (Lou et al., 2009). Research conducted by other scientists revealed the presence of the luteolin, by using the HPLC-MS technique. The first study on the quantification of lignans in burdock root was conducted by Liu and collab., which used lyophilized roots from six different genotypes of Chinese origin (Liu et al., 2015). This study determined the concentration of arctinine present in the dried root and root bark, which ranged from (20-40) mg/100 g and from (130-210) mg/100 g, respectively. In the case of arctiin and arctigenin, Liu and collab. have indicated the HPLC techniques, as a method to identifying

these compounds (Liu et al. 2014). Predes et al. have used the HR-ESI-MS techniques to determine the presence of quercetin and arctigenin in the hydroethanolic extract from burdock root (Predes et al. 2011). Thus, the concentration of arctigenin (1.27 mg/100 g), caffeic acid (2.18 mg/100 g), chlorogenic acid (0.68 mg/100g) and quercetin (1.82 mg/100 g) were determined. Haghi et al have examined possible differences between cultivated burdock root (BC) and wild root, which grows freely in the wild (BS) (Haghi et al., 2013). The authors used HPLC and UPLC as techniques for the quantitative evaluation of chlorogenic acids. Jiang and collab. highlighted the fact that the extraction of polysaccharides is carried out mainly in an aqueous solvent, (hot water) (Jiang et al., 2019). It has thus been determined that high temperature and long extraction time can degrade polysaccharides, thereby reducing their pharmacological activity. Thus, the ultrasonic-assisted extraction (UAE) variant was proposed, due to the accelerated process, and minimal effects on the structure of the molecules. According to these studies, polysaccharides obtained by the ultrasonic method present an antioxidant activity higher than those obtained by other methods. By applying a combined method (microwave and ultrasonic oven) for extraction of polysaccharides fractions, the extraction time was reduced from 15 min to 1 min (Lou et al 2009). Other authors have used these technologies who have conducted comparative extraction studies for the two processes. The result obtained consisted of increasing the yield of the extraction process of polysaccharides from burdock root from 12% to 24%. Most of the compounds identified were caffeic quinic acids, of which four were mono caffeoylquinic acids, six dicafeoylchinic acids, and two tricafeoylchinic acids. (Milani et al., 2012). Similarly, given that burdock roots are a valuable source of fructooligosaccharides (17%) (Zhang et al., 2018), Milani and collab. have demonstrated the efficiency of the inulin extraction process from burdock roots in the presence of high-intensity ultrasound, with an extraction yield of 12% inulin (Milani et al., 2012). Microwave-assisted extraction has been used for the extraction of fructans, inulin, and ten sugars, all from burdock roots (Liu et al., 2014). The UPLC technique was used to identify the major caffeine compounds from burdock root, 5-CQA, and 1,5-DCQA (esters consisting of one molecule of quinic acid with one or two units of caffeic acid, respectively). These identified compounds exhibit an inhibitory effect on viruses like HSV-1; HSV-2; ADV-3, ADV-11, or HIV-1 (Haghi, et al. 2013; Chiang et al., 2002; Yang et al., 2005).

BIOLOGICAL ACTIVITIES OF BIOMOLECULES OF INTEREST CONTAINED IN *Arctium lappa*

Studies performed have shown that the biological activities of bioproducts derived from burdock are due to lignans, arctiin, arctigenin, and polysaccharides (Table 1). These, in combination with polyphenols (flavonoids and polyphenolcarboxylic acids), exert antitumor, antibacterial, antiviral, hepatoprotective, anti-urolytic activities (Jingvi et al., 2012; Chan et al., 2011).

ANTIMICROBIAL ACTIVITY

Chlorogenic acid isolated from burdock leaves inhibits the development of microorganisms E. coli, *S. aureus*, and *M. luteus* (Lin et al., 2004). Antimicrobial activity exhibits the volatile fractions obtained from leaves and seed of A. lappa, for microorganisms such as *B. subtilis, E. coli, A niger*, and *C. albicans* (Aboutabl et al., 2013). Solid bioproduct obtained from *A. lappa* leaves by lyophilization, inhibits the microorganisms such as *B. subtilis, C. albicans, L. acidophilus,* and *P. aeruginosa* (Pereira et al., 2005; Oliveira et al. 2014). The bioproducts obtained by *A. lappa* by extraction with EtOAc, are useful in treating stomatological infections with *C. albicans, E. coli, L. acidophilus*, *P. aeruginosa*, *S. aureu*s, and *M. luteu*s (Gentil et al., 2006; Tita et al. 2009). *Arctium lappa* root extract inhibits the development of *Klebsiella pneumonia*; the mechanism of action is probably due to the inhibition of β-lactamases (Rajasekharan et al., 2017). Studies have confirmed that burdock bioproducts exhibit antiviral activities against A/NWS/33, H1N1; IFV (arctiin and arctigenin) (Hayashi et. al., 2010); HSV-1; HSV-2; ADV-3; ADV-11; HIV-1 (Matsumoto et al., 2006; Liu and Tang, 1997). A study conducted in 2012 showed that 3.7 billion people (approximately 67% of the

world's population) had HSV-1 infection (Bacon et al., 2003).

ANTIOXIDANT ACTIVITY

In a study performed by Lou and collab., antioxidant capacity (A.A.) was associated with the content of the polyphenolic compound from burdock (Lou et al., 2009). Biomolecules such as chlorogenic acid, o-hydroxybenzoic acid, caffeic acid, p-coumaric acid, and rutin, found in extracts from *A. lappa* leaves, are involved in biological activities as A.A. The bioproducts obtained from burdock leaves are effective in the treatment of some eye disorders (Lee et al., 2020). Studies performed on the polysaccharide fractions isolated from the root of *A. lappa* (called *Bardanae radix*) have shown beneficial effects on patients with diabetes, especially due to their antioxidant effect. In this context, the co-presence of caffeoylquinic derivatives, (chlorogenic acids), responsible for the strong antioxidant activity of burdock root extracts, has been demonstrated (Ravini Ayoddhia et al., 2019). Similarly, starting from the observation that a high level of glucose favors the activation of PKC (protein kinase, enzyme with a role in gene expression and regulation responsible with inflammation and disturbance of lipid metabolism in diabetic lab animals), Liu and collab discovered that the polysaccharides from burdock can influence the PKC activity, modifying the lipidic metabolism in lab animals with induced diabetes (Li et al., 2019). In the context of the benefits of antioxidant compounds, oleamide was identified as the bioactive compound responsible for the antiallergic activity of burdock roots. Studies performed on lab animals were reported attenuation of levels of histamine, TNF-α, and interleukins (Yang et al., 2016; Ayoddhia et al., 2019). The authors conclude that the identification of oleamide may contribute to further research to reduce the allergic response by burdock root administration. Possible therapeutic effects of burdock root have been reported by other scientists (Jiang et al., 2019; Maghsoumi et al., 2016). The hepatoprotective effect of burdock roots, in close connection with the antioxidant effect, has also been reported in two studies, both with similar doses (300 mg/kg body weight) using different extracts. Predes and collab. have studied the effects of the administration of a burdock ethanolic extract in case of hepatotoxicity generated by the administration of cadmium and reported the complete restoration of biochemical functions. Previous studies performed on lab animals with paracetamol-induced liver damage (800 mg/kg in rats) showed that in administering a burdock extract, normalization of liver function in rats is achieved after 30 days. Both results indicated that burdock roots contributed to the recovery of liver function (Predes et al., 2014). The antioxidant effect has been proven in studies performed by Tian and collab. who investigated the possible neuroprotective activity of burdock root extract in human neuroblastoma affected by H_2O_2. Increased cell viability was observed for burdock extract at concentrations of 40-80 µg/ml. The antioxidant capacity and antiapoptotic activity of burdock extract have been associated with the neuroprotective effect (Tian et al., 2015).

THE MECHANISM OF ACTION OF THE MOLECULES OF INTEREST CONTAINED IN *Arctium lappa*

The active biomolecules of *A. lappa* are arctiin; arctigenin; caffeic acid; chlorogenic acid; cinnarine; benzoic acid; p-coumaric acid; rutin; quercitrin; quercetin and luteolin. All these, together or separately, are associated with the medicinal properties of this species (Thaisa et al., 2020). As previously mentioned, the major active chemical compound, distinctive for *Arctium lappa* is arctigenin (AR), and its glucosylated form arctiin (Gao et al., 2018). In the Chinese Pharmacopoeia, arctiin is listed as a chemical marker and major active chemical ingredient in Fructus arctii (Chinese Pharmacopoeia, 2010). Regarding the pharmacological properties, arctigenin has antitumor and antidiabetic activities, while the derivatives isolappaol C, lappaol (C, D, F), and diarctigenin have anti-inflammatory activities. Biological tests revealed that arctigenin has A.A. and reduces both inflammation and level of lipids (Thaisa et al., 2020; Chen et al, 2020; Chena et al., 2020). Arctigenin (AR) and arctiin inhibit microorganisms and viruses; the mechanism of the anti-influenza effect of AR has been linked to the direct inhibitory effect on

viral replication (Hayashi et al., 2010; Yuan et al., 2008; Swarup et al., 2008). Inflammatory responses play an important role in the progression of many chronic diseases, so AR can serve as adjuvants in the treatment of these.

The main anti-inflammatory mechanism of AR is achieved by inhibiting the inducible synthesis of nitric oxide (iNOS), along with the modulation of proinflammatory cytokines (Tabas et al., 2013). The compound named arctigenin (AR), the most potent bioactive component found in *A. lappa* represents a promising therapeutic compound that can be used in the management of inflammation (Yao et al., 2011; Gao et al., 2018). Concerning the mechanism involved, studies performed with arctigenin on MH60 cell lines indicated that this compound stimulates the process of apoptosis (IC50 = 1.0 μM) as the most likely mechanism of action (Tousch et al., 2014; Ferracane et al., 2010). The mixture of arctigenin and polyphenols inhibit the development of the malign cells and decrease the apparition of metastases (Tousch et al., 2014); the compounds arctiin, caffeine, and chlorogenic acid all taken together have antimutagenic properties (Ferracane et al., 2010). Research performed *in vitro* with bioproducts obtained in CH_2Cl_2 from *A. lappa*, are cytotoxic for tumoral cells lines which have not received nutrients, at a concentration of 50μg brut extract/mL (Awale et al., 2006). The cell tumor cell lines type PANC-1 had a significantly higher resistance to nutrient deprivation and survived in these conditions for more than 48 hours. The researchers found that in the presence of arctigenin in the culture medium at a concentration of 0.01μ/mL tumor cells enter the process of apoptosis (Awale et al., 2006). Regarding the mechanism of action, preclinical studies performed in laboratory, animals with induced pancreatic cancer indicate that arctigenin (0.1 μg/ml) acts as an inhibitor of the Akt (Protein kinase B) phosphorylation process stimulated by the deprivation of glucose (Awale et al., 2006). Other studies confirmed the anti-tumor activity of *A. lappa* on tumor cell lines of the type: HepA; S180; MCF-7; BGC-823 (Agha et al., 2020). Matrix metallopeptidases (MMPs, zinc-dependent enzymes), play a central role in metastases (Lou et al., 2017). If the tumor cell lines type MDA-MB-231 (breast cancer) are exposed to AR the MMP-2 and MMP-9 activities are inhibited (Lou et al., 2017). Arctigenin enhances the processes of apoptosis in tumoral cell lines exposed to cisplatin, by acting as a sensitizer of cancer cells to cisplatin (Yao et al., 2011). Another study found that blood pressure was reduced in arctigenin-treated hypertensive rats (Liu et al., 2015). Ravini and collab. reported that arctigenin represents a cytotoxic compound for tumor lines, inducing their necrosis (Ayoddhia et al., 2019). The hydromethanolic extract made from *A. lappa* fruits exhibits a strong antitumor effect on MH60 cell lines, due to the presence of AR. Other researchers found that arctiin has a strong cytotoxic effect on the following tumor cell lines: HepG2; A549; K-OV-3; SK-MEL-2; XF498; HCT15 (Ferracane et al., 2010). Clinical trials were performed in Japan regarding the toxicity and safety profile of GBS-01 (an oral drug derived from *A. lappa* fruit which contains a high level of AR). In this study, the patients with pancreatic cancer with resistance at gemcitabine were received AR in doses ranging between (3÷12) g/day. In the first 4 weeks, the established degree of toxicity was 4, with non-hematological toxicity, Following the studies performed, it was observed a slight increase of the levels of the gamma-glutamyl transferase, serum glucose level, and total bilirubin (Strimpakos et al., 2013). A greater number of studies over time have focused on polysaccharides extracted from *A. lappa*, especially fructans, which have been used as a source of inulin (Watanabe et al., 2020). Recently, natural lignans from *A. lappa* have been found to have promising antitumor potential. They can induce apoptosis in cancer cells, suppressing tumor growth by decreasing tumor tolerance to glucose, leading to starvation (Watanabe et al., 2020). A recent study found that a bioproduct derived from *A. lappa*, obtained by extraction in an aqueous solution of 70% EtOH, exerted inhibitory effects on atherogenic diet-induced thickening of the vascular wall in the aorta (Song et al., 2018).

CONCLUSIONS

Data from the literature have confirmed that *Arctium lappa* contains a large number of bioactive components, with definite beneficial biological effects. In this context, arctigenin

(AR), the most potent bioactive component found in *A. lappa*, is a promising therapeutic compound that can be used in the management of both acute inflammation and chronic inflammation. Therefore, studies have shown a wide range of possible clinical uses of this plant due to its anti-inflammatory, antitumor, antiviral and antimicrobial effects.

ACKNOWLEDGEMENTS

This research work was funded by the Ministry of Research, Innovation and Digitization Romania, POC-G-2015 ID P_40_406, SMIS 105542, Contract D no. D02/23.03.2021.

REFERENCES

Aboutabl, E., El-Tantawy, M., Shams, M. (2013). Chemical composition and antimicrobial activity of volatile constituents from the roots, leaves, and seeds of *Arctium lappa* L. (*Asteraceae*) grown in Egypt. *Egypt. Pharm. J.,* 12(2), 173–176.

Agha, T. M., Baharetha, H.M., Al-Mansoub, M.A. (2020). Proapoptotic and Antiangiogenic Activities of *Arctium Lappa L*. on Breast Cancer Cell Lines. *Scientifica* , 1-14.

Al-Snafi, A.E. (2014). The Pharmacological Importance and Chemical Constituents of *Arctium Lappa*. A Review. *International Journal for Pharmaceutical Research Scholars,* 3(1-1), 663–670.

Awale, S., Lu, J., Kalauni, S.K. (2006). Identification of arctigenin as an antitumor agent having the ability to eliminate the tolerance of cancer cells to nutrient starvation. *Cancer Res*., 66(3), 1751–1757.

Ayoddhia, R., Don, S.G., Yap, M.K.K. (2019). *Arctium lappa* L. root extract induces cell death *via* mitochondrial-mediated caspase-dependent apoptosis in Jurkat human leukemic T cells. *Biomedicine & Pharmacoteherapy*, 110, 918–929.

Bacon, T.H., Levin, M.J., Leary, J.J. (2003). Herpes simplex virus resistance to acyclovir and penciclovir after two decades of antiviral therapy. *Clin. Microbiol. Rev*. 16, 114–128.

Carlotto, J., Silva, M.L., Dartora, N., Ferreira, D.M. (2015). Identification of a dicaffeoylquinic acid isomer from *Arctium lappa* with potent anti-ulcer activity. *Talanta*, 135, 50–57.

Chan, Y.S., Cheng, L.N., Wu, J.H. (2011). A review of the pharmacological effects of *Arctium lappa* (burdock). *Inflammopharmacology*, 19, 245–254.

Chen, J., Li, W., Jin, E., He, Q, Yan, W., Yang, H., 2016. The antiviral activity of arctigenin in traditional Chinese medicine on porcine circovirus type 2. *Research in Veterinary Science.* 106, 159–164.

Chen, H.,Tang, L.J.,Tu, H., et al., (2020).Arctiin protects rat heart against ischemia/reperfusion injury via a mechanism involving reduction of necroptosis. *Eur J Pharmacol*.,*875*, 173053.

Chen, M., Xu, J., Wang, Y., Wang, Z., Guo, L., Li, X., & Huang, L. (2020). Arctium lappa L. polysaccharide can regulate lipid metabolism in type 2 diabetic rats through the SREBP-1/SCD-1 axis. *Carbohydrate research*,*494*, 108055. https://doi.org/10.1016/j.carres.2020.108055

Chiang, L.C., Chiang, W., Chang, M.Y. (2002). Antiviral activity of Plantago major extracts and related compounds in vitro. *Antiviral Res* 55, 53–62

Custódio-Souzaa, A. R., Guedesa, A.R. Rodrigueza, J. M. (2018). Extraction of *Arctium Lappa* leaves using supercritical CO_2+ethanol: Kinetics, chemical composition, and bioactivity assessments. *The Journal of Supercritical Fluids*, 4, 137–146.

Dias, M.M., Zuza, O., Riani, L.R., Pinto, P.F. (2017). In vitro schistosomicidal and antiviral activities of *Arctium lappa L. (Asteraceae*) against Schistosoma mansoni and Herpes simplex virus*., Biomed Pharmacother*, 94, 489–498.

Ferracane, R., Graziani, G., Gallo, M. (2010). Metabolic profile of the bioactive compounds of burdock (*Arctium lappa*) seeds, roots, and leaves. *Journal of Pharmaceutical and Biomedical Analysis*, 51(2), 399–404.

Gao, Q., Yang, M., Zuo, Z. (2018). Overview of the anti-inflammatory effects, pharmacokinetic properties, and clinical efficacies of arctigenin and arctiin from *Arctium lappa* L. *Acta Pharmacologica Sinica* 39, 787–801.

Gao, H., Jiang, X.W., Yang, Y., Liu, W.W. (2020). Isolation, structure elucidation and neuroprotective effects of caffeoylquinic acid derivatives from the roots of *Arctium lappa L. Phytochemistry,* 177, 112432.

Gentil, M., Pereira, J., Silva Sousa, Y. (2006). In *vitro* evaluation of the antibacterial activity of *Arctium lappa* as a phytotherapeutic agent used in intracanal dressings. *Phytotherapy Research,* 20, 184–186.

Gilioli A.M.H., Missau, F.C., Brighente, I.M. (2007). Avaliação do teor de fenólicos e flavonóides em extratos de Arct*ium lappa. 30ª Reunião Anual da Sociedade Brasileira de Química* http://sec.sbq.org.br/cdrom/30ra/resumos/T1231-1.pdf

Haghi, G., Hatami, A., Mehran, M. (2013). UPLC and HPLC of caffeoyl esters in wild and cultivated *Arctium lappa* L. *Food chem*, 138(1), 321–326.

Hayashi, K., Narutaki, K., Nagaoka, Y. (2010). Therapeutic effect of arctiin and arctigenin in immunocompetent and immunocompromised mice infected with influenza A virus. *Biol. Pharm. Bull*. 33, 1199–1205.

Jiang, X. W., Bai, J. P., Zhang, Q., Hu, X. L. (2016). Caffeoylquinic acid derivatives from the roots of *Arctium lappa L*. (burdock) and their structure–activity relationships (SARs) of free radical scavenging activities. *Phytochemistry Letters*, 15, 159–163.

Jiang, Y.Y, Li, Y., Wang, L., Hu, L. (2019). Extraction and antioxidant activities of polysaccharides from roots of *Arctium lappa* L. Int. J. Biol. Macromol.,123, 531–538.

Jingvi, L., Yi-Zhong, C., Ricky, N.S.W. (2012). Comparative Analysis of Caffeoylquinic Acids and Lignans in Roots and Seeds among Various Burdock (Arctium lappa) Genotypes with High Antioxidant Activity, *J. Agric. Food Chem.,* 60, 4067–4075.

Kim, D.H., Choi, Y.R., Shim, J. et al., 2020. Suppressive Effect of *Arctium lappa L*. Leaves on Retinal Damage against A2E-Induced ARPE-19 Cells and Mice. *Molecules*, 25(7), 1737.

Lee, J., Hab, S.J., Park, J., Kimb, Y.H., Lee, N.H. (2020). *Arctium lappa* root extract containing L-arginine prevents TNF-α-induced early atherosclerosis in vitro and in vivo. *Nutrition Research,* 77, 85–96.

Lee, C.J., Park, S.K., Kang, J.Y., Kim, J.M., Yoo, S.K., Han, H.J., Kim, D., Heo, H.J. (2019). Melanogenesis regulatory activity of the ethyl acetate fraction from *Arctium lappa L.* leaf on α-MSH–induced B16/F10 melanoma cells. *Ind. Crops Prod.*, 138, 111581.

Li, X., Zhao, Z., Kuang, P., Shi X., Wang, Z. (2019). Regulation of lipid metabolism in diabetic rats by *Arctium lappa L.*olysaccharide through the PKC/NF-κB pathway. *Int J. Biol Macrom*ol.136, 115–122.

Li, K., Zhu, L., Li, H., Zhu, Y. Q., Pan, C., Gao, X. D., & Liu, W.(2019). Structural characterization and rheological properties of pectin with anti-constipation activity from the roots of *Arctium lappa L., Carbohydr. Polym.*, 215, 119–129.

Liang, X., Gao, Y., Fei, Y., Zou, Y., He, M., Yin, L., Yuan, Z., Yin, Z., Zhang, W. (2018). Chemical characterization and antioxidant activities of polysaccharides isolated from the stems of *Parthenocissus tricuspidata*. *Int. J. Biol. Macromol*. 119, 70–78.

Lin, L.Z., Harnly, J.M. (2008). Identification of hydroxycinnamoylquinic acids of arnica flowers and burdock roots using a standardized LC-DAD-ESI/MS profiling method. *Journal of Agricultural and Food Chemistry*, 56, 10105–10114.

Lin, X.C., Liu, C.Y., Chen, K. (2004). Extraction and content comparison of chlorogenic acid in *Arctium lappa* L. leaves collected from different terrain and its restraining bacteria test. *Nat Prod Res Dev.,* 16, 328–330.

Liu, L., Tang, L. (1997). Studies on antimutagenicity of Burdock, *Acta Academiae Medicine Nanjing*, 4, 343–345.

Liu, J.J., Wang, Z.Z., Zhang, J.N. (2014). In vitro and in vivo antioxidant activity of fructan from the roots of *Arctium lappa* L. *Int. J. Biol. Macromol*. 65 446–453,

Liu, Y., Wang, G., Yang, M., Chen, H. (2015). Arctigenin reduces blood pressure by modulation of nitric oxide synthase and NADPH oxidase expression in spontaneously hypertensive rats. *Biochem Biophys Res Commun,* 468 (4), 837–842.

Lou, C., Zhu, Z., Zhao, Y., Zhu, R. (2017). Arctigenin, a lignan from *Arctium lappa L*., inhibits metastasis of human breast cancer cells through the downregulation of MMP-2/-9 and heparanase in MDA-MB-231 cells. *Oncol Rep* 37 (1), 179–184.

Lou, Z., Wang, H., Wang, D. (2009). Preparation of inulin and phenol-rich dietary fiber powder from the burdock root. *Carbohydrate Polymers, 78*(4), 666–671.

Machado, F.B., Yamamoto, R.E., Zanoli, K. (2012). Evaluation of the antiproliferative activity of the leaves from *Arctium lappa* by bioassay-guided fractionation. *Molecules,* 17(2), 1852–1859.

Maghsoumi-Norouzabad, L., Alipoor, B., Abed, R. (2016). Effects of *Arctium lappa* L. (Burdock) root tea on the inflammatory status and oxidative stress in patients with knee osteoarthritis. *Int J Rheum Dis*., 19, 255–261.

Maghsoumi-Norouzabada, L., Shishehborb, F., Abed, R. (2019). Effect of *Arctium lappa* linne (Burdock) root tea consumption on lipid profile and blood pressure in patients with knee osteoarthritis. *Journal of Herbal Medicine,* 17-18, 100266.

Matsumoto, T., Hosono-Nishiyama, K., Yamada, H. (2006). Antiproliferative and Apoptotic Effects of Butyrolactone Lignans from *Arctium lappa* on Leukemic. *Planta Med*., 72, 276–278.

Milani, E., Hosseini, F., Zaerzadeh, E. (2012). Optimization of Inulin Extraction from Burdock Tuber by Using Response Surface Methodology (RSM). *J. Med. Plants,* 11, 149–158.

Nema, A.M., Abdoul, H.B., Eldeen, R.S.S. (2019). The Role of *Arctium lappa L.* Extracts against Zinc Oxide Nanoparticles Induced Hepato-Cardiotoxicity and Hyperlipidemia in Male Rats. *Biology* 9(2), 1–16.

Olennikov, D. N., & Tankhaev, L. M. (2011). A quantitative assay for total fructans in burdock (*Arctium sp*p.) roots. *Russian Journal of Bioorganic Chemistry*, *37*(7), 893–898.

Oliveira, J.F., Almeida, R.B.A., Figueiredo, V. (2014). Control of microorganisms of oral health interest with *Arctium lappa L.* (burdock) extract non-cytotoxic to the cell culture of macrophages (RAW 264.7). *Archives of Oral Biology*. 59, 808–814.

Pereira, J.V., Bergamo, D.C., Pereira, J.O. (2005). Antimicrobial activity of *Arctium lappa* constituents against microorganisms commonly found in endodontic infections. *Braz Dent J*., 16, 192–196.

Petkova, N., Ivanov, I. , Mihaylova, D., Lante, A. (2020). Effect of pressure liquid extraction and ultrasonic irradiation frequency on inulin, phenolic content, and antioxidant activity in burdock (*Arctium lappa* L.) roots. *Acta Sci. Pol. Hortorum Cultu*s, 19(3), 125–133

Predes, F.B., Ruiz, A.L., Carvalho, J.E. (2011). Antioxidative and in vitro antiproliferative activity of *Arctium lappa* root extracts*. BMC Complement Altern Med*., 11, 25.

Predes, F.B., Silva Diamante M.A., Foglio, M.A. (2014). Hepatoprotective Effect of *Arctium lappa* Root Extract on Cadmium Toxicity in Adult Wistar Rats. Biol Trace Elem Res., 160, 250–257.

Qin, Z.B., Dinga, L.F, Wanga, X., Huanga, L.J. (2019). Lignans from the seeds of *Arctium lappa L*. (burdock) and their inhibitory effects on nitric oxide production, *Phytochemistry Letters*, 34, 43–49.

Rajasekharan S.K., Ramesh, S., Satish, A.S., Lee J., (2017). Antibiofilm and Anti-(-Lactamase Activities of Burdock Root Extract and Chlorogenic Acid against *Klebsiella pneumonia. J. Microbiol. Biotechnol.,* 27(3), 542–551.

Savina, A. A., Sheichenko V. I., Stikhin Y. (2006). Sesquiterpene lactones in the juice of great burdock leaves. *Pharm. Chem. J.,* 40, 624–626.

Skowronska, W., Granica, S., Dziedzic, M. (2021). *Arctium lappa* and *Arctium tomentosum*, Sources of Arctii radix: Comparison of Anti-Lipoxygenase and Antioxidant Activity as well as the Chemical Composition of Extracts from Aerial Parts and Roots. *Plants*, 10(1), 78.

Song, H.S., Koo, H.J., Park, B.K. (2018). The suppressive effect of the three-herb extract mixture on vascular and liver inflammation in atherogenic diet with high fructose-fed mice. *Pharm Biol,* 56, 32–42.

Strimpakos, A.S., Saif, M.W. (2013). Update on phase I studies in advanced pancreatic adenocarcinoma. *Hunting in darkness JOP* 14(4), 354–358.

Swarup, V., Ghosh, J., Mishra, M.K., (2008). Novel strategy for the treatment of Japanese encephalitis using arctigenin, a plant lignan. *J. Antimicrob. Chemother.,* 61, 679–88.

Tabas, I., Glass, C.K. (2013). Anti-inflammatory therapy in chronic disease: challenges and opportunities. *Science*, 339, 166–72.

Thaísa, M.A.M, Maria, T.P.S.C (2020). Burdock (*Arctium lappa L*) roots as a source of inulin-type fructans and other bioactive compounds: Current knowledge and future perspectives for food and non-food applications. Food Res. Int., 141, 109889.

Tian, X., Guo, L., Hu, X. (2015). Protective effects of *Arctium lappa L*. roots against hydrogen peroxide-induced cell injury and potential mechanisms in SH-SY5Y cells. *Cellular and Molecular Neurobiology*, 35(3), 335–344.

Tita, I., Mogosanu, G.D., Tita, M.G. (2009). Ethnobotanical inventory of medicinal plants from the southwest of Romania. *Farmacia*, 57, 141–156.

Tousch, D., Bidel, L.P.R., Cazals, G. (2014). Chemical Analysis and Antihyperglycemic Activity of an Original Extract from Burdock Root (*Arctium lappa*), *J. Agric. Food Chem.,* 62, 7738–7745.

Watanabe A., Hiroyuki, S., Hiroki, M. (2020). Effect of Dose and Timing of Burdock (*Arctium lappa*) Root Intake on Intestinal Microbiota of Mice. *Microorganisms*, *8*(2), 220.

Yang, Z., Liu N., Huang, B. (2005). Effect of anti-influenza virus of arctigenin *in vivo*. *Zhongyaocai,* 28(11), 1012–1014

Yang, W., Lee S., Jeong, Y. J. (2016). Antiallergic activity of ethanol extracts of *Arctium lappa L*. undried roots and its active compound, oleamide, in regulating FcεRI-mediated and MAPK signaling in RBL-2H3 cells. *Journal of Agricultural and Food Chemistry*, 64(18), 3564–3573.

Yao, X., Zhu, F., Zhao, Z., Liu, C. (2011). Arctigenin enhances the chemosensitivity of cancer cells to cisplatin through inhibition of the STAT3 signaling pathway. *J Cell Biochem* 112 (10), 2837–2849.

Yuan, Y., Dou, D., Kang, T. (2008). Evaluating the quality of *Arctiin Lappa L* with different origins, 2008. *Shijie Kexue Jishu-Zhongyiyao Xiandaihua*, 10, 75–7.

Zhang, N., Wang, Y., Kan, J. (2019). In vivo and in vitro anti-inflammatory effects of water-soluble polysaccharides from *Arctium lappa. Int J Biol Macromol.*,135, 717-24.

Zhang, H., Chen, K., Zhang, P. (2018). Synthesis of acetylation burdock fructooligosaccharide (BFO). IOP Conf. Ser.: Earth Environ. Sci., 108, 042085

Zheng, Z., Wang, X., Liu, P. (2018). Semi-Preparative Separation of 10 Caffeoylquinic Acid Derivatives Using High-Speed Counter-Current Chromatography Combined with Semi-Preparative HPLC from the Roots of Burdock (*Arctium lappa* L.). *Molecules*, 23(2), 429.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

MICROBIAL BIOSTIMULANTS INCREASE BIOACTIVE COMPOUNDS IN MEDICINAL PLANTS - A REVIEW

Lavinia-Diana-Nicoleta BARBU[1, 2], Oana-Alina BOIU-SICUIA[*1, 2], Călina Petruța CORNEA[1], Narcisa BĂBEANU[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Biotechnology, 59 Marasti Blvd., District 1, Bucharest, Romania
[2]Research-Development Institute for Plant Protection, 8 Ion Ionescu de la Brad Blvd., District 1, Bucharest, Romania

Corresponding author email: sicuia_oana@yahoo.com

Abstract

Functional foods are gaining popularity among consumers because they contain biologically active compounds involved in human health and wellness. Although some plant species are already known for their beneficial properties if properly consumed, certain cultivation technologies can increase their content of bioactive compounds. For this purpose, plant probiotics could be applied as biostimulants, which are approved in organic agriculture. Plant probiotics are beneficial microorganisms that can be found in the rhizosphere, or as endophytes. They can be applied as single strains or as consortia. These beneficial microorganisms could increase the production of biologic active compounds in their host plants, such as: flavonoids, organic acids, phenols, tannins, and vitamins. This review focuses on various plant probiotics and their role in the bioactive compounds synthesis in their hosts. Among plant probiotics mediating bioactive compounds synthesis in plants are mentioned Azospirillum, Azotobacter, Bacillus, Phyllobacterium, Pseudomonas, Rhizobium and several other genera of plant beneficial microorganisms.

Key words*: plant probiotics, beneficial bacteria, endophytes, medicinal bioactive compounds, functional foods.*

INTRODUCTION

Functional foods, nutraceuticals, and designer foods are edible products containing supplements which complete diets and improve consumer's health. The functional foods, beside their nutritional value, provide unexpected health benefits for the consumer. In comparison, designer foods are fortified with health promoting ingredients, while nutraceuticals are derived from food sources and used as additional health improvers to complete the basic nutritional value found in foods. All these became increasingly popular in our times, due to the fact they bring additional fiber, vitamins, minerals, biologic active compounds, prebiotics and probiotics in our diets. Nowadays, some spontaneous herbs also acquired unexpected credentials. Less popular plant species, such as amaranth (*Amaranthus* spp.), sorrel (*Rumex* spp.), purslane (*Portulaca oleracea*). dandelion greens (*Taraxacum* spp.), watercress (*Nasturtium officinale*) and others, are gaining popularity as functional foods, as they contain health beneficial bioactive compounds, vitamins, minerals or essential oils.

Other supplements with proven health benefits are the prebiotics and probiotics. The prebiotics are indigestible fibers that stimulate the intestinal microbiota in humans and animals. And the probiotics are viable microorganisms, mostly bacteria and some yeast, which are helping digestion and food transformation, with various beneficial traits for their host organisms. Although most studied probiotics are those for human and animal health, there is a special category of probiotics associated to plants, which bring beneficial attributes to these hosts. These plant probiotics are beneficial microorganism living in symbiotic relationship with the plant hosts or in free-living association with plants. They can act in different ways to increase plant development, to protect plant against different stress factors or increase their production of biologically active compounds

(de Souza Vandenberghe et al., 2017). Therefore, selected plant probiotics could be applied as bioprotectants, biocontrollers, biofertilizers or biostimulants, especially in organic gardening. These plant probiotic microorganisms could be found on plant surfaces, as epiphytes, root surface, as rhizospheric microorganisms, or inside plant tissues, as endophytes (Whipps et al., 2008; Dutta & Bora, 2019). The contact between plants and microorganisms is made at the rhizosphere, endosphere and phillosphere level. Plant roots penetrate various layers of soil substrate in search of nutrients and water. During soil exploration, they encounter millions of different microorganisms and have developed advanced genetic and metabolic mechanisms to both recruit and defend against microorganisms. Root colonization involves a complex molecular communication between microorganism and roots. Attracted by root exudates, the microorganisms migrate towards roots via chemotaxis and may colonize the root surface, and soil aggregates that form around roots, or both. Best studied microbial colonizers of the root system, with beneficial effects on their hosts, are PGPR (plant growth promoting rhizobacteria) and mycorrhiza. Most beneficial interactions between plants and their symbionts begin at the rhizosphere level and should be considered the first application management of plant probiotics (Walker et al., 2020). The phillosphere is considered a hostile environment for microorganisms due to the limited nutrient sources, and UV radiation, but microorganisms found at this level were able to regulate various processes in plants, such as production of biologic active compounds including organic acids, ascorbic acid (vitamin C), and diverse phenolic compounds, flavonoids and essential oils (Lindow & Leveau, 2002; Flores-Felix et al., 2008). Endophytes are more involved in plant metabolic activity than other plant associated microorganisms. Thanks to their intra-tissue colonization they have an intimate relationship with their host. Although plants are not revealing any symptomatology due to endophytic colonization, many processes in plants are improved (Fadiji & Babalola, 2020).
This review focuses on various plant probiotics and other plant associated microorganisms with beneficial traits for their host. Mostly, the role of plant associated microorganisms in the bioactive compounds synthesis in their hosts.

MATERIALS AND METHODS

This review, is presenting plants probiotics effect, as single population or consortia of microorganisms, on bioactive compounds production in their hosts.
Data collected in this study derives from research articles published since 1995, featuring the importance of plant associated microorganisms for their hosts (Table 1).
The beneficial aspects documented in this review are focused on the improved plant quality, regarding their nutritional and medicinal value, if properly colonized by certain microorganisms. The most studied bioactive compounds are the antioxidants, especially ascorbic acid (vitamin C); flavonoids, such as anthocyanin; chlorophyll; alkamides and essential oils.

Table 1. Plant probiotics used in agriculture
(adapted after Jiménez-Gómez et al., 2017)

Inoculated plants	Microbial inoculants & Plant probiotics	Triggered effects on bioactive compound composition	Reference
Begonia malabarica	*Glomus mosseae, Bacillus coagulans, Trichoderma viridae*	Increased various secondary metabolites, such as total phenols, ortho-dihydroxy phenols, tannins, flavonoids, and alkaloids	Selvaraj et al., 2017
Borago officinalis	AMF inoculated soil	Increased content of carotenoids and chlorophyll	Rahimi et al., 2017

Inoculated plants	Microbial inoculants & Plant probiotics	Triggered effects on bioactive compound composition	Reference
Brassica oleracea	*Bacillus megaterium, Pantoea agglomerans* and *B. subtilis*	Increase chlorophyll content	Turan et al., 2014
Capsicum annuum	*Rhizobium leguminosarum* PETP01	Increased antioxidant activity	Silva et al., 2014
Cymbopogon citratus	*Azotobacter* sp. and *Pseudomonas* sp.	Increased total flavonoid and total phenol content, improved the antioxidant capacity of the essential oils and helped lemongrass plants to tolerate better the abiotic stress	Mirzaei et al., 2020
Echinacea purpurea	*Glomus intraradices*	Increased production of secondary phytomedicinal metabolites	Araim et al., 2009
	Bacterial endophytes of *E. purpurea*	Increased concentration of alkamides	Maggini et al., 2017
Fagopyrum esculentum	*Azospirillum* spp. *and Azotobacter* spp. *inoculants* applied to buckwheat plants	Increased grain yield and concentrations of total flavonoid and phenol content in buckwheat	Singh et al., 2015
Fragaria x ananassa	*Bacillus* sp. RC23*, B. cereus* RC18, *B. megaterium* RC01 or *Paenibacillus polymyxa* RC05	Vitamin C enhancement	Erturk et al., 2012
	Phyllobacterium sp. PEPV15		Flores-Félix et al., 2015
Hibiscus sabdariffa	AMF or PGRP	Increased content of total chlorophyll and carotenoids in roselle	Sanayei et al., 2021
Hyoscyamus niger	*Pseudomonas putida* 168 or *P. fluorescens* 187 strains	Simulated antioxidant enzymes activity, increased proline accumulation, improved tropane alkaloid production and yield of root and shoot organs.	Ghorbanpour et al., 2013
Lactuca sativa	Endophytic plant growth promoting selenobacteria with or without mycorrhizal fungi	Increased content of total chlorophyll and carotenoids in lettuce	Durán et al., 2016
Lycopersicon esculentum	*Bacillus amyloliquefaciens* FZB42	Vitamin C enhancement	Gül et al., 2008
	Bacillus amyloliquefaciens and *B. megaterium*		Shen et al., 2016
	Pseudomonas sp. 19Fv1T		Bona et al., 2017
	Mixture of PGPR (*Pseudomonas putida* 41, *Azotobacter chroococcum* 5, and *Azospirillum lipoferum* OF strains) and AMF (*Glomus intaradics*, *G. mossea*, and *G. etanicatum*)	Increased antioxidant activity and lycopene content	Ordookhani et al., 2010
	Mixture of PGPR (*A. chroococcum* 5, and *A. lipoferum* OF strains) and AMF (*G. intaradics*, *G. mossea*, and *G. etanicatum*)		
	Mixture of the PGPR strains: *P. putida* 41, *A. chroococcum* 5, and *A. lipoferum* OF strain		
	Bacillus licheniformis	Improved total flavonoids content	Ochoa-Velasco et al., 2016
Mentha piperita	*Pseudomonas fluorescens*	Increased essential oils amount	Banchio et al., 2008

Inoculated plants	Microbial inoculants & Plant probiotics	Triggered effects on bioactive compound composition	Reference
Mentha piperita	Plant growth promoting rhizobacteria	Increased amount of essential oils	Santoro et al., 2011
Ocimum basilicum	*Bacillus subtilis* GB03	Elevated α-terpenol and eugenol accumulation	Banchio et al., 2009
	Mixture of *P. putida* 41 strain, *A. chroococcum* 5 strain, *A.lipoferum* OF strain	Increased levels of essential oils antioxidant activity and microelements content	Ordookhani, 2011
	Bacillus lentus, Pseudomonas sp., *Azospirillum brasilense*	Increased antioxidant activity and chlorophyll leaf content	Heidari & Golpayegani, 2012
	Diazotrophs	Increased carotenoids and chlorophyll content in purple basil	Mariotti et al., 2021
Origanum majorana	*Pseudomonas fluorescens Bradyrhizobium sp.*	Increased amount of essentials oils	Banchio et al., 2008
Nasturtium officinale	*Bacillus subtilis*	Increased antioxidant capacity and total phenols of watercress	Pignata et al., 2016
Pelargonium graveolens	*Bacillus subtilis, Pseudomonas fluorescens*	Enhanced essential oil yield	Mishra et al., 2010
Pelargonium species	*Glomus intraradices* AMF and phosphate solubilizing bacteria	Enhanced yield and composition of essential oil (citronellol, geraniol, geranial, and eudesmol) in rose-scented geranium	Prasad et al., 2012
Ribes nigrum	ProbioHumus (Baltic Probiotics, Latvia) based on *Saccharomyces cerevisiae* yeast, *Bacillus subtilis* sporulated bacteria, *Bifidobacterium animalis, B. bifidum, B. longum, Lactobacillus casei, L. diacetylactis, L. delbrueckii, L. plantarum, Lactococcus lactis, Streptococcus thermophiles* lactic acid bacteria, *Rhodopseudomonas palustris,* and *R. sphaeroides* phototropic bacteria	Ascorbic acid and anthocyanins increased content	Jurkonienė et al., 2021
	NaturGel containing also microorganisms of *Azotobacter, Bacillus, Rhizobium, Bradyrhizobium, Lactobacillus,* and *Trichoderma* genera		
	Probiotic strains and mycorrhizal fungi	Enhanced antioxidant activity of berries and increased level of anthocyanins in fruits	Lingua et al., 2013
Rubus sp.	*P. fluorescens* N21.4	Increase and stabilize total flavonoid content in blackberry	Ramos-Solano et al., 2014
Spinacia oleracea	*Rhizobium* sp. PEPV12	Increase chlorophyll content	Jiménez-Gómez et al., 2016
Stevia rebaudiana	PGPR (such as *Azotobacter chroococcum, Bacillus polymixa, Pseudomonas putida)* with or without *Glomus intraradices* AMF	Increased chlorophyll content and augmented stevioside amount	Vafadar et al., 2014
Tagetes minuta	*Pseudomonas fluorescens, Azospirillum brasilense*	Increased monoterpene and phenolic compounds	del Rosario Cappellari at al., 2013

RESULTS AND DISCUSSIONS

The interaction between medicinal plants and their endophytic community was studied in order to review the increased production of biologic active compounds in microbial fortified plants. A significant increase of vitamin C level in plants was observed if microbial inoculated. The vitamin C is also known as ascorbic acid.

The results obtained by Flores-Félix et al. (2015) on strawberries showed that vitamin C levels were significantly higher in the fruits of plants inoculated with *Phyllobacterium* sp. PEPV15 strain, with approximately 79% more, compare to the uninoculated control. Previous studies performed by Erturk et al. (2012) showed high levels of vitamin C content in strawberry fruits after plants were inoculated with *Paenibacillus polymyxa* RC05 strain, as well as with *Bacillus megaterum* RC01, *Bacillus cereus* RC18 and *Bacillus* RC23 single strains, but not significant when treated with *Bacillus* RC03. Less significant differences regarding vitamin C content were observed in strawberry after foliar, and/or root application of *Pseudomonas* BA-8, *Bacillus* OSU-142 and *Bacillus* M-3 strains (Pirlak et al., 2009). Although these studies are revealing a wide variation regarding vitamin C levels in fruits of microbial inoculated strawberry plants, all bacteria used stimulated plant growth and increased different parameters of growth and productivity. This means that proper inoculants should be properly selected in order to increase the levels of bioactive compounds in plants.

Beside strawberries, blackcurrant berries are also known for their beneficial impact on human health (Hannum, 2004; Gopalan et al., 2012). Blackcurrant berries are rich in anthocyanins, polyphenolic substances, antioxidants, vitamin C and gamma-linolenic acid. A recent study performed on blackcurrant grown in organic farming system showed that commercial plant probiotic products, like ProbioHumus and NaturGel increased the contents of ascorbic acid and anthocyanins. Moreover, when sprayed as single treatments or in combination, they significantly improved fruit yields with 38, 25, and 16%, respectively, compared with uninoculated control (Jurkonienė et al., 2021).

Increased levels of vitamin C are also detected in vegetables after bacterial inoculation. In tomato fruits, the vitamin C content varies depending on production system (autumn or spring), nutrient system (open or closed-loop fertigation), concentrations of nutrient solution (full or half amount) and microbial treatments (PGPR inoculated or control). For instance, Gül et al. (2008) obtained best levels of vitamin C in tomato fruits (19.43 mg/100 ml) produced in spring growing season, when plants grown in perlite pots were inoculated with *Bacillus amyloliquefaciens* FZB42, in closed-loop fertigation system, with half amount of the normally used concentration of nutrient solution. Additionally, it was shows that vermicompost combined with plant probiotics *Bacillus megaterium* and *B. amyloliquefaciens* inoculation also increases vitamin C contents in fruits and tomato yield (Shen et al., 2016). Inoculation with *Pseudomonas* sp. 19Fv1T strain not only enhanced yield of tomato plants but also increased vitamin C concentration in fruits compared with the control treatment (Bona et al., 2017).

Beside vitamin C content, the antioxidant activity was also analyzed. On basil (*Ocimum basilicum* L.) studies were performed with various mixtures of *Pseudomonas putida* 41, *Azotobacter chroococcum* 5, and *Azospirillum lipoferum* OF strains presented increased levels of essential oils antioxidant activity and microelements content, compared to the control treatment (Ordookhani, 2011). Inoculants based on *Pseudomonas* sp., *Bacillus lentus* and *Azospirillum brasilense* on water stressed basil also increased antioxidant activity. Catalase and guaiacol peroxidase activity were higher as well as leaf chlorophyll content (Heidari & Golpayegani, 2012). The antioxidant activity was higher also in tomato fruits when plants were treated with the PGPR mixture of *Pseudomonas putida* 41, *Azotobacter chroococcum* 5, and *Azospirillum lipoferum* OF strains, with and without AMF (arbuscular mycorrhizal fungi) colonized soil (Ordookhani et al., 2010).

Bacterial inoculation with *Bacillus subtilis* increased the antioxidant capacity and total phenols of watercress (*Nasturtium officinale* R. Br.) medicinal plant (Pignata et al., 2016)

The antioxidant activity of berries can also be increased by plant microbial inoculation. Probiotic strains and mycorrhizal fungi are able to increase the level of anthocyanins in fruits (Lingua et al., 2013).

In addition to vitamin production and antioxidant activity, some plant probiotics are able to stimulate also carotenoids production. Such pigments are involved in photosynthesis, photoprotection, and act as stress hormones and signaling molecules in plants (Shumskaya & Wurtzel, 2013). Carotenoids are also revealing human health, nutrition and wellbeing attributes and some are precursors of vitamin A or act as antioxidants (Park et al., 2017). Beside the very well-known beta-carotene, lycopene is another known carotenoid. In tomato fruits, lycopene amount can be increased by proper PGPR inoculation of tomato plants. The combined treatment of PGPR and AMF on tomatoes also revealed higher levels of lycopene and potassium in tomato fruits (Ordookhani et al., 2010). Plants inoculation with *Pseudomonas putida* 41, *Azotobacter chroococcum* 5, and *Azospirillum lipoferum* OF strains of PGPR along with a mixture of *Glomus lipoferum, G. mossea* and *G. etunicatum* AMF increased not only lycopene amount in fruits but also antioxidant levels and potassium content in fruits and shoots (Ordookhani et al., 2010).

Carotenoids and chlorophyll content was increased in lettuce plants inoculated with endophytic plant growth promoting selenobacteria with or without mycorrhizal fungi (Durán et al., 2016). Similar effects were seen also in the medicinal plants *Borago officinalis* grown in AMF inoculated soil (Rahimi et al., 2017), in roselle (*Hibiscus sabdariffa*) inoculated either with AMF or PGRP (Sanayei et al., 2021), in purple basil (*Ocimum basilicum* L. cv. Red Rubin) inoculated with diazotrophs (Mariotti et al., 2021).

In *Stevia rebaudiana*, not only the chlorophyll content was increased but also the stevioside amount was augmented in biofertilised plants. Best results were obtained with the combined treatment of *Azotobacter chroococcum* PGPR + *Glomus intraradices* AMF, followed by other mixed treatments, such as *Bacillus polymyxa* + *G.intraradices*, or *A. chroococcum* + *Pseudomonas putida*. Although, the other nine microbial biofertilizers also revealed improved NPK, chlorophyll and steviosede content compared to the untreated control (Vafadar et al., 2014).

Azospirillum spp. and *Azotobacter* spp. inoculants applied to buckwheat plants (*Fagopyrum esculentum*) increased grain yield and concentrations of total flavonoid and phenol content (Singh et al., 2015). In water stressed lemongrass (*Cymbopogon citratus*), *Azotobacter* sp. and *Pseudomonas* sp. inoculants not only that increased total flavonoid and total phenol content, but also improved the antioxidant capacity of the essential oils and helped plants to tolerate better the abiotic stress (Mirzaei et al., 2020).

Pseudomonas fluorescens N21.4 inoculation in *Rubus* sp. var. Lochness increase and stabilize total flavonoid content in blackberry (Ramos-Solano et al., 2014).

Effect of root colonization with selected microorganisms was studied to different medicinal plants in order to determine the composition and amount of essential oils. *Pseudomonas fluorescens* inoculation of peppermint (*Mentha piperita*), as well as *P. fluorescens* + *Bradyrhizobium* sp. inoculation of oregano (*Origanum majorana*) increased total essential oil content without modifying the composition (Banchio et al., 2008). Later studies on microorganisms' effect on peppermint showed that the volatile organic compounds of rhizobacteria can increase biosynthesis of essential oils and plant growth parameters (Santoro et al., 2011).

Phytosterols are essential biologic active compounds present in high concentrations in vegetable oils (Granado et al., 1995). Some studies suggest that application of plant probiotics can increase the levels of sterols in plants. Silva et al. (2014) inoculated two strains of *Rhizobium* (TVP08 and PEPT01) in pepper (*Capsicum annuum*) and evaluated their effect on sterols. The rhizobia inoculation produced a positive effect on the ripening of the pepper fruit, in addition to an improvement in several primary and secondary metabolites, which improved the nutritional value of the plant. Moreover, the aqueous extracts of *Capsicum*

annuum leaves, after bacterial inoculation with *Rhizobium* TVP08 strain, presented a significant acetylcholinesterase (AchE) inhibitory activity, with an important relevance in the treatment of Alzheimer's disease (Silva et al., 2014).

Alkamides from *Echinacea* have significant anti-inflammatory and immunomodulatory properties. Maggini et al. (2017) reported an increased concentration of alkamides in bacterial colonized plants of *Echinacea purpurea*.

Microbial inoculants perspectives

Studies on microbial inoculants are in continuous process, either for new strains selection, analyzing their mechanisms of action, or understanding their implications on plants or environment. Microbial inoculants development is taking place at a global level. In agriculture, most of them are used as microbial fertilizers, biostimulants or plant protection products. However, in most countries, commercialization depends on specific established standards, and government approval. However, according to policy initiatives addressed at the global level (e.g. Horizon 2020), it is absolutely necessary that the quantities of chemical fertilizers used be kept to a minimum and that "green products" be introduced (García-Fraile et al., 2015). Many countries have developed government policies for the use of biofertilizers, and biostimulants, although, the regulations are still in progress (García-Fraile et al., 2017).

In this context, collaboration between researchers and industry is a key factor in establishing the basis of a global biofertilizer market (García-Fraile et al., 2015).

Microbial inoculants or plant probiotics must meet a number of characteristics, but most importantly they must be safe for the environment and humans. Another important feature is their stability, and viability, also in terms of survival in certain biotic or abiotic conditions. Inoculants should include the most effective microbial strains that promote plant growth and productivity (Glick, 2012). Selected microbial strains should survive in wide range environmental conditions, as well as in certain abiotic stress factors (Chauhan et al., 2015). Various formulation types are available for microbial inoculant used in agriculture most commons being the granules, powders, liquids, suspensions, emulsions and effervescent products (Kamilova et al., 2015; Lesueur et al., 2016; Zamfiropol-Cristea et al., 2017; Macik et al., 2020).

CONCLUSIONS

Microbial inoculants are having an important role in agriculture, stimulating plant growth and development. Their beneficial traits were demonstrated in both normal and stressful environmental conditions of plant production. This review documented important knowledge regarding microbial inoculants and their role in plant stimulation to produce phytomedicinal metabolites and nutritional compounds.

Such microbial inoculants can be considered plant probiotics, as they are able to improve plant physiological status in various environmental conditions. The applications of plant probiotics in agricultural practices enhance plants yield and quality.

REFERENCES

Araim, G., Saleem, A., Arnason, T., & Charest, C. (2009). Root colonization by arbuscular mycorrhizal (AM) fungus increases growth and secondary metabolism of purple cone flower, *Echinacea purpurea* (L.) Moench. *Journal of Agricultural and Food Chemistry*, *57* (6), 2255-2258. DOI: 10.1021/jf803173x.

Banchio, E., Bogino, P.C., Zygadlo, J., & Giordano, W. (2008). Plant growth promoting rhizobacteria improve growth and essential oil yield in *Origanum majorana* L. *Biochemical Systematics and Ecology, 36* (10)*,* 766-771.

Banchio, E., Xie, X., Zhang, H., & Paré, P. W. (2009). Soil bacteria elevate essential oil accumulation and emissions in sweet basil. J. Agric. Food Chem. 57, 653-657. DOI: 10.1021/jf8020305.

Bona, E., Cantamessa, S., Massa, N., Manassero, P., Marsano, F., Copetta, A., Lingua, G., D'Agostino, G., Gamalero, E., & Berta, G. (2017). Arbuscular mycorrhizal fungi and plant growth-promoting pseudomonads improve yield, quality and nutritional value of tomato: a field study. *Mycorrhiza, 27*(1), 1-11. https://doi.org/10.1007/s00572-016-0727-y.

Chauhan, H., Bagyaraj, D.J., Selvakumar, G., & Sundaram, S. (2015). Novel plant growth promoting rhizobacteria - Prospects and potential. *Applied Soil Ecology, 95*, 38-53.

del Rosario Cappellari, L., Valeria Santoro, M., Nievas, F., Giordano, W., & Banchio, E. (2013). Increase of secondary metabolite content in marigold by inoculation with plant growth-promoting

rhizobacteria. *Applied Soil Ecology*, 70, 16-22. https://doi.org/10.1016/j.apsoil.2013.04.001.

de Souza Vandenberghe, L. P., Garcia, L., Rodrigues, C., Camara, M. C., de Melo Pereira, G. V., de Oliveira, J., & Soccol, C. R. (2017). Potential applications of plant probiotic microorganisms in agriculture and forestry. *AIMS microbiology*, *3*(3), 629-648. https://doi.org/10.3934/microbiol.2017.3.629.

Durán, P., Acuña, J.J., Armada, E., López-Castillo, O.M., Cornejo, P., Mora, M.L., & Azcón, R., (2016). Inoculation with selenobacteria and arbuscular mycorrhizal fungi to enhance selenium content in lettuce plants and improve tolerance against drought stress. Journal of soil science and plant nutrition, 16 (1), 211-225. http://dx.doi.org/10.4067/S0718-95162016005000017.

Dutta, J., & Bora, U. (2019). Chapter 18 - Rhizosphere microbiome and plant probiotics. In J.S. Singh (Ed.), *New and Future Developments in Microbial Biotechnology and Bioengineering,* 273-281. Elsevier, https://doi.org/10.1016/B978-0-12-818258-1.00018-2.

Erturk, Y., Ercisli, S., & Cakmakci, R. (2012). Yield and growth response of strawberry to plant growth-promoting rhizobacteria inoculation. *Journal of Plant Nutrition, 35*(6), 817-826.

Fadiji, A.E., & Babalola, O.O. (2020). Exploring the potentialities of beneficial endophytes for improved plant growth. *Saudi Journal of Biological Sciences*, *27* (12), 3622-3633.

Flores-Félix, J. D., Velázquez, E., García-Fraile, P., González-Andrés, F., Silva, L. R., & Rivas, R. (2018). *Rhizobium* and *Phyllobacterium* bacterial inoculants increase bioactive compounds and quality of strawberries cultivated in field conditions. *Food research international (Ottawa, Ont.), 111*, 416-422. https://doi.org/10.1016/j.foodres.2018.05.059.

Flores-Félix, J. D., Silva, L. R., Rivera, L. P., Marcos-García, M., García-Fraile, P., Martínez-Molina, E., Mateos, P. F., Velázquez, E., Andrade, P., & Rivas, R. (2015). Plants probiotics as a tool to produce highly functional fruits: the case of *Phyllobacterium* and vitamin C in strawberries. *PloS One, 10*(4), e0122281.

García-Fraile, P., Menéndez, E., & Rivas, R. (2015). Role of bacterial biofertilizers in agriculture and forestry. *AIMS Bioengineering, 2* (3),183-205.

García-Fraile, P., Menéndez, E., Celador-Lera, L., Díez-Méndez, A., Jiménez-Gómez, A., Marcos-García, M., Cruz-González, X.A., Martínez-Hidalgo, P., Mateos, P.F., & Rivas, R. (2017). Chapter 6 - Bacterial Probiotics: A Truly Green Revolution. In: Kumar V., Kumar M., Sharma S., Prasad R. (eds). *Probiotics and Plant Health* (pp. 131-162). Springer, Singapore. https://doi.org/10.1007/978-981-10-3473-2_6.

Ghorbanpour, M., Hatam, M., & Khavazi, K. (2013). Role of plant growth promoting rhizobacteria on antioxidant enzyme activities and tropane alkaloid production of Hyoscyamus niger under water deficit stress. Turkish Journal of Biology, 37, 350-360. DOI:10.3906/biy-1209-12.

Glick, B.R. (2012). Plant growth-promoting bacteria: mechanisms and applications. *Scientifica*, 963401. http://dx.doi.org/10.6064/2012/963401.

Gopalan, A., Reuben, S.C., Ahmed, S., Darvesh, A.S., Hohmannb, J., & Bishayee, A. (2012). The health benefits of blackcurrants. *Food & Function, 3*, 795-809.

Granado, J., Felix, G., & Boller, T. (1995). Perception of fungal sterols in plants (subnanomolar concentrations of ergosterol elicit extracelular alkalinization in tomato cells). *Plant Physiology, 107*(2), 485-490. https://doi.org/10.1104/pp.107.2.485.

Gül, A., Kidoglu, F., Tüzel, Y., & Tüzel, I.H. (2008). Effects of nutrition and *Bacillus amyloliquefaciens* on tomato (*Solanum lycopersicum* L.) growing in perlite. *Spanish Journal of Agricultural Research, 6*(3), 422-429. https://doi.org/10.5424/sjar/2008063-335.

Hannum S. M. (2004). Potential impact of strawberries on human health: a review of the science. Critical Reviews in Food Science and Nutrition, 44(1), 1-17. https://doi.org/10.1080/10408690490263756.

Heidari, M., & Golpayegani, A. (2012). Effects of water stress and inoculation with plant growth promoting rhizobacteria (PGPR) on antioxidant status and photosynthetic pigments in basil (*Ocimum basilicum* L.). *Journal of the Saudi Society of Agricultural Sciences, 11*(1), 57-61.

Jiménez-Gómez, A., Celador-Lera, L., Fradejas-Bayón, M., & Rivas, R. (2017). Plant probiotic bacteria enhance the quality of fruit and horticultural crops. *AIMS microbiology, 3(3)*. 483-501. https://doi.org/10.3934/microbiol.2017.3.483.

Jiménez-Gómez, A., Menéndez, E., Flores-Félix, J.D., García-Fraile, P. Mateos, P.F., & Rivas, R. (2016). Effective Colonization of Spinach Root Surface by Rhizobium. In: F.González-Andrés & E. James (Eds.) *Biological nitrogen fixation and beneficial plant-microbe interaction*, 109-122, Springer International Publishing. https://link.springer.com/ chapter/ 10.1007/978-3-319-32528-6_10

Jurkonienė, S., Mockevičiūtė, R., Jankauskienė, J., Jankovska-Bortkevič, E., Armalytė, G., & Gavelienė, V. (2021). Application of Commercial Plant Probiotics Improves the Berry Yield and Quality of Field-Grown Blackcurrant. *ACS Agricultural Science & Technology, 1*(6), 615-622. https://doi.org/ 10.1021/acsagscitech.1c00115.

Kamilova, F., Okon, Y., de Weert, S., & Hora, K. (2015) Commercialization of microbes: Manufacturing, inoculation, best practice for objective field testing, and registration. In: Lugtenberg B. (Ed.). *Principles of Plant-Microbe Interactions*, 319-328. Springer, Cham. https://doi.org/10.1007/978-3-319-08575-3_33.

Lesueur, D., Deaker, R., Herrmann, L., Bräu, L., & Jansa, J. (2016) The production and potential of biofertilizers to improve crop yields. In: Arora N., Mehnaz S., Balestrini R. (Eds.) *Bioformulations: for sustainable agriculture.* 71-92. Springer, New Delhi. https://doi.org/10.1007/978-81-322-2779-3_4.

Lindow, S.E., & Leveau, J.H. (2002). Phyllosphere microbiology. *Current Opinion in Biotechnology 13*. 238-243.

Lingua, G., Bona, E., Manassero, P., Marsano, F., Todeschini, V., Cantamessa, S., Copetta, A., D'Agostino, G., Gamalero, E., & Berta, G. (2013). Arbuscular mycorrhizal fungi and plant growth-promoting pseudomonads increases anthocyanin concentration in strawberry fruits (*Fragaria* x *ananassa* var. Selva) in conditions of reduced fertilization. *International Journal of Molecular Sciences, 14*(8), 16207-16225. https://doi.org/10.3390/ijms140816207.

Macik, M., Gryta, A., & Frac, M. (2020). Biofertilizer in agriculture: An overview on concepts, strategies and effects on soli microorganism. Advances in Agronomy, 162, 31-76.

Maggini, V., De Leo, M., Mengoni, A., Gallo, E. R., Miceli, E., Reidel, R. V. B., Biffi, S., Pistelli, L., Fani, R., Firenzuoli, F., & Bogani, P. (2017). Plant-endophytes interaction influences the secondary metabolism in *Echinacea purpurea* (L.) Moench: an *in vitro* model. *Scientific Reports, 7*(1), 16924. https://doi.org/10.1038/s41598-017-17110-w.

Mariotti, L., Scartazza, A., Curadi, M., Picciarelli, P., & Toffanin, A. (2021). *Azospirillum baldaniorum* Sp245 induces physiological responses to alleviate the adverse effects of drought stress in purple basil. Plants, 10, 1141. DOI: 10.3390/plants10061141.

Mirzaei, M., Ladan Moghadam, A., Hakimi, L., & Danaee. E. (2020). The Plant growth promoting rhizobacteria (PGPR) improve plant growth, antioxidant capacity, and essential oil properties of lemongrass (*Cymbopogon citratus*) under water stress. *Iranian Journal of Plant Physiology 10*(2), 3155-3166.

Mishra, R. K., Prakash, O., Alam, M., & Dikshit, A. (2010). Influence of plant growth promoting rhizobacteria (PGPR) on the productivity of *Pelargonium graveolens* L. Herit. *Recent Research in Science and Technology 2*, 53-57.

Ochoa-Velasco, C.E., Valadez-Blanco, R., Salas-Coronado, R., Sustaita-Rivera, F., Hernández-Carlos, B., García-Ortega, S., & Santos-Sánchez, N.F. (2016). Effect of nitrogen fertilization and *Bacillus licheniformis* biofertilizer addition on the antioxidants compounds and antioxidant activity of greenhouse cultivated tomato fruits (*Solanum lycopersicum* L. var. Sheva). *Scientia Horticulturae, 201*, 338-345.

Ordookhani, K. (2011). Investigation of PGPR on antioxidant activity of essential oil and microelement contents of sweet basil. *Advances in Environmental Biology, 5*, 1114-1120.

Ordookhani, K., Khavazi, K., Moezzi, A., & Rejali, F. (2010). Influence of PGPR and AMF on antioxidant activity, lycopene and potassium contents in tomato. *African Journal of Agricultural Research, 5*(10), 1108-1116.

Park, Y. J., Park, S. Y., Valan Arasu, M., Al-Dhabi, N. A., Ahn, H. G., Kim, J. K., & Park, S. U. (2017). Accumulation of carotenoids and metabolic profiling in different cultivars of tagetes flowers. *Molecules (Basel, Switzerland), 22*(2), 313. https://doi.org/10.3390/molecules22020313.

Pignata, G., Niñirola, D., Casale, M., Lo Turco, P.E., Egea-Gilabert, C., Fernández, J.A., & Nicola, S. (2016).Inherent quality and safety of watercress grown in a floating system using *Bacillus subtilis*. *The Horticulture Journal, 85*(2), 148-153. doi: 10.2503/hortj.MI-091.

Pırlak, L., & Köse, M. (2009). Effects of plant growth promoting rhizobacteria on yield and some fruit properties of strawberry. *Journal of Plant Nutrition, 32*(7), 1173-1184.

Prasad, A., Kumar, S., Pandey, A., & Chand, S. (2012). Microbial and chemical sources of phosphorus supply modulate the yield and chemical composition of essential oil of rose-scented geranium (Pelargonium species) in sodic soils. Biology and Fertility of Soils, 48(1), 117-122.

Ramos-Solano, B., Garcia-Villaraco, A., Gutierrez-Mañero, F. J., Lucas, J. A., Bonilla, A., & Garcia-Seco, D. (2014). Annual changes in bioactive contents and production in field-grown blackberry after inoculation with *Pseudomonas fluorescens*. *Plant Physiology and Biochemistry, 74*, 1-8. https://doi.org/10.1016/j.plaphy.2013.10.029.

Rahimi, A., Jahanbin, Sh., Salehi, A., & Farajee, H. (2017). Changes in content of chlorophyll, carotenoids, phosphorus and relative water content of medicinal plant of borage (*Borago officinails* L.) under the influence of mycorrhizal fungi and water stress. *Journal of Biological Sciences, 17*, 28-34. DOI: 10.3923/jbs.2017.28.34

Sanayei, S., Barmaki, M., Ebadi, A., & Torabi-Giglou, M. (2021). Amelioration of water deficiency stress in roselle (*Hibiscus sabdariffa*) by arbuscular mycorrhizal fungi and plant growth-promoting rhizobacteria. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 49*(2), 11987. DOI: 10.15835/nbha49211987.

Santoro, M.V., Zygadlo, J., Giordano, W., & Banchio, E. (2011). Volatile organic compounds from rhizobacteria increase biosynthesis of essential oils and growth parameters in peppermint (*Mentha piperita*). *Plant Physiology and Biochemistry, 49*, 1177-1182.

Selvaraj, T., Rajeshkumar, S., Nisha, M.C., Wondimu, L. & Tesso. M. (2008). Effect of *Glomus mosseae* and plant growth promoting rhizomicroorganisms (PGPR's) on growth nutrients and content of secondary metabolites in *Begonia malabarica* Lam. *International Journal of Science and Emerging Technologies, 2,* 516-525.

Shumskaya, M., & Wurtzel, E.T. (2013). The carotenoid biosynthetic pathway: Thinking in all dimensions. *Plant Science, 208*, 58-63.

Shen, F., Zhu, T-B., Teng, M-J., Chen, Y., Liu, M-Q., Hu, F., & Li, H-X. (2016). [Effects of interaction between vermicompost and probiotics on soil nronerty, yield and quality of tomato.] *Ying Yong Sheng Tai Xue Bao, 27*(2), 484-490.

Silva, L. R., Azevedo, J., Pereira, M. J., Carro, L., Velazquez, E., Peix, A., Valentão, P., & Andrade, P. B. (2014). Inoculation of the nonlegume *Capsicum annuum* (L.) with *Rhizobium* strains. 1. Effect on bioactive compounds, antioxidant activity, and fruit

ripeness. *Journal of Agricultural and Food Chemistry, 62* (3), 557-564. DOI: 10.1021/jf4046649.

Singh, R., Babu, S., Avasthe, R.K., Yadav, G. S., Chettri, T.K., Phempunadi, C. D. & Chatterjee, T. (2015). Bacterial inoculation effect on soil biological properties, growth, grain yield, total phenolic and flavonoids contents of common buckwheat (*Fagopyrum esculentum* Moench) under hilly ecosystems of North-East India. *African Journal of Microbiology Research, 9*(15), 1110-1117.

Turan, M., Ekinci, M., & Yildirim, E. (2014). Plant growth-promoting rhizobacteria improved growth, nutrient, and hormone content of cabbage (*Brassica oleracea*) seedlings. *Turkish Journal of Agriculture and Forestry, 38*, 327-333.

Vafadar, F., Amooaghaie, R., & Otroshy, M. (2014). Effects of plant-growth-promoting rhizobacteria and arbuscular mycorrhizal fungus on plant growth, stevioside, NPK, and chlorophyll content of *Stevia rebaudiana*. *Journal of Plant Interactions, 9* (1), 128-136, DOI: 10.1080/17429145.2013.779035.

Walker, R., Otto-Pille, C., Gupta, S., Schillaci, M., & Roessner, U. (2020). Current perspectives and applications in plant probiotics. *Microbiology Australia 41*. 95-99. DOI: 10.1071/MA20024.

Whipps, J.M., Hand, P., Pink, D., & Bending, G.D. (2008). Phyllosphere microbiology with special reference to diversity and plant genotype, *Journal of Applied Microbiology, 105*, 1744-1755.

Zamfiropol-Cristea V., Răut I., Șeșan T.E., Trică B., & Oancea F. (2017). Surface response optimization of submerged biomass production for a plant biostimulant Trichoderma strain. *Scientific Bulletin. Series F. Biotechnologies, 21*, 56-65.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

RHIZOBIAL EXOPOLYSACCHARIDES: STRUCTURE AND APPLICATIONS

Roxana-Mădălina STOICA, Angela CĂȘĂRICĂ, Ana-Despina IONESCU

National Institute for Chemical-Pharmaceutical Research and Development – ICCF Bucharest, 112 Vitan Avenue, District 3, Bucharest, Romania

Corresponding author email: roxym_stoica@yahoo.com

Abstract

Microbial exopolysaccharides (EPS) represent an important group of biologically active compounds produced and secreted by bacteria and fungi, which accumulate outside the cells. Recently, research has been focused on the exploration and discovery of new exopolysaccharides of microbial origin, due to their various biotechnological applications. Rhizobium strains produce a wide diversity of exopolysaccharides, with different structures at the species level, and a large field of applications, such as pharmaceutical, food, and cosmetics industries. In this context, this article aims to present a mini-review of the main EPS synthesized by Rhizobium strains, highlighting their structures and potential applications.

Key words*: applications, exopolysaccharides, Rhizobium spp., structure.*

INTRODUCTION

In recent years, interest in the exploitation of microorganisms for production and obtaining of valuable polysaccharides has significantly increased (Shanmugam & Abirami, 2019).

These biopolymers produced by a variety of microorganisms present commercially relevant properties which are suitable and attractive for a wide range of applications, ranging from several chemical industries to biomedicine and cosmetics (Freitas et al., 2014; Ventorino et al., 2019).

In general, bacterial polysaccharides can be grouped into intracellular polysaccharides, cell wall polysaccharides, and those that are released into the cell culture medium called extracellular polysaccharides (EPS) (Jeong et al., 2022).

In the rhizosphere, polysaccharides play a key role in cell signaling reactions between plants and microorganisms, like plant root nodulation for nitrogen fixation, and also, biofilm formation (Jeong et al., 2022).

Rhizobium genus comprises symbiotic nitrogen-fixing species associated with the roots of legume plants (Gonzalez et al., 2019). *Rhizobium* species are Gram-negative, aerobic, rod shaped bacteria and non-spore forming (Zakhia & de Lajudie, 2001; Ribeiro & Burkert, 2016).

Rhizobial strains can be considered unexplored sources of microbial exopolysaccharides, promising for industrial applications, due to their high morphological, genetic and phylogenetic diversity (Bomfeti et al., 2011; Donot et al., 2012; Sethi et al., 2019). Exopolysaccharides produced by *Rhizobium* species can be obtained through submerged fermentation processes with high yields and controllable operation parameters (temperature, agitation, aeration, pH, dissolved oxygen level, pH correction, substrate addings) (Jeong et al., 2022). In this mini-review, we summarize the current knowledge about exopolysaccharides produced by several *Rhizobium* species, focusing on its structures and possible applications of these biopolymers in industry.

Rhizobial EPS structure

Rhizobium species produce a wide range of EPS, with different types of sugars and their linkages in a single subunit, repeat unit size, and polymerization degree (Bomfeti et al., 2011).

Rhizobial EPS repeating units consist of a variable number of hexose and uronic acid residues linked by alpha or beta glycosidic connections. It can either be linear or side branched. In addition to sugars, non-carbohydrate substituents, such as succinate, pyruvate, or acetate can be found, all of which

contribute to the polysaccharide's acidic nature (Acosta-Jurado et al., 2021).

Several studies regarding the EPS produced by *Rhizobium* spp. have been published in recent years: *Rhizobium radiobacter* S10 (Zhou et al., 2014), *Rhizobium tropici* CIAT899 (commercially known as SEMIA 4077) (Staudt et al., 2011), SEMIA 4080 (Castellane et al., 2014), R. *radiobacter* CAS (Andhare et al., 2017), *Rhizobium* sp. PRIM-18 (Priyanka et al., 2015), and *Rhizobium* sp. M2 (Urai et al., 2017), *Rhizobium tropici* LBMP-C01 (Moretto et al., 2015), *Rhizobium* sp. KYGT 207 (Kaci et al., 2005), *Rhizobium* sp. VMA301 (Mandal et al., 2007), *Rhizobium leguminosarum* bv. *trifolii* (Janczarek et al., 2015) and *Rhizobium leguminosarum* ATCC 10004 (Sellami et al., 2015).

Rhizobium tropici, a legume-symbiont soil bacterium, has the ability to produce extracellular polysaccharides (EPS) (Castellane et al., 2014). *R. tropici* CIAT 899 synthesize an extracellular polysaccharide with a single octasaccharide repeating unit composed of 6D-glucose, 2 D-galactose, 3 pyruvic acid, and 1 acetic acid molecule in the molar ratio of 6:2:1.5:1. Pyruvic acid groups replace half of the terminal groups on 4,6-galactose at position 3, while acetyl O-acetyl groups replace the other half (Oliveira et al., 2012).

Another strain, *Rhizobium meliloti*, which causes nodule formation in alfalfa (*Medicago sativa*) plants, produces two structurally unique EPS: succinoglycan (EPS I) and galactoglucan, which is formed under phosphate deficiency (EPS II) (Janczarek, 2011). EPS I is composed of repeating units of seven D-glucose residues and one D-galactose residue, linked by β-1,3, β-1,4, and β-1,6 glycosidic linkages and replaced with acetyl, pyruvyl, and succinyl groups (Castellane et al., 2015a; Ruiz et al., 2015), while EPS II consists of disaccharide repeating units that are linked by α-1,3 and β-1,3 bonds and include D-glucose and D-galactose in a 1:1 molar ratio. The majority of the glucosyl residues are 6-*O*-acetylated, and all of the galactosyl residues have 4,6-*O*-pyruvyl groups replaced (Janczarek, 2011). The chemical structure of succinoglycan produced by *Rhizobium* is shown in Figure 1. Both EPS I and II are produced in two major fractions: High Molecular Weight (HMW), which consists of hundreds to thousands of repeating units, and Low Molecular Weight (LMW), which consists of monomers, dimers, and trimers in the case of EPS I and oligomers (15-20) in the case of EPS II (Ghosh & Maiti, 2016).

Also, *Rhizobium leguminosarum* is a rhizobial specie that can be divided into three biovars based on the type of legumes infected: *trifolii, viciae*, and *phaseoli*. *R. leguminosarum* strains produce EPS with structures that are similar, but not identical, being composed of repetitive units containing D-glucose, D-glucuronic acid, and D-galactose in a molar ratio of 5:2:1, linked by β-1,3 and β-1,4 glycosidic linkages and modified by acetyl, pyruvyl, and 3-hydroxybutanoyl groups (Janczarek, 2011).

In most *R. leguminosarum* bv. *trifolii* strains, this polymer is composed of octasaccharide repeating units containing D-glucose, D-glucuronic acid, and D-galactose in a molar ratio of 5:2:1, connected by β-1,3 and β-1,4 glycosidic bonds, and are modified by non-sugar (acetyl and pyruvyl) groups, but in other strains, the galactose residue is not present (Skorupska et al., 2006). The EPS repeating unit of *R. leguminosarum* bv. *viciae* is similar to the *R. leguminosarum* bv. *trifolii* octasaccharide, but with an extra D-glucuronic acid residue (Acosta-Jurado et al., 2021).

In the case of *R. leguminosarum* bv. *trifolii* 4S, an EPS subunit consist of seven sugars, but the galactose molecule is absent in this chain, while the EPS subunit of *R. leguminosarum* bv. *viciae* 248 possesses an additional glucuronic acid.

Becker & Pühler (1998) showed that succinoglycan produced by *Rhizobium* sp. NGR234 is composed of repeating units having one galactose and seven glucose molecules coupled β-1,3, β-1,4 and β-1,6 linkages, and succinyl, acetyl, and pyruvyl residues.

Another type of EPS was produced by this strain, which consisted of alternating units of glucose and galactose with α-1,3 and β-1,3 linkages, and residues of acetyl and pyruvyl.

In the study of Guentas et al. (2001), the molecular structure of the EPS produced by a strain of *Rhizobium sp.* B isolated from nodules of alfafa was analysed, and it was shown that it contained high amounts of glucose and rhamnose (1:2), as well as traces of 2-deoxy-D-arabino-hexuronic acid.

Staehelin et al. (2006) found that the acidic EPS produced by *Rhizobium* sp. NGR234 contained glucosyl, galactosyl, glucuronosyl, and 4,6-pyruvylated galactosyl residues with glycosidic linkages of β-1,3, β-1,4, β-1,6, α-1,3 and α-1,4 respectively.

The effect of several carbon sources (sucrose, glucose, glycerol, and galactose) on the composition of EPS generated by *Rhizobium tropici* 4077 and 4080 was investigated by Castellane & Lemos (2007). They found mainly units of glucose, galactose, and glucuronic acid, with differences in their ratios depending on the carbon source utilised. Mannose, rhamnose, and galacturonic acid were also detected.

Zhao et al. (2010) investigated the chemical structure of the EPS produced by *Rhizobium* sp. N613 isolated from the Korshinsk pea shrub. The molecular weight and monosaccharide composition of *Rhizobium* sp. N613 EPS showed that are glucans composed of glucose and β-sugar units.

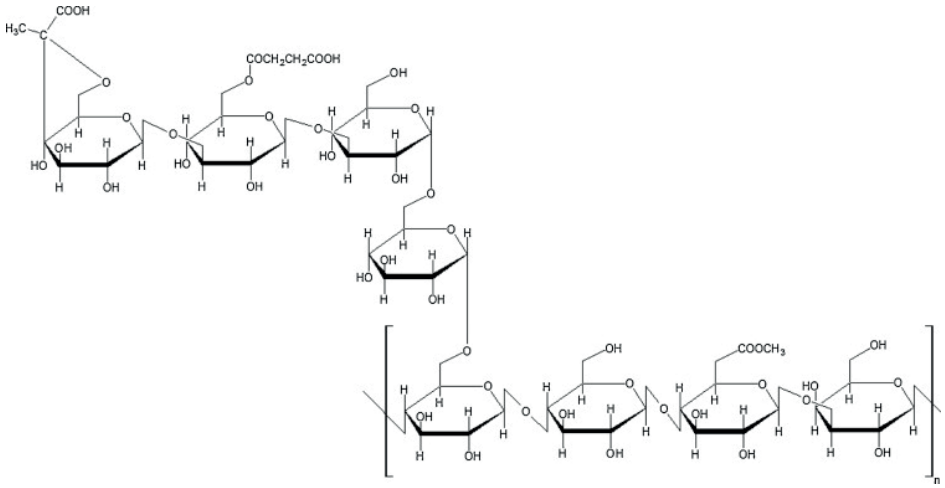


Figure 1. Chemical structure of succinoglycan produced by *Rhizobium*

Potential applications of rhizobial EPS

Succinoglycan produced by *Rhizobium* strains has a significant potential for commercial use as a water-soluble thickener, and the polysaccharide solution has a distinctive high viscosity due to the presence of about 10% succinic acid. It has also been reported to have superior properties under extreme operational situations, such as high temperature, pressure, salt or ionic concentration, and high shear rate (Andhare et al., 2017). These characteristics make the polysaccharide suitable for the use as a thickening, gelling, stabilizing, texturizing, and emulsifying agent in the food, pharmaceutical, and cosmetics industries (Zhou et al., 2014), and as a thickener for oil recovery (Gao et al., 2021). According to Yang et al. (2019) it was demonstrated that dietary succinoglycan successfully reduced diet-induced hypercholesterolemia in rats. The polysaccharide exhibits remarkable anti-inflammatory effects in vivo and in vitro (Cheng et al., 2019). These findings suggest that succinoglycan may be used as a health-promoting dietary ingredient (Gao et al., 2021).

Succinoglycan synthesized by *R. radiobacter* CAS has recently been demonstrated to exhibit useful properties for cosmetic applications, including 95% water solubility at room temperature and binding ability of 4.35 g/g and 3.68 g/g with soybean and peanut oils (Kavitake et al., 2019). Water-in-oil milky lotion, sun screen cream, water-in-oil foundation, sun screen milky lotion, and others are examples of commonly produced succinoglycan composition in cosmetic preparations. Because of its oil-in-water emulsification, thickening, and plasticizing properties, it's a popular cosmetic addition (Halder et al., 2017).

Such properties of rhizobial EPS are important in the production of cosmetic formulations, with hydrating potential, rhizobial polymers being a

good option to substitute industrial glycerin derivatives for the development of cosmetics (Vieira et al., 2017).

Furthermore, succinoglycan has been also used as a polymer material in biosensing and drug delivery (Barman et al., 2020).

In another study, Castellane et al. (2014) showed that the wild-type *Rhizobium tropici* SEMIA 4080 strain and the mutant strain (MUTZC3) produce an extracellular polysaccharide with specific properties, that is used as an emulsifying agent.

Rhizobial EPS also showed promising properties, such as cytotoxicity against cancer cells. Zhao et al. (2010) investigated the anticancer activity of the EPS produced by *Rhizobium sp.* N613 isolated from the Korshinsk pea shrub. EPS inhibited the growth of transplantable sarcoma 180 (S180), hepatoma 22 (H22), and Ehrlich ascites carcinoma (EAC) as compared to control, with inhibitory rates of 44.17 % and 55.80% against S180 and H22, respectively, at a dose of 10 mg/kg. At a dosage of 60 mg/kg, the inhibition rate against EAC was found to be 53.10 %.

Also, in the pharmaceutical field, a dermo-pharmaceutical formulation containing a biopolymer produced by *R. meliloti* NCIMB 40472 and an extract of the microalgae *Haematococcus pluvialis* that assures skin nutrition, care, and regeneration has been described (patent number WO 1999013855A1) (Lintner, 1999).

In another study, Priyanka et al. (2015) found that the EPS produced by *Rhizobium* sp. PRIM-18 can be efficiently functionalized to promote cell proliferative and wound healing activity. Therefore, rhizobial EPS could be further explored for its applications in regenerative medicine.

Table 1 presents some information about EPS produced by *Rhizobium.*

Table 1. Overview of production, composition and applications of some EPS produced by *Rhizobium* sp.

Microorganism	EPS production	Monomer units	Potential applications	References
Rhizobium radiobacter S10	2.834 mg L^{-1}	Galactose Glucose Glucosamine Mannose	Food industry	Zhou et al., 2014
Rhizobium radiobacter CAS	-	Glucose Galactose	Food processing and product development sector	Kavitake et al., 2019
Rhizobium tropici LBMP-C01	3.48 g L^{-1}	Rhamnose Glucose Galactose	Candidate for food industry	Moretto et al., 2015
Rhizobium tropici Semia 4080, MUTZC3, JAB1, JAB6	-	Glucose Galactose Mannose Rhamnose Glucuronic acid Galacturonic acid	non-Newtonian and pseudoplastic fluid flow	Castellane et al., 2014
Rhizobium tropici Semia 4077	7.45 g L^{-1}	Mannose Rhamnose Glucuronic acid Galacturonic acid Glucose Galactose	It is a good water-solubility, viscous aqueous solutions with shear thinning behaviour, film-forming capacity, and emulsifier agent.	Castellane et al., 2015b
Rhizobium undicola strain N37	-	Galactose Mannose	Good stability Newtonian, fluid behavior	Ribeiro & Burkert, 2016
Rhizobium sp. KYGT207	2.5 g L^{-1}	Glucose Galactose Mannuronic acid	Thickening agent with polyelectrolyte properties	Kaci et al., 2005
Rhizobium sp. PRIM-18	-	Glucose Galactose Mannose	High emulsifying activity, enhanced HDF cell proliferation and wound healing *in vitro*	Priyanka et al., 2015
Rhizobium sp. N613	-	-	Anticancer properties: sarcoma 180 (S180), hepatoma 22 (H22), and Ehrlich ascites carcinoma (EAC)	Zhao et al., 2010

CONCLUSIONS

This mini-review gives an insight into EPS produced by several *Rhizobium* strains.
Rhizobial EPS exhibit significant structural diversity, with novel properties that make them valuable sources of natural polymers for use in a variety of industrial sectors, including medicine.

ACKNOWLEDGEMENTS

This study was funded by the Ministry of Research, Innovation and Digitization, in the frame of the "NUCLEU" Program, Project code: 19-41 03 01, and with the support of the National Institute for Chemical-Pharmaceutical Research and Development-ICCF, Bucharest.

REFERENCES

Acosta-Jurado, S., Fuentes-Romero, F., Ruiz-Sainz, J. E., Janczarek, M., & Vinardell, J.M. (2021). Rhizobial exopolysaccharides: genetic regulation of their synthesis and relevance in symbiosis with legumes. *International Journal of Molecular Sciences*, *22,* 1-27.

Andhare, P., Delattre, C., Pierre, G., Michaud, P., & Pathak, H. (2017). Characterization and rheological behaviour analysis of the succinoglycan produced by *Rhizobium radiobacter* strain CAS from curd sample. *Food Hydrocolloids*, *64*, 1-8.

Barman, B., Kumar, D., Gopmandal, P.P., & Ohshima, H. (2020). Electrokinetic ion transport and fluid flow in a pH-regulated polymer-grafted nanochannel filled with power-law fluid. *Soft Matter*, *16*(29), 6862-6874.

Becker, A., & Pühler, A. (1998). Production of exopolysaccharides. In: Spaink, H.P., Kondorosi, A., Hooykaas, P.J.J. (eds) The Rhizobiaceae. Springer, Dordrecht.

Bomfeti, C.A., Florentino, L.A., Guimarães, A.P., Cardoso, P.G., Guerreiro, M.C., & de Souza Moreira, F.M. (2011). Exopolysaccharides produced by the symbiotic nitrogen-fixing bacteria of leguminosae. *Revista Brasileira de Ciencia do Solo*, *35*, 657-671.

Castellane, T.C.L., & Lemos, E.G.M. (2007). Composição de exopolissacarídeos produzidos por estirpes de rizóbios cultivados em diferentes fontes de carbono. *Pesquisa Agropecuária Brasileira*, *42*(10). 1503-1506.

Castellane, T.C.L., Lemos, M.V.F., & Lemos, E.G.D.M. (2014). Evaluation of the biotechnological potential of *Rhizobium tropici* strains for exopolysaccharide production. *Carbohydrate Polymers*, *1*(111), 191-197.

Castellane, T.C.L., Otoboni, A.M.M.B., & Lemos, E.G.M. (2015a). Characterization of exopolysaccharides produced by *Rhizobia species*. *Revista Brasileira de Ciencia do Solo*, *39*, 1566-1575.

Castellane, T.C.L., Persona, M.R., Campanharo, J.C., & Lemos, E.G.M. (2015b). Production of exopolysaccharide from rhizobia with potential biotechnological and bioremediation applications. *International Journal of Biological Macromolecules*, *74*, 515-522.

Cheng, R., Wang, L., Li, J., Fu, R., Wang, S. & Zhang, J. (2019). *In vitro* and *in vivo* anti-inflammatory activity of a succinoglycan Riclin from *Agrobacterium* sp. ZCC3656. *Journal of Applied Microbiology, 127*, 1716-1726.

Donot, F., Fontana, A., Baccou, J.C., & Schorr-Galindo, S. (2012). Microbial exopolysaccharides: Main examples of synthesis, excretion, genetics and extraction. *Carbohydrate Polymers*, *87*, 951-962.

Freitas, F., Alves, V.D., Reis, M.A., Crespo, J.G., & Coelhoso, I.M., (2014). Microbial Polysaccharide-Based Membranes: Current and Future Applications, *Journal of Applied Polymer Science*, 40047, 1-11.

Gao, H., Yang, L., Tian, J., Huang, L., Huang, D., Zhang, W., Xie, F., Niu, Y., Jin, M., Jia, C., Zou, C., Huang, J., Chang, Z., Yang, X., & Jiang, D. (2021). Characterization and rheological properties analysis of the succinoglycan produced by a high-yield mutant of *Rhizobium radiobacter* ATCC 19358. *International Journal of Biological Macromolecules*, *166*, 61-70.

Ghosh, P., & Maiti, T. (2016). Structure of extracellular polysaccharides (EPS) produced by Rhizobia and their functions in legume–Bacteria symbiosis: - A *Review. Achievements in the Life Sciences, 10*(2)*,* 136-143.

Gonzalez, V., Santamaria, R.I., Bustos, P., Perez-Carrascal, O.M., Vinuesa, P., Judrez, S., Martinez-Flores, I., Cevallos, M.A., Brom, S., Martinez-Romero, E., & Romero, D. (2019). Phylogenomic *Rhizobium* species are structured by a continuum of diversity and genomic clusters. *Frontiers in Microbiology*, *10*(910)*, 1-15.*

Guentas, L., Pheulpin, P., Michaud, P., Heyraud, A., Gey, C., Courtois, B., & Courtois, J. (2001). Structure of a polysaccharide from a *Rhizobium* species containing 2-deoxy-β-D-arabino-hexuronic acid. *Carbohydrate Research*, *332*(2). 167-173.

Halder, U., Banerjee, A., & Bandopadhyay, R. (2017). Structural and functional properties, biosynthesis, and patenting trends of bacterial succinoglycan: A Review. *Indian Journal of Microbiology*, *57*(3), 278-284.

Janczarek, M., Rachwal, K., Ciesla, J., Ginalska, G., & Bieganowski, A. (2015). Production of exopolysaccharide by *Rhizobium leguminosarum bv. trifolii* and its role in bacterial attachment and surface properties. *Plant Soil*, *388*, 211-227.

Janczarek., M. (2011). Environmental signals and regulatory pathways that influence exopolysaccharide production in *Rhizobia*, *International Journal of Molecular Sciences, 12*, 7898-7933.

Jeong, J.P., Kim, Y., Hu, Y., & Jung, S. (2022). Bacterial succinoglycans: structure, physical properties, and applications. *Polymers*, *14*(276), 1-21.

Kaci, Y., Heyraud, A., Barakat, M., & Heulin, T., (2005). Isolation and identification of an EPS-producing *Rhizobium* strain from arid soil (Algeria): characterization of its EPS and the effect of inoculation on wheat rhizosphere soil structure. *Research in Microbiology*, *156*, 522-531.

Kavitake D., Delattre C., Devi, PB, Pierre, G., Michaud, P., Shetty, P.H., & Andhare, P. (2019). Physical and functional characterization of succinoglycan

exopolysaccharide produced by *Rhizobium radiobacter* CAS from curd sample. *International Journal of Biological Macromolecules, 1*(134), 1013-1021.

Lintner K. (1999). Composition for cosmetic or dermopharmaceutical use containing a combination of algae extract and exopolysaccharides. WO Patent 1999013855A1.

Mandal, M.S., Ray, B., Dey, S., & Pati, B. (2007). Production and composition of extracellular polysaccharide synthesized by a *Rhizobium* isolate of *Vigna mungo* (L.) Hepper. *Biotechnology Letters*, *29*, 1271-1275.

Moretto, C., Castellane, T.C.L., Lopes, E.M., Omori, W.P., Sacco, L.P., & Lemos, E.G.M. (2015). Chemical and rheological properties of exopolysaccharides produced by four isolates of rhizobia. *International Journal of Biological Macromolecules*, *81*, 291-298.

Oliveira, J., Figueiredo, M., Silva, M., Malta, M., Vendruscolo, C., & Almeida, H. (2012). Production of extracellular biopolymers and identification of intracellular proteins and *Rhizobium tropici*. *Current Microbiology*, *65*, 686-691.

Priyanka, P., Arun, A.B., Ashwini, P., & Rekha, P.D., (2015). Versatile properties of an exopolysaccharide R-PS18 produced by *Rhizobium* sp. PRIM-18. *Carbohydrate Polymers*, *126*, 215-221.

Ribeiro V.A., & Burkert C.A.V. (2016). Exopolysaccharides produced by *Rhizobium*: production, composition and rheological properties. *Journal of Polymer and Biopolymer Physics Chemistry*, *4*(1), 1-6.

Ruiz, S.P., Martinez, C.O., Noce, A.S., Sampaio, A.R., Baesso, M.L., & Matioli, G. (2015). Biosynthesis of succinoglycan by *Agrobacterium radiobacter* NBRC 12665 immobilized on loofa sponge and cultivated in sugar cane molasses. Structural and rheological characterization of biopolymer. *Journal of Molecular Catalysis B: Enzymatic*, *122*, 15-28.

Sellami, M., Oszako, T., Miled, N., & Ben Rebah, F. (2015). Industrial wastewater as raw material for exopolysaccharide production by *Rhizobium leguminosarum*. *Brazilian Journal of Microbiology, 46*(2), 407-413.

Sethi, D., Mohanty, S., & Pattanayak, S.K. (2019). Effect of different carbon, nitrogen and vitamine sources on exopolysaccharide production of *Rhizobium* species isolated from root nodule of redgram. *Indian Journal of Biochemistry & Biophysics*, *56*, 86-93.

Shanmugam, M., & Abirami, R.G. (2019). Microbial Polysaccharides - Chemistry and Applications. *Journal of Biologically Active Products from Nature*, *9*(1), 73-78.

Skorupska, A., Janczarek, M., Marczak, M., Mazur, A., & Król, J. (2006). Rhizobial exopolysaccharides: genetic control and symbiotic functions. *Microbial Cell Factories*, *5*(7), 1-19.

Staehelin, C., Forsberg, L.S., D'Haeze, W., Gao, M.Y., Carlson, R.W., Xie, Z.P., Pellock, B.J., Jones, K.M., Walker, G.C, Streit, W.R., & Broughton, W.J. (2006). Exo-oligosaccharides of *Rhizobium sp.* strain NGR234 are required for symbiosis with various legumes. *Journal of Bacteriology*, *188*(17), 6168-6178.

Staudt, A.K., Wolfe, L.G., & Shrout, J.D. (2011). Variations in exopolysaccharide production by *Rhizobium tropici*. *Archives of Microbiology*, 194, 197-206.

Urai, M., Aizawa, T., Imamura, K., Hamamoto, H., & Sekimizu, K. (2017). Characterization of the chemical structure and innate immune-stimulating activity of an extracellular polysaccharide from *Rhizobium sp.* strain M2 screened using a silkworm muscle contraction assay. *Drug Discoveries and Therapeutics*, *11*(5), 238-245.

Ventorino, V., Nicolaus, B., Di Donato, P., Pagliano, G., Poli, A., Robertiello, A., Iavarone, V., & Pepe, O. (2019). Bioprospecting of exopolysaccharide-producing bacteria from different natural ecosystems for biopolymer synthesis from vinasse. *Chemical and Biological Technologies in Agriculture*, *6*(18), 1-9.

Vieira, I.R.S., Sales, J.S., Cerqueira-Coutinho, C.S., Hellmann, T., de Sousa, B.F.S., Lopes, J.T., Camara, A.L., Costa, M.C.P., Ricci-Junior, E., & dos Santos, E.P. (2017). Development and in vivo evaluation of the moisturising potential of cosmetic formulations containing Babassu (*Orbignya phalerata Martius*) oily extract. *Biomedical and Biopharmaceutical Research*, *14*(2), 204-219.

Yang, Y., Sun, Q., Xu, X., Yang, X., Gao, Y.,Sun, X., Zhao, Y., Ding, Z., Ge, W., Cheng, R., & Zhang, J. (2019).Oral administration of succinoglycan riclin improves diet-induced hypercholesterolemia in Mice. *Journal of Agricultural and Food Chemistry. 67*(48), 13307-13317.

Zakhia, F., & de Lajudie, P. (2001). Taxonomy of rhizobia. *Agronomie, 21*, 569-576.

Zhao, L., Chen, Y., Ren, S., Han, Y., & Cheng, H. (2010). Studies on the chemical structure and antitumor activity of an exopolysaccharide from *Rhizobium* sp. N613. *Carbohydrate Research*, *345*(5), 637-643.

Zhou, R., Wu, Z., Chen, C., Han, J., Ai, L., & Guo, B. (2014). Exopolysaccharides produced by *Rhizobium radiobacter* S10 in whey and their rheological properties. *Food Hydrocolloids*, *36*, 362-368.

MISCELLANEOUS

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVI, No. 1, 2022
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

NUTRITION SENSITIVE AGRICULTURE: FROM PLANT HEALTH TO HUMAN HEALTH

Jennifer DeLacey

University of Maryland, School of Public Health, 4200 Valley Dr, College Park, MD 20742

Corresponding author email: jdelacey@umd.edu

Abstract

WHO estimated over 700 million people were hungry in 2020, 22% of children were stunted, and nearly 30% of women were anemic. Undernutrition accounts for as much as 45% of child mortality. Global food production must increase to feed growing populations while food security is threatened by plant disease and climate change. Women and children in rural areas of low- and middle-income countries (LMICs) are the most vulnerable to undernutrition and poor health. This paper aims to review nutrition sensitive agriculture interventions for maternal and child health at the global level with a focus on the nexus of plant health and food security, and to recommend directions for future research and policy. This topic links environmental and human health within the One Health approach.

Key words*: maternal and child health, nutrition sensitive agriculture, plant disease.*

INTRODUCTION

The United Nations (UN) projection for the global population in 2050 is over 9 billion people and much of this increase will occur in LMICs (UN, 2019). Alarmingly, the Food and Agriculture Organization of the UN (FAO) estimated the prevalence of undernourishment in the world increased in 2020 during the COVID-19 pandemic, affecting as many as 811 million people (FAO, 2021). To meet the UN's Sustainable Development Goal (SDG) 2, agricultural production must quickly increase to eliminate hunger in the face of challenges including climate change, water availability, plant diseases, and pests (UN, n.d.; Tomich et al., 2011; Binns et al., 2021). This review argues for a greater focus within One Health on the pathway from plant and environmental health to human health. One Health has potential to improve outcomes in non-zoonotic areas like plant health to human health, though this potential has been neglected until recently (Rizzo et al., 2021).

Women and children in LMICs are the most vulnerable to undernutrition (Tirado et al., 2013). Nutrition sensitive agriculture (NSA) interventions are promising means to improve maternal and child health (Ruel et al., 2018). Yet, the opportunity for such interventions to incorporate agroecology and a One Health perspective has not been realized (Rizzo et al., 2021).

Integrated One Health efforts to manage emerging zoonotic diseases, particularly those applied in chicken wet markets as a platform for zoonotic disease control can be transferred to plant disease/non-zoonotic areas (Chan, 2002; Gongal et al., 2020). Surveillance, monitoring, and control of plant pests and diseases alongside human and animal diseases in LMICs can help prevent escalation of crop failures to humanitarian crises (Rizzo et al., 2021). Multisectoral, integrated infrastructure for maternal and child health through sustainably increased food production could benefit from acknowledging the critical importance of plant and environmental health.

This review examines agroecology and NSA interventions for maternal and child health on smallholder farms and rural households in LMICs. The search terms for identifying NSA interventions were modeled on (Ruel et al., 2018). Articles were analyzed by the consideration for plant health and disease.

In this paper, biotic stresses including plant pests and disease are emphasized rather than abiotic stresses such as temperature, salinity, and drought. Research on the impacts of climate change is available (Binns et al., 2021).

Limitations of this paper include a narrow scope, as it is intended neither to be comprehensive nor to address climate change or infectious diseases, particularly zoonoses. Regarding gender, females are the focus, following the trends in literature. Adequately addressing gender identity, sexual orientation, or health issues specific to men is outside this paper's scope.

FOOD SECURITY AND PLANT DISEASE

Global food security is an issue evidenced in the almost 5 million child deaths each year that are attributable to undernutrition (Binns et al., 2021). As the population grows, with the largest increases in LMICs, food insecurity will grow if food production does not increase to meet demand (Conway & Wilson, 2012; Binns et al., 2021). While the largest population increases are expected in Africa and Asia, these regions are already home to the most undernourished people (FAO, 2021).

Several factors threaten crop production and global food security, including biotic stress from plant diseases and plant pests and abiotic stress from effects of climate change such as increased temperatures, more frequent or severe droughts or flooding, and increased levels of greenhouse gases (Tomich et al., 2011; Binns et al., 2021). For the common crops of wheat, rice, maize, potato, and soybean the proportion lost due to plant diseases and pests has been estimated at 17 to 30% (Raymaekers et al., 2020). Not all crop production is intended for human consumption; livestock feed and biofuel production are also major outlets for agricultural products (Conway & Wilson, 2012).

Plants are vulnerable to diseases caused by bacteria, fungi, oomycetes, parasites, and viruses as well as plant pests including insects (Raymaekers et al., 2020). Strategies to control these threats or increase yields by eliminating weeds have traditionally included the use of chemical fungicides, pesticides, and herbicides, but these chemicals can be harmful to the environment, animal health, and human health (Raymaekers et al., 2020; Rizzo et al., 2021). In addition, heavy continued input of these chemicals constitutes selective pressure on plants, microbes, and pests resulting in populations with decreased susceptibility to chemical inputs (Rizzo et al., 2021; Wielgosz et al., 2014). This can lead producers to apply greater amounts of the chemicals in hopes of recovering lost yields, further increasing resistance in plants and potentially jeopardizing human health through overexposure (Rizzo et al., 2021). In addition, the use of insecticides in agriculture can have spillover effects on animal and human health via influencing the development of insecticide resistance among insect vectors of disease (Wielgosz et al., 2014).

NUTRITION AND MATERNAL AND CHILD HEALTH

Undernutrition accounts for as much as 45% of child mortality (Black et al., 2013). The role of nutrition throughout the life course influences maternal and child health, as female undernourished children who survive to reproductive age and become pregnant are at increased risk of poor maternal health outcomes (Black et al., 2013).

There are numerous challenges to improving MCH; improved nutrition alone is not sufficient to close the gaps in morbidity and mortality between LMICs and HICs. However, improving nutrition is a necessary step in improving MCH and population health in LMICs.

Some of the most vulnerable populations include women and children in rural areas in LMICs (FAO, 2021). A majority of the world's 149 million stunted children and 45 million wasted children in 2020 lived in sub-Saharan Africa and Central and Southern Asia (FAO, 2021). Globally, anemia affects about a third of females aged 15-49, but this condition is three times more prevalent in Africa than in higher income regions (FAO, 2021).

In some areas, poverty, lack of education, and traditional gender views limit women's abilities to improve their own and their children's health (Tirado et al., 2013; Kerr et al., 2019). By focusing on this population in these rural areas, NSA interventions can be part of a sustainable solution to end the cycle of food insecurity, poor nutrition, and poor health (Kerr et al., 2019). Action is needed because this population is also at high risk for adverse

social, environmental, and economic effects of climate change (Tirado et al., 2013). In fact, SDG 13 includes a target emphasizing the need to develop capacity to adapt to and overcome the negative effects of climate change among women, children, and other marginalized groups (UN, n.d.).

A study of dietary intake in rural women and children in LMICs found the species level biodiversity of foods consumed was associated with adequate nutrient intake (Lachat et al., 2018). Biodiversity is an important link between human and environmental health, protecting plants from threats and supporting nutrient cycling in the environment (Altieri et al., 2017).

NUTRITION SENSITIVE AGRICULTURE

NSA interventions enable household heads, usually women, to improve their own and their families' nutritional status through raising livestock or growing crops (Mosha et al., 2018). The products can be consumed by the household or sold to generate income, which can offset costs related to education and health care (Mosha et al., 2018).

Limitations of NSA programs include the prerequisite of access to and control over land; a study in India found larger land size was correlated with improved dietary diversity but not BMI (Harris-Fry et al., 2020). However, constraints on women's time may influence the effect of NSA interventions on women's health and the researchers proposed increased land size could reduce women's free time (Harris-Fry et al., 2020).

Situated within a network of intersectoral programs, improved agricultural production was cited as a factor in reducing child stunting in Ethiopia between 2000 and 2016 (Tasic et al., 2020). Along with higher food security through increased yields, better sanitation, more health care workers, poverty reduction and education for girls contributed to decreased stunting (Tasic et al., 2020).

NSA interventions can be placed on a spectrum from rarely mentioning control of plant diseases or pests to complete integration of agroecological approaches. While some programs do not acknowledge these agricultural challenges, others noted their existence but did not include strategies to overcome them. For example, in a program in Thailand that supplied participants with hens and gardening materials, some participants reported the plants did not grow because they were eaten by insects (Roesler et al., 2021).

Insufficient research on plant disease and pest management for smallholder producers has been cited as a limitation of some NSA interventions, including one in South Africa that reported an association between crop production and increased dietary diversity (Hendriks et al., 2020).

Commonly, NSA interventions involve agricultural education on weed, insect, or pest management, but do not document or analyze the interaction between plant health and overall study outcomes. A study from Ghana which integrated gardening, keeping hens for egg production, and education falls into this category (Marquis et al., 2018). More examples come from the Hellen Keller International's Enhanced Homestead Food Production (EHFP), which has reached many women and children in LMICs (Haselow et al., 2016). This program trains female model farmers in gardening practices to act as resources in their communities (Haselow et al., 2016).

A promising example involving a home garden intervention in Guatemala incorporated education on weeding, composting, and pest management in agricultural classes and home visits (Guzmán-Abril et al., 2021). Unfortunately, the sustainability of this intervention and its effects could not be ascertained due to the COVID-19 pandemic (Guzmán-Abril et al., 2021).

Some NSA programs have been modeled on principles of agroecology, respecting the nexus of plant, human, and environmental health. In one such program implemented in Tanzania, participants had increased use of sustainable soil conservation and pest management practices along with decreased household food insecurity and probable depression among women (Santoso et al., 2021).

AGROECOLOGY

Agroecology respects the idea of One Health by integrating multisectoral approaches to improving ecosystems and the health of their

stewards, who are often poor people (Altieri et al., 2017). Perhaps in contrast with mainstream One Health, agroecology is rooted as a social movement arguing for a fundamental shift away from the harmful practices of corporate and agro-industrial companies (Altieri et al., 2017).

Sustainable agriculture lies at the core of agroecology, which respects agrobiodiversity and practices such as integrated pest management (IPM) and crop rotation to decrease risk of crop yield loss to plant disease or pests while improving soil health (Tomich et al., 2011). Agroecology encompasses local and indigenous knowledge as well as social, political, and economic components of the food system (Kerr et al., 2019).

Viewing agricultural production as a component of local ecosystems, the interdependence of human and environmental health is clear (Tomich et al., 2011). In the dominant conventional agriculture system, farmers are incentivized to increase yields by whatever means necessary, altering the original ecosystem by applying the nutrients nitrogen and phosphorous or by introducing irrigation (Tomich et al., 2011; Santoso et al., 2021). Monocultures can be profitable, giving farmers purchasing power which can be used to buy food or more agricultural inputs, but monoculture also increases the crop's susceptibility to pests and disease, leading the farmer to increase pesticide application (Tomich et al., 2011). Crop rotation can alleviate the disruption to nutrient cycles and help control pests and diseases, but the choice not to grow a cash crop may be perceived as an economic loss even if it benefits the ecosystem (Tomich et al., 2011; Altieri et al., 2017).

Instead of conventional agricultural practices which deplete land and favor larger, wealthier producers over smallholders, agroecology advocates a more self-sustained approach to farming (Altieri et al., 2017; Kerr et al., 2019). For example, in agroecology livestock and crop production can be integrated to provide beneficial nutrients for crops, rather than the industrial method of producing large amounts of cereals to feed animals in factories and relying on antibiotics to prevent animal disease and promote growth (Altieri et al., 2017; Tomich et al., 2011).

Addressing plant and environmental health through agroecology should not be at odds with improving human health, rather these concepts should be recognized as linked and mutually reinforcing. One instance of this plant-human health nexus is the beneficial practice of intercropping with legumes, which return nitrogen to the soil and provide protein and iron for the human diet (Kerr et al., 2019). Given the high prevalence of anemia in women and children in LMICs, legume cultivation could be a valuable asset to improve maternal and child health status (FAO, 2021).

Women who provide a significant amount of the labor in smallholder farming in LMICs while caring for young children would benefit from sustainable investment and education in the principles of agroecology (Altieri et al., 2017; Kerr et al., 2019; Santoso et al., 2021). NSA interventions that embrace agroecology should be part of an integrated approach to improving health equity while respecting the ecosystems of smallholder farms in LMICs (Kerr et al., 2019).

ONE HEALTH

One Health has traditionally focused on zoonotic disease, though risks for emerging infectious diseases such as deforestation, increasing agricultural intensification, and antimicrobial resistance also apply to plant diseases (Gongal et al., 2020). It aims to respond to emergent public health threats through multisectoral engagement and emphasizes the relationships between the environment, animals, and humans (Gongal et al., 2020). As part of the environment, plants have been included in One Health but the salience of risks to plant health has been a lower priority than zoonoses such as avian influenza (Gongal et al., 2020; Rizzo et al., 2021).

The H5N1 highly pathogenic avian influenza (HPAI) outbreak of 1997 led to a highly coordinated response and effort to prevent future outbreaks involving FAO, the World Health Organization (WHO), and the World Organization for Animal Health (OIE) (Gongal et al., 2020). Authorities in Hong Kong took drastic actions in 1997 after detecting human HPAI infections, culling all 1.5 million

chickens in their jurisdiction and suspending live poultry imports from China (Chan, 2002). Following this outbreak, the Hong Kong government implemented segregation and testing of poultry prior to importation, licensing and surveillance requirements for farms (Chan, 2002). These measures accompany continuous human disease monitoring that relies on joint efforts by health care workers, scientists, and public health authorities (Chan, 2002).

Notably, the FAO was included in the avian influenza response because zoonoses constitute a threat to the food chain, either through decreased food security due to loss of livestock or through decreased food safety due to foodborne pathogens associated with animal source foods (Gongal et al., 2020). Full consideration of these same threats to food security and safety requires inclusion of plant health because plant disease decreases crop yields and plant foods can also harbor foodborne pathogens (Rizzo et al., 2021). Examples of these cases include banana Xanthomonas wilt (BXW) caused by *Xanthomonas campestris* pathovar *musacearum*, aflatoxin contamination in food and feed crops, and lettuce as a source of Shiga-toxin producing *Escherichia coli* O157:H7 (Rizzo et al., 2021).

Although One Health is not as focused on the link between environmental and social inequity as agroecology, both concepts hold promise for progressing towards global health equity (Altieri et al., 2017; Rizzo et al., 2021). BXW is not only harmful to banana plants which protect against soil erosion and provide shade for other crops; the disease is also associated with food insecurity among the poorest households as banana production decreases and prices increase (Rizzo et al., 2021). The economic and related health effects to farmers could be mitigated while supporting disease surveillance and monitoring as in One Health. When an outbreak of avian influenza was detected in Lebanon in 2016, the containment measures of culling birds, disinfecting farms, and properly disposing of remains were accompanied by providing indemnity to farmers (Farah et al., 2018). Incorporating economic protection into the preparedness plan was in the best interests of the public, animal health, and the producer (Farah et al., 2018). Safety nets for farmers are one way One Health can contribute to SDG 10 which calls for reducing social and health inequalities (UN, n.d.).

In addition to FAO, WHO, and OIE, the international organizations United Nations Children's Emergency Fund (UNICEF), the World Bank, and the United Nations System Influenza Coordination (UNSIC) acted together to respond to and develop a framework for reducing the risk of infectious diseases (Killewo et al., 2017). The reliance on government actors and international organizations within One Health underlies its links to SDG 17 and strengthening partnerships among international, national, and local institutions (UN, n.d.). People affected by policies and institutions while living at the zones of animal, human, and environmental health interaction are also important stakeholders in One Health. Smallholder farmers could benefit from integrated approaches to address food insecurity and poor health through sound management of ecosystems and environmental resources including measures to prevent and manage plant pest and disease risks (Altieri et al., 2017). Women smallholder farmers in LMICs are a resource for improving maternal and child health through partnerships with international organizations like FAO and WHO; nongovernmental organizations such as Hellen Keller International, and state and local agricultural extensions and educational institutions. These partnerships should mirror the linkages between plant health, One Health, agroecology, nutrition sensitive agriculture, and food security and nutrition, as seen in Figure 1 below.

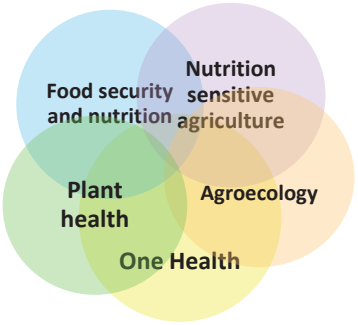


Figure 1. One Health linkages

Though One Health has been dominated by the impact of zoonotic diseases on human health, it should sharpen the focus on plant health for a more holistic view of global health equity for people, animals, and the environment (Rizzo et al., 2021).

CONCLUSIONS

More research is needed on the impact of plant pests and diseases in NSA interventions and the effect of such programs for maternal and child health. Still, these interventions show promise in reducing the burden of poor MCH in rural areas in LMICs through integration of NSA and agroecology. They represent a step toward integration of agriculture with other sectors such as health, education, transportation, and information technology. Future work on the threat of plant diseases to food security and incorporating mitigation strategies into agricultural production at the smallholder level should address socioeconomic and cultural factors such as the role of poverty, women's rights, and education. It is vital for future generations that we reform agricultural practices and refocus on preserving the health of plants and the environment as ends in themselves and because of their interdependence with human health.

Strategies, interventions, and infrastructure commonly employed in One Health for management of zoonotic diseases should be transferred to the management of plant diseases that threaten biodiversity and human nutrition. Similar to One Health collaborations for zoonotic disease prevention, the FAO and WHO should coordinate with international and national environmental bodies to protect plant health through enhanced surveillance and monitoring. These partnerships should be paired with support for agricultural research that centers agroecology rather than assimilating it into current practices.

National and local agricultural extension programs need to reach, listen to, and work with all farmers, regardless of gender or education level (Kerr et al., 2019). These extension programs should be a chance for dialogue with communities in the interest of crafting sustainable local solutions instead of reinforcing the dominance of agricultural intensification that exploits environmental and human resources (Altieri et al., 2017; Kerr et al., 2019).

Nations and regional organizations should work with rural communities to reinvent agricultural extension programs, with the help of research scientists, public health, environmental protection, and education sectors. One Health University Networks in Africa (AFROHUN) and Southeast Asia (SEAOHUN) are two resources for workforce training, integration of agroecology and plant health into university programs, and collaboration among students, scientists, and professionals from different disciplines (Killewo et al., 2017; SEAOHUN, 2021). Program-level transformations will need to be outlined in policy and reinforced by dedicated funding, with governments willing to place more power and resources for ecosystem stewardship in the hands of farmers (Altieri et al., 2017). Doing so at the national level may require action by the executive, legislative, and judicial branches to support social, political, human, and environmental rights with a focus on smallholder farmers and rural communities.

ACKNOWLEDGEMENTS

This review paper was written under the supervision of University of Maryland, School of Public Health Faculty.

REFERENCES

Altieri, M. A., Nicholls, C. I., & Montalba, R. (2017). Technological Approaches to Sustainable Agriculture at a Crossroads: An Agroecological Perspective. *Sustainability*, 9(3), 349. https://doi.org/10.3390/su9030349

Binns, C. W., Lee, M. K., Maycock, B., Torheim, L. E., Nanishi, K., & Duong, D. T. T. (2021). Climate Change, Food Supply, and Dietary Guidelines. *Annual Review of Public Health*, 42, 233–255. https://doi.org/10.1146/annurev-publhealth012420-105044

Black, R. E., Victora, C. G., Walker, S. P., Bhutta, Z. A., Christian, P., de Onis, M., Ezzati, M., Grantham-McGregor, S., Katz, J., Martorell, R., & Uauy, R. (2013). Maternal and child undernutrition and overweight in low-income and middle-income countries. *The Lancet*, 382(9890), 427–451. https://doi.org/10.1016/S0140-6736(13)60937-X

Chan, P. K. S. (2002). Outbreak of Avian Influenza A(H5N1) Virus Infection in Hong Kong in 1997.

Clinical Infectious Diseases, 34(Supplement_2), S58–S64. https://doi.org/10.1086/338820

Conway, G., & Wilson, K. (2012). One Billion Hungry: Can We Feed the World? (1st ed.). *Cornell University Press*. https://www.jstor.org/stable/10.7591/j.cttq43v4

FAO. (2021). The State of Food Security and Nutrition in the World 2021. FAO, IFAD, UNICEF, WFP and WHO. https://doi.org/10.4060/cb4474en

Farah, Z. E., Khatib, O., Hamadeh, S., Ahmad, K., El Bazzal, B., Zalloua, P., Anmar, W., & Ghosn, N. (2018). Containment of Highly Pathogenic Avian Influenza A(H5N1) Virus, Lebanon, 2016. *Emerging Infectious Diseases,* 24(2), 374–376. https://doi.org/10.3201/eid2402.171276

Gongal, G., Ofrin, R., de Balogh, K., Oh, Y., Kugita, H., & Dukpa, K. (2020). Operationalization of One Health and tripartite collaboration in the Asia Pacific region. *WHO South-East Asia Journal of Public Health*, 9(1), 21. https://doi.org/10.4103/2224-3151.282991

Guzmán-Abril, A., Alajajian, S., Rohloff, P., Proaño, G. V., Brewer, J., & Jimenez, E. Y. (2021). Academy of Nutrition and Dietetics Nutrition Research Network: A Home Garden Intervention Improves Child Length-for-Age Z Score and Household-Level Crop Count and Nutritional Functional Diversity in Rural Guatemala. *Journal of the Academy of Nutrition and Dietetics*, S22122672(21)00230-6. https://doi.org/10.1016/j.jand.2021.04.002

Harris-Fry, H., Krishnan, S., Beaumont, E., Prost, A., Gouda, S., Mohanty, S., Pradhan, R., Rath, S., Rath, S., Pradhan, S., Mishra, N. K., Allen, E., & Kadiyala, S. (2020). Agricultural and empowerment pathways from land ownership to women's nutrition in India. *Maternal & Child Nutrition*, 16(4), e12995. https://doi.org/10.1111/mcn.12995

Haselow, N. J., Stormer, A., & Pries, A. (2016). Evidence-based evolution of an integrated nutrition-focused agriculture approach to address the underlying determinants of stunting. *Maternal & Child Nutrition*, 12 Suppl 1, 155–168. https://doi.org/10.1111/mcn.12260

Hendriks, S. L., Viljoen, A., Marais, D., Wenhold, F. a. M., McIntyre, A. M., Ngidi, M. S., Annandale, J. G., Kalaba, M., & Stewart, D. (2020). Considerations for the design of nutrition-sensitive production programmes in rural South Africa. *Bmc Public Health*, 20(1), 1383. https://doi.org/10.1186/s12889-020-09445-3

Kerr, R. B., Young, S. L., Young, C., Santoso, M. V., Magalasi, M., Entz, M., Lupafya, E., Dakishoni, L., Morrone, V., Wolfe, D., & Snapp, S. S. (2019). Farming for change: Developing a participatory curriculum on agroecology, nutrition, climate change and social equity in Malawi and Tanzania. *Agriculture and Human Values,* 36(3), 549–566. http://dx.doi.org/10.1007/s10460-018-09906-x

Killewo, J., Bazeyo, W., & Mdegela, R. (2017). One Health Central and Eastern Africa: Historical and Future Perspectives. In S. R. Quah (Ed.), *International Encyclopedia of Public Health* (Second Edition) (pp. 342–347). Academic Press. https://doi.org/10.1016/B978-0-12-803678-5.00512-9

Lachat, C., Raneri, J. E., Smith, K. W., Kolsteren, P., Van Damme, P., Verzelen, K., Penafiel, D., Vanhove, W., Kennedy, G., Hunter, D., Odhiambo, F. O., Ntandou-Bouzitou, G., De Baets, B., Ratnasekera, D., Khy, H. T., Remans, R., & Termote, C. (2018). Dietary species richness as a measure of food biodiversity and nutritional quality of diets. *Proceedings of the National Academy of Sciences of the United States of America,* 115(1), 127–132. https://doi.org/10.1073/pnas.1709194115

Marquis, G. S., Colecraft, E. K., Kanlisi, R., Aidam, B. A., Atuobi-Yeboah, A., Pinto, C., & Aryeetey, R. (2018). An agriculture-nutrition intervention improved children's diet and growth in a randomized trial in Ghana. *Maternal & Child Nutrition*, 14 Suppl 3, e12677. https://doi.org/10.1111/mcn.12677

Mosha, D., Canavan, C. R., Bellows, A. L., Blakstad, M. M., Noor, R. A., Masanja, H., Kinabo, J., & Fawzi, W. (2018). The impact of integrated nutrition-sensitive interventions on nutrition and health of children and women in rural Tanzania: Study protocol for a cluster-randomized controlled trial. *Bmc Nutrition*, 4(1). https://doi.org/10.1186/s40795-018-0238-7

Raymaekers, K., Ponet, L., Holtappels, D., Berckmans, B., & Cammue, B. P. A. (2020). Screening for novel biocontrol agents applicable in plant disease management – A review. *Biological Control*, 144, 104240. https://doi.org/10.1016/j.biocontrol.2020.104240

Rizzo, D. M., Lichtveld, M., Mazet, J. A. K., Togami, E., & Miller, S. A. (2021). Plant health and its effects on food safety and security in a One Health framework: Four case studies. *One Health Outlook*, 3(1), 6. https://doi.org/10.1186/s42522-021-00038-7

Roesler, A., Smithers, L. G., Wangpakapattanawong, P., & Moore, V. (2021). A Nutrition-Sensitive Agriculture Initiative in Ethnic Communities of Northern Thailand: Local Perspectives and Future Prospects. *Food and Nutrition Bulletin*, 03795721211025129. https://doi.org/10.1177/03795721211025129

Ruel, M. T., Quisumbing, A. R., & Balagamwala, M. (2018). Nutrition-sensitive agriculture: What have we learned so far? *Global Food Security*, 17, 128–153. https://doi.org/10.1016/j.gfs.2018.01.002

Santoso, M. V., Bezner Kerr, R. N., Kassim, N., Martin, H., Mtinda, E., Njau, P., Mtei, K., Hoddinott, J., & Young, S. L. (2021). A Nutrition-Sensitive Agroecology Intervention in Rural Tanzania Increases Children's Dietary Diversity and Household Food Security But Does Not Change Child Anthropometry: Results from a Cluster-Randomized Trial. *The Journal of Nutrition*, 151(7), 2010–2021. https://doi.org/10.1093/jn/nxab052

SEAOHUN. (2021). SEAOHUN Annual Report 2020. SEAOHUN.

Tasic, H., Akseer, N., Gebreyesus, S. H., Ataullahjan, A., Brar, S., Confreda, E., Conway, K., Endris, B. S., Islam, M., Keats, E., Mohammedsanni, A., Wigle, J., & Bhutta, Z. A. (2020). Drivers of stunting reduction in Ethiopia: A country case study. *The American*

Journal of Clinical Nutrition, 112(Suppl 2), 875S-893S. https://doi.org/10.1093/ajcn/nqaa163

Tirado, M. C., Crahay, P., Mahy, L., Zanev, C., Neira, M., Msangi, S., Brown, R., Scaramella, C., Costa Coitinho, D., & Mueller, A. (2013). Climate change and nutrition: Creating a climate for nutrition security. *Food and Nutrition Bulletin*, 34(4), 533–547. https://doi.org/10.1177/156482651303400415

Tomich, T. P., Brodt, S., Ferris, H., Galt, R., Horwath, W. R., Kebreab, E., Leveau, J. H. J., Liptzin, D., Lubell, M., Merel, P., Michelmore, R., Rosenstock, T., Scow, K., Six, J., Williams, N., & Yang, L. (2011). Agroecology: A Review from a Global-Change Perspective. *Annual Review of Environment and Resources,* 36(1), 193–222. https://doi.org/10.1146/annurev-environ-012110-121302

UN (n.d.). Sustainable Development Goals. United Nations Sustainable Development. Retrieved October 14, 2021, from https://www.un.org/sustainabledevelopment/sustainable-development-goals/

UN (2019). World population prospects Highlights, 2019 revision Highlights, 2019 revision.

Wielgosz, B., Kato, E., & Ringler, C. (2014). Agro-ecology, household economics and malaria in Uganda: Empirical correlations between agricultural and health outcomes. *Malaria Journal*, 13, 251. https://doi.org/10.1186/1475-2875-13-251

ISSN 2285 – 1364
ISSN-L 2285 – 1364