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Review

Food additives and bioactive substances from in vitro systems of edible plants from the Balkan peninsula

During the last few years there is an increasing demand to the natural biologically active compounds. According to the World Health Organization (WHO) about 11% of the conventional medicines are of plant origin. Nowadays, plant biotechnologies are modern and reliable tool for producing valuable bioactive compounds. Recently, the potential of plant cells as foods also was confirmed. The advantages of plant in vitro systems over the intact plants are well known: growing under controlled and optimized laboratory conditions; independence of climatic and soil differences; preservation of rare and endangered plant species; cultivation in diverse bioreactor systems for increasing production yields of target metabolites. There have been developed many in vitro systems for production of various plant bioactive compounds with potential application in food industries. But potential for industrial implementation of this technology depends on solving problems with the scale-up of bioreactor cultivation, development of additional approaches for improving/modification of bioactivities of the target plant secondary metabolites, and to find way to exclude or replace in the culture media the carcinogenic plant growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) with its safety analogs, such as α-naphtaleneacetic acid (NAA) and/or indole-3-butyric acid (IBA). The aim of the current mini review is to summarize information about different in vitro systems of edible plants from the Balkan Peninsula with potential for producing food additives and biologically active substances and to describe prospects for successful industrial implementation of this technology.

Keywords: Biologically active substances / Edible plants / Natural food additives / Plant cultures

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1 Introduction

Since ancient times people used plants both as food and as sources of various valuable bioactive compounds. The extracts of medicinal and aromatic plants are used as food and cosmetic additives (e.g. essential oils, rosmarinic acid, vanillin), natural dyestuffs (e.g. betalains, anthocyanins), biopesticides (e.g. nicotine, rotenone, ryonidine), as well as phytotherapeutics (terpenic acids, phenolic acids, alkaloids: vinblastine, vincristine, caffeine, nicotine, ephedrine) [1–3].

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **DW**, dry weight; **FW**, fresh weight; **IBA**, Indole-3-butyric acid; **LS**, Linsmaier and Skoog medium; **NAA**, α-Naphtaleneacetic acid; **RA**, rosmarinic acid

Currently plants remain an essential source of biologically active compounds in spite of development of chemical or microbial technologies [2]. According to the World Health Organization (WHO) 11% of the conventional medicines are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors [4].

According to Turrill, 1929 [5], Balkans are richer in flora than any comparable area in Europe. The flora in the region is characterized by high percentage of endemism. For example, in Bulgaria there are 270 higher plant species that are endemic to Balkan Peninsula, and other 174 species occur only on the territory of Bulgaria [6]. These figures are of similar magnitude for the other Balkan countries, except for Greece, where about 750 species are endemics to the country [7].

The ethnobotanical studies in the recent decades revealed the peculiarities of traditional and contemporary plant use and the most popular wild plant species. Redzic, 2006 [8] reported 308 plants used in nutrition and diet of local people in Bosnia

and Herzegovina. Considerably lower number of species – 88 were noted by Nedelcheva, 2013 [9] for Bulgaria. Luczaj et al., 2014 [10] reviewed the wild food plants in Dalmatia (Southern Croatia) and concluded that the species used in the region were almost the same like in the other Mediterranean countries.

While many studies focused on the use of wild edible plants, relatively little is known about their cultivation. Currently, mostly aromatic and medicinal plants, having commercial value, are subjected to cultivation (for example, Mentha, Origanum, Melissa, Coriandrum), while the majority of wild edible plant species are collected from nature. This could cause disturbance of their natural populations and plant communities. Therefore, the cultivation could be a very promising tool for preventing the exhaustion of the natural resources and genepool of wild edible plants and could contribute to the conservation of their natural habitats [11, 12].

However, natural plant-derived food additives and bioactive compounds extracted through conventional methods from intact plants are available only seasonally and are affected by environmental and geographical factors. Plant secondary metabolites are synthesized in minor amounts in specialized tissues (like trichomes) in variable yields year to year, that makes their extraction, isolation, and purification from intact plants difficult. Due to their very complex structure and/or chirality, in many cases organic synthesis is not possible or cost ineffective. These disadvantages can be overcome by plant in vitro cultures. Nowadays, plant biotechnology is modern and reliable tool for producing plant bioactive compounds. The advantages of plant in vitro systems over the intact plants are well known: year-round growing under controlled and optimized laboratory conditions (pH, temperature, medium components, and others environmental factors); independence of climatic and soil differences; conservation of rare and endangered plant species; cultivation in diverse bioreactor systems for increasing production yields of target metabolites; separation of target compounds is much easier due to lower complexity of the cultured materials [3, 13, 14].

In the current mini review we summarize available information about in vitro plant systems of edible plants from the Balkan Peninsula producing food additives and biologically active substances.

2 In vitro systems of edible plants from the Balkan Peninsula producing food additives and bioactive substances

The capacity of plant cell, tissue, and organ cultures to produce and accumulate many valuable chemical compounds similar to the parent plant in nature has been highlighted since the inception of in vitro technology. The strong and growing demand in today's marketplace for natural, renewable products has refocused attention to in vitro plant materials as potential sources for secondary phytochemical products, and has paved the way for new research exploring secondary product expression in vitro [15].

According to the degree of differentiation of the cultivated plant tissues, plant in vitro systems could be classified as undifferentiated in vitro cultures (calli and cell suspensions) and differentiated in vitro cultures (shoots, hairy roots, adventitious roots, bulbs, somatic embryos) [3, 16].

2.1 Undifferentiated in vitro systems

Undifferentiated in vitro systems consist mainly of callus cultures and cell suspensions. Valuable bioactive substances from callus and cell suspension cultures have been successfully obtained from edible plants naturally grown in the Balkan Peninsula. Most of them belong to the Lamiaceae and Vitaceae families (Table 1).

Keskin and Kunter, 2010 [17] successfully obtained callus culture of *Vitis vinifera* L. cv. Öküzgözü, grown in Turkey that produce resveratrol - a valuable stilbene. This secondary metabolite has been found to possess a number of health benefits: antiviral, antioxidant, anti-inflammatory, cancer chemopreventive and therapeutic effects, prevent heart-artery diseases by reducing cholesterol and harmful blot clots [18–21]. Due to these health benefits, there is an increasing demand for effective approaches to produce resveratrol. When 12-day-old culture of *V. vinifera* L. cv. Öküzgözü was exposed to UV light (254 nm) for 15 min. the amount of accumulated trans-resveratrol was 26-fold higher than the control calluses [17].

Except trans-resveratrol, suspension cultures obtained of grapevine grown in Slovak Republic and Italy have been reported to accumulate a wide range of catechins, flavonoids and phenolic acids possessing multiple biological properties such as antioxidant, antibacterial, anticancer, estrogenic and heart-protecting activities [22–25]. Most of these researches have been focused on improvement of in vitro production of the target compounds by adding elicitors, such as fungal pathogen *Phaeomoniella chlamydospora*, metyljasmonate [23], chitosan [24], dimethyl-β-cyclodextrin [25] or cultivation in batch and fed-batch bioreactors [24]. The elicited with dimethyl-β-cyclodextrin in vitro culture of *V. riparia x V. berlandieri* produced in the medium about 2400 times higher amount of trans-resveratrol (911.25 mg/L) compared to untreated cells (0.37 mg/L) [25]. Ferri et al., 2014 [24] reported for 20-fold higher production of free and mono glucosylated resveratrol (32.72 μmol/g DW or 7.47 μg/g DW) in fed-batch cultivation of *Vitis vinifera* cv. Barbera treated with chitosan.

Beside by suspension cultures of grapevine, resveratrol was successfully obtained by many engineered microorganisms (*Saccharomyces cerevisiae, Escherichia coli)* [26–30]. The highest yield of resveratrol was obtained by Mingji et al., 2016 [30]. They used metabolic engineering strategies, synthetic biology techniques and system biology approaches and constructed engineered strain of *S. cerevisiae* produced 811.50 and 754.70 mg/L resveratrol in fed-batch fermentation on a minimal medium with glucose or ethanol, respectively. Besides, this strain produced resveratrol from low cost carbon sources in short process time with high purity that is a base for its commercial application. Additionally, this engineered strain of *S. cerevisiae* synthesized higher amount of resveratrol compared to the suspension cultures of grapevine [23, 25].

Cell suspension cultures of sweet basil (*Ocimum basilicum* L.), grown in Greece were successfully developed for production of rosmarinic acid (RA) – one of the widespread natural antioxidant in the Lamiaceae family [31, 32]. Double volume of the

Table 1. In vitro systems of edible plants from the Balkan Peninsula producing food additives and bioactive substances

Type of in vitro culture	Plant species (Family)	Bioactive compounds	Yield	References
Callus	*Vitis vinifera* L. cv. Öküzgözü (Vitaceae)	*trans*-resveratrol	62.23 μg/g FW	[17]
Cell suspension	*Vitis vinifera* L. cv. St. Laurent (Vitaceae)	Catechin	1.48 mg/g FW	[23]
		Epicatechin	2.05 mg/g FW	
		trans -resveratrol	0.45 mg/L	
		Rutin	1.32 mg/g FW	
		p-coumaricacid	0.09 mg/L	
		Vanillic acid	0.73 mg/L	
Cell suspension	*Vitis vinifera* cv. Barbera (Vitaceae)	Free and mono glucosylated resveratrol	32.72 μmol/g DW	[24]
Cell suspension	*V. vinifera* cv. Pinot Noir (Vitaceae)	*trans*-resveratrol	0.51 mg/L, 5.80 μg/g	[25]
Cell suspension	*V. vinifera* cv. Merzling (Vitaceae)	*trans*-resveratrol	4.31 mg/L, 3.91 μg/g	[25]
Cell suspension	*V. amurensis* (Vitaceae)	*trans*-resveratrol	225.22 mg/L, 187.35 μg/g	[25]
Cell suspension	*V. riparia* x *V. berlandieri* (Vitaceae)	*trans*-resveratrol	911.25 mg/L, 622.90 μg/g	[25]
Cell suspension	*Ocimum basilicum* L. (Lamiaceae)	Rosmarinic acid	10 mg/g DW	[31]
Imobilized cell culture	*Ocimum basilicum* L. (Lamiaceae)	Rosmarinic acid	20 mg/g DW	[32]
Callus	*Satureja hortensis* L. (Lamiaceae)	Rosmarinic acid	82.3 4 $\pm$ 0.26 mg/g FW	[33]
Cell suspension	*S. tomentosa* Mill. (Lamiaceae)	Oleanolic acid,	71.89 $\pm$ 1.23 μg/mL	[35]
		Ursolic acid	256.28 $\pm$ 2.68 μg/mL	
Rhizogenic callus	*S. scabiosifolia* Lam. (Lamiaceae)	Oleanolic acid	829.14 mg/g DW	[37]
Cell suspension	*Helianthus annuus* L. (Asteraceae)	α - tocopherol	11.26 μg/g FW	[39]
Cell suspension	*Arabidopsis thaliana* L. (Brassicaceae)	α - tocopherol	9.00 μg/g FW	[39]
Cell suspension	*Helianthus annuus* L. (Asteraceae)	α - tocopherol	24 μg/g FW	[40]
Cell suspension	*Helianthus annuus* L. (Asteraceae)	α - tocopherol	77.00 μg/g DW	[41]
Cell suspension	*Helianthus annuus* L. (Asteraceae)	Linoleic acid	14.1% of the identified total volatiles compound	[42]
Hairy roots	*Salvia tomentosa* Mill. (Lamiaceae)	Gallic acid	39*	[44]
		Protocatechuic acid	188	
		Salicylic acid	446	
		Chlorogenic acid	331	
		Vanillic acid	332	
		Caffeic acid	131	
		Syringic acid	167	
		p-Coumaric acid	44	
		Sinapic acid	521	
		Ferulic acid	232	
		Cinnamic acid	27	
		Myrecetin	47	
		Hesperidin	21	
		Quercetin	25	
		Luteolin	4	
		Kaempferol	11	
			*Adsorbed on Amberlite XAD-4, μg/RITA apparatus (200 ml medium)	
Hairy roots	*Beta vulgaris* L.	Betanin	53 mg/L	[45]
Shoots	*Vitis vinifera* cv. Feteasca Neagra, (Vitaceae)	trans-resveratrol	41.30 μg/g DW	[50]
Shoots	*Vitis vinifera* Cabernet Sauvignon (Vitaceae)	trans-resveratrol	7.94 μg/g DW	[50]
Shoots	*S. officinalis* L. (Lamiaceae)	Carnosic acid,	2.75 $\pm$ 0.02 mg/g DW	[51]
		Carnosol,	0.50 $\pm$ 0.07 mg/g DW	
		Rosmarinic acid	12.3 $\pm$ 0.1 mg/g DW	
Hairy roots	*S. officinalis* L. (Lamiaceae)	Rosmarinic acid	30.9 $\pm$ 1.0 mg/g DW	[51]

immobilization matrix of cell suspension culture of sweet basil increased RA production hundred times. In addition, RA was secreted into the culture medium, where it was collected without terminating the culture of immobilized cells [31,32].

Tepe and Sokmen, 2007 [33] developed a callus culture of one of the most spread edible plant in the territory of Balkan Peninsula *Satureja hortensis* L. In this study, production and optimization of RA was investigated on Gamborg's B5 [34] basal medium supplemented with different indole-butyric acid (IBA) and N6-benzyl aminopurine (6-BA) combinations and sucrose concentrations.

Protocol for efficient cultivation of cell suspension culture of *Salvia tomentosa* Mill. (Lamiaceae), naturally grown in Bulgaria and production of valuable pentacyclic triterpenes was reported by Marchev et al., 2011 [35]. *S. tomentosa* Mill. suspension synthesized high amounts of oleanolic (71.89 μg/mL) and ursolic acid (256.28 μg/mL). However, the nutrient media used for its cultivation were supplemented with 2,4-D, which makes the use of these valuable metabolites in food systems under discussion, concerning the safety of this plant growth regulator and possible cancer risk in humans [36].

It is well known that undifferentiated cell cultures have unstable growth and varying secondary metabolites yields because of the high level of somaclonal variation and unpredictable changes in endoreduplication pattern [37]. To minimize those risks, the so called rhizogenic callus cultures - fast growing undifferentiated transformed plant cells with a stable ploidy pattern have been developed and studied for secondary metabolites production [37]. Such type of callus culture was initiated from *S. scabiosifolia* Lam. after genetic transformation with agropine-type strain *Agrobacterium rhizogenes* ATCC 15834 and subculture of transformed roots on Linsmaier and Skoog (LS) medium [38]. The obtained rhizogenic callus was used to initiate cell suspension culture with stable growth characteristics in liquid medium and predominant accumulation of oleanolic acid (829.14 μg/g DW) [37]. The stable growth, the predictable yields, and cultivation in plant growth regulators free nutrient media of rhizogenic cell suspension culture of *S. scabiosifolia* Lam. seems to be a possible approach for producing oleanolic acid for foods [37].

After optimization of medium composition and culture conditions, cell suspension cultures of *Helianthus annuus* L. (Asteraceae) and *Arabidopsis thaliana* L. (Brassicaceae) were established for the production of the most active component of vitamin E, α-tocopherol. A considerable increase (49 and 66%, respectively) of α-tocopherol production was obtained in both cultures, after a treatment with jasmonic acid [39]. Higher yeld of α-tocopherol (24 μg/g FW) was obtained by standardized suspension of *H. annuus* L [40]. Besides, Geipel et al., 2014 [41] described an efficient protocol for α-tocopherol production from photomixotrophic suspension culture of *H. annus.* Georgiev et al., 2010 [42] also reported for cell suspension culture of *Helianthus annuus* L. (Asteraceae) that produced 14.1% linoleic acid of the identified total volatile compounds.

The homogeneity of an in vitro cell population, the large availability of material, the high rate of cell growth in controlled and reproducible bioreactor conditions make cell suspension cultures a valuable platform for the production of high-value secondary metabolites and other substances of commercial interest [15]. Further benefits include improved safety of the biosynthesized products based on the absence of environmental pollutants and agrochemicals, which is important for products legal registration as food ingredients [43].

2.2 Differentiated in vitro systems

Undifferentiated plant in vitro systems have several disadvantages as producers of food additives and bioactive metabolites. The main concern is that the biosynthesis of some metabolites requires a complex structural and physiological (cellular and tissues) compartments that only specialized and differentiated cells and organs can provide [3]. Other important points are the low and variable yields and the high level of genetic instabilities of fast growing undifferentiated plant cells [16]. The development of production processes based on differentiated plant in vitro systems could be a suitable solution to overcome those concerns.

Transformed hairy root cultures, obtained after genetic transformation with *Agrobacterium rhizogenes* strains are the preferable production systems for valuable bioactive substances among the differentiated plant in vitro systems. This is because transformed hairy roots usually have comparatively high growth rates, stable metabolite profiles, low level of somaclonal variability, high potential to accumulate secondary metabolites, as well as cultivation without exogenous growth regulators. These in vitro systems also can be easily cultivated in various bioreactor types and temporary immersion systems [3].

Marchev and co-workers, 2011 [44] developed hairy root culture of *S. tomentosa* Mill. by *Agrobacterium* transformation and cultivation in two-phase temporary immersion RITA system with a presence of adsorbent resin Amberlite XAD-4 as a second phase. The application of this approach had some important biotechnological advantages, such as an effective separation of the resin nets from the explants and removing of the biosynthesized phenolics from the cultivation medium. The authors [44] established that about 85% of released phenolic acids and 100% of released flavonoids were removed from the medium and 100% of the explants formed strong and healthy hairy roots for two weeks. This new transformation protocol was proved to be very effective and could be widely applied in other plant species, overproducing and releasing phytotoxic phenolics in high concentrations [44].

Production of the valuable food colorant betanin by Croatian *Beta vulgaris* L. hairy roots has been investigated by Kriznik and co-workers, 2010 [45]. The authors reported volumetric yield of 53 mg/L betanin. This yield could be base for further industrially visible production process. Furthermore, there is available a huge amount of information about all bioprocess engineering aspects (media optimization, elicitation, bioreactor design improvement and etc.) for development of such process reported by Pavlov and co-workers in the beginning of this century [46–49].

It was established that shoot cultures of *Vitis vinifera* cv. Feteasca Neagra and *Vitis vinifera* cv Cabernet Sauvignon were

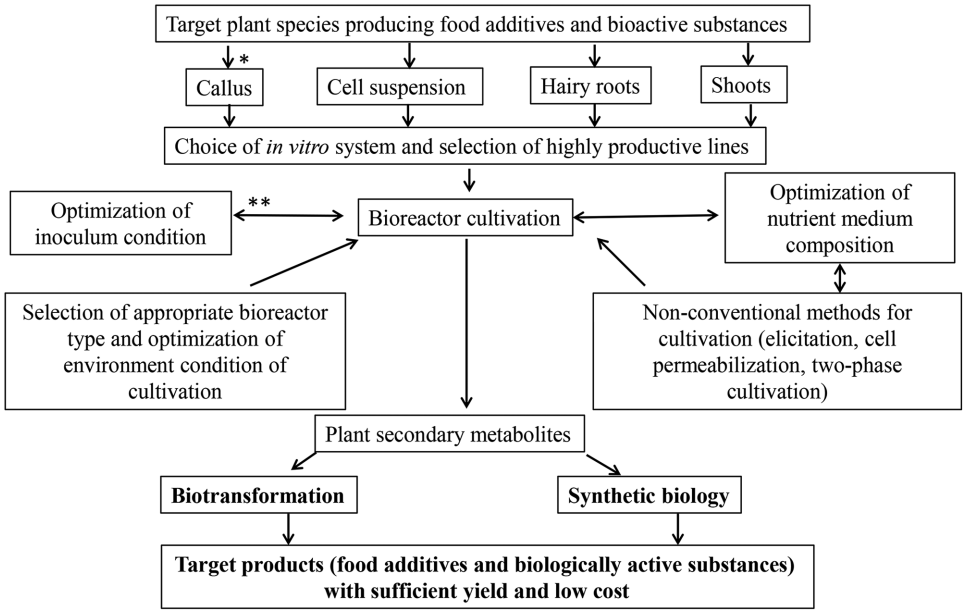

Figure 1. Integrated bioprocess approach for improving the production of food additives and bioactive compounds by plant in vitro systems (∗ consecutive steps; ∗∗ interconnected steps).

suitable in vitro systems for trans-resveratrol production. Elicitated cultures (with $AlCl_3$) accumulated 41.30 μg/g DW and 97.94 μg/g DW trans-resveratrol, respectively [50]. These yields are higher than above described yields achieved by cell suspension culture of *Vitis vinifera* L. cv. Öküzgözü and *Vitis vinifera* cv. Barbera [17, 24], but lower than those reported from genetically modified yeasts [30].

It seems the differentiated in vitro cultures of *S. officinalis* L. (Lamiaceae) are suitable production systems for carnosic acid, carnosol and rosmarinic acid [51]. Grzegorczyk and co-workers, 2007 [51] established that shoot culture produced carnosic acid, carnosol, rosmarinic acid, as the hairy root culture synthesized only rosmarinic acid. Reported volumetric yields for these compounds (Table 1) could be base for further development of economically sufficient processes, Furthermore hairy roots accumulated two fold higher quantities of rosmarinic acid than the suspension culture of the same edible plant [51].

Nevertheless achieved progress, still a challenge and a critical step that limits the industrial implementation of differentiated plant in vitro systems is their complicated scale up, as the main problem is transfer of biomasses at the inoculation processes [3]. The growth of plant tissues or organs under controlled submerged conditions brings other engineering problems related to construction of vessels, realization of mass transfer, mixing and etc. [3]. With the advance of bioprocess engineering, most of these technological issues have been solved in acceptable level and nowadays many bioreactor systems, as well as cultivation strategies have been developed and successfully applied for cultivation of various differentiated plant in vitro cultures including adventitious roots, transformed hairy roots, embryos, shoots and seedlings. Some of the processes have been implemented in commercial scale, as well [3, 52].

3 Conclusion and future prospects of bioprocess engineering aspects of production of bioactive compounds by plant in vitro systems

The objectives of many biotechnological industries are to develop plant in vitro culture techniques to the stage that the production of target bioactive compounds is cheaper either than extracting the naturally grown plants or the chemically synthesized product.

Conventional approaches for increasing the productivity of the plant in vitro cultures are based on media optimization, selection of a suitable plant tissue culture, elicitation by different biotic (microorganism-derived elicitors; plant cell wall compounds; peptides, cyclodextrins) and abiotic elicitors (metal salts, UV light), as well as optimization of bioreactor design and environmental conditions of cultivation [53–55].

Despite a significant progress, the experience showed that the low yields and the complex scale-up of bioreactor cultivation remain major limitations for industrial implementation of plant cell and tissue cultures. Therefore, there is an increasing necessity to find additional approaches for solving these limitations in yield, cost and bioprocessing conditions [56].

Biotransformation was proposed as a novel element of the integrated bioprocess approach for optimization of the production processes of food additives and bioactive compounds by plant in vitro systems (Fig. 1). It is based on modification bioactivity, bioavailability and toxicity of the produced secondary metabolites. Transformations of the structures of plant secondary metabolites are performed by bacteria and filamentous fungi as an adaptive response to their environment at physiological and/or biochemical level. They have ability to convert molecules at positions that are either difficult or impossible

by chemical methods or are economically inexpedient. Besides, biotransformation proceeds at mild conditions regarding pH, temperature and pressure in contrast to the complicated steps required for their chemical synthesis. Another advantage of microbial transformations is that the obtained derivate is pure and with sufficient yield [57].

During the last years synthetic biology has been remarkably developed. So, it could be proposed as a second novel element in the integrated bioprocess approach for production of plant bioactive compounds with sufficient yields and low cost (Fig. 1). Synthetic biology is a promising engineering tool for controlling and programming cellular behavior and biosynthesis of target compounds [58]. Most frequently, the yeasts are used as an appropriate biosynthetic matrix in this approach [59], but they also could be used for tailoring the metabolite pathways in the plant cells themselves. Omics studies and metabolic modeling enhance the understanding of yeast and plant cells metabolic networks. Nowadays, the challenges associated with synthetic biology are discovery or program of a biochemical pathway toward the desired compound, efficient and rapid assembly of this pathway in the host, optimization of enzymatic activity and expression levels of individual enzymes in the pathway, and optimization of the host cell for efficient supply of precursors, co-factors, and energy for the process [60]. Nevertheless of these limitations, the future development of the production of bioactive plant secondary metabolites will be based on the development of synthetic biological platforms – from one side for tailoring plant cell metabolism and from other for development of plant secondary metabolites production systems based on the yeasts.

Regardless of all described limitations of plant cells biotechnologies, the first commercial processes for production of cosmetics and food additives in Bulgaria are visible in the near future. Last year appeared the first Utility Model (Phytochemical profile of dedifferentiated cells of *Calendula officinalis* L.) developed by Prof. Atanas Pavlov and Dr. Vasil Georgiev, 2017 [61] in collaboration with INOVA BM Ltd., on which base nowadays the development of the industrial processes is at the stage of the commercialization of the products.

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4 References

[1] Mulabagal, V., Tsay, H.-S., Plant cell cultures - an alternative and efficient source for the production of biologically important secondary metabolites. *Int. J. Appl. Sci. Eng.* 2004. *2*, 29–48.

[2] Sheludko, Y. V., Recent advances in plant biotechnology and genetic engineering for production of secondary metabolites. *Cytology and Genetics.* 2010, *44*, 52–60.

[3] Steingroewer, J., Bley, T., Georgiev, V., Ivanov, I., et al., Bioprocessing of differentiated plant in vitro systems. *Eng. Life Sci.* 2013, *13*, 26–38.

[4] Sahoo, N., Manchikanti, P., Dey, S., Herbal drugs: Standards and regulation. *Fitoterapia.* 2010, *81*, 462–471.

[5] Turrill, W. B., The plant-life of the Balkan peninsula: a phytogeographical study (Oxford Memoirs on Plant Geography). *Geol. Mag.* 2009, *66*, 188–189.

[6] Petrova, A., Vladimirov, V., Balkan endemics in the Bulgarian flora. *Phytol. Balcan.* 2010, *16*, 293–311.

[7] Strid, A., Andonovski, A., Andonovski, V., The high mountain vegetation of the Balkan peninsula, in: Nagy, L., Grabherr, G., Körner, Ch., Thompson, D.B.A. (Eds.), *Alpine Biodiversity in Europe. Ecological Studies*, Springer-Verlag Berlin Heidelberg 2003, pp. 113–121.

[8] Redzic, S., Wild edible plants and their traditional use in the human nutrition in Bosnia and Herzegovina, *Ecol. Food Nutr.* 2006, *45*, 189–232.

[9] Nedelcheva, A., An ethnobotanical study of wild edible plants in Bulgaria, *Eurasia J. Biosci.* 2013, *7*, 77–94.

[10] Łuczaj, Ł., Dolina, K., Fressel, N., Perković, S., Wild food plants of Dalmatia (Croatia), in: Pieroni, A., Quave, C. (Eds), *Ethnobotany and Biocultural Diversities in the Balkans*, Springer, New York, NY 2014, pp. 137–148.

[11] Hardalova, R., Evstatieva, L., Gussev, Ch., Wild medicinal plant resources in Bulgaria and recommendation for their long-term development, in: Meine, C. (Ed.), *National Strategy for Biodiversity Conservation.* Pensoft, Sofia 1998, pp. 527–561.

[12] Turner, N., Łuczaj, Ł., Migliorini, P., Pieroni, A., et al., Edible and tended wild plants, traditional ecological knowledge and agroecology. *Crit. Rev. Plant Sci.* 2011, *30*, 198–225.

[13] Kim, Y., Wyslouzil, B. E., Weathers, P. J., Secondary metabolism of hairy root cultures in bioreactors. *In vitro Cell Dev-Pl.* 2002, *38*, 1–10.

[14] Srivastava, S., Srivastava, A. K., Hairy root culture for massproduction of high-value secondary metabolites. *Crit. Rev. Biotechnol.* 2007, *27*, 29–43.

[15] Bharati, A. J., Bansal, Y. K., In vitro production of flavonoids: a review. *World J. Pharm. Pharm. Sci.* 2014, *3*, 508–533.

[16] Georgiev, V., Schumann, A., Pavlov A., Bley, T., Temporary immersion systems in plant biotechnology. *Eng. Life Sci.* 2014, *14*, 607–621.

[17] Keskin, N., Kunter, B., Production of trans-resveratrol in callus tissue of Ökűzgőzű (Vitis vinifera L.) in response to ultraviolet-C irradiation. *J. Anim. Plant. Sci.* 2010, *20*, 197–200.

[18] Bradamante, S., Barenghi, L., Villa, A., Cardiovascular protective effects of resveratrol. *Cardiovasc. Drug Rev.* 2004, *22*, 169–188.

[19] Szekeres, T., Saiko, P., Fritzer-Szekeres, M., Djavan, B., Jager, W., Chemopreventive effects of resveratrol and resveratrol derivatives. *Ann. N. Y. Acad. Sci.* 2011, *1215*, 89–95.

[20] Huang, T. C., Lu, K. T., Wo, Y. Y., Wu, Y. J., et al., Resveratrol protects rats from A-induced neurotoxicity by the reduction of iNOS expression and lipid per-oxidation, *PLoS One.* 2011, *6*, e29102.

[21] Baur, J. A., Sinclair, D. A., Therapeutic potential of resveratrol: the in vivo evidence. *Nat. Rev. Drug Discov.* 2006, *5*, 493–506.

[22] Guerrero, R. F., Garcia-Parrilla, M. C., Puertas, B., Cantos-Villar, E., Wine, resveratrol and health: a review. *Nat. Prod. Commun.* 2009, *4*, 635–658.

[23] Sak, M., Dokupilova, I., Mihalik, D., Lakatošova, J., Gubišova, M. et al., Elicitation of phenolic compounds in cell culture

of Vitis vinifera L. by Phaeomoniella chlamydospora. *Nova Biotechnol. Chim.* 2014, *13*, 162–171.

[24] Ferri, M., Dipalo, S. C., Bagni, N., Tassoni, A., Chitosan elicits mono-glucosylated stilbene production and release in fed-batch bioreactor cultures of grape cells. *Food Chem.*. 2014, *124*, 1473–1479.

[25] Zamboni, A., Vrhovsek, U., Kassemeyer, H. H., Mattivi, F. U., Elicitor-induced resveratrol production in cell cultures of different grape genotypes (Vitis spp.). *Vitis.* 2006, *45*, 63–68.

[26] Becker, J. V., Armstrong, G. O., van der Merwe, M. J., Lambrechts, M. G., et al., Metabolic engineering of *Saccharomyces cerevisiae* for the synthesis of the wine-related antioxidant resveratrol. *FEMS Yeast Res.*. 2003, *4*, 79–85.

[27] Beekwilder, J., Wolswinkel, R., Jonker, H., Hall, R., et al., Production of resveratrol in recombinant microorganisms. *Appl. Environ. Microbiol.* 2006, *72*, 5670–5672.

[28] Shin, S. Y., Han, N. S., Park, Y. C., Kim, M. D., et al., Production of resveratrol from p-coumaric acid in recombinant Saccharomyces cerevisiae expressing 4-coumarate: coenzyme A ligase and stilbene synthase genes. *Enzyme Microb. Technol.* 2011, *48*, 48–53.

[29] Watts, K. T., Lee, P. C and Schmidt-Dannert, C., Biosynthesis of plant-specific stilbene polyketides in metabolically engineered Escherichia coli. *BMC Biotechnol.*. 2006. *6*, http://doi.org/10.1186/1472-6750-6-22.

[30] Mingji, L., Engineering of Saccharomyces cerevisiae for production of resveratrol and its derivatives, The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, 2016, PhD thesis.</bib>

[31] Kintzios, S., Makri, O., Panagiotopoulos, E., Scapeti, M., In vitro rosmarinic acid accumulation in sweet basil (Ocimum basilicum L.). *Biotechnol. Lett.* 2003, *25*, 405–408.

[32] Moschopoulou, G., Kintzios, S., Achievement of thousand-fold accumulation of rosmarinic acid in immobilized cells of sweet basil (Ocimum basilicum L.) by ten-fold increase of the volume of the immobilization matrix. *J. Biol. Res.* 2011, *15*, 59–65.

[33] Tepe, B., Sokmen, A., Production and optimisation of rosmarinic acid by Satureja hortensis L. callus cultures. *Nat. Prod. Res.* 2007, *21*, 1133–1144.

[34] Gamborg, O. L, Miller R. A., Ojima, K., Nutrient requirements of suspension culture of soybean root cells. *Ex. Cell. Res.* 1968, *50*, 15–158.

[35] Marchev, A., Ivanov, I., Georgiev, V., Pavlov, A., Determination of di- and triterpenes in Salvia tomentosa Mill. cell suspension culture by high performance liquid chromatography. *Scientific works of University of Food Technologies* "*Food science, engineering and technologies*", 2012, *59*, 229–233.

[36] Loomis, D., Guyton, K., Grosse, Y., El Ghissasi, F. et al., Carcinogenicity of lindane, DDT, and 2,4-dichlorophenoxyacetic acid. *Lancet Oncol.*. 2015, *16*, 891–892.

[37] Marchev, A., Georgiev, V., Badjakov, I., Kondakova, V., et al., Triterpenes production by rhizogenic callus of Salvia scabiosifolia Lam. obtained via Agrobacterium rhizogenes mediated genetic transformation. *Biotechnol. Biotechnol. Eq.* 2011, *25*, 30–33.

[38] Linsmaier, E. M., Skoog, F., Organic growth factor requirements of tobacco tissue cultures. *Physiol. Plant.* 1965, *18*, 100–127.

[39] Gala, R., Mita, G., Caretto, S., Improving α-tocopherol production in plant cell cultures. *J. Plant Physiol.*. 2005, *162*, 782–784

[40] Caretto, S., Nisi, R., Paradiso, A., Laura, D., Tocopherol production in plant cell cultures. *Mol. Nutr. Food Res.* 2010, *54*, 726–730.

[41] Geipel, K., Song, X., Socher, M. L., Kümmritz, S., et al., Induction of a photomixotrophic plant cell culture of Helianthus annuus and optimization of culture conditions for improved α-tocopherol production. *Appl. Microbiol. Biotechnol.* 2014, *98*, 2029–2040.

[42] Georgiev, M., Georgiev, V., Penchev, P., Antonova, D., et al., Volatile metabolic profiles of cell suspension cultures of *Lavandula vera*, *Nicotiana tabacum* and *Helianthus annuus*, cultivated under different regimes. *Eng. Life Sci.*. 2010, *10*, 148–157.

[43] Angelov, A., Gotcheva, V., Safety assessment and regulations or food ingredients derived from plant in vitro systems, in; Pavlov, A., Bley, T. (Eds.), *Bioprocessing of Plant In vitro Systems, Reference Series in Phytochemistry*, Springer International Publishing AG 2016, pp. 1–14.

[44] Marchev, A., Georgiev, V., Ivanov, I., Badjakov, I., et al., Two phase temporary immersion system for Agrobacterium rhizogenes genetic transformation of sage (Salvia tomentosa Mill.). *Biotechnol. Lett.* 2011, *33*, 1873–1878.

[45] Kriznik, B., Pavokovic, D., Enhancement of betanin yield in transformed cells of sugar beet (Beta vulgaris L.). *Acta Bot. Croat.* 2010, *69*, 173–182.

[46] Pavlov, A., Georgiev, V., Ilieva, M., Betalain biosynthesis by red beet (Beta vulgaris L.) hairy root culture. *Process Biochem.* 2005, *40*, 1531–1533.

[47] Pavlov, A., Georgiev, V., Kovatcheva, P., Relationship between type and age of the inoculum cultures and betalains biosynthesis by *Beta vulgaris* hairy root culture. *Biotechnol. Lett.* 2003, *25*, 307–309,

[48] Pavlov, A., Georgiev, M., Bley, T., Batch and fed-batch production of betalains by red beet (Beta vulgaris) hairy roots in a bubble column reactor. *Z. Naturforsch.* 2007, *62c*, 439–446.

[49] Georgiev, V., Ilieva, M., Bley, T., Pavlov, A., Betalain production in plant in vitro systems. *Acta Physiol. Plant.* 2008, *30*, 581–593.

[50] Bejan, C., Visoiu, E., Resveratrol biosynthesis on in vitro culture conditions in grapevine (cv. Feteasca Neagra and Cabernet Sauvignon) under the action of AlCl3 as elicitor agent. *Rom. Biotechnol. Lett*, 2011, *16*, 97–101.

[51] Grzegorczyk, I., Matkowski, A., Wysokin´ska, H., Antioxidant activity of extracts from in vitro cultures of Salvia officinalis L. *Food Chem.*. 2007, *104*, 536–54.

[52] Georgiev, M.G., Weber, J., Bioreactors for plant cells: hardware configuration and internal environment optimization as tools for wider commercialization. *Biotechnol. Lett.* 2014, *36*, 1359–1367.

[53] Zhao, J., Davis, L.C., Verpoorte, R., Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnol. Adv.* 2005, *23*, 283–333.

[54] Georgiev, M., Weber, J., Maciuk, A., Bioprocessing of plant cell cultures for mass production of targeted compounds. *Appl. Microbiol. Biotechnol.* 2009, *83*, 809–823.

[55] Hussain, M., Fareed, S., Ansari, S., Rahman, M. A., et al., Current approaches toward production of secondary plant metabolites. *J. Pharm. Bioallied Sci.* 2012, *4*, 10–20.

[56] Pavlov, A., Plant cells and algae in bioreactors. *Eng. Life Sci.* 2014, *14*, 548–549.
[57] Mutafova, B., Fernandes, P., Mutafov, S., Berkov, S., et al., Microbial transformations of plant secondary metabolites, in: Pavlov, A., Bley, T. (eds.), *Bioprocessing of Plant In vitro Systems, Reference Series in Phytochemistry*, Springer International Publishing AG 2016, pp. 1–41.
[58] Derek, J. M., Valiante, V., Unkles, S. E., Brakhage, A. A., Synthetic biology of fungal natural products. *Front Microbiol.* 2015, *6*, 775.
[59] Sharma, K.K., Exploiting fungi in synthetic biology: commentary. *Curr. Synthetic Sys. Biol.* 2015, *3*, 123–124.
[60] Mingji, L., Borodina, I., Application of synthetic biology for production of chemicals in yeast Saccharomyces cerevisiae. *FEMS Yeast Res.*. 2015, *15*, 1–12.
[61] Pavlov, A., Georgiev, V., Phytochemical profile of dedifferentiated cells of Calendula officinalis L. BG Patent 2773, 2017.