PRODUCTION OF SECONDARY METABOLITIES IN PLANT TISSUE CULTURES

Angelika FILOVÁ
Department of Plant Physiology, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 01 Nitra, Slovak Republic
e-mail: angela.filova@uniag.sk

Abstract: *Plant cell and tissue cultures hold great promise for controlled production of myriad of useful secondary metabolites. In the search for alternatives to production of medicinal compounds from plants, biotechnological approaches, specifically plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites. Exploration of the biosynthetic capabilities of various cell cultures has been carried out by a group of plant scientists and microbiologists in several countries during the last decade. A number of medicinally important alkaloids, anticancer drugs, recombinant proteins and food additives are produced in various cultures of plant cell and tissues. Advances in the area of cell cultures for the production of medicinal compounds has made possible the production of a wide variety of pharmaceuticals like alkaloids, terpenoids, steroids, saponins, phenolics, flavanoids and amino acids. Some of these are now available commercially in the market for example shikonin and paclitaxel (Taxol). Until now 20 different recombinant proteins have been produced in plant cell culture, including antibodies, enzymes, edible vaccines, growth factors and cytokines. Advances in scale-up approaches and immobilization techniques contribute to a considerable increase in the number of applications of plant cell cultures for the production of compounds with a high added value. Some of the secondary plant products obtained from cell suspension culture of various plants. Cell suspension culture systems are used now days for large scale culturing of plant cells from which secondary metabolites could be extracted. A suspension culture is developed by transferring the relatively friable portion of the callus into liquid medium and is maintained under suitable conditions of aeration, agitation, light, temperature and other physical parameters. Cell cultures cannot only yield defined standard phytochemicals in large volumes but also eliminate the presence of interfering compounds that occur in the field-grown plants. The advantage of this method is that it can ultimately provide a continuous, reliable source of natural products. The major advantage of the cell cultures include synthesis of bioactive secondary metabolites, running in controlled environment, independently from climate and soil conditions. A number of different types of bioreactors have been used for mass cultivation of plant cells. The first commercial application of large scale cultivation of plant cells was carried out in stirred tank reactors to produce shikonin by cell culture of Lithospermume rythrorhizon. Cell of Catharanthus roseus, Dioscorea deltoidea, Digitalis lanata, Hypericum perforatum, Maackia amurensis, Panaxnoto ginseng, Taxus wallichiana and Sophora flavescens have been cultured in various bioreactors for the production of secondary plant products. Plant tissue culture represents the most promising areas of application at present time and giving an out look into the future.*

Key words: *flavonoides, plant tissue cultures, production, secondary metabolites*

INTRODUCTION

Bioactive compounds extracted from plants are widely used. The natural habitats for a large number of plants are rapidly destroyed leading to extinction of many valuable and even endemic species. Studies on the production of plant metabolites by callus and cell suspension cultures have been carried out on an increasing scale since the end of the 1950's. The prospect of using such culturing techniques is for obtaining secondary metabolites, such as active compounds for pharmaceuticalss and cosmetics, hormones, enzymes, proteins, antigens, food additives and natural pesticides from the harvest of the cultured cells or tissues (TERRIER *et al.*, 2007).

Biotechnology offers an opportunity to exploit cells, tissues, organs or entire organisms by growing them in vitro and to genetically manipulate to get desired compounds (RAO and RAVISHANKAR, 2002). Secondary metabolities can be produced by using different biotechnological approaches, such as casus cultures, cell suspension cultures and/or organ cultures. In the following sections we will briefly review the individual in vitro culture techniques with regard to the attempts to use them for production of secondary metabolities. Since it was observed, that production of secondary metabolites is generally higher in differentiated plant tissues, there were attempts to cultivate whole plant organs, i.e. shoots or roots in in vitro conditions with the aim to produce medicinally important compounds (BIONDI *et al*., 2002). As it was expected, such organ cultures produced similar patterns of secondary metabolites as intact plants. The advantage of using the organ cultures is that they are relatively more stable in production of secondary metabolites than cultures of undifferentiated cells, such as cells in callus or suspension culture (RAO and RAVISHANKAR, 2002). For the objective of production of plant secondary products, generally two types of organ cultures are considered, i.e. root cultures and shoot cultures.

PRODUCTION OF SECONDARY METABOLITIES IN ORGAN CULTURES

Root cultures are valuable sources of medicinbal compounds (PENCE, 2011; LI *et al*., 2002) Many of the secondary compounds, for example, the tropane alkaloids hyoscyamine and scopolamine were produced quite well in root cultures (FAZILATUN et al., 2004). Root systems of higher plants, however, generally exhibit slower growth than cultures of undifferentiated plant cells and are difficult to harvest. Therefore, alternative methods for production of compounds synthesized in plant roots were investigated. The most promising one of them is the use of plant hairy root cultures (PENCE, 2011).

As with roots, it is also possible to cultivate plant aerial parts – shoots – for production of secondary metabolites (BOURGAUD *et al.,* 2001; NOGUEIRA and ROMANO, 2002; SMITH *et a*l., 2002). Shoot cultures are usually used to overcome the dependency of commercial production of certain secondary compounds on the natural plants (KHANAM *et al.*, 2000) or to induce somaclonal variation in vitro and to select high secondary product yielding clones (DHAWAN *et al*., 2003).

Major problem of organ cultures is the large-scale cultures (KAIMOYO *et al*., 2008). Different types of bioreactors have been used for the culture of plant roots and/or shoots (KAŠPAROVÁ, 2009; KIM *et al*., 2002). Compared to the cell suspension cultures, organ cultures generally display a lower sensitivity to shear stress, but they show a high degree of spatial heterogeneity in biomass production. Another problem is the quite high cost of these bioreactor systems for commercial large-scale production of plant secondary metabolites. As they have to compete with the cultivation of the whole plant, such a process in most cases is not economically viable (ZHAO *et al*., 2010). Up to date, the only commercial example of the use of plant organ cultures for secondary metabolite production is the cultivation of ginseng roots (HIBINO and USHIYAMA, 1999).

PRODUCTION OF SECONDARY METABOLITIES IN CALLUS CULTURES

Callus culture is the culture of dedifferentiated plant cells induced on media usually containing relatively high auxin concentrations or a combination of auxin and cytokinin in in vitro conditions. Callus cultures can be embryogenic or non-embryogenic. Embryogenic calli contain differentiated embryogenically competent cells that can regenerate complete plants through the process called somatic embryogenesis (PTAK, 2013). The main uses of somatic embryogenesis include clonal propagation of plants, regeneration of haploid or transgenic

plants and fundamental study of the process of embryogenesis in plants. Non-embryogenic callus cultures, containing more or less homogenous clumps of dedifferentiated cells, are used for secondary metabolite production. Of the tissue culture means, this approach is relatively frequently used for production of flavonoids. In this section, we summarize literature data on the production of different groups of flavonoids by callus culture. Some examples of flavonoid production in callus culture are presented in Table 1.

MADHAVI *et al.* (1998) studied the isolation of bioactive constituents from *Vaccinium myrtillus* fruits and cell cultures. Fruits and casus cultures were extracted and fractionated. Major fractions contained flavonoids, such as cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside and proanthocyanidins. Anthocyanin accumulation in callus was lower (0.08 mg/g dry cell weight; DCW) than in the fruit (27.3 mg/g DCW). Callus cultures accumulated both oligomeric (178 mg/g DCW) and polymeric (436 mg/g DCW) proanthocyanidins; proanthocyanidins were similarly present in fruit extracts (oligo- and polymeric, 202 and 1613 mg/g DCW, respectively).

DIAS *et al.* (1998) published the isolation of a new naturally occurring compound 6-C-prenyl luteolin, together with luteolin-5,3'-dimethyl ether, luteolin-5-glucoside and luteolin-3'-glucoside from the callus of *Hypericum perforatum* var. *angustifolium*. The total flavonoid content of callus, around 0.05-0.7 mg/g (DCW), was much lower than that found in wild growing *H. perforatum* plants 14-70 mg/g (DCW). FEDOREYEV *et al.* (2004) established callus cultures from the different parts of *Maackia amurensis* and analyzed for isoflavonoids. The isoflavones daidzein, retuzin, genistein and formononetin and the pterocarpans maakiain and medicarpin were found to be produced by these cultures. The content of isoflavones and pterocarpans was essentially the same in cultures derived from leaf petioles, inflorescences and apical meristems of the plant. The maximal yield of isoflavones and pterocarpans in calluses was 20.8 mg/g (DCW), approximately four times higher than the content of the heartwood of *M. amurensis* plants. Moreover, LI *et al.* (2002) established six callus cultures of Genista species with the objective to produce isoflavones of phytoestrogenic activity. The cultures were optimized for their growth and isoflavonoid production by changing various media in the presence or absence of light. The best growth and the highest isoflavone production was obtained under constant light regime on SH basal medium containing 22.6 μmol/L 2,4-dichlorophenoxyacetic acid (2,4-D), 23.2 μmol/L kinetin and 3% (w/v) of sucrose.

Callus cultures of all species produced more isoflavones than the parent herbs. In vitro cultures had lower contents of genistein esters than the herbs. The callus with the highest isoflavone content was obtained from *G. tinctoria*, producing 6586.5 mg of total isoflavones per 100 g DCW, in which the HPLC analysis identified 3016.3 mg of genistin. The effect of the potential elicitors (killed cells of *Pseudomonas aeruginosa*, linoleic acid, chromium trichloride, jasminic acid, substituted anilides of pyrazine-2-carboxylic acids and iodoacetic acid) on the production of flavonoids in callus culture of *Ononis arvensis* L. was examined by TUMOVA and co-workers (TUMOVA, 2010; TUMOVA and TUMA, 2010). All the tested elicitors markedly increased the production of flavonoids in comparison with the control.

Stable and optimized callus cultures are a logical step in the first phase of the cell culture production of plant secondary metabolites, i.e. preparing the inoculum for liquid suspension cultures. Production of secondary metabolities in cell suspension cultures have been widely published and it was proposed as a technology to overcome problems of variable product quantity and quality from whole plants due to the effects of different environmental factors, such as climate, diseases and pests (YAMAMOTO *et al.*, 2002; ZHANG *et al.*, 2002; RAO and RAVISHANKAR, 2002). During the past decades, this technology therefore attracted much academic and industrial interest. The approach of using plant cell suspension

cultures for secondary metabolite (including flavonoids) production is based on the concept of biosynthetic totipotency of plant cells, which means that each cell in the cultures retains the complete genetic information for production of the range of compounds found in the whole plant. Cell suspension cultures are initiated from established callus cultures by inoculating them into liquid media. The cultures are then kept in glass flasks under continual agitation on horizontal or gyratory shakers and eventually they can be transferred to a specialized bioreactor (BOURGAUD *et al.*, 2001).

YAMAMOTO *et al.* (2002) showed the effect of polysaccharides on the production of prenylated flavanones (sophoraflavanone G and lehmanin) in *Sophora flavescens* callus culture. The production of these flavanones was stimulated up to 5 times by addition of 2 mg/mL yeast extract. More-over, the production of prenylated flavanones also can be increased by 2-5 times by addition of cork pieces. The effect of different elicitors, such as killed cells of *Pseudomonas aeruginosa*, chromium trichloride, jasminic acid, substituted anilides of pyrazine-2-carboxylic acids and iodoacetic acid, on the production of flavonoids in cell suspension cultures of *Ononis arvensis* L. was examined by TUMOVA *et al.* (2002). They showed a marked increase of the production of flavonoids in comparison with the control by all the tested elicitors. MONACHE *et al.* (1995) isolated flavonoids from callus and cell cultures of *Maclura pomifera*. Among the flavonoids, flavones and flavanones were produced preferentially by suspended cells, but with the prenyl substituents exclusively on ring A, while the isoflavones did not show the 3', 4'-dihydroxyl substitution pattern found in the products isolated from fruits. The *M. pomifera* cell suspension culture showed a greater level of metabolite accumulation (0.91%) than stems (0.26%), leaves (0.32%) and fruits (0.08%) of the parent plant. ZHANG *et al.* (2002) studied the temperature effect on anthocyanin production in cell suspension cultures of *Fragaria ananassa* at a temperature range of 15-35◦C. The maximum anthocyanin production was obtained at 20◦C. Anthocyanin production of 270 mg/L on day 9 was increased 1.8, 3 and 4-fold over that of cultures at 20, 25 and 30 ◦C, respectively. In addition, ZHAO *et al.* (2010) reported also anthocyanin accumulation in cell suspension cultures of *Vitis vinifera*. Following either the addition of jasmonic acid or light irradiation, the anthocyanin biosynthesis was enhanced, whereas cell growth was inhibited. The maximum anthocyanin accumulation of 13.8 CV (color value)/g FCW (fresh cell weight) occurred on day 7 when jasmonic acid was added to the cultures at a final concentration of 20 μM on day 0. This represented an 8.5-fold increase compared with the control culture in the dark. Following the continuous light irradiation of 8000-8300 lux, the maximum anthocyanin accumulation reached was 6.8 CV/g FCW on day 10, which was 4.8-fold that of the control. A process, that combined jasmonic acid treatment and light irradiation, resulted in a significant synergistic enhancement of anthocyanin accumulation up to 22.62 CV/g FCW on day 7. This value was 13.9-fold that of the control. As a result, the maximum anthocyanin production of 2200 CV/ L was achieved on day 10, representing a 5.8-fold increase compared with the control. Moreover, PARK *et al.* (1995) studied cell cultures of *Pueraria lobata* for elicitor-induced enzymatic and genetic activation of isoflavonoid production. Addition of yeast extract to the cell cultures stimulated the accumulation of isoflavones and daidzein dimers. Culture productivity is critical to the practical application of cell suspension culture technology to production of flavonoids. Until now, variol strategies have been developed to improve the production of secondary metabolites in in vitro cultures, such as the manipulating the parameters of the environment and medium, selecting high yielding cell clones, precursor feeding and elicitation (reviewed in COLLIN, 2001; RAO and RAVISHANKAR, 2002; TUMOVÁ *et al.*, 2010).

Cell cultures of *Taxus sp.* presents a potential alternative source of taxol and related taxanes used in cancer chemotherapy. The quantification and distribution of taxol in explantate callus

culture of selected chemotypes of *Taxus baccata, Taxus canadensis, Taxus brevifolia* and *Taxus cuspidate* studied FILOVÁ (2011). Selected chemotypes of *Taxus sp*. was cultivated on modification Westvaco medium WV with auxine 2,4-D (1, 2 a 5 mg.l^{-1}) for induction callus. Callusu culture of *T. canadensis* produced the most amount of taxol in 56- day of cultivation. Callus cultures of *T. baccata* produced a half amount of taxol *T. canadensis*. Growth of biomass and production secondary metabolites of taxanes characters of yew are dependent of types explantate cultures, of concentration auxine in medium, of chemotypes and time cultivation.

Table 1.

Flavonoid production in callus cultures.

Flavonoid family	Compound name	Name of the plant	Type of material [a]	Yield of production	Susp. pharm.activity [c]	References
Flavanones	Silymarin Silybin Silychristin Silydianin	*Silybum marianum*	Cotyledons	0.35±0.03 mg/g (DCW) 0.20±0.02 mg/g (DCW) 0.10±0.01 mg/g (DCW) 0.05±0.01 mg/g (DCW)	Anti-hepatotoxic, hepatoprotective, antioxidant	CACHO et al., 1999
	6-C-prenyl luteolin luteolin-5,3'-dimethyl ether luteolin-5-glucoside luteolin-3'-glucoside	*Hypericum perforatum var. angustifolium*	Shoots	140±46 µg/g (DCW) 95±39 µg/g (DCW) 110±39 µg/g (DCW) 110±39 µg/g (DCW)	Anti-inflammatory, antiviral	DIAS et al., 1998
Flavones	Vitexin Isovitexin Orientin Isoorientin	*Drosophyllum lusitanicum*	Shoots	0.8mg/57g (FCW) 1.5mg/57g (FCW) 0.4 mg/57g (FCW) 1.4 mg/57g (FCW)	antioxidant	BUDZIANOWSKI, 2002
	Daidzein Retuzin Genistein Formononetin	*Maackia amurensis*	Leaf petioles, inflorescences, leaves, apical meristems	0.52±0.18mg/g (DCW) 0.91±0.34mg/g (DCW) 2.53±0.42mg/g (DCW) 4.23±1.01mg/g (DCW) ND[b]	Hepatoprotective, cardioprotective, anticancer, antioxidant	FEDOREYEV et al., 2000
	Lehmanin	Sophora flavescens	Roots	ND[b]	anti-inflammatory, antibacterial	KIM et al. 2002
Antho-cyanins	Anthocyanin	*Hyoscyamus muticus L.*	Cotyledon, hypocotyl	0.05 % g of (DCW)	Antioxidant, cardioprotective, anticancer	BASU & CHAND, 1996; HOU, 2003
	cyanidin 3-(6"-malonylglucoside)	*Taraxacum officinale*	Red-purple cells	0.08 mg/g (DCW)	Antioxidant cardioprotective, anticancer	HOU, 2003
	cyanidin-3-galactoside,	*Vaccinium myrtillus L.*	Hypocotyl		Antioxidant, astrigent,	MADHAVI et al., 1998

[a] Plant material used for suspensions culture establishment [b] ND-not defined [c] Suspected pharmaceutical activity

PRODUCTION OF SECONDARY METABOLITES BY HAIRY ROOTS

Hairy roots grow rapidly, show plagiotropic growth, and are highly branched on phytohormone-free medium (HU AND DU, 2006). *Agrobacterium rhizogenes*-derived hairy roots and plants have application for many different areas. Hairy root cultures have been tested extensively in root nodule research, for production of plant secondary metabolites. Transformed roots have been widely studied for *in vitro* production of secondary metabolites in many plant species and for artificial seed production. Hairy root cultures produce secondary metabolites over successive generations without losing genetic or biosynthetic stability (GIRI VE NARASU, 2000). Many aspects of plant secondary metabolite biosynthesis have been studied using transformed root cultures (KUZOVKINA AND SCHNEIDER, 2006). The strong corelation between secondary metabolite production and morphological differentiation gives more impetus to the application of organized cell cultures for large scale production of phytochemicals. Intergeneric co-culture of genetically transformed hairy roots and shooty teratomas is effective for improving tissue specific secondary metabolites.

This mimics the situation observed in the whole plant where a localized metabolite synthesis is translocated throughout the organs for further bioconversion (GIRI AND NARASU, 2000). Also, production of two different secondary metabolites is possible simultaneously by adventitious root cocultures (WU *et al.,* 2008). This vast potential of hairy root cultures (CETIN *et al*. 2005) as a stable source of biologically active chemicals has provided the exploitation of *in vitro* system through up scaling in novel bioreactors (MEHROTRA *et al*., 2008). Hairy root cultures of *Lithospermum erythrorhizon, Harpagophytum procumbens* (LUDWIG-MULLER *et al*., 2008) and adventitious roots of *Panax ginseng* (JEONG *et al*., 2008) and *Scopolia parviflora* (MIN *et al*., 2007) were studied in various volumes of bubble column bioreactors to obtain shikonin, harpagide, ginsenosides and alkoloids respectively. Ginsenoside was also produced in 5 L stirred tank bioreaktor using adventitious root culture (JEONG *et a*l., 2008). Hairy root culture of *Stizolobium hassjo* to yield 3,4-dihydroxyphenylalanine was reported using 9L mist bioreactor. Present scale-up technology dictates the use of stainless steel tanks for growth of plant cells on an industrial scale. The usage of bioreactors equipped with special hangers inside the vessel is reported. Hairy root cultures continue to attract interest as a potential resource for large-scale production of commercially valuace compounds.

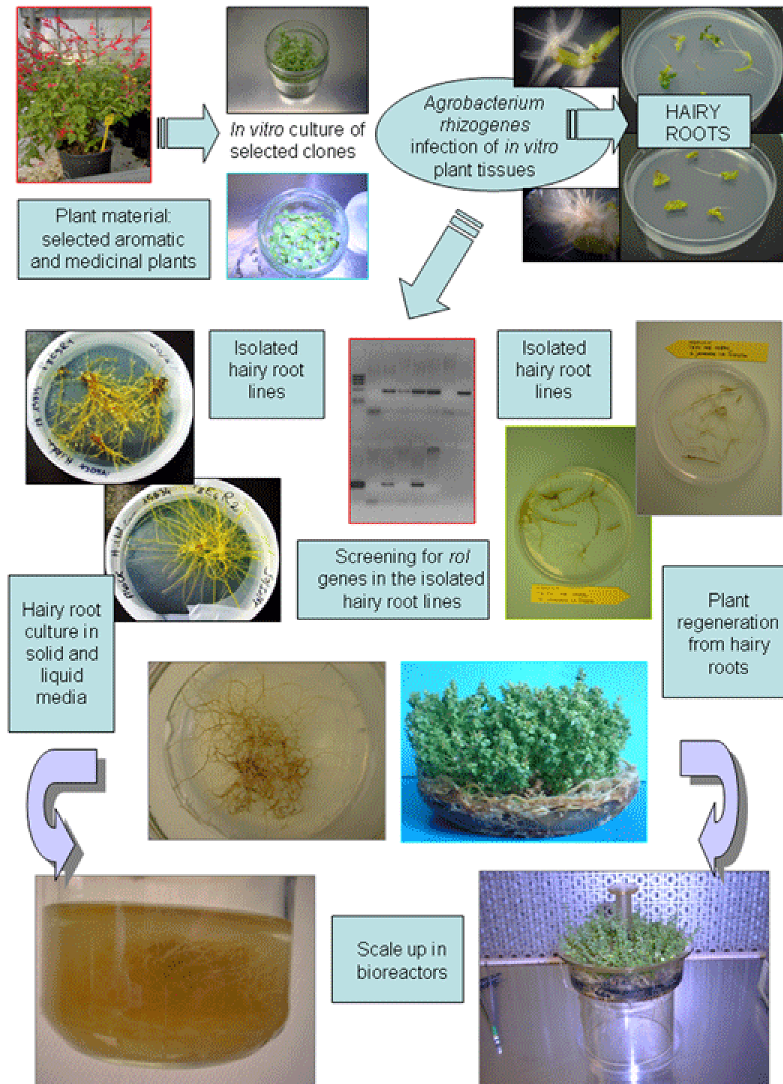

Figure 1: Biotechnological applications of hairy roots research

CONCLUSIONS

This review briefly summarized the possible sources of secondary metabolities for their perspective biotechnological production. Plant tissue culture techniques have been tried for large-scale production of secondary metabolites in plants species that have medicinal importance or those that is generally difficult to cultivate. The entire biotechnological potential of secondary metabolities has not yet been exploited. The technology of plant tissue culture has its origin in the first half of the 20th century, with the work of pioneers, such as Haberlandt,

White, Nobécourt and Gautheret. Plant tissue cultures were suggested for the first time for production of phytochemicals as early as in 1956. Since then, the development of plant tissue culture-based systems, as an alternative to conventional whole plant or synthetic production, become a challenge for research scientists worldwide. Despite of great progress in the organic synthesis of many plant secondary metabolites and related compounds, the extraction of plant secondary metabolites is still commercially required and actual. Moreover, most of these compounds are very difficult to synthesize chemically. Taking into account the food consumers' preferences, natural compounds are better accepted than synthetic ones in general. These facts lead to the development of procedures for growing plant tissues and cells in a manner similar to that used for microorganisms, i.e. under controlled conditions in culture vessels and utilization of high producing cultures on industrial scale. Plant tissue cultures are able to transform natural and synthetic compounds using the potential of their enzymes in processes such as hydrogenation, dehydrogenation, isomeration, glycosylation, hydroxylation or transfer of short carbon backbones. The production of secondary metabolities via tissue culture techniques have been reported in both callus and cell suspension cultures. The spectrum of the produced compounds and their yields depended on the proper selection of plant species, explant types and culture conditions.

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ACKNOWLEDGEMENT

This study was supported by project: Vybudovanie výskumného centra „AgroBioTech“, ITMS kód: 26220220180.