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Approaches to flavonoid production in plant tissue cultures

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Flavonoids are low molecular-weight polyphenolic compounds found throughout the plant kingdom. They have a wide range of structure-dependent biological effects and functions in the biochemistry, physiology and ecology of plants. Flavonoids may be exploited in many ways in food processing, cosmetic and pharmaceutics industry. The evolving commercial importance of flavonoids and a need for renewable resources of valuable chemicals has lead to attempts in developing alternative systems for their production. Different *in vitro* systems have been developed for production of flavonoids, e.g., in callus cultures, cell suspension cultures, root cultures or shoot cultures. The selection of highly productive lines as well as the optimalization of chemical and physical culture environments of cells for maximum productivity is the prerequisite for the commercial application of these systems.

Key words: flavonoids, *in vitro* culture, plant cell culture, organ culture.

Introduction

Flavonoids are plant secondary metabolites found in most terrestrial vascular plants. They belong to a group of natural phenolic substances with variable chemical structures and in plants they are found in fruits, vegetables, grains, tree barks, roots, stems, flowers, as well as tea and wine (Hermann, 1976; Stafford, 1991; Harborne & Williams, 2000). More than 6000 different flavonoids have been identified, many of which are responsible for the attractive colors of flowers, fruits and leaves (Nijveldt et al., 2001). The interesting biological activities of flavonoids have prompted the intensive research on physiological properties of these compounds and their effects on human health (Rusak et al., 2002). Their wide occurrence, complex diversity and manifold functions have made flavonoids a very attractive system for research on a molecular-biological level. Up to date, vast amount of knowledge on flavonoids has been accumulated. This has provided the tools and the know-how for successful metabolite engineering of the flavonoid pathway

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(Forkmann & Martens, 2001). Flavonoids possess significant antihepatotoxic, antiallergic (Di carlo et al., 1999; Mojžiš & Mojžišová, 2001), anti-inflammatory, antiosteoporotic, and antiatherogenic effects (Davilla et al., 1989; Ishimi et al., 1999; Chul-Ho et al., 2001), antitumor, antiproliferative, and anticancer activities (Kuo, 1996; Lee, 1999; Birt et al., 2001; Horvathová et al., 2001; Lopez-Lazaro, 2002; Manthey & Guthrie, 2002), as well as antioxidant (Rice-Evans et al., 1996; Hollman & Katan, 1999; Lamuela-Raventós, 1999; Eberhardt et al., 2000; Mojžiš & Mojžišová, 2001; Havsteen, 2002), cardioprotective (Hollman & Katan, 1997; Narayana et al., 2001), antiviral (Jassim & Naji, 2003) and inhibition activity against many mammalian enzyme systems *in vitro* (Middleton et al., 2000). Their pharmacological properties could explain why, when consumed regularly in the human diet, these compounds have pleiotropic health promoting and disease-preventing activities (Gantet & Memelink, 2002). On the other hand, there is also an information on the possible toxic effects of some flavonoids (Dunnick & Hailey, 1992), and therefore more investigations are needed to elucidate the suggested health promoting effects of these compounds. The available knowledge on this topic should not be regarded sufficient yet, and as a result there is no single flavonoid approved as pharmaceutical drug up to now (Havsteen, 2002).

Biosynthesis and chemistry of flavonoids

Flavonoids are low-molecular-weight compounds and theirbiosynthesis ranks among the best described plant secondary metabolic pathways, and genes encoding flavonoid biosynthetic enzymes have been cloned and characterized in various species (Winkel-Shirley, 1998). These secondary metabolites belong to phenylpropanoid group of compounds, which are derived from the products of aromatic amino acid biosynthesis – phenylalanine and the Krebs cycle – acetyl CoA (Winkel-Shirley, 1998; Gantet & Memelink, 2002). The first committed step in the formation of flavonoids is conjugation of malonyl-CoA and coumaroyl-CoA molecules (Fig. 1)tochalcones, catalysed by the enzyme chalcone synthase (CHS) (Holton & Cornish, 1995). Chalcones are converted to flavanones by the action of chalcone isomerase (CHI). Flavanones are precursors of all classes of flavonoids (Winkel-Shirley, 2002). Flavonoids are usually characterized by a C_6-C_3-C_6 carbon skeleton (Peterson & Dwyer, 1998) and consistof a three-ring structure A, B, C (Fig. 2). Ring A and ring B are connected by a three-carbon unit and linked by an oxygen-containing heterocycle (ring C). The ring B and C are biosynthesized by way of shikimic acid pathway. The ring A originates from the acetate-malonate pathway (Formica & Regelson, 1998). The general chemical structure of flavonoids and the numbering of ring atoms is shown in Figure 2. The structural difference in each flavonoid family results from the variation in the number and substitution pattern of hydroxyl groups and the extent of glycosylation (Amić et al., 2003). Subclasses of flavonoids are: flavonoles, isoflavonoles, flavones, isoflavones, flavanones, isoflavanones, flavanols, isoflavanols, flavanes, isoflavanes, anthocyanidines, aurones and coumarins (Fig. 3).

Biotechnological approaches for production of flavonoids

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 & Ravishankar, 2002). Flavonoids can be produced by using different biotechnological approaches, such as callus 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 flavonoid production.

Production of flavonoids in organ cultures

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 & 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.

Root cultures are valuable sources of medicinal compounds (Flores et al., 1987; Sevon & Oksman-Caldentey, 2002; Yu et al., 2002)

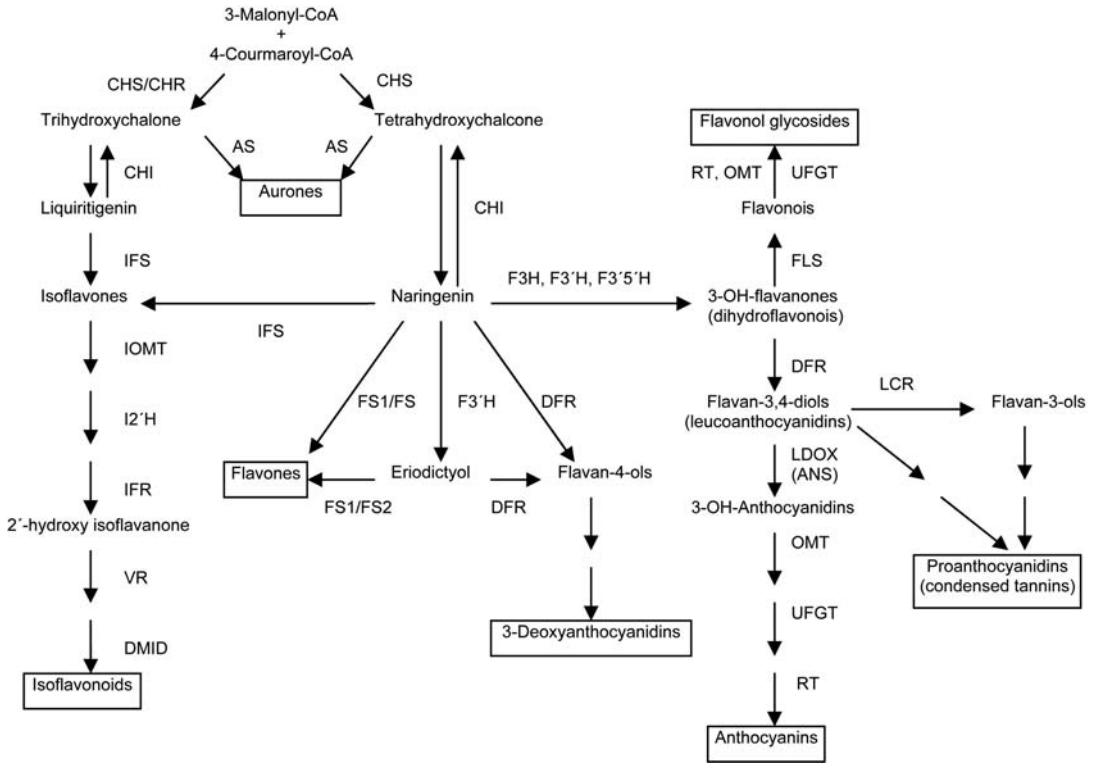

Fig. 1. Biosynthesis of flavonoids (adapted from WINKEL-SHIRLEY, 2002). CHS, chalcone synthase; CHR, chalcone reductase; AS, aureusidin synthase; CHI, chalcone isomerase; IFS, isoflavone synthase; IOMT, isoflavone *O*-methyltransferase; I2'H, isoflavone 2'-hydroxylase; IFR, isoflavone reductase; VR, vestitone reductase; DMID ,7,2'-dihydroxy, 4'-methoxyisoflavanol dehydratase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3'5'-hydroxylase; LDOX, leucoanthocyanidin dioxygenase; ANS, anthocyanidin synthase; OMT, *O*-methyltransferase; UFGT, flavonoid glucosyl transferase; RT, rhamnosyl transferase; FS1/FS2, flavone synthase; LCR, leucoanthocyanidin reductase. The names of the major classes are boxed.

and also flavonoids (ASADA et al., 1998; BOURGAUD et al., 1999; GAO et al., 1999; TUMOVÁ L., 1999). Many of the secondary compounds, for example, the tropane alkaloids hyoscyamine and scopolamine were produced quite well in root cultures (ENDO & YAMADA, 1985; HASHIMOTO et al., 1986). 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 (SEVON & OKSMAN-CALDENTEY, 2002).

Hairy roots result from the successful transfer and integration of the genes located on the root inducing plasmid *Ri* of *Agrobacterium rhizogenes* into the plant genome and their expression therein. The T-DNA of *Ri* plasmid is "split" into two different parts, called the T_L-DNA (containing *rol* genes) and T_R-DNA (containing *aux* genes for auxin synthesis). Genes of Ri T_L-DNA direct the synthesis of substances that recruits the cells to differentiate into roots under the influence of endogenous auxin synthesis (NILSSON

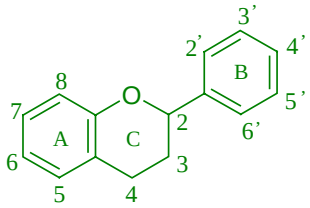

Fig. 2. Basic structure of flavonoids.

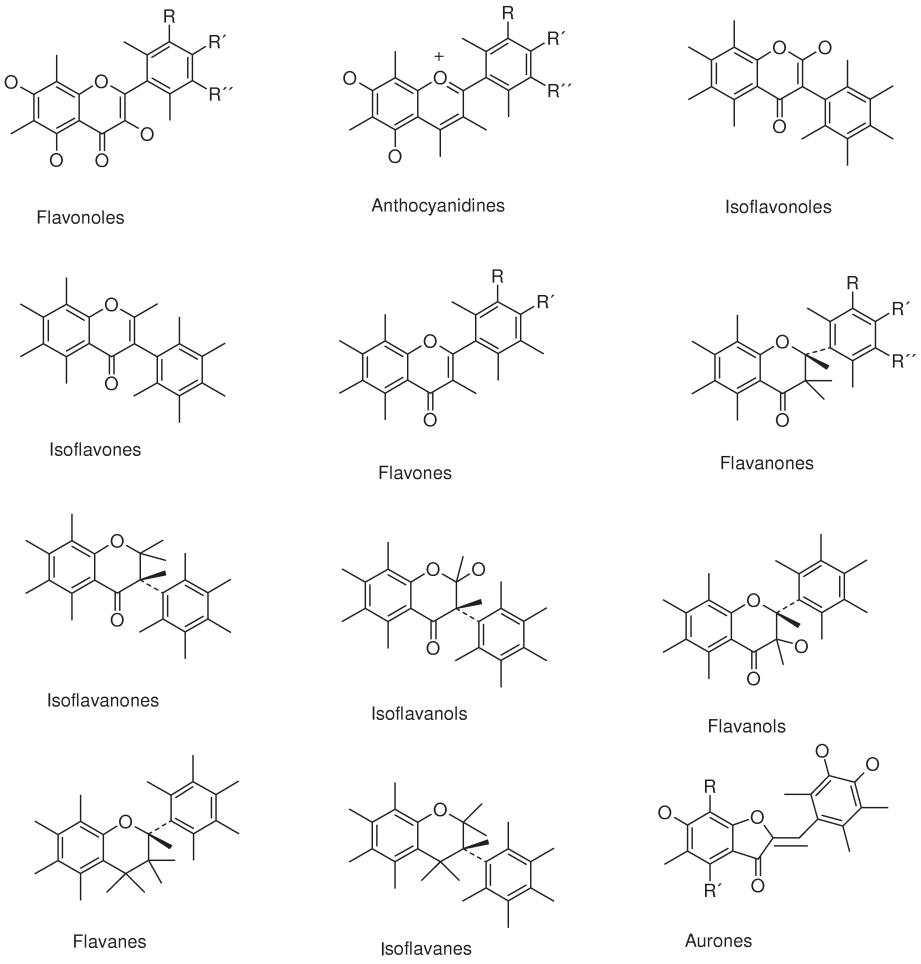

Fig. 3. Chemical structures of flavonoids.

& Olsson, 1997; Altamura, 2004). The characteristic capacity of hairy roots for secondary metabolite production, their inherent genetic stability reflected in stable productivity, and the possibility of genetic manipulation to increase biosynthetic capacity, have initiated a considerable interest, both as a fundamental research tool and as a source of valuable products (Sevon & Oksman-Caldentey, 2002). These types of roots are characterized by fine structure, fast growth, frequent branching, plagiotropism and they can be subcultured and indefinitely propagated on a synthetic medium without phytohormones (Chilton et al., 1982; Sevon & Oksman-Caldentey, 2002). Many times, they do not need incubation under light. In addition to their growth capacities, hairy roots display interesting properties regarding the production of secondary metabolites, i.e. the metabolite pattern found in hairy roots is similar or identical to that of non-transformed plant roots (Parr & Hamill, 1987). Important property of hairy roots is also their ability to produce secondary metabolites concomitantly with growth. That means there is a possibility to get a continuous source of secondary compounds from actively growing hairy roots (Kim et al., 2002). Because of these advantages, many of the root-derived plant products, once not considered feasible for production by cell suspension cultures, are being reinvestigated for production using the hairy root culture technology (for a review, see Rao & Ravishankar, 2002). Different flavonoids have also been successfully produced in hairy root cultures.

Zhou et al. (1997) published the isolation

of a new flavone glucoside, 5,7,2',6'-tetrahydroxy flavone 2'-O-β-glucopyranoside and 15 known flavonoids from the hairy root cultures of skullcap (*Scutellaria baicalensis*). KUZOVKINA et al. (2001) showed that elicitation with methyl jasmonate increased the content of flavonoids wogonin, baicalein and baicalin up to 2.3-times in hairy root cultures of skullcap. BOURGAUD et al. (1999) established several transformed root cultures from *Psoralea* spp. with the objective of producing daidzein and related flavonoids. ASADA et al. (2000) studied the biosynthesis of the hemiterpene moiety of glabrol, the main prenylated flavanone of *Glycyrrhiza glabra*, using transformed hairy root cultures.LI et al. (2002) produced several flavonoids [licoagrosides D, E, and F, medicarpin 3-O-glucoside, calycosin 7-O-glucoside, formononetin 7-O-(6"-malonylglucoside) and 2'-hydroxyformononetin 7-O-glucoside] in hairy root cultures of *G. pallidiflora.* To increase the metabolite production in hairy root cultures, the same strategies, as developed for cell cultures, can be used, for example, the modifications of the growth media composition, the use of elicitors or biotransformation of precursors to products (BOURGAUD et al., 2001).

As with roots, it is also possible to cultivate plant aerial parts – shoots – for production of secondary metabolites (BOURGAUD et al., 2001; NOGUEIRA & ROMANO, 2002; SMITH et al., 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). 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. Shoot cultures can be transgenic, if they are obtained after infection with a soil bacterium *A. tumefaciens* (SAITO et al., 1985; SPENCER et al., 1993), or non-transgenic, when they are cultured on media with appropriate hormonal balance (MASSOT et al., 2000). The knowledge of the various biosynthetic pathways and availability of a wealth of genes for biosynthesis of flavonoids has been used already for transgenic manipulation of flavonoid biosynthesis (DAVIES, 2000; FORKMANN & MARTENS, 2001). The goals for production of transgenic plants with altered biosynthesis of flavonoids were for example the flower colour modification (TANAKA et al., 1998; DAVIES, 2000), nutriceutical production (DE VOS et al., 2000) or male sterility induction for the development of hybrid seed systems (TAYLOR & JORGENSEN, 1992).

Common properties of hairy root cultures and shoot cultures are their genetic stability and a good capacity for secondary metabolite production. Similarly to hairy root cultures, shoot cultures have also the ability to produce secondary compounds concomitantly with growth (MASSOT et al., 2000). Difficulties accompanying the use of plant shoot cultures for production of secondary metabolites concern their relatively slower growth rate, the necessity to expose shoot cultures to light and some differences in the metabolic patterns, because some of the biosynthetic pathways are localized in specific organs, such as glandulae (BOURGAUD et al., 2001).

Major problem of organ cultures is the large-scale cultures (VERPOORTE et al., 2002). Different types of bioreactors have been used for the culture of plant roots and/or shoots (TAYA et al., 1989; WEATHERS et al., 1997; 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 (WILLIAMS &DORAN, 2000). 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 (VERPOORTE et al., 2002). 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 & USHIYAMA, 1999).

Production of flavonoids 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 (ZIMMERMAN, 1993). 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 ap-

proach 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 callus 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. (2000) 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, LUCZKIEWICZ et al. (2003) 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, 1999; TUMOVA & DUSEK, 2000; TUMOVA & OSTROZLIK, 2002; TUMOVA et al., 2003). All the tested elicitors markedly increased the production of flavonoids in comparison with the control.

Production of flavonoids in cell suspension cultures

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 flavonoids 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., 1995; ZHANG et al., 1997, 2002; RAO & 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 (RAO & RAVISHANKAR, 2002), 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). As with callus cultures, production of several classes of flavonoids in cell suspension cultures have been reported (Tab. 2).

YAMAMOTO et al. (1995) 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 yest extract. More-

over, the production of prenylated flavanones also can be increased by 2-5 times by addition of cork pieces (YAMAMOTO et al., 1996). 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 and co-workers (TUMOVA & BLAZKOVA, 2002; TUMOVA & ZAPALKOVA, 2002; TUMOVA et al., 2003). 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. (1997) studied the temperature effect on anthocyanin production in cell suspension cultures of *Fragaria ananassa* at a temperature range of 15-35 ℃. The maximum anthocyanin production was obtained at 20 ℃. 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 ℃, respectively. In addition, ZHANG et al. (2002) 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, various 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 & RAVISHANKAR, 2002;VERPOORTE et al., 2002).

Conclusions

Flavonoids are a large group of low-molecular-weight polyphenolic secondary metabolites that are widespread among plants and are used as common dietary components and have many potent biological properties. The use of flavonoids for prevention and cure of human diseases is already widespread (GANTET & MEMELINK, 2002). These aspects made flavonoids an interesting object for industrial production. This review briefly summarized the possible sources of flavonoids for their perspective biotechnological production. The entire biotechnological potential of flavonoids 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 (GAUTHERET, 2002). Plant tissue cultures were suggested for the first time for production of phytochemicals as early as in 1956 (ROUTIEN et al., 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 cell

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	Valenzula et al., 1986; Morazzoni & Bombardelli, 1995; Cacho et al., 1999
	Sophoraflavone G	*Sophora flavescens*	Roots	ND[b]	anti-inflammatory, antibacterial	Yamamoto et al., 1995.; Tsuchiya & Iinuma, 2000
	Wogonin	*Scutellaria columnae*	Hypocotyl	15mg/50g of the lyophylized callus tissue	Antioxidant, cytotoxic, antithrombotic	Ryu et al., 1985; Gabrielska et al., 1997; Kimura et al., 1997; Stojakowska, 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 et al., 1991; 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)	Hepatoprotective, cardioprotective, anticancer, antioxidant	Fedoreyev et al.; 2000, Birt et al., 2001

Table 1. continued.

Flavonoid family	Compound name	Name of the plant	Type of material [a]	Yield of production	Susp. pharm.activity [c]	References
	Lehmanin	*Sophora flavescens*	Roots	ND[b]	anti-inflammatory, antibacterial	Yamamoto et al, 1995, Tsuchiya & Iinuma, 2000; Kim et al. 2002
Antho -cyanins	cyanidin 3-O-(6-O-(E)-ferulyol-β-D-gluco-pyranosyl)-2-O-β-D-xylopyranosyl-β-D-glucopyranoside)	*Glehnia littoralis*	Petiole	14.2% g^{-1} of (DCW)	Antioxidant, cardioprotective, anticancer	Miura et al., 1998; Hou 2003; Ng et al., 2004
	Anthocyanin	*Hyoscyamus muticus* L.	Cotyledon, hypocotyl	ND[b]	Antioxidant, cardioprotective, anticancer	Basu & Chand, 1996; Hou 2003
	cyanidin-3-lathyroside [cyanidin-3-O {β-D-xylopyranosyl (1>2) β-D-galacto pyranoside}], cyanidin 3-β-D-glucopyranoside	*Daucus carota*	Seeds	ND[b]	Antioxidant, cardioprotective, anticancer	Narayan, 2000; Ravindra & Narayan, 2003; Hou 2003
	cyanidin 3-(6"-malonylglucoside)	*Taraxacum officinale*	Red-purple cells	0.05 % g of (DCW)	Antioxidant, cardioprotective, anticancer	Akashi et al., 1997; Hou 2003
	cyanidin-3-galactoside, cyanidin-3-glucoside,cyanidin-3-arabinoside	*Vaccinium myrtillus* L.	Hypocotyl	0.08 mg/g (DCW)	Antioxidant, astrigent, antiseptic	Madhavi et al., 1998

[a] Plant material used for suspension culture establishment. [b] ND – not defined. [c] Suspected pharmaceutical activity

Table 2. Production of flavonoids by suspension cultures.

Flavonoid family	Compound name	Name of the plant	Type of material [a]	Yield of production	Susp. pharm.activity [c]	References
	Silymarin Silybin Silychristin Silydianin	*Silybum marianum*	Cotyledons	3.15±0.08 mg/g (DCW) 2.21±0.04 mg/g (DCW) 0.79±0.03 mg/g (DCW) 0.15±0.02 mg/g (DCW)	Anti-hepatotoxic, hepatoprotective, antioxidant	Cacho et al., 1999, Morazzoni & Bombardelli 1995, Valenzula et al., 1986
Flavanones	Prenylnaringenin, Lupininifolin, Erythrisenegalone 4',5-dihydroxy-2",2"-dimethylpyrano-[5",6"; 7,8]-flavanone	*Maclura pomifera*	Plantlet	ND[b]	Antioxidant, cardioprotective, anticancer	Monache et al., 1995, Birt et al., 2001
	Sophoraflavone G	*Sophora flavescens*	Roots	7 $mg \cdot g^{-1}$(FCW)	anti-inflammatory, antibacterial	Yamamoto et al., 1996., Yamamoto et al., 2002, Kim et al., 2002, Tsuchiya & Iinuma 2000
Flavones	Aromadendrin, Steppogenin, Morin, Naringenin, 8.8'-bis-naringenin, Apigenin, Cycloartocarpesin, Artocarpein	*Maclura pomifera*	Plantlet	ND[b]	Antioxidant, cardioprotective, anticancer,	Monache et al., 1995, Birt et al., 2001

Table 2. continued.

Flavonoid family	Compound name	Name of the plant	Type of material [a]	Yield of production	Susp. pharm.activity [c]	References
Isoflavonoids	Parvisoflavone A, Wighteone, Alpinumisoflavone, Osajin, Warangalone	*Maclura pomifera*	Plantlet	ND[b]	Antimicrobial, cardioprotective, anticancer, antioxidant	Monache et al. 1995, Birt et al., 2001
	Lupalbigenin	*Sophora flavescens*	Roots	4 mg · g^{-1} FCW	anti-inflammatory, antibacterial	Yamamoto et al., 1996, Yamamoto et al, 2002, Kim et al. 2002, Tsuchiya & Iinuma 2000
Catechins	Epicatechin, Epicatechin gallate, Epigallocatechin gallate, Catechin, Catechin gallate, Gallocatechin, Epigallocatechin	*Camellia sinensis*	Seeds	150 mg/g (DCW)	antioxidative, antibacterial, antiallergic	Shibasaki-Kitakawa et al., 2003, Middleton et al. 2000
	(+)-catechin (−)-epicatechin	*Vitis vinifera*	Pulp of fruits	2 mg · g^{-1} (FCW) 3 mg · g^{-1}(FCW)	Antioxidant, Cardioprotective	TEGUO et al., 1996 Fauconneau et al., 1997
Anthocyanins	Anthocyanin	*Vaccinium pahalae*	Stock	19 mg/g (DCW)	Cardioprotective, antioxidant, antitumor	Meyer et al , 2002, Cook et al ., 1996 Castonguay et al., 1997 Cao et al., 1997

[a] Plant material used for callus culture establishment. [b] ND – not defined. [c] Suspected pharmaceutical activity.

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 flavonoids 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. Of the different flavonoid types, production of anthocyanins in glycosidic form and catechins as aglycons is reported most frequently. Production of flavonoids in tissue culture was reported to be more effective in callus culture. In this case, it has been shown that production of both forms of flavonoids, i.e. aglycons and their glycosylated forms, is possible. There are also reports on the production of prenylated and acetylated flavonoids. Nevertheless, published yields are frequently more than one order less than in the case of isolation of these compounds from native plants, i.e. usually in a range of micrograms to milligrams per liter of culture medium. However, as our understanding of the factors involved in biosynthetic pathway expression will grow, so will the ability to control the secondary metabolite production in these systems.

The ability of manipulating flavonoid biosynthesis in plant species is gaining rapidly in importance as new economically important uses emerged, such as in the areas of food and feed quality and nutraceuticals. Metabolic engineering, i.e. the modulation of metabolic and biosynthetic networks of an organism with the intention to direct metabolic flux into the biochemical pathway of a certain valuable molecule, will provide in this context an important tool to improve the plant cell factory for the production of desired flavonoids. Introduction of new or altered genes into plants through genetic transformation, either via *A. tumefaciens* or *A. rhizogenes*, can be used for the metabolic engineering purposes. A wealth of flavonoid genes have been identified as yet and used for metabolic engineering of flavonoid composition and content (Tanaka et al. 1998; Dixon & Steele 1999; De Vos et al., 2000). There were three types of genes used successfully for transgenic modification of the flavonoid pathway (Forkmann & Martens, 2001; Memelink et al., 2001): (i) structural genes that control single, biosynthetic steps of various flavonoid classes or steps of flavonoid modification; (ii) regulatory genes coding for the transcription factors that switch on or off the whole pathway or parts of it; and (iii) genes that act indirectly (e.g. through vacuolar pH modification, interaction with metal ions or transcription factors) on the accumulation of flavonoids in plant cells. As it can be used both for plants and cell cultures, metabolic engineering represents a powerful tool to improve the plant cell factory for the production of the desired phytochemicals, including flavonoids.

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References

Akashi, T., Saito, N., Hirota, H., & Ayabe, S. 1997. Phytochemistry **46:** 283–287.

Amić, D., Davidović-Amić, D., Bešlo, D. & Trinajstić, N. 2003. Croatica Chemica Acta **76:** 55–61.

Altamura, M. M. 2004. Plant Cell Tissue Organ Cult.**77:** 89–101.

Asada, Y., Li, W. &Yoshikawa, T. 1998. Phytochemistry **47:** 389–392.

Asada, Y., Li, W. &Yoshikawa, T. 2000. Phytochemistry **55:** 323–326.

Basu, P. & Chand, S. 1996. J. Biotechnol. **52:** 151–159.

Biondi, S., Scaramagli, S., Oksman-Caldentey, K. M. & Poli, F. 2002. Plant Sci. **163:** 563–569.

Birt, D. F., Hendrich, S. & Wang, W. 2001. Pharm. Therap. **90:** 157–177.

Bourgaud, F., Bouque, V. & GUCKERT, A. 1999. Plant Cell Tissue Organ Cult. **56:** 97–104.

Bourgaud, F., Gravot, A., Milesi, S. & Gontier, E. 2001. Plant Sci. **161:** 839–851.

Budzianowski, J., Budzianowska, A. & Kromer, K. 2002. Phytochemistry **61:** 421–425.

Budzianowski, J., Pakulski, G., Robak, J. 1991. Pol. J. Pharmacol. Pharm. **43:** 395–401.

Cacho, M., Morán, M., Corchete, P. & Fernández–Tárrago, J. 1999. Plant Sci. **144:** 63–68.

Cao, G., Sofic, E., Prior, R. 1997. Free Radic. Biol. Med. **22:** 749–760.

Castonguay, A., Gali, H., Perchellet, E., Gao, X., Boukharta, M., Jalbert, G., Okuda, T., Yoshida, T., Hatano, T., Perchellet, J.-P. 1997. Int. J. Oncol. **10:** 367–373.

Chilton, M.D., Tepfer, D.A., Petit, A., Casse-Delbart, F. & Tempé, J. 1982. Nature **295:** 432–433.

Chul-Ho, L., Tae-Sook, J., Yang-Kyu, Ch., Byung-Hwa, H., Goo-Taeg, O., Eun-Hee, K., Ju-Ryoung, K., Jang-Il, H. & Song-Hae, B. 2001. Biochem. Biophys. Res. Commun. **284:** 681–688.

Cook, N. C., Samman, S. 1996. Nutr. Biochem. **7:** 66–76.

Collin, H. A. 2001. Plant Growth Regul. **34:** 119–34.

Davies, K.M. 2000. Plant colour and fragrance, pp. 127–163. In: Verpoorte, R. & Alfermann, A.W. (eds) Metabolic Engineering of Plant Secondary Metabolism, Kluwer Academic Publishers, Dordrecht-Boston-London.

Davilla, J. C., Lenherr, A. & Acosta, D. 1989. Toxicology **57:** 267–268.

De Vos, R., Bovy, A., Busink, H., Muir, S., Collins, G. & Verhoyen, M. 2000. Polyphenols Commun. **1:** 25–26.

Dhawan, S., Shasany, A.K., Naqvi, A.A., Kumar, S. & Khanuja, S.P.S. 2003. Plant Cell Tissue Organ Cult. **75:** 87–94.

Dias, A. C. P., Tomás-Barberán, F. A., Fernandes-Ferreira, M. & Ferreres, F. 1998. Phytochemistry **48:** 1165–1168.

Di Carlo, G., Mascolo, N., Izzo, A. A. & Capasso, F. 1999. Life Sci. **65:** 337–353.

Dixon, R. A. & Steele, C. L. 1999. Trends Plant Sci. **4:** 394–400.

Do, C. B. & Cormier, F. 1990. Plant Cell Rep. **9:** 143–146.

Dunnick, J. K. & Hailey, J.R. 1992. Fundam. Appl. Toxicol. **19:** 423–31.

Eberhardt, M. V., Lee, C. Y. & Liu, R. H. 2000. Nature **405:** 903–904.

Endo, T. & Yamada, Y. 1985. Phytochemistry **24:** 1233–1236.

Fauconneau, B., Teguo, P., Huguet, F., Barrier, L., Decendit, A. & Mérillon, J.M. 1997. Life Sci. **61:** 2103–2110.

Fedoreyev, S. A., Pokushalova, T. V., Veselova, M. V., Glebko, L. I., Kulesh, N. I., Muzarok, T. I., Seletskaya, L. D., Bulgakov, V. P. & Zhuravlev, Y. N. 2000. Fitoterapia **71:** 365–372.

Flores, H. E., Hoy, M. W. & Pickard, J. J. 1987. Trends Biotechnol. **5:** 64–68.

Forkmann, G., Martens, S. 2001. Curr. Opin. Biotechnol. **12:** 155–160.

Formica, J. V. & Regelson, W. 1995. Food Chem. Toxicol. **33:** 1061–1080.

Gabrielska, J., Oszmiański, J., Żyłka, R. & Komorowska, M. 1997. Z. Naturforsch. **52:** 307–317.

Gantet, P. & Memelink, J. 2002. Trends Pharmacol. Sci. **23:** 563–569.

Gao, Z, Huang, K, Yang, X & Xu, H 1999. Biochim. Biophys. Acta **1472:** 643–650.

Gautheret, R. J. 2002. Plant tissue culture: the history, pp. 105–113. In: Laimer, M., Rücker, W. (eds): Plant Tissue Culture: 100 Years Since Gottlieb Haberlandt, Springer-Verlag, Wien-New York.

Harborne, J. B. & Williams, C. A. 2000. Phytochemistry **55:** 481–504.

Hashimoto, T., Yukimune, Y. & Yamada, Y. 1986. J. Plant Physiol. **124:** 61–75.

Havsteen, B. H. 2002. Pharmacol Ther. **96:** 67–202.

Hermann, K. 1976. J. Food Technol. **11:** 433–448.

Hibino, K. & Ushiyama, K. 1999: Commercial production of Ginseng by plant cell culture technology, pp. 215–224. In: Fu, T. J., Singh, G. & Curtis, W. R. (eds) Plant Cell and Tissue Culture for the Production of Food Ingredients, Kluwer Academic Publishers, Dordrecht.

Hollman, P. C. & Katan, M. B. 1997. Biomed Pharmacother. **51:** 305–310.

Hollman, P. C. & Katan, M. B. 1999. Food Chem. Toxicol. **37:** 937–942.

Holton, T. A. 1995. Plant Cell **7:** 1071–1083.

Horvathová, K., Vachalková, A. & Novotný, L. 2001. Neoplasma **48:** 435–441.

Hou, D. X. 2003. Curr. Mol. Med. **3:**149–59.

Ishimi, Y., Miyaura, C., Ohmura, M., Onoe, Y., Sato, T., Uchiyama, Y., Ito, M., Wang, X., Suda, T. & Ikegami, S. 1999. Endocrinology **140:** 1893–1900.

Jassim, S.A.A & Naji, M.A. 2003. J. App. Microbiol. **95:** 412–427.

Kamei, H., Kojima, T., Hasegawa, M., Koide, T., Umeda, T., Yukawa, T. & Terabe, K. 1995. Cancer Invest. **13**: 590–4.

Khanam, N., Khoo, C. & Khan, A.G. 2000. Plant Cell Tissue Organ Cult. **62:** 125–133.

Kim, D. W., Chi, Y. S., Son, K. H., Chang, H. W., Kim, J. S., Kang, S. S. & Kim, H. P. 2002. Arch. Pharm. Res. **25:** 329–35.

Kim, Y., Wyslouzil, B.E. & Weathers, P.J. 2002. In Vitro Cell. Dev. Biol. Plant **38:** 1–10.

Kimura, Y., Yokoi, K., Matsushita, N. & Okuda, H. 1997. J Pharm Pharmacol **49:** 816–822.

Kitamura, Y., Ohta, M., Ikenaga, T. & Watanabe, M. 2002. Phytochemistry **59:** 63–68.

Kuo, S. M. 1996. Cancer Lett. **110:** 41–48.

Kuzovkina, I. N., Guseva, A. V., Alterman, I. E. & Karnachuk, R. A. 2001. Russ. J. Plant Physiol. **48:** 448–452.

Lamuela-Raventós, R. M., Covas, M. I., Fitó, M., Marrugat, J. & de la Torre-Boronat, M. C. 1999. Clin. Chem. **45:**1870–1872.

Lee, K.H. 1999. Med. Res. Rev. **19:** 569–596.

Li, W., Koike, K., Asada, Y., Hirotani, M. Rui, H., Yoshikawa, T. & Nikaido, T. 2002. Phytochemistry **60:** 351–355.

Lopez-Lazaro, M. 2002. Curr. Med. Chem. Anti-Canc. Agents **2:** 691–714.

Madhavi, D. L., Bomser, J., Smith, M. A. L. & Singletary, K. 1998. Plant Sci. **131:** 95–103.

Manthey, J. A. & Guthrie, N. 2002. J. Agric. Food. Chem. **50:** 5837–5843.

Massot, B., Milesi, S., Gontier, E., Bourgaud, F. & Guckert, A. 2000. Plant Cell Tissue Organ Cult. **62:** 11–19.

Memelink, J., Kijne, J. W., van der Heijden, R. & Verpoorte, R. 2001. Adv. Biochem. Engn/Biotechnol. **72:** 103–125.

Meyer, J. E., Pépin, M. F. & Smith, M. A. 2002. J. Biotechnol. **93:** 45–57.

Miura, H., Kitamura, Y., Ikenaga, T., Mizobe, K., Shimizu, T., Nakamura, M., Kato, Y., Yamada, T., Maitani, t. & Goda, Y. 1998. Phytochemistry **48:** 279–283.

Middleton, E., Kandaswami, C. & Theoharides, T. C. 2000. Pharmacol. Rev. **52:** 673–751.
Morazzoni, P. & Bombardelli, E. 1995. Fitoterapia **66:** 3–42.
Mojžiš, J. & Mojžišová, G. 2001. Flavonoidy a ich biologické účinky. Vienala, Košice, 133 pp.
Monache, G. D., De Rosa, M.C., Scurria, R., Vitali, A., Cuteri, A., Monacelli, B., Pasqua, G. & Botta. B. 1995. Phytochemistry **39:** 575–580.
Narayan, M. S. & Venkataraman, L. V. 2000. FoodChem. **70:** 361–363.
Narayana, R. K., Reddy, S. M., Chaluvadi, M. R. & Krishna, D. R. 2001. Ind. J. Pharmacol. **33:** 2–16.
Ng, T. B., Liu, F., & Wang, H. X. 2004. J. Ethnopharmacol. **93:** 285–8.
Nijveldt, R.J., van Nood, E., van Hoorn , D. E. C., Boelens, P. G., van Norren, K. & van Leeuwen, P. A. M. 2001. Am J Clin Nutr. **74:** 418–425.
Nilsson, O. & Olsson, O. 1997. Physiol. Plant.**100:** 463–473.
Nogueira, J. M. F. & Romano, A. 2002. Phytochem. Anal. **13:** 4–7.
Park, H. H., Hakamatsuka, T., Sankawa, U. & Ebizuka, Y. 1995. Phytochemistry **38:** 373–380.
Parr, A. D. & Hamill, J. D. 1987. Phytochemistry **26:** 3241–3245.
Peterson, J. & Dwyer, J. 1998. Nutrition Res. **18:** 1995–2018.
Rao, S. M. & Ravishankar, G. A. 2002. Biotechnol. Adv. **20:** 101–153.
Ravindra, P. V. & Narayan, M. S. 2003. Int. J. Food Sci. Nutr. **54:** 349–355.
Rice-Evans, C. A., Miller, N. J. & Paganga, G. 1996. Free Radic. Biol. Med. **20:** 933–956.
Routien, J. B., Tenafly, N. J. & Nickell, L. G. 1956. Pfizer & Co., Patent US-02747334.
Rusak, G., Gutzeit, H. O. & Ludwig-MÛller, J. 2002. Food Technol. Biotechnol. **40:** 267–273.
Ryu, S. H., Ahn, B. Z. & Pack, M. Y. 1985. Planta Med.**51:** 355.
Saito, K., Murakoshi, I., Inzé, D. & van Montagu, M. 1985. Plant Cell Rep. **7:** 607–610.
Sevon, N. & Oksman–Caldentey, K. M. 2002. Planta Medica **68:** 859–868.
Shibasaki-Kitakawa, N., Takeishi, J. & Yonemoto, T. 2003. Biotechnol. Prog. **19**: 655–668.
Smith, M. A. L., Kobayashi, H., Gawienowski, M. & Briskin, D. P. 2002. Plant Cell Tissue Organ Cult. **70:** 105–111.
Spencer, A., Hamill, J. D. & Rhodes, M. J. C. 1993. Phytochemistry **32:** 911–919.
Stafford, H A. 1991. Plant Physiol. **96:** 680–685.
Stojakowska, A. & Kisiel, W. 1999. Fitoterapia **70:** 324–325.
Tanaka, Y., Tsuda, S. & Kusumi, T. 1998. Plant Cell Physiol. **39:** 1119–1126.
Taya, M., Yoyama, A., Kando, O., Kobayashi, T. & Matsui, C. 1989. J. Chem. Engn. Jpn. **22:** 74–89.
Teguo, P. W., Decendit, A., Vercauteren, J., Deffieux, G. & Mérillon, J. M. 1996. Phytochemistry **42:** 1591–1593.
Tsuchiya, H. & Iinuma, M. 2000. Phytomedicine **7:** 161–5.
Tumova, L. 1999. Ceska Slov Farm. **48:** 262–264.
Tumova, L., Bartakova, M. & Zabloudilova, J. Ceska Slov. Farm. **52:** 189–192.
Tumova, L. & Blazkova, R. 2002. Ceska Slov. Farm. **51:** 44–46.
Tumova, L. & Dusek, J. 2000. Ceska Slov. Farm. **49:** 78–81.
Tumova, L. & Ostrozlik, P. 2002. Ceska Slov. Farm. **51:** 173–176.
Tumova, L. & Zapalkova, L. 2002. Ceska Slov. Farm. **51:** 96–98.
Valenzuela, A., Guerra, R. & Videla, L. A. 1986. Planta Med. **52:** 438–440.
Verpoorte, R., Contin, A. & Memelink, J. 2002. Phytochem. Rev.**1:** 13–25.
Weathers, P., Wyslouzil, B.E. & Whipple, M. 1997. Laboratory-scale studies of nutrient mist reactors for culturing hairy roots, pp. 191–200. In: Doran, P. M. (ed.): Hairy Rroots, Harwood Academic Publishers, Amsterdam.
White, F. & Nester, E. W. 1980. J. Bacteriol. **141:** 1134–1141.
Wiiliams, G. R. C. & Doran, P. M. 2000. Biotechnol. Prog. **16:** 391–401.
Winkel-Shirley, B. 1998. Seed Sci. Res. **8:** 415–422.
Winkel-Shirley, B. 2002. Curr. Opin. Plant Biol. **5:** 218–223.
Zimmerman, J. L. 1993. Plant Cell **5:** 1411–1423.
Yamamoto, H., Yamaguchi, M. & Inoue, K. 1995. Phytochemistry **40:** 77–81.
Yamamoto, H., Yamaguchi, M. & Inoue, K. 1996. Phytochemistry **43:** 603–608.
Yamamoto, H., Zhao, P. & Inoue, K. 2002. Phytochemistry **60:** 263–267.
Yu, K. W., Gao, W. Y., Hahn, E. J. & Paek, K. Y. 2002. Biochem. Engn. J. **11:** 211–215.
Zhang, W., Seki, M. & Furusaki, S. 1997. Plant Sci. **127:** 207–214.
Zhang, W., Curtin, C., Kikuchi, M. & Franco, C. 2002. Plant Sci. **162:** 459–468
Zhou, Y., Hirotani, M. Yoshikawa, T & Furuya, T. 1997. Phytochemistry **44:** 83–87.

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