Research review paper

Plant in vitro culture for the production of antioxidants — A review

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ARTICLE INFO

Article history:
Received 4 April 2008
Received in revised form 1 July 2008
Accepted 10 July 2008
Available online 16 July 2008

Keywords:
Antioxidant
In vitro culture
Medicinal plants
Biosynthesis

ABSTRACT

Plants in vitro cultures are able to produce and accumulate many medicinally valuable secondary metabolites. Antioxidants are an important group of medicinal preventive compounds as well as being food additives inhibiting detrimental changes of easily oxidizable nutrients. Many different in vitro approaches have been used for increased biosynthesis and the accumulation of antioxidant compounds in plant cells. In the present review some of the most active antioxidants derived from plant tissue cultures are described; they have been divided into the main chemical groups of polyphenols and isoprenoids, and several examples also from other chemical classes are presented. The strategies used for improving the antioxidants in vitro production efficiency are also highlighted, including media optimization, biotransformation, elicitation, Agrobacterium transformation and scale-up.

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1. Introduction

The use of plant cell and tissue culture methodology as a means of producing medicinal metabolites has a long history (Rout et al., 2000; Verpoorte et al., 2002). Since plant cell and tissue culture emerged as a discipline within plant biology, researchers have endeavored to utilize plant cell biosynthetic capabilities for obtaining useful products and for studying the metabolism (Misawa, 1994; Verpoorte et al., 2002).

Cultured plant cells synthesize, accumulate and sometimes exude many classes of metabolites. Medicinal compounds are of particular interest and much effort has been devoted to obtaining some of the most active and precious therapeutics. Numerous alkaloids, saponins, cardenolides, anthraquinones, polyphenols and terpenes have been reported from in vitro cultures and reviewed several times (Misawa, 1994; Verpoorte et al., 2002; Vanisree and Tsay, 2004).

In recent decades, interest in chemopreventive plant natural products has grown rapidly. The etiology of several degenerative and aging-related diseases has been attributed to oxidative stress, and numerous studies have been undertaken to search for the most effective antioxidants (Halliwell, 1995; Aruoma, 2003; Soobrattee et al., 2005).
The present review summarizes the achievements of plant cell and tissue culture technology for the production of secondary metabolites which have significant potential as antioxidants. The focus will be on the major chemical classes of antioxidants and on the approaches used to improve the in vitro methods for producing these compounds.

1. What do we call an antioxidant?

Broadly defined, an antioxidant is a compound that inhibits or delays the oxidation of substrates even if the compound is present in a significantly lower concentration than the oxidized substrate (Halliwell, 1995; Halliwell and Gutteridge, 2007). The scavenging of reactive oxygen species (ROS) is one of possible mechanisms of action. Others include the prevention of ROS formation by metal binding or enzyme inhibition. Chain breaking antioxidants prevent damage by interfering with the free radical propagation cascades.

The antioxidant compounds can be recycled in the cell or are irreversibly damaged, but their oxidation products are less harmful or can be further converted to harmless substances (Halliwell and Gutteridge, 2007). At the cellular and organism level the antioxidant protection is provided by numerous enzymes and endogenous small molecular weight antioxidants such as ascorbic acid, uric acid glutathione, tocopherols and several others. Many compounds contain antioxidant activity in addition to their specialized physiological function, and their importance as antioxidants in vivo is sometimes ambiguous (Azzi et al., 2004). The antioxidant activity of plant secondary metabolites has been widely established in vitro systems and involves several of the above mentioned mechanisms of action. Therefore, in this review, only those classes of compounds with established antioxidant properties will be considered.

1.2. Why hunt for antioxidants in vitro?

The natural resources of both potential and established antioxidants are vast. Some antioxidant compounds are extracted from easily available sources, such as agricultural and horticultural crops (maize, buckwheat, grapevine, carrots, beetroot, citrus hesperidin, hops, apples, berries, tea leaves etc.), or medicinal plants such as pine, skullcap, sage, rosemary, tormentil, and many others. Recently, the use of wine and olive industry waste products has been considered as a potential source of dietary or food preserving antioxidants (González-Paramás et al., 2004; Obied et al., 2005). This leads to the question: why is the complex and rather expensive in vitro technology needed for products that may be obtained from abundant and cheap sources? The necessary conditions that make using biotechnological methods for the production of secondary plant metabolites economically viable have been firmly established: high economic value, sufficient abundance in intact plants, limited availability from natural sources (rare/endangered/overexploited species), and difficult cultivation (Misawa, 1994; Verpoorte et al., 2002). When taking these factors into consideration, in vitro technology offers some or all of the following benefits: simpler extraction and purification from interfering matrices, novel products not found in nature, independence from climatic factors and seasons, more control over biosynthetic routes for obtaining the most desired variants (e.g. enantiomers or glycosides) or a proportion of given compounds, shorter and more flexible production cycles, easier fulfillment of the high-profile pharmaceutical production demands (such as GMP and GLP), and last but not least, the exploitation of the genetic engineering potential for avoiding legal restrictions against GMO introduction into the natural environment. As will be shown in this paper, some of the listed conditions are likely to be fulfilled with respect to antioxidants, whereas others will not, due to the ubiquitous presence of the main classes of antioxidants and the abundance of the aforementioned inexpensive sources. On the other hand, growing knowledge about the specific mechanisms of their activity will create the demand for more specific products that could be used in accurately defined therapeutic and nutritional situations. Finally, as was inherent in plant in vitro cultures from the very beginning, investigation into the biosynthetic routes advances our understanding of the function and regulation of plant metabolites. Moreover, it is still barely known what the actual roles are of many antioxidant secondary metabolites in a real plant (Grassmann et al., 2002; Misawa, 1994).

1.3. Methods used for assessment of antioxidant properties

In a majority of tissue culture papers reporting the production of secondary metabolites, their antioxidant activity has not been actually determined. The knowledge of their properties usually comes from studies involving dietary antioxidants and compounds isolated from ethnomedical plants with both food conservation and chemoprevention in mind. Thus, in most cases the antioxidant power of many secondary metabolites is so well established, that real time monitoring of activity during the culture seems to be redundant. On the other hand, the enormous variability among antioxidants, and their complex structure–activity relationships suggest that antioxidant and any other biological activities should be paid more attention during research. There are a number of simple, slightly more complex, and quite sophisticated methods for antioxidant testing. The matter has been reviewed by many authors with respect to different aspects of the problem (Halliwell, 1995; Aruoma, 2003; Sanchez-Moreno, 2002; Matkowski, 2006).

The antioxidant testing can reveal various mechanisms of action, depending on features of the particular assay. Simple methods include free radical scavenging with use of colored, artificial stable free radicals such as 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS used in the TEAC assay — Trolox equivalent antioxidant capacity) (Re et al., 1999) and DPPH&Hz.rad; (1,1-diphenyl-2-picrylhydrazyl free radical) (Molyneux, 2004), as well as transition metal reduction that can be monitored by colorimetry. The metal-based methods include the reduction of ferric ions: FRAP — (ferric reducing ability of plasma) and ferric thiocyanate assays (Halliwell, 1995; Aruoma, 2003), or molybdenum ion — phosphomolybdenum (P-Mo) assay (Prieto et al., 1999). These tests are easy and affordable and can be used in high throughput screening. Their main drawback is that their relevance to the real oxidizing life is somewhat limited. The first issue is the chemical context of the assays, which use artificial compounds or are conducted in unrealistic conditions. This problem is eliminated in methods based on naturally occurring reactive oxygen species, but the fate of a free radical is observed either indirectly with chromatophore reagents or with more expensive ESR techniques (electron spin resonance). The scavenging of superoxide radical anion, hydroxyl radical, or nitric oxide can be observed (Darmon et al., 1992; Aruoma, 2003; Sreejayan and Rao, 1997).

Several assays based on a substrate degradation inhibition are available which can be used to find out whether the tested compound can really protect biomolecules from oxidative damage. Polyunsaturated lipids, proteins, components of nucleic acids, cellular membranes, microsomal fractions from different organs can serve as model systems to be protected. The oxidation can be initiated by various chemical, physicochemical or biological approaches. The degradation products are monitored by spectrophotometry (thiobarbituric reactive substances — TBARS, carotene bleaching), fluorescence (ORAC assay — for oxygen radical absorption capacity) or chromatography (Halliwell, 1995; Matkowski, 2006; Aruoma, 2003; Sanchez-Moreno, 2002).

Some of the described methods have been used for testing the antioxidant activity of compounds from in vitro cultures of several plants and will be referred to later in this paper as well as being specified in Table 1.
2. Chemical classes of antioxidant secondary metabolites from in vitro cultures

2.1. Polyphenols

It is ironic from the point of view of the tissue culturist, that the easily oxidizable phenolics, which are often regarded as nuisance and the reason for experiment failure, are at the same time highly desirable as dietary and therapeutic free radical scavengers. Considerable attention has been paid to minimizing the "phenolization" of cultures, frequently by adding antioxidants, such as ascorbic acid or glutathione (GSH), or phenol absorbing polymers like PVP (polyvinylpyrrolidone). The excessive production of polyphenols results from unfavorable or suboptimal culture conditions. At the onset of senescence, the decrease in free phenolics and a simultaneous increase in cell-wall bound and oxidized forms is observed in callus cultures (Lopez Ardullos et al., 2001), which reflects the natural processes during plant–pathogen interactions (e.g. hypersensitive response) (Hammerschmidt, 2005).

Polyphenols in plants derive mainly from the shikimic acid pathway through aromatic carboxylic acids — cinnamic or benzoic. Most of them possess antioxidant properties, albeit to varying degrees. The most powerful of the flavonoids are the flavanols and their oligomeric forms called condensed tannins or proanthocyanidins, flavones and flavonols and anthocyanins. Some isoflavones are also known as antioxidants. Among other groups of antioxidant polyphenols there are: phenolic acids (caffeic acid derivatives), lignans, hydrolysable tannins (gallotannins and ellagitannins), stilbenes, and xanthones. Their physiological functions in plants are versatile and have been reviewed recently (Harborne, 2001; Matkowski, 2006).

Table 1

<table>
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<tr>
<th>Species</th>
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</tr>
</tbody>
</table>
Phenolic acids occur in plants in free form as glycosides and can be integrated into larger molecules in an ester form. They are common as depsides — the intermolecular ester of two or more units composed of the same or different phenolic acids such as: caffeic, coumaric, ferulic, gallic, and syringic.

Depsides are, for example, a ubiquitous chlorogenic as well as isochlorogenic, ellagic, lithospermic and rosmarinic acids. Due to the presence of a high number of hydroxyl groups and a carboxyl moiety, their antioxidant properties are very pronounced (Rice-Evans et al., 1996; Sroka, 2005).

Special attention should be paid to rosmarinic acid (RA), a depside composed of two caffeic acid molecules (Fig. 1), found in many Lamiaceae and Boraginaceae species. Its biochemistry and pharmacology has been extensively reviewed by Petersen and Simmonds (2003). The antioxidant properties of rosmarinic acid are also well established. Several plants and various techniques have been used for in vitro production of this compound (Shetty, 1997). Rosmarinic acid can accumulate in cell cultures to amounts greater than those in intact plants. The suspension cultures producing rosmarinic acid were generated from Anchusa officinalis (De-Eknamkul and Ellis, 1984, 1985; Su and Humphrey, 1990; Su et al., 1993), Eritrichum sericeum (Fedoreyev et al., 2005), Lithospermum erythrorrhizon (Yamamoto et al., 2000) (Boraginaceae), and Coleus blumei (Petersen et al., 1993), Lavandula vera (Pavlov et al., 2005a,b; Georgiev et al., 2006a,b), Ocimum basilicum (Kintzios et al., 2004), Salvia officinalis (Hippolyte et al., 1992), Zataria multiflora (Mohagheghzadeh et al., 2004) (Lamiaceae). Depending on the species, the yield of rosmarinic acid from suspension cultures can exceed 10% of cell mass and 6 g/l of medium. An interesting example is also the production of rosmarinic acid of over 5% of cell dry mass in Anthoceros agrestis (Vogelsang et al., 2006). Another system used for rosmarinic acid production are transformed roots of Salvia miltiorrhiza (Chen et al., 1999a,b, 2001) and S. officinalis (Grzegorczyk et al., 2006, 2007). In the second species, the accumulation was much higher reaching 4.5% of dry weight and its antioxidant activity has been confirmed with several assays. Extracts from rosmarinic acid accumulating lavender cells also have superior radical scavenging properties (Kovacheva et al., 2001, 2006). Even larger amounts (over 7% dw) of rosmarinic acid accumulated in Agrobacterium transformed callus of C. blumei (Bauer et al., 2004) and transformed roots Hyssopus officinalis, but only when cultured on WP.
medium which was much more superior to both MS and B5 (Murakami et al., 1998). Razaque and Ellis (1977) report a yield of 8–11% of rosmarinic acid in C. blumei cell cultures. An extremely high level of RA production was reported by Hippolyte et al. (1992) in S. officinalis reaching 6.4 mg/l of suspension culture, although the result has not appeared in any other reference.

A closely related antioxidant is licoispermic acid B, a tetramer depside (Fig. 1), accumulating especially in the aforementioned hairy root cultures of H. officinalis and elicited S. miltiorrhiza as well as in Lithospermum erythrorhizon suspension cultures (Yamamoto et al., 2000, 2002). Oligomeric caffeic acid depsides such as DCQAs (dicaffeoylquinic acids) — potent antioxidants and antivirals, accumulate also in cell cultures of sweet potato storage roots, where they are accompanied by anthocyanins (Konczak et al., 2004). Chlorogenic acid and cyanin (another DCQA) are present in cardoon (Cynara cardunculus) callus in quantities two–times greater than in the leaves — the usual medicinal crude drug. The antioxidant capacity of the cardoon callus was confirmed by the TBARS method (Trajtenberg et al., 2006).

**Flavonoids** are one of the largest groups of secondary metabolites based on a common structure. Chemically they possess a tricyclic phenylenzopyran (with the exception of chalcones) structure but biosynthetically come from phenylalanine and malonyl-CoA in the phenylpropanoid pathway.

Subclasses of flavonoid include flavonols, flavones, isoflavonoids, flavanons, flavanones, proanthocyanidins (catechins), and anthocyanidins. Substitution patterns include the hydroxylation of rings A and B, methylation of hydroxyl groups, prenylation, and glycosylation. Other modifications can form biflavonoids, lignans, oligomeric proanthocyanidins, glycoside esters with other phenolics etc.

Antioxidant properties of flavones depend on the hydroxylation pattern. Substitution with hydroxyl, methoxyl or other groups, and glycosylation also influences hydrophilicity and many biological activities. Among the vast variety of structures, several flavones from the genus Scutellaria (Lamiaceae) are distinguished by their unsubstituted B-ring and common occurrence in the form of glucuronides (Fig. 1). Scutellaria baicalensis, the favorite natural drug of traditional Oriental medicines, contains in the roots probably the highest known amount of flavones (up to 20% dw) of which the strong lipophilic antioxidants: baicalin, baicalein, wogonoside and wogonin are the most abundant. In cell suspension and hairy root cultures of S. baicalensis the accumulation of these compounds was reported (Morimoto et al., 1998; Nishikawa et al., 1999; Stojakowska and Malarz, 2000). The *in vivo* antioxidant function of baicalein in metabolizing hydrogen peroxide has been proposed as a result of experiments using cell cultures (Morimoto et al., 1998).

Flavone C-glycosides are of particular interest because of their limited occurrence in plants and they have important therapeutic properties, including the prevention of cardiovascular disorders related to oxidative stress. In tissue cultures of *Passiflora quadrangularis* several C-glycosylated flavones such as isoorientin, orientin (Fig. 1), vitexin and isovitexin have been induced in varied amounts, and their antioxidant activity determined by DPPH assay (Antognoni et al., 2007). Crataegus monogyna has also been reported (Terahara et al., 2001). This observed simplification and alteration of the metabolic profile, when compared to natural conditions, is typical for *in vitro* produced anthocyanins. In the extensively studied *I. batatas*, in the tissue cultures induced from storage roots the accumulation of anthocyanins can even exceed the tubers by several times. The production of valuable di-acylated cyanidin glycosides from callus and cell suspension as well as their regulation towards overproduction and their more desirable profile has also been reported (Terahara et al., 2000, 2004; Konczak et al., 2005). The superior stability and antioxidant activity of anthocyanin-rich extracts have also been confirmed. In this species the extraordinary high amount of anthocyanins was recorded in selected cell lines along with a greater proportion, in comparison to the tubers, of cyanidin–based to peonidin–based.

Another example of flavonoid polyphenols frequently studied for *in vitro* production is anthocyanins. Pigments are useful as products because their accumulation can easily be visually evaluated. Moreover, their beneficial health promoting properties as well as their growing importance as bioactive and natural food colorants additionally increases their attractiveness for plant biotechnology. The versatile values of anthocyanins have been reported and reviewed in countless publications. One of their most pronounced features is their substantial antioxidant capacity determined in various assays, but their biological activity varies greatly, due to their structural diversity. Analogously to other polyphenols, more hydroxylated compounds express larger free radical scavenging capacity, whereas methylation of the hydroxyl groups decreases this effect partially (Stintzing and Carle, 2004). Glycosylation can also reduce the ability to scavenge free radicals, but on the other hand enhances the stability of the molecule. Acylation of the sugar moieties with carboxylic, usually hydroxycinnamic acids (an example of a glycosylated and acylated cyanidin is shown in Fig. 1) additionally boosts the antioxidant potential and color stability. For that reason, not all anthocyanins are equally desirable as natural medicines. Cyanidin and delphinidin glycosides acylated with phenol carboxylic acids are preferred over less substituted versions and petunidin, pelargonidin, peonidin or malvidin derivatives. This is one of the main reasons for conducting tissue culture experiments. In abundant natural sources, the anthocyanin profile is rarely optimal and the raw material often requires complex extraction and purification procedures. In several species listed below the level of anthocyanins in *in vitro* equals or exceeds the natural sources and their metabolic profile is more useful. Thus, even if anthocyanins are not so expensive, the ability to obtain products with useful structural and functional properties may become economically feasible (Konczak et al., 2005).

Anthocyanins are obtained from *in vitro* cultures of *Prunus* sp. (Durzan et al., 1991; Blando et al., 2005), Daucus carota (Ravindra and Narayan, 2003), Clethra litoralis (Miura et al., 1998), *V. pahalae* (Meyer et al., 2002), Ipomoea batatas (Konczak-Islam et al., 2000, 2004) Fragaria ananassa (Edahiro et al., 2005), V. vinifera (Decdent and Méribel, 1990), Rudbeckia hirta (Luczkiewicz and Cisowski, 2001) and Ajuga reptans (Callebaut et al., 1990; Terahara et al., 2001) and others.

Interestingly, two species used medicinally due to their antitumor alkaloids, Catharanthus roseus and Campotheca acuminata, produce significant amounts of anthocyanins of over 200 μg/g fresh weight in cell cultures (Filippini et al., 2003; Pasqua et al., 2005). In *C. roseus*, p-coumaroyl glucosides of highly methoxylated malvidin or hirsutidin predominated, unlike in field-grown flowers. In *C. acuminata* a rare cyanidin galactoside accounted for over 95% of all anthocyanins. In *A. reptans* flower-derived cell cultures the anthocyanin composition was altered from delphinidin–based in flowers towards stronger anthocyanin – di–acylated cyanidin glycosides (Callebaut et al., 1997; Terahara et al., 2001). This observed simplification and alteration of the metabolic profile, when compared to natural conditions, is typical for *in vitro* produced anthocyanins. In the extensively studied *I. batatas*, in the tissue cultures induced from storage roots the accumulation of anthocyanins can even exceed the tubers by several times. The production of valuable di-acylated cyanidin glycosides from callus and cell suspension as well as their regulation towards overproduction and their more desirable profile has also been reported (Terahara et al., 2000, 2004; Konczak et al., 2005). The superior stability and antioxidant activity of anthocyanin-rich extracts have also been confirmed. In this species the extraordinary high amount of anthocyanins was recorded in selected cell lines along with a greater proportion, in comparison to the tubers, of cyanidin–based to peonidin–based.
pigments (Konczak et al., 2005). Given the aforementioned ability of cell culture from this species to accumulate medicinally active dephides, this is one of the most promising systems for the biotechnological production of health-promoting polyphenols.

*Silybum marianum*, milk thistle, the famous hepatic drug contains in the achenes an isomeric flavonoid lignans complex called silymarin that is a strong antioxidant (e.g. silybin in Fig. 1). The untreated cell cultures contain low and variable amounts of flavonolignans, but after they have been elicited their production can be increased 6-fold (Sanchez-Sampedro et al., 2005a). In another study, the same authors reveal the pivotal role of calcium deprivation in stimulating silymarin biosynthesis (Sanchez-Sampedro et al., 2005b). The role of peroxidases in both the synthesis by oxidative coupling of precursors and the degradation of silymarin compounds has also been explored (Sanchez-Sampedro et al., 2007a).

Stilbenes are bicyclic polyphenols represented by the well-known resveratrol (Fig. 1), a phyttoalexin and the red wine antioxidant. Although present in a considerable amount of natural sources like grapes and some *Polygonaceae* species, it has been also induced in elicited hairy root cultures of *Arachis hypogea* and recovered efficiently from the liquid medium (Medina-Bolivar et al., 2007). The same species was used to enhance production of a rare stilbene — piceatannol in cell culture (Ku et al., 2005). This approach seems quite promising for the production of a purified single antioxidant which the pharmaceutical industry prefers to use. In cell cultures of *V. vinifera*, the influence of elicitors on the stilbene biosynthesis and the stilbene synthase activity has been studied (Tassoni et al., 2005) as well as the antioxidant capacity of resveratrol and astringin from the stilbene synthase activity has been studied (Tassoni et al., 2006). The antioxidant activity of *in vitro* cultures of common sage, especially in lipophilic systems such as the inhibition of lipid peroxidation, can be attributed to abietane diterpenoids rather than to phenolics (Grzegorczyk et al., 2007). Light plays an important role in stimulating the diterpene biosynthesis in *vitro* (Caruso et al., 2000; Kužma et al., 2006) even though in natural conditions they can only be found in the underground parts of plants.

Volatile terpenoids responsible for fragrant properties of plant organs serve as a multipurpose chemical defense weapon against fungal and bacterial pathogens as well as a means of long distance chemical communication e.g. attracting pollinators. Moreover, the role of a monoterpenic β-thujaplicin in the alleviation of oxidative damage in cell cultures of *cypress* has been postulated (Zhao et al., 2005), but this does not imply that this is its direct function as an antioxidant. The antioxidant properties of several monoterpens, such as γ-terpinene and limonene have been reported. This suggests that this group of compounds may receive more attention although at present they are underestimated as antioxidants. Tissue cultures are able to produce volatile constituents of essential oils and many reports have been produced on that subject (Mulder-Krieger et al., 1988; Hrazdina, 2006). However, *in vitro* production of essential oils has not been aimed at the antioxidant properties of the products.

### 2.2. Isoprenoids

Isoprenoids are produced in plant cells from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). These basic units are synthesized via cytoplasmic mevalonate or plastidic deoxyxylulose (or methylerythritol) phosphate pathways. IPP and DMAPP are subsequently fused by sets of prenyltransferases and terpene synthases and a variety of modifying enzymes to eventually yield enormously variable compounds in numerous groups of terpenoids (mono, sesqui, di, and triterpenes and their derivatives), carotenoids, and steroids (Bouvier et al., 2005; Thorn, 2006). Each of these groups contains metabolites of different chemical characters such as alcohols, acids, aldehydes, or a combination of these; they can also be aromatic, glycosylated and form more complex structures. The most powerful antioxidants among them are carotenoids and abietane diterpenoids (some of them containing a phenolic structure within a molecule). In comparison to most phenopropanoid polyphenols, isoprenoids are generally more lipophilic, hence their important role in protecting membrane lipids. Both β-carotene and lycopene are recognized as dietary chemopreventive agents, protecting against cancerogenesis. The participation in an antioxidant cascade is likely to contribute to these prophylactic properties. Crocin derived from saffron (stigma of *Crocus sativus*) has been confirmed as a powerful antioxidant (Ochiai et al., 2004), stronger than α-tocopherol, and being a glycoside of an apocarotenoid acid, crocetin, it is water soluble (Fig. 2). Whereas other carotenoids are readily available from abundant natural sources such as carrots or tomatoes as well as by means of microbial (Bhosale and Bernstein, 2005) and fungal biotechnology (Böhme et al., 2006), it is reasonable to obtain crocin from the plant tissue cultures (Chen et al., 2003, 2004) of *C. sativus*. In this case, the *in vitro* production could be profitable due to the high cost of the raw material (about 200 000 flowers are needed to get 1 kg of dried stigma).

Abietane diterpenes are present in many *Lamiaceae* medicinal plants as well as in some gymnosperms. Some of these compounds demonstrate substantial antioxidant potential in various systems. Carnosic acid (Fig. 2) and carnosol, rosmanol and ferruginol (Fig. 2) are responsible for the antioxidant activity of sage (*Salvia* sp.) and rosemary (*Rosmarinus officinalis*) along with the phenolic rosmarinic and salvianolic acids, but the organ distribution differs between the two mentioned chemical classes. However, the published tissue culture studies in *Lamiaceae* aimed at enhancing the rosmarinic acid production rather than at abietane diterpenoids. The tissue cultures as a source of abietane diterpenes have been established for *R. officinalis* (Caruso et al., 2000), *S. officinalis* (Grzegorczyk et al., 2007) and *Salvia sclarea* (Kužma et al., 2006). The antioxidant activity of *in vitro* cultures of common sage, especially in lipophilic systems such as the inhibition of lipid peroxidation, can be attributed to abietane diterpenoids rather than to phenolics (Grzegorczyk et al., 2007). Light plays an important role in stimulating the diterpene biosynthesis in *vitro* (Caruso et al., 2000; Kužma et al., 2006) even though in natural conditions they can only be found in the underground parts of plants.

*Volcanic* terpenoids, widely used in human nutrition as vitamin E and in food conservation, are powerful free radical scavengers and protect plant cells against oxidative damage in a lipophilic environment (Kruk et al., 2005). Plants are the primary source of natural tocopherols isolated from vegetable oils or maize embryos. Their biosynthesis integrates the aromatic aminoacids (shikimic acid) and plastidic isoprenoid deoxyxylulose phosphate (pentose) pathways. The first pathways create the aromatic (phenolic) head while the second gives rise to the hydrophobic tail of a tocochromanol skeleton (DellaPenna, 2005). α-Tocopherol (Fig. 2) is the most active form of vitamin E, while the extraction from plant oils usually yields a mixture of β-, γ-, δ- and α-tocopherols and tocotrienols. Therefore there is a demand for alternative sources of pure α-tocopherol from plant tissues. Various culture systems of *Helianthus annuus* (sunflower) have been established for this purpose. A 100% increase in cell suspensions has been achieved by media optimization, elicitation and precursor feeding (Caretto et al., 2004; Gala et al., 2005). A photomixotrophic cell line has also established a cultured on sucrose-deprived medium (Fachechi et al., 2007). An attempt has been also made to select the most efficient genotype of hazel (*Corylus avellana*) for the production of tocopherols in bioreactors (Sivakumar et al., 2005).
Betalains — the purple betacyanins and yellow betaxanthines pigments functionally replace anthocyanins in 13 taxons grouped in Caryophyllales (previously Centrospermae) order, such as the families: Chenopodiaceae, Amaranthaceae, Portulacaceae, Cactaceae, Phytolaccaceae and others. These nitrogen containing compounds (Fig. 2) although widely used as non-toxic food colorants, remain understudied in terms of their antioxidant potential (Gliszczyńska-Swigło et al., 2006; Kanner et al., 2001). These properties of betalains have been recently reviewed by Stintzing and Carle (2004). The major advantages of betalains as dietary antioxidants are their bioavailability, which is greater than most flavonoids, and their superior stability in comparison to anthocyanins (Kanner et al., 2001; Stintzing and Carle, 2004).

Red beetroot (Beta vulgaris) is a rich natural source of betacyanins (with betanin predominating — Fig. 2) and is also an object of metabolic in vitro studies on their biosynthesis. They possess the useful properties of pigments and are therefore preferred as a model system despite their low commercial value. The production of beetroot pigments has been performed using a variety of techniques such as cell suspensions and transformed roots in bioreactors (Hunter and Kilby, 1999; Pavlov et al., 2002, 2005b). Being ideal metabolites for direct observation they have also been used for compound recovery experiments using various permeabilization and extraction methods (Hunter and Kilby, 1999). Red beet betalains from hairy roots have been shown to be efficient free radical scavengers (Pavlov et al., 2002, 2005b). The growing knowledge of their therapeutic and preventive properties is likely to further increase the research interest in their in vitro biosynthesis.

Indole glucosinolates are a tryptophane containing group from the diverse class of thioglycoside compounds typical to the Brassicaceae. Upon hydrolysis by myrosinase they generate biologically active antioxidants and carcinogenesis inhibitors such as indole-3-carbinol derived from glucobrassicin (Fig. 2). In cruciferous plants they are thought to contribute to the regulation of auxin biosynthetic routes (Bak et al., 2001). Although they are present in common cabbage related vegetables (broccoli in particular) their instability and uneven or unpredictable distribution would make their biotechnological production an interesting option. Yet, to date there has been very little data available on that topic. The horseradish (Armoracia rusticana) cells, both in suspension and immobilized in polyurethane

![Fig. 2. Structures of antioxidant metabolites from plant in vitro cultures from isoprenoid and other chemical classes.](image_url)
I. batatas biomass increase and the accumulation of the desired metabolite. In the latter species, several factors had been optimized (e.g. CO2 monitoring of secondary metabolites production instrumentation development and the trend towards a metabolomic just one dominant antioxidant, differing in the absorption maxima 2000). On the other hand, this approach is only adequate if there is can also be applied non-destructively (Durzan et al., 1991; Smith, 1999). Laboratories that lack phytochemical facilities need other equivalent substitution methods. For operation on a large scale, time saving is also a consideration. These faster and less elaborate techniques involve spectrophotometry that can be used for some groups of compounds, while for other types it is rather unsuitable. The first group contains colored compounds — flavonoid, carotenoid, and quinoid pigments or UV absorbing phenolics.

Anthocyanins are commonly estimated by this technique, which can also be applied non-destructively (Durzan et al., 1991; Smith, 2000). On the other hand, this approach is only adequate if there is just one dominant antioxidant, differing in the absorption maxima from any interfering compounds. The rapid progress in phytochemical instrumentation development and the trend towards a metabolomic approach and high throughput screening is likely to also influence the monitoring of secondary metabolites production in vitro. Indeed, in recent publications the chromatographic determination of antioxidants predominates, frequently accompanied by bioactivity guided fractionation, spectral structure elucidation of isolated chemicals by MS or NMR. The use of hyphenated techniques such as LC-MS and LC-NMR or 2D-NMR is considered to be the best means for structure determination when novel compounds are present in vitro that have not been known from intact plants (Tian et al., 2005; Farag et al., 2007; Sanchez-Sampedro et al., 2007b).

3. Strategies for increased production

The continuous monitoring of a chosen metabolite is a prerequisite for the successful development of production technology and can be carried out using a number of different methods. For accurate measurements, chromatographic methods such as HPLC and GC with the appropriate detection are sufficient. Laboratories that lack phytochemical facilities need other equivalent substitution methods. For operation on a large scale, time saving is also a consideration. These faster and less elaborate techniques involve spectrophotometry that can be used for some groups of compounds, while for other types it is rather unsuitable. The first group contains colored compounds — flavonoid, carotenoid, and quinoid pigments or UV absorbing phenolics.

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3.1. Optimization of biosynthesis by culture conditions

A range of environmental and nutritional factors are known to influence the biosynthetic pathways of secondary metabolites. Light induction of anthocyanin pigments is a well documented example (Harborne, 2001; Stintzing and Carle, 2004). In cultured cherry (Prunus cerasus) callus and suspensions, the light exposure causes a rapid change of the tissue color from white to purple (Blando et al., 2005). The anthocyanidin profile was different both from intact leaves and fruits with cyanidin-3-glucoside as the single dominant compound, absent delphinidin (unlike in fruits) and only a small amount of cyanidin-3-rutioside (dominant in the leaves). This is a good example of a distinct metabolic profile under in vitro conditions. However, the irradiation of cultures can lead to an increase in cost and can generate undesirable temperature gradients in the culture vessels. Therefore, several examples of constitutive, light-independent systems for anthocyanin production were established such as Glehnia littoralis (Miura et al., 1998), l. batatas (Konczak et al., 2005) or Aralia cordata (Kobayashi et al., 1993). In the latter species, several factors had been optimized (e.g. CO2 supplementation) to obtain a final yield of over 17% of the dry mass.

The media composition has to be optimized for both an intensive biomass increase and the accumulation of the desired metabolite. Auxins and cytokinins are crucial for the proper stimulation of biosynthetic pathways, unless we deal with a hormone-independent transformed root culture. In anthocyanin producing G. littoralis cultures, NAA at 1 mg/l was the preferred auxin, superior to IAA and 2,4-D and the addition of 0.1 mg/l kinetin further improved cell growth and pigment biosynthesis (Miura et al., 1998). The higher concentration of NAA (5 mg/l) was optimal for R. hirta (Luczkiewicz and Cisowski, 2001). In the same study, the highest accumulation was achieved by adding cysteine, which is one of the constituents of CoA, a crucial factor in flavonoid biosynthesis.

A two or three-stage growth system can be also useful for optimizing metabolite production. The first stage is used for the initiation and/or the maintenance of a culture with the aim of obtaining the highest percentage of induction and the fastest biomass growth. The second phase aims at the accumulation of a product. It has been successfully applied to anthocyanins in R. hirta (Luczkiewicz and Cisowski, 2001) where the cultures in the optimized second stage media contained up to 5% anthocyanins in dry mass.

In C. sativus cultures the two-stage system leads to a substantial accumulation of crocin (430 mg/l) simply by changing the hormone composition from NAA at 2 mg/l, BAP at 1 mg/l for biomass growth to IAA at 2 mg/l and BAP at 0.5 mg/l for crocin production (Chen et al., 2003).

3.2. Production in differentiated tissues

In some cases, full development in natural conditions is useful for producing a considerable amount of secondary products, especially if the undifferentiated cell culture is either unable to or barely accumulating antioxidant compounds. This has been reported for several classes of metabolites, especially for alkaloids, and has also been published for some antioxidant compounds. Ellagic acids present in Rubus chamaemorus plants was 3 times lower in shoot cultures and over 10 times lower in callus (Thiem and Krawczyk, 2003). Similarly in O. basilicum cell cultures (Kintzios et al., 2004) the accumulation of rosmarinic acid was markedly lower than in regenerated plantlets. In S. officinalis (Grzegorczyk et al., 2007) and rosemary (Caruso et al., 2000) in vitro cultures, the abietane diterpene antioxidants (carnosol and carnosic acid) were present only in shoot cultures and not in callus, suspension or hairy roots. On the other hand, undifferentiated cell suspensions are able to accumulate great amounts of phenolic acids. Moreover, the cells cultured in suspension tend to form larger aggregates upon differentiation, therefore increasing their shear stress resistance. The potential of fast growing somatic embryos cultures can be also utilized for the production of medicinal metabolites, but reports on that topic are scarce and deal chiefly with alkaloid production such as paclitaxel (Lee and Son, 1995). For example, phenol glycosides, lignans and flavonoid accumulation has been mentioned in Siberian ginseng somatic embryos (Shoahehl et al., 2006).

Even in dedifferentiated cells, some biosynthetic potential typical for the developed organs from which they were initiated, can be conserved. In Pueraria lobata callus cultures, the bioactive isoflavonoid content depended on the source organ, reflecting relations in the mature plant (Matkowski, 2004). The selection of proper donor plants and organs should already be considered when starting the culture, unless it can be overcome by a suitable treatment. However, in most circumstances the dedifferentiation apparently also involves some of the biochemical properties of the cells. The modification of relative biosynthesis to degradation ratios of a desired product can also influence the final levels of a desired compound in the culture.

3.3. Selection of high-producing cell lines

The super-efficient clones of cultured cell or tissues can be selected by monitoring the level of the compound of interest, or can be complemented by a selecting agent facilitating the process. The
increased anthocyanins in *Rudbeckia* plant, was not found in that study, but other authors have succeeded neither are antioxidants. Salidroside, the antioxidant present in the phenylalanine, caffeic acid or cucumber juice (Liu et al., 2007).

Production when compared to feeding individually with tyrosine, phenylethanoid glycosides precursors results in a doubling of 

In contrast, the feeding of naringenin or phenylalanine significantly increased anthocyanin accumulation of *C. litoralis* selected from a single purple spot in a white, dark grown callus (Miura et al., 1998; Kitamura et al., 2002). Similarly, a selected cell line of purple sweet potato (PL) accumulates a large amount of acylated anthocyanins without light exposure (Konczak et al., 2005).

3.4. Precursor feeding and biotransformation

When starting an in vitro culture of a medicinal plant, that in its intact form accumulates substantial amounts of valuable products, it sometimes happens that a dedifferentiated cell mass is unable to complete the biosynthesis. This is due to the uncoupling of the enzymatic machinery, or an insufficient expression of developmentally regulated biosynthetic genes, or a lack of environmental stimuli. If biosynthetic enzymes are expressed in part of the pathway, the exogenous delivery of precursors can solve the problem. It can be also helpful in omitting the metabolic channeling that directs the precursors to a pathway other than that desired.

In *Rhodiola rosea* compact callus aggregate culture no secondary products were detected until the medium was supplemented with cinnamyl alcohol that was efficiently transformed into a pharmacologically active rosin and rosavin (György et al., 2004). However, neither are antioxidants. Salidroside, the antioxidant present in the plant, was not found in that study, but other authors have succeeded in antioxidant production in this species (Furmanowa et al., 1998). In contrast, the feeding of naringenin or phenylalanine significantly increased anthocyanins in *Rudbeckia* (Luczkiwicz and Cisowski, 2001) and strawberry (Edahiro et al., 2005). Similarly, a selected cell line of purple sweet potato (PL) accumulates a large amount of acylated anthocyanins without light exposure (Konczak et al., 2005).

3.5. Elicitation and stress induced production

One of the best established roles of secondary metabolites is the involvement in stress responses (Grassmann et al., 2002). Phytoalexins are synthesized in reaction to a pathogen attack, other compounds are associated with abiotic disturbances.

The upregulation of biosynthesis of secondary metabolism upon exposure to stress factors has been used to obtain a high yield of many medicinal compounds (Verpoorte et al., 2002; Vanisree et al., 2004). Exposure to stress factors results in an excessive level of oxidizing molecules such as ROS due to the disturbances in redox state equilibrium. The increased ROS production may help to combat the invasive pathogens but can also be triggered by harmful environmental factors such as UV radiation, xenobiotics, heavy metals, drought, and mechanical damage. Therefore, antioxidant properties of some metabolites along with antioxidant enzymes can aid the plant in restoring the redox homoeostasis (Matkowski, 2006). Apart from direct antioxidant activity, some compounds — e.g. flavones can serve as substrates for cellular peroxidases (Morimoto et al., 1998).

In vitro cultured material can be elicited by bacterial or fungal lysates or stress response mediators such as salicylate or jasmonic acid/methyl jasmonate and hydrogen peroxide as well as by environmental factors like metals or irradiation. The stress related growth regulators, jasmonic acid and its esters, especially methyl jasmonate (MeJA) have been widely used in promoting the biosynthesis of both inducible and constitutive secondary metabolites, including medicinal compounds such as anticancer alkaloids (Vanisree and Tsay, 2004; Verpoorte et al., 2002). With respect to the antioxidants, jasmonic acid enhanced α-tocopherol production by sunflower and *A. thaliana* cell cultures (Gala et al., 2005) and cyanidin glucosides in cherry callus (Blando et al., 2005). On the other hand, in the resveratrol producing grapevine cell cultures (Tassoni et al., 2005), jasmonic acid proved to be a weaker elicitor than its methyl ester. Besides, MeJA promoted the accumulation of trans-resveratrol, whereas JA resulted in a modest increase of the less desirable cis-resveratrol. MeJA and salicylic acid also stimulated the curcumin glycosylation by *C. roseus* cell cultures (Kaminaga et al., 2003).

Using natural biotic elicitors also results in enhanced secondary metabolism. Yeast extracts (YE — also read as yeast elicitor), but neither chitin nor chitosan, have a positive impact on the production of silymarin, the effect being potentiated when combined with JA. However, it proved more effective when supplemented with MeJA in this system (Sanchez-Sampedro et al., 2005a). Chitin elicited the production of several new flavonoids in cactus (*Cephalocereus senilis*) cell cultures (Liu et al., 1993). YE applied to the hairy root culture of *S. miltiorrhiza* improved both the growth of the roots and the accumulation of rosmarinic and lithospermic B acids, preceded by a significant rise in tyrosine aminotransferase activity (Chen et al., 2001; Yan et al., 2006). Compared to the effect of an abiotic elicitor — Ag+, YE was superior (Yan et al., 2006). Rosmarinic acid production was also enhanced in *O. basilicum* hairy roots by Phytophthora cell walls, while salicylic and jasmonic acids or chitosan suppressed it (Bais et al., 2002).

In peanut hairy roots (Medina-Bolivars et al., 2007) sodium acetate and chitosan were the most effective of the five tested elicitors causing a 60-fold increase in resveratrol accumulation.

The use of metal-based elicitors gives positive results as well. Vanadium salts increase the accumulation of rosmarinic acid in lavender cell culture nearly three-fold (Georgiev et al., 2006a), while in *Vitis* cells only the cis isomer of resveratrol was induced (Tassoni et al., 2005).

Rare earth elements such as La**3+** and Ce**3+** can also serve as elicitors to promote crocin (Chen et al., 2004), phenylethanolamides from *C. deserticola* (Ouyang et al., 2003), and silymarin (Sanchez-Sampedro et al., 2005b) production. The mechanism of these lanthanides actions involves interference with cellular calcium signaling (Sanchez-Sampedro et al., 2005b).

Irradiation with UV has been also used for making the plant cells synthesize more antioxidants. In particular, flavonoids and other polyphenols are known to protect plants from harmful UV impact. In *P. quadrangularis* callus subjected to UV-B treatment, the irradiation
stimulated a large increase in the amount of flavonoid, corroborating the higher radical scavenging activity (Antognoni et al., 2007). Similarly, the callus of *A. hypogea* was induced by UV radiation to accumulate much greater amounts of stilbenes (Ku et al., 2005).

The use of a downstream mediator of elicitation such as MeJa may be more effective than biotic elicitors. The latter bring about a broad spectrum of responses, not all of which are necessarily desirable. On the other hand, biotic elicitors are of some use due to their lower cost and are particularly appropriate at the initial stage of research when the pathways of induction for a particular compound are not yet determined. In addition, different elicitors can diversely act on the biosynthetic competence of the cells, as illustrated by *V. vinifera* cultures resveratrol isomers production (Tassoni et al., 2005).

### 3.6. Transformation with *Agrobacterium rhizogenes* — hairy roots

This is a commonly used method to enhance the production of secondary metabolites. Thousands of species have been transformed with *A. rhizogenes* with the aim of achieving a transformed roots induction. If the intact plant is known to synthesize large amounts of antioxidant compounds, one can expect a substantial increase in their level in the cultured roots and sometimes also in plantlets regenerated from transformed roots. The subject of transformed root methodology, its recent developments and the prospects for production of medicinal compounds have been addressed in several reviews (Georgiev et al., 2007; Guillon et al., 2006; Uozumi, 2004). The manipulation and optimization of transformed roots productivity are generally the same as those of other culture systems with perfect culture conditions, cell line selection, precursor feeding and elicitation. Thus, only a few examples that specifically report the production of antioxidant compounds by hairy roots will be mentioned in this chapter.

Several species from the Lamiaceae have been transformed with *A. rhizogenes* for the production of polyphenolic antioxidants. The very popular rosmanarinic acid is again a good example of this. In *S. officinalis* hairy roots, the antioxidant activity and RA level were much higher than in untransformed organs (Grzegorczyk et al., 2006, 2007). This depside has also been efficiently produced by the hairy roots of other species such as: *S. militortitia* (Chen et al., 2001; Yan et al., 2006), *C. blumei* (Bauer et al., 2004), *O. basilicum* (Bais et al., 2002), *H. officinalis* (Murakami et al., 1998; Kochan et al., 1999).

Baikal skullcap (*S. baicalensis*) is one of the richest natural sources of flavones reaching up to 20% in the root. Transformed roots produced baikalein and wogonin glucuronides, but also a phenylethanoid acetieside, a compound unknown from plants growing in natural conditions (Nishikawa et al., 1999; Stojakowska and Malarz, 2000). In *S. scarea* hairy roots some abietane diterpenoids are also reported, although not in large enough amounts for them to be considered as a source of antioxidants (Kuźma et al., 2006). An effective system for rutin production has been established with the hairy roots of buckwheat (*Fagopyrum esculentum*) whose leaves are a rich natural source of rutin (Lee et al., 2007). A high yield of antioxidant betalains can be achieved from the transformed roots of *B. vulgaris* (Pavlov et al., 2002, 2005b).

An innovative transgenic approach with respect to antioxidant production has been applied to *Sauussurea involucrata* transformed roots overexpressing heterologous chalcone isomerase from a related species (Li et al., 2006). This has led to the enhanced production of the flavone apigenin, even in comparison to the selected wild type cell lines or transformed roots not bearing foreign chalcone isomerase gene.

Besides the numerous positive results of transformed roots producing secondary metabolites, there is one example that achieved the opposite result in relation to antioxidants. Specifically, in two boraginaceous species: *E. sericeum* and *L. erythrolzihon*, having introduced the rolC gene exhibited greatly reduced levels of the two antioxidants: rabdosin and rosmanarin acid in comparison to the lines without the introduced rolC (Bulgakov et al., 2005).

### 3.7. Scale-up or the never-ending bioreactor story

The ultimate goal of the biotechnology of medicinal plants is the industrial production of useful natural products. However, most of the published work deals with laboratory experiments on a small scale, even if there are liquid cultures involved. When thinking of industrial exploitation of the potential of plant cell factory, systematic scale-up studies must be carried out. However, the literature on that is relatively limited. One of the few established commercial systems is the well-known shikonin technology using *L. erythrolzihon* (Miswa, 1994; Bulgakov et al., 2001). Additional papers have been produced on experimental bioreactor systems in which cells, aggregates or differentiated organs are sources of antioxidant compounds. A few pilot-scale studies have been published on the production of α-tocopherol from hazel (*Sivakumar et al., 2005*), rosmanarin acid from lavender (*Pavlov et al., 2005a* and basil (*Kintzios et al., 2004*), betalains from *B. vulgaris* (*Pavlov et al., 2007*). Several types of vessels are applied, but the most popular are classic designs like stirred, airlift or mist bioreactors (*Pavlov et al., 2007*). Temporary immersion systems are also used, e.g. for betalains (*Pavlov and Bley, 2006*) and semi-continuous perfusion high density cultures for rosmanarin acid production by *A. officinalis* (*Su et al., 1993*; *Su and Humphrey, 1990*). The scale-up study on *P. pahalae* cell cultures proved the importance of physical factors such as illumination of the bioreactor interior for sustained production of anthocyanins (*Meyer et al., 2002*). Similarly, in the aforementioned *A. cordata* cultures, the upscale in 500 l fermentor allowed the production of over 500 g of mixed anthocyanins during a 16 day period (*Kobayashi et al., 1993*).

Nonetheless, the bioreactor technology in relation to above mentioned antioxidants, though sometimes remarkably efficient in terms of production (as in the case of rosmanarin acid), does not seem likely to approach factory-scale industrial application in the near future. Hopefully, at least some of the mentioned research on antioxidants biotechnology will finally result in market implementation.

### 3.8. Concluding remarks and future perspective

Despite the immense variety of antioxidant compounds obtained from in vitro cultured plant, their cells, tissues and organs, not many examples of practically applicable technologies can be named. The few that are available include rosmanarin acid, α-tocopherol from sunflower cells and improved anthocyanins from several sources.

There are, however, numerous inspiring and promising reports on such compounds as resveratrol, kinobeaon A, lithospermic acid B and other oligomeric caffeic acid depsides or crocin, which should provoke and encourage more research focused on the regulation of biosynthesis and its increased economic feasibility. Finally, the burgeoning metabolomic and metabolic engineering approaches may add a new fascinating dimension to the possibilities of antioxidant-making plant cell factories that have been presented in this paper.

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