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REVIEW

Sage in vitro cultures: a promising tool for the production of bioactive terpenes and phenolic substances

Andrey Marchev · Christiane Haas · Sibylle Schulz · Vasil Georgiev · Juliane Steingroewer · Thomas Bley · Atanas Pavlov

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Abstract Extracts of *Salvia* species are used in traditional medicine to treat various diseases. The economic importance of this genus has increased in recent years due to evidence that some of its secondary metabolites have valuable pharmaceutical and nutraceutical properties.The bioactivity of sage extracts is mainly due to their content of terpenes and polyphenols. The increasing demand for sage products combined with environmental, ecological and climatic limitations on the production of sage metabolites from field-grown plants have led to extensive investigations into biotechnological approaches for the production of *Salvia* phytochemicals. The purpose of this review is to evaluate recent progress in investigations of sage in vitro systems as tools for producing important terpenoids and polyphenols and in development of methods for manipulating regulatory processes to enhance secondary metabolite production in such systems.

Keywords Elicitation · Hairy roots · Manipulation of metabolic pathways · Plant cell cultures · Sage *Salvia* spp. · Secondary metabolites

Andrey Marchev and Christiane Haas contributed equally to this work.

A. Marchev · V. Georgiev · A. Pavlov (✉)
Laboratory of Applied Biotechnologies, Department of Industrial Microbiology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, 139 Ruski Blvd., 4000 Plovdiv, Bulgaria
e-mail: at_pavlov@yahoo.com

C. Haas · S. Schulz · J. Steingroewer · T. Bley
Institute of Food Technology and Bioprocess Engineering, Technische Universität Dresden, 01062 Dresden, Germany

V. Georgiev
Center for Viticulture and Small Fruit Research, Florida A&M University, 6505 Mahan Drive, Tallahassee, FL 32317, USA

A. Pavlov
Department of Organic Chemistry and Microbiology, University of Food Technologies, 26 Maritza Blvd., 4002 Plovdiv, Bulgaria

Introduction

Salvia is the largest genus in the *Lamiaceae* family and represents nearly 1,000 species that are widely distributed around the world. Herbal infusions of many *Salvia* species have been used in traditional medicine by different cultures to treat oral inflammation, throat and headaches, and digestive disturbances. Moreover, their antispasmodic, antiseptic and hypoglycemic effects were recognized by ancient healers long before the development of modern medicine (Nadir et al. 2013). Even since the discovery of antibiotics, treatment with *Salvia* extracts has been recommended to patients with tuberculosis, chronic

bronchitis, fever, mental and nervous disorders, as well as sexual debility (Akin et al. 2010).

Many investigations have confirmed the pharmaceutical importance of the genus *Salvia* due to its diverse bioactivities (Alizadeh and Shaabani 2012). These stem from the wide range of compounds found in *Salvia* including sterols, phenolics and terpenoids (Aşkun et al. 2010). Moreover several *Salvia* species (*S. sclarea* L., *S. officinalis* L., *S. triloba* L. and *S. lavandulaefolia* Vahl.) are generally recognized as safe (GRAS) by the Food and Drug Administration in USA (FDA 2012) and drugs and tinctures prepared from these plants are listed in several official Pharmacopoeias (for example: European Pharmacopoeia 6th edn; Pharmacopoeia Hungarica 8th edn). Hence, it is not surprising that *Salvia* species are economically important and widely used in the food, pharmaceutical, cosmetic and perfumery industries (Böszörményi et al. 2009).

In the last few years, many natural *Salvia* habitats have shrunk or fallen under threat due to the growing demand for plant-derived ingredients (Cui et al. 2012). This has led to the development of biotechnological methods for producing standardized valuable phytochemicals (e.g. food additives, pharmaceuticals and pesticides) based on plant in vitro cultures, which offer the advantages of continuous production under controlled conditions, independently of environmental factors (geographical latitude, climatic change, and seasonal variation) and are harmless to natural plant populations (Steingroewer et al. 2013). Plant tissue cultivation techniques are based on the totipotency of plant cells, which enables every cultured cell to produce in vitro wide range of metabolites synthesized by mature plant (Zhao et al. 2011). In this review, we summarize recent achievements of investigation of *Salvia* in vitro systems as biological matrixes for the production of pharmaceutically important secondary metabolites.

Salvia in vitro systems as a source of pharmaceutically important terpenoids and polyphenols

The potential of in vitro *Salvia* systems as tools for controlled production of valuable secondary metabolites has been extensively investigated. In particular, considerable attention has been paid to cell suspensions and hair root cultures. Table 1 summarizes some of the more recent findings concerning the potential of *Salvia* in vitro systems to accumulate terpenoids and polyphenols.

Cell suspension cultures

Only a few reports have described cell suspensions from *Salvia* as a source of secondary metabolites. This is primarily because of their low metabolite yields, slow growth, low biomass density, high tendency to form aggregates and higher degree of genetic and metabolic instabilities during sub-cultivation cycles. Most studies have been conducted using calli and/or cell suspension cultures of Danshen (*Salvia miltiorrhiza* Bunge). The state of the art in this field has been reviewed by Wang and Wu (2010). Different explants from this species, including leaves, roots, stems, petioles, anthers and seedlings, readily initiate callus formation on MS medium, supplied with the appropriate combination of growth regulators (Wang and Wu 2010). Once initiated, some callus lines can be maintained and cultivated on media without growth regulators (Zhao et al. 2010a; Dong et al. 2010). Although some *S. miltiorrhiza* cell lines can produce tanshinones, cryptotanshinone and/or ferruginol, the yields were highly variable, and the accumulated amounts were unpredictable and often significantly lower compared to those obtained from mature plants (Wang and Wu 2010). Tanshinones and cryptotanshinone are important diterpene quinines with anti-cancer, anti-inflammatory and neuroprotective effects (Gong et al. 2011; Zhang et al. 2013), while ferruginol is a meroterpene compound that has tumor-reducing properties (Tayarani-Najaran et al. 2013) (Fig. 1, Table 1).

It is clear that additional studies on the regulatory mechanisms that govern secondary metabolite biosynthesis in *Salvia* plants have to be done in order to establish cell suspension cultures that provide high and consistent yields of valuable secondary metabolites. In a comparative study of various *S. officinalis* in vitro systems with different levels of differentiation, it was found that hairy roots and cell suspensions were the best systems for production of rosmarinic acid, whereas the shoot cultures were the only system that produced carnosic acid (Fig. 1) and carnosol (Grzegorczyk et al. 2007). Bolta et al. (2003) observed that the biosynthesis of ursolic acid by an *S. officinalis* cell

Table 1 Recently reported bioactive terpenoids and polyphenols from *Salvia* in vitro systems

Secondary metabolite	Producer; [type in vitro system]	Biological activity	References
Triterpenes	*S. tomentosa*; [C]		Georgiev et al. (2011)
Ursolic acid	*S. scabiosifolia*; [C]		Marchev et al. (2011a)
Oleanolic acid	*S. officinalis*; [CS]		Bolta et al. (2003)
Abietane diterpenoids	*S. officinalis*; [SH]	Antioxidant	Grzegorczyk et al. (2007)
Royleanone	*S. sclarea*; [HR]	Antimicrobial	Kuźma et al. (2008)
15-Deoxyfuerstione	*S. austriaca*; [HR]	Anti-biofilm	Kuźma et al. (2009)
Taxodione		Anti-inflammatory	Kuźma et al. (2011)
Ferruginol		Cytotoxic	
Salvipisone		Anti-carcinogenic	
Aethiopinone			
1-Oxoaethiopinone			
Carnosic acid			
Carnosol			
Tanshinones	*S. miltiorrhiza*; [CS]	Anti-cancer	Wu and Shi (2008)
Tanshinone I	*S. miltiorrhiza*; [HR]	Anti-inflammatory	Yuan et al. (2009)
Tanshinone IIA		Antioxidant	Zhao et al. (2010a, 2011)
Cryptotanshinone		Neuroprotective	Gupta et al. (2011)
Dihydrotanshinone I			Yan et al. (2011)
			Yang et al. (2012a, 2012b, 2012c)
			Kai et al. (2011, 2012)
			Gu et al. (2012)
			Liang et al. (2012)
Hydrophilic phenolic compounds	*S. miltiorrhiza*; [HR]	Antioxidant	Yuan et al. (2009)
Salvianolic acid A	*S. miltiorrhiza*; [CS]	Anti-inflammatory	Xiao et al. (2009b)
Salvianolic acid B (lithospermic acid B)		Anti-carcinogenic	Dong et al. (2010)
		Hepatoprotective	Gu et al. (2012)
		Neuroprotective	Hao et al. (2012)
		Cardioprotective	
		Immunomodulatory	
Phenolic acids	*S. officinalis*; [HR]	Antioxidant	Grzegorczyk et al. (2007)
Rosmarinic acid	*S. officinalis*; [CS]	Anti-inflammatory	Xiao et al. (2009b)
Caffeic acid	*S. miltiorrhiza;* [HR]	Anti-carcinogenic	Dong et al. (2010)
Salvianic acid (danshensu)	*S. miltiorrhiza*; [CS]	Hepatoprotective	Zhao et al. (2011)
	S. tomentosa; [HR]	Neuroprotective	Xiao et al. (2011)
			Gu et al. (2012)

HR hairy roots; *CS* cell suspension; *SH* shoot culture; *C* callus

culture was adversely effected by cell differentiation: the single-cell fraction of the *S. officinalis* cell suspension accumulated almost 50-fold higher amount of ursolic acid than highly aggregated (particles sizes larger than 1,100 μm) suspension culture (Bolta et al. 2003). However, the tested *S. officinalis* cell suspensions exhibited negligible biomass accumulation when forced to grow as single cells (Bolta et al. 2003).

Because of the heterogeneous growth patterns and highly variable secondary metabolites production,

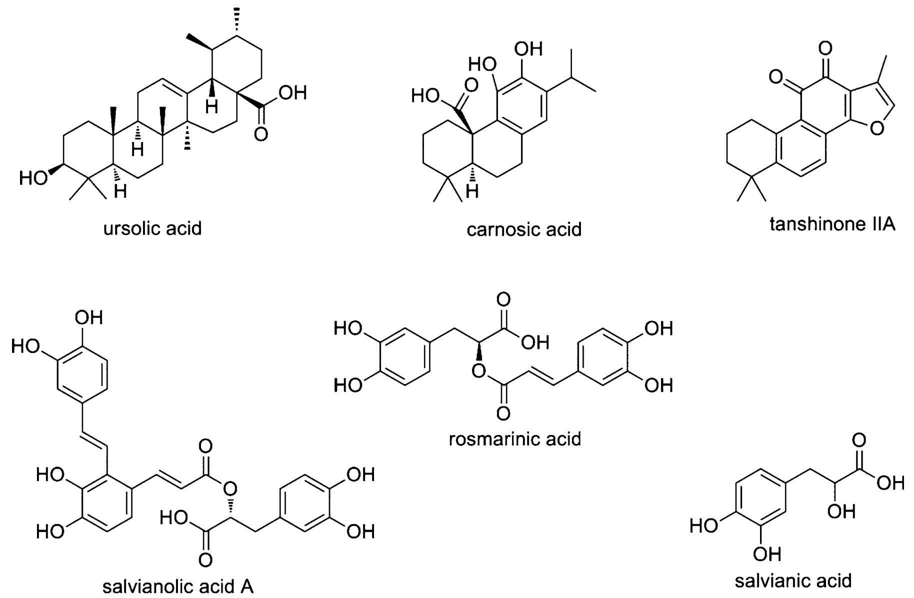

Fig. 1 Structures of some important biologically active compounds, produced by *Salvia* species

advanced alternative monitoring systems such as the RAMOS cultivation system (Geipel et al. 2013) and a laccase based biosensor (Eremia et al. 2013) were developed and adapted to enable the non-destructive on-line measurement of biomass and rosmarinic acid accumulation by *Salvia* cell suspensions. It seems that not only the lack of cell differentiation but any efforts made to increase secondary metabolite yields in *Salvia* cell cultures were accompanied with significant suppress of cell growth. We have recently demonstrated that the type and the proportion of used auxins and cytokinins could have significant impact on the levels of ursolic and oleanolic acids production by calli of *S. tomentosa* Mill. (Georgiev et al. 2011). Flow cytometry analyses revealed that growth regulators had impact not only on the triterpenes formation, but also induced several endoreduplication cycles in *S. tomentosa* cells, producing mixoploid calli with a higher degree of polyploidy than that observed in mature plant (Georgiev et al. 2011). To avoid this negative effect of exogenous growth regulators, a rhizogenic callus culture of *Salvia scabiosifolia* Lam. was obtained through genetic transformations with *Agrobacterium rhizogenes* (Marchev et al. 2011a). The resulting line exhibited acceptable growths rate and levels of terpenoid production when cultivated in the absence of exogenous growth regulators (Marchev et al. 2011a). However, despite of efforts done so far, sage cell suspensions currently seem to be more useful as model systems for biochemical, biosynthetic and genetic studies. However, they should still be considered and potentially developed further in order to fully explore the potential of plant in vitro systems as tools for the commercial production of bioactive phytochemicals from *Salvia* species.

Hairy root cultures

Hairy root cultures are the most effective in vitro systems for the production of valuable secondary metabolites whose biosynthesis occurs primarily in the roots in mature plants (Dai et al. 2011). As renewable source of phytochemicals, hairy root cultures possess several advantages relative to known plant in vitro systems but also require more complicated equipment to ensure their growth (Steingroewer et al. 2013). *Salvia* species are attractive subjects for hairy roots induction mainly because of their potential to produce a wide range of biologically active compounds, that accumulate in the roots of mature plants (Yuan et al. 2009; Xiao et al. 2011; Zhao et al. 2011; Gu et al. 2012; Hao et al. 2012). However, only a few *Salvia* species have been used to generate hairy roots (Kuźma et al. 2008; Grzegorczyk and Wysokińska 2010; Kai et al. 2011). It seems that sterile young plants grown in vitro are the best sources of explants for transformation (Kuźma et al. 2008; Dai et al. 2011; Kai et al. 2011; Hao et al. 2012). Notably, very low

transformation efficiencies were observed when using leaves from sage plants grown in vivo as explants (Gupta et al. 2011).

Our recent research demonstrated that the observed difficulties during transformation of in vivo *Salvia* plants were due to the extensive release of phenolic compounds that have strong antimicrobial and allelopathic activities by the damaged plant tissue (Marchev et al. 2011b). By using a temporary immersion system in combination with a two-phase cultivation protocol that incorporates Amberlite XAD-4 resin, we were able to remove these compounds and neutralize their negative effects on the formation of hairy roots (Marchev et al. 2011b). This procedure allowed us to achieve 100 % transformation efficiency using leaves of mature *S. tomentosa* Mill. plants (Marchev et al. 2011b). Beside the transformation method, the efficiency of hairy root induction also depends on the type of used *Agrobacterium* strain and activation of its virulent genes (Gupta et al. 2011). Since single or multi-copies of T-DNA can be randomly integrated at non-specific sites of the plant genome, *S. miltiorrhiza* hairy root lines, initiated from the same explants, exhibited significant variations in root phenotypes, growth rates and tanshinones accumulation (Dai et al. 2011).

Salvia hairy roots have been extensively used as model systems for studies on the biosynthesis of tanshinones and phenolic acid derivatives (Xiao et al. 2009a; Dai et al. 2011; Yang et al. 2012a). The production of these specific metabolites may be significantly up-regulated in hairy roots in contrast to the field-growing plants or other plant in vitro systems, which facilitates their study (Kuźma et al. 2008). For example, hairy roots of *S. officinalis* were found to accumulated higher amount of rosmarinic acid (31 mg rosmarinic acid/g DW) than mature plant roots, in vitro shoots or cell suspension cultures, and therefore their extracts exhibited higher antioxidant activity (Grzegorczyk et al. 2007). The total diterpenoid content of *S. sclarea* hairy roots was ninefold higher than that in the roots of two year-old field-grown plants (Kuźma et al. 2008). Significant differences were observed also in distribution patterns of individual diterpenoids. For example, aethiopinone was found to be the major diterpene in *S. sclarea* hairy roots (48 % of the total diterpenoids content), but its concentration in the roots of field-grown plants was rather low (up to 8 % of the total diterpenoids content) (Kuźma et al. 2008). Modified metabolism of sage hairy roots may also results in production of novel biologically active compounds that are not typically found in the field-growing plants. Thus, in addition to the typical abietane-type diterpenoids, *S. austriaca* Jacq. hairy roots were found to produce 7-(2-oxohexyl)-11-hydroxy-6,12-dioxo-7,9(11),13-abietatriene, a new diterpenoid that is structurally related to taxodione. This compound proved to have strong antimicrobial and anti-biofilm activities against methicillin-resistant *Staphylococcus aureus* strains, as well as remarkably high in vitro cytotoxic activity against three cancer cell lines: human skin melanoma MW-115, human leukemia promyelocitic HL-60 and lymphoblastic NALM-60 (Kuźma et al. 2012).

One of the most complicated problems concerning industrial application of hairy roots remains bringing the yields of produced phytochemicals to economically-feasible levels (Steingroewer et al. 2013). Research of *Salvia* hairy roots faced the same challenge and many strategies for improving the yields of produced diterpenoids have been evaluated in recent years, including genetic engineering and elicitation (Xiao et al. 2011; Gu et al. 2012; Yang et al. 2012c). In addition, bioreactor designs may impose significant bottlenecks in the scaling-up of hairy root cultivation (Steingroewer et al. 2013). *S. officinalis* hairy roots accumulated up to 1.6-fold more biomass when cultivated in nutrient sprinkle bioreactor in comparison with shake-flasks cultivation (Grzegorczyk and Wysokińska 2010). At these bioreactor systems, the amount of accumulated rosmarinic acid was up to 5-fold higher than that detected in mature *S. officinalis* plants (Grzegorczyk and Wysokińska 2010). A hairy root culture of *S. sclarea* grown in a nutrient sprinkle bioreactor in combination with elicitation using 125 µm methyl jasmonate (MJ) exhibited enhanced diterpenoid production with a maximum yield of 330 mg diterpenoids/l after 30 days of cultivation (Kuźma et al. 2009). It is clear that integrated approaches may attribute to more effective optimization of secondary metabolite yields from sage hairy root cultures. However, commercialization of *Salvia* hairy roots seems to be a complex process requiring much more efforts to combine the achievements of in vitro manipulation, genetic engineering, biosynthetic elucidation and bioprocess engineering.

Techniques for manipulating the regulation of biosynthesis to enhance secondary metabolite production

Elicitation

Elicitation techniques have been applied to most of the sage in vitro cultures that have been reported in order to increase their secondary metabolism. Wang and Wu (2010) recently published extensive review of successful elicitation strategies applied to *S. miltiorrhiza* in vitro cultures. The most widely used elicitors are Ag^{+}, yeast extract (YE) or the polysaccharide fraction of YE and MJ. Osmotic stress becomes an important factor affecting not only plant growth, development, and secondary metabolite production but also can serve as an abiotic elicitor in plant cell and tissue cultures. While sorbitol alone is only a moderately effective elicitor of tanshinones production in *Salvia* cultures, much stronger responses were observed when it was used in combination with YE (Shi et al. 2007) or chitosan (Zhao et al. 2010a). Moreover, no growth inhibition was observed in *S. miltiorrhiza* hairy roots treated between 30 and 100 g sorbitol/l. Since plant roots are mainly involved in water uptake regulation, hairy root cultures could be more sensitive to osmotic agents than undifferentiated cell cultures. A particularly interesting compound is sucrose because it can induce osmotic stress while simultaneously serving as the main carbon source for the cultured plant cells. Polyethylene glycol was investigated as an alternative osmotic agent but proved to be less effective at eliciting secondary metabolite production by *S. miltiorrhiza* hairy root cultures due to its high viscosity at the concentrations used (Wu and Shi 2008).

Some metal ions also elicit secondary metabolite production in sage. For example, La^{3+} applied to *S. miltiorrhiza* hairy root culture increased tanshinones production around 50 % (up to 27 mg tanshinones/g) (Zhou et al. 2012). While this tanshinones concentration is very high, no volumetric yield was published. It is important to consider this parameter because elicitation can influence the growth negatively (Zhao et al. 2010a) and therefore the optimization of elicitor concentration as well as time point of addition is important (Shi et al. 2007; Wu et al. 2007). *S. miltiorrhiza* hairy root cultures can produce a volumetric yield of 27.8 mg tanshinones/l after combined elicitation with the polysaccharide fraction of YE and osmotic stress (sorbitol). Repeated nutrient feeding and elicitor application improved further the volumetric yield up to 144 mg tanshinones/l and even supported biomass formation (Wu and Shi 2008). Elicitors have also been reported to increase the production of valuable phenolic acids by *S. miltiorrhiza* in vitro cultures. MJ, Ag^{+} and YE enhanced the concentrations of rosmarinic acid and lithospermic acid (Xiao et al. 2009b, 2010), while salicylic acid effectively stimulated the production of salvianolic and caffeic acids (Dong et al. 2010) but suppressed growth. There are two biosynthetic pathways (the phenylpropanoid and tyrosine derived pathways) in the production of rosmarinic acid, which is a precursor of salvianolic acid B (Xiao et al. 2009b, 2010). Studies on gene transcription and the activities of key enzymes in both pathways (phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-transferase (TAT) showed different responses to elicitors. Elicitation with MJ increased PAL and TAT transcripts in *S. miltiorrhiza* hairy roots (Xiao et al. 2009b), but treatment with Ag^{+} and YE reduced PAL activity while increasing that of TAT (Yan et al. 2006). These opposing effects of Ag^{+} elicitation on PAL and TAT activity were confirmed by analyzing the transcription of the corresponding genes in *S. miltiorrhiza* hairy roots (Xiao et al. 2010). A study on *S. miltiorrhiza* cell suspensions revealed an increase in caffeic acid after SA elicitation which closely resembled the increase in PAL activity. (Dong et al. 2010). However, no corresponding increase in TAT was observed in this case.

In order to understand the effects of elicitors, it is important to examine the synthesis of both the target metabolite(s) and various side-pathway products. The production of rosmarinic acid competed with that of homogenistic acid (an intermediate along a side-pathway) in both control and MJ-elicited *S. miltiorrhiza* hairy roots culture. However, the elicited system produced lithospermic acid B (a dimer of rosmarinic acid) in much larger quantities than the control (Xiao et al. 2009b). Similar effect on biosynthesis of lithospermic acid was also observed in response to elicitation with Ag^{+} (Xiao et al. 2010). Because the metabolic consequences of elicitation have not yet been fully elucidated, more comprehensive studies should be performed in order to explain why in some cases, elicitation-induced increases in the activity or

transcription of key biosynthetic enzymes are accompanied by decreases in the abundance of target metabolites.

Process modeling is likely to play a key role in the development of efficient elicitation protocols for use with sage in vitro cultures. A simulation-based investigation into tanshinones production by *S. miltiorrhiza* hairy roots using YE elicitation and resins for product recovery has been reported (Yan et al. 2011). These authors found that the elicitor increased tanshinones production while the resin had no effect, either by itself or synergistically with the elicitor. They also showed that the adsorption capacity of the resin had no effect on tanshinones accumulation and that the rate at which the compounds were released from the roots into the medium was the limiting factor in the process (Yan et al. 2011).

In summary, the results discussed above demonstrate that the combined use of biotic and abiotic methods of elicitation represents a powerful tool for increasing secondary metabolite production from sage in vitro cultures and in other cases as well. By itself, osmotic stress is only moderately effective at increasing target compound yields. However, when used in combination with other elicitors, it is an extremely effective way of increasing metabolite yields in *Salvia* in vitro systems. At present, the greatest barrier to the commercial use of *Salvia* cultures for secondary metabolite production is their low and variable productivity of the desired compounds. The most effective way of addressing this issue is likely to involve improved methods of fed-batch cultivation and the development of efficient elicitor recycling systems (Wu and Shi 2008). The introduction of such methods will be crucial in unlocking the potential of sage cell cultures and will significantly increase their commercial viability.

The manipulation of metabolic pathways in *Salvia* by genetic engineering

The biosynthetic potential of *Salvia* in vitro systems can be improved by over-expressing, up-regulating, silencing or introducing new genes that encode rate-limiting enzymes or key transcription factors responsible for secondary metabolite formation (Zhou et al. 2011). Several genes that are involved in the biosynthesis of phenolics and terpenoids in *Salvia* species have been cloned over the last few years (Wu et al. 2009; Xiao et al. 2009a, b; Song and Wang 2009; Dai et al. 2011; Hua et al. 2012; Jin et al. 2012). Over-expression of single genes, encoding enzymes from the phenylpropanoid or tyrosine-derived pathways (e.g. cinnamic acid 4-hydroxylase, TAT and 4-hydroxyphenylpyruvate reductase) can boost the production of rosmarinic and lithospermic acids in *S. miltiorrhiza* hairy root cultures (Xiao et al. 2011). The co-expression of the genes encoding TAT and 4-hydroxyphenylpyruvate reductase had a particularly strong effect on phenolic acids production (4.3- and 3.2-fold increase in rosmarinic and lithospermic acids) (Xiao et al. 2011).

In general, the simultaneous over-expression of multiple genes seems to be the most powerful strategy for increasing secondary metabolite yields in *Salvia* cells. The so-called "push and pull" strategy whereby genes encoding key enzymes from both the upstream and downstream sections of the targeted biosynthetic pathway are over expressed together (Fig. 2) has recently been used to increase tanshinones production in *S. miltiorrhiza* hairy roots (Kai et al. 2011). The authors found that co-expression of *SmHMGR* (the gene encoding 3-hydroxy-3-methylglutaryl-CoA reductase, an enzyme from the upstream mevalonate pathway) and *SmGGPPS* (gene encoding geranyldiphosphate synthase, a key enzyme from the downstream tanshinones biosynthetic pathway) caused a 4.7-fold increase in tanshinones production relative to a control culture (Kai et al. 2011). In addition, the authors showed that the over expression of *SmDXS* (gene encoding 1-deoxy-D-xylulose-5-phosphate synthase, an enzyme from the upstream methylerythritol phosphate pathway) had significantly stronger pushing effect than *SmHMGR* in tanshinones production, which supported the suggestion that tanshinones are primarily produced via the methylerythritol phosphate pathway rather than the mevalonate pathway (Kai et al. 2011).

Because terpenoid biosynthesis in *Salvia* cells involves multiple reaction steps that are catalyzed by a complex enzyme network (Fig. 2), there is a need for more detailed information on the enzymes involved and associated regulatory genes. A complex systematic analysis of genes, participating in terpenoid formation in *S. miltiorrhiza* plants has recently been conducted and led to the identification of 40 genes, 27 of which were previously unknown (Ma et al. 2012). The authors demonstrated that some of the key

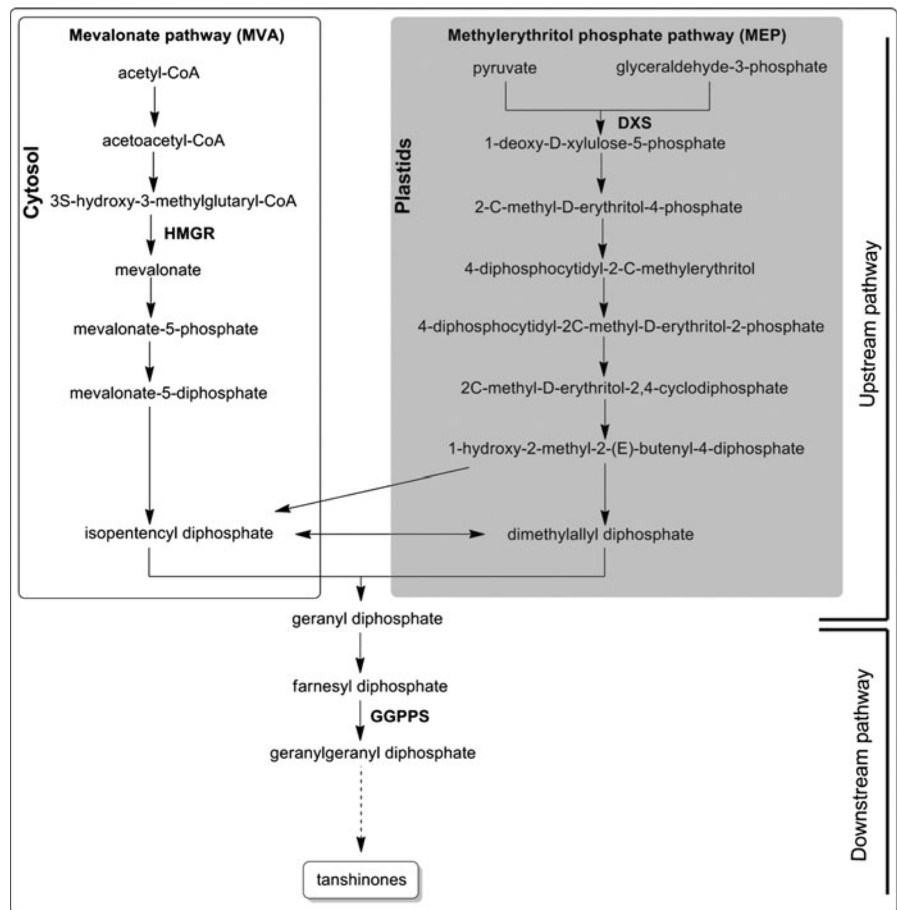

Fig. 2 Biosynthetic pathway for tanshinones production in *Salvia*. HMGR—3-hydroxy-3-methylglutaryl-CoA reductase; DXS—1-deoxy-D-xylulose-5-phosphate synthase; GGPPS—geranylgeranyldiphosphate synthase

enzymes in terpenoid biosynthesis (such as DXS, HMGR and GGPPS) are encoded by multiple genes and have different expression patterns and subcellular localizations (Ma et al. 2012). The results of this investigation have provided important new insights that will be useful for the engineering and manipulation of terpenoid biosynthesis in *Salvia* in vitro systems.

Hairy root/bacteria co-cultures

Because secondary metabolites play integral roles in the defense mechanisms of plant cells, their biosynthesis can be significantly up-regulated in response to microbial attack. Under controlled environmental conditions, such effects can be achieved by using biotic elicitors by co-culturing appropriate bacteria with the plant cells. Live bacteria are constant source of biotic elicitors which can have very strong effect on secondary metabolites accumulation in plant cells (Wu et al. 2007; Zhao et al. 2010b). However, the bacteria may outcompete slow-growing plant cells under poorly-chosen conditions. It is therefore necessary to carefully optimize and analyze several factors when establishing plant-bacteria co-cultures in vitro including the composition of the nutrient medium, the nature of the plant–microbe interactions, the microbial culture density, and the timing of the addition of the microbial culture. Root-colonizing bacteria (rhizobacteria) are harmless to plants and naturally stimulate their growth by facilitating the nutrient uptake. The most efficient such species for the establishment of in vitro co-cultures of bacteria and *Salvia* hairy roots seems to be *Bacillus cereus* (Zhao et al. 2010b).

A recent study examined the effects of *B. cereus* cell suspensions, bacterial extracts and supernatant on production of tanshinones by *S. miltiorrhiza* hairy roots. The bacterial polysaccharide stimulated tanshinones biosynthesis while the bacterial protein fraction promoted hairy roots growth. However, the bacterial

extracts and supernatant had less pronounced effects on tanshinones accumulation in hairy roots (Zhao et al. 2010b). The inoculation of *S. miltiorrhiza* hairy roots with various concentrations of *B. cereus* cells at the beginning of cultivation process had a very strong positive effect on tanshinones accumulations (12- to 18-fold increase), but also significantly suppressed roots growth (up to 50 % less biomass) and the overall yield remained low (Wu et al. 2007; Zhao et al. 2010b). After optimization of the inoculation timing, a 12-fold increase in the volumetric tanshinones yield was achieved by adding a suspension of *B. cereus* cells (2.5 % v/v) to an 18-day-old *S. miltiorrhiza* hairy root culture (Zhao et al. 2010b). Obviously, hairy root-bacteria co-cultures are potentially powerful and cost-effective tools for boosting the yields of target secondary metabolites from sage in vitro systems. However, more experimental work will be required to develop fully optimized culture conditions and protocols.

Conclusions and future prospective

Over the last decade there has been considerable commercial and academic interest in the biological activity of tanshinones produced Danshen and an overview of relevant patents has been produced by Tian and Wu (2013). The demand for Danshen biomass is expected to increase in the next few years because the extracts of *S. miltiorrhiza* are currently undergoing clinical trials in the United States (Hatfield et al. 2013). Similar increases in demand are expected for other *Salvia* species since they produce other valuable compounds such as rosmarinic, carnosic, ursolic and oleanolic acids. Ursolic and oleanolic acids have been recommended for skin cancer therapy in Japan (Kuttan et al. 2011), while tanshinone IIA (in the form of a sodium sulfonate derivate) has already been approved for the treatment of cardiovascular diseases in China (Tian and Wu 2013). In addition, a new microemulsion technique has been developed to enhance the delivery efficiency of tanshinone IIA, which significantly increase its antitumor activity (Ma et al. 2013). Plant in vitro systems are eco-friendly for the production of biomass and secondary metabolites from *Salvia* species and have great commercial potential. However, a deeper understanding of the regulatory processes that govern the biosynthesis of secondary metabolites in *Salvia* in vitro systems will be required to enable their exploitation. Innovative approaches based on genetic engineering (Kai et al. 2011), two-phase cultivation systems (Marchev et al. 2011b) and the combination of elicitation with two-phase cultivation (Yan et al. 2011) can be used to increase and stabilize the yields of desired pharmacologically valuable compounds from *Salvia* in vitro systems. In addition, modern cultivation and monitoring technologies such as RAMOS systems (Geipel et al. 2013) can be used to expedite optimization procedures and accelerate the development and scale-up of the entire production process.

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