Rosmarinic Acid and Related Metabolites

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Abbreviations

ATP	Adenosine triphosphate
BAP	6-benzylaminopurine
C4H	Cinnamic acid 4-hydroxylase
CoA	Coenzyme A
DHPL	3,4-dihydroxyphenyllactic acid
DMSO	Dimethyl sulfoxide
DW	Dry weight
2,4-D	2,4-dichlorophenoxyacetic acid
3′-H	Hydroxycinnamoyl-hydroxyphenyllactate 3′-hydroxylase
3-H	Hydroxycinnamoyl-hydroxyphenyllactate 3-hydroxylase
4CL	4-coumarate CoA-ligase
HdhA	Hydroxyacid dehydrogenase
HpaBC	4-hydroxyphenylacetate 3-hydroxylase
HPPD	Hydroxyphenylpyruvate dioxygenase
HPPR	Hydroxyphenylpyruvate reductase
HQT	Hydroxycinnamoyl-CoA quinate hydroxycinnamoyltransferase
HR	Hairy roots
HST	Hydroxycinnamoyl-CoA shikimate hydroxycinnamoyltransferase
IAA	Indole-3-acetic acid
MeJA	Methyl jasmonic acid
NAA	1-naphthaleneacetic acid
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate), reduced
PAL	Phenylalanine ammonia-lyase

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pHPL	4-hydroxyphenyllactic acid
pHPP	4-hydroxyphenylpyruvic acid
RA	Rosmarinic acid
RAS	Rosmarinic acid synthase, hydroxycinnamoyl-CoA:hydroxyphenyllactate hydroxycinnamoyltransferase
SA	Salicylic acid
TAL	Tyrosine ammonia-lyase
TAT	Tyrosine aminotransferase
YE	Yeast extract

2.1 Occurrence and Structures of Rosmarinic Acid and Related Metabolites

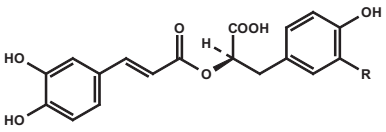
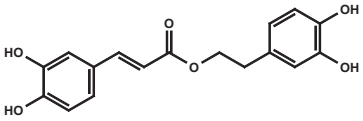
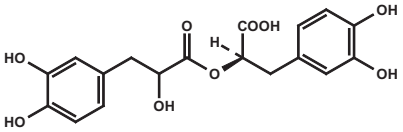
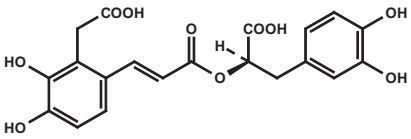
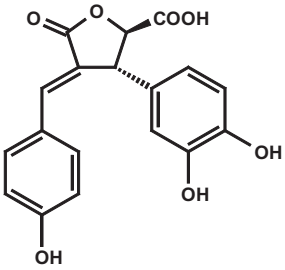
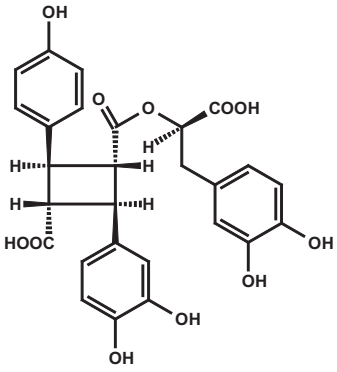
Rosmarinic acid (RA) (Table 2.1) was first described in 1958 as an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (DHPL) extracted from rosemary (*Rosmarinus officinalis*) [140], but has since then been detected in plant species across the plant kingdom from hornworts to mono- and dicotyledonous plants (for reviews see [126, 129]). Hotspots of RA presence are the sub-family Nepetoideae of the Lamiaceae and the family Boraginaceae. In other plant taxa, RA often only occurs sporadically and may not occur in all species of the same genus [126]. RA and related caffeic acid esters have been isolated from hornworts (species of the genera *Anthoceros*, *Folioceros*, *Nothothylas*, *Phaeoceros*, *Dendroceros*, *Megaceros*; [6, 155, 156, 162, 163]) as well as fern species (*Blechnum* spec.; [68, 70, 169]). Furthermore, grasses [32, 109] and species of the so-called basal orders (*Sarcandra glabra*, [191]; *Chloranthus* spec., [129]) contain RA, whereas there are – up to now – no reports from leafy mosses, liverworts and gymnosperms.

A larger number of derivatives of RA have been described, many of them occurring in *Salvia* species (Table 2.1; [18, 79, 103, 168]). These derivatives generally contain RA as core structure. Metabolites often incorrectly described as caffeic acid oligomers contain additional 4-coumaric or caffeic acid moieties or a second RA molecule. Further derivatization can occur by glycosidation (e.g. RA glucosides; [43, 95, 165]) (**see also Chap. 9 of this book**), methylation (e.g. methyl rosmarinate, methyl lithospermate [90], methylmelitric acid [105]) or the addition of ethyl and butyl or hydroxycinnamoyl moieties.

Whereas the biosynthesis of RA in Lamiaceae and Boraginaceae (e.g. *Coleus blumei*, *Salvia miltiorrhiza*, *Melissa officinalis, Anchusa officinalis, Lithospermum erythrorhizon*) is well investigated [106, 126, 128, 129], it is less well analyzed in other plant taxa. The same is true for the formation of most of the above-mentioned RA derivatives.

There are thousands of publications on the diverse biological activities of RA and derivatives such as the salvianolic acids. Recent reviews on this topic have been published by e.g. Shetty [142], Wang [168], Bulgakov et al. [18] and Amoah et al. [5], and this topic will therefore not be covered in this article.

Table 2.1 Examples for rosmarinic acid and related compounds

Structure	Common name	Reference
	R = H isorinic acid	[139]
	R = OH rosmarinic acid	[140]
	Teucrol	[45]
	Salvianic acid C	[28]
	Salvianolic acid D	[4]
	Megacerotonic acid	[155]
	Anthocerotonic acid	[155]

(continued)

Table 2.1 (continued)

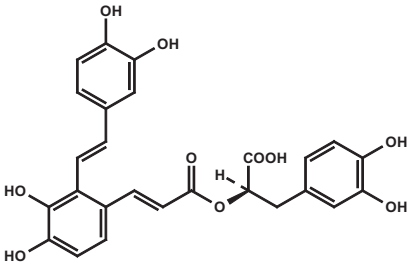
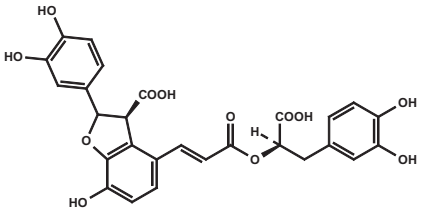
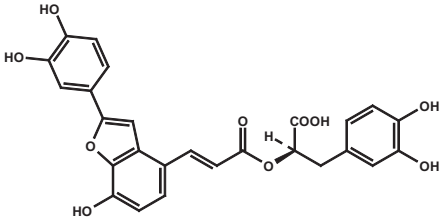
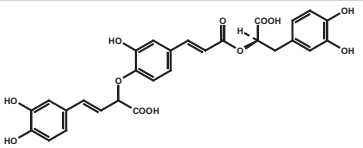
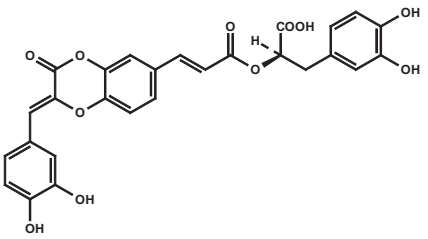
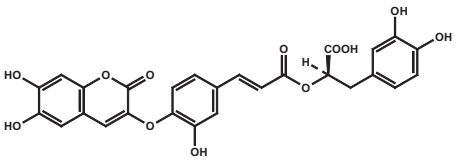
Structure	Common name	Reference
	Salvianolic acid A	[96]
	Lithospermic acid	[82, 167]
	[Monardic acid A = (7*R*,8*R*)-stereoisomer of lithospermic acid]	[114]
	Salvianolic acid C	[3]
	Melitric acid A	[2]
	Melitric acid B	[2]
	Sagecoumarin	[105]

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Table 2.1 (continued)

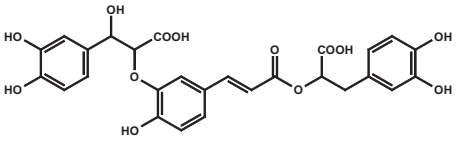
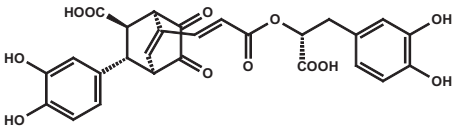
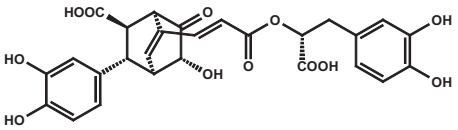
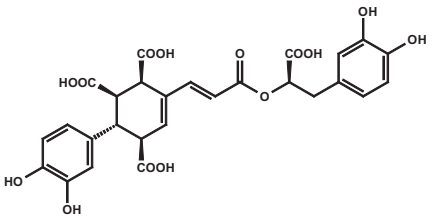
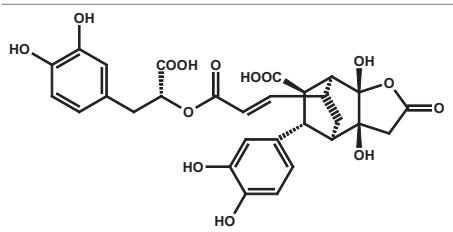
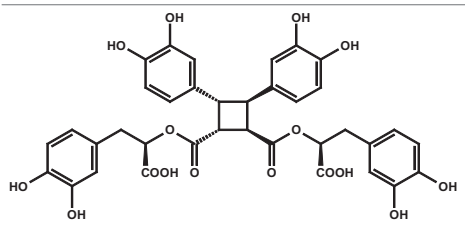
Structure	Common name	Reference
	Salvianolic acid K	[102]
	Yunnaneic acid C	[158]
	Yunnaneic acid D	[158]
	Yunnaneic acid E	[159]
	Yunnaneic acid F	[159]
	Sagerinic acid	[102]

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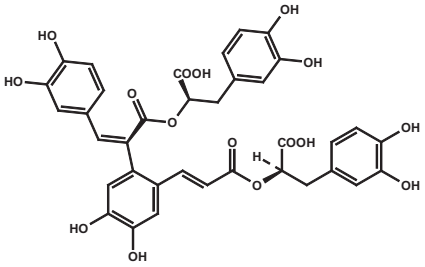
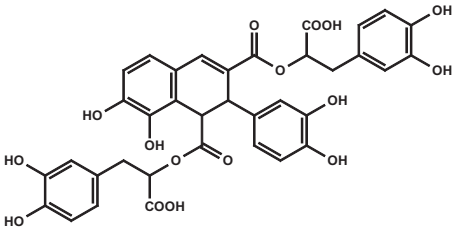
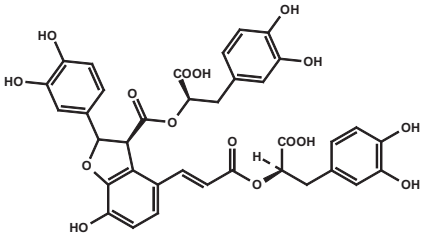
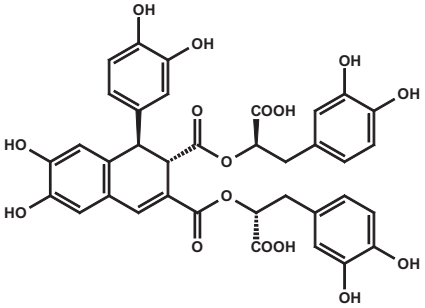
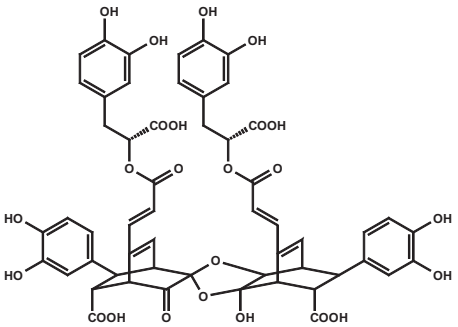
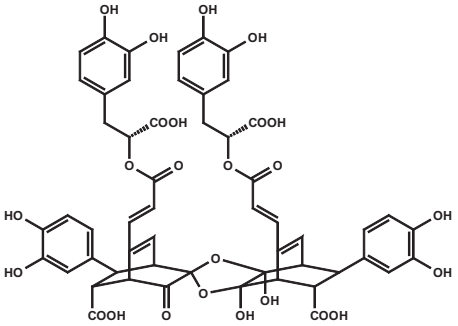
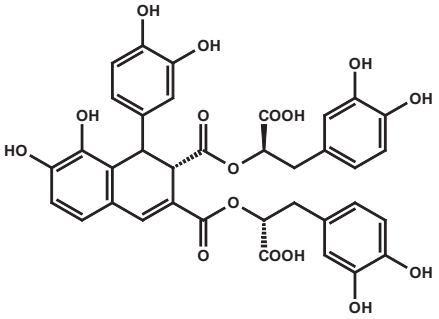
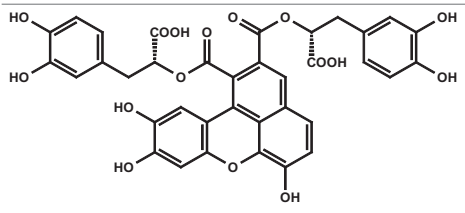
Table 2.1 (continued)

Structure	Common name	Reference
	Salvianolic acid E	[4]
	Salvianolic acid L	[104]
	Salvianolic acid B = lithospermic acid B	[3, 157]
	[Monardic acid B = (7*R*,8*R*)-stereoisomer of lithospermic acid B]	[114]
	(−)-Rabdosiin	[1] [103]

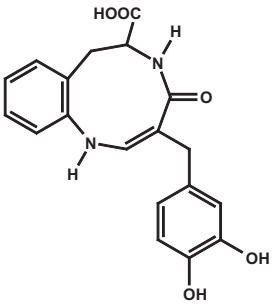
(continued)

Table 2.1 (continued)

Structure	Common name	Reference
	Yunnaneic acid A	[158]
	Yunnaneic acid B	[158]
	Yunnaneic acid G	[159]
	Yunnaneic acid H	[159]

(continued)

Table 2.1 (continued)

Structure	Common name	Reference
	Anthocerodiazonin	[163]

2.2 Biosynthetic Pathway of Rosmarinic Acid

RA is derived from two distinct pathways: The general phenylpropanoid pathway provides the caffeic acid moiety, while DHPL is produced by a tyrosine-derived pathway [130, 131]. Both pathways are dependent on the shikimate pathway, which generates the aromatic amino acid precursors L-phenylalanine and L-tyrosine. The biosynthetic pathway (Fig. 2.1) has first been elucidated in *Coleus blumei* [130], a member of the family Lamiaceae, and *Anchusa officinalis* [36], and to a great part confirmed in *Melissa officinalis* [171, 172].

The general phenylpropanoid pathway starts with L-phenylalanine as precursor. The enzyme phenylalanine ammonia-lyase (PAL) is responsible for the transformation of the amino acid to *trans*-cinnamic acid [134]. A cytochrome P450-dependent enzyme, cinnamic acid 4-hydroxylase (C4H), introduces the first hydroxyl group to the aromatic ring in *para* position to form 4-coumaric acid [124]. Then, the ATP-dependent coenzyme A (CoA) activation of 4-coumaric acid to 4-coumaroyl-CoA is catalyzed by the enzyme 4-coumarate CoA-ligase (4CL) [81].

L-Tyrosine is the precursor in the formation of the second intermediary precursor in RA biosynthesis. Tyrosine aminotransferase (TAT) catalyzes the transamination of tyrosine and 2-oxoglutarate to 4-hydroxyphenylpyruvate (pHPP) and glutamate [36]. In a NAD(P)H-dependent step, the enzyme hydroxyphenylpyruvate reductase (HPPR) reduces pHPP to 4-hydroxyphenyllactic acid (pHPL) [69, 127].

The trans-esterification of the two precursors is catalyzed by rosmarinic acid synthase (RAS). This enzyme forms an ester of 4-coumaric acid and pHPL [127] and belongs to the BAHD acyltransferase superfamily in the subgroup hydroxycinnamoyltransferases [14]. The product 4-coumaroyl-4′-hydroxyphenyllactic acid is hydroxylated at the 3- and 3′- positions by two cytochrome P450-dependent enzyme activities, caffeoyl-4′-hydroxyphenyllactate 3′-hydroxylase and 4-coumaroyl-3′,4′-dihydroxyphenyllactate 3-hydroxylase (3'H, 3H) (Fig. 2.1) [124]. The product, RA, is then stored in the vacuole. For comprehensive reviews on biosynthesis, distribution and evolution of RA biosynthesis see e.g. Petersen and Simmonds [128] and Petersen [126].

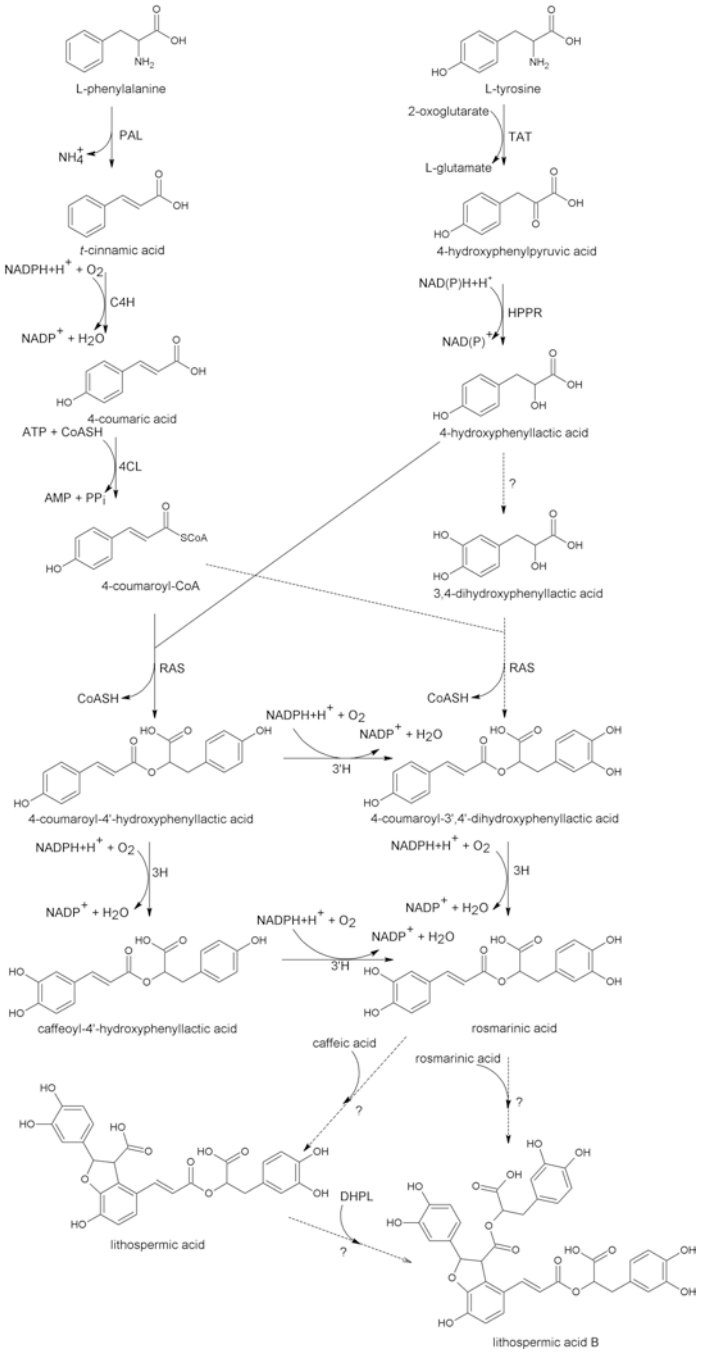


Fig. 2.1 Biosynthetic pathway of rosmarinic acid and derivatives as evaluated in *Plectranthus scutellarioides* (syn. *Coleus blumei*) [130] and *Salvia miltiorrhiza* [39, 177]. Reactions specifically described in or proposed for *Salvia miltiorrhiza* are shown by dashed lines and arrows. *PAL* phenylalanine ammonia-lyase, *C4H* cinnamic acid 4-hydroxylase, *4CL* 4-coumarate CoA-ligase, *TAT* tyrosine aminotransferase, *HPPR* hydroxyphenylpyruvate reductase, *RAS* "rosmarinic acid synthase" (4-hydroxycinnamoyl-CoA:4′-hydroxyphenyllactate hydroxycinnamoyltransferase), *3H, 3'H* 3- and 3′-hydroxylases

Di et al. [39] suggested an alternative pathway in *Salvia miltiorrhiza*. They propose that an additional hydroxylation of pHPL to DHPL occurs prior to the esterification. Accordingly, the product 4-coumaroyl-3′,4′-dihydroxyphenyllactate undergoes a single hydroxylation. While Di et al. [39] furthermore propose a direct formation of lithospermic acid B by coupling of two molecules of RA, Xiao et al. [177] suggest a sequential formation by addition of caffeic acid and DHPL in two separate reactions (Fig. 2.1).

2.3 Production of RA in Untransformed Aseptic *In Vitro* Cultures

Cell cultures of species of the families Lamiaceae and Boraginaceae have been established for the biotechnological production of RA and related compounds. However, although these efforts were successful at pilot scale, an industrial-scale production process for RA has never been established. Efforts to optimize RA production in *in vitro* cultures are summarized in the following paragraphs.

2.3.1 Species from the Family Lamiaceae

Coleus blumei **(syn.** *Solenostemon scutellarioides***,** *Plectranthus scutellarioides***)** The first reports on the formation of high amounts of RA in plant callus and cell suspension cultures are from 1977. Razzaque and Ellis [134] as well as Zenk et al. [187] both used *Coleus blumei*, the painted nettle, to establish suspension cultures that accumulated up to 15% of the cell dry weight (DW) as RA. The latter authors also described the influence of the sucrose concentration of the medium on the outcome of RA production as well as the incorporation of exogenously fed L-phenylalanine. The same species was used by Ulbrich et al. [164] in the first biotechnological production process, a two-phase culture system with a growth and a production phase. In the latter phase, a 5% sucrose solution was used for cultivation and a yield of 21% RA in the cell DW was achieved. Since then, *in vitro* cultures of *Coleus blumei* have been the most prominent system to elucidate the biosynthetic pathway of RA and to isolate and characterize the respective enzymes and genes (see below and review articles by [126, 128, 129]).

The influence of the carbohydrate source and concentration was investigated in more detail by Gertlowski and Petersen [54] and Petersen et al. [132]. They showed that sucrose is quickly cleaved into glucose and fructose. The optimal sucrose concentration was at 5%. Glucose as sole carbohydrate source was nearly as effective as sucrose while fructose led to a lower RA accumulation. The stimulating effect of higher sucrose concentrations on RA biosynthesis and accumulation is not due to an osmotic effect since partial replacement by mannitol could not promote RA formation. The onset of RA biosynthesis is independent of the sugar concentration and correlates with the depletion of mineral nutrients (e.g. phosphate) from the medium. Medium optimization was also done by Ju et al. [80] in order to establish a

two-phase culture system with a growth phase and a production phase for callus as well as suspension cultures. Essentially the same results were obtained as described before: higher sucrose concentrations increased the formation and accumulation of RA. With 6% sucrose in the medium calli contained 33.7% RA in the DW, suspension cells accumulated RA to 10.1%.

Permeabilization was investigated in order to isolate RA from the medium instead of the cells and thus enable a continuous production process. For this purpose, Park and Martinez [118] added dimethyl sulfoxide (DMSO) to the suspension cultures. This, however, resulted in loss of cell viability. Preconditioning at a lower DMSO concentration (0.1%) ensured cell viability at higher DMSO levels (0.5–1.5%) and resulted in a prominent release of RA to the medium. With 0.5% DMSO, 2.85 g RA per 100 g cell DW was found in the medium, which was 66.4% of the total RA production.

Immobilization of *Coleus blumei* cells was performed by adding luffa cubes to a suspension culture in order to capture the cells within the sponge-like luffa material [107]. Luffa is the dry fibrous material of berry endocarp of *Luffa cylindrica*, Cucurbitaceae. After 33 days of growth, the cell-inhabited cubes were placed into a glass column and fed with medium by spraying it from the top. The cells were viable to a high percentage until 52 days but showed strongly reduced growth. RA production was higher (2% of the cell DW) than in the respective parent suspension culture (1.2%).

Approaches to further increase the production of RA were based on elicitation or transformation of *Coleus blumei* cell cultures; the latter will be described in the chapter "Hairy roots". Fungal elicitor preparations (*Pythium aphanidermatum*) or methyl jasmonate (MeJA) were added to suspension cultures of *Coleus blumei* and resulted in increased activities of some biosynthetic enzymes as well as about a three-fold RA accumulation [153]. Interestingly, an effect of the volatile MeJA could also be seen when it was applied via the gas phase.

Bauer et al. [12] investigated RA accumulation in different callus cell lines transformed by *Agrobacterium tumefaciens* and cultivated on hormone-free media. Growth and RA accumulation varied between different lines. The highest RA accumulation was 11% of the cell DW.

Whole *in vitro* grown plants of *Solenostemon scutellarioides* were investigated by Dewanjee and coworkers [37, 38]. Feeding of precursors (Phe and Tyr alone and in combination) could increase RA levels (up to 3.1-fold) as well as the activities of PAL, TAT and RAS. On the other hand, phytopathogenic fungi were applied with best results using *Alternaria alternata*. This increased RA accumulation up to 1.6-fold (18.5 mg/100 g fresh weight).

Salvia officinalis Various varieties of culinary sage (*Salvia officinalis*) were compared with respect to their RA accumulation in leaves and suspension cultures. The RA content varied between 0.89% and 7.82% of the DW, the best variety being "Dwarf". In all varieties except one, leaves contained less RA than suspension cells [174]. A similar approach was followed by Grzegorcyzk et al. [58] who compared the RA content in seed-derived and *in vitro* regenerated sage plants as well as shoot

callus and cell suspension cultures after different numbers of passages. Here the highest RA content was found in suspension cells with around 1.9% of the cell DW. Hippolyte et al. [71, 72] characterized RA production in suspension cultures of sage further. The optimal sucrose concentration was 5%, which resulted in an RA content of 19% of the DW at the end of the culture period. Feeding of L-phenylalanine as precursor shortened the production period and enhanced RA production at 0.1 g/l phenylalanine in 5% sucrose medium. The highest RA accumulation of 36% of the cell DW could be observed in a low osmolarity medium (Heller-medium with 5% sucrose).

A number of studies reported on shoot cultures of sage as a source for RA. MS agar medium supplemented with different concentrations of the long chain saturated primary alcohol triacontanol showed positive effects with respect to shoot multiplication as well as diterpene and RA content. Highest RA concentrations of approximately 2% of the DW were found after addition of 20 µg/l triacontanol [59]. Shoots grown in liquid medium accumulated around 3% RA in the DW irrespective of the triacontanol concentration [55]. MeJA (50 and 100 µM) stimulated RA levels in liquid cultivated shoots even further to 4.1% of the DW on the fifth day after elicitation [56]. Shoot cultures were also cultivated in a laboratory scale sprinkle bioreactor with a 43-fold increase in biomass after 3 weeks and a RA content of 2.6% of the DW [57].

Shoot cultures on solidified MS medium were used to test the effect of sodium salicylate on diterpene (carnosol, carnosic acid) and RA production. Although the amount of diterpenes was stimulated by elicitiation, the RA levels remained largely unaffected and growth was decreased [93]. In contrast, Ejtahed et al. [46] showed a two-fold increase in RA production to 1.8% of the DW in shoot cultures after addition of 250 µM salicylic acid (SA).

Salvia miltiorrhiza *Salvia miltiorrhiza* is an important Asian medicinal plant which is very well investigated with respect to its accumulation of tanshinones and phenolic acids, among them RA, lithospermic acids and salvianolic acids (Table 2.1). Many investigations have been performed with hairy root cultures and are described below. In addition, undifferentiated cell cultures were the basis for the production of phenolic acids [42]. Morimoto et al. [112] showed that callus cultures accumulated 1.24% of the DW as RA and 0.1% as lithospermic acid B. In shoot cultures regenerated from these calli, both phenolic acids accumulated in considerably higher amounts (6.96% RA and 6.05% lithospermic acid B). The same compounds were detected in Ti-transformed suspension cells (4.59% RA and 0.81% lithospermic acid in the cell DW) [26]. In a special 6,7-V-medium, the same cell line produced phenolic acids (RA 530 mg/l and lithospermic acid B 216 mg/l) as well as tanshinones (220 mg/l), the latter being excreted to the medium to a considerable extent [21]. In an attempt to increase secondary metabolite production, yeast extract (YE) was used. This resulted in nearly doubled tanshinone production, while RA accumulation was strongly reduced [22, 23]. MeJA (10 µmol/l) also stimulated the activities of PAL and TAT as well as RA accumulation [179]. Addition of Ca^{2+} ions

(10 mM) enhanced the accumulation of RA up to 2% of the DW. This was also coupled to enhanced PAL and TAT activities [101].

Stems and leaves of *Salvia milthiorrhiza* were used by [175] to establish callus cultures and to compare their efficacy to produce RA and salvianolic acid B. Stem callus contained more phenolic acids (1.27 ± 0.38% RA and 0.87 ± 0.20% salvianolic acid B in the DW) than leaf callus (0.28 ± 0.02% RA and 0.07 ± 0.03% salvianolic acid B).

SA (22.5 mg/l) was used as elicitor to increase the production of phenolic acids. Addition of SA resulted in a doubled RA accumulation 2 days after addition (to approximately 0.03% of the DW). At the same time, H_2O_2 levels increased. External addition of H_2O_2 (10 mM) also induced RA formation and it was suggested that H_2O_2 is a mediator in elicitation processes by SA [66]. Besides increase in RA formation (to 1.1%), SA addition also induced Ca^{2+} mobilization. Extracellular addition of calcium ions (10 mM) or the calcium ionophore A23187 also enhanced RA levels [64]. SA was shown to lower the cytoplasmic pH by inhibition of the plasma membrane H^+-ATPase. The RA content was increased to about 2.25-fold of the control level [99].

Ocimum basilicum Undifferentiated *in vitro* cultures of basil (*Ocimum basilicum*) were investigated by Kintzios et al. [88]. Leaf-derived suspension cultures accumulated about 10% of the cell DW as RA. Immobilization in calcium alginate resulted in a dramatic decrease of the RA level. Immobilization in test tubes at high cell density (25×10^4 cells/ml; approximately 4 ml volume; mini-bioreactor), in contrast, resulted in highly enhanced RA production and RA concentrations of 2% of the cell DW could be achieved. RA was also determined in the medium with about 5 mg/ml in the first week of the experiment [113]. Nodal shoot explants and suspension-cultured cells of *Ocimum basilicum* were incubated in a small bioreactor by [87]. They reported increased growth and RA accumulation in the bioreactor-cultivated plant material. Highest RA levels (0.02% of the DW) were found in the organized plant material.

Addition of YE (0–5 g/l) positively influenced RA formation in basil callus cell lines from 0.67% in controls to 2.3% in the DW on medium with 5 g/l YE [63].

A red-colored cell line of basil accumulated RA and anthocyanins, both of which arise from the general phenylpropanoid pathway. Strazzer et al. [144] chose a stable anthocyanin-producing cell line that also accumulated 0.8 mg/g fresh weight RA and subjected these cells to mechanical stress (enhanced agitation) and light stress. Both treatments led to increased RA accumulation (up to 1.9 mg/g fresh weight for combined light and mechanical stress), and in parallel anthocyanin accumulation was enhanced as well. Since both biosynthetic pathways require phenylpropanoid precursors, the overall flux into the phenylpropanoid pathway must have increased. The authors also propose that both stressors might increase the formation of reactive oxygen species which can be quenched by both, RA and anthocyanins.

In vitro shoot regeneration from basil nodal explants was performed by [84]. They found highest RA levels (approximately 40 mg/g DW) in fully acclimatized plantlets. The effect of benzyladenine on RA accumulation was dependent on the

basil cultivar. In the anthocyanin-producing variety, the accumulation of anthocyanin and RA were inversely correlated with the benzyladenine concentration. The same group used different culture vessel types for the micropropagation of basil shoots. A prominent difference with respect to RA accumulation (approximately 16% of the cell DW compared to 4% in other culture systems) was observed, which was inversely correlated with biomass accumulation [83].

Orthosiphon aristatus *Orthosiphon aristatus* (Java tea) was first used as suspension culture for the production of RA by Sumaryono et al. [152] and Sumaryono and Proksch [151]. These cells synthesized about 1–2 μmol RA per g fresh weight. After elicitation with YE (4–6 g/l), RA accumulation increased to 10 μmol/g fresh weight 3–4 days after elicitor addition; decarboxylated RA was found as well.

Cell cultures of *Orthosiphon aristatus* established from plants from different locations were analysed with respect to their growth characteristics. Highest RA contents ranged between 4.5% and 5.0% of the cell DW [100].

Glechoma hederacea A suspension culture of *Glechoma hederacea* accumulated up to 25.9% RA in the cell DW in CB2-medium [41] in only 7 days of culture. Besides, lower amounts of caffeic acid and chlorogenic acid were detected. This is one of the highest levels of RA accumulation described so far.

Lavandula vera Several aspects of medium optimization and elicitation have been evaluated in the course of investigations on *Lavandula vera* suspension cultures. RA was identified as the main phenolic metabolite [92]. Linsmayer and Skoog medium was used as the basic medium. Several medium components were varied and finally an optimized medium presented [77, 123]. Raising the sucrose content of the medium from 3% to 7% strongly reduced the biomass accumulation to 45% of the control but at the same time dramatically enhanced the RA yield to more than sevenfold of the control [75]. Doubling the phosphate concentration resulted in enhanced growth (131%) and enhanced RA production (206% compared to the control) [74]. Reduction of the medium's ammonium concentration to ¼ enhanced RA accumulation to 2.7 times of the control level (1.5% of the DW) but still ensured growth. Increasing the level of ammonium ions delayed the onset of RA biosynthesis and reduced the overall accumulation. Higher nitrate levels in the medium were reported to be beneficial for RA accumulation [76]. A combination of optimized medium parameters (NH_4NO_3, KNO_3, and KH_2PO_4) resulted in a 27-fold RA accumulation (17.9% of the cell DW) [123]. Feeding of the precursor phenylalanine strongly increased the amounts of caffeic acid and raised RA accumulation to 128% of the control level [119].

RA is mostly accumulated intracellularly. Adding the resin Amberlite XAD4 or a mixture of 4% polyethylene glycol and 7.5% dextran to the liquid medium as a two-phase culture system resulted in a release of RA to the extracellular phase. The total RA accumulation in presence of XAD4 was slightly increased (115% of controls), but only 6.4% of the total amount of RA was adsorbed to the resin. Cultivation with polyethylene glycol and dextran as second phase strongly reduced biomass

accumulation, although the content of RA per cell remained unchanged. About 12% of the total RA amount was found in the extracellular phase [122].

Further optimization of RA production by *Lavandula vera* cell cultures was done in 3 l-bioreactors with respect to dissolved oxygen concentration, agitation speed and temperature with the result of doubling the RA production (3.5 g/l) compared to shake flask cultures [52, 120, 121] .

A selection of putatively high producing cell lines was achieved by applying a fluorinated phenylalanine derivative. As the best result, an enhanced RA accumulation from 0.5% of the cell DW to approximately 1% was observed [53].

A way of enhancing secondary metabolite production is elicitation which was also applied to *Lavandula vera* cultures. Different biotic elicitors such as bacterial homogenates and cell wall preparations did not result in increased RA accumulation [91]. An abiotic elicitor, vanadyl sulfate, was added to the culture 11 days after inoculation. The highest RA accumulation (280% of the control level) was observed with 25 mg/l vanadyl sulfate after 12 h. As an additional effect more RA was found extracellularly [49]. The addition of benzothiadiazole had only small effects, whereas elicitation with MeJA (50 μM) on day 11 enhanced RA accumulation 2.4 times [50]. Here, the best elicitation result in *Lavandula vera* suspension cultures was about 12.6% RA in the dry cell biomass (calculated with the published data).

Lavandula officinalis Common lavender cultivated as *in vitro* culture was investigated by Nitzsche et al. [115]. Suspension cultures contained about six to ten times the amount of RA as normal plants. Interestingly, here RA was also secreted to the medium, which has not been described frequently. Usually, secreted RA is quickly decomposed, e.g. by peroxidases (own unpublished observations) and thus cannot be identified as RA anymore. Application of jasmonic acid or stress by oxygen depletion changed the profile of phenolic metabolites but did not increase the RA content.

Satureja khuzistanica The Iranian species *Satureja khuzistanica* was used to establish a callus culture for RA production. On B5 medium with 5% sucrose, callus cells accumulated 7.5% RA in the DW [136]. Suspension cultures of the same species showed much higher RA contents (18% of DW) after 21 days [137]. It was shown that reducing the nitrogen content to ¼ decreased growth slightly and RA accumulation severely to 3.8% of the cell DW.

Melissa officinalis Although *Melissa officinalis*, lemon balm, is one of the most important RA-containing medicinal plants, *in vitro* cultures of *Melissa officinalis* are barely investigated. Extracts of lemon balm are used against *Herpes simplex* infection due to their content of phenolic compounds; the most important of them is RA. Besides RA, melitric acids A and B (Table 2.1) have been detected in *Melissa officinalis* [2]. Suspension cultures of lemon balm have been characterized and used as source for the isolation of cDNAs and genes for PAL, 4CL and RAS. Suspension cultures accumulated up to 6.7% of the cell DW as RA after 6 days of cultivation. The effect of increased sucrose concentrations was not as prominent as observed for

suspension cultures of *Coleus blumei* [171, 172]. Hot water extracts of whole *in vitro* cultured lemon balm plants were analysed by Barros et al. [9]. They showed a wide variety of phenolic acids in the plant material, of which sagerinic acid (Table 2.1) was dominant followed by lithospermic acid and RA (which commonly is named as the dominant phenolic acid). Attempts to increase the RA content in *Melissa officinalis* shoot cultures by treatment with 200 ppb ozone for 3 h resulted in a transient increase of the RA content (30 mg/g fresh weight) at 2 h after starting the ozone treatment [161] .

Ocimum sanctum Holy basil (*Ocimum sanctum*, syn. *O. tenuiflorum*) is cultivated for medicinal and religious purposes because of its essential oil composed of several phenolic compounds (e.g. eugenol, isoeugenol, estragol). It also contains other phenolic antioxidants, mainly RA (0.012–0.025% of the DW). Callus cultures derived from different plant organs showed RA concentrations of 0.14–0.27% of the DW [65].

Rabdosia rubescens The effect of the sucrose concentration and the ratio of NO_3^- to NH_4^+ on specialized metabolism and plant regeneration were tested by Dong et al. [40]. The best result with respect to RA was achieved with 5% sucrose and a NO_3^-/NH_4^+ ratio of 2:1.

Agastache rugosa The effect of MeJA on RA accumulation was investigated in suspension cultures of *Agastache rugosa* (Korean or Indian mint). 50 μM MeJA proved to be optimal for the stimulation of RA accumulation from 7.8 to 36.6% of the cell DW. Also other phenolic acids were present in higher levels. The expression levels of PAL, C4H and 4CL correlated well with the increase in the RA level [86].

2.3.2 Species of the Family Boraginaceae

Anchusa officinalis *Anchusa officinalis* was one of the first species taken into culture for the production of RA and the investigation of its biosynthesis. Suspension cultures accumulated up to 6% of the cell DW as RA and the accumulation phase correlated with the linear growth phase. Early biosynthetic investigations established that 20–30% of exogenously applied, radioactively labelled phenylalanine or tyrosine was incorporated into RA [33]. Microspectrophotometric investigations suggested that RA is accumulated in the vacuoles [20]. Ellis [44] also studied the accumulation of RA in clonal cell lines derived from single cells with known productivity. This showed that high-producing mother cells did not result in high-producing clonal cell lines. After several subcultures each cell line established a quite stable level of RA production, which was not related to the RA production level of the mother cell. The optimization of the culture medium with respect to macronutrients (sucrose, alternative sugars, nitrate, phosphate and Ca^{2+}) and phytohormones (2,4-D, NAA, IAA and 2-chloro-4-fluorophenoxy-

acetic acid as auxins; BAP, kinetin and zeatin as cytokinins) was undertaken by De Eknamkul and Ellis [34, 35]. Surprisingly, a combination of all optimized levels of single macronutrients did not result in increased growth and RA production. Variations of phytohormone contents were performed in standard B5-medium. The highest RA levels of 12% of the DW were achieved in medium with 0.25 mg/l NAA as auxin, while medium with 2,4-D showed a decrease in RA accumulation. In contrast to previous results, the onset of RA synthesis was shifted to the exponential growth phase.

Su and Humphrey [145] established a high density culture of *Anchusa officinalis* with perfusion and tested several growth media. Using this technique the RA yield was doubled in comparison to control cultures. This, however, was only based on higher cell densities (38 g DW/l compared to approximately 14 g/l) while the RA content in the cells (approximately 3.3% of the DW) decreased slightly. The principle of perfusion culture was transferred to a membrane-aerated bioreactor. Here, the cell density was at 26 g/l and the calculated cellular RA content approximately 4.6% of the DW [146]. Optimization of the perfusion strategy in shake flasks led to higher productivity with respect to RA. The best result was obtained by growing the culture as batch culture in B5 medium with 3% sucrose and 0.25 mg/l NAA for 10 days, followed by perfusing the culture with B5 medium containing 6% sucrose and the same NAA concentration at a constant perfusion rate of 0.1/day. The obtained cell density was 35 g/l and 11.3% RA were found in the cell DW [149]. This procedure has been transferred to a stirred-tank bioreactor with similar productivity. However, the suspension cells proved to be very sensitive to agitation, aeration conditions and the dissolved oxygen concentration [148]. The inoculum size strongly influenced the productivity with best results at 4 g DW/l [147]. The results with a perfusion culture of *Anchusa officinalis* have been summarized by Su et al. [150].

Lithospermum erythrorhizon Suspension cultures of *Lithospermum erythrorhizon* were mainly investigated with respect to their accumulation of the red pigment shikonin. However, unpigmented cell cultures also accumulate RA (0.55% of the DW) and lithospermic acid [48]. Interestingly, the accumulation of phenolic acids and shikonin cannot occur under the same culture conditions but require different culture media. Elicitors such as YE and MeJA were added to increase the RA amount up to 0.22% of the cell fresh weight [110, 111]. Elicited cell cultures were mainly used to investigate the biosynthetic pathway for RA in this species. Besides RA, Yamamoto et al. [181, 182] identified RA-related compounds in *Lithospermum erythrorhizon* such as rhabdosiin, lithospermic acid and lithospermic acid B as well as lithospermic acid B glucoside (Table 2.1). Among these, lithospermic acid B was the predominant compound. Addition of MeJA or YE strongly increased the formation of RA by factors of 10- and 4-fold of the control cells, respectively. At the same time, the activities of PAL, 3H and 3'H were increased while RAS activity remained at a rather low level [117].

2.3.3 Non-vascular Plant Species

Anthoceros agrestis **(Anthocerotaceae)** The occurrence of RA in non-vascular plants like the hornworts was first described by Takeda et al. [155, 156]. Hornworts are among the earliest land plants to evolve. Nevertheless, hornworts contain RA and related compounds like anthocerotonic acid, megacerotonic acid and anthocerodiazonin (Table 2.1) as well as other phenolic compounds [162, 163]. Cell cultures of *Anthoceros agrestis* have been established by Binding and Mordhorst [15] and further investigated with respect to RA accumulation and biosynthesis by Petersen and coworkers (e.g. [125, 165]). Not all enzymes found in Lamiaceae for RA biosynthesis have to date been found in *Anthoceros* as well and thus the biosynthetic pathway is still under investigation. Suspension cultures of *Anthoceros agrestis* can accumulate quite high levels of RA. Pezeshki has measured up to 9% RA in the cell DW in a hormone-free B5-derived medium with 1% sucrose after 2 weeks of cultivation [133], whereas higher sugar content (2%) resulted in a lower RA accumulation. In the latter medium, however, an accumulation of RA 3′-*O*-β-D-glucoside at the beginning of the culture period was observed [165] (**see also Chap. 9 of this book**). With respect to the intracellular RA concentrations *Anthoceros agrestis* suspension cultures are in no way inferior to cell cultures of many higher plant species. It must, however, be mentioned that the cell mass increase of these cultures is lower.

2.4 Production of Rosmarinic Acid in Hairy Roots

Hairy roots have become a common type of axenic plant *in vitro* culture due to their easy maintenance and rapid biomass increase. Usually hairy roots are established by infecting plant material with *Rhizobium rhizogenes* (formerly *Agrobacterium rhizogenes*) strains, which transfer genes of their Ri plasmid to the plant cells. These are stably integrated into the plant genome and direct the plant cells to produce roots. The developing roots often carry high numbers of root hairs that give the roots a "hairy" appearance [108]. In recent years, efforts have been made to optimize the production of plant metabolites in hairy root cultures of plants that contain the very same metabolites or to insert new pathways for small molecules or proteins of interest into model plants [51, 62, 141, 160].

Hairy root cultures of members of both, the Boraginaceae and Lamiaceae, have been used for the production of RA and other caffeic acid derivatives. As of April 2016, 36 scientific articles had been published on this topic.

2.4.1 Hairy Roots of Lamiaceae Species

The production of RA in hairy root cultures of plants in the Lamiaceae is well documented (Tables 2.2 and 2.3). Hairy roots have several advantages with respect to undifferentiated cell cultures or plants. The hairy root material contains mostly the same metabolites as the source plant but is more stable than undifferentiated plant

Table 2.2 Hairy root cultures of Lamiaceae species established for the production of RA and related compounds. For experiments with *Salvia miltiorrhiza* see Table 2.3

Plant species	Compound	Experiment	Reference
Agastache foeniculum	RA	Establishment of HR, 4-fold higher production of RA (0.02% DW) than in non-transformed roots	[116]
Coleus blumei	RA	Establishment of HR and normal roots, comparison of biomass and RA content. HR had 2.8-fold higher RA content (5% DW). Effects of MeJA and YE on HR	[11]
Coleus blumei	RA, caffeic acid, chlorogenic acid	Transformation of HR with the *Arabidopsis thaliana* PAL gene under the control of the constitutive CaMV 35S promotor decreases the formation of RA and chlorogenic acid, but enhances caffeic acid levels	[10]
Coleus blumei	RA and other phenolic acids	Endogenously synthesized elicitor β-cryptogein causes excretion of RA from the cells to the medium	[166]
Coleus blumei	RA	Establishment of HR, RNAi-mediated suppression of HPPR or RAS reduced RA by 92%, overexpression led to RA levels of 176% of the control HR lines (1.73% DW)	[73]
Coleus blumei	RA	Comparison of different tissues revealed high stability of production of RA in HR	[13]
Coleus forskohlii	RA and other natural products	Analysis of various media for HR cultures with respect to biomass and RA accumulation. Comparison of the elicitors YE, SA and MeJA. Increase of RA content up to 3.4-fold higher with MeJA than control	[98]
Dracocephalum kotschyi	RA and flavonoids	Establishment of HR, up to 15-times higher production of RA in HR than in non-transformed roots (max. 0.15% DW)	[47]
Dracocephalum moldavica	RA	Establishment of HR, analysis of different media, tenfold higher RA content than in untransformed roots (7.8% DW)	[173]
Hyssopus officinalis	RA and other phenolic acids	Comparison of different media with respect to RA amount. Highest value was 6% DW, 60% higher than in callus, cell suspension culture and 1 year old field plant roots. Detection of nine other phenolic acids in HR	[89]

(continued)

Table 2.2 (continued)

Plant species	Compound	Experiment	Reference
Nepeta cataria	RA	Elicitation of HR cultures with auxins and polyamines led to increase in biomass and RA accumulation (1.92% DW)	[185]
Ocimum basilicum	RA and related phenolic acids	Comparison of various clones of HR cultures with respect to RA amount. Highest amount was 14.1% DW	[154]
Ocimum basilicum	RA	Increased production of RA in HR and elicited HR compared with untreated or untransformed roots. Exudation of RA into medium upon treatment with *Pythium ultimum*	[8]
Ocimum basilicum	RA and other antioxidants	Production of RA as major antioxidant, dependent on cultivar (up to 7.6% DW)	[143]
Salvia officinalis	RA	Comparison of two lines of HR cultures transformed with different strains of *Agrobacterium* and with untransformed HR, up to 2.3-fold increase in RA accumulation (approx. 4.5% DW)	[60]
Salvia officinalis	RA	Comparison of shoot and HR cultures with respect to accumulation of antioxidants and biomass (shoot 2.6%, HR 3.5% DW)	[57]
Salvia wagneriana	RA	Establishment of culture, no elicitation of RA with JA	[135]

DW dry weight, *HR* hairy roots, *JA* jasmonic acid, *MeJA* methyl jasmonic acid, *SA* salicylic acid, *YE* yeast extract

cells. Moreover, the yield of RA and other caffeic acid derivatives can be increased by eliciting with e.g. MeJA or SA.

A problem remains during the downstream processing of the phenolic acids: the extraction of RA from cells and organs is a tedious process. For biotechnological use, exudation of the phenolic metabolites into the medium would be an important step for a simpler and cheaper production. Two publications deal with this problem. In 2002, Bais and coworkers treated hairy roots of RA-producing *Ocimum basilicum* with *Pythium ultimum*. Upon this fungal *in situ* challenge, the hairy roots produced droplets on the roots tips with concentrated RA solutions. This behavior was absent with other fungi or in untreated roots. It has been hypothesized that this strategy might be useful for the plant root to prevent infections with soil pathogens, as RA showed effective antimicrobial activity [8].

The oomycete *Phytophthora cryptogea* produces ß-cryptogein. This proteinaceous elicitor causes activation of phenylpropanoid metabolism via stimulation of calcium-dependent pathways. By transforming *Rhizobium rhizogenes* with the coding sequence for β-cryptogein, Vuković and her colleagues obtained modified

Coleus blumei hairy root clones with the ability to produce the elicitor endogenously. These cultures were able to secrete RA and caffeic acid into the culture medium [166].

A modulation of product amounts can not only be achieved by changing the medium or eliciting the root culture, but also by manipulating the expression pattern of genes for enzymes of RA biosynthesis. Hücherig and Petersen [73] used techniques of RNAi suppression and overexpression with a constitutive promotor to modulate gene expression for HPPR and RAS in hairy roots of *Coleus blumei*. They showed that the insertion of interfering hairpin RNA of both genes led to decreased expression values of HPPR and RAS and accordingly to reduced RA accumulation. One HPPR-RNAi-line accumulated only about 8% of the RA amount found in control lines (1.73% of DW in controls). In contrast, an overexpression of these genes led to a 1.8-fold increase in RA accumulation compared to control lines.

By far the most publications on RA production in hairy roots are dealing with the plant *Salvia miltiorrhiza* (Table 2.3), the red or Chinese sage, named for its red ochre-colored roots. It is an important plant in traditional Chinese medicine, also known as Danshen, Dan Shen or Tan Shen. Two substance groups dominate the constituents of the plant extracts, namely phenolic acids (RA, lithospermic acids, and salvianolic acids) and diterpenes (tanshinones). Danshen is employed for the treatment of various diseases associated with malfunctioning blood flow, cardiovascular and cerebrovascular diseases, such as coronary heart disease, hypertension, angina pectoris, ischemic strokes and hyperlipidemia. It is used in various phytopharmaceutical forms, for oral application or injection, as solids, liquids or aerosols, as single preparation or in combination with other drugs. Clinical and pharmacological studies of bioactive metabolites isolated from Danshen have focused on Danshensu, which is DHPL, salvianolic acid B and tanshinone IIA [30, 190].

Xiao et al. [177] investigated the production of lithospermic acid B in hairy roots. It has been hypothesized that lithospermic acid B is directly derived from RA. After elicitation of hairy root cultures of *S. miltiorrhiza* with silver ions (Ag^+), they investigated accumulation of RA, lithospermic acid B and intermediates of the RA biosynthetic pathway as well as gene expression of enzymes involved in this pathway and found an inverse proportionality of RA and lithospermic acid accumulation after elicitation. This finding, combined with metabolic profiling and gene activity measurements, led to the conclusion that RA is the precursor of lithospermic acid B.

Other publications presented a genetic engineering approach to stimulate the accumulation of phenolic acids in *S. miltiorrhiza*. Xiao et al. [178] used an overexpression/suppression approach to manipulate the expression patterns of genes of the RA biosynthetic pathway. The upregulation of the single genes for *c4h*, *tat* and *hppr* as well as suppression of the *4-hydroxyphenylpyruvate dioxygenase* gene (*hppd*) led to an increase of RA, lithospermic acid B or both. The gene product HPPD participates in the tyrosine catabolic pathway by catalyzing the conversion of 4-hydroxphenylpyruvate to homogentisate. A co-overexpression of *tat* and *hppr* resulted in the highest accumulation of both RA and lithospermic acid, 4.3 and 3.2-fold higher than in the wild type, respectively.

Table 2.3 Hairy root cultures of *Salvia miltiorrhiza* (Lamiaceae) established for the production of RA and related compounds

Compounds	Experiment	Reference
Lithospermic acid B, RA and related compounds	Establishment of HR, comparison of different media with respect to accumulation of lithospermic acid B (between 0.73 and 1.61% DW) and RA (0.48% DW) and increase of biomass	[25]
Phenolic acids	Methyl viologen inhibited biomass production and decreased content of phenolic acids in HR	[24]
Phenolic acids, RA and lithospermic acid B, other natural products	Increase of phenolic acids and other natural products and biomass upon elicitation with YE (up to 2.89% lithospermic acid B and 2.98% RA in DW)	[27]
RA and related compounds	Comparison of two elicitors, YE and silver ions. Increase of RA accumulation and gene expression for enzymes of RA biosynthesis for both elicitors, effects with YE higher (up to 8% DW)	[183]
RA and lithospermic acid B	Elicitation of HR with MeJA increased RA and lithospermic acid B approx. 2–8-fold higher than untreated control (RA up to 6.02% DW, lithospermic acid B up to 19.3% DW). Gene expression was elevated for RA biosynthesis genes	[176]
RA, salvianolic acid B, DHPL (danshensu)	Dependence of phenolic acid content on concentration of MeJA elicitor in medium and growth stage of HR. Accumulation in DW, RA 14.35%, salvianolic acid B 1.59%, DHPL 0.51%	[29]
RA and lithospermic acid B	Elicitation of HR cultures with Ag^+ led to approx. 3-fold increase of lithospermic acid B (to 18.8% DW), while RA content decreased. Analysis of gene expression and intermediates suggest RA as precursor for lithospermic acid B	[177]
RA and lithospermic acid B	Overexpression of genes of RA biosynthesis and suppression of genes for by-products led to 3.2–4.3-fold increase of phenolic acids in HR compared to untransformed wildtypes	[178]
RA, salvianolic acids	Effects of MeJA and YE on accumulation of RA and salvianolic acids. MeJA elevated the accumulation of salvianolic acid B up to 7.11% and RA up to 3.38% DW, YE increased RA content up to 5.71% DW but suppressed salvianolic acids	[189]
Salvianolic acids and RA	Effects of sugar and other nutrients of the medium on accumulation of salvianolic acids in whole plants, seedlings and HR	[170]
Phenolic acids, RA and lithospermic acid B	Effects of various concentrations of abscisic acid and fluridone on growth and accumulation of phenolic acids in HR	[31]

(continued)

Table 2.3 (continued)

Compounds	Experiment	Reference
RA, lithospermic acid B and other natural products	Overexpression of allene oxide cyclase promoted biosynthesis of natural products in HR, RA increased 2.1-fold compared to wildtype (up to 0.28% DW), lithospermic acid B accumulated 1.8-fold more than wildtype and 2.3-fold more than blank vector control (up to 1.90% DW)	[61]
RA, salvianolic acid B and caffeic acid	Gene expression study of RA biosynthetic genes after elicitation with MeJA, LC-MS-analysis of phenolic acids. Both expression and accumulation were elevated several hours after induction	[97]
RA, lithospermic acid B	[Ring-^{13}C]-labeled phenylalanine and UPLC/Q-TOF measurement to analyze the biosynthetic pathway of phenolic acids	[39]
RA, salvianolic acid B and tanshinones	Endophytic bacteria decrease the production of phenolic acids and biomass and increase the production of tanshinones	[184]
RA and salvianolic acid B	Treatment of HR with MeJA and fungal extracts, expression and activity analysis of phenylpropanoid and tyrosine-derived pathway (RA max 4.5% DW)	[188]
RA, salvianolic acid B and tanshinones	Silver ions as elicitor for secondary metabolites, analysis of gene expression (up to 1.5% DW)	[180]
RA and salvianolic acid B	Study on transcription factors for RA biosynthesis	[67]

DW dry weight, *HR* hairy roots, *JA* jasmonic acid, *MeJA* methyl jasmonic acid, *SA* salicylic acid, *YE* yeast extract

Using the overexpression of allene oxide cyclase, Gu et al. [61] were also able to enhance the accumulation of secondary metabolites, namely tanshinone IIA, RA and lithospermic acid B in hairy roots of *Salvia miltiorrhiza*. Allene oxide cyclase catalyzes a reaction in the pathway toward jasmonates, which are a group of phytohormones that are induced in response to various stresses [16]. Jasmonates are known to trigger plant defence mechanisms, especially the production of secondary metabolites. MeJA, for instance, is an important elicitor. Overexpression of the *Salvia miltiorrhiza* allene oxide cyclase gene in hairy root cultures led also to an increase in RA biosynthetic genes encoding PAL, HPPR and 4CL.

2.4.2 Hairy Roots of Boraginaceae Species

Two species of the Boraginaceae family (*Lithospermum erythrorhizon* and *Eritrichium sericeum*) have been used to establish hairy roots for the production of RA and related compounds (Table 2.4). *Lithospermum erythrorhizon*, the purple gromwell, accumulates RA, lithospermic acid and rabdosiin, a condensation product of two molecules of RA (Table 2.1). Another interesting natural substance from this species is shikonin, a prenylated naphthoquinone. The content of phenolic

Table 2.4 Hairy root cultures of Boraginaceae species established for the production of RA and related compounds

Plant species	Compounds	Experiment	Reference
Lithospermum erythrorhizon	Lithospermic acid B, RA, rabdosiin and other natural products	Analysis of HR in M-9 medium for caffeic acid derivatives and other natural products	[181]
Eritrichium sericeum and *Lithospermum erythrorhizon*	Rabdosiin and RA	Presence of *rolC* in HR inhibits production of phenolic acids compared to control cultures. MeJA-triggered (1 μM) *E. sericeum* HR can accumulate up to 3.41% and 6.92% of the DW as rabdosiin and RA, respectively	[19]

DW dry weight, *HR* hairy roots, *MeJA* methyl jasmonic acid

compounds in hairy roots was considerably lower than in suspension cultures and RA was hardly detectable [181]. Thus, this culture system is inferior for biotechnological uses.

Bulgakov et al. [19] described interesting effects of the agrobacterial *rolC* gene. This gene is located on the Ri plasmid, which is transferred during infection of the plant with *Rhizobium rhizogenes*. RolC causes inhibition of phenolic acid production (namely RA and rabdosiin) in *Lithospermum erythrorhizon* and *Eritrichium sericeum* callus and hairy root cultures, leading to depletion of both substances to a level two- to three-fold lower than in untransformed plant material. Yet, the effects are reversible with cantharidin, an inhibitor of serine/threonine phosphatases, which has led to the hypothesis, that *rolC* affects shikimate metabolism via a set of regulatory phosphatases, which in return can be affected by cantharidin. This finding was unexpected because several publications had demonstrated that transgenic hairy roots bearing the *rolC* gene can produce more secondary metabolites without further treatment than untransformed cultures.

2.5 Production of Rosmarinic Acid and Related Caffeic Acid Esters in Microorganisms

In recent years, several efforts to introduce a biosynthetic pathway for RA and related phenolic metabolites into *Escherichia coli* have been reported. The first step was taken by Kim et al. [85] who inserted coding sequences for 4CL and hydroxycinnamoyl-CoA shikimate hydroxycinnamoyltransferase (HST) or hydroxycinnamoyl-CoA quinate hydroxycinnamoyltransferase (HQT) into *E. coli* and fed different hydroxycinnamic acids to the bacteria. These were capable to produce hydroxycinnamoylshikimate or hydroxycinnamoylquinate, metabolites closely related to RA. They circumvented the necessity to introduce enzymes necessary to hydroxylate the benzene ring, which, in plants, requires cytochrome P450s.

Therefore, their approach can be viewed as a biotransformation rather than a *de novo* synthesis of hydroxycinnamic acid esters. To increase the amount of acceptor substrates, the authors mutated different enzymes of the shikimate pathway, leading either to the production of quinate or shikimate esters [85].

The next step was taken by Bloch and Schmidt-Dannert in 2014 (Fig. 2.2). They took advantage of the fact that RAS, the key enzyme for RA production and responsible for esterification of 4-coumaroyl-CoA and pHPL, can also use caffeoyl-CoA and DHPL as substrates, since the enzyme has a broad substrate promiscuity regarding the hydroxylation in *meta* position [94, 138]. In plants, these hydroxyl groups are added after the RAS reaction by cytochrome P450 reactions. The engineered pathway starts for both, the acceptor and the donor, with pHPP from the bacterial shikimate pathway. The acceptor molecule DHPL is produced by addition and overexpression of two enzymes, a dehydrogenase (HdhA; hydroxyacid dehydrogenase from *Lactobacillus delbrueckii* ssp. *bulgaricus*) and a hydroxylase complex (HpaBC; 4-hydroxyphenylacetate 3-hydroxylase from *E. coli*), using $FADH_2$ (and $NAD(P)H + H^+$) as cofactors. The donor is synthesized by using three enzymes. In the bacterial pathway to aromatic amino acids, pHPP is transaminated to tyrosine. An inserted tyrosine ammonia-lyase (TAL from *Rhodobacter sphaeroides*) deaminates tyrosine to 4-coumaric acid, which is hydroxylated with the HpaBC complex described above to build caffeic acid. After CoA activation with an inserted 4CL (At4CL2 from *Arabidopsis thaliana*), an introduced RAS (CbRAS from *Coleus blumei*) produces RA. Alongside RA, isorinic acid (ester of caffeic acid and pHPL) was observed. The introduction of RAS from other plants species, namely *Lavandula angustifolia* or *Melissa officinalis*, resulted in higher production of RA and isorinic acid (1.8 ± 0.3 μM RA, 5.3 ± 0.7 μM isorinic acid with MoRAS; approximately 2.5 mg phenolic acids/l). Both metabolites were released into the medium and the amount of product was increased when appropriate precursors were fed to the medium. The authors stated, however, that an industrial use of this modified *E. coli* needs either feeding of expensive precursors like pHPL or DHPL, which would elevate production costs into unprofitable ranges or to use bacterial strains with upregulated shikimic acid and tyrosine biosynthetic pathways, so that the precursors would be produced autotrophically [17].

A similar approach was followed by Jiang et al. [78] using a tyrosine-overproducing *E. coli* strain as a platform [7]. Furthermore, coding sequences for an *Arabidopsis thaliana* 4CL, a mutated D-lactate dehydrogenase (LDH^{Y52A}) from *Lactobacillus pentosus* [186], the HpaBC complex from *E. coli* BW25113 and a synthetic CbRAS sequence (optimized for expression in *E. coli*) were used. The final transformed *E. coli* strain was able to produce approximately 133 mg RA per litre of culture besides approximately 55 mg/l caffeoyl-phenyllactate.

Recently, Zhuang et al. [192] achieved the formation of 18 RA analogues by feeding *E. coli* BLRA1 transformed with a 4CL from *Arabidopsis thaliana* and RAS from *Coleus blumei* with different donor substrates (4-coumaric acid, caffeic acid, ferulic acid, 3,4-dihydroxyphenylpropanoic acid and 4-hydroxyphenylpropanoic acid) and various acceptors (pHPL, DHPL, phenyllactic acid, mandelic acid and tyrosol).

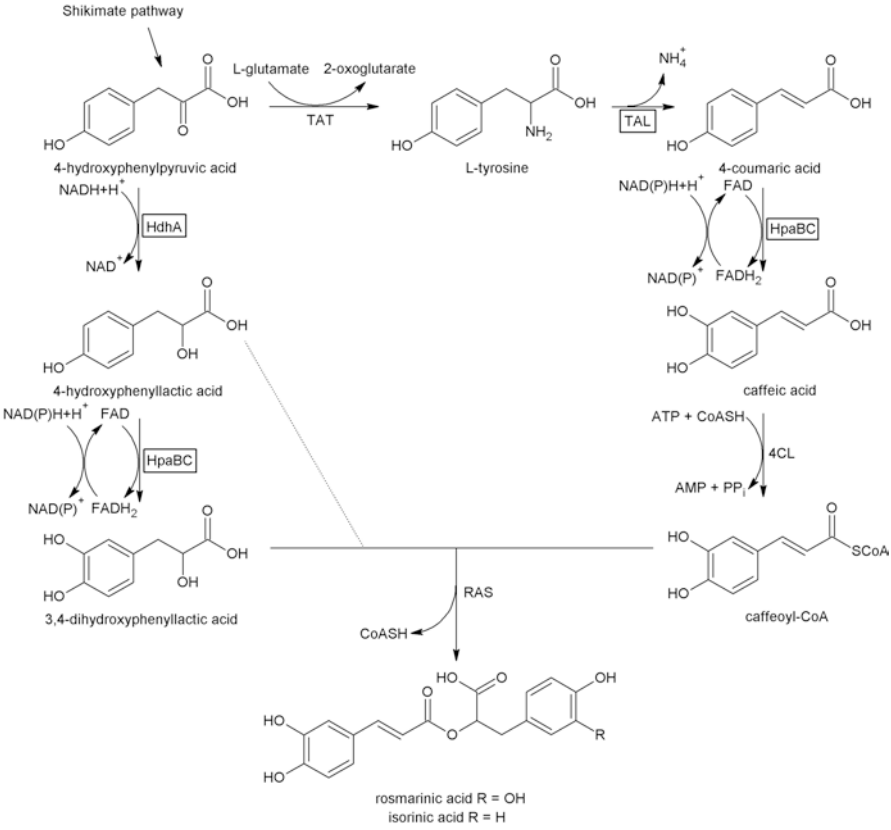


Fig. 2.2 Formation of isorinic acid and rosmarinic acid in *Escherichia coli* as established by Bloch and Schmidt-Dannert [17]. *HdhA* hydroxyacid dehydrogenase from *Lactobacillus delbrueckii* ssp. *bulgaricus*, *HpaBC* 4-hydroxyphenylacetate 3-hydroxylase from *E. coli*, *TAT* tyrosine aminotransferase (endogenous), *TAL* tyrosine ammonia-lyase from *Rhodobacter sphaeroides*, *4CL* 4-coumarate CoA-ligase (At4CL2) from *Arabidopsis thaliana*, *RAS* rosmarinic acid synthase from *Coleus blumei*. Microbial enzymes are marked by boxes

2.6 *In Vitro* Formation of Non-natural Hydroxycinnamic Acid Esters and Amides by "Rosmarinic Acid Synthase"

RAS is the essential ester-forming enzyme in the biosynthetic pathway towards RA [130]. *In vivo*, this enzyme couples a hydroxycinnamoyl unit activated as CoA thioester (4-coumaroyl-CoA, caffeoyl-CoA) to the aliphatic OH-group of a phenylpyruvate derivate. RAS proteins from lavender as well as *Coleus blumei* heterologously expressed in *Escherichia coli* displayed unexpected substrate promiscuity. The recombinant proteins were shown to form esters as well as amides and accepted a considerable variety of compounds leading to products that had not yet been described, e.g. hydroxycinnamoyl-D-phenylalanine, hydroxycinnamoyl-D-tyrosine, hydroxycinnamoyl-D-DOPA, hydroxycinnamoyl-phenethylamine, hydroxycinnamoyl-tyramine, and hydroxycinnamoyl-tryptamine [94, 138].

2.7 Conclusion and Outlook

RA and related metabolites are among those specialized metabolites in plants that are produced at the highest levels. Often, the contents in undifferentiated cells, such as callus and suspension cells, are considerably higher (sometimes exceeding 30% of the DW) than in the source plants. Undifferentiated cells, however, often lose their production capacity with increasing numbers of subcultivations. This disadvantage is less pronounced in differentiated organs. Here HR cultures are the most often established production systems. Up to now, however, the RA production levels in HR are lower (<20% of the DW) than in undifferentiated cells. Many production systems have been established, mostly at laboratory scale. With the exception of early attempts in the 1980s [164], these have not been developed further to semi-industrial or industrial scale. This may be due to the lack of commercial demand for these phenolic acids, since, despite the many biological effects of RA and related phenolic acids, medicinal applications have not been developed, perhaps with exception of *Salvia miltiorrhiza* and its extracted ingredients as traditional Chinese medicines.

Very recent approaches have shown that RA and similar metabolites can also be produced in genetically modified *E. coli*. Here, a combination of bacterial and plant genes have been used and the necessity of membrane-bound cytochrome P450 enzymes circumvented. The amount of RA produced in prokaryotic systems (133 mg/l; [78]) is, however until now, not competitive with plant cell cultures (e.g. 6.4 g/l in *Salvia officinalis* [72]).

References

1. Agata I, Hatano T, Nishibe S, Okuda T. Rabdosiin, a new rosmarinic acid dimer with a lignan skeleton from *Rabdosia japonica*. Chem Pharm Bull. 1988;36:3223–5.
2. Agata I, Kusakabe H, Hatano T, Nishibe S, Okuda T. Melitric acids A and B, new trimeric caffeic acid derivatives from *Melissa officinalis*. Chem Pharm Bull. 1993;41:1608–11.
3. Ai C, Li L. Stereostructure of salvianolic acid B and isolation of salvianolic acid C from *Salvia miltiorrhiza*. J Nat Prod. 1988;51:145–9.
4. Ai C, Li L. Salvianolic acids D and E: two new depsides from *Salvia miltiorrhiza*. Planta Med. 1992;58:197–9.
5. Amoah SKS, Sandjo LP, Kratz JM, Biavatti MW. Rosmarinic acid – pharmaceutical and clinical aspects. Planta Med. 2016;82:388–406.
6. Asakawa Y. Chemical constituents of the bryophytes – chemical constituents of Anthocerotae (hornworts). In: Herz W, Kirby GW, Moore RE, Steglich W, Tamm C, editors. Progress in the chemistry of organic natural products. Wien/New York: Springer; 1995. p. 460–4.
7. Bai YF, Bi HP, Zhuang YB, Liu C, Cai T, Liu XN, Zhang XL, Liu T, Ma YH. Production of salidroside in metabolically engineered *Escherichia coli*. Sci Rep. 2014;4:6640.
8. Bais HP, Walker TS, Schweizer HP, Vivanco JM. Root specific elicitation and antimicrobial activity of rosmarinic acid in hairy root cultures of *Ocimum basilicum*. Plant Physiol Biochem. 2002;40:983–95.
9. Barros L, Duenas M, Dias MI, Sousa MJ, Santos-Buelga C, Ferreira ICFR. Phenolic profiles of cultivated, in vitro cultured and commercial samples of *Melissa officinalis* L. infusions. Food Chem. 2013;136:1–8.

10. Bauer N, Fulgosi H, Jelaska S. Overexpression of phenylalanine ammonia-lyase in transgenic roots of *Coleus blumei* alters growth and rosmarinic acid synthesis. Food Technol Biotechnol. 2011;49:24–31.
11. Bauer N, Kiseljak D, Jelaska S. The effect of yeast extract and methyl jasmonate on rosmarinic acid accumulation in *Coleus blumei* hairy roots. Biol Plant. 2009;53:650–6.
12. Bauer N, Leljak-Levanic D, Jelaska S. Rosmarinic acid synthesis in transformed callus culture of *Coleus blumei* benth. Z Naturforsch. 2004;59c:554–60.
13. Bauer N, Vukovic R, Likic S, Jelaska S. Potential of different *Coleus blumei* tissues for rosmarinic acid production. Food Technol Biotechnol. 2015;53:3–10.
14. Berger A, Meinhard J, Petersen M. Rosmarinic acid synthase is a new member of the superfamily of BAHD acyltransferases. Planta. 2006;224:1503–10.
15. Binding H, Mordhorst G. Gametophyte regeneration and apospory from archegoniate protoplasts under conditions devised for higher plants. Bot Acta. 1991;104:330–5.
16. Bishop G, Sakakibara H, Seo M, Yamaguchi S. Biosynthesis of hormones. In: Buchanan BB, Gruissem W, Jones RL, editors. Biochemistry & molecular biology of plants. Chichester: Wiley; 2015. p. 769–833.
17. Bloch SE, Schmidt-Dannert C. Construction of a chimeric biosynthetic pathway for the de novo biosynthesis of rosmarinic acid in *Escherichia coli*. Chembiochem. 2014;15:2393–401.
18. Bulgakov VP, Inyushkina YV, Fedoreyev SA. Rosmarinic acid and its derivatives: biotechnology and applications. Crit Rev Biotechnol. 2012;32:203–17.
19. Bulgakov VP, Veselova MV, Tchernoded GK, Kiselev KV, Fedoreyev SA, Zhuravlev YN. Inhibitory effect of the *Agrobacterium rhizogenes* rolC gene on rabdosiin and rosmarinic acid production in *Eritrichium sericeum* and *Lithospermum erythrorhizon* transformed cell cultures. Planta. 2005;221:471–8.
20. Chaprin N, Ellis BE. Microspectrophotometric evaluation of rosmarinic acid accumulation in single cultured plant cells. Can J Bot. 1984;62:2278–82.
21. Chen H, Chen F. Kinetics of cell growth and secondary metabolism of a high-tanshinone-producing line of the Ti transformed *Salvia miltiorrhiza* cells in suspension culture. Biotechnol Lett. 1999;21:701–5.
22. Chen H, Chen F. Effects of yeast elicitor on the growth and secondary metabolism of a high-tanshinone-producing line of the Ti transformed *Salvia miltiorrhiza* cells in suspension culture. Process Biochem. 2000a;35:837–40.
23. Chen H, Chen F. Effect of yeast elicitor on the secondary metabolism of Ti-transformed *Salvia miltiorrhiza* cell suspension cultures. Plant Cell Rep. 2000b;19(7):710.
24. Chen H, Chen F. Induction of phytoalexin formation in crown gall and hairy root culture of *Salvia miltiorrhiza* by methyl viologen. Biotechnol Lett. 2000c;22:715–20.
25. Chen H, Chen F, Zhang YL, Song JY. Production of lithospermic acid B and rosmarinic acid in hairy root cultures of *Salvia miltiorrhiza*. J Ind Microbiol Biotechnol. 1999b;22:133–8.
26. Chen H, Chen F, Zhang YL, Song JY. Production of rosmarinic acid and lithospermic acid B in Ti-transformed *Salvia miltiorrhiza* cell suspension cultures. Process Biochem. 1999a;34:777–84.
27. Chen H, Chen F, Chiu FCK, Lo CMY. The effect of yeast elicitor on the growth and secondary metabolism of hairy root cultures of *Salvia miltiorrhiza*. Enzyme Microb Technol. 2001;28:100–5.
28. Chen Z, Gu W, Huang H, Yang X, Sun C, Chen W, Dong Y, Ma H. Studies on the water-soluble phenolic acid constituents of *Salvia miltiorrhiza*. Yaoxue Tongbao. 1981;16:24–5.
29. Chen Z, Zhang J, Zhao S, Wang Z, Hu Z. Effects of methyl jasmonate on accumulation of phenolic acids in *Salvia miltiorrhiza* hairy root. Zhongguo Yaoxue Zazhi. 2010;45:970–4.
30. Cheng TO. Cardiovascular effects of Danshen. Int J Cardiol. 2007;121:9–22.
31. Cui B, Liang Z, Liu Y, Liu F, Zhu J. Effects of ABA and its biosynthetic inhibitor fluridone on accumulation of phenolic acids and activity of PAL and TAT in hairy root of *Salvia miltiorrhiza*. Zhongguo Zhongyao Zazhi. 2012;37:754–9.
32. da Silva Pinto L, de Souza FHD, Nascimento IR, Lopes LMX. Phenylpropanoids from *Paspalum atratum* (Poaceae). Biochem Syst Ecol. 2015;63:68–71.

33. De Eknamkul W, Ellis BE. Rosmarinic acid production and growth characteristics of *Anchusa officinalis* cell suspension cultures. Planta Med. 1984;50:346–50.
34. De Eknamkul W, Ellis BE. Effects of macronutrients on growth and rosmarinic acid formation in cell suspension cultures of *Anchusa officinalis*. Plant Cell Rep. 1985a;4:46–9.
35. De Eknamkul W, Ellis BE. Effects of auxins and cytokinins on growth and rosmarinic acid formation in cell suspension cultures of *Anchusa officinalis*. Plant Cell Rep. 1985b;4:50–3.
36. De Eknamkul W, Ellis BE. Tyrosine aminotransferase: the entrypoint enzyme of the tyrosine-derived pathway in rosmarinic acid biosynthesis. Phytochemistry. 1987;26:1941–6.
37. Dewanjee S, Gangopadhyay M, Das U, Sahu R, Khanra R. Enhanced rosmarinic acid biosynthesis in *Solenostemon scutellarioides* culture: a precursor-feeding strategy. Nat Prod Res. 2014b;28:1691–8.
38. Dewanjee S, Gangopadhyay M, Das U, Sahu R, Samanta A, Banerjee P. Signal transducer and oxidative stress mediated modulation of phenylpropanoid pathway to enhance rosmarinic acid biosynthesis in fungi elicited whole plant culture of *Solenostemon scutellarioides*. Enzyme Microb Technol. 2014a;66:1–9.
39. Di P, Zhang L, Chen J, Tan H, Xiao Y, Dong X, Zhou X, Chen W. ^{13}C tracer reveals phenolic acids biosynthesis in hairy root cultures of *Salvia miltiorrhiza*. ACS Chem Biol. 2013;8:1537–48.
40. Dong C, Su X, Wang W. Effects of sucrose concentration, nitrogen on the growth and main secondary metabolites accumulated of regeneration plant of *Rabdosia rubescens* (Hemsl.) Hara. Xibei Zhiwu Xuebao. 2009;29:494–8.
41. Döring AS, Petersen M. Production of caffeic, chlorogenic and rosmarinic acids in plants and suspension cultures of *Glechoma hederacea*. Phytochem Lett. 2014;10:cxi–cxvii.
42. Dreger M, Krajewska-Patan A, Gorska-Paukszta M, Pieszak M, Buchwald W, Mikolajczak P. Production of the secondary metabolites in *Salvia miltiorrhiza* in vitro. Herba Polonica. 2010;56:78–90.
43. Duan Y, Dai Y, Gao H, Ye W, Yao X. Chemical constituents of *Sarcandra glabra*. Zhongcaoyao. 2010;41:29–32.
44. Ellis BE. Characterization of clonal cultures of *Anchusa officinalis* derived from single cells of known productivity. J Plant Physiol. 1985;119:149–58.
45. El-Mousallamy AMD, Hawas UW, Hussein SAM. Teucrol, a decarboxyrosmarinic acid and its 4′-O-triglycoside, teucroside from *Teucrium pilosum*. Phytochemistry. 2000;55:927–31.
46. Ejtahed RS, Radjabian T, Hoseini Tafreshi SA. Expression analysis of phenylalanine ammonia lyase gene and rosmarinic acid production in *Salvia officinalis* and *Salvia virgata* shoots under salicylic acid elicitation. Appl Biochem Biotechnol. 2015;176:1846–58.
47. Fattahi M, Nazeri V, Torras-Claveria L, Sefidkon F, Cusido RM, Zamani Z, Palazon J. A new biotechnological source of rosmarinic acid and surface flavonoids: hairy root cultures of *Dracocephalum kotschyi* Boiss. Ind Crop Prod. 2013;50:256–63.
48. Fukui H, Yazaki K, Tabata M. Two phenolic acids from *Lithospermum erythrorhizon* cell suspension cultures. Phytochemistry. 1984;23:2398–9.
49. Georgiev M, Kuzeva S, Pavlov A, Kovacheva E, Ilieva M. Enhanced rosmarinic acid production by *Lavandula vera* MM cell suspension culture through elicitation with vanadyl sulfate. Z Naturforsch. 2006b;61C:241–4.
50. Georgiev MI, Kuzeva SL, Pavlov AI, Kovacheva EG, Ilieva MP. Elicitation of rosmarinic acid by *Lavandula vera* MM cell suspension culture with abiotic elicitors. World J Microbiol Biotechnol. 2007a;23:301–4.
51. Georgiev MI, Pavlov AI, Bley T. Hairy root type plant in vitro systems as sources of bioactive substances. Appl Microbiol Biotechnol. 2007b;74:1175–85.
52. Georgiev M, Pavlov A, Ilieva M. Rosmarinic acid production by *Lavandula vera* MM cell suspension: the effect of temperature. Biotechnol Lett. 2004;26:855–6.
53. Georgiev M, Pavlov A, Ilieva M. Selection of high rosmarinic acid producing *Lavandula vera* MM cell lines. Process Biochem. 2006a;41:2068–71.
54. Gertlowski C, Petersen M. Influence of the carbon source on growth and rosmarinic acid production in suspension cultures of *Coleus blumei*. Plant Cell Tiss Org. 1993;34:183–90.

55. Grzegorczyk I, Wysokinska H. Liquid shoot culture of *Salvia officinalis* L. for micropropagation and production of antioxidant compounds; effect of triacontanol. Acta Soc Bot Pol. 2008;77:99–104.
56. Grzegorczyk I, Wysokinska H. The effect of methyl jasmonate on production of antioxidant compounds in shoot cultures of *Salvia officinalis* L. Herba Pol. 2009;55:238–43.
57. Grzegorczyk I, Wysokinska H. Antioxidant compounds in *Salvia officinalis* L. shoot and hairy root cultures in the nutrient sprinkle bioreactor. Acta Soc Bot Poloniae. 2010;79:7–10.
58. Grzegorcyzk I, Bilichowski I, Mikiciuk-Olasik E, Wysokinska H. In vitro cultures of *Salvia officinalis* L. as a source of antioxidant compounds. Acta Soc Bot Poloniae. 2005;74:17–21.
59. Grzegorcyzk I, Bilichowski I, Mikiciuk-Olasik E, Wysokinska H. The effect of triacontanol on shoot multiplication and production of antioxidant compounds in shoot cultures of *Salvia officinalis* L. Acta Soc Bot Poloniae. 2006;75:11–5.
60. Grzegorczyk I, Krolicka A, Wysokinska H. Establishment of *Salvia officinalis* L. hairy root cultures for the production of rosmarinic acid. Z Naturforsch. 2006;61c:351–6.
61. Gu XC, Chen JF, Xiao Y, Di P, Xuan HJ, Zhou X, Zhang L, Chen WS. Overexpression of allene oxide cyclase promoted tanshinone/phenolic acid production in *Salvia miltiorrhiza*. Plant Cell Rep. 2012;31:2247–59.
62. Guillon S, Trémouillaux-Guiller J, Pati PK, Rideau M, Gantet P. Hairy root research: recent scenario and exciting prospects. Curr Opin Plant Biol. 2006;9:341–6.
63. Guirgis AA, Abd El-Kawi MA, Abbas HN, Araffa AMS, Maksoud AI. High rosmarinic acid content in induced mutants and in *in vitro* elicited sweet basil (*Ocimum basilicum* L.) callus. Asian J Plant Sci. 2007;6:1058–64.
64. Guo H, Zhu N, Deyholos MK, Liu J, Zhang X, Dong J. Calcium mobilization in salicylic acid-induced *Salvia miltiorrhiza* cell cultures and its effect on the accumulation of rosmarinic acid. Appl Biochem Biotechnol. 2015;175:2689–702.
65. Hakkim FL, Shankar CG, Girija S. Chemical composition and antioxidant property of holy basil (*Ocimum sanctum* L.) leaves, stems, and inflorescence and their *in vitro* callus cultures. J Agric Food Chem. 2007;55:9109–17.
66. Hao W, Guo H, Zhang J, Hu G, Yao Y, Dong J. Hydrogen peroxide is involved in salicylic acid-elicited rosmarinic acid production in *Salvia miltiorrhiza* cell cultures. Sci World J. 2014, Article ID 843764, 7 pages.
67. Hao G, Jiang X, Feng L, Tao R, Li Y, Huang L. Cloning, molecular characterization and functional analysis of a putative R2R3-MYB transcription factor of the phenolic acid biosynthetic pathway in *S. miltiorrhiza* Bge. f. *alba*. Plant Cell Tiss Org Cult. 2016;124:151–68.
68. Harborne JB. Caffeic acid ester distribution in higher plants. Z Naturforsch. 1966;21b:604–5.
69. Häusler E, Petersen M, Alfermann AW. Hydroxyphenylpyruvate reductase from cell suspension cultures of *Coleus blumei* Benth. Z Naturforsch. 1991;46c:371–6.
70. Häusler E, Petersen M, Alfermann AW. Rosmarinsäure in *Blechnum*-Spezies. In: Haschke HP, Schnarrenberger C, editors. Botanikertagung, vol. 1992. Berlin: Akademie-Verlag; 1992. p. 507.
71. Hippolyte I, Marin B, Baccou JC, Jonard R. Influence du milieu d'entretien et de la concentration en saccharose sur la production d'acide rosmarinique par des suspensions cellulaires de Sauge (*Salvia officinalis* L.). CR Acad Sci Paris. 1991;313:365–71.
72. Hippolyte I, Marin B, Baccou JC, Jonard R. Growth and rosmarinic acid production in cell suspension cultures of *Salvia officinalis* L. Plant Cell Rep. 1992;11:109–12.
73. Hücherig S, Petersen M. RNAi suppression and overexpression studies of hydroxyphenylpyruvate reductase (HPPR) and rosmarinic acid synthase (RAS) genes related to rosmarinic acid biosynthesis in hairy root cultures of *Coleus blumei*. Plant Cell Tiss Org Cult. 2013;113:375–85.
74. Ilieva M, Pavlov A. Rosmarinic acid by *Lavandula vera* MM cell suspension: phosphorus effect. Biotechnol Lett. 1996;18:913–6.
75. Ilieva M, Pavlov A. Rosmarinic acid production by *Lavandula vera* MM cell-suspension culture. Appl Microbiol Biotechnol. 1997;47:683–8.

76. Ilieva M, Pavlov A. Rosmarinic acid production by *Lavandula vera* MM cell suspension culture: nitrogen effect. World J Microbiol Biotechnol. 1999;15:711–4.
77. Ilieva-Stoilova MP, Pavlov AI, Kovatcheva-Apostolova EG. Further research into *Lavandula* species: cell cultures of *L. vera* and rosmarinic acid production. Med Aromat Plants Ind Profiles. 2002;29:214–26.
78. Jiang J, Bi H, Zhuang Y, Liu S, Liu T, Ma Y. Engineered synthesis of rosmarinic acid in *Escherichia coli* resulting production of a new intermediate, caffeoyl-phenyllactate. Biotechnol Lett. 2016;38:81–8.
79. Jiang RW, Lau KM, Hon PM, Mak TCW, Woo KS, Fung KP. Chemistry and biological activities of caffeic acid derivatives from *Salvia miltiorrhiza*. Curr Med Chem. 2005;12:237–46.
80. Ju Q, Liu G, Liu X, Han X, Liu H. Influence of growth regulators and sucrose concentrations on growth and rosmarinic acid production in calli and suspension cultures of *Coleus blumei*. Nat Prod Res. 2009;23:127–37.
81. Karwatzki B, Petersen M, Alfermann AW. Transient activity of enzymes involved in the biosynthesis of rosmarinic acid in cell cultures of *Coleus blumei*. Planta Med. 1989;55:663–4.
82. Kelley CJ, Mahajan JR, Brooks LC, Neubert LA, Breneman WR, Carmack M. Polyphenolic acids of *Lithospermum ruderale* Dougl. ex Lehm. (Boraginaceae). 1. Isolation and structure determination of lithospermic acid. J Org Chem. 1975;40:1804–15.
83. Kiferle C, Lucchesini M, Maggini R, Pardossi A, Mensuali-Sodi A. *In vitro* culture of sweet basil: gas exchanges, growth, and rosmarinic acid production. Biol Plantarum. 2014;58:601–10.
84. Kiferle C, Lucchesini M, Mensuali-Sodi A, Maggini R, Raffaelli A, Pardossi A. Rosmarinic acid content in basil plants grown in vitro and in hydroponics. Cent Eur J Biol. 2011;6:946–57.
85. Kim BG, Jung WD, Mok H, Ahn JH. Production of hydroxycinnamoyl-shikimates and chlorogenic acid in *Escherichia coli*: production of hydroxycinnamic acid conjugates. Microb Cell Factories. 2013b;12:15.
86. Kim YB, Kim JK, Uddin M, Xu H, Park WT, Tuan PA, Li X, Chung E, Lee JH, Park SU. Metabolomics analysis and biosynthesis of rosmarinic acid in *Agastache rugosa* Kuntze treated with methyl jasmonate. PLoS One. 2013a;8:e64199.
87. Kintzios S, Kollias H, Straitouris E, Makri O. Scale-up micropropagation of sweet basil (*Ocimum basilicum* L.) in an airlift bioreactor and accumulation of rosmarinic acid. Biotechnol Lett. 2004;26:521–3.
88. Kintzios S, Makri O, Panagiotopoulos E, Scapeti M. *In vitro* rosmarinic acid accumulation in sweet basil (*Ocimum basilicum* L.). Biotechnol Lett. 2003;25:405–8.
89. Kochan E, Wysokinska H, Chmiel A, Grabias B. Rosmarinic acid and other phenolic acids in hairy roots of *Hyssopus officinalis*. Z Naturforsch. 1999;54c:11–6.
90. Kohda H, Takeda O, Tanaka S, Yamasaki K, Yamashita A, Kurokawa T, Ishibashi S. Isolation of inhibitors of adenylate cyclase from Dan-shen, the root of *Salvia miltiorrhiza*. Chem Pharm Bull. 1989;37:1287–90.
91. Kojuharova L, Pavlov A, Ilieva M, Mincheva M, Kovatcheva E. Enhancement of phenolic compounds synthesis from plant cell culture of *Lavandula vera* MM. Proceedings of bioprocess engineering '95 1995:24–27.
92. Kovatcheva E, Pavlov A, Koleva I, Ilieva M, Mihneva M. Rosmarinic acid from *Lavandula vera* MM cell culture. Phytochemistry. 1996;43:1243–4.
93. Kracun-Kolarevic M, Dmitrovic S, Filipovic B, Peric M, Misic D, Simonovic A, Todorovic S. Influence of sodium salicylate on rosmarinic acid, carnosol and carnosic acid accumulation by *Salvia officinalis* L. shoots grown in vitro. Biotechnol Lett. 2015;37:1693–701.
94. Landmann C, Hücherig S, Fink B, Hoffmann T, Dittlein D, Coiner HA, Schwab W. Substrate promiscuity of a rosmarinic acid synthase from lavender (*Lavandula angustifolia* L.). Planta. 2011;234:305–20.
95. Lee JH, Park KH, Lee MH, Kim HT, Seo WD, Kim JY, Baek IY, Jang DS, Ha TJ. Identification, characterisation, and quantification of phenolic compounds in the antioxidant activity-containing fraction from the seeds of Korean perilla (*Perilla frutescens*) cultivars. Food Chem. 2013;136:843–52.

96. Li L, Tan R, Chen W. Salvianolic acid A, a new depside from roots of *Salvia miltiorrhiza*. Planta Med. 1984;50:227–8.
97. Li W, Gao W, Zhao J, Cui G, Shao A, Huang L. Research on mechanism of secondary metabolites of phenolic acids in *Salvia miltiorrhiza* hairy root induced by jasmonate. Zhongguo Zhongyao Zazhi. 2012;37:13–6.
98. Li W, Koike K, Asada Y, Yoshikawa T, Nikaido T. Rosmarinic acid production by *Coleus forskohlii* hairy root cultures. Plant Cell Tiss Org Cult. 2005;80:151–5.
99. Li X, Guo H, Qi Y, Liu H, Zhang X, Ma P, Liang Z, Dong J. Salicylic acid-induced cytosolic acidification increases the accumulation of phenolic acids in *Salvia miltiorrhiza* cells. Plant Cell Tiss Org Cult. 2016;126:333–41.
100. Liang LF, Keng CL, Lim BP. Selection of cell lines for the production of rosmarinic acid from cell suspension cultures of *Orthosiphon stamineus* Benth. In Vitro Cell Dev-Pl. 2006;42:538–42.
101. Liu L, Dong J, Zhang J, Dang X, Xing B, Yang X. Effects of calcium on synthesis of rosmarinic acid and related enzymes in suspension cultures of *Salvia miltiorrhiza*. Shengwu Gongcheng Xuebao. 2012;28:1359–69.
102. Lu Y, Foo LY. Rosmarinic acid derivatives from *Salvia officinalis*. Phytochemistry. 1999;51:91–4.
103. Lu Y, Foo LY. Polyphenolics of *Salvia* – a review. Phytochemistry. 2002;59:117–40.
104. Lu Y, Foo LY. Salvianolic acid L, a potent phenolic antioxidant from *Salvia officinalis*. Tetrahedron Lett. 2001;42:8223–5.
105. Lu Y, Foo LY, Wong H. Sagecoumarin, a novel caffeic acid trimer from *Salvia officinalis*. Phytochemistry. 1999;52:1149–52.
106. Ma XH, Ma Y, Tang JF, He YL, Liu YC, Ma XJ, Shen Y, Cui GH, Lin HX, Rong QX, Guo J, Huang LQ. The biosynthetic pathways of tanshinones and phenolic acids in *Salvia miltiorrhiza*. Molecules. 2015;20:16235.
107. Martinez BC, Park CH. Immobilization of *Coleus blumei* cells in a column reactor using a spray feeding system. Biotechnol Tech. 1994;8:301–6.
108. Mehrotra S, Srivastava V, Rahman LU, Kukreja AK. Hairy root biotechnology – indicative timeline to understand missing links and future outlook. Protoplasma. 2015;252:1189–201.
109. Míka V, Kubáň V, Klejdus B, Odstrčilová V, Nerušil P. Phenolic compounds as chemical markers of low taxonomic levels in the family Poaceae. Plant Soil Environ. 2005;51:506–12.
110. Mizukami H, Ogawa T, Ohashi H, Ellis BE. Induction of rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures by yeast extract. Plant Cell Rep. 1992;11:480–3.
111. Mizukami H, Tabira Y, Ellis BE. Methyl jasmonate-induced rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures. Plant Cell Rep. 1993;12:706–9.
112. Morimoto S, Goto Y, Shoyama Y. Production of lithospermic acid B and rosmarinic acid in callus tissue and regenerated plantlets of *Salvia miltiorrhiza*. J Nat Prod. 1994;57:817–23.
113. Moschopoulou G, Kintzios S. Achievement of thousand-fold accumulation of rosmarinic acid in immobilized cells of sweet basil (*Ocimum basilicum* L.) by ten-fold increase of the volume of the immobilization matrix. J Biol Res. 2011;15:59–65.
114. Murata T, Oyama K, Fujiyama M, Oobayashi B, Umehara K, Miyase T, Yoshizaki F. Diastereomers of lithospermic acid and lithospermic acid B from *Monarda fistulosa* and *Lithospermum erythrorhizon*. Fitoterapia. 2013;91:51–9.
115. Nitzsche A, Tokalov SV, Gutzeit HO, Ludwig-Müller J. Chemical and biological characterization of cinnamic acid derivatives from cell cultures of lavender (*Lavandula officinalis*) induced by stress and jasmonic acid. J Agric Food Chem. 2004;52:2915–23.
116. Nourozi E, Hosseini B, Hassani A. A reliable and efficient protocol for induction of hairy roots in *Agastache foeniculum*. Biologia. 2014;69:870–9.
117. Ogata A, Tsuruga A, Matsuno M, Mizukami H. Elicitor-induced rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures: activities of rosmarinic acid synthase and the final two cytochrome P450-catalyzed hydroxylations. Plant Biotechnol. 2004;21:393–6.

118. Park CH, Martinez BC. Enhanced release of rosmarinic acid from *Coleus blumei* permeabilized by dimethyl sulfoxide (DMSO) while preserving cell viability and growth. Biotechnol Bioeng. 1992;40:459–64.
119. Pavlov A, Ilieva M. The influence of phenylalanine on accumulation of rosmarinic and caffeic acids by *Lavandula vera* MM cell culture. World J Microbiol Biotechnol. 1999;15:397–9.
120. Pavlov A, Georgiev MI, Ilieva MP. Production of rosmarinic acid by *Lavandula vera* MM cell suspension in bioreactor: effect of dissolved oxygen concentration and agitation. World J Microbiol Biotechnol. 2005a;21:389–92.
121. Pavlov AI, Georgiev MI, Panchev IN, Ilieva MP. Optimization of rosmarinic acid production by *Lavandula vera* MM plant cell suspension in a laboratory bioreactor. Biotechnol Prog. 2005b;21:394–6.
122. Pavlov A, Ilieva M, Mincheva M. Release of rosmarinic acid by *Lavandula vera* MM cell suspension in two-phase culture systems. World J Microbiol Biotechnol. 2001;17:417–21.
123. Pavlov AI, Ilieva MP, Panchev IN. Nutrient medium optimization for rosmarinic acid production by *Lavandula vera* MM cell suspension. Biotechnol Prog. 2000;16:668–70.
124. Petersen M. Cytochrome P450-dependent hydroxylation in the biosynthesis of rosmarinic acid in *Coleus*. Phytochemistry. 1997;45:1165–72.
125. Petersen M. Cinnamic acid 4-hydroxyase from cell cultures of the hornwort *Anthoceros agrestis*. Planta. 2003;217:96–101.
126. Petersen M. Rosmarinic acid: new aspects. Phytochem Rev. 2013;12:207–27.
127. Petersen M, Alfermann AW. Two new enzymes of rosmarinic acid biosynthesis from cell cultures of *Coleus blumei*: hydroxyphenylpyruvate reductase and rosmarinic acid synthase. Z Naturforsch. 1988;43c:501–4.
128. Petersen M, Simmonds MSJ. Rosmarinic acid. Phytochemistry. 2003;62:121–5.
129. Petersen M, Abdullah Y, Benner J, Eberle D, Gehlen K, Hücherig S, Janiak V, Kim KH, Sander M, Weitzel C, Wolters S. Evolution of rosmarinic acid biosynthesis. Phytochemistry. 2009;70:1663–79.
130. Petersen M, Häusler E, Karwatzki B, Meinhard J. Proposed biosynthetic pathway for rosmarinic acid in cell cultures of *Coleus blumei* Benth. Planta. 1993;189:10–4.
131. Petersen M, Häusler E, Meinhard J, Karwatzki B, Gertlowski C. The biosynthesis of rosmarinic acid in suspension cultures of *Coleus blumei*. Plant Cell Tiss Org Cult. 1994;38:171–9.
132. Petersen M, Szabo E, Meinhard J, Karwatzki B, Gertlowski C, Kempin B, Fuss E. Biosynthesis and accumulation of rosmarinic acid in suspension cultures of *Coleus blumei*. Plant Cell Tiss Org Cult. 1995;43:89–92.
133. Pezeshki S. Biosynthese von Kaffeesäuremetaboliten im Ackerhornmoos *Anthoceros agrestis* und im Kleinen Blasenmützenmoos *Physcomitrella patens*. PhD thesis, Philipps-Universität Marburg. 2016.
134. Razzaque A, Ellis BE. Rosmarinic acid production in *Coleus* cell cultures. Planta. 1977;137:287–91.
135. Ruffoni B, Bertoli A, Pistelli L, Pistelli L. Micropropagation of *Salvia wagneriana* Polak and hairy root cultures with rosmarinic acid production. Nat Prod Res. 2016;4:1–7.
136. Sahraroo A, Babalar M, Mirjalili MH, Moghaddam MRF, Ebrahimi SN. *In-vitro c*allus induction and rosmarinic acid quantification in callus culture of *Satureja khuzistanica* Jamzad (Lamiaceae). Iranian J Pharm Res. 2014;13:1447–56.
137. Sahraroo A, Mirjalili MH, Corchete P, Babalar M, Moghaddam MRF. Establishment and characterization of a *Satureja khuzistanica* Jamzad (Lamiaceae) cell suspension culture: a new in vitro source of rosmarinic acid. Cytotechnology. 2015;68:1415–24.
138. Sander M, Petersen M. Distinct substrate specificities and unusual substrate flexibilities of two hydroxycinnamoyltransferases, rosmarinic acid synthase and hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase, from *Coleus blumei* Benth. Planta. 2011;233:1157–71.
139. Satake T, Kamiya K, Saiki Y, Hama T, Fujimoto Y, Kitanaka S, Kimura Y, Uzawa J, Endang H, Umar M. Studies on the constituents of fruits of *Helicteres isora* L. Chem Pharm Bull. 1999;47:1444–7.

140. Scarpati ML, Oriente G. Isolamento e costituzione dell'acido rosmarinico (dal *rosmarinus off*.). Ric Sci. 1958;28:2329–33.
141. Shanks JV, Morgan J. Plant 'hairy root' culture. Curr Opin Biotechnol. 1999;10:151–5.
142. Shetty K. Rosmarinic acid biosynthesis and mechanism of action. Funct Foods Biotechnol. 2007;165:187–207.
143. Srivastava S, Conlan XA, Adholeya A, Cahill DM. Elite hairy roots of *Ocimum basilicum* as a new source of rosmarinic acid and antioxidants. Plant Cell Tiss Org Cult. 2016;126:19–32.
144. Strazzer P, Guzzo F, Levi M. Correlated accumulation of anthocyanins and rosmarinic acid in mechanically stressed red cell suspensions of basil (*Ocimum basilicum*). J Plant Physiol. 2011;168:288–93.
145. Su WW, Humphrey AE. Production of rosmarinic acid in high density perfusion cultures of *Anchusa officinalis* using a high sugar medium. Biotechnol Lett. 1990;12:793–8.
146. Su WW, Humphrey AE. Production of rosmarinic acid from perfusion culture of *Anchusa officinalis* in a membrane-aerated bioreactor. Biotechnol Lett. 1991;13:889–92.
147. Su WW, Lei F. Rosmarinic acid production in perfused *Anchusa officinalis* culture: effect of inoculum size. Biotechnol Lett. 1993;15:1035–8.
148. Su WW, Lei F, Kao NP. High density cultivation of *Anchusa officinalis* in a stirred-tank bioreactor with in situ filtration. Appl Microbiol Biotechnol. 1995;44:293–9.
149. Su WW, Lei F, Su LY. Perfusion strategy for rosmarinic acid production by *Anchusa officinalis*. Biotechnol Bioeng. 1993;42:884–90.
150. Su WW, Asali EC, Humphrey AE. *Anchusa officinalis*: production of rosmarinic acid in perfusion cell cultures. In: Bajaj YPS, editor. Biotechnology in agriculture and forestry, medicinal and aromatic plants VI. Berlin/Heidelberg: Springer; 1994. p. 1–20.
151. Sumaryono W, Proksch P. *Orthosiphon aristatus* (Java tea): *in vitro* culture and elicitation of rosmarinic acid in cell suspensions. In: Biotechnology in agriculture and forestry, medicinal and aromatic plants V. Berlin\Heidelberg: Springer; 1993. p. 287–99.
152. Sumaryono W, Proksch P, Hartmann T, Nimtz M, Wray V. Induction of rosmarinic acid accumulation in cell suspension cultures of *Orthosiphon aristatus* after treatment with yeast extract. Phytochemistry. 1991;30:3267–71.
153. Szabo E, Thelen A, Petersen M. Fungal elicitor preparations and methyl jasmonate enhance rosmarinic acid accumulation in suspension cultures of *Coleus blumei*. Plant Cell Rep. 1999;18:485–9.
154. Tada H, Murakami Y, Omoto T, Shimomura K, Ishimaru K. Rosmarinic acid and related phenolics in hairy root cultures of *Ocimum basilicum*. Phytochemistry. 1996;42:431–4.
155. Takeda R, Hasegawa J, Shinozaki M. The first isolation of lignans, megacerotonic acid and anthocerotonic acid, from non-vascular plants, Anthocerotae (hornworts). Tetrahedron Lett. 1990a;31:4159–62.
156. Takeda R, Hasegawa J, Shinozaki K. Phenolic compounds from Anthocerotae. In: Zinsmeister HD, Mues R, editors. Bryophytes: their chemistry and chemical taxonomy. Proceedings of the phytochemical Society of Europe, vol. 29. Oxford: Oxford Science Publications; 1990b. p. 201–7.
157. Tanaka T, Morimoto S, Nonaka G, Nishioka I, Yokozawa T, Chung HY, Oura H. Magnesium and ammonium-potassium lithospermates B, the active principles having a uremia-preventive effect from *Salvia miltiorrhiza*. Chem Pharm Bull. 1989;37:340–4.
158. Tanaka T, Nishimura A, Kouno I, Nonaka G, Young TJ. Isolation and characterization of yunnaneic acids A-D, four novel caffeic acid metabolites from *Salvia yunnanensis*. J Nat Prod. 1996;59(9):843.
159. Tanaka T, Nishimura A, Kouno I, Nonaka G, Yang CR. Four new caffeic acid metabolites, yunnaneic acids E-H, from *Salvia yunnanensis*. Chem Pharm Bull. 1997;45:1596–600.
160. Tian L. Using hairy roots for production of valuable plant secondary metabolites. In: Krull R, Bley T, editors. Filaments in bioprocesses. Advances in biochemical engineering/biotechnology, vol. 149. Cham: Springer International Publishing; 2015. p. 275–324.

161. Tonelli M, Pellegrini E, D'Angiolillo F, Petersen M, Nali C, Pistelli L, Lorenzini G. Ozone-elicited secondary metabolites in shoot cultures of *Melissa officinalis* L. Plant Cell Tiss Org Cult. 2015;120:617–29.
162. Trennheuser F. Phytochemische Untersuchung und *in vitro* Kultur ausgewählter Vertreter der Anthocerotopsida. PhD thesis, University of Saarbrücken. 1992.
163. Trennheuser F, Burkhard G, Becker H. Anthocerodiazonin, an alkaloid from *Anthoceros agrestis*. Phytochemistry. 1994;37:899–903.
164. Ulbrich B, Wiesner W, Arens H. Large-scale production of rosmarinic acid from plant cell cultures of *Coleus blumei* Benth. In: Neumann KH, Barz W, Reinhard E, editors. Primary and secondary metabolism of plant cell cultures. Berlin\Heidelberg: Springer; 1985. p. 293–303.
165. Vogelsang K, Schneider B, Petersen M. Production of rosmarinic acid and a new rosmarinic acid 3′-*O*-β-D-glucoside in suspension cultures of the hornwort *Anthoceros agrestis* Paton. Planta. 2006;223:369–73.
166. Vukovic R, Bauer N, Curkovic-Perica M. Genetic elicitation by inducible expression of β-cryptogein stimulates secretion of phenolics from *Coleus blumei* hairy roots. Plant Sci. 2013;199–200:18–28.
167. Wagner H, Wittmann D. Zur chemischen Struktur der Lithospermsäure aus *Lithospermum officinale* L. Tetrahedron Lett. 1975;(8):547–50.
168. Wang BQ. *Salvia miltiorrhiza*: chemical and pharmacological review of a medicinal plant. J Med Plants Res. 2010;4:2813–20.
169. Wang CZ, Davin LB, Lewis NG. Stereoselective phenolic coupling in *Blechnum spicant*: formation of 8-2′ linked (−)-cis-blechnic, (−)-trans-blechnic and (−)-brainic acids. Chem Commun. 2001:113–4.
170. Wang CL, Liang ZS, Li DR, Liu Y, Liu FH. Effects of growth-regulating substances and soluble sugar contents on accumulation of salvianolic acids in *Salvia miltiorrhiza* Bunge. Zhiwu Shengli Xuebao. 2012;48:181–8.
171. Weitzel C, Petersen M. Enzymes of phenylpropanoid metabolism in the important medicinal plant *Melissa officinalis* L. Planta. 2010;232:731–42.
172. Weitzel C, Petersen M. Cloning and characterisation of rosmarinic acid synthase from *Melissa officinalis* L. Phytochemistry. 2011;72:572–8.
173. Weremczuk-Jezyna I, Grzegorczyk-Karolak I, Frydrych B, Krolicka A, Wysokinska H. Hairy roots of *Dracocephalum moldavica*: rosmarinic acid content and antioxidant potential. Acta Physiol Plant. 2013;35:2095–103.
174. Whitaker RJ, Hashimoto T, Evans DA. Production of the secondary metabolite, rosmarinic acid, by plant cell suspension cultures. Ann N Y Acad Sci. 1984;435:364–6.
175. Wu CF, Karioti A, Rohr D, Bilia AR, Efferth T. Production of rosmarinic acid and salvianolic acid B from callus culture of *Salvia miltiorrhiza* with cytotoxicity towards acute lymphoblastic leukemia cells. Food Chem. 2016;201:292–7.
176. Xiao Y, Gao S, Di P, Chen J, Chen W, Zhang L. Methyl jasmonate dramatically enhances the accumulation of phenolic acids in *Salvia miltiorrhiza* hairy root cultures. Physiol Plant. 2009;137:1–9.
177. Xiao Y, Gao SH, Di P, Chen JF, Chen WS, Zhang L. Lithospermic acid B is more responsive to silver ions (Ag^+) than rosmarinic acid in *Salvia miltiorrhiza* hairy root cultures. Biosci Rep. 2010;30:33–40.
178. Xiao Y, Zhang L, Gao S, Saechao S, Di P, Chen J, Chen W. The c4h, tat, hppr and hppd genes prompted engineering of rosmarinic acid biosynthetic pathway in *Salvia miltiorrhiza* hairy root cultures. PLoS One. 2011;6:e29713.
179. Xing BY, Dang XL, Zhang JY, Wang B, Chen ZY, Dong JE. Effects of methyl jasmonate on the biosynthesis of rosmarinic acid and related enzymes in *Salvia miltiorrhiza* suspension cultures. Zhiwu Shengli Xuebao. 2013;49:1326–32.
180. Xing B, Yang D, Guo W, Liang Z, Yan X, Zhu Y, Liu Y. Ag^+ as a more effective elicitor for production of tanshinones than phenolic acids in *Salvia miltiorrhiza* hairy roots. Molecules. 2015;20:309–24.

181. Yamamoto H, Inoue K, Yazaki K. Caffeic acid oligomers in *Lithospermum erythrorhizon* cell suspension cultures. Phytochemistry. 2000a;53:651–7.
182. Yamamoto H, Yazaki K, Inoue K. Simultaneous analysis of shikimate-derived secondary metabolites in *Lithospermum erythrorhizon* cell suspension cultures by high-performance liquid chromatography. J Chromatogr B. 2000b;738:3–15.
183. Yan Q, Shi M, Ng J, Wu JY. Elicitor-induced rosmarinic acid accumulation and secondary metabolism enzyme activities in *Salvia miltiorrhiza* hairy roots. Plant Sci. 2006;170:853–8.
184. Yan Y, Zhang S, Zhang J, Ma P, Duan J, Liang Z. Effect and mechanism of endophytic bacteria on growth and secondary metabolite synthesis in *Salvia miltiorrhiza* hairy roots. Acta Physiol Plant. 2014;36:1095–105.
185. Yang YK, Lee SY, Park WT, Park NI, Park SU. Exogenous auxins and polyamines enhance growth and rosmarinic acid production in hairy root cultures of *Nepeta cataria* L. plant. OMICS. 2010;3:190–3.
186. Yao YF, Wang CS, Qiao J, Zhao GR. Metabolic engineering of *Escherichia coli* for production of salvianic acid A via an artificial biosynthetic pathway. Metab Eng. 2013;19:79–87.
187. Zenk MH, El-Shagi H, Ulbrich B. Production of rosmarinic acid by cell-suspension cultures of *Coleus blumei*. Naturwiss. 1977;64:585–6.
188. Zhang S, Yan Y, Wang B, Liang Z, Liu Y, Liu F, Qi Z. Selective responses of enzymes in the two parallel pathways of rosmarinic acid biosynthetic pathway to elicitors in *Salvia miltiorrhiza* hairy root cultures. J Biosci Bioeng. 2014;117:645–51.
189. Zhao S, Zhang J, Yang L, Wang Z, Hu Z. Determination and biosynthesis of multiple salvianolic acids in hairy roots of *Salvia miltiorrhiza*. Yaoxue Xuebao. 2011;46:1352–6.
190. Zhou L, Zuo Z, Chow MSS. Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. J Clin Pharmacol. 2005;45:1345–59.
191. Zhu L, Li Y, Yang J, Zuo L, Zhang D. Studies on chemical constituents of *Sarcandra glabra*. Zhongguo Zhongyao Zazhi. 2008;33:155–7.
192. Zhuang Y, Jiang J, Bi H, Yin H, Liu S, Liu T. Synthesis of rosmarinic acid analogues in *Escherichia coli*. Biotechnol Lett. 2016;38:619–27.