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MINI REVIEW ARTICLE

Hairy roots as a potential source for the production of rosmarinic acid from genus *Salvia*

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Abstract

The genus *Salvia*, a member of the Lamiaceae family, exhibits a rich array of secondary metabolites, including di- and triterpenoids, polyphenols, and essential oil compounds. These constituents contribute to valuable pharmacological activities such as antibacterial, antiviral, anti-inflammatory, and antioxidant properties. Among these metabolites, rosmarinic acid stands out as a particularly promising compound, deriving from the precursors phenylalanine and tyrosine. It belongs to the phenolic compound class and acts as an ester of caffeic acid, showcasing diverse therapeutic potentials like antifungal, antibacterial, antiviral, antioxidant, anticancer, anti-ageing, anti-inflammatory, and anti-diabetic effects. To facilitate the production of such secondary metabolites, plant tissue culture techniques have played a pivotal role, with hairy root cultures being one of the preferred methods. This review provides an extensive examination of the biosynthetic pathway of rosmarinic acid and its successful generation using hairy root cultures. Additionally, the review highlights the utilization of genetic modification tools and various biotic and abiotic elicitors, including yeast extract, methyl jasmonate, and silver ion (Ag+), in hairy root cultures of diverse *Salvia* species to enhance the production of rosmarinic acid.

Keywords

Salvia; secondary metabolites; rosmarinic acid; hairy root cultures; *Agrobacterium rhizogenes*; genetic engineering; elicitors

Introduction

Salvia is the largest genus in the Lamiaceae family, comprising around 1000 species of herbs and shrubs distributed globally (1,2). Lamiaceae is commonly known as the mint or sage family, and its plants are valued for their aromatic properties, easy cultivation via stem cuttings, edible leaves, and ornamental foliage (3). Some well-known ornamental members include *Coleus, Stachys, Thymus,* and *Salvia* (4,5). These plants have been used medicinally since ancient times due to the presence of various beneficial compounds such as alkaloids, terpenes, glycosides, phenols, and polyphenols (6–8). *Salvia*, in particular, contains diverse secondary metabolites like polyphenols, diterpenoids, triterpenoids, and essential oils with notable anti-viral, anti-microbial, anti-inflammatory, and antioxidant activities (9–11). Additionally, it contains phenolic compounds like rosmarinic acid and salvianolic acids (13–15). Rosmarinic acid is a phenolic compound first identified in *Salvia rosmarinus* (Rosemary) and found in over 35 plant families, from primitive hornworts to advanced monocotyledons and dicotyledons, but not in gymnosperms (16,21). It is stored in the vacuoles and cytoplasm as an anion within plant cells (17,18).

Tissue culture techniques, particularly hairy root culture, are widely

utilized for enhancing the production of secondary metabolites like rosmarinic acid (22–24). Hairy root cultures have been found to yield higher amounts of metabolites compared to callus and cell suspension cultures, which may exhibit variation in cell growth and erratic secondary metabolite yield due to genetic and epigenetic changes (25,26). Conversely, hairy root cultures grown rapidly without exogenous plant growth regulators, display genetic and biosynthetic stability, and accumulate equivalent or greater amounts of secondary metabolites (30–32). Hairy root cultures have proven successful in producing various phytochemicals, including artemisinin from *Artemisia*, forskolin from *Coleus,* indole alkaloids from *Catharanthus* and *Cinchona*, shikonin from *Lithospermum*, withanolides from *Withania*, verbascoside from *Gmelina arborea* (33), diosgenin from *Trigonella*, ajmaline, ajmalicine from *Rauvolfia micrantha* (34), and sanguinarine and coniferin from *Linum flavum* (35,36). Hairy roots result from infection by *Agrobacterium rhizogenes* bacteria, which transfer their root-inducing Ri plasmid into the plant, encoding enzymes capable of modifying hormonal metabolism (37). Additionally, hairy root cultures are employed in biotransformation to produce valuable biochemicals for pharmaceutical purposes, exhibiting improved solubility in biological systems, enhanced pharmacokinetics, and cost-effectiveness (35,38).

This review focuses on rosmarinic acid production using hairy root cultures of *Salvia*, its biosynthesis, genetic engineering, and metabolic engineering of genes involved in rosmarinic acid production, as well as elicitation strategies using various biotic and abiotic components.

Methodology

Multiple academic databases, including Google Scholar, Scopus, and Web of Science, were utilized to retrieve scholarly papers and research articles. The search strategy employed specific keywords such as "salvia," "secondary metabolites," "hairy roots," "rosmarinic acid," "metabolic engineering," and "elicitors.

Physicochemical Properties of Rosmarinic acid:

Rosmarinic acid, a caffeic acid ester with a molecular weight of 360 Da, belongs to the hydroxyl cinnamic acids family. It is formed by the esterification of 3,4-dihydroxycinnamic acid (caffeic acid) and 3,4-dihydroxyphenyllactic acid (DHPL) (39–41). This compound possesses a hydroxylated cinnamic acid moiety at the ortho-, meta-, or para- position of the benzene ring. The IUPAC name of rosmarinic acid is (2R)-3-(3,4-dihydroxyphenyl)-2-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxypropanoic acid (42). Structure of the compound rosmarinic acid is shown in Fig. 1.

It exhibits antioxidant properties by donating hydrogen to acceptors and preventing reactions with dioxygen and peroxides, thereby trapping free radicals (43). While being soluble in organic solvents such as ethanol, dimethyl sulfoxide, and dimethyl formamide at approximately 25mg/mL, it only shows limited solubility in water (42,44). This crystalline solid compound has a red-orange color, a melting point of -171 to -175℃, a boiling point

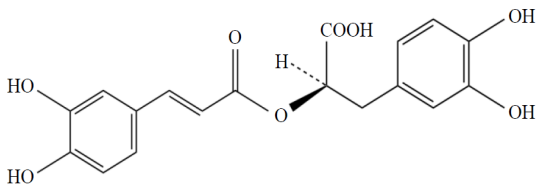

Fig. 1. Rosmarinic Acid ($C_{18}H_{16}O_8$) drawn using ChemDraw Ultra version 12.0.2

of 694.71℃ at 760 mm Hg, a density of 1.547 g/cm^3, a vapor pressure of 1.1x10^{-13} mmHg at 25℃, and a polarity represented by log P=1.82 (42,44,45).

Biosynthetic Pathway of Rosmarinic acid Synthesis:

Phenylalanine serves as a precursor to rosmarinic acid (RA) (46,47). Phenylpropanoid pathway enzymes convert phenylalanine to an activated hydroxycinnamic acid (48). Phenylalanine ammonia-lyase (PAL) facilitates the deamination of l-phenylalanine to t-cinnamic acid (49). Cinnamate 4-hydroxylase (C4H), a cytochrome P450 monooxygenase, hydroxylates t-cinnamic acid at position 4 to produce 4-coumaric acid (50,51). 4-coumaroyl:coenzyme ligase (4CL) then activates hydroxycinnamic acid through a two-step process, forming hydroxycinnamoyl-AMP and subsequently hydroxycinnamoyl-CoA (51,52). The other precursor, L-tyrosine, undergoes transamination using 2-oxoglutarate as a second substrate, catalyzed by tyrosine aminotransferase (TAT) (21,47). This reaction leads to the formation of 4-hydroxyphenylpyruvate and glutamate. Hydroxyphenylpyruvate reductase (HPPR) reduces 4-hydroxyphenylpyruvate to D-4-hydroxyphenyllactate, accepting both NADH and NADPH as cosubstrates (47,51,53,54).

Rosmarinic acid synthase (RAS), also known as 4-hydroxycinnamoyl-CoA:4-hydroxyphenyllactate hydroxycinnamoyltransferase, transfers the 4-coumaroyl moiety from 4-coumaroyl-CoA to the aliphatic hydroxyl group of hydroxyphenyllactate, releasing coenzyme A and forming 4-coumaroyl-4'-hydroxyphenyllactate (pC-pHPL) (21,53,55). Subsequently, two cytochrome P450 monooxygenases (50) hydroxylate pC-pHPL at either position 3 or 3' of the aromatic rings. One of these enzymes (3-H or hydroxycinnamoyl-hydroxyphenyllactate 3-hydroxylase) catalyzes the 3-hydroxylation of pC-pHPL to form caffeoyl-4'-hydroxyphenyllactic acid, while the other enzyme (3'-H or hydroxycinnamoyl-hydroxyphenyllactate 3'-hydroxylase) hydroxylates pC-pHPL at 4-coumaric acid or a 4-coumaroyl moiety, yielding 4-coumaroyl-3',4'-dihydroxyphenyllactic acid (50,56). Further hydroxylation of caffeoyl-4'-hydroxyphenyllactic acid and 4-coumaroyl-3',4'-dihydroxyphenyllactic acid by 3'-H and 3-H, respectively, results in the formation of rosmarinic acid (Fig. 1) (46). The complete biosynthetic pathway is depicted in Figure 2 (46).

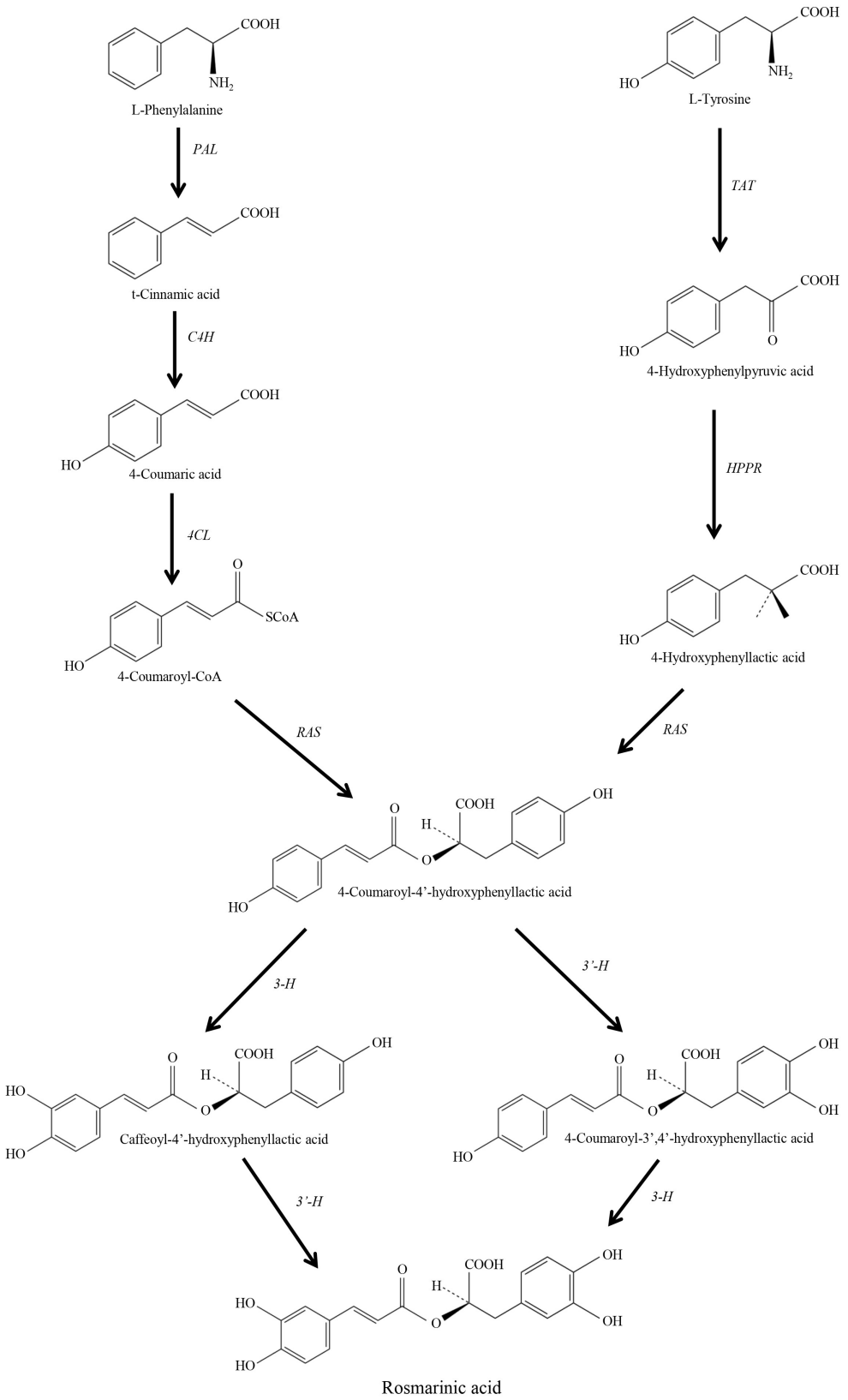

Fig. 2. Biosynthetic pathway of rosmarinic acid production (ChemDraw Ultra version 12.0.2). (The involved enzymes are abbreviated: PAL: Phenylalanine ammonia lyase; C4H: Cinnamate 4-hydroxylase; 4CL: 4-coumaroyl: coenzyme ligase; RAS: Rosmarinic acid synthase; 3-H: Hydroxycinnamoyl-hydroxyphenyllactate 3-hydroxylase; 3'-H: Hydroxycinnamoyl-hydroxyphenyllactate 3'-hydroxylase; TAT: Tyrosine aminotransferase; HPPR: Hydroxyphenylpyruvate reductase)

Pharmacological Significance of Rosmarinic acid:

Rosmarinic acid exhibits diverse pharmacological properties, including antiviral, anticancer, anti-aging, anti-inflammatory, antimicrobial, antifungal, antioxidant (57,58), antidiabetic, neuroprotective (59), and hepatoprotective activities (46,60,61). These effects are attributed to its modulation of cell signaling pathways and gene expression (62).

Anti-inflammatory activity

Rosmarinic acid exhibits anti-inflammatory properties, protecting the skin from UVB-induced DNA damage (63). Moreover, it inhibits the overproduction of high mobility group box 1 (HMGB1) nuclear protein, known to contribute to tissue damage in certain diseases (64). Furthermore, it displays strong anti-inflammatory effects in cases of lipopolysaccharide-induced acute lung injury (65).

Anti-microbial activity

Rosmarinic acid has demonstrated inhibitory and bactericidal effects against several pathogenic bacteria, such as *Staphylococcus epidermidis* 5001, *Pseudomonas aeruginosa* ATCC 27583, *Mycobacterium smegmatis 5003*, *Stenotrophomonas maltophilia, Enterococcus faecalis* C159 -6, *Corynebacterium T25-17*, and *Staphylococcus warneri* T12A12, as well as *Pantoea agglomerans*, *Klebsiella* sp., and *Streptomyces* sp (66,67).

Anti-fungal activity

Rosmarinic acid shows strong inhibitory effects on the growth of several fungi such as *Alternaria kikuchiana, Pestalotiopsis mangiferae, Botrytis cinerea,* and *Penicillium citrinum* (68).

Anti-cancer activity

Rosmarinic acid treatment has demonstrated apoptosis induction in human leukemia U937 cells and growth inhibition of breast cancer cell line MCF7 by activating hypermethylated tumor suppressor genes (69,70). Moreover, it exhibits anti-metastatic and tumor weight-reducing effects (71), as well as impedes cell proliferation in human ovarian cancer cells (72).

Anti-viral activity

Rosmarinic acid exhibits antiviral activity against virus replication in human lymphocyte MT-4 cells. Additionally, it reacts with nitrite ions, forming nitrorosmarinic acid, which also possesses antiviral properties. Furthermore, nitrorosmarinic acid specifically inhibits HIV-1 integrase activity, thereby preventing the integration of viral DNA into the host genome (73).

Anti-oxidant activity

Rosmarinic acid exerts its antioxidant properties by scavenging free radicals and safeguarding cellular membranes against lipid peroxidation (74). Furthermore, it effectively mitigates oxidative stress by inhibiting nitric oxide synthase activity (75) and decreasing intracellular reactive oxygen species (ROS) generation (76).

Anti-proliferative activity

Rosmarinic acid exhibits antiproliferative and proapoptotic properties in human colon carcinoma-derived cell lines (77). Moreover, it demonstrates efficacy in treating glomerular sclerosis by impeding mesangial cell proliferation (78).

Neuroprotective activity

Rosmarinic acid, an effective inhibitor of ciguatoxin (CTX), a cytotoxin from microalgae, shows promise in treating CTX-induced neurological impairment (79). Furthermore, rosmarinic acid and its derivatives exhibit cognitive-enhancing properties and potential for Alzheimer's disease prevention (80).

Rosmarinic acid in food industry

Rosmarinic acid and its derivatives are extensively utilized for their antioxidant properties in preserving fried food products, delaying oxidation, and preventing the formation of undesirable compounds, thereby extending shelf life (81,82). Additionally, these compounds serve as co-pigments in beverages such as juices and wines, aiding in the stabilization and enhancement of color due to the presence of unstable natural colorants (83,84).

Production of rosmarinic acid using tissue culture studies

The implementation of *in vitro* methodologies has resulted in the synthesis and accumulation of valuable plant secondary metabolites through cell, tissue, and organ culture. Techniques like callus, cell suspension, and organ cultures (shoot, root, somatic embryos) are employed for phytochemical production. Undifferentiated cultures generally exhibit lower yields (85). However, certain secondary metabolites can only be synthesized in organized structures, leading to higher yields in differentiated tissues. The economic feasibility of scaling up such techniques remains a challenge (86). Genetic transformation, especially the production of hairy roots through *Agrobacterium rhizogenes* and optimization of cultural conditions, has significantly boosted secondary metabolite yield (22, 23, 27, 37).

Sterile shoots of *Salvia officinalis* at 5 weeks of age were used as explants and cultured on Murashige and Skoog (MS) medium supplemented with 2.22µM 6-Benzylaminopurine (BAP) and 0.57µM Indole-3-acetic acid (IAA). Hairy roots were induced by wounding the second node of stems and midvein of leaves with a sterile needle dipped in a bacterial culture of ATCC 15834 *Agrobacterium rhizogenes* strain. The hairy roots were transferred to Woody Plant (WP) liquid medium containing 500mg/L ampicillin and incubated in the dark. Ampicillin concentration was gradually reduced until it was eliminated. Higher rosmarinic acid production (45 mg/g DW) was observed compared to A4-induced lines (87).

In another study, 3-week-old *Salvia officinalis* seedlings were used as explants and maintained on MS medium with 0.45mg/L BAP and 0.1mg/L IAA, sub-cultured every 3 weeks. Hairy roots were induced by infecting shoot tips with ATCC 15834 *Agrobacterium rhizogenes* strain. The good cultures were sub-cultured every 40 days in hormone -free WP medium in 300mL flasks (80mL medium). Rosmarinic acid accumulation was 34.7 ± 1.07mg/g DW (88).

For *Salvia wagneriana*, 4-week-old aseptic shoots on MS medium were used as explants. Hairy roots were

induced by infecting leaf lamina or petiole fragments with ATCC 15834 and NCPPB 1855 *Agrobacterium rhizogenes* strains. Rosmarinic acid accumulation was 173µg/g FW (89).

Similarly, 5-week-old aseptic shoots of *Salvia viridis* on MS medium with 0.1mg/L IAA and 0.5mg/L BAP were used as explants. Hairy roots were induced by infecting leaves and shoots with a needle dipped in A4 *Agrobacterium rhizogenes* strain. After infection, explants were transferred to hormone-free MS medium and then to liquid WP medium with 500mg/L ampicillin. Rosmarinic acid accumulation was 35.8mg/g DW (90). In *Salvia bulleyana*, 5-week-old aseptic shoots on MS medium with 0.1mg/L IAA and 0.5mg/L BAP were used as explants. Hairy roots were induced by infecting shoots and leaves with a needle dipped in A4 *Agrobacterium rhizogenes* strain. After infection, explants were transferred to hormone-free MS medium and then to liquid WP medium with 500mg/L ampicillin. Rosmarinic acid accumulation ranged from 31.2 to 39.6mg/g DW (91).

Genetic Engineering/Metabolic Engineering Studies for Rosmarinic acid Production

Genetic engineering allows alteration of an organism's DNA to manipulate cellular metabolism for desired traits (92). Gene transfer techniques are being developed to understand and regulate genes responsible for secondary metabolite synthesis in different plants (93). Consequently, genetic engineering enables manipulation of plant secondary metabolism to enhance production of rosmarinic acid, a valuable compound used across various industries. Altered genes in specific pathways have been employed to modify rosmarinic acid production.

Modification in phenylpropanoid pathway and tyrosine -derived pathway

Genetic engineering of phenylpropanoid and tyrosine-derived pathways was performed to modulate rosmarinic acid production in *S. miltiorrhiza* hairy root cultures (94, 95). Intermediate plasmid p1304+ was constructed by integrating c4h, tat, hppr, and hppd DNA sequences between P35S and TNOS in p1304+. Subcloning of hppr in p1304+-tat resulted in p1304+-tat-hppr plasmid for the tat-hppr binary expression vector. Transformation of *S. miltiorrhiza* leaf disc explants with positive clones was carried out. Culturing hairy root clones in dark at 25°C, using half strength liquid B5 medium with hygromycin and cefotaxime. Metabolite analysis at day 45 showed wild type produced 211mg/L rosmarinic acid, the vector control 56.1mg/L. c4h transformed roots had 201mg/L, tat-transformed comparable to wild type, hppr transformed produced 616mg/L, and antisense-hppd transformed produced 542mg/L. Co-expression of tat and hppr resulted in 906mg/L rosmarinic acid, indicating their synergistic effect. Tyrosine-derived pathway genes (tat, hppr, and hppd) had more impact on rosmarinic acid biosynthesis than phenylpropanoid pathway gene c4h (95).

In *Antirrhinum majus*, the DEL/ROS1 protein complex (Delila and Rosea1 transcription factors) triggers the expression of essential genes, including PAL, involved in the phenylpropanoid pathway (96). A control vector, pBI121-CAMBIA1302 (pBC), and a coexpression vector, pBC -AmDEL-AmROS1 (pDR), were constructed and introduced into *Agrobacterium tumefaciens* strain EHA105 using the heat-shock method. *S. miltiorrhiza* leaves were infected with *A. tumefaciens* and placed on MS selection medium. Upon shoot development, they were transferred to half strength MS medium with hygromycin B (10mg/L) and cefotaxime (200mg/L). The root system developed within 2 weeks, and the plantlets were multiplied in MS basal medium. Rosmarinic acid content was measured at three stages, showing a significant increase after 60 days compared to the wild type and vector-transformed control (pBC). Increased rosmarinic acid content was also observed after 210 days (97, Table 1).

In the tyrosine-derived pathway for rosmarinic acid, 4-coumaroyl-CoA acts as a precursor for both flavonoids and rosmarinic acid synthesis. Chalcone synthase (CHS) and RAS enzymes utilize 4-coumaroyl-CoA as substrates for flavonoid and rosmarinic acid production, respectively. Decreasing flavonoid synthesis in *S. miltiorrhiza* impacts rosmarinic acid and related phenolic acids' production. CHS is a key enzyme in flavonoid synthesis (98). Silencing the CHS transcript through RNAi-mediated silencing in *S. miltiorrhiza* hairy root cultures, followed by treatment with salicylic acid (SA), increased phenolic acid production efficiently. The CHS cDNA sequence was cloned into the pKANNIBAL vector in both sense and antisense directions. Fragments with the CaMV 35S promoter, octopine synthase (OCS) terminator, and PDK intron were ligated into the pART27 vector. *Agrobacterium tumefaciens* ATCC15834 was electroporated to introduce the recombinant plasmid into hairy root lines. Transformant root lines displaying kanamycin resistance were cultured in modified Gamborg's medium (67-V liquid medium) (99) at 25°C in darkness. On day 18 after inoculation, SA elicitor (50µM) was applied to the transgenic hairy root cultures and controls. In SA-treated wild-type lines, rosmarinic acid production was 20.42mg/g DW, 1.87 times higher than wild -type lines. CHS-silenced lines produced 21.09mg/g DW rosmarinic acid, 1.93 times that of wild-type lines. SA-treated CHS silencing lines showed the highest rosmarinic acid content (42.45mg/g DW). These findings suggest that combining both techniques is more effective for enhancing phenolic acid yield in *S. miltiorrhiza* hairy root cultures compared to genetic alteration or elicitor therapy (100).

Through RAS catalysis, 4-coumaric acid CoA and 3,4 -dihydroxyphenyllactic acid were converted into 4-coumaroyl-4'-hydroxyphenyllactic acid, which was further converted into rosmarinic acid by CYP98A14 (21). To enhance phenolic acid accumulation, RAS and CYP98A14 genes were individually duplicated into pCAMBIA1304+ vector with CaMV35S promoter using SpeI and BstEII restriction sites. The resulting constructs, pCAMBIA2300+-RAS and pCAMBIA2300+-CYP98A14, were ligated into pCAMBIA2300 vector at PstI, and transformed into *A. tumefaciens* strain C58C1 for infecting *S. miltiorrhiza* explants. Hairy roots were grown on decreasing carbenicillin levels and then on half strength MS liquid medium. Transgenic lines overexpressing RAS and CYP98A14 showed higher rosmarinic acid production

(22.57-26.07mg/g DW and 30.35-48.72mg/g DW, respectively) compared to control (15.97mg/g) (101). Overexpression of SmMYB2 gene in *S. miltiorrhiza* hairy roots also increased rosmarinic acid production by driving CYP98A14 expression (102). Similarly, overexpression of SmMYB98 gene increased total salvianolic acid production, including rosmarinic acid, while SmMYB98-KO (kaurene oxidase) gene decreased total salvianolic acid production (103).

Modification in jasmonate biosynthesis pathway

MeJA application boosts rosmarinic acid production in *S. miltiorrhiza* hairy root cultures (104). AOC gene targeting for internal JA overexpression was compared to exogenous JA treatment (105). AOC's specificity influences the stereochemical composition of jasmonates, making it a potential regulator of plant metabolite biosynthesis (106). The AOC cDNA from *S. miltiorrhiza* was cloned into the pCAMBIA1304 vector (p1304-SmAOC) with the hygromycin phosphotransferase gene (hpt) (105). Transgenic hairy roots were established using *A. rhizogenes* strain C58C1, showed a 2.1-fold increase in rosmarinic acid (2.3mg/g DW) compared to wild type (1.3mg/g DW) when transferred to half strength B5 liquid media at 25°C in the dark (105).

Modulation of transcription factors

SmMYC2 was overexpressed in *S. miltiorrhiza* through the construction of pMD19T–SmMYC2 I construct, which, upon transfer to the destination vector, yielded pEarleyGate201-SmMYC2 (97). The vector was then introduced into *Agrobacterium tumefaciens* strain GV3101, and explants were co-cultured and subsequently transferred to MS selection medium. Shoots were developed and subjected to root induction on half strength MS medium containing 10mg/L glufosinate-ammonium and 200 mg/L cefotaxime, followed by propagation on half strength MS basal medium. After 2 months, the rosmarinic acid content in the roots was assessed, revealing a 2.46-fold increase in the overexpressed SmMYC2 roots (6.36 ± 0.21mg/g) compared to the vector transformed control (2.59 ± 0.04mg/g) (107).

The basic helix-loop-helix (bHLH) superfamily constitutes the second-largest family of transcription factors in plants. In the context of secondary metabolism regulation in plants, these bHLH transcription factors have shown significant efficacy (108). Notably, the isolation and functional characterization of SmbHLH148 from *S. miltiorrhiza* revealed its role in the accumulation of phenolic acids and tanshinones in hairy roots. To achieve

Table. 1. Genetic engineering /metabolic engineering studies for rosmarinic acid production from genus *Salvia*

S. No.	Name of species	Media used + PGR	Control yield	Vector + Gene of interest	Elicited yield	Reference
1.	*S. miltiorrhiza*	Half-strength B5 medium + 100mg/L hygromycin and 500mg/L cefotaxime	Wild type (211mg/L) Vector transformed control- ck (56.1mg/L)	p1304^{+} -c4h	201mg/L	(95)
				p1304^{+} -tat	similar levels to those of wild type	
				p1304^{+} -hppr	616mg/L	
				p1304^{+} - antisense-hppd	542mg/L	
				P1304^{+}- tat-hppr	906mg/L	
2.	*S. miltiorrhiza*	Half strength MS medium + 10mg/L hygromycin B + 200 mg/L cefotaxime	Wild type (1.58 ± 0.64 mg) Vector transformed control- pBC (2.32 ± 0.47 mg)	pBC-DEL-ROS1	4.92 ± 1.34 mg	(97)
3.	*S. miltiorrhiza*	67-V liquid medium	SA treated wild-type lines (20.42mg/g DW) (1.87-fold of the wild-type lines)	CHS silencing lines	21.09mg/g DW (in line RNAi-23)	(100)
				SA treated CHS silencing lines	42.45mg/g DW (in line RNAi-2)	
4.	*S. miltiorrhiza*	Half-strength MS medium + carbenicillin	pCAMBIA2300 empty vector (15.971mg/g)	pCAMBIA2300+-RAS	22.573 to 26.072mg/g DW	(101)
				pCAMBIA2300+-CYP98A14	30.351 to 48.720mg/g DW	
5.	*S. miltiorrhiza*	Half-strength B5 medium + 10mg/L hygromycin	Wild type (1.3mg/g DW)	p1304-SmAOC	2.8mg/g DW	(105)
6.	*S. miltiorrhiza*	Half strength MS medium + 10mg/L glufosinate-ammonium + 200 mg/L cefotaxime	2.59 ± 0.04mg/g	pEarleyGate201-SmMYC2	6.36 ± 0.21mg/g	(107)
7.	*S. miltiorrhiza*	67-V liquid medium	4.00- fold of the control	SmbHLH148-1300 (*SmbHLH148* + *pCAMBIA1300 binary vector)*	5.28mg/g DW	(109)

this, SmbHLH148 was amplified, cloned into the pCAMBIA1300 binary vector harboring the CaMV35S promoter and NOS terminator using Xba I and Sac I restriction sites, and introduced into *A. rhizogenes* (ATCC 15834). Subsequent infection of *S. miltiorrhiza* leaves with this construct led to the growth of hairy roots on 67-V liquid medium. The overexpression of SmbHLH148 demonstrated a substantial increase of 300% (5.28 mg/g DW) in rosmarinic acid content compared to the control. Moreover, this overexpression induced activation of phenylpropanoid pathway genes (109).

Elicitation Strategies for Rosmarinic acid Production

Plants display morphological and physiological alterations in response to microbiological, physical, and chemical stimuli termed "elicitors." To enhance competitiveness, persistence, and survival, plants elevate the synthesis of secondary metabolites through elicitation. Elicitors, whether biotic or abiotic, act as signals recognized by specific receptors on the plant cell membrane, triggering defense and stress-induced responses that induce and enhance the production and storage of secondary metabolites (110). Elicitation serves as a promising approach to address challenges associated with large-scale production of secondary metabolites, such as low productivity. Table 2 and Table 3 provide a list of elicitors employed in obtaining rosmarinic acid from hairy root culture of *Salvia* species.

Biotic elicitors

Biotic elicitors can originate from pathogens (fungi, bacteria, or yeast) or the host plant itself, existing as unprocessed extracts or partially purified substances like polysaccharides, glycoproteins, inactivated enzymes, and purified chitosan (CHI), among others (111). In a study with *Salvia miltiorrhiza* hairy root cultures grown in 67-V liquid medium, the addition of 0.2mL yeast elicitor led to a significant increase in rosmarinic acid accumulation, reaching 3.23±0.15% DW after 21 days, compared to the control with only 1.90±0.18% DW. When exposed to yeast along with 500µM H_2O_2, the rosmarinic acid content further increased to 3.65±0.26% DW (112). Similarly, in another experiment, *S. miltiorrhiza* hairy roots elicited with 1mL yeast extract (YE) on the 7th day of cultivation exhibited an elevated rosmarinic acid content of 2.97% DW, compared to 1.24% DW in the control (113). Furthermore, it was found that yeast extract was more effective than Ag+ in promoting rosmarinic acid accumulation. Hairy roots treated with 200mg/L yeast extract in MS medium showed a rosmarinic acid content of 74.1mg/g DW on day 8 after elicitation, 1.6 times higher than the control (46.1mg/g DW) (114).

The impact of *Sclerotium rolfsii* Sacc. strains on rosmarinic acid accumulation has been documented. In a study involving *S. miltiorrhiza* hairy root culture, treatment with 280µg/mL fungal extract of *S. rolfsii* Sacc. in MS media resulted in a significant reduction in rosmarinic acid levels. Specifically, there was a decrease of 35.8%, 42.4%, and 28.8% on days 5, 7, and 9 after treatment, respectively (115). Furthermore, *Rhizobium radiobacter* (endophytic bacteria) derived from *S. miltiorrhiza* roots were utilized as an elicitor. Hairy roots were cultured on solid MS media supplemented with 0.5g/L casein hydrolysate and 0.025% bacterial suspension, which caused a significant reduction in rosmarinic acid content. The highest decrease, amounting to 94.5%, was observed on day 9 (116). Another investigation demonstrated that *S. virgata* hairy roots elicited with 50ppm yeast extract produced the highest amount of rosmarinic acid after 5 days of treatment (15.58±0.01mg/g DW), which was 1.44 times higher than the control. This was achieved in half-strength MS liquid medium (117). In the context of *S. bulleyana* hairy root cultures maintained on hormone-free WP medium, the addition of yeast extract at 250mg/L resulted in approximately a twofold increase in rosmarinic acid levels, elevating it from 12.5mg/g DW to 29.0mg/g DW (118). Table 2 provides a summary of the biotic elicitors used in the production of rosmarinic acid in hairy root cultures and

Table 2. Effect of various biotic elicitors in the production of rosmarinic acid from various species of *Salvia*

S. No.	Name of species	Media used+ PGR+ parameters for biotic elicitors	Yield of metabolite		Reference
			Control yield	Elicited yield	
1.	*S. miltiorrhiza*	67-V medium + 0.2mL yeast extract (day 6)	1.90±0.18 % DW	3.23±0.15 % DW	(112)
2.	*S. miltiorrhiza*	67-V medium + 1mL YE (day 7)	1.24 % DW	2.97% DW	(113)
3.	*S. miltiorrhiza*	MS Medium + 200mg/L YE (day 8)	Not applicable (NA)	74.1mg/g DW (1.6-fold of control)	(114)
4.	*S. miltorrhiza*	67-V liquid medium + 280µg/ mL *Sclerotium rolfsii* extract (day 7)	NA	Reduced by 42.4%	(115)
5.	*S. miltiorrhiza*	MS medium + 0.5g/L casein hydrolysate + *Rhizobium radiobacter* (day 9)	NA	Reduced by 94.5%	(116)
6.	*S. virgata*	Half strength MS medium + 50ppm YE	NA	15.58±0.01mg/g DW (1.44- fold of control)	(117)
7.	*S. bulleyana*	Half strength SH medium + 250mg YE	12.5mg/g DW	29mg/g DW	(118)

*NA - not applicable

their corresponding yields (115, 116, 117, 118).

Abiotic elicitors

Abiotic elicitors are comprised of physical elements and chemical substances that do not have a biological origin, such as, heavy metal salts, intercellular signaling molecules (methyl jasmonate [MeJa], jasmonic acid, salicylic acid), temperature shift, UV irradiation (111). Further, abiotic elicitors can be divided into organic and inorganic elicitors.

MeJA, a well-known organic elicitor, was employed to stimulate rosmarinic acid production in *S. miltiorrhiza* hairy roots cultured in half strength B5 medium. After 18 days of cultivation, MeJA treatment resulted in a significant 1.9-fold increase in rosmarinic acid content, rising from 3.25% to 6.02% of dry weight (DW) (104). In a similar fashion, the application of 50µM ABA to 67-V liquid media-grown hairy root cultures of *S. miltiorrhiza* led to a remarkable elevation in rosmarinic acid content from 3.66mg/g to 7.45mg/g (119). Interestingly, treatments involving fluridone or paclobutrazol alone exhibited minimal influence on rosmarinic acid levels in the hairy roots. However, when combined with abscisic acid (ABA), paclobutrazol resulted in a drastic reduction of rosmarinic acid content, reaching only 15% of the control levels (119). Moreover, treatment with 150mM $CoCl_2$ yielded the lowest rosmarinic acid levels compared to the effect of 50mM ethylene, causing an 83% decrease (119). Another study focusing on *S. wagneriana* HRD3 line demonstrated that treatment with 3.3mg/L JA led to a significant enhancement in rosmarinic acid production, reaching 213µg/g fresh weight (FW) compared to the control's 173µg/g FW (89). Additionally, the use of casein hydrolysate, a rich source of organic carbon, nitrogen, phosphate, and other amino acids, as an elicitor, resulted in a remarkable twofold increase in rosmarinic acid production (89,120). In liquid media containing 67-V supplemented with 100µM MeJA, rosmarinic acid levels significantly increased at day 3, reaching 20.3mg/g DW, which was 1.5-fold higher than the control (121). *Salvia przewalskii* hairy root growth responded positively to low SA concentrations but was inhibited at high SA levels. MeJA stimulated hairy root growth from 0 to 400µM, but at 600µM, growth inhibition occurred. Treatment with 50µM SA elevated rosmarinic acid content by 1.41 times, while 400µM MeJA increased it by 1.27 times (122). Among *S. virgata* hairy roots induced by ATCC15834 strain, the highest rosmarinic acid content, 18.45±0.8mg/g DW, was observed after elicitation with 22.4ppm MeJA (117). Post MeJA treatment, converted roots showed a rosmarinic acid level of 110.2mg/g DW (118), which was 13 times higher than in roots from 2-year-old plants cultivated in field conditions (123). Abiotic elicitors used for rosmarinic acid production in *Salvia* hairy root cultures are presented

Table 3. Effect of various abiotic elicitors in the production of rosmarinic acid from various species of *Salvia*

S. No.	Name of species	Media used+ PGR+ parameters for abiotic elicitors	Yield of metabolite		Reference
			Control yield	Elicited yield	
		Organic abiotic elicitors			
1.	*S. miltiorrhiza*	Half-strength B5 medium + 100µM methyl jasmonate (day 6)	3.25% of DW	6.02% of DW	(104)
2.	*S. miltiorrhiza*	67-V medium + 50µM ABA	3.66mg/g	7.45mg/g	(119)
3.	*S. wagneriana*	Hormone-free liquid MS medium + 3.3mg/L JA	173µg/g FW	213µg/g FW	(89)
4.	*S. miltiorrhiza*	67-V liquid medium + 100µM MeJA (day 3)	NA	20.3mg/g DW (1.5-fold of control)	(121)
5.	*S. przewalskii*	67-V medium + 400µM MeJA (day 3)	NA	67.1273 ± 0.41mg/g DW (1.27- fold increase)	(122)
6.	*S. przewalskii*	67-V medium + 50µM SA (day 3)	NA	44.0306 ± 0.08 mg/g DW (1.41- fold increase)	(122)
7.	*S. bulleyana*	Half strength SH medium + 100µM MeJA	8.34 ± 0.14mg/g DW	110.2mg/g DW	(118,123)
		Inorganic abiotic elicitors			
8.	*S. miltiorrhiza*	67-V medium with 0.0124mM phosphate (day 6)	NA	2.283-fold of control	(124)
9.	*S. virgata*	Half strength MS medium + 2.5ppm Ag^+ (day 5)	NA	16.01±0.09mg/g DW (1.54- fold of control)	(117)
10.	*S. miltiorrhiza*	1: 500 Smoke-water (day 3)	NA	32.99-folds of the control	(125)

*NA - not applicable

in Table 3. *S. miltiorrhiza* biomass and phenolic acid content responded to phosphate levels in inorganic abiotic elicitation. Hairy roots were exposed to 67-V medium with varied phosphate concentrations for 6 days. Optimal rosmarinic acid accumulation occurred at 0.0124mM phosphate, showing a 2.283-fold increase over the control (124). *S. virgata* hairy roots, induced by the ATCC15834 strain, exhibited enhanced rosmarinic acid production (16.01±0.09mg/g dry weight) when cultivated in half-strength MS liquid medium with 2.5ppm Ag+ as an elicitor (117). Utilizing smoke water derived from the combustion of *Crataegus pinnatifida* and *Magnolia denudata* plant materials, a 1:500 dilution treatment on 18 -day-old *S. miltiorrhiza* hairy roots resulted in a significant 32.99-fold increase in rosmarinic acid accumulation compared to the control (125).

Large scale production or bioreactor studies for rosmarinic acid production

Bioreactors are specialized vessels, typically made of glass or stainless-steel, employed for the large-scale propagation of cells or tissues to yield significant metabolites (126). Various bioreactor types exist, such as stirred-tank reactors, bubble-column reactors, and trickle-bed reactors. In a previous study, a nutrient sprinkle bioreactor was utilized to scale up the production of rosmarinic acid. The hairy root culture of *Salvia officinalis* was established through infection with *Agrobacterium rhizogenes* ATCC 15834. Regular subculturing every 40 days in hormone-free WP liquid medium was conducted, with maintenance under cool white fluorescent lamps at a temperature of 26±2°C. Upon reaching 40 days of growth, the hairy root culture was inoculated into a 5L nutrient sprinkle bioreactor composed of glass, consisting of two vessels: one for plant material growth (internal volume: 5L) and the other for nutrient medium storage (volume: 1.5L). The bioreactor operated by spraying liquid MS or WP media through a nozzle onto the plant material, with unused media returning to the reservoir. The transformed roots demonstrated substantial levels of rosmarinic acid in the sprinkling bioreactor culture, achieving an average yield of 477.13±14.73 mg/L (126). This concentration was higher (4-5 times) than those found in the roots of organically grown plants and the samples of dried *S. officinalis* leaves that were sold commercially (88).

Conclusion and Prospects

Rosmarinic acid is a promising plant secondary metabolite known for its significant anti-oxidant, anti-cancer, anti-aging, anti-inflammatory, and anti-diabetic properties. While various plant species, including *Salvia* genus, produce it, field harvesting is impractical due to continuous processing, low content, and potential harm to native plant populations. Therefore, precise selection of in -vitro systems and cultivation conditions is crucial for mass-producing rosmarinic acid. Among tissue culture methods, hairy roots show great potential, being involved in synthesizing essential commercial metabolites. Biosynthetically, rosmarinic acid originates from tyrosine and phenylalanine, and leveraging biotechnological approaches such as metabolic engineering can boost its production. Manipulating genes in the tyrosine-derived pathway and suppressing the competing biosynthetic route of flavonoids facilitate enhanced rosmarinic acid synthesis. Combining genetic engineering and elicitation strategies involving biotic and abiotic components can enhance rosmarinic acid production. Among biotic elicitors, only YE has demonstrated a yield increase, while abiotic elicitors like MeJa and smoke-water generate elevated levels of rosmarinic acid. Employing both approaches in hairy root cultures of *Salvia* holds promise for high yield. Utilizing biotechnological tools like metabolomics and hetero-host technology in other Salvia species can yield significant outcomes, fostering extensive research on rosmarinic acid production. Employing bioreactors under carefully chosen cultivation conditions and operational modes offers potential for large-scale, sustainable production of this vital biomolecule.

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Authors' contributions

SS, KPA collected the literature and drafted the review manuscript, WNS and PN critically revised the manuscript, PN supervised the work and all the authors gave the final approval.

Compliance with ethical standards

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