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Review

Salvia Species: Biotechnological Strategies Applied to In Vitro Cultures for the Controlled Production of Bioactive Diterpenoids

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Abstract: Plant secondary metabolites have great applications in the nutritional and cosmetic aspects of human health. Terpenes, and in particular bioactive diterpenoids, represent an important group of compounds found in *Salvia* species. Their production in plants is often limited, and chemical synthesis is often not economically feasible. Biotechnological approaches using plant cell and tissue cultures can be routinely established under sterile conditions from explants for biomass production and the extraction of secondary metabolites. The biosynthesis and accumulation of bioactive diterpenoids in vitro in *Salvia* cells and tissues can be enhanced by strain improvement, techniques to select high-producing cell lines, optimisation of the growth medium, use of specific precursors or elicitors, induction of their release into the culture medium, and the overexpression of genes. This review analyses the biotechnological techniques applied to the in vitro culture of *Salvia* cells and tissues to enhance the production and accumulation of bioactive diterpenoids and summarises their biological activities.

Keywords: diterpenoids; *Salvia*; elicitation; in vitro culture; hairy root; cell culture; biological activity

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1. Introduction

Salvia L. is a large genus of the Lamiaceae, with about 980 species. It is mainly distributed in Mesoamerica/South America, the Mediterranean region, and Southwest and East Asia [1–5]. *Salvia* species have long been known for their wide variety of medicinal uses in folk medicine for pain relief, protection against oxidative stress, free radical damage, angiogenesis, inflammation, and bacterial and virus infection [6–10]. *Salvia* are cultivated for their aromatic properties and abundance of secondary metabolites, which are used to produce food additives and essential oils, pharmaceuticals, dyes, cosmetics, and biocides [8,11]. Due to their widespread ethnic use, some *Salvia* species (*S. sclarea*, *S. officinalis*, and *S. fruticosa*) are recognised as safe in the USA [12], and their drugs are included in the European Pharmacopoeia VI. From a phytochemical point of view, *Salvia* spp. have a complex chemical composition with terpenoids [7,13–16] and polyphenols [17–20] being the main compounds. By the beginning of 2011, a total of about 773 compounds had been isolated from 134 *Salvia* species and classified into seven main groups: sesquiterpenoids, diterpenoids, sesterterpenoids, triterpenoids, steroids, polyphenols, and others. According to their structure, diterpenoids can further be divided into a number of groups [8,21]. The most

representative are abietane [22–24], clerodane [25–28], pimarane [29], icetexane [30–32], and labdane [13]. Extensive research into biotechnological approaches to the production of *Salvia* phytochemicals has been driven by the increasing demand for sage products and the environmental, ecological, and climatic constraints of producing sage metabolites from field-grown plants [33]. Growing demand has led to the overexploitation of natural habitats, and complex climate-related stressors can affect many wild *Salvia* species [34]. Alternative and renewable sources of Salvia biomass are urgently needed to prevent ecological crisis. In vitro plant culture technology is a potential approach for the continuous production of secondary metabolites in *Salvia* species under controlled conditions. In vitro systems are environmentally friendly and can be grown independently of natural plant populations, environmental conditions, latitude, climatic variations, or seasonal changes. In vitro plant tissues and cell culture methods may represent an alternative strategy for the production of highly valuable plant metabolites, and could even be adopted by the pharmaceutical industry for the development of new drugs and formulations, the production of pharmaceuticals and cosmetics, as food additives in the food industry, and for crop protection in agriculture [35,36]. Biotechnological techniques have greatly facilitated plant propagation and the production of some important bioactive compounds from the genus *Salvia.* Various in vitro systems such as shoots, cell culture, and hairy roots are used to produce bioactive compounds [33,37–44]. However, many issues remain to be addressed before large-scale biotechnological production based on *Salvia* in vitro systems can be developed [41].

This review, which originated from the first author's PhD thesis [45], focuses on the main diterpenoids obtained from in vitro cultures of *Salvia* species. The aim of the present review is to provide an overview of promising biotechnological strategies for the precise control of growth conditions and the optimisation of growth regulators, nutrient supply, and elicitation strategies to stimulate diterpenoid biosynthesis in in vitro *Salvia* species. Furthermore, genetic engineering techniques to modify metabolic pathways to enhance the production of specific bioactive diterpenoids have also been reported. To the best of our knowledge, this is the first review article dealing with the in vitro production of different classes of diterpenoids in different *Salvia* species.

2. Search Strategy

The present paper provides an overview of the most important results reported in the literature on the in vitro production of bioactive diterpenes from cell and tissue cultures of *Salvia* species. A systematic search was conducted in English language databases (Medline (PubMed), Web of Science, and Scopus) from inception to January 2024, with no restriction on publication date. In addition, report papers and reviews were manually retrieved, and traced references in the included literature were tracked down using the references found to retrieve additional material retrospectively. The articles used in this review were selected from journals with a high impact factor, based on their scientific relevance and the reliability of their data. The search was carried out independently by three researchers and then cross-checked. Keywords and phrases used in the search included the following: "*Salvia*", "diterpenoids", "bioactive diterpenes in *Salvia*", "biological activity", "elicitation", "biotic elicitors", "abiotic elicitors", "combination of elicitors", "in vitro cultures", "hairy root", "cell culture", "growth conditions", "in situ adsorption", "medium renewal", and "gene overexpression". In total, 19 *Salvia* species were considered. For a better understanding of the studies presented in this review, tables in the Supplementary Materials (Tables S1–S9) were prepared to show the main results related to the influence of the elicitation on producing and accumulating specific bioactive diterpenes.

3. Structure and Biological Activity of the Main Diterpenoids Obtained from In Vitro Cultures of *Salvia* Species

Diterpenoids [16,46–62] are composed of four isoprene units and 20 carbon atoms in their chemical structures. The various bioactive diterpenoids found in the in vitro culture

of tissues or cells in *Salvia* species are detailed in Table 1, and the structures of the major bioactive diterpenoids found in in vitro *Salvia* species are reported in Figure 1.

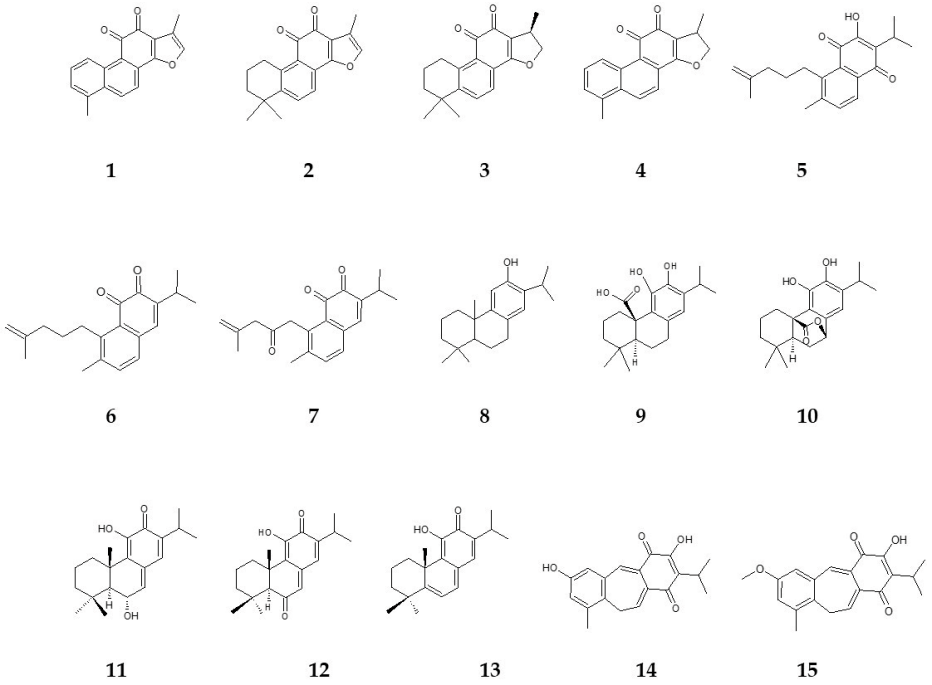

Figure 1. Bioactive diterpenoids obtained from in vitro cell and tissue cultures of *Salvia* species. Reproduced from [45].

Table 1. Diterpenoids produced by the in vitro culture of various *Salvia* species. Reproduced from [45].

Compound Number	Name	*Salvia* Species	In Vitro Culture	References
1	tanshinone I	*S. miltiorrhiza*	HR	[63]
			CC	[64]
		S. castanea	HR	[65]
2	tanshinone IIA	*S. miltiorrhiza*	HR	[63]
			CC	[64]
		S. castanea	HR	[65]
3	cryptotanshinone	*S. miltiorrhiza*	HR	[63]
			CC	[64]
		S. castanea	HR	[65]
4	dihydrotanshinone I	*S. miltiorrhiza*	HR	[63]
		S. castanea	HR	[65]
5	salvipisone	*S. sclarea*	HR	[66]
6	aethiopinone	*S. sclarea*	HR	[66]
7	1-oxoaethiopinone	*S. sclarea*	HR	[66]
8	ferruginol	*S. sclarea*	HR	[66]
		S. miltiorrhiza	HR	[63]
9	carnosic acid	*S. officinalis*	C	[67]
			CC	[67]
			MP	[37]
10	carnosol	*S. officinalis*	C	[67]
			CC	[67]
			MP	[37]
11	taxodone	*S. austriaca*	HR	[68]
12	taxodione	*S. austriaca*	HR	[68]
13	15-deoxy-fuerstione	*S. austriaca*	HR	[68]
14	demethylfruticuline A	*S. corrugata*	RS	[38]

Table 1. *Cont.*

Compound Number	Name	*Salvia* Species	In Vitro Culture	References
15	fruticuline A	*S. corrugata*	MP	[38]
			apex	[38]
			leaves	[38]
			RS	[38]
			apex	[38]
			leaves	[38]

HR: hairy roots, CC: cell culture, C: callus, MP: micropropagated plants, RS: regenerated shoots.

3.1. Tanshinones: Tanshinone I, Tanshinone IIA, Cryptotanshinone, Dihydrotanshinone I

Tanshinones are widely distributed in the genus *Salvia* [7,69–71]. The dried root of *S. miltiorrhiza* is commonly used in Traditional Chinese Medicine (TCM) to improve body function (e.g., stimulate circulation and improve blood flow) [72]. Several hydrophobic bioactive diterpenoids have been isolated from this drug [9,70,72–79]. Since the 1990s, several researchers have exploited the in vitro culture of *S. miltiorrhiza* including the hairy roots and cell culture to produce tanshinones. Due to their remarkable activities in the clinical treatment of cardiovascular diseases, these compounds have received much attention. The roots of *S. castanea*, *S. tomentosa*, and *S. przewalskii* have been used as a substitute for *S. miltiorrhiza* roots because of their tanshinone content [80–82]. Several years later, roots of micropropagated and in vitro regenerated *S. przewalskii* plants were assayed for tanshinone I (T-I) and tanshinone IIA (T-IIA). The results showed that the highest tanshinone levels, 3.8 mg/g dry weight (DW) of TI and 7.6 mg/g DW of TIIA, were produced by 2-year-old roots in in vitro regenerated plants grown in the field and harvested at the flowering stage [83]. Tanshinones have also been found in the roots of *S. abrotanoides* (ex *Perovskia abrotanoides*) [84], and the amounts are increased by biotic and abiotic elicitors [85]. Nowadays, these compounds have been found to possess a wide range of pharmacological activities such as antibacterial, antioxidant, anti-inflammatory, and antitumour properties [86].

3.2. Abietane Diterpenoids: Aethiopinone, Salvipisone, Ferruginol, and 1-Oxoaethiopinone

The rearranged abietane diterpenoids salvipisone (**5**) and aethiopinone (**6**) as well as ferruginol have been extracted from the roots of *S. aethiopis* [87–89]. These compounds are mainly present in the roots of some *Salvia* species and have also been isolated from several species such as *S. argentea* [90], *S. candidissima* [91], *S. ceratophylla* [92], and from the aerial parts of *S. cyanescens* [93]. All of these diterpenoids as well as the rearranged abietane 1-oxoaethiopinone (**7**) have been isolated from the roots of *S. sclarea* [94]. Only *S. sclarea* has been investigated for producing these abietane diterpenoids using hairy root transformation [95,96]. As previously described, ferruginol (**8**), salvipisone, aethiopinone, and 1-oxo-aethiopinone were produced by the in vitro hairy root transformation of *S. sclarea* using *Agrobacterium rhizogenes* strain LBA 9402 [95]. Ferruginol has recently been purified from the hairy roots of *S. corrugata* [97]. These compounds have been reported to have various biological activities. Among them, ferruginol exhibited potent anti-acanthamoeba [98] and in vitro antiplasmodial activities [99]. Aethiopinone has been shown to have analgesic and anti-inflammatory properties [100]. It has also been shown to be cytotoxic to several solid tumour cell lines. The most potent effect was on the human melanoma A375 cell line (IC_{50} 11.4 μM) [66]. Salvipisone and aethiopinone have shown antibacterial activity against selected methicillin-susceptible and methicillin-resistant *S. aureus* and *S. epidermidis* strains [101–103]. These compounds exhibited relatively high cytotoxicity against HL-60 and NALM-6 leukaemia cells (IC_{50} range 0.6–7.7 μg/mL, corresponding to 2.0–24.7 μM), whereas 1-oxoaethiopinone and ferruginol were less active [104].

3.3. Abietane Diterpenoids: Carnosic Acid and Carnosol

Carnosic acid (**9**) is one of the most studied phenolic diterpenoids [105,106]. It is important in the food, cosmetic, and pharmaceutical industries because of its potent antioxidant, anti-inflammatory, and anticancer properties [107–115]. Carnosic acid and carnosol (**10**) have been isolated from various *Salvia* species [87,116]. Carnosic acid is mainly found in the aerial parts of the plant, and its biosynthesis in the leaves has been extensively studied in *S. fruticosa* and *S. rosmarinus* [106,117,118]. The biological activity of carnosic acid and carnosol has been the subject of extensive investigation [119]. Carnosic acid and carnosol exhibited anti-inflammatory activities by acting on mPGES-1 and 5-LO, resulting in suppressing pro-inflammatory eicosanoid formation [114]. Later on, these diterpenoids from the leaves of *S. officinalis* were found to have antioxidant and antibacterial activities [108]. Recently, carnosic acid was shown to suppress the development of oral squamous cell carcinoma via the mitochondrial apoptotic pathway [120] and to prevent the biofilm formation of methicillin-resistant *Staphylococcus aureus* (MRSA) [121]. Recent studies have demonstrated its potential as a preventive agent against soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* on slices of potato tubers [122].

3.4. Abietane Diterpenoids: Taxodone, Taxodione, and 15-Deoxyfuerstione

Taxodone (**11**), taxodione (**12**), and 15-deoxyfuerstione (**13**) were isolated from the roots of *S. spinosa* [123] and the hairy roots of *S. austriaca* [68]. Taxodione showed activity against human acetylcholinesterase and cytotoxic activity against the A549 cell line [124]. This compound was found to be active against *Trypanosoma brucei rhodesiense* (IC_{50} = 0.05 µM with high selectivity, SI = 38) and inhibited the growth of *Plasmodium falciparum* and *T. cruzi* by 50% at concentrations of 1.9 and 7.1 µM, respectively (SI values of 1.0 and 0.27) [68]. Taxodione from the roots of *S. deserta* was found to be leishmanicidal with an IC_{50} value of 1.46 µM (0.46 mg/L) against *Leishmania donovani* [125]. Taxodione from *S. chorassanica* exerted a protective effect in ischaemic injury induced by serum/glucose deprivation and the putative role of apoptosis as an underlying mechanism [126]. Taxodone and 15-deoxy-fuerstione are potent antimicrobial agents. In addition to direct biocidal/biostatic activity, they also interfered with the primary virulence factors/mechanisms of *S. aureus* and *Candida albicans*. In addition, both diterpenoids significantly inhibited microbial adhesion and biofilm formation when used at sub-inhibitory concentrations [127]. 15-Deoxyfuerstione from the roots of *S. lachnocalyx* showed a cytotoxic activity against MOLT-4, HT-29 and MCF-7 cells and significant anticancer activity with IC_{50} values in the range of 0.54–11.82 mg/mL [123].

3.5. Icetexane Diterpenoids: Demethylfruticuline A and Fruticuline A

Demethylfruticuline A (**14**) and fruticuline A (**15**) have been reported as the main constituents of the aerial part of *S. corrugata* [30]. Fruticuline A is an icetexane diterpenoid first isolated from *S. fruticulosa* [128], and later from *S. arizonica* [19], *S. corrugata* [30], and *S. lachnostachys* [129]. As previously described for the establishment of an in vitro culture of *S. corrugata*, analysis of the regenerated shoots showed the presence of both icetexanes. The yield of fruticuline A was higher in the methanolic extract than in those of fresh leaves and fresh shoot tips. However, only fruticuline A was found in micropropagated plants, whereas trace amounts of both diterpenoids were found in callus [38]. The biosynthetic pathway of demethylfruticuline A, fruticuline A has not been reported. However, biosynthetic relationships have shown that the icetexane skeleton originates from a rearrangement of the more common abietane skeleton, resulting in a 6-7-6 tricyclic scaffold with the systematic name 9(10/20)-*abeo*-abietane [23]. Consistent with this hypothesis, the majority of icetexane natural products discovered to date have been found in plant species that also produce abietane diterpenoids as secondary metabolites [31]. The icetexanes discovered to date vary widely in the degree of oxygenation and oxidation in each ring, resulting in a range of structures and biological activities [32]. Fruticuline A and demethylfruticuline A have shown potent antibacterial activity against Gram-positive bacteria [130,131], and

antioxidant activity [132]. Fruticuline A has also been reported to have anti-inflammatory and analgesic properties [133]. Demethylfruticuline A induces anoikis, a type of apoptosis induced in mammalian cells by the loss of cell adhesion mediated by CD36 [134], and also causes apoptosis by the induction of reactive oxygen species in mitochondria [135]. Recently, fruticuline A and demethylfruticuline A have shown remarkable binding affinity to CDK-2 compared to known CDK-2 inhibitors, leading to the consideration of these compounds as excellent natural CDK-2 inhibitors [136].

4. Strategies to Increase the In Vitro Production of Diterpenoids

Several parameters should be considered to control the optimal in vitro production of bioactive secondary metabolites from plants. Medium composition (sucrose level, exogenous growth hormone, type of nitrogen source, and their relative amounts) and growth conditions (light, temperature, and the presence of chemicals) can influence the growth, total biomass yield, and secondary metabolite production [137,138]. Specific treatments of the in vitro culture including precursor feeding and elicitor application can be used to increase plant metabolite production and improve its qualitative value as an ingredient in the pharmaceutical industry [139,140]. All of these approaches have opened up a new area of research that could have important economic benefits. For *Salvia* species, the differentiation or not of tissue culture, the chemical composition of the culture medium, the use of elicitors, the interaction with microorganisms (bacteria or fungi), the reduction in extracellular secondary metabolites by absorption, and transgenic culture with genes involved in the biosynthetic pathway are some in vitro strategies to increase the production of bioactive diterpenoids.

4.1. Medium Composition and Effect of Nutrients (Mineral, Hormone, and Sucrose Composition)

The in vitro growth of plant organs, tissues, and cells takes place in an appropriate medium that contains all of the necessary elements (salt composition, percentage of sucrose, and the presence of plant growth regulators). These elements have an influence on the accumulation of biomass and the formation of secondary metabolites. External factors such as carbon source, nitrogen source, growth regulators, medium pH, temperature, light, and oxygen are considered to easily regulate the expression of plant secondary metabolite pathways [141,142].

4.1.1. Effect of Salts

The most commonly used media are Murashige and Skoog (MS) [143], with very high concentrations of nitrate (NO_3^-), potassium (K^+), and ammonia (NH_4^+), Gamborg (B5) [144] and 67-V [145], with lower levels of inorganic nutrients than MS. Salt composition can affect biomass growth and secondary metabolite production. In general, high concentrations of ammonium ions inhibit the formation of secondary metabolites, while lowering the ammonium nitrogen increases it [146]. Zhi et al. [63] showed that the accumulation of diterpenoids in the hairy roots of *S. miltiorrhiza* was higher in the absence of ammonium nitrate (about 220 mg dry roots and 19 mg diterpenoids/g DW) than in its presence (about 140 mg dry roots and 7 mg diterpenoids/g DW). In the *Ti*-transformed cell suspension cultures, the B5 medium supported the best growth while the 67-V medium promoted tanshinone production [64]. Compared to the control, changing the KH_2PO_4 concentration could promote adventitious growth, but a high KH_2PO_4 concentration inhibited T-IIA biosynthesis in adventitious roots of *S. miltiorrhiza* [147].

4.1.2. Effect of Hormones

Cytokinin types and concentrations have different effects on different metabolites. Abscisic acid (ABA) and thidiazuron (TDZ) promote the increase in T-I and cryptotanshinone (CT) 5-fold and 7.5-fold, respectively, over the control in *S. miltiorrhiza* hairy root cultures [148]. In *S. officinalis*, the content of carnosol reached the highest value of 30.5 ± 0.42 mg/g DW in suspension cultures supplemented with 1.5 mg/L zeatine (ZEA),

and carnosic acid reached 12.7 ± 2.96 mg/g DW in callus cultures supplemented with 1.5 mg/L 6-benzylaminopurine (BA). However, the amount of these compounds was at a trace level in the suspension cultures supplemented with 0.5 mg/L of kinetine (KIN) [67].

4.1.3. Effect of Sucrose

The initial concentration of sucrose, which is the main carbon source in plant culture, has a significant effect on the growth of transformed cell cultures of *S. miltiorrhiza*. The fastest growth was observed in medium containing 30 g/L sucrose; it was significantly reduced at 60 g/L sucrose, and completely inhibited at 100 g/L sucrose [64]. The level of sucrose affects the productivity of secondary metabolite accumulating cultures [149]. In Ti-transformed *S. miltiorrhiza* cell suspension cultures, tanshinone production was better in the 30 g/L sucrose medium than in the 20 g/L or 40 g/L sucrose medium. However, a much higher tanshinone production was observed in the 100 g/L sucrose medium, although the cell growth was almost completely inhibited under these conditions [64].

4.1.4. Effect of Environment Factors

Light plays an important role in promoting plant growth and inducing or regulating plant metabolism [150–153]. On the other hand, light has an inhibitory effect on the accumulation of secondary metabolites such as nicotine and shikonin [154]. The green cell aggregates of *S. miltiorrhiza* transferred to the fresh B5 medium containing 4 g/L yeast extract and cultivated in darkness produced more tanshinones than their counterparts cultivated under continuous illumination, suggesting the inhibitory effect of light on tanshinone production in the cell cultures [64].

4.2. Elicitation

Plants and in vitro cultured plant cells show physiological and morphological responses to microbial, physical, or chemical factors, which are known as "elicitors" [155]. The term elicitor was originally used to describe molecules capable of inducing the production of phytoalexins. It is now defined as a substance that, when applied in small amounts to a living system, can induce or enhance the biosynthesis of specific compounds that are critical for plant adaptation to stress conditions [156]. Table 2 classifies elicitors according to their nature and may include abiotic elicitors such as metal ions and inorganic compounds as well as biotic elicitors from fungi, bacteria, viruses, or herbivores, plant cell wall components, and chemicals released by plants at the site of infection when attacked by pathogens or herbivores [157]. Elicitation is a widely used method that aims to enhance the production of secondary metabolites and has been described for many plant species. The discovery of elicitors has opened up a new approach to secondary metabolite production. All culture types (cell, callus, root, hairy root, whole plant, shoots, and seedling) can be exposed to elicitor treatments. Methyl jasmonate (MJ), jasmonic acid (JA), and salicylic acid (SA) are the most commonly used elicitors. In addition, polysaccharides such as pectin, dextran, chitin, chitosan, and alginate are often used as elicitors to induce secondary metabolism in plant cell and tissue cultures [158]. For an appropriate effect, several parameters such as elicitor concentration and selectivity, duration of elicitor exposure, age of culture, cell line, growth regulation, nutrient composition, and quality of the cell wall materials are also important factors influencing the successful production of secondary metabolites [159].

Table 2. Elicitors classification based on their nature. Adapted from Naik and Al–Khayri [160].

Biotic Elicitors	**Abiotic Elicitors**			
	Physical	**Chemical**	**Hormones**	**Plant Signal Compounds**
Polysaccharide Yeast extract Fungi Bacteria	UV radiation Osmotic stress Salinity Drought Thermal stress	Heavy metals Mineral salts Gaseous toxins	ABA TDZ	Jasmonic acid Salicylic acid

The activation of the biosynthesis of the plant product requires the perception of an extracellular or intracellular signal by a receptor on the surface of the plasma membrane. Elicitor perception initiates a signal transduction cascade, leading to the activation or de novo biosynthesis of transcription factors. These in turn directly regulate the expression of biosynthetic genes involved in secondary metabolism [161].

4.2.1. Biotic Elicitors

Microorganism—Roots Interaction

The elicitor-induced production of secondary metabolites in culture has been tested in a variety of microorganisms (viruses, bacteria, algae, and fungi), not necessarily pathogens. Rhizosphere microbes are best known to act as biotic elicitors that can induce the synthesis of secondary metabolites in plants [157]. These microorganisms are mainly endophytes (microorganisms that colonise the inside of host plants without causing any apparent disease) and have been used by some researchers in co-culture with their host plant tissues as efficient elicitors leading to the production of secondary metabolites. In some cases, these microorganisms have a direct interaction with the plant tissues. The amount of inoculum is important for inducing the elicitation effect. However, not all endophytes can co-culture with their host plant tissues for a long time, depending on the toxicity of the fungal isolate. In most cases, these fungi have been produced as elicitors with their toxicity removed, which can also stimulate the secondary metabolism of the host plants [162]. The endophyte has a symbiotic relationship with the plants, which may have a long evolutionary and interactive communication. Table S1 (Supplementary Materials) reports the effect of microorganism interaction and their extracts on diterpenoid production in in vitro *Salvia* hairy root cultures.

Actinomycetes are an abundant and widespread group of soil microbes, constituting approximately 10 to 50% of the soil microbial community. Tyc et al. [163] and Adegboye et al. [164] and others have reported them to be important producers of secondary metabolites [165]. These microorganisms have been widely studied for their ability to modulate the relationships between plants and biotic/abiotic stresses, often producing valuable secondary metabolites that can affect host physiology [166]. A soil actinomycete strain, designated *Streptomyces pactum* Act12, has been isolated from the drought, low temperature, and high latitude environment of the Qinghai-Tibetan Plateau [167]. Most *Streptomyces* are efficient rhizosphere and rhizoplane colonisers. They can also be endophytes, colonising the internal tissues of host plants [168]. Yan et al. [169] showed that hairy roots of *S. miltiorrhiza* treated with 2% and 4% *Streptomyces pactum* Act12 for 14 days strongly promoted the synthesis of tanshinones, most significantly increasing the concentration of CT 13.21 and 33.63 times more than that of the control at 2% and 4% *Streptomyces* treatment as well as 8.42 and 15.31 for DT-I, 11.77 and 10.36 for T-IIA, and 5.58 and 5.67 for T-I concentrations, respectively. The stimulatory effect of ACT12 on T-I was less than on some of the other tanshinones because the concentrations of the four substances were significantly increased. The total tanshinone concentrations in the hairy roots of *S. miltiorrhiza* were 9.19 and 12.61 higher in the 2% and 4% ACT12 treatments, respectively.

Plant growth promoting rhizobacteria (PGPR) are rhizosphere bacteria that can colonise the root system (rhizosphere, rhizoplane, or roots) of the plants. They are beneficial to plant growth by facilitating nutrient uptake, producing and releasing growth-promoting substances such as phytohormones, and providing plant protection [170,171]. *Bacillus cereus* is one of the most common rhizobacterial species that has been shown to increase plant resistance to bacterial and fungal pathogens [170,172]. Research by Wu and co-workers [173,174] showed that live *B. cereus* cells inoculated into the *S. miltiorrhiza* hairy root culture on day 0 dramatically stimulated the total root tanshinone accumulation but suppressed hairy root growth. The culture inoculated with 0.2% (OD value of 0.5) bacteria on day 0 reached the highest tanshinone content of 2.67 mg/g DW and the highest volumetric yield of 10.4 mg/L (on day 28), which were 13.5 and 7.6 times higher than those of the control at 0.20 mg/g DW and 1.40 mg/L, respectively [174]. A few years later, the tanshinone accumulation was

most dramatically enhanced by a bacterial concentration of 2.5% (with an OD value of 0.5) inoculated on day 0 for 7 days to 2.78 mg/g DW, which was about 18-fold higher than in the control culture at 0.15 mg/g DW. In addition, the highest volumetric tanshinone yield of 22.4 mg/L, which was approximately 12-fold higher than the control yield of 1.82 mg/L, was achieved with the same bacterial concentration inoculated on day 18 [173].

Yan et al. [175] described that *Pseudomonas brassicacearum* sub sp. *neoaurantiaca* (B1), isolated from both the phloem and xylem of healthy *S. miltiorrhiza*, inoculated into *S. miltiorrhiza* hairy root culture at 0.025% (OD value of 0.5) on day 21 and maintained in co-culture for 9 days, promoted the biomass growth. In addition, it promoted the increase in the total tanshinone content in hairy roots, reaching a 3.7-fold increase compared to the control, and most obviously increased the content of dihydrotanshinone I (DT-I) and CT content (19.2-fold and 11.3-fold, respectively) compared to the control. Consequently, hairy roots of *S. miltiorrhiza* have inhibitory effects on the growth of endophytic bacteria B1, which is mainly due to the antibacterial effect of tanshinone substances in the hairy roots.

Zhai et al. [176] investigated the effects of the live endophytic fungus *Chaetomium globosum* D38 on tanshinone biosynthesis in *S. miltiorrhiza* hairy roots and showed that administration of D38 on day 18 significantly increased the levels of DT-I and CT by 8-fold and 14.9-fold, respectively, compared to the control.

Microorganism Extracts and Constituents

Biotic elicitors produced by pathogens have been mainly used in *Salvia* species to induce the plant defence responses and secondary metabolite production.

Trichoderma species control fungal pathogens both by microbial antagonism and by inducing local and systemic responses [177]. The endophytic fungus *Trichoderma atroviride* D16 from the root of *S. miltiorrhiza* was first reported to produce T-I and T-IIA in mycological medium [178]. A few years later, Ming et al. [179] showed that both the mycelium extract (EM) and polysaccharide fraction (PSF) from *Trichoderma atroviride* D16 treatment were responsible for promoting hairy root growth and causing a significant change in the abundance of the four tanshinones. Among the four tanshinone species, DT-I and CT were most dramatically stimulated by EM and PSF on day 18. The content of DT-I in hairy roots treated with 300 mg/L EM was ~35-fold higher than the control (1.338 mg/g DW versus 0.039 mg/g DW), and the content of CT treated with 150 mg/L EM was almost 83-fold higher than that of the control (3.061 mg/g DW versus 0.037 mg/g DW). Similarly, the levels of DT-I and CT in hairy roots treated with 180 mg/L PSF were ~23-fold (1.216 mg/g DW versus 0.052 mg/g DW) and ~66-fold (3.496 mg/g DW versus 0.053 mg/g DW) higher, respectively, than the control. Furthermore, under the influence of *C. globosum* D38 EM, the content of DT-I and CT reached the highest levels. These were 21-fold and 19.8-fold higher than the control group at doses of 60 mg/L and 90 mg/L, respectively [176].

Chitosan and chitin are structural components of cell walls found in many fungi. However, chitosan is a hydrophilic biopolymer obtained by the *N*-deacetylation of chitin and can be used as an antimicrobial agent [180]. Zhao et al. [181] showed that the application of 100 mg/L chitosan in *S. miltiorrhiza* cell culture induced the production of T-I 3.4-fold (0.27 mg/g versus 80 μg/g of the control). The authors also found that its stimulating effect on tanshinone accumulation (about 6-fold) was stronger than SA, MJ, and sorbitol, but much weaker than Ag^+, Cd, and YE.

Bacterial extracts are biological mixtures prepared from autoclaved and centrifuged microorganism cultures without identification of the active compounds [182]. The *Bacillus cereus* bacterial extract slightly enhanced both the hairy root growth and tanshinone biosynthesis when it was fed to the *S. miltiorrhiza* hairy root culture growing on MS medium on day 21. Wu et al. [174] described that the bacterial extract of 100 mg/mL applied on day 0 and harvested 28 days after the application increased the root weight by almost 50% and the TT content of roots increased by about 2-fold compared to the control. Furthermore, Zhao et al. [173] showed that the 10-fold higher concentration of bacterial extract (1 g/L) applied on day 0 and harvested 7 days later dramatically reduced the hairy root biomass by

5.21 g DW/L and 7.54 g DW/L compared to the control at 12.2 g DW/L and significantly increased the tanshinone content by about 12-fold (2.04 mg/g DW versus 0.17 mg/g DW in the control).

In addition, coronatine (Cor), an analogue of methyl jasmonic acid, a polyketide effector molecule produced by *Pseudomonas syringae* pv *tomato* strain DC3000 (Pst DC3000), bound the same receptor and acted as a structural agonist of JA-Ile [183]. It was first described as an elicitor by Weiler et al. [184]. Vaccaro et al. [185] showed that 0.1 μM Cor, applied to *S. sclarea* hairy root cultures for 28 days, allowed the extraction of 103.32 ± 2.10 mg/L aethiopinone, corresponding to approximatively a 24-fold increase over the basal content of the control hairy roots (4.40 ± 0.13 mg/L). This level was higher than the 16-fold increase (73.29 ± 0.11 mg/L) induced by elicitation with MJ for the same elicitation time, and higher than the final yield obtained after 7 days with this elicitor (24.08 ± 0.79 mg/L). Interestingly, Cor increased the content of carnosic acid, with the maximum yield obtained after 28 days of elicitation (36.75 ± 2.20 mg/L), representing an approximately 18-fold increase over the basal content of the control hairy roots (Table S2, Supplementary Materials).

For decades, scientists have been used yeast extract as one of the biotic elicitors. Despite limited knowledge of the composition and mechanism of action of yeast, autoclaved solutions providing cell wall fragments have been widely used as elicitors to enhance plant secondary metabolite production, mainly in plant cell or hairy root cultures [182]. Treatments of transformed and nontransformed *S. miltiorrhiza* cell cultures with a yeast extract mainly resulted in an inhibition of biomass accumulation (Table S3, Supplementary Materials). Nevertheless, Ti transformed cells cultured in fresh 6,7-V medium containing 20 g/L sucrose with 4 g/L yeast extract for 8 days stimulated the production of the total tanshinone (CT and T-IIA) productivity of 22.2 mg/L compared to trace amounts in the control cultured in fresh B5 medium [64]. When cultured in a MS-NH_4 medium (MS without ammonium nitrate, containing 30 g/L sucrose) yeast elicitor (4 g/L) for 18 days, this production increased from trace levels in the control to 12.23 mg/L [186]. Treatment of an 18-day-old nontransformed *S. miltiorrhiza* cell culture with yeast extract at 100 mg/L for 7 days reduced biomass production by no more than 50% (5.1–5.5 g/L versus 8.9 g/L), but increased the total tanshinone content to 2.30 mg/g, about 11.5 times that of the control (0.20 mg/g), and more drastically, the CT content was about 2.01 μg/g, which was 34 times that of the control (60 μg/g) [181]. This last result is in agreement with that of the normal cell suspensions of *S. miltiorrhiza* in which CT production was stimulated only after cell growth had been suppressed [187]. However, when 4-day-old Ti transformed cells were treated with 0.1% (*v*/*v*) YE, the biomass accumulation decreased compared to the control (13.1 versus 14.4 g DW/L at day 5) while the CT production increased significantly (11.5 versus 0 mg/L) [188].

Table S4 (Supplementary Materials) illustrates the effects of the yeast extract on diterpenoid production in in vitro *Salvia* hairy root cultures. In contrast to the cell cultures, applying the yeast extract to the hairy roots did not only inhibit the biomass production. Treatment of 18-day-old hairy roots of *S. miltiorrhiza* for 9 days with YE 100 mg/L increased the volumetric total tanshinone (CT, T-I, and T-IIA) of 7.62 mg/L about 4.3-fold over the control [189], and a treatment for 4 days increased the total tanshinone by about ~2.2 mg/g DW, which was 3.1-fold over the control [190]. However, the treatment with YE 25 mg/L increased this amount to 9.92 mg/L, approximately 5-fold over the control [191]. The total tanshinone (CT, T-I, T-IIA) increased significantly when 18-day-old hairy roots were treated with 100 mg/L for 12 days, reaching 13.7 mg/L, about 3.8 times the control [192]. Yang et al. [193] investigated the production of tanshinones when *S. miltiorrhiza* hairy roots were treated with 200 mg/L YE and showed that CT was the most produced diterpenoid. Chen et al. [194] reported that the intracellular content of CT increased from 0.001% to as much as 0.096% of DW.

Treatment of *S. castanea f. tomentosa* with an optimum concentration of 200 mg/L yeast extract improved both the growth status and tanshinone accumulation. The CT content was increased to a maximum of 2.84 ± 0.33 mg/g DW in the hairy root cultures. Furthermore,

an apparent activation of T-IIA showed a sustained promotion to 2.52 ± 0.67 mg/g DW and DT-I to 1.95 ± 0.09 mg/g DW [65]. The T-I content was slightly responsive to elicitation [65]; however, it was drastically increased to approximately 37.14 times the control level [193].

4.2.2. Abiotic Elicitors

Heavy Metals

In abiotic elicitation, chemical or physical stimuli are used to trigger the synthesis of plant metabolites [195].

Various heavy metal elements have been used to induce specialised metabolites in *S. miltiorrhiza* such as lanthanum, cerium, silver, cobalt, and cadmium [173,196–198], as reported in Table S5 (Supplementary Materials). Among them, Ag^+ was the most frequent elicitor. However, it caused a dose-dependent depression of *S. miltiorrhiza* hairy roots and cell growth [173,198]. A *S. miltiorrhiza* cell suspension exposed to Ag^+ 25 μM for 7 days significantly increased the total tanshinone (CT, T-I, and T-IIA) content to 2.04 mg/g, about 10-fold of the control (0.20 mg/g), with a large amount of CT at 1817.5 μg/g, about 30-fold of the control (59.9 μg/g) [173]. Recently, Yu et al. [199] showed that on day 7 after treatment with Ag^+ 60 μM, CT was 18.07 times higher than the control.

Plant Signal Compounds

Jasmonates are defined as "hormones" because they elicit cellular responses at low concentrations far from their site of synthesis [200,201]. MJ acts as an efficient elicitor of secondary metabolite production throughout the plant kingdom, particularly those involved in developmental processes and defence responses [202]. It has been used by some researchers to increase the in vitro production of diterpenoids in some *Salvia* species. Some studies on the elicitation of hairy roots of *S. miltiorrhiza* by MJ showed that 100 to 150 μM is the ideal concentration to ensure that the production of various tanshinones is at least four times higher than that of the control [203–205]. However, these results were very different from the previous ones that reported that after elicitation with MJ, the CT and T-IIA were 23.8-fold and 6.2-fold higher, respectively, than those of the control [206]. In cell culture, MJ showed only a moderate or insignificant stimulating effect on tanshinone accumulation in normal and transformed *S. miltiorrhiza* cell cultures [181,188]. MJ showed only a moderate stimulating effect on tanshinone accumulation in *S. castanea* Diels *f. tomentosa Stib.* hairy root culture [65]. Kuźma et al. [207] reported that the total diterpenoid content of *S. sclarea* hairy roots in shake flasks elicited with 125 μM MJ for 7 days was twice that of the control roots. In addition, the diterpenoid content of the same treatment, using a bioreactor as a growth system (67.5 mg/g DW), was about 6-fold higher than that of the non-elicited roots and 2.4-fold higher than that of the MJ-treated roots maintained in shake flasks (28.16 ± 1.2 mg/g DW). Aethiopinone was found to be the major diterpenoid synthesised after MJ treatment among all of the diterpenoids present in *S. sclarea* roots. Its content was 40 mg/g DW, 9.1-fold higher than the control and about 60% of the total diterpenoids. Vaccaro et al. [185] found that 100 μM MJ applied for 7 days to the hairy roots of *S. sclarea* seemed to be more effective in inducing the accumulation of aethiopinone (9.72 ± 0.08 mg/g DW), corresponding to a 25-fold increase over the content of the untreated hairy roots (0.38 ± 0.07 mg/g DW).

Few studies on the genus *Salvia* have used SA elicitation to enhance diterpenoid production (Tables S3 and S6a, Supplementary Materials). SA has been reported as an effective elicitor for the hairy roots and cell cultures of *S. miltiorrhiza.* Hao et al. [204] showed that the total tanshinone production after the application of SA to *S. miltiorrhiza SmGGPPS*, the overexpression of hairy roots was only 1.63-fold that of the mimic treatment control. Zhao et al. [173] and Yu et al. [199] treated the *S. miltiorrhiza* non-transformed cell culture with 100 and 200 μM for 7 days, and the content of CT produced was 6.5-fold and 4.39-fold higher than the control, respectively (Table S3, Supplementary Materials). Kračun-Kolarević et al. [208] reported a significant increase in carnosol and carnosic acid in the shoot cultures of *S. officinalis.* The carnosol and carnosic acid contents were higher

(3.8 and 1.4 times, respectively) in the 4-week-old controls than in 1-week-old explants. SA treatment increased the carnosol production from 2 mg/g DW (in 1-week-old control explants) to 14 mg/g DW (in 4-week-old shoots growing at 150 µM).

Other Elicitors

Table S6b (Supplementary Materials) report the effect of other chemical compounds on diterpenoid production in in vitro *S. miltiorrhiza* hairy root cultures. The application of 100 µM sodium nitroprusside (SNP), a donor of NO, resulted in a significant increase in the production levels of T-I, CT, DT-I, and T-IIA in the hairy roots by 80, 170, 60, and 180% above the control level, respectively [209]. The application of β-aminobutyric acid as an elicitor at a dose of 2 mM caused a significant increase in the total tanshinone content of 1.09 mg/g DW, about 4.5 times that of the control at 0.24 mg/g DW [190]. The addition of PEG 2% increased the T-I, CT, DT-I, and T-IIA in the hairy roots to 0.9, 2.1, 3.9, and 2.0 mg/L, respectively, compared to the control at 0.7, 1.3, 3.2, and 1.5 mg/L, respectively [209]. Smoke–water (SW) 1:1000 (v/v) was found to be very effective in enhancing the T-I accumulation in hairy roots after 3 days of elicitation [210].

Effect of Light Irradiation

The spectral quality, intensity, and duration of light irradiation may affect plant cell and tissue cultures. Few studies have demonstrated the involvement of light irradiation in the accumulation of secondary metabolites in *Salvia* (Table S7, Supplementary Materials). The hairy roots of *S. miltiorrhiza* exposed to UV-B irradiation showed an increasing production of three tanshinones (CT, T-IA, and T-IIA) and reached a peak (0.38 mg/g DW) that was 1.8 times higher than that of the control. Among the three tanshinones, CT was the most abundant and reached the maximum (0.13 mg/g DW), 3.4 times that of the control, after 40 min of UV-B irradiation [211]. Recently, it was shown that different 1- and 3-week LED light spectrum treatments could regulate tanshinones in the hairy roots of *S. miltiorrhiza*. However, blue light decreased the T-IIA content via the downregulation of key enzymes involved in the biosynthesis process [212].

4.2.3. Combination or Synergic Effect of Elicitors

Some studies have shown that the production of secondary metabolites in *S. miltiorrhiza* hairy root cultures can be enhanced or potentiated by incorporating multiple elicitor treatments over the culture period rather than a single treatment [204,211,213,214]. Many studies have investigated the combination of yeast extract with other elicitors (Tables S8 and S9, Supplementary Materials) in *Salvia* hairy root and cell cultures. The Ti-transformed *S. miltiorrhiza* cell cultures treated with the combination of yeast extract and SA for 15 days increased the total tanshinone production (CT and T-IIA) to the highest levels, with 15.07 mg/L. The yeast extract treatment had 12.23 mg/L and the control and SA treatments had barely detectable amounts of tanshinone [186]. A few years earlier, Chen and Chen [188] showed that in cell suspension, 200 µM SA added one day before the yeast elicitor increased the CT formation from 13.8 mg/L to 18.9 mg/L (an increase of 37%). Combinations of two elicitors (YE + Ag^+ or YE + Cd^{2+}) and three elicitors (YE + Ag^+ + Cd^{2+}) in *S. miltiorrhiza* cell culture increased the tanshinone content by about 20% and 40%, respectively, compared to a single elicitor [173].

Sorbitol, widely used as an osmotic in plant cell and tissue cultures, has a slight enhancing effect on root growth [85] and can have a positive effect on the production of secondary metabolites [215]. The combination of yeast extract with sorbitol (100 mg/L + 50 g/L, respectively) added on day 21 and maintained for 9 days in suspension in a hairy root culture of *S. miltiorrhiza* decreased the biomass production, and more significantly, increased tanshinone (CT, T-I, and T-IIA) production (16.3 versus 1.77 mg/L) compared to the control [189]. One year later, Wu et al. [191] reported that the combination of yeast extract 25 mg/L with sorbitol +50 g/L increased the biomass production and more drastically increased the tanshinone (CT, T-I, and T-IIA) production by 7-fold (from 0.2 to

1.6 mg/g DW) and the volumetric yield by 13-fold (from 1.95 to 27.4 mg/L) compared to the batch control culture.

Good tanshinone production was obtained by the combination of yeast extract pre-treated for 3 days with of β-aminobutyric acid, reaching the amount of 20.1 mg/L [190]. Treatment for 2 days with Ag^+ with CT dramatically increased production [216]. The combination of MJ with a pre-treatment of 40 min with UV-B on hairy roots of *S. miltiorrhiza* significantly increased the total tanshinone content (CT, T-I, and T-IIA) to 28.21 mg/L, 4.9 times higher than that of the control [211].

4.2.4. Elicitation and Nutrient or Medium Feeding or Renewal

Precursor feeding is an obvious and popular approach to increasing the secondary metabolite production in plant cell cultures. Attempts have been made to induce or increase the production of plant secondary metabolites by feeding precursors [149]. In principle, elicitation has been used to induce or stimulate the secondary metabolism of plant cells, but it is not favourable for cell growth (primary metabolism). However, suppressed biomass growth has been observed in hairy root cultures treated with abiotic and biotic elicitors, especially at relatively high doses. This unfavourable effect could be reduced or eliminated during the elicitation process by nutrient or medium supplementation or renewal, thus improving the secondary metabolite production more effectively [192,217–219]. Treatment with 15 μM Ag^+ caused a depression of *S. miltiorrhiza* hairy root (9.6 g DW/L versus 12.1 g DW/L of the control). Supplementation of sucrose and fresh medium prior to the addition of Ag^+ to the culture medium maintained the hairy root growth at 18.9 g DW/L and 22.3 g DW/L versus 12.1 g DW/L of the control, respectively, and the total tanshinone production at 36.6 mg/L and 55.7 mg/L versus 24.4 mg/L and 7.3 mg/L of the Ag^+ treatment and control, respectively [198]. It was found that sorbitol was mainly a strong stimulator of tanshinone production and had beneficial effects on the hairy root growth of *S. miltiorrhiza* [189,191]. To maintain secondary metabolite production throughout the extended fed-batch process without affecting the biomass, both frequent elicitor challenge (sorbitol + YE) and sufficient nutrient supply to the hairy roots of *S. miltiorrhiza* (from 10 mL every 5 days) were essential. Of note was the extremely high tanshinone content achieved in the OS + YE treated culture of 18.1 mg/g DW (equivalent to 1.8% *w*/*w* or 143.9 mg/L TT yield). This was approximately 11.5 times higher than in the single treatment process (1.57 mg/g DW) and almost 100 times higher than in the absence of any elicitor treatment (0.2 mg/g DW). Moreover, the root fragments released from the roots in the OS + YE treated culture had an even higher tanshinone content (110 mg/g DW or 11% *w*/*w*) [191].

4.3. Production with In Situ Adsorption

Some secondary metabolites are hydrophobic and are stored intracellularly either in the cytosol or in the cell vacuole. They are minimally secreted into the culture medium and may appear in the culture broth as a result of cell lysis [155,220]. The production of target metabolites can be limited by both the feedback inhibition of accumulated synthesised metabolites and their degraded compounds [221]. However, some secondary metabolites produced in the cell may be released into the culture medium. Miyasaka et al. [222] found that in immobilised cultured cells of *S. miltiorrhiza*, much of the cryptotanshinone was released into the medium, whereas most of the ferruginol was retained in the cells. The introduction of an in situ product removal mechanism such as a solid adsorbent into the culture medium can often effectively induce product release from plant cells and increase productivity [223].

For the enhancement of metabolite production in plant cell culture, different adsorbents for the solid/liquid two-phase system have been used. The addition of 2 g of X-5 resin in a nylon bag to each 200 mL flask recovered a large portion of tanshinones (CT, T-I, and T-IIA) from the roots (70–94%). A significant increase in tanshinone yield was also obtained by combining the macroporous polystyrene resin (X-5 adsorption) with double YE elicitation (added to the culture at 100 mg/L on days 30 and 40). To prolong biomass

growth and secondary metabolite production, medium renewal, YE elicitation, and resin replacement were performed every 10 days for a total of three times (days 30, 40, and 50). Interestingly, the root biomass was increased to 30 g/L DW (versus 8–10 g/L DW in batch mode) and the volumetric tanshinone yield to 87 mg/L (about a 15-fold increase), with 76% adsorbed on the resin. The volumetric productivity of total tanshinone reached 1.46 mg/L per day, more than seven times that of the batch culture [192].

4.4. Gene Overexpression

The overexpression of *SmMDS* in transgenic *S. miltiorrhiza* hairy roots significantly increased the tanshinone yield compared to the control. The total tanshinone content increased in the SmMDS-overexpressing lines after elicitor treatment [224]. The overexpression of SmWRKY1 in *S. miltiorrhiza* significantly increased the transcripts of genes coding for enzymes in the MEP pathway, especially 1-deoxy-D-xylulose-5-phosphate synthase (SmDXS) and 1-deoxy-D-xylulose-5-phosphate reductoisomerase (SmDXR), resulting in a more than 5-fold increase in tanshinone production in the transgenic lines (up to 13.7 mg/g DW) compared to the control lines [225]. Overexpression of *SmMYB9b* in the hairy roots of *S. miltiorrhiza* increased the tanshinone concentration to 2.16 ± 0.39 mg/g DW, a 2.2-fold improvement over the control [226]. The overexpression of *SmGGPPS* and *SmDXSII* in the hairy roots produced higher levels of tanshinone than the control and single-gene transformed lines; tanshinone production in the double-gene transformed line GDII10 reached 12.93 mg/g DW [227]. Overexpression of *SmAOC* in the hairy roots of *S. miltiorrhiza* significantly increased the yield of T-IIA [228]. Overexpression of the ethylene response factor *SmERF6* in the hairy roots of *S. miltiorrhiza* increased their tanshinone accumulation [229], while the overexpression of the heterologous TFs *At*WRKY18, *At*WRKY40, and *At*MYC2 in the hairy roots of *S. sclarea* resulted in the expression of several genes encoding enzymes of the MEP-dependent pathway, in particular DXS, DXR, GGPPS, and CPPS. The final yield of aethiopinone was significantly increased by a factor of four in HR lines overexpressing *At*WRKY40 TF [230]. The engineering of the plastidial 2-C-methyl-D-erythritol 4-phosphate-derived isoprenoid pathway in *S. sclarea* hairy roots by the ectopic expression and plastid targeting of cyanobacterial genes encoding the 1-deoxy-D-xylulose 5-phosphate synthase or 1-deoxy-D-xylulose-5-phosphate reductoisomerase gene, the first two enzymatic steps of the plastidial MEP pathway and the plastid-targeted expression of these proteins, significantly increased the yield of aethiopinone by 3-fold and about 6-fold, respectively. The accumulation of other abietane-type diterpenoids (ferruginol, salvipisone, and carnosic acid) was also increased [231].

5. Conclusions

Salvia species are an important source of secondary metabolites and most of these species have been studied in vitro by tissue and cell culture for the controlled production of bioactive diterpenoids. Several techniques have been used to increase the production of cell lines and to optimise the growth medium by using specific precursors or elicitors. This review analysed the biotechnological approaches applied to the in vitro cultures of *Salvia* cells and tissues to enhance the production and accumulation of bioactive diterpenoids and summarised their biological activities. The study showed that culture type, growth medium composition, nutrient supply, elicitors, and in situ uptake can influence both the growth biomass and production of bioactive diterpenoids. All classes of elicitors had a stimulating effect on diterpenoid production in the in vitro cultures of *Salvia* species. *S. miltiorrhiza* was the most studied species for in vitro diterpenoid production, and cryptotanshinone was one of the most stimulated diterpenoids by elicitation. The effects of different elicitors on diterpenoid production in plant tissues and cell cultures were dependent on their concentration, time, and duration of application as well as on the specificity of the secondary metabolites. However, the biomass of *Salvia* cell cultures was more negatively affected by the elicitors compared to tissues. The combination of the overexpression of key genes in a suitable tissue, growing on an ideal medium treated with the best elicitor, with nutrient

feeding and in situ uptake could be a strategy for the production of diterpenoids in Salvia species. In conclusion, the application of biotechnological strategies to in vitro cultures of *Salvia* species represents a promising approach for the controlled production of bioactive diterpenoids. These techniques have the potential to revolutionise the pharmaceutical and nutraceutical industries by providing a sustainable and reliable source of valuable medicinal compounds with further research and development including system scale-up. However, further studies are needed to overcome existing challenges and to fully exploit the benefits of these innovative approaches.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14040835/s1, Table S1. Effect of microorganism interaction and their extracts on diterpenoid production in in vitro *Salvia* hairy root cultures; Table S2. Effect of methyl jasmonate and coronatine on diterpenoid production in in vitro *Salvia* hairy root cultures; Table S3. Effect of elicitors on diterpenoid production in in vitro *Salvia* cell cultures; Table S4. Effect of yeast extract on diterpenoid production in in vitro *Salvia* hairy root cultures; Table S5. Effect of Ag+ on diterpenoid production in in vitro *Salvia* hairy root cultures; Table S6a. Effect of other chemical compounds on diterpenoid production in in vitro *Salvia* hairy root cultures; Table S6b. Effect of other elicitors on diterpenoid production in in vitro *Salvia* hairy root cultures; Table S7. Effect of physical elicitors on diterpenoid production in in vitro *Salvia* hairy root cultures; Table S8. Effect of the combination of elicitors on diterpenoid production in in vitro *Salvia* hairy root cultures; Table S9. Effect of the combination of elicitors on diterpenoid production in in vitro *Salvia* cell cultures.

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