Chapter 10

Abiotic and Biotic Elicitors–Role in Secondary Metabolites Production through In Vitro Culture of Medicinal Plants

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Additional information is available at the end of the chapter

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Abstract

Plant secondary metabolites are having the great application in human health and nutritional aspect. Plant cell and organ culture systems are feasible option for the production of secondary metabolites that are of commercial importance in pharmaceuticals, food additives, flavors, and other industrial materials. The stress, including various elicitors or signal molecules, often induces the secondary metabolite production in the plant tissue culture system. The recent developments in elicitation of plant tissue culture have opened a new avenue for the production of secondary metabolite compounds. Secondary metabolite synthesis and accumulation in cell and organ cultures can be triggered by the application of elicitors to the culture medium. Elicitors are the chemical compounds from abiotic and biotic sources that can stimulate stress responses in plants, leading to the enhanced synthesis and accumulation of secondary metabolites or the induction of novel secondary metabolites. Elicitor type, dose, and treatment schedule are major factors determining the effects on the secondary metabolite production. The number of parameters, such as elicitor concentrations, duration of exposure, cell line, nutrient composition, and age or stage of the culture, is also important factors influencing the successful production of biomass and secondary metabolite accumulation. This chapter reviews the various abiotic and biotic elicitors applied to cultural system and their stimulating effects on the accumulation of secondary metabolites.

Keywords: Cell culture, elicitor, organ culture, secondary metabolites, stress

1. Introduction

The total mankind is dependent on plants as a source of carbohydrates, proteins, vitamins, food, and shelter. Plants are studied for their important constituents and the nutritional factors

for over decades. Along with the essential primary metabolites, higher plants are also capable to produce a number of low molecular weight compounds. A diverse group of organic compounds that are produced by plants to facilitate interaction with the biotic environment and the establishment of a defense mechanism are called as plant secondary metabolites [1–3]. The production of these metabolites is very low (less than 1% dry weight) and depends greatly on the physiological and developmental stage of the plant [4,5]. Plant natural products have been an important part of medicine throughout human history. In recent years, the use of herbal medicines has steadily increased worldwide [6]. With this increasing demand comes growing concerns about the safety and efficacy of herbal medicines. Although the potential for medicinal plants seems almost limitless, there are a few major obstacles that hinder large-scale utilization by the western medical system. Among them is the lack of reproducibility common in testing many plant extracts (up to 40%), which has limited the enthusiasm for developing plant-based pharmaceuticals [7]. Unlike standardized single-entity pharmaceutical drugs, herbal medicines consist of complex mixtures with multiple compounds responsible for therapeutic activity, making standardization difficult [8]. Further complicating the issue is the fact that plants, unlike synthetic medicines, are living organisms, with inherent biological variation [9]. Just because plant material originates from the same species, it does not necessarily mean that the chemical content will be identical. This lack of reproducibility may be due to two main factors, genetic variability and differences in growing conditions.

In addition, plants are a valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides, and food additives. Plants are producing new compounds and in future new chemical models are drawing for new drugs because the most of the plants chemistry is yet to be explored [10]. The characterization of molecular structures and chemical analysis helped us to pinpoint the activities of plants under controlled conditions. Although all these advancements, we still depend on the secondary metabolites of biological sources including pharmaceuticals [11].

Due to various agro alimentary, perfumes, flavors, colors, and pharmacological effects, the secondary metabolites are having extensive demand and various commercial preparations are available in the market. Besides, the appeal of using natural products for medicinal purposes is increasing, and metabolic engineering can alter the production of pharmaceuticals and help to design new therapies. The evolving commercial importance of secondary metabolites has in recent years resulted in a greater interest in secondary metabolism, particularly in the possibility of altering the production of bioactive plant metabolites [12]. Secondary metabolites are separated into nitrogen compounds (alkaloids, nonprotein amino acids, amines, alcamides, cyanogenic glycosides, and glucosinolates) and nonnitrogen compounds (monoterpenes, diterpenes, triterpenes, tetraterpenes, sesquiterpenes, saponins, flavonoids, steroids, and coumarins).

The plant tissue culture plays an important role in the rapid clonal propagation, regeneration of genetically manipulated superior clones, conservation of germplasm, production of secondary metabolites, and ex vitro conservation of valuable phytodiversity [13,14]. The plant,

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cell, tissue and organ culture techniques have come up with an escapable tool with the possibilities of acclaiming and supplementing the conventional method in plant breeding, plant improvement, and biosynthetic pathways. This technique has several potential applications in crop improvement, and efficient regeneration is a prerequisite in such improvement programs. The biotechnological production of secondary metabolites in plant cell and organ cultures is an attractive alternative to the extraction of the whole plant material [15]. In particular, plant–specific important compounds are obtained by using the plant cell and organ cultures [2]. The faster proliferation rates and shorter biosynthetic cycle of cell and organ cultures leads to have a higher rate of metabolism when compared to field grown plants [16]. Further, plant cell/organ cultures are under controlled conditions proliferates at their optimum growth rates when compared to the cultivated plants, which are facing environmental, ecological, and climatic variations. In recent years, various strategies have been developed for use in biomass accumulation and the synthesis of secondary compounds, such as strain improvement, optimization of medium, and culture environments, elicitation, precursor feeding, metabolic engineering, permeabilization, immobilization, and biotransformation methods, bioreactor cultures, and micropropagation [17]. The focus of the present chapter is the influence of abiotic and biotic elicitors on the secondary metabolite production in the in vitro cultured medicinal plants.

2. Classification of elicitors and secondary metabolite production via in vitro culture of medicinal plants

Stress is an important factor in determining the chemical composition and therapeutic activity of medicinal plants. Actively stimulating, or eliciting, the plant stress response to induce the desired chemical response is called elicitation, harnessing the connection between plant stress and phytochemistry. "Elicitor may be defined as a substance for stress factors which, when applied in small quantity to a living system, it induces or improves the biosynthesis of specific compound which do have an important role in the adaptations of plants to a stressful conditions" [18]. Elicitation is the induced or enhanced biosynthesis of metabolites due to addition of trace amounts of elicitors [18]. Several biotechnological strategies have been hypothesized and applied for the productivity enhancement, and elicitation is recognized as the most practically feasible strategy for increasing the production of desirable secondary compounds from cell, organ, and plant systems [19–21].

On the basis of nature, elicitors can be divided into two types abiotic and biotic (Figure 1). Abiotic elicitors comprise of substances that are of nonbiological origin and are grouped in physical, chemical, and hormonal factors. Biotic elicitors are the substances of biological origin that include polysaccharides originated from plant cell walls (e.g. chitin, pectin, and cellulose) and micro–organisms.

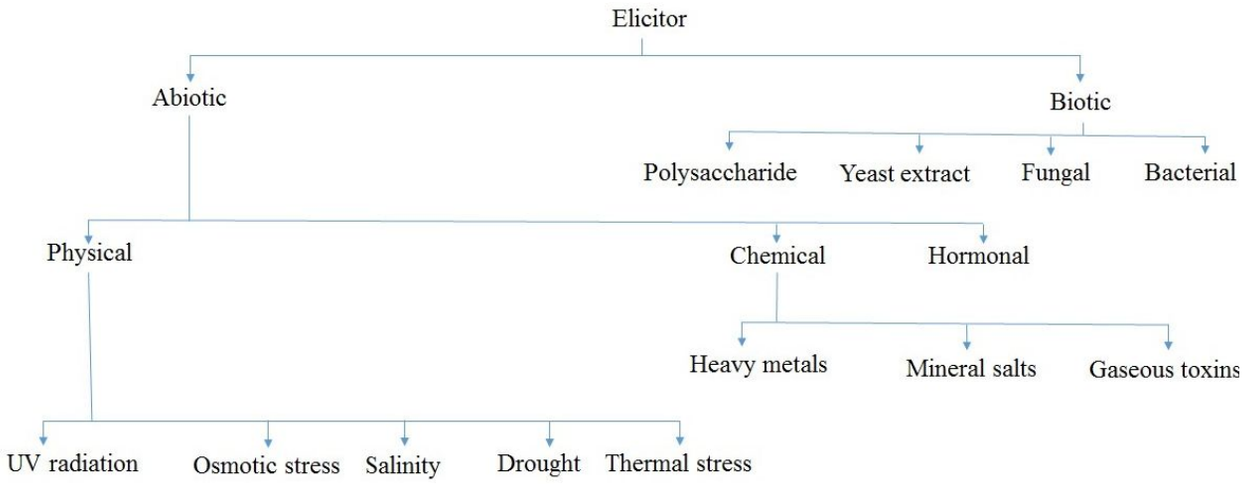

Figure 1. Elicitors Classification Based on their Nature

3. Abiotic elicitors

As mentioned above, the abiotic elicitors are categorized into physical, chemical, and hormonal elicitors. Abiotic elicitors have wide range of effects on the plants and in the production of secondary metabolites (Table 1).

Elicitor	Plant species	Nature of culture	Compounds	References
Ozone (O_3)	*Melissa officinalis*	Shoot	Rosmarinic acid	[163]
	Hypericum perforatum	Cell suspension	Hypericin	[164]
	Pueraria thomsnii	Cell suspension	Puerarin	[165]
pH	*Bacopa monnieri*	Shoot	Bacoside A	[166]
	Withania somnifera	Hairy root	Withanolide A	[167]
	Withania somnifera	Cell suspension	Withanolide A	[168]
Sucrose	*Hypericum adenotrichum*	Seedling	Hypericin and pseudohypericin	[41]
	Corylus avellana	Cell suspension	Paclitaxel	[169]
	Bacopa monnieri	Shoot	Bacoside A	[166]
	Withania somnifera	Cell suspension	Withanolide A	[168]
Ultraviolet C	*Vitis vinifera*	Cell suspension	Stilbene	[34]
Proline	*Stevia rebaudiana*	Callus and suspension	Steviol glycoside	[40]
Polyethylene glycol	*Stevia rebaudiana*	Callus and suspension	Steviol glycoside	[40]

Elicitor	Plant species	Nature of culture	Compounds	References
	Hypericum adenotrichum	Seedling	Hypericin and pseudohypericin	[41]
Jasmonic acid	*Bacopa monnieri*	Shoot	Bacoside A	[170]
	Plumbago indica	Hairy root	Plumbagin	[88]
	Plumbago rosea	Cell suspension	Plumbagin	[86]
Methyl jasmonate	*Salvia miltiorrhiza*	Hairy root	Tanshinone	[94]
	Perovskia abrotanoides	Adventitious roots	Cryptotanshinone and tanshinone IIA	[78]
	Vitis vinifera	Cell suspension	Stilbene	[34]
	Bacopa monnieri	Shoot	Bacoside	[96]
	Salvia officinalis	Shoot	Diterpenoid	[171]
	Silybum marianum	Cell suspension	Silymarin	[172]
	Salvia castanea	Hairy root	Tanshinone	[173]
	Gymnema sylvestre	Cell suspension	Gymnemic acid	[148]
	Withania somnifera	Hairy root	Withanolide A, withanone, and withaferin A	[95]
	Andrographis paniculata	Cell suspension	Andrographolide	[97]
	Vitis vinifera	Cell suspension	trans-Resveratrol	[91]
	Taverniera cuneifolia	Root	Glycyrrhizic acid	[135]
Gibberelic acid	*Salvia miltiorrhiza*	Hairy root	Tanshinones	[174]
	Echinacea pupurea	Hairy root	Caffeic acid derivatives	[175]
Salicylic acid	*Salvia miltiorrhiza*	Hairy root	Tanshinone	[94]
	Vitis vinifera	Cell suspension	Stilbene	[34]
	Digitalis purpurea	Shoot	Digitoxin	[176]
	Hypericum hirsutum	Shoot	Hypericin and pseudohypericin	[177]
	Gymnema sylvestre	Cell suspension	Gymnemic acid	[148]
	Withania somnifera	Hairy root	Withanolide A, withanone, and withaferin A	[95]
	Datura metel	Root	Hyoscyamine and scopolamine	[178]
	Glycyrrhiza uralensis	Adventitious root	Glycyrrhizic acid	[179]

Elicitor	Plant species	Nature of culture	Compounds	References
Sodium salicylate	*Salvia officinalis*	Shoot	Carnosol	[180]
Sodium chloride	*Catharanthus roseus*	Embryogenic tissues	Vinblastine and vincristine	[55]
Sorbitol	*Perovskia abrotanoides*	Adventitious roots	Cryptotanshinone and tanshinone IIA	[78]
Silver (Ag)	*Perovskia abrotanoides*	Adventitious roots	Cryptotanshinone and tanshinone IIA	[78]
	Vitis vinifera	Cell suspension	Resveratrol	[67]
	Salvia castanea	Hairy root	Tanshinone	[173]
	Datura metel	Hairy root	Atropine	[150]
Cadmium (Cd)	*Vitis vinifera*	Cell suspension	Resveratrol	[67]
	Datura stramonium	Root	Sesquiterpenoid	[79]
Cobalt (Co)	*Vitis vinifera*	Cell suspension	Resveratrol	[67]
Copper (Cu)	*Ammi majus*	Shoot	Xanthotoxin	[181]
	Bacopa monnieri	Shoot	Bacoside	[170]
	Datura stramonium	Root	Sesquiterpenoid	[79]

Table 1. Effect of Different Abiotic Elicitors on the Production of Various Secondary Metabolites in Plants

3.1. Physical elicitors

Physical elicitors include light, osmotic stress, salinity, drought, and thermal stress.

3.1.1. Light

The light is a physical factor that can affect the metabolite production. Light can stimulate such secondary metabolites include gingerol and zingiberene production in *Zingiber officinale* callus culture [22]. The effect of light irradiation on anthocyanin production in cell suspension cultures of *Perilla frutescens* was reported [23]. The effect of light and hormones on the digitoxin accumulation in *Digitalis purpurea* L. was reported by Hagimori et al. [24]. Moreover, in hairy root cultures of *Artemisia annua*, the effect of light irradiation influenced the artemisinin biosynthesis [25]. The effect of white light on taxol and baccatin III accumulation in cell cultures of *Taxus cuspidate* was reported by Fett–Neto et al. [26]. Ultraviolet (UV) radiation stimulates secondary metabolite production. Increasing UV–B exposure in field–grown plants not only increased the total essential oil and phenolic content but also decreased the amount of the possibly toxic beta–asarone [27]. These findings are to be expected as phenolics are known UV protectants [28]. *Catharanthus roseus* plants, exposed to UV–B light, show significant increases in the production of vinblastine and vincristine, which have proven effective in the treatment of leukemia and lymphoma [29]. UV–C irradiation promotes the phenylpropanoid pathway

and stimulates flavonoid synthesis [30]. UV–C irradiation is an effective method to enhance stilbene production in *Vitis vinifera* berries [31], *V. vinifera* leaves [32], and *V. vinifera* callus of different genotypes [33]. UV–C together with methyl jasmonate (MeJA) or salicylic acid (SA) also used to enhance stilbene production in *V. vinifera* cell cultures [34].

3.1.2. Osmotic stress

Osmotic stress (water stress) is an abiotic physical elicitor [35] and is one of the important environmental stresses that can alter the physiological and biochemical properties of plants and increase the concentration of secondary metabolites in plant tissues [36]. Proline acts as an osmolyte, as protective agent for cytoplasmic enzymes, as a reservoir of nitrogen and carbon sources for post–stress growth, or even as a stabilizer of the machinery for protein synthesis, regulation of cytosolic acidity and scavenging of free radicals [37]. However, the various roles of proline have been proposed, but the main role could be the osmotic adjustment in osmotically stressed plant tissues and the protection of plasma membrane integrity [38]. Polyethylene glycol (PEG) is an osmotic agent (nonpenetrating osmoticum) that has been used for induction of water stress in many plants [39]. The proline and PEG enhanced the production of steviol glycosides content in both callus as well as suspension culture of *Stevia rebaudiana* [40]. PEG elicited the pharmacologically active compounds, such as hypericin and pseudohypericin, in *Hypericum adenotrichum* [41]. Sucrose is a typical osmotic stress agent used for the induction of water stress in plants that also serves as a vital carbon and energy source [42]. It has been shown that water and osmotic imbalance can strongly influence the synthesis of hypericin and hyperforin in *Hypericum perforatum* plants [43]. In addition, it has been reported that both hypericin and pseudohypericin concentrations decreased, while hyperforin concentration increased significantly in the plants grown under water stress conditions [36].

3.1.3. Salinity

Salinity reduces plant growth and development and alters a wide array of physiological and metabolic processes [44,45]. Plants have developed complex mechanisms for adaptation to the osmotic, ionic, and oxidative stresses that are induced by the salt stress. Exposure to salinity is known to induce or stimulate the production of secondary plant products, such as phenols, terpenes, and alkaloids [46–48]. *C. roseus* grown under salt stress showed increased levels of the alkaloid vincristine [49]. In *Grevillea*, a significant increase in anthocyanin concentration was reported under salinity exposure in both the salt–tolerant *Grevillea ilicifolia* and the salt–sensitive *Grevillea arenaria* [50]. In contrast to this, salt stress decreased the anthocyanin level in the salt–sensitive species [51]. In *Datura innoxia*, salt treatment increased the total alkaloid content in young leaves, and the results indicated that at the organ level, tropane alkaloid accumulation was related to plant growth [52]. Glycine betaine was increased under salinity in numerous species including *Triticum aestivum* [53] and *Trifolium repens* [54]. Salinity also increased the diamine and polyamine content in *Oryza sativa* [53]. An improved synthesis of vinblastine and vincristine was observed in *C. roseus* embryogenic tissue culture by using NaCl as an elicitor [55].

3.1.4. Drought stress

One of the most important abiotic stress is drought, which affect plant growth and their developmental process [56]. The available water in the soil is reduced to such critical levels, and atmospheric conditions add to the continuous loss of water; the situation is called drought stress. The high temperature in the environment and solar radiations add up the water deficit in the soil, which leads to drought stress. Drought stress tolerance is observed in all types of plants, but its extent varies from species to species [56]. Drought stress, which can also greatly reduce plant growth, can increase secondary metabolite content. Mild water stress significantly increased the content of the anti–inflammatory saikosaponins in *Bupleurum chinense* [57]. Moderate water stress increased the content of salvianolic acid in roots of *Salvia miltiorrhiza*, although the content of other bioactives, including tanshinone, was lowered [58]. Moderate drought stress also increased the production of rosmarinic, ursolic, and oleanolic acid in *Prunella vulgaris* [59]. A weak water deficit greatly increased the glycyrrhizic acid content in roots of *Glycyrrhiza uralensis* [60]. In *Hypericum brasiliense*, the amounts of various phenols and betulinic acid were drastically increased under drought stress [61].

3.1.5. Thermal stress

Although thermal stress can greatly reduce plant growth and induce senescence, elevated temperatures (heat stress) or low temperatures (cold stress) have also been shown to increase secondary metabolite production. Temperature strongly influences metabolic activity and plant ontology, and high temperatures can induce premature leaf senescence [62]. Elevated temperatures increase leaf senescence and root secondary metabolite concentrations in the herb *Panax quinquefolius* [63]. A 5°C increase in temperature significantly increased the ginsenoside content in roots of *P. quinquefolius* [63]. A temperature variation has multiple effects on the metabolic regulation, permeability, and rate of intracellular reactions in plant cell cultures [62]. Temperature range of 17–25°C is normally used for the induction of callus tissues and growth of cultured cells [16]. The temperature and light quality influences on the production of ginsenoside in hairy root culture of *Panax ginseng* [64]. The *Melastoma malabathricum* cell cultures incubated at a lower temperature range (20 ± 2°C) grew better and had higher anthocyanin production than those grown at 26 ± 2°C and 29 ± 2°C [65]. Fifteen days at 35°C significantly increased the hypericin and hyperforin content in shoots of *Hypericum perforatum* [66].

4. Chemical elicitors

Heavy metals have become one of the main abiotic stress agents for living organisms because of their increasing use in the developing fields of industry and agrotechnics and high bioaccumulation and toxicity [67]. Although a lot of information is available concerning the effects of heavy metals on plant growth and physiology, much less is known regarding their effects on the production of secondary metabolites. Heavy metal–induced

changes in metabolic activity of plants can affect the production of photosynthetic pigments, sugars, proteins, and nonprotein thiols. These effects can result from the inhibition of enzymes involved in the production of these natural products, likely through impaired substrate utilization [68]. Metals may alter the production of bioactive compounds by changing aspects of secondary metabolism [2]. Metals including Ni, Ag, Fe, and Co have been shown to elicit the production of secondary metabolites in a variety of plants [69].

An increased oil content up to 35% in *Brassica juncea* was seen due to the effective accumulation of metals (Cr, Fe, Zn, and Mn) [70]. The highest accumulations of secondary metabolites such as shikonin [71] and also the production of digitalin [72] were observed by treating Cu^{2+} and Cd^{2+}. The production of betalains in *Beta vulgaris* also stimulated by Cu^{2+} [73]. Co^{2+} and Cu^{2+} have a stimulatory effect on the production of secondary metabolites in *Beta vulgaris* [73]. The betalaines production was enhanced by exposing the hairy root culture to metal ions [74]. The stimulatory effects of Cu^{2+} on the accumulation of betacyanins in callus cultures of *Amaranthus caudatus* were reported by Obrenovic [75]. The addition of Zn^{2+} (900 μM) improved the yield of lepidine in cultures of *Lepidium sativum* [76]. However, Cu proved more effective than Zn in enhancing the yield product [76]. In hairy root cultures of *Brugmansia candida*, silver nitrate ($AgNO_3$) or cadmium chloride ($CdCl_2$) elicited the overproduction of two tropane alkaloids, scopolamine, and hyoscyamine [20]. The production of taxol in cell culture of *Taxus* sp. was enhanced by the rare–earth metal (lanthanum) [77]. $AgNO_3$ stimulated the production of tanshinone in the root culture of *Perovskia abrotanoides* [78]. The treatment of root cultures of *Datura stramonium* with cadmium salts at external concentrations of approximately l mM has been found to induce the rapid accumulation of high levels of sesquiterpenoid–defensive compounds, notably lubimin and 3–hydroxylubimin, but not alkaloid [79].

5. Hormonal elicitors

Various plant hormones have been extensively used in elicitation studies. The most studied, because of their key roles in the plant defense response, are jasmonic acid (JA) and SA and its derivatives.

5.1. Jasmonates

Jasmonates, including JA and MeJA, are a family of cyclopentanone compounds that modulate a wide range of plant responses [80,81] and act as effective elicitors to enhance secondary metabolites in in vitro cultures. They constitute an important class of elicitors for many plant secondary metabolic pathways, which are typically manifested by the elicitation of secondary metabolite biosynthesis when plants face particular environmental stresses [82]. JA is an important signal molecule of plant in response to wound and pathogen attack [83]. JA and its more active derivative MeJA can induce the production of a wide range of plant secondary metabolites such as rosmarinic acid, terpenoid indole alkaloid, and plumbagin in various cell

cultures [84–86]. JA elicitation are reported to induce the production of rosmarinic acid in *Mentha piperita* [84], anthocyanin in *V. vinifera* [87], and plumbagin in hairy roots of *Plumbago indica* [88]. JA and MeJA have been used as elicitors for stilbene biosynthesis in *V. vinifera* foliar cuttings [89], *V. vinifera* cell cultures [90,91], and *Vitis rotundifolia* hairy root cultures [92]. The addition of MeJA to *V. vinifera* cell cultures also promoted anthocyanin accumulation [93]. MeJA with transgenic technology highly enhanced the production of tanshinones in *Salvia miltiorrhiza* hairy roots [94]. In the hairy root culture of *Withania somnifera*, MeJA elicited the production of withanolide A, withanone, and withaferin A [95]. The MeJA enhanced the production of bacoside A, a valuable triterpenoid saponin having nootropic therapeutic activity in in vitro shoot cultures of *Bacopa monnieri* [96]. In *Andrographis paniculata* cell culture, the MeJA induced the production of andrographolide content [97]. In *Glycyrrhiza glabra*, methyl jasmonate induced the production of the saponin soyasaponin [98]. It enhanced the production of paclitaxel in *Tacus canadensis* and *T. cuspidate* [99], and in *Rubus idaeus*, it stimulated the production of the raspberry ketone benzalacetone [100].

5.2. Salicylic acid

Salicylic acid, well known for the systemic acquired resistance it induces in the plant response to many pathogens, can also elicit the production of secondary metabolites in plants [101,102]. SA with transgenic technology highly enhanced the production of tanshinones in *S. miltiorrhiza* hairy roots [94]. The higher production of withanolide A, withanone, and withaferin A was reported in the elicited–hairy roots of *W. somnifera* [95]. SA induced the stilbene production in the cell suspension of *V. vinifera* [34]. It stimulated the production of alkaloids such as vincristine and vinblastine in periwinkle [103], the tropane alkaloid scopolamine in hairy root cultures of *Brugmansia candida* [77], and pilocarpine in jaborandi leaves [104]. Anthraquinone production was greatly increased in *Rubia cordifolia* after a SA treatment [105]. SA also affects terpenoid secondary metabolism in plants. It induced accumulation of the triterpenoids ginsenosides in ginseng and glycyrrhizin in licorice [106,107]. Recent evidence demonstrated that suitable concentrations of SA can also promote monoterpene production [108].

5.3. Gibberellic acid

Gibberellin (GA), a phytohormone, is also well known as an effective elicitor for the production of secondary metabolites [109].

6. Biotic elicitors

In the production of secondary metabolites from plants, the use of biotic elicitors had an important role (Table 2).

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Elicitor	Plant species	Nature of culture	Compounds	References
Chitin	*Hypericum perforatum*	Shoot	Hypericin and pseudohypericin	[182]
	Hypericum perforatum	Cell suspension	Phenylpropanoid and naphtodianthrone	[183]
	Vitis vinifera	Cell suspension	trans-Resveratrol and viniferins	[91]
Pectin	*Hypericum perforatum*	Shoot	Hypericin and pseudohypericin	[182]
Dextran	*Hypericum perforatum*	Shoot	Hypericin and pseudohypericin	[182]
Yeast extract	*Perovskia abrotanoides*	Adventitious roots	Cryptotanshinone and tanshinone IIA	[78]
	Plumbago rosea	Cell suspension	Plumbagin	[86]
	Silybum marianum	Cell suspension	Silymarin	[172]
Trichoderma atroviride	*Salvia miltiorrhiza*	Hairy root	Tanshinone	[184]
Protomyces gravidus	*Ambrosia artemisiifolia*	Hairy root	Thiarubrine A	[134]
Claviceps purpurea	*Azadirachta indica*	Hairy root	Azadirachtin	[136]
Mucor hiemalis	*Taverniera cuneifolia*	Root	Glycyrrhizic acid	[135]
Fusarium oxysporum	*Hypericum perforatum*	Cell suspension	Phenylpropanoid and naphtodianthrone	[183]
Phoma exigua	*Hypericum perforatum*	Cell suspension	Phenylpropanoid and naphtodianthrone	[183]
Botrytis cinerea	*Hypericum perforatum*	Cell suspension	Phenylpropanoid and naphtodianthrone	[183]
Aspergillus niger	*Gymnema sylvestre*	Cell suspension	Gymnemic acid	[149]
Saccharomyces cerevisiae	*Gymnema sylvestre*	Cell suspension	Gymnemic acid	[149]
Agrobacterium rhizogenes	*Gymnema sylvestre*	Cell suspension	Gymnemic acid	[149]
Bacillus subtilis	*Gymnema sylvestre*	Cell suspension	Gymnemic acid	[149]
Escherichia coli	*Gymnema sylvestre*	Cell suspension	Gymnemic acid	[149]
	Datura metel	Hairy root	Atropine	[150]
Bacillus cereus	*Datura metel*	Hairy root	Atropine	[150]
Staphylococcus aureus	*Datura metel*	Hairy root	Atropine	[150]
Rhizobium leguminosarum	*Taverniera cuneifolia*	Root	Glycyrrhizic acid	[135]

Table 2. Effect of Different Biotic Elicitors on the Production of Various Secondary Metabolites in Plants

6.1. Polysaccharide

The biotic elicitors have been utilized to increase secondary metabolite production in medicinal plants. In a *Panax ginseng* cell suspension, the cell wall–derived elicitor oligogalacturonic acid significantly increased the ginseng saponin content [110]. The treatment of cultured cells of *Lithospermum erythrorhizon* with the polysaccharide agropectin induced the production of the naphthoquinone shikonin [111]. The chitosan treatment of cultures of *Plumbago rosea* increased the plumbagin content [112]. The application of chitin or chitosan induced the production of coumarins and fluoroquinolone alkaloids in shoot cultures of *Ruta graveolens* [113]. Chitosan enhanced the production of trans–resveratrol and viniferins in the cell system of *V. vinifera* [91]. Chitin induced the phenylpropanoid and naphtodianthrone production in cell suspension cultures of *H. perforatum* [114].

6.2. Yeast origin

For decades, scientists are using yeast extract as one of the biotic elicitors. Yeast extracts stimulated ethylene biosynthesis in tomato [115] and bacterial resistance in bean (*Phaseolus vulgaris*) [116]. Yeast extract elicited the production of tanshinone in the root culture of *Perovskia abrotanoides* [78].

6.3. Fungal origin

Biotic elicitors produced by pathogens have mainly been used to induce the plant defense response. In the past, biological mixtures were prepared from pathogens without identification of the active compounds. The use of pathogenic and nonpathogenic fungal preparations as elicitors has become one of the most effective strategies to induce phenylpropanoid/flavonoid biosynthetic pathways in plant cells [117,118]. Necrotrophic pathogens such as *Botrytis* sp. usually kill the host cells often through secretion of toxins before deriving nutrients from them [119]. On the other hand, biotrophic pathogens *Fusarium* sp. or *Phoma* sp. try to avoid killing the host cells, and derive their nutritional benefits from extensive contact with them and by altering the host metabolism and secretion systems [120,121]. An early defense reaction of the plant cell attacked by fungal pathogen includes the rapid and transient production of reactive oxygen species (ROS). Plant cells are usually protected against the detrimental effects of ROS by a complex of nonenzymatic and enzymatic antioxidant systems [122]. It has been demonstrated that the phenylalanine ammonia lyase (PAL) enzyme that catalyses the entry of L–phenylalanine into the phenylpropanoid pathway has reputedly a crucial role in the synthesis of antioxidant/defense–related compounds [117]. The mycelia extracts from the above mentioned fungi induced partitioning of the phenylpropanoid pathway and a rapid stimulation of the monolignol pathway in *Linum usitatissimum* cultured cells [123]. Cultures of *Phytophthora* elicited microbial resistance in soybean [124] and potato [125]. Extracts from microbial–enriched composts stimulated systemic resistance to *Phytophthora* in pepper (*Capsicum annuum*) [126]. As the plant defense response and the production of secondary metabolites are closely related, it is not surprising that a number of elicitors have also been shown to increase the production of secondary metabolites in medicinal plant cell culture. Similar to plant defense, initial work on secondary metabolite elicitation was performed using

biological mixtures. Fungal cell wall fragments increased the production of the indole alkaloids ajmalicine, serpentine, and catharanthine by up to five times in cell suspensions of *C. roseus* [69,127] and the 12–oxo–phytodienoic acid, raucaffrincine, in *Rauwolfia canescens* [128]. Fungal mycelia increased the diosgenin content in *Dioscorea deltoidea* cells by 72% [129]. In *Papaver somniferum*, fungal spores increased the content of codeine, morphine, and sanguinarine by over eightfold [130,131]. A mixture of fungal polysaccharides increased the amount of the antimicrobial alkaloid acridone epoxide up to 100–fold in cultures of *R. graveolens* [132]. *Taxus chinensis* cells treated with an endophytic fungus found in the bark of the *T. chinensis* tree produced three times as much taxol as nonelicited cells [133].

The content of thiarubrine A was enhanced 3–fold in *Ambrosia artemisiifolia* hairy root cultures through the utilization of autoclaved cell wall filtrates from the fungus *Protomyces gravidus*, a pathogen of *Ambrosia artemisiifolia* [134]. The fungal challenged root cultures of *Taverniera cuneifolia*, increased the glycyrrhizic acid content [135]. Moreover, maximum increase in glycyrrhizic acid was noticed in *Mucor hiemalis* treated cultures. In case of *Fusarium moniliforme* and *Aspergillus niger*, threefold increase in glycyrrhizic acid was observed as compared to control unchallenged root culture. However, marginal increase in glycyrrhizic acid content was noticed, in *Penicillium fellutanum* and *Aspergillus tenuis* challenged cultures [135]. Similarly, biotic elicitors from *Claviceps purpurea* were included in *Azadirachta indica* hairy root cultures, leading to a 5–fold increase in the production of azadirachtin [136]. The transformed cell suspension cultures of *W. somnifera* when treated with the dual elicitation of copper sulfate (100 μM) and the cell extract of *Verticilium dahaliae* (5% v/v) showed highest production of withaferin A content when compared with the individual elicitors [137].

6.4. Bacterial origin

The bacterial elicitors stimulated the biosynthesis of scopolamine in adventitious hairy root cultures of *Scopolia parviflora* via the inhibition of H6H (hyoscyamine 6β–hydoxylase) expression [138]. In bacterial elicitation, the maximum glycyrrhizic acid increase was observed in *Rhizobium leguminosarum* challenged culture as compared to unchallenged control roots of *Taverniera cuneifolia* [135]. Furthermore, in *Bacillus aminovorans*, *Agrobacterium rhizogenes,* and *Bacillus cereus* challenged cultures, significant increase in glycyrrhizic acid content was observed. However, root culture challenged by *Agrobacterium tumefaciens* did not show any significant increase in glycyrrhizic acid content [135]. The gradual increase in hypericin and pseudohypericin was observed in seedlings of *H. perforatum* after challenging with Rhizobacterium [139]. Coronatine, phytotoxin produced by the *Pseudomonas syringae* species significantly induced taxane synthesis in taxane media cell cultures [140], also induced the viniferins production in the cell culture of *V. vinifera* [91].

7. Parameters of elicitors

Elicitation has been widely used to increase the production or to induce de novo synthesis of secondary metabolites in in vitro plant cell cultures [141]. This opened up a new area of

research that could have important economic benefits for pharmaceutical industry. Several parameters such as elicitor concentration and selectivity, duration of elicitor exposure, age of culture, cell line, growth regulation, nutrient composition, and quality of cell wall materials are also important factors influencing the successful production of secondary metabolite [142]. Some of these parameters were highlighted on elicitation of some medicinal plants for the production of secondary metabolites.

7.1. Elicitor concentration

Elicitor concentration plays a very important role in elicitation process. High dosage of elicitor has been reported to induce hypersensitive response leading to cell death, whereas an optimum level was required for induction [143–145]. At 0.1% (w/v) sodium chloride, ginseng saponin content and productivity were increased to approximately 1.15 and 1.13 times control values, respectively [146]. In the cell culture of *S. miltiorrhiza*, the effects of different concentrations of SA were affected the accumulation of salvianolic acid B and of caffeic acid. The increased accumulation of salvianolic acid B and of caffeic acid was observed in the applications of 3.125–25.0 mg/L of SA at 8 and 96 h when compared to the 32.0–50.0 mg/L of SA. After 96 h treatments with 3.125–25.0 mg/L of SA, the concentration of the phenolic acids decreased drastically compared to the amount 8 h after the treatments but still accumulated the higher concentrations of compound than that of the control [147]. The various concentrations (50, 100, 150, 200, and 250 μM) of MeJA and SA were used in the cell suspension cultures of *Gymnema sylvestre*. The MeJA at 150 μM and SA at 200 μM enhanced the accumulation of gymnemic acid content [148]. In the hairy root culture of *W. somnifera*, the MeJA (15 μM) and SA (150 μM) enhanced the production of withanolide A, withanone, and withaferin A content [95]. In the cell suspension culture of *V. vinifera*, the cobalt at all three used concentrations (5.0, 25, and 50 μM), Ag, and Cd at low concentration (5.0 μM) were most effective to stimulate the phenolic acid production, increasing the 3–O–glucosyl–resveratrol up to 1.6–fold of the control level (250.5 versus 152.4 μmol/g), 4 h after the treatments [67]. In the *A. paniculata* cell culture, MeJA at 5 μM showed 5.25 higher accumulation of andrographolide content compared with control [97]. The root cultures of *Taverniera cuneifolia* treated with increasing concentrations of MeJA (1.0, 2.5, 5, 10, 100, and 1000 μM) [135]. The glycyrrhizic acid content increased gradually with increase in MeJA (1–100 μM) concentration. Approximately 2.5–fold increase in glycyrrhizic acid production was noticed in MeJA (100 μM) treated roots, as compare to the unchallenged root culture. However, further increase in MeJA (1000 μM) concentration resulted in decrease in glycyrrhizic acid production [135].

7.2. Duration of elicitor exposure

The cell suspension culture of *G. sylvestre* was treated with MeJA and SA for 24 h, 48 h, and 72 h. With the MeJA treatment, the maximum gymnemic acid production was recorded 72 h after treatment with 150 μM (135.41 ± 0.43 mg/g DCW). The gymnemic acid content was 15.4–fold higher than the control cultures that were free of the elicitor. When the MeJA concentration exceeded 150 μM, there was a drastic fall (36.3%) in the gymnemic acid accumulation [148]. A high concentration of 200 μM SA was required to induce substantial quantities of gymnemic

aid (43.27 ± 0.80 mg/g DCW) in the suspensions that reached a maximum after 48 h treatment. The SA–induced response toward gymnemic acid accumulation resulted in a 4.9–fold increase in comparison to the control cultures [148]. The different biotic elicitors (*A. rhizogenes*, *Bacillus subtilis*, *Escherichia coli, Aspergillus niger*, and *Saccharomyces cerevisiae*) required different duration of time (24, 48, 48, 72, and 72 h, respectively) to elicit the gymnimic acid in the cell suspension culture of *G. sylvestre* [149]. The MeJA and SA for 4 h exposure time enhanced the production of withanolide A, withanone and withaferin A content in the hairy root culture of *W. somnifera* [95]. The yields of atropine content in the *Datura metel* hairy roots were increased by nanosilver as an elicitor after 12, 24, and 48 h, but atropine accumulation in *D. metel* hairy roots was reduced by $AgNO_3$, *Bacillus cereus*, and *Staphylococcus* after 12, 24, and 48 h [150]. In the cell culture of *Andrographis paniculata,* the MeJA induced the highest accumulation of andrographolide at 24 h compared with 48 and 72 h of treatments [97]. In the cell system of *V. vinifera*, a rapid accumulation of trans–resveratrol was recorded with MeJA treatment, starting from 2 h and reaching its maximum value at 96 h and the highest levels of viniferins recorded in cell cultures elicited with chitin (chitosan) for 144 h [91]. The MeJA produced the highest amount of bacoside A, 1.5–fold higher than the control shoots in the *B. monnieri* shoot culture after 48 h [96]. MeJA elicitation can cause an initial rapid increase in amount of various secondary metabolites from 24 to 72 h compared to controls after which a subsequent decrease can be found [151].

7.3. Age of culture

Age of culture plays is an important parameter in the production of bioactive compounds by elicitation. The treatment with MeJA and SA in the hairy root culture of *W. somnifera* showed highest accumulation of withanolide A, withanone, and withaferin A content after 40 days of culture [95]. In a study, 20–day–old cell cultures of *C. roseus* showed higher yields of ajmalicine on elicitation. The optimum level of ajmalicine (166 μg/g DW) was observed in 20–day–old cells elicited with extracts of *Trichoderma viride* followed by 90 and 88 μg/g DW ajmalicine in cells elicited with *A. niger* and *F. moniliforme*, respectively [127,152]. A similar type of observation was noticed from various workers Rijhwani and Shanks [153] and Ganapathi and Kargi [142]. The selenium addition at inoculum time did not significantly affect ginseng saponin accumulation. However, the addition of 0.5 mM selenium as an elicitor, after 21 days of culture, ginseng saponin content and productivity increased to about 1.31 and 1.33 times control levels, respectively [146]. The MeJA, at a concentration of 10 μM and 100 μM when introduced to cell suspension of *C. roseus* on day 6 of cell growth increased ajmalicine and serpentine production, respectively, re–elicitation showed a negative effect on both growth and alkaloid synthesis [154].

7.4. Nutrient composition

The composition of the medium or selection of medium also played a vital role in elicitation process. In the callus culture of *Erythroxylum coca*, the amounts of cocaine, cinnamoylcocaine, chlorogenic acid (CGA), and 4–coumaroyl quinate (CQA) were significantly affected by the

culture medium [155]. Cocaine production was nearly an order of magnitude greater on Anderson rhododendron medium (ARM) [156], Gamborg B5 (GB5) [157], and Murashige–Tucker medium (MMT) [158], but the amounts produced on MMT and GB5 were not significantly different from each other. Cinnamoylcocaine was affected in the same way. The major factor controlling tropane alkaloid (TA) accumulation was medium composition, with cocaine levels on ARM being nearly an order of magnitude greater than on the other media. Many nutrients, including cobalt, copper, molybdenum, calcium, magnesium, iron, boron, iodine, manganese, zinc, and myo–inositol, and the growth regulators and the ammonium:nitrate ratio are at equivalent levels in ARM as in one of the other media, and can therefore be excluded as factors promoting TA accumulation. However, a number of factors differed between ARM and the other media, and might be responsible for the elevated TA content. Total ion concentration is lower in ARM, and could be an important factor given the importance of salt content in controlling secondary metabolism [155]. Nitrate concentration was also lower in ARM, and there are numerous reports in the literature of an inverse relationship between nitrate availability and accumulation of secondary metabolites in many plant species, including *Arabidopsis thaliana* [159], *Hordeum vulgare* [160], and *Nicotiana tabacum* [161]. Similarly, the reduction of nitrate concentration in the culture medium of *Atropa belladonna* hairy roots increases alkaloid content [162]. In regards to CGA, the media were all significantly different from each other with the lowest production on ARM and highest on MMT. Less CQA was produced on ARM than on either of the other two media, which did not differ from each other [155]. Apart from these characteristics, the efficiency of elicitation also depends on elicitor specificity, cell line or clones of microbial elicitor used the presence of growth regulators, nutrient composition of the medium, and the environmental conditions.

8. Conclusion

The development of plant tissue cultures for the production of secondary metabolites has been underway for more than three decades. Although there are well–established plant tissue culture techniques, their application to large scale production is still limited to a few processes. Various stimulation and process strategies have been exercised to improve secondary metabolite production in plant tissue cultures. Elicitation has been widely applied for enhancement of secondary metabolite production in plant cell and organ cultures. The effects of various abiotic and biotic elicitors on secondary metabolite production in plant tissue cultures are dependent on the specific secondary metabolites. The exploration of the production of useful secondary metabolites through regulation of biosynthetic pathway of the various plant cell and tissue cultures of medicinal plants has been carried out by a group of plant scientists in several countries during the last decade. Although, elicitation enhances secondary metabolism in plant cells in vitro, but the exact mechanism is not exactly understood. There is a tremendous scope for the large–scale production of secondary metabolites in the plant tissue culture system by using the elicitors as an agent.

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