Chapter 4
Elicitation: An Alternative Approach Towards Commercialization of Secondary Metabolite Production

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4.1 Introduction

Plants are known for the production of a large array of natural products, also referred to as secondary metabolites. Plant secondary metabolites represent a huge number of natural compounds with a wide diversity in chemical structure. They are economically important to man due to their multiple applications, such as pharmaceuticals, flavors, fragrances, insecticides, dyes, food additives, toxins, etc. However, it is well known that their production is frequently low and depends on the physiological and developmental stage of the plant. The majority of pharmaceutically important secondary metabolites are obtained from wild or cultivated plants, although some attempts have been made, but their chemical synthesis in most cases has not been economically feasible. Therefore, production of plant secondary metabolites by cultivation of plants and chemical synthesis are important agronomic and industrial objectives. As a promising alternative to produce plant secondary metabolites, plant cell culture technology has many advantages over traditional field cultivation and chemical synthesis, particularly for many natural compounds that are either derived from slow growing plants or difficult to be synthesized with chemical methods (Zhao and Verpoorte 2007; Zarate and Verpoorte 2007).

Plants exhibit a wide array of defense strategies against pathogen attack. The resistance against pathogen is performed by both pre-existing (constitutive) and induced defense systems. Inducible defense responses are triggered following recognition of a range of chemical factors termed 'elicitors' (Hammond-Kosack and Jones 1997). Originally the term elicitor was used for molecules capable of

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inducing the production of phytoalexins, but it is now commonly used for compounds stimulating any type of plant defense (Ebel and Cosio 1994; Ramachandra and Ravishankar 2002). Eventually, the induction of defence responses may lead to enhanced resistance. They might be both of biotic and abiotic origin.

The first biotic elicitors were described in the early 1970 (Keen 1975). Since then, numerous publications have accumulated evidence for pathogen-derived compounds that induce defense responses in intact plants or plant cell cultures. They comprise distinct compounds among either oligosaccharides or lipo and glycol-proteins. Such biotic elicitors often originate from the pathogen (exogenous elicitors) but in some cases are liberated from the attacked plant by the action of enzymes of the pathogen (endogenous elicitors) (Boller 1995; Ebel and Cosio 1994).

Elicitors act as signal compounds at low concentrations, providing information for the plant to trigger defence, distinguishing elicitors from toxins, which may act only at higher concentrations and/or affect the plant detrimentally without active plant metabolism (Boller 1995). The terms are overlapping, however, as exemplified by certain fungal compounds like fumonisin B1. While fumonisin B1 can be seen as a phytotoxin in the interaction of the necrotrophic Pathogen *Fusarium verticillioides* with its host maize (Desjardins and Plattner 2000), it acts as a elicitor switching on active plant defence and cell death programmes in the model plant *Arabidopsis* (Stone et al. 2000).

Elicitors are usually capable to induce various modes of plant defense including the production of ROS (reactive oxygen species), the hypersensitive response and the production of phytoalexins i.e., antimicrobial secondary compounds (Montesano et al. 2003). The induction of phytoalexin biosynthesis has gained special importance in biotechnological approaches to improve the production of secondary metabolites. Many of these compounds are of high value as therapeutics or otherwise biologically active agents. An example is the bio-production of taxol, a diterpenoid found in the bark of *Taxus* trees. This compound is approved by the Food and Drug administration (FDA) for the treatment of ovarian and breast cancer. There is a high demand for taxol, but its synthesis production is extremely costly, so biosynthesis in *Taxus* spp. Cell culture has become the focus of extensive research (Heinrich 2002). In general, plant cell cultures are rich sources of valuable pharmaceuticals and other biologically active compounds (Chen et al. 2002), however, relatively few cultivars and derived cell cultures synthesize secondary metabolites over extended and in amounts suitable for commercial exploitation. Elicitation studies have shown promise in increasing yields and cutting production costs (Miao et al. 2000; Zhang and Wu 2003).

In recent research into in vitro culture systems, a wide variety of elicitors have been employed in order to modify cell metabolism. These modifications are designated to enhance the productivity of useful metabolites in the cultures of the plant cells/tissues. The cultivation period, in particular can be reduced by the application of elicitors, although maintaining high concentrations of product (Ramachandra and Ravishankar 2002).

4.2 Classification of Elicitors

According to Radman et al. (2003) elicitors are classified as physical or chemical, biotic or abiotic and complex or defined depending on their origin and molecular structure.

4.2.1 Biotic Elicitors

Biotic elicitors are molecules of either pathogen or host origin that can induce defense responses in plant tissue. Often complex biological preparations have been used as elicitors, where the molecular structure of the active ingredients is unknown. Examples of such elicitors are yeast extract and microbial cell-wall preparations.

In recent years, the exact molecular structure of an increasing number of elicitors has been elucidated, including various polysaccharide, oligosaccharides, proteins, glycoproteins and fatty acids (Anderson 1989; Hahn et al. 1989).

4.2.1.1 Proteins and Glycoproteins as Elicitor

Protein elicitors have been used to elucidate the role of ion channels in plant cell membranes in the signal transfer triggered by external stimuli. Proteins and enzymes are another class of elicitors that triggers defense reactions. e.g., in plant cell cultures. Cellulase cause rapid accumulation of phytoalexins in *Nicotiana tabacum* cell cultures, an increase in the production of capsidol and debneyol and production of two previously unknown phytoalexins (Threlfall and Whithed 1988).

The pathogenic fungus *Phytophtora drechleri* secretes elicitins (protein elicitors) that induce necrosis in tobacco leaves: holo-proteins (proteins involved in the phototropic signal perception) cause concentration-dependent leaf necrosis. At least three isoforms of this elicitor are produced by *P. drechleri* (Huet et al. 1992).

Glycoproteins have also been shown to elicit phytoalexins in plant cell cultures. A glycoprotein of molecular mass 46 kDa isolated from *Phytophthora nicotianae* was shown to elicit phytoalexin accumulation in tobacco callus (Farmer and Helgeson 1987). A 42 kDa glycoprotein purified from *P. megasperma* f. sp. *glycinea* was shown to stimulate various defense responses in cultured parsley cells including ion fluxes, oxidative burst, expression of defense-related genes and phytoalexin accumulation (Nürnberger et al. 1994a). However, parsley leaves did not respond to the pure glycoprotein (Parker et al. 1991). A protein of molecular mass 60 kDa isolated from the bacteria *Pseudomonas solanacearum* was described as an inducer of the hypersensitive response in potato based on its capacity to elicit callus browning when applied at very low concentrations (Huang et al. 1989). Harpins and PopA1 are proteins isolated from *Pseudomonas* species which induce necrotic zones when infiltrated into tobacco leaves, similar to the hypersensitive necrosis induced by the incompatible bacteria from which they are secreted

(Arlat et al. 1994; He et al. 1993). Elicitins are small extracellular holoproteins produced by many species of *Phytophthora*. Upon application to tobacco plants they were shown to induce tissue necrosis (Billard et al. 1988; Ricci et al. 1989), as well as production of ethylene and the phytoalexin capsidiol (Milat et al. 1991).

4.2.1.2 Oligosaccharides as Elicitor

Oligosaccharides derived from cell walls of fungi and plants, including β-glucans, chitin, chitosan, and pectin, are inducers of the synthesis of a wide spectrum of defensive chemicals in plant tissues. These oligosaccharides are generated at infection or wound sites and may be early signals to activate genes whose products, such as antibiotic phytoalexins, extensin, proteinase inhibitors, pathogenesis-related proteins (PR proteins), and lignin, enhance the plants' defenses against pathogens and herbivores. Unlike the N-linked oligosaccharides that are involved in recognition systems in animals and yeast, the well-characterized carbohydrates that activate plant defensive genes are not covalently attached to proteins. They are relatively small oligomers that are hydrolytic fragments derived from cell walls of attacking pathogens or pests or from the cell walls of the plant itself (Darvill and Albersheim 1984; Ryan 1987). The ability of these oligosaccharides to alter gene expression patterns has stimulated renewed interest in intracellular and intercellular signaling processes in plants.

Chitosan is a mostly acetylated β-1, 4-linked D-glucosamine polymer, which acts as a structural component of the cell wall of several plant fungal pathogens, such as *Fusarium* species. Orlita et al. (2008) used chitin and chitosan as elicitor of coumarins and furoquinolone alkaloids in *Ruta graveolens* (common rue). There was a significant increase in the growth rate of *R. graveolens* shoots in the presence of either chitin or chitosan. Moreover, the results of the elicitation of coumarins and alkaloids accumulated by *R. graveolens* shoots in the presence of chitin and chitosan show that both compounds induced a significant increase in the concentrations of nearly all the metabolites. Adding 0.01 % chitin caused the increase in the quantity (μg/g dry weight) of coumarins (pinnarin up to 116.7, rutacultin up to 287.0, bergapten up to 904.3, isopimpinelin up to 490.0, psoralen up to 522.2, xanhotoxin up to 1531.5 and rutamarin up to 133.7). The higher concentration of chitosan (0.1 %) induced production of simple coumarins (pinnarin up to 116.7 and rutacultin up to 287.0), furanocoumarins (bergapten up to 904.3, isopimpinelin up to 490.0, psoralen up to 522.2, xanhotoxin up to 1531.5) and dihydrofuranocoumarins (chalepin up to 18 and rutamarin up to 133.7). Such a dramatic increase in the production of nearly all metabolites suggests that these compounds may be participating in the natural resistance mechanisms of *R. graveolens*. Whereas, Putalum et al. (2007) used chitosan in hairy root cultures of *Artemisia annua*. Artemisinin production by hairy roots of *A. annua* was increased six-fold to 1.8 μg mg^{-1} dry weight over 6 days by adding 150 mg chitosan l^{-1}.

Elicitor-like substances are involved in the symbiosis between leguminous plants and rhizobia (Cullimore et al. 2001). The bacterial partner produces

chemical signals-nodulation (Nod) factors-that are responsible for appropriate recognition of the bacteria by the plant partner and subsequent nodulation. The Nod factors are lipo-chito-oligosaccharides and there are pieces of evidence suggesting that the perception of Nod factors has evolved from recognition of more general elicitors of plant defence such as chitin fragments or LPS (Boller 1995; Cullimore et al. 2001). Interestingly, LPS from plant growth-promoting rhizobacteria trigger induced systemic resistance (ISR) to subsequent infections of plant pathogens without eliciting the accumulation of PR proteins or phytoalexin (van Loon et al. 1998).

Cline and Coscia (1988) reported the stimulation of Sanguinarine production by combined fungal elicitation and hormonal deprivation in cell suspension cultures of *Papaver bracteatum*. The fungal elicitor preparations from either homogenized mycelia of *Dendryphion penicilatum*, a specific pathogen of *Papaver* species or conidia of *Verticillium dakliae*, a general pathogen, were added to 14-day-old suspension cultures of *Papaver bracteatum*. Dendryphion extracts elicited an accumulation of the benzophenanthridine alkaloid, sanguinarie, which was not greatly influenced by hormone deprivation. Millimolar concentrations of dopamine were detected under all conditions. The baine was found when cells were cultured in hormone-free media, but it was not elicitor dose dependent. Verticillium-elicited cultures accumulated sanguinarine in an elicitor dose-dependent manner only under conditions of hormonal deprivation, resulting in an elevation of sangirine levels 5- to 500-fold greater than controls (2–10 % dry weight).

Wang et al. (2001) reported a protocol for the enhancement of Taxol production and excretion in *Taxus chinensis* cell culture by fungal elicitation and medium renewal. An endophytic fungus, *Aspergillus niger*, isolated from the inner bark of a *Taxus chinensis* tree, was used as an elicitor to stimulate the Taxol (paclitaxel) production in a *Taxus chinensis* cell suspension culture. Different elicitor doses and elicitation times were tested in a batch culture; and the highest volumetric Taxol yield was achieved when 40 mg/l of the fungal elicitor (carbohydrate equivalent) was added to the culture during the late exponential-growth phase. The elicitation resulted in a more than two-fold increase in the Taxol yield and about a six-fold increase in total secretion. The Taxol yield was further improved substantially by applying medium renewal and re-elicitation to the culture. In particular, with repeated medium renewal (in a way similar to medium perfusion) and a second elicitation of the culture, the volumetric Taxol yield was increased to 67.1 mg/l, which was about seven times the amount obtained in the non-elicited batch culture.

4.2.2 Abiotic Elicitors

Abiotic elicitors or stress agents, on the other hand, include ultraviolet radiation, heavy metal salts, and other chemical compounds with diverse mechanisms of action (Eilert 1987). The use of abiotic elicitors in plant cell cultures has received less attention compared with the biotic elicitors (Radman et al. 2003).

4.2.2.1 Heavy Metal Salts as Elicitor

Some heavy metal salts are often found to trigger phytoalexin production. For example, $AgNO_3$ increased significantly scopolamine release (three-fold) and scopolamine and hyoscyamine accumulation (5- to 8-fold) in hairy root culture of *Brugmansia candida*. While, $CaCl_2$ had little effect on accumulation or release of either alkaloid (scopolamine and hyoscyamine). $CdCl_2$ acted positively on the release of both alkaloids (3- to 24-fold), but was highly detrimental to growth. (Pitta-Alvarez et al. 2000). Zhao et al. (2010) examined the effects of biotic and abiotic elicitors on the production of diterpenoid tanshinones in *Salvia miltiorrhiza* cell culture. Four classes of elicitors were tested, heavy metal ions (Co^{++}, Ag^{+}, Cd^{++}), polysaccharides (yeast extract and chitosan), plant response-signaling compounds (salicylic acid and methyl jasmonate), and hyperosmotic stress (with sorbitol). Of these, Ag (silver nitrate), Cd (cadmium chloride), and polysaccharide from yeast extract (YE) were most effective to stimulate the tanshinone production, increasing the total tanshinone content of cell by more than ten-fold (2.3 mg/g versus 0.2 mg/g in control). The stimulating effect was concentration-dependent, most significant at 25 μM of Ag and Cd and 100 mg/l (carbohydrate content) of YE. Of the three tanshinones detected, cryptotanshinone was stimulated most dramatically by about 30-fold and tanshinones I and IIA by no more than five-fold. Meanwhile, most of the elicitors suppressed cell growth, decreasing the biomass yield by about 50 % (5.1–5.5 g/l versus 8.9 g/l in control) (Zhao et al. 2010).

4.2.2.2 Ultrasound as Elicitor

The low-energy ultrasound (US) can act as an abiotic elicitor to induce plant defense responses and stimulate secondary metabolite production in plant cell cultures (Wu and Lin 2002). In addition, US can induce cell membrane permeabilization so as to enhance intracellular product release. This cell-permeabilizing effect may be complementary to the two-phase culture to accomplish product release from the cells and removal from the medium. In the study of Lin and Wu (2002), the combination of US stimulation and in situ solvent extraction in a *Lithospermum erythrorhizon* cell culture led to 2- to 3-fold increases in the yield of shikonin. While, in *Taxus chinensis* Wu and Lin (2003) achieved 1.5- to 1.8-fold increase in taxol yield with 2 min US treatment once or twice during a week-culture period. Ramani and Jayabaskaran (2008) reported enhanced catharanthine and vindoline production in suspension cultures of *Catharanthus roseus* by ultraviolet-B light. A dispersed cell suspension culture from *C. roseus* leaves in late exponential phase and stationary phase were irradiated with UV-B for 5 min. The stationary phase cultures were more responsive to UV-B irradiation than late exponential phase cultures. Catharanthine and vindoline increased 3-fold and 12-fold, respectively, on treatment with a 5-min UV-B irradiation.

4.2.2.3 Light as Elicitor

Light plays a role in both growth and secondary metabolite production. Many hairy root lines when exposed to light, turn green, and develop mature chloroplasts fully capable of photosynthesis (Flores et al. 1993). Green roots have metabolic capabilities distinct from their non-green counterparts. For example, *Ipomoea aquatica* hairy roots grown in the light produce twice as much biomass and four times as much peroxidase as roots grown in the dark (Taya et al. 1994).

Roots do not have to turn green, however, to show profound alterations in secondary metabolism in response to light. For example, increasing light intensity to about 200 mmol m^{-2} s^{-1} doubles the growth yield of *A. annua* hairy roots. In contrast, light inhibited the growth of *Tagetes patula* hairy roots and significantly altered the type of thiophenes produced compared to dark-grown cultures (Mukundan and Hjortsø 1991). Sauerwein et al. (1992) also found that the alkaloid content of both normal and hairy roots of *Hyoscyamus albus* was greater in roots grown in the light compared to roots grown in the dark. Bhadra et al. (1998) studied the effect of light on growth as well as indole alkaloid accumulations of *Catharanthus roseus* hairy root cultures. The total yield of alkaloids was significantly different between light-adapted and dark-grown roots. More interestingly, they demonstrated that production of some alkaloids during a specific growth phase shifts when light conditions are altered. Light-adapted roots show growth-associated production of serpentine, and non-growth-associated production of tabersonine. When roots are shifted from dark to light, production of ajmalicine shifts from being growth-associated to non-growth-associated. Taken together these responses are not surprising since considerable evidence now shows that many of the enzymes in the terpenoid and other alkaloid pathways are regulated by light.

4.3 Active Mechanism of Elicitation in Plant Cells

The active mechanisms employed by elicitors are complex and distinctive. As little is known regarding the biosynthetic pathways of most secondary plant metabolites, the effects of elicitation on a plant cell/tissue cultures are difficult to predict. Therefore, elicitation approaches tend to be empirical steps. The effects of elicitors rely on a host of factors, including the concentration of the elicitor, the growth stage of the culture at the time of elicitation and the contact duration of elicitation (Lu et al. 2001).

Plant cells respond to various biotic and abiotic elicitors by activating a wide array of reactions viz., ion fluxes across the plasma membrane, synthesis of reactive oxygen species (ROS) and phosphorylation and dephosphoryaltion of proteins. These are all putative components of signal transduction pathways that lead to elicitor-induced defense response, e.g. the activation of defense genes and hypersensitive cell death (Dietrich et al. 1990; Nürnberger et al. 1994b). It has been suggested that ROS alone cannot mediate a sufficient disease resistance response

in plants, but in combination with Nitric oxide (NO) can function synergistically to activate a stronger response (Wang and Wu 2005). NO is a diffusible, bioactive signaling molecule (Beligni and Lamattina 2000; Neill et al. 2003; Romero-Puertas et al. 2004). In cell suspension cultures, NO plays a crucial role in the synthesis of secondary meatbolites via chemical (e.g., methyl jasmonate, Wang and Wu 2005), physical ultrasound (Wang et al. 2006), or microbial elicitors (Wang and Wu 2005), physical ultrasound (Wang and Wu 2004; Xu et al. 2005).

Wu et al. (2007) reported the involvement of NO in elicitation, accumulation of secondary metabolites and antioxidant defence in adventitious roots of *Echinacea purpurea*. When roots were treated with 100 mM sodium nitropruside (SNP), an exogenous NO producer, the accumulation of phenolics, flavonoids, and caffeic acid derivatives was enhanced.

Intensive research has been devoted to establishing the mechanism of elicitation in plants. Research was focused mainly on the biotic, particularly carbohydrate elicitors and the effects of abiotic elicitors on the over production of secondary metabolites in plants is poorly understood.

A general mechanism for biotic elicitation in plants may be summarized on the basis of elicitor-receptor interaction. When a plant or plant cell culture is challenged by the elicitor an array of biochemical activities occurs which are as follows;

- Binding of the elicitor to a plasma membrane receptor (Cheong and Hahn 1991; Nürnberger et al. 1994b; Leburun-Garcia et al. 1999).
- Altered ion fluxes across the plant cell membrane i.e., Cl^- and K^+ efflux, Ca^{++} influx (Zimmermann et al. 1999; Ivashkina et al. 2001).
- Increased activity of the plant phospholipases was found in some plant tissues and cultured cells after elicitor contact (Munnik 2001; Wang 2001; Laxalt and Munnik 2002); synthesis of secondary messengers Ins (Hammond-Kosack and Jones 2000) P_3 and diacylglycerol (DAG) (Mahady et al. 1998) mediating intracellular Ca^{++} release, nitric oxide (Delledone et al. 2002; Huang et al. 2002) and octadecanoid signaling pathway (Piel et al. 1997).
- Rapid changes in protein phosphorylation patterns have been observed upon elicitor treatment of a variety of cell cultures (Boller 1995; Siegrist et al. 1998)
- G-protein activation (Mahady et al. 1998; Luan 1998) which are also involved in the early responses to elicitors.
- Activation of NADPH oxidase responsible for AOS and cytosol acidification (Leburun-Garcia et al. 1999).
- Cytoskeleton reorganization (Kobayashi et al. 1995).
- Generation of active oxygen species (Apostol et al. 1989; Levine et al. 1994).
- Accumulation of pathogenesis-related proteins such as chitinases and glucanases, endo-polygalacturonases, hydroxyproline-rich glycoproteins, protease inhibitors (Stintzi et al. 1993; Benhamou 1996).
- Cell death at the infection sit (hypersensitive response), (Luan 1998; Mahady et al. 1998).
- Structural changes in the cell wall (lignifications of the cell wall, callus deposition), (Kauss et al. 1989).

- Transcriptional activation of the corresponding defense response genes (Memelnik et al. 2001; Cormack et al. 2002).
- Plant defence molecules such as tannins and phytoalexins are detected 2–4 h after stimulation with the elicitor (Ito and Shibuya 2000; Pedras et al. 2002).
- Synthesis of jasmonic and salicylic acids as secondary messenger (Katz et al. 2002).
- Systematic acquired resistance (Leburun-Garcia et al. 1999).

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Glossary

Elicitor An elicitor, in biology, is a molecule that enhances the production of another.

Phytoalexins Any of various antimicrobial chemical substances produced by plants to combat infection by a pathogen (as a fungus).

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