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Strategies in Enhancing Secondary Metabolites Production in Plant Cell Cultures

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ABSTRACT

Conventionally, pharmaceutically important secondary metabolites such as flavonoids were extracted directly from whole plants collected from the wild. This conventional method is not cost effective and could even lead to extinction of some endangered plant species. Furthermore, the production of uniform quantity and quality secondary metabolites will be affected as their production is greatly influenced by geographical, seasonal and environmental variations. Biotechnological approaches, specifically, plant tissue culture techniques, have been considered as an attractive solution to the problems of extracting secondary metabolites for industrial applications. Nevertheless, the commercial implementation of pilot scale plant cell suspensions for chemical production is still in the development stage with a few exceptional cases. The major setback is the failure of cell cultures to accumulate significant amounts of secondary metabolites compared to whole plants or organ cultures. Even so, several strategies can be applied in order to substantially increase the yields of secondary metabolites in plant cell cultures. This paper discussed the strategies of nutrient manipulation, precursor feeding and elicitation in enhancing the production of secondary metabolites.

Keywords: callus culture, cell suspension culture, plant biotechnology, secondary metabolites
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **B5**, Gamborg medium; **MS**, Murashige and Skoog; **IAA**, indole-3-acetic acid; **NAA**, 1-naphthaleneacetic acid; **PGR**, plant growth regulator

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INTRODUCTION

With the increasing concern on the side effects caused by modern synthetic or chemical drugs, medicinal plants remain the main source of a large range of basic healthcare and pharmaceutical products. Successful attempts to produce some of the valuable pharmaceuticals in relatively large quantities by cell cultures have been reported. Production of taxol, a popular anticancer drug, by cell cultures of various *Taxus* species is one of the most extensively explored and promising areas of plant cell cultures (Christen *et al.* 1989; Tabata 2004) owing to the enormous commercial value of taxol, the scarcity and slow growth of the *Taxus* tree, low contents of taxol and the costly synthetic process (Cragg *et al*. 1993; Tabata 2004; Croteau *et al.* 2006; Guo *et al.* 2006; Zhang *et al.* 2011). Another notable example is the large-scale production of pharmaceutically important compounds, ginsenoside and vinblastine, in the cell suspension cultures of *Panax notoginseng* and *Catharanthus roseus*, respectively (Smith *et al*. 1987). **Table 1** shows a list of other secondary metabolites that have been successfully produced in cell suspension cultures of various medicinal plants. Other examples include shikonin production by cell suspension cultures of *Lithospermum erythrorhizon* and berberine production by *Coptis japonica* (Fujita and Tabata 1987). Rosmarinic acid production by cell cultures of *Coleus blumeli* has also been achieved on a large scale. Similarly, sanguinarine, which has market potential as oral hygiene products has been produced in *Papaver somniferum* (Eilert *et al*. 1985; Ulbrich *et al*. 1985). Production of ajmalicine from *Catharanthus roseus* were successfully upscaled from shake-flask culture to bioreactor (Ten Hoopen *et al.* 1994). Recent study by Amdoun *et al.* (2009), production of tropane alkaloids, which are important natural compounds used as pharmaceuticals ingredients, were reported to have produced from hairy root culture of *Datura stramonium* L.

Nevertheless, the commercial implementation of pilot

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Review

Table 1 Bioactive secondary metabolites production from cell suspension culture of representative plant species.

Species	Active ingredient	Culture medium	References
Cassia acutifolia	Anthraquinones	MS + 2,4-D (4.52 μM), kinetin (0.47 μM), sucrose (0.088 M), *myo*-inositol (555.06 μM)	Nazif *et al.* 2000
Catharanthus roseus	Indole alkaloids	MS + sucrose (0.088 M)	Moreno *et al.* 1993
Catharanthus roseus	Catharanthine	MS + NAA (10.74 μM), IAA (11.42 μM), kinetin (0.47 μM), sucrose (0.088 M)	Zhao *et al.* 2001
Cinchona robusta	Robustaquinones	B5 + 2,4-D (9.04 μM), kinetin (0.93 μM), cystine (208.07 μM), sucrose (0.058 M)	Schripsema *et al.* 1999
Fragaria ananassa	Anthocyanin	LS + 2,4-D (4.52 μM), BA (0.44 μM), phenylalanine (100 mM)	Edahiro *et al.* 2005
Isoplexis isabellina	Anthraquinones	MS + 2,4-D (5 μM), kinetin (10 μM)	Arrebola *et al.* 1999
Lycium chinense	Cerebroside	MS + 2,4-D (1.0 ppm), kinetin (0.1 ppm)	Jang *et al.* 1998
Morinda citrifolia	Anthraquinones	¼ MS + IBA (24.60 μM), sucrose (0.088 M)	Baque *et al*. 2010
Polygonum hydropiper	Flavonoids	MS + 2,4-D (10 μM), kinetin (10 μM), casamino acid (0.1%), sucrose (3%)	Nakao *et al.* 1999
Rauwolfia sellowii	Alkaloids	B5 + 2,4-D (4.52 μM), kinetin (0.93 μM), sucrose (0.088 M)	Rech *et al.* 1998
Taxus baccata	Taxol baccatin III	B5 (salts) + 3X B5 vitamins, 2,4-D (20 μM), kinetin (4 μM) + GA_3 (1 μM)	Cusido *et al.* 1999
Taxus spp.	Taxol	B5 medium + 2,4-D (0.91 μM), BA (2.22 μM), casein hydrolysate (200 mg/L), sucrose (0.088 M)	Wu *et al.* 2001
Taxus wallichiana	Taxol	½ WPMSH, IBA (9.84 μM), SH vitamins	Datta *et al*. 2006
Torreya nucifera var. *radicans*	Diterpenoids	MS + 2,4-D (45.24 μM), casamino acid (1 g/L), coconut milk (7%) and K^+ instead of NH_4^+	Orihara *et al.* 2002

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid; NAA: 1-naphthaleneacetic acid; GA_3: gibberellic acid; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid; BA: 6-benzyl adenine; B5: Gamborg's medium; LS: Linsmaier and Skoog medium; MS: Murashige and Skoog medium; WPMSH: Lloyd and McCown's medium supplemented with Schenk Hilderbrandt vitamins

scale plant cell suspension for chemical production is still in the development stage with a few exceptional cases (Eibl and Eibl 2008). For most of the compounds of interest such as morphine, quinine, vinblastine, atropine, scopolamine and digoxin, they are yet to come to a commercially feasible process (Verpoorte *et al*. 1994). The major setback is the failure of cell cultures to accumulate significant amounts of secondary metabolites compared to whole plants or organ cultures. Collin and Watts (1984) ascribed this to the lack of morphological and cellular differentiation that is necessary for the expression of many plant secondary metabolites in cell cultures. In addition, low growth rate as well as the difficulty in isolating a hyper-producing mutant line limited the number of large-scale industrial production using plant cell culture technology (Shinmyo *et al*. 1998). Even so, many biotechnological strategies have been hypothesized and experimented for enhanced production of secondary metabolites from plants. Some of these include screening of high yielding cell line, medium modification, precursor feeding, elicitation, large-scale cultivation in bioreactor system, hairy root culture, plant cell immobilization, biotransformation, engineered bacterium platform approach, gamma irradiation and microporpagation, among others (Dornenburg and Knorr 1995; Bhalsingh and Maheshwari 1998; Rao and Ravishankar 2002; Vanisree *et al*. 2004; Karuppusamy 2009; Jan *et al.* 2011; Reinsvold *et al.* 2011). There are also attempts to cultivate shoot and root cultures for the production of medicinally important compounds as these organ cultures are relatively more stable (Bourgaud *et al*. 2001).

MANIPULATION OF CULTURE MEDIUM

Many of the constituents of plant cell culture medium are important determinants of growth and accumulation of secondary metabolites. A number of chemical and physical factors affecting cultivation have been tested extensively with various plant cells. These factors include media components, plant growth regulators, pH, temperature, aeration, agitation, light etc. Medium manipulation is the most fundamental approach in plant cell culture technology. As an example, **Table 2** shows the effects of different medium formulation on biomass and serpentine production in cell suspension cultures of *Catharanthus roseus*.

Inorganic salts

One of the most commonly used media for plant tissue cultures is that developed by Murashige and Skoog (MS) (1962) for tobacco tissue culture. The significant feature of MS medium is its very high concentration of nitrate, potassium and ammonia. B_5 medium established by Gamborg *et al*. (1968) is also used by many researchers. The levels of inorganic nutrients in B5 medium are lower than in MS medium. Studies on the influence of various basal media on *Catharanthus roseus* demonstrated that the amount of serpentine produced depends on the composition of the basal medium used (Misawa 1994).

Table 2 Effects of different media on growth and serpentine production in cell suspension cultures of *Catharanthus roseus.*

Basal medium	Cell yield (g DW/L)	Serpentine (mg/L)	Serpentine content (% DW)
Blaydes	7.6	4.4	0.06
Gamborg + 1 mg/L 2,4-D	4.6	0.5	0.01
Gamborg + 2 mg/L 2,4-D	5.2	0	0
Gamborg + 1.86 mg/L NAA	7.6	1.2	00.02
Gamborg	5.1	0	0
Heller + 0.175 mg/L IAA + 1.13 mg/L BA	5.4	6.6	0.12
Linsmaier and Skoog	8.9	0	0
Murashige and Skoog	2.3	10.4	0.12
Nitsch and Nitsch	2.0	2.0	0.09
Velicky and Martin	5.0	0	0
White	0.8	0	0

Source: Misawa 1994

Nitrogen concentration was found to affect the level of secondary metabolites in cell suspension culture as it regulates the expression of specific proteins through mechanisms affecting the transcription and mRNA stability (Sugiharto and Sugiyama 1992). The nitrogen sources are important for secondary metabolites synthesis of compounds such as alkaloids, anthocyanins and shikonins from cell suspension cultures (Kim and Chang 1990; Zhong 2001). The plant tissue culture medium such as MS or B5 has both nitrate and ammonium as sources of nitrogen. However, the ratio of the ammonium/nitrate-nitrogen and overall levels of total nitrogen have been shown to markedly affect the production of secondary plant products (Rao and Ravishankar 2002; Smetanska 2008). It is a general trend that a lower ammonium (NH_4^+) to nitrate (NO_3^-) ratio is more favorable for plant tissue and cell growth (Wu and Zhong 1999), such as those reported in *Panax ginseng* cells (Ushiyama 1991). Meanwhile, complete elimination of nitrate in cultures of *Chrysanthemum cinerariaefolium* induced a two-fold increase in pyrethrin accumulation during the second phase of the culture (Rajasekaran *et al*. 1991). As reported by Lee *et al.* (2011), growing root culture of mulberry (*Morus alba* L.) in medium containing lower NH_4^+/NO_3^- ration resulted in a greater rutin production than growth in a medium containing a higher ratio of nitrogen sources.

Phosphorus is another key nutrient for plant cell growth. Its concentration in the medium can also have a major effect

on the production of secondary metabolites in plant cell cultures. It is a key role in all metabolic processes including energy transfer, signal transduction, biosynthesis of macromolecules, photosynthesis and respiration (Raghothama 2000). Higher levels of phosphate were found to enhance cell growth but caused a negative influence on secondary product accumulation (Rao and Ravishankar 2002). Zhang and Zhong (1997) found that an increase in initial phosphate from 1.25 to 3.75 mM enhanced both cell growth and saponin yield in suspension cultures of *Panax notoginseng*. However, Choi *et al*. (1994) reported an adverse observation in *P. notoginseng* callus cultures, in which the saponin content was lowered with an increase in phosphate concentration from 3.7 to 11 mM. Maximum production of selinan from cell culture of *Silene vulgaris* was observed at 1.25-3.75 µM phosphate (Gunter and Ovodov 2005). Similar concentrations were also shown to be optimal for pectin production by cell cultures of *Panax notoginseng* (Liu and Zhong 1998).

Vitamins

The basal MS medium includes vitamins such as *myo*-inositol, nicotinic acid, pyridoxine-HCl and thiamine-HCl. Among these vitamins, thiamine is essential for many plant cells and other vitamins stimulate the growth of cells in some cases. Thiamine is also involved in cell biosynthesis and metabolism (Willims 1995). However, Gamborg's B5 vitamins differed from the vitamins of MS in having a high concentration of thiamine. It is also well known that isolated root cultures are very much dependent on thiamine supply for continuous growth *in vitro* (White 1937). Root culture of *Solanum khasianum* Clarke introduced to Gamborg's B5 media with vitamins at normal concentrations had showed maximum fresh growth index (FGI). However increased vitamin concentration reduced FGI but solasodine production was increased by 2 times of concentration (Jacob and Malpathak 2005).

Myo-inositol has been described as a natural constituent of plants which involved in cell membrane permeability (Loewus and Murthy 2000; Stevenson *et al*. 2000), stimulation of cell division when added at low concentrations to the culture medium (Thorpe *et al*. 2010). However, high concentrations of *myo*-inositol may encourage the formation of calcium-inositol and ferrous inositol complexes in the medium. Uptake of these complexes by plant cell is difficult and, therefore, osmotic potential of the culture medium is increased (Drobak and Watkins 2000). The high osmotic potential of the culture medium will therefore limit the plant biosynthesis processes and growth (Zhu 2001). In study conducted by Perez *et al*. (2004) with pineapple culture, the proteolytic activity of the culture significantly decreased when the *myo*-inositol concentration was higher than 1.10 mM. Depending on the species of plant, there were also examples where higher concentrations of *myo*-inositol were favorable for secondary metabolite production. In the callus culture of *Rheum ribes*, *myo*-inositol at 100 mg/L increased the anthraquinone content (Sepehr and Ghorbanli 2002).

Carbon sources and concentrations

Plant cell cultures are usually grown heterotrophically using simple sugars as carbon source (Rao and Ravishankar 2002). Generally, sucrose or its component monosaccharides, glucose or fructose at 2 to 4% is the best carbon source for the growth of most plant cell cultures (Abdullah *et al*. 1998). Besides supporting growth, sugars in the culture medium serves as signaling molecules regulation division, differentiation and metabolism of the cells (Sherson *et al.* 2003; Rolland *et al.* 2006; Wang and Weathers 2007). Other sugars such as maltose also supported the growth of various plant cells. It has been established that, among these carbon sources, sucrose is energetically the most advantageous for the cultivation of plant cell cultures, particularly with regard to biosynthesis of secondary metabolites. Nevertheless, the cell cultures differ in the sequence in which they consume the inversion products of sucrose (Wu and Zhong 1999). The disaccharide, sucrose may be hydrolyzed to glucose and fructose during high temperature sterilization or can be hydrolyzed by invertase located in the cell wall (Paek *et al*. 1996; Zhang *et al*. 1996). In *Morinda elliptica*, higher biomass was yielded in 5% glucose as compared to 5% sucrose (Abdullah *et al*. 1998). In most cases, glucose has been reported as a preferred form of monosaccharide taken by cell compared to fructose (Kino-Oka *et al*. 1994; Kretzchmar *et al.* 2007). However, catharanthine production was doubled in *Catharanthus roseus* cultures when fructose was supplied as carbon source (Kim *et al*. 2001).

Misawa (1994) revealed that the most suitable carbon source and its optimal concentration for growth as well as the secondary metabolites accumulation are plant species and products dependent. Increased sucrose concentration usually resulted in increased biomass and secondary metabolites production as observed in *Catharanthus roseus* callus (Zhao *et al*. 2001). Nevertheless, cell growth was repressed by relatively higher initial sucrose concentration, which led to a relatively higher osmotic pressure in cell cultures of *Panax notoginseng* (Zhang *et al*. 1996), *Holarrhena antidysenterica* (Panda *et al*. 1992) and *Vitis vinifera* (Do and Cormier 1990). Zhang and Zhong (1997) discovered that in increasing the biomass and secondary product yield, constant or intermittent feeding of sucrose or other sugars was more effective than raising the initial concentration. However, in a number of plant cells, production of secondary metabolites was affected by initial sugar concentration. For instance, cell culture of *Melastoma malabathricum* showed the highest pigment content (anthocyanin production) of 0.69 ± 0.22 colour value per gram fresh cell mass in medium supplemented with 4.5% (w/v) sucrose (See *et al.* 2011). Other examples includes the accumulation of saponin by *Panax notoginseng* (Zhang *et al*. 1996) and *Panax ginseng* (Choi *et al*. 1994), carotenoid and anthocyanin by *Perilla frutescens* (Zhong *et al*. 1994; Zhong and Yoshida 1995), rosmarinic acid by *Coleus blumei* (Gertlowski and Petersen 1993), anthocyanin by *Aralia cordata* (Sakamoto *et al*. 1996) as well as shikonin formation by *Lithospermum erythrorhizon* (Srinivasan and Ryu 1993). Zhang *et al*. (1996) indicated high osmotic pressure caused by high sugar level as the contributing factor in high saponin production from *Panax notoginseng*.

A dual role of sucrose as carbon source and osmotic agent was also observed in *Solanum melongena*, where mannitol was added as osmoticum during the study (Rao and Ravishankar 2002). The results shown hypothesize that there could be a complex interaction between the sucrose and the level of osmoticum in the medium (Mukherjee *et al.* 1991).

Medium pH

The pH of the medium is usually adjusted between 5 and 6 before autoclaving and extremes of pH are avoided. Medium pH generally drops by 0.6 to 1.3 units after autoclaving (Sarma *et al*. 1990; Nagella and Murthy 2010). The concentration of hydrogen ions in the medium changes during the development of the culture. This is due to the uptake of compounds required as essential nutrients or as buffer components (NH_4^+, NO_3^-, PO_4^{3+}), as well as secretion of acids (lactate, malate, succinate), which is particularly pronounced during the stationary growth stage (Endress 1994). The medium pH decreased during ammonia assimilation and increases during nitrate uptake (McDonald and Jackman 1989). Photoautotropic cell suspension cultures of *Chenopodium rubrum* showed that the increase in the external pH from 4.5 to 6.3 increased the cytosolic pH by 3.0 units and the vacuolar pH by about 1.3 units (Rao and Ravishankar 2002).

The alkaloid productivity and the storage capacity were found to be influenced by the size of the pH gradient

between the medium and vacuoles (Moreno *et al*. 1995). Shifting the pH of the medium between low and high values can change the permeability of cell membrane and was used to release intracellular alkaloids into the culture medium (Asada and Shuler 1989). Cultures of *Daucus carota* excreted less than 90% of anthocyanin when grown at pH 5.5 than at pH 4.5 because anthocyanin degrades at higher pH (Ramawat 1999). According to Nagella and Murthy (2010), high and low pH did not stimulate the biomass production and withanolide A production in *Withania somnifera* cell culture. In *Bacopa monnieri* shoot cultures, initial medium pH at 4.5 was found optimum for biomass accumulation and bacoside A production (Naik *et al.* 2010).

Plant growth regulators

Plant growth regulators (PGRs) play an important role in controlling and regulating plant differentiation, development and growth (Zhao *et al*. 2001). The PGRs usually have profound effects on both cell growth and product formation (Whitmer *et al*. 1998; Wu and Zhong 1999). The type and concentration of auxin or cytokinin or the auxin/cytokinin ratio alters dramatically both the growth and the product formation in cultured plant cells (Rao and Ravishankar 2002). Effects induced by plant growth regulators are not uniform with each species requires different kinds and levels of PGRs for callus induction, its growth and metabolites production (Endress 1994). Some important PGRs are auxins, cytokinins, abscisic acid, giberellin and ethylene (Moreno *et al*. 1995).

1. Auxins

Auxins constitute a group of plant hormones capable of promoting several aspects of plant growth and development, such as cell division, cell extension, vascular differentiation, adventitious root formation and apical dominance (Centeno *et al*. 1999). Individual cultures differ in their sensitivity to these hormones. Collin (2001) concluded that secondary product accumulation, in general, was suppressed by the presence of high auxin levels, particularly 2,4-D (Lee *et al.* 2011). An increase in the auxin level often leads to a dedifferentiation through stimulation of cell division and consequently diminishes the level of secondary metabolites (Becker and Sauerwein 1990). This is true in the cases of indole alkaloid and anthocyanin production from *Catharanthus roseus* (Zhao *et al*. 2001) and *Oxalis reclinata* (Makunga *et al*. 1997), respectively.

The absence of production with 2,4-D was proposed by Abdullah *et al*. (1998) due to either a lack of induction or repression/inhibition of enzymes needed for production and also due to the lack of sufficient carbon-skeletons for the secondary metabolites pathway which might prevent an 'overflow' in the direction of synthesis. On the other hand, stimulations by 2,4-D have been observed in anthocyanin production from callus cultures of *Oxalis linearis* (Meyer and van Staden 1995) as well as in formononetin accumulation by suspension cultures of *Glycyrrhiza glabra* (Arias-Castro *et al*. 1993). Misawa (1994) also reported that the production of L-DOPA by *Mucuna pruriens*, ubiquionone-10 by *Nicotiana tabacum* and diosgenin by *Dioscorea deltoidea* were stimulated by high levels of 2,4-D. Meanwhile, elimination of 2,4-D or replacement of 2,4-D by 1-naphthaleneacetic acid (NAA) or indole-3-acetic acid (IAA) has been shown to enhance the production of anthraquinones in *Morinda elliptica* (Abdullah *et al.* 1998) and *Morinda citrifolia* (Hagendoorn *et al.*1994; Van der Plas *et al*. 1995).

2. Cytokinins

Cytokinins have been shown to affect the expression of specific genes by both increasing and decreasing the abundance of particular proteins or mRNAs (Taha *et al.* 2008). According to their chemical structure, cytokinins can be classified into at least two broad groups, namely adenine derivatives and phenylurea derivatives. Zheng and co-researchers (1999) categorized kinetin, 6-benzylaminopurine, N^6-(2-isopentenyl) adenine, zeatin and zeatin riboside as the adenine-type cytokinins while thidiazuron is a representative of the phenylurea derivative group. As described by Endress (1994), the mechanism of the cytokinin effect is that it captures free radicals or reduces their formation to very low levels. These free radicals reduce membrane integrity by their effect on phospholipases. Thus, cytokinins, stabilize membranes and indirectly affect the exportation of essential amino acids (Endress 1994).

Cytokinins have different effects depending on the type of metabolite and species concerned. Cytokinins were found to stimulate alkaloid biosynthesis in some tumorous cell lines of *Catharanthus roseus* (Moreno *et al*. 1995). It is also able to amplify the increase in alkaloid production caused by removal from the culture medium of non-tumorous *C. roseus* cell lines (Decendit *et al*. 1992). Zia *et al.* (2007) reported that 8.88 μM BAP produced 3.05 μg/g artemisinin when compared to same amount of NAA and kinetin, only 1.95 μg/g and 1.7 μg/g artemisinin were produced, respectively in callus of *Artemisia absinthium*. According to Rao and Ravishankar (2002), kinetin stimulated the production of anthocyanin in *Haplopappus gracilus* but inhibited the formation of anthocyanins in *Populus* cell cultures. Although kinetin is one of the most popular cytokinins, zeatin was reported as the only cytokinin that was able to stimulate production of an isoflavonoid formononetin from *Glycyrrhiza glabra* cells (Arias-Castro *et al*. 1993).

3. Auxin/cytokinin ratio

Plants regulate their degree of differentiation by means of auxin/cytokinin ratios. By using appropriate combinations, the composition and concentration of secondary compounds produced by processes bound to particular differentiation patterns may be influenced. The high auxin: cytokinin ratio of 5 mg/L IBA with 0.1 mg/L thidiazuron (TDZ) had demonstrated the enhancement of the biomass as well as the content of the total phenolics and flavonoids when compared to the control, which was without cytokinin supplementation in root culture of *Eleutherococcus koreanum* (Lee *et al.* 2010). Similar results were also attained by Shim *et al.* (2010) on the secondary metabolite (total anthraquinones, phenolics and flavonoid) accumulation in cell suspension culture of *Morinda citrifolia* were greatly enhanced in media supplemented with 3.0 mg/L NAA and 0.9 mg/L BA, but suppressed when only auxin was present. Wu and Zhong (1999) demonstrated that not only the individual phytohormone level but also their combination had a significant influence on cell growth and ginsenoside accumulation in *Panax notoginseng*. Similar observation was also attained in the production of camptothecin by *Nothapodytes foetida* (Thengane *et al*. 2003), anthraquinone by *Morinda elliptica* (Abdullah *et al*. 1998) and *Rheum ribes* (Sepehr and Ghorbanli 2002), jaceosidin by *Saussurea medusa* (Zhao *et al*. 2001) as well as indole alkaloid by *Catharanthus roseus* (Zhao *et al*. 2001).

ELICITATION

An elicitor may be defined as a substance which, when introduced in small concentration to a living cell system, initiates or improves the biosynthesis of specific compounds (Radman *et al*. 2003). Elicitors can be classified on the basis of their 'nature' like abiotic elicitors (e.g. jasmonic acid) or biotic elicitors (e.g. yeast extract and enzyme), or on the basis of their 'origin' like exogenous elicitors (e.g. glucans and glycoproteins) and endogenous elicitors (e.g. alginate oligomers and hepta-β-glucosides) (Namdeo 2007). Elicitation, which refers to the treatment of plant cells with biotic and abiotic elicitors, has been one of the most effective means for enhancing secondary metabolite production in plant tissue cultures (Roberts and Shuler 1997; Rao and Ravishankar 2002). This strategy works on the basis

that the accumulation of most secondary metabolites in plants is part of the defense responses of plants to pathogen infection and environmental stimuli (stress).

Biotic elicitors include polysaccharides derived from plant cell walls or microorganisms, glycoproteins and low molecular weight organic acids (Dörnenburg and Knorr 1995). Yeast extract is one of the most common biotic elicitor studied in plant-microbe interaction. In the study of isoflavonoid accumulation in *Pueraria candollei* var. *candollei* and *P. candollei* var. *mirifica* suspension culture, yeast extract enhanced the early accumulation of isoflavonoids after elicitation (Korsangruang *et al*. 2010). A crude extract from *Fusarium oxysporum* increased the taxol production in suspension cultures of *Taxus chinensis* var. 'Mairei' by 3-fold (Yuan *et al*. 2002). Park *et al*. (1995) who studied the cell cultures of *Pueraria lobata,* discovered that by the addition of yeast extract to the cell cultures, it stimulated the accumulation of isoflavones and daidzein dimers. Meanwhile, extracts of *Aspergillus niger* and *Rhizopus oryzae* showed a positive effect on shikonin production in suspension cultures of *Arnebia euchroma* (Fu and Lu 1999).

Abiotic elicitors are substances of non-biological origin, predominantly inorganic salts, and physical factors acting as elicitors like Cu and Cd ions, Ca^{2+} or high pH, environmental stress factors such as osmotic shock, presence of heavy metal ions or other chemicals and UV radiation. For example, treating hairy root cultures of *Beta vulgaris* with up to 10-fold calcium higher than that present in the basal medium increased the production of betalains by 3-fold (Savitha *et al*. 2005). Likewise, the addition of 0.05M of potassium chloride increased the ajmalicine production in *Catharanthus roseus* by 4-fold as compared to the untreated cells (Zhao *et al*. 2001a). Current study conducted by Li *et al.* (2011) on the addition of sodium nitroprusside (SNP), a donor of nitric oxide, had shown to stimulate terpenoid indole alkaloids formation at growth phase of *Catharanthus roseus* hairy root culture.

PRECURSOR FEEDING

With the basis of the knowledge on biosynthetic pathways, several organic compounds have been added to the culture medium in order to enhance the synthesis of secondary metabolites (Namdeo *et al*. 2007). Precursor feeding has been an obvious and popular approach to increase secondary metabolites production in plant cell cultures. Precursor feeding is based on the idea that any compound, which is an intermediate, in or at the beginning of a secondary metabolite biosynthetic route, stands a good chance of increasing the yield of the final product (Rao and Ravishankar 2002).

Many strategies were attempted to synthesize the desired secondary metabolites in appreciable quantity and at competitive economic value but failed to gain commercial exploitation (Namdeo *et al*. 2007). This may reflect the poor understanding of basic secondary metabolic regulation in plant cell cultures. Precursor feeding to some plant cell culture systems may be promising as it showed favorable results. For example, precursors of alkaloid biosynthesis, the amino acids, have been added to cell suspension cultures for the production of tropane alkaloids, indole alkaloids, etc. Addition of phenylalanine to *Salvia officinalis* cell suspension cultures stimulated the production of rosmarinic acid (Ellis and Towers 1970). Addition of the same precursor resulted in stimulation of taxol production in *Taxus* cultures (Fett-Neto *et al*. 1993, 1994). Similarly, feeding ferulic acid to cultures of *Vanilla planifolia* resulted in an increase in vanillin accumulation (Romagnoli and Knorr 1988). Recent study done by Chamnipa *et al.* (2010) on the cell culture of *Vitex glabrata* R.Br. using 5 mg/L cholesterol as precursor had significant improvements on the cell growth and production of 20-hydroxyecdysone. Another study conducted by Palacio *et al.* (2011) on *Larrea divaricata* Cav. plant cell cultures had reported feeding of 0.5 mM of L-phenylalanine resulted in an increase of nordihydroguaiaretic acid (NDGA) by 2.2-fold higher than in the control. Studies also revealed that squalene, one of the precursors in triterpenes biosynthetic pathway, was found to increase the production of madecassoside and asiaticoside in the callus of *Centella asiatica* (Ling *et al*. 2005). The successful example of precursor feeding is still very limited as the key to successful protocol using precursor feeding lies in the identification of cheapest by product of other process which can be converted to desired secondary metabolite by selected plant cell line (Sevón and Oksman-Cakdentey 2002; Verpoorte and Memelink 2002).

FUTURE PERSPECTIVES

Despite the many studies conducted over the years on the production of secondary metabolites via *in vitro* approach, some challenges still persist until today mainly in the understanding of biochemistry mechanisms of the small molecule production and the vast diversity of plant species and its products. Recent improvements on the analytical methods and bioreactors design may push forward the pace of commercialization of plant produced products. In addition, the advancement in modern biotechnology has led to the enhancement as well as 'manufacturing' of molecules trough metabolic engineering. Plant metabolic engineering refers to the purposeful alteration of genes and metabolic pathways within the plant cells in order to increase the production of a specific substance. Nevertheless, concerted efforts are still required in this aspect of research.

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