Strategies in Enhancing Secondary Metabolites Production in Plant Cell Cultures

Anna P. K. Ling1* • S. L. Ong2 • H. Sobri3

1 Department of Human Biology, International Medical University (IMU), Kuala Lumpur, Malaysia
2 Department of Science, Faculty of Engineering and Science, Tun Hussein Onn University of Malaya (UTHM), Setapak, Kuala Lumpur, Malaysia
3 Agrotechnology and Biosciences Division, Malaysian Nuclear Agency, Bangi, 43000 Kajang, Selangor, Malaysia

Corresponding author: anna_ling@imu.edu.my

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

CONTENTS

INTRODUCTION ................................................................................................................................. 94
MANIPULATION OF CULTURE MEDIUM .................................................................................... 95
Inorganic salts................................................................................................................................. 95
Vitamins........................................................................................................................................ 96
Carbon sources and concentrations......................................................................................... 96
Medium pH................................................................................................................................ 96
Plant growth regulators............................................................................................................ 97
ELICITATION .............................................................................................................................. 97
PRECURSOR FEEDING ............................................................................................................ 98
FUTURE PERSPECTIVES ......................................................................................................... 98
REFERENCES ............................................................................................................................ 98

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 pharmacologically 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 blumelli has also been achieved on a large scale. Similarly, sanguinarine, which has market potential as oral hygiene products has been produced in Papaver somniferum (Ellert et al. 1985; Ulbrich et al. 1985). Production of ajmalicine from Catharanthus roseus were successfully upscaled from shake-flask culture to bioreactor (Ten Hooopen 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
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, scopalamine 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 (Shimmyo 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 micropropagation, among others (Dornenburg and Knorr 1995; Bhalsingh and Maheshwari 1998; Dornen 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 cultures are relatively more stable (Bourgaud et al. 2001). The number of large-scale industrial production using plant cell cultures. Collin and Watts (1984) ascribed this to the lack of secondary metabolites compared to whole plants or organ cultures. Many biotechnological strategies have been hypothesized for the expression of many plant secondary metabolites in cell cultures. A number of chemical and physical factors affecting cultivation have been tested extensively with 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 (NH₄⁺) to nitrate (NO₃⁻) ratio is more favorable as those reported in Catharanthus roseus. Meanwhile, complete elimination of nitrate in cultures of Catharanthus roseus demonstrated that the amount of serpentine produced depends on the composition of the basal medium used (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 (Sugiarto 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 nitrogen and ammonium. B₅ 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).

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<table>
<thead>
<tr>
<th>Species</th>
<th>Active ingredient</th>
<th>Culture medium</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cassia acutifolia</td>
<td>Anthraquinones</td>
<td>MS + 2,4-D (4.52 μM), kinetin (0.47 μM), sucrose (0.088 M), myo-inositol (555.06 μM)</td>
<td>Nazif et al. 2000</td>
</tr>
<tr>
<td>Catharanthus roseus</td>
<td>Indole alkaloids</td>
<td>MS + sucrose (0.088 M)</td>
<td>Moreno et al. 1993</td>
</tr>
<tr>
<td>Catharanthus roseus</td>
<td>Catharantine</td>
<td>MS + NAA (10.74 μM), IAA (11.42 μM), kinetin (0.47 μM), sucrose (0.088 M)</td>
<td>Zhao et al. 2001</td>
</tr>
<tr>
<td>Cinchona robusta</td>
<td>Robustusquinones</td>
<td>B5 + 2,4-D (9.04 μM), kinetin (0.93 μM), cystine (208.07 μM), sucrose (0.058 M)</td>
<td>Schipperma et al. 1999</td>
</tr>
<tr>
<td>Fragaria ananassa</td>
<td>Anthocyanin</td>
<td>LS + 2,4-D (4.52 μM), BA (0.44 μM), phenylalanine (100 mM)</td>
<td>Edahiro et al. 2005</td>
</tr>
<tr>
<td>Ipomoea alba var. latifolia</td>
<td>Anthraquinones</td>
<td>MS + 2,4-D (5 μM), kinetin (10 μM)</td>
<td>Arrebola et al. 1999</td>
</tr>
<tr>
<td>Lycium chinense</td>
<td>Cerebroside</td>
<td>MS + 2,4-D (1.0 ppm), kinetin (0.1 ppm)</td>
<td>Jang et al. 1998</td>
</tr>
<tr>
<td>Morinda citrifolia</td>
<td>Anthraquinones</td>
<td>½ MS + IBA (24.60 μM), sucrose (0.088 M)</td>
<td>Baque et al. 2010</td>
</tr>
<tr>
<td>Polygonum hydropiperoides</td>
<td>Flavonoids</td>
<td>MS + 2,4-D (10 μM), kinetin (10 μM), casamino acid (0.1%), sucrose (3%)</td>
<td>Nakao et al. 1999</td>
</tr>
<tr>
<td>Rauwolfia selloii</td>
<td>Alkaloids</td>
<td>B5 + 2,4-D (4.52 μM), kinetin (0.93 μM), sucrose (0.088 M)</td>
<td>Rech et al. 1998</td>
</tr>
<tr>
<td>Taxus baccata</td>
<td>Taxol baccatin III</td>
<td>B5 (salts) + 3X B5 vitamins, 2,4-D (20 μM), kinetin (4 μM) + GA₃ (1 μM)</td>
<td>Cuadso et al. 1999</td>
</tr>
<tr>
<td>Taxus spp.</td>
<td>Taxol</td>
<td>B5 medium + 2,4-D (0.91 μM), BA (2.22 μM), casein hydrolysate (200 mg/L),</td>
<td>Wu et al. 2001</td>
</tr>
<tr>
<td>Taxus wallichiana</td>
<td>Taxol</td>
<td>½ WPMSH, IBA (9.84 μM), SH vitamins</td>
<td>Datta et al. 2006</td>
</tr>
<tr>
<td>Torreya nucifera var. radicans</td>
<td>Diterpenoids</td>
<td>MS + 2,4-D (45.24 μM), casamino acid (1 g/L), coconut milk (7%) and K⁺ instead of NH₄⁺</td>
<td>Orihara et al. 2002</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Basal medium</th>
<th>Cell yield (g DW/L)</th>
<th>Serpentine content (mg/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladley</td>
<td>7.6</td>
<td>4.4</td>
</tr>
<tr>
<td>Gamborg + 1 mg/L 2,4-D</td>
<td>4.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Gamborg + 2 mg/L 2,4-D</td>
<td>5.2</td>
<td>0</td>
</tr>
<tr>
<td>Gamborg + 1.86 mg/L NAA</td>
<td>7.6</td>
<td>1.2</td>
</tr>
<tr>
<td>Gamborg 5 mg/L</td>
<td>5.1</td>
<td>0</td>
</tr>
<tr>
<td>Heiler + 0.175 mg/L IAA + 1.13 mg/L BA</td>
<td>5.4</td>
<td>6.6</td>
</tr>
<tr>
<td>Linsmaier and Skoog</td>
<td>8.9</td>
<td>0</td>
</tr>
<tr>
<td>Murashige and Skoog</td>
<td>2.3</td>
<td>10.4</td>
</tr>
<tr>
<td>Nitsch and Nitsch</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Velicky and Martin</td>
<td>5.0</td>
<td>0</td>
</tr>
<tr>
<td>White</td>
<td>0.8</td>
<td>0</td>
</tr>
</tbody>
</table>

Source: Misawa 1994

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid; NAA: 1-naphthylacetic acid; GA₃: 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 McCowin’s medium supplemented with Schenk Hildebrandt vitamins.
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 macro-molecules, 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 to 3.75 mM enhanced shoot cell growth. The 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 selenin 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 selenin 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 (Williams 1995). However, Gamborg’s B5 vitamins differed from the vitamins of MS in having a high concentration of myo-inositol has been described as a natural constituent in 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 uptake of these compounds by the growing cells (Zhou 2001). A 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 Ghorbani 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 serve 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 tobiosynthesis 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 (Kino-Oka et al. 1994) and glucose has been reported as a preferred form of monosaccharide taken by cell compared to fructose (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 cultures (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), Holarthera antarctica (Pan et al. 1992) and Catharanthus roseus (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 Yoshiida 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). It was hypothesized 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 (NH₄⁺, NO₃⁻, PO₄³⁻), 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). Photoautotrophic 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 concentration of auxin or cytokinin or the auxin/cytokinin ratio. Plant growth regulators (PGRs) usually have profound effects on both cell growth and product formation. 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, gibberellin 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 de-differentiation 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, ubiquinonone-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-naphthyleneacetic acid (NAA) or indole-3-acetic acid (IAA) has 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\(^2\)-(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 Haploppappus gracilis 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 (e.g. cytokinin) has 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 Ghorbani 2002), jacosidin 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 metabolism 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 (Dörnburg and Knorr 1995). Yeast extract is one of the most common biotic elicitor studied in plant-microbe interaction. In the study of elicitation on [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++ 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 metabolism biosynthetic route, stands a good chance of increasing the yield of the final product (Rao and Ravisankar 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 cultures (Fett-Neto et al. 1993, 1994). Similarly, feeding f erulic acid to cultures of Vanilla planifolia resulted in an increase in vanillin accumulation (Romagnoli and Knorr 1988). Recent study done by Channipa et al. (2010) on the cell culture of Vitis labrata 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 nor-dihydroguaiaretic 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 asaitoside 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 of “clean” or “green” 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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