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Review

Current approaches for enhancing secondary plant production *in vitro*

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Abstract

Higher plants are an important source of secondary metabolites such as flavours, fragrances, dyes, pigments, food additives, terpenoids, alkaloids, and glycosides with medicinal importance. Several of these secondary plant products also form the basis for drug development. Today, several countries use plant derived chemicals as drugs with modifications. Unfortunately, these secondary plant products are synthesized in very low quantities. Therefore, cultured plant cells, tissues and organs such as hairy roots become an alternative source for enhancing the bioactive compounds. Elevated levels of secondary plant products have been reported from tissue cultures of various medicinal plants and bioreactors are vital for the commercial production. Also, in several instances, biochemistry of the biosynthetic pathway/s is not known, in case if it is known, the corresponding genes have not yet been isolated. In such a scenario, there is an urgent need to integrate large scale sequencing data, metabolomics, transcriptomics and proteomics. But, perhaps functional genomics holds the key to link the gene with the metabolite and subsequently genetic engineering of plant secondary metabolism for enhancing the secondary metabolites.

Key words: Cell cultures, elicitation, genetic engineering, genomics, secondary plant products

1. Introduction

Secondary plant products are regarded for their crucial role in the survival of the plant in its ecosystem, often protecting plants against pathogen attack, insect bite, mechanical injury, biotic and abiotic stresses (Hartmann, 2007). Secondary plant products are classified according to their biosynthetic pathway (Horborne, 1999). Plant produce an enormous diversity of secondary plant products, most of them may have an impact on humans.While all terpenoids (25,000) are derived from the five-carbon precursor isopentenyldiphosphate, alkaloids (around 12,000 known) contain one or more nitrogen atoms and are biosynthesized from amino acids. Approximately 8,000 phenolic compounds are formed in plants, either from shikimic acid pathway or the malonate/acetate pathway (Rodney *et al.*, 2000). Based on NAPRALERT database, it is estimated that about 15% of the approximately 250,000 known plant species have been subjected for some sort of phytochemical analysis, but less than 5% of them have been studied to the presence of biological activities (Verpoorte, 2000). About 25% of all prescriptions sold in the USA and Europe are from natural products. Approximately one third of the ~980 new pharmaceuticals in the past two and half decades originated from or was inspired by natural products. About 119 drugs are obtained now commercially from higher plant systems and 74% of them are found from ethnobotanical information. Medicinal plants will continue to provide novel products as well as chemical models for new drugs, because the chemistry of the majority of the plant products is complex or yet to be characterized. Despite many advances in chemical synthesis of drugs, we still depend largely on plant sources for a number of secondary metabolites including pharmaceuticals. Some of the complex structures of the secondary plants products are shown in the Figure 1.

2. Why are cell cultures important for enhancing secondary plant products?

Plant cell cultures have been proved as a good source of secondary plant products (Komaraiah *et al.*, 2004; Johnson *et al.*, 2013). Each cell in culture retains complete genetic information and hence, the cells in culture are chemically totipotent like the mother plants. Plant cells in culture are known to produce many important alkaloids like berberine, palmitine, hyoscyamine, camptothecin, vinblastine, terpenoids such as carotenes, mono, di, tri and sesquiterpenes, saponins and sapogenins (ginsenosides), quinones like anthraquinones, benzoquinones and naphthoquinones, steroids like cardiac glycosides, protease inhibitors and plant virus inhibitors (peptides), food additives (pigments, sweetening steviosides and phenylpropanoids like flavonoids, isoflavonoids, stilbens, tannins,

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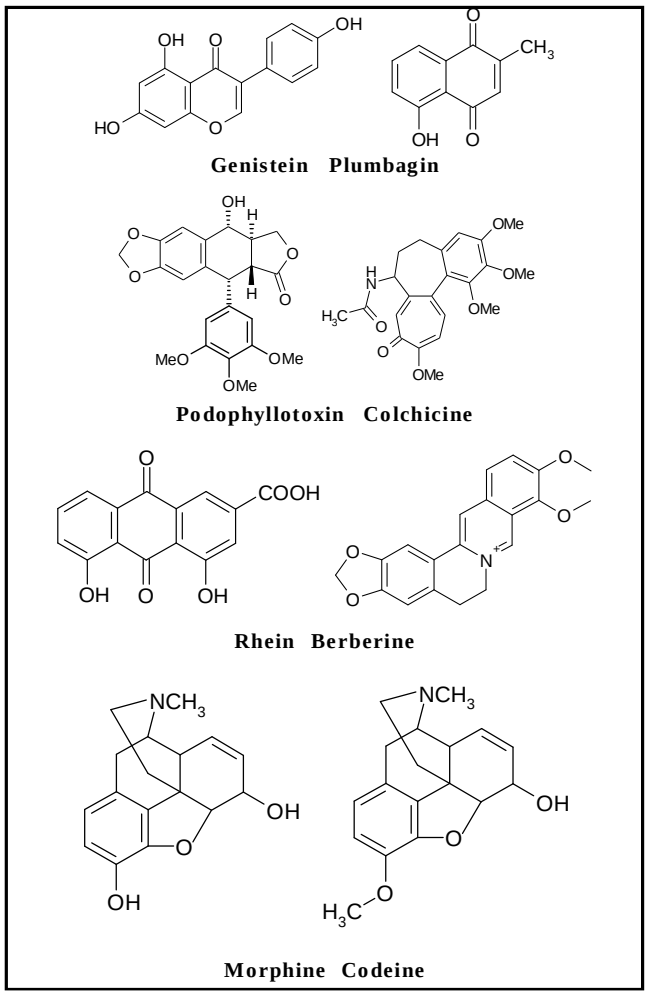

Figure 1: Chemical structures of few important secondary metabolites

lignans, coumarins, anthocyanins, *etc*.) (Rao and Ravishankar, 2002; Johnson *et al.*, 2013). There are many advantages of cell culture techniques over that of conventional techniques. Following are some of the advantages and disadvantages of cell culture systems for production of secondary plant products.

- Cultured cells will be free of microbes and insects.
- Cell culture technique offers defined production system, which ensures the continuous supply of products, uniform quality and yield.
- Cells of any plant and region can be cultured and manipulated *in vitro* to yield high amounts of specific metabolites.
- Cell culture systems also produce novel compounds that are not produced or found in parent plant.
- Rational regulation of metabolic processes is possible *in vitro*.
- It offers also efficient downstream processing.
- Plant cells perform stereo- and regio-specific biotransformation for the production of novel compounds from cheap precursors.
- While some of the secondary plant products cannot be detected in cultured cells, the biosynthesis of others is possible only in low quantities despite many efforts.
- Many plant cell cultures are painfully slow growing and cannot be used in fermenters on an industrial scale.

Several plant cell culture systems have been reported to produce higher amounts of secondary metabolites than in the intact plants. For example, shikonin has been improved by several-folds in cell cultures of *Lithospermum erythrorhizon* (Fujita *et al.*, 1984), berberine in *Thalictrum minus* (Smolko and Peretti, 1994), *Capsicum* in cell cultures of *capsaicin* (Johnson and Ravishankar, 1996), plumbagin in hairy roots and suspension cultures of *Plumbago rosea* (Komaraiah *et al*., 2002, 2003), anthraquinones in cell cultures of *Morinda citrifolia* (Komaraiah *et al*., 2005), psoralen, a furanocoumarin in callus cultures of *Cullen corylifolium* (Sreelakshmi *et al*., 2007) and withanolides in hairy root cultures of *Withania somnifera* (Madhavi *et al*., 2012). Despite significant progress, it is not always possible to produce secondary metabolites in high concentration from cultured cells/hairy roots. The biosynthetic pathways are not yet established in many cases and this has become a major bottleneck in our understanding of the production of the compounds. Some of the factors that modulate the accumulation of these compounds *in vitro* are discussed below.

3. Effect of elicitation on secondary plant production

It is believed that secondary plant products have a role to play in the defense of the plant system. Therefore, biotic stress due to infection by virus, bacteria, fungi, nematodes, and insects enhances the *de novo* synthesis of secondary metabolites. This phenomenon is known as elicitation. Compounds that elicit such a response in plant cells are called as elicitors. Biotic elicitors prepared from fungal extracts or toxins, fungal mycelial extracts, bacterial extracts or toxins, culture filtrates, fractions or compounds obtained from microbial cell walls, and yeast extracts have been used for enhancing the compounds. Similarly, abiotic elicitors like ultraviolet light radiations, exposure of cells or tissues to low or high temperatures, fungicides, antibiotics, salts of heavy metals have greatly influenced the accumulation of secondary products. Though both biotic and abiotic elicitors have been shown to enhance the secondary metabolites, no elicitor has been found to have a general effect on many or all cultured cells/hairy roots. Optimum production of secondary products from medicinally important plants will depend on:

- elicitor specificity
- elicitor concentration
- duration of elicitor treatment
- growth stage of culture
- time course of elicitation.

Out of many elicitors that have been tested so far, fungal elicitors appear to be better in enhancing the secondary metabolite. The concentration of elicitor also plays a vital role in the process of elicitation. Elicitors of fungal, bacterial and yeast origin, *viz*., polysaccharides, glycoproteins, inactivated enzymes, purified curdlan, xanthan and chitosan (extracted from shell), and salts of heavy metals were reported for the production of various secondary metabolites (Rao *et al*., 1996). In *Thalictrum minus*, yeast extract has enhanced the synthesis of berberine. Brodelius (1988) conducted several experiments on the effects of yeast elicitors and demonstrated

improved accumulation of benzophenanthridine alkaloids in cell cultures of *Thalictrum* and *Escholtzia californica*. Cell cultures of *Plumbago rosea* when treated with the elicitors prepared from fungi, bacteria, yeast extract and chitosan enhanced the synthesis of plumbagin, a naphthoquinone in suspension cultures (Komaraiah *et al*., 2002, 2003). *Papaver somniferum* cell suspensions treated with homogenate of *Botrytis mycelium* resulted in a remarkable increase in accumulation of sanguinarine of up to 3% of the cell dry weight. It is now clear that the flux of metabolites in a number of secondary pathways can be influenced by elicitor treatment, by induction or amplification of various enzymes associated in the biosynthetic pathway. We will have an advantage if the secondary metabolite is produced extracellularly since it can be adsorbed to an inert material such as XAD-7 and down-stream processing becomes easier. Further, we can harvest the spent medium and have semi-continuous production process (Komaraiah *et al*., 2003). Kurtz *et al*. (1987) showed that for the production of sanguinarine in poppy cell cultures, that a continuous process is feasible in which a sequence of elicitation and medium change can be used to produce the alkaloid. Also, in case of the production of paclitaxel, elicitation has been shown to cause a clear increase in productivity. Table 1 shows the list (not a comprehensive list) of secondary metabolites that have been enhanced using elicitation method. It appears therefore, elicitation has been used successfully in several tissue culture systems for increasing the product yield and for subsequent commercialization (Fujita *et al*., 1984; Kurtz *et al*., 1987; Rao and Ravishankar, 2002; Komaraiah *et al*., 2005; Johnson *et al*., 2012).

4. Effect of immobilization on secondary plant product accumulation

Immobilization technique has improved the accumulation of secondary plant products in cultured cells and the list (not a complete list) is provided in Table 2. Plant cell cultures are grown in liquid medium for better growth and nutrient absorption. But for industrial production of any compound, large scale cultivation of cells is needed in a fermenter. Unfortunately, cells are washed out during this operation. Hence, the rate of reactions become slow and consequently the product rates. In a suspension culture, cells grow faster and, hence, the secondary product accumulation is slow. Further, cell aggregates have the better ability to accumulate metabolites than the free floating cells. Therefore, it is vital to anchor the plant cells onto some solid support or entrap the cells in a matrix such as gel, agar, alginate, polyacrylamide or polyurethane foam, *etc*. This process of anchoring the plant cells is known as immobilization.

Table 1: Effect of elicitation on the accumulation of secondary metabolites *in vitro*

Name of plant species	Secondary metabolite	Elicitor	Reference
Catharanthus roseus (L.) G. Don	Catharanthine	Vanadylsulphate	Smith *et al*., 1987
Capsicum frutescens L.	Capsaicin	Phycocyanin	Rao *et al*., 1996
Plumbago rosea L.	Plumbagin	Chitosan	Komaraiah *et al*., 2002
Pueraria candollei var. *mirifica*	Isoflavonoid	Methyl jasmonate	Korsangruang *et al*., 2010
Gloriosa superba L.	Colchicine	Tyrosine	Ghosh *et al*., 2002
Nicotiana tabacum L.	Nicotin	Methyljasmonate, quercetin	Zayed and Wink, 2009
Glycyrrhiza uralensis Fisch.	Flavanoids	Methyljasmonates Phenylalanine	Guo *et al*., 2013
Thalictrum minus L. *var. hypoleucum*	Berberine	Spermidine	Hara *et al*., 1991
Digitalis lanata Ehrh.	Digoxin	Chitoplant, Silioplant Methyl jasmonate	Perez - Alonso *et al*., 2012
Morinda citrifolia L.	Anthraquinone	Arachidonic acid	Komaraiah *et al*., 2005
Taxus media L.	Paclitaxel and Baccatin III	Methyl jasmonate	Yukimune *et al*., 1996
Taxus cuspidata Siebold & Zucc.	Taxol	Phenylalanine, Benzoic acid	Fett-Neto *et al*., 1994
Hypericum perforatum L.	Adhyperforin	L-isoleucine	Karppinen *et al*., 2007
Salvia miltiorrhiza Bunge	Tanshinone	Ag^{+} and yeast extract	Cheng *et al*., 2013
Centella asiatica L.	Asiaticoside	Methyl jasmonate	Kim *et al*., 2004
Catharanthus roseus (L.) G. Don	Vindoline and Vinblastin	Artemisinic acid	Liu *et al*., 2014
Scopolia parviflora Lam.	Scopolamine	Silver nitrate and Methyl jasmonate	Jung et al., 2003
Vitis vinifera L.	Anthrocyanin	Methyl jasmonate	Qu *et al*., 2011
Hyoscyamus muticus L.	sesquiterpene	Methyl jasmonates and fungal elicitor	Singh *et al*., 1998

4.1 Influence of cell aggregate size on immobilization

In immobilized bioreactors, plant suspensions are trapped - perhaps stuck to a sticky surface - while nutrient flows over them. Among the many factors that affect product accumulation in cultures, cell aggregate size is important. Rama Rao *et al.* (2008) studied the effect of growth regulators and cell aggregate size on berberine production from cell cultures of *Tinospora cordifolia*. While cell aggregates of 500 μm in diameter promoted production of biomass (9.6 g dry cell wt/l), larger cell aggregates (above 500 μm) favoured berberine accumulation with 3.8 mg/g dry weight of tissue. The yield of berberine in cell suspensions of *Tinospora* was 5-14-folds higher than that of intact plants (Rama Rao *et al*., 2008). Even in the immobilized state, cell aggregate size plays an important role in the accumulation of the secondary products (Komaraiah *et al*., 2002, 2003). Plant cells may be immobilized in sodium alginate beads by complexing the gel with calcium. The sodium alginate bead formation is carried out in the growth chamber by dripping an alginate-cell gelatin suspension into a calcium solution contained in the growth chamber. Secondary metabolites of viable plant cells are produced with the cells immobilized in a porous inorganic support. Immobilization includes the steps of (a) preparing a support comprising of substantially uniform and porous matrix of inorganic material having a tensile strength of at least 500 MPa, (b) introducing a culture of viable plant cells into the process of said matrix, (c) entrapping the plant cells by coating the matrix with a solution or colloidal suspension not interfering with the cell viability, and (d) immobilizing the entrapped cells within the matrix with a reactive gas including a carrier gas saturated with volatile SiO_2 or organic modified SiO_2 precursors. However, secondary metabolites are rarely released from the suspended or immobilized cell cultures, and they have very low solubility in water due to their hydrophobicity (Johnson *et al*., 2012).

4.2 *In situ* removal of the product for easy down-stream processing

For using the immobilized cells more effectively and economically, it is necessary that the water insoluble products should be removed from the culture medium without disturbing the cell metabolic activities. The concentration of the secondary plant product of interest may be toxic to the cultured cells (except in the case of *Lithospermum* which is a pigment). To avoid cell toxicity, secondary metabolites are separated from the site of synthesis (usually by storage in the vacuole, sometimes in specialized cells) in many medicinal plants. Thus, the feedback regulation of the secondary plant product is common in many plants and even cultured cells. In this regard, *in situ* adsorption or extraction of metabolites by using hydrophobic materials received great importance. Berlin *et al.* (1984) first reported the use of adsorbents to retain volatile compounds from cell cultures of *Thuja*. Increased shikonin was noticed from suspensions of *Lithospermum*, and plumbagin content from *Plumbago rosea* cell suspensions by *in situ* product removable by charcoal, XAD-7 and other inert materials (Yeoman and Yeoman 1996; Komaraiah *et al*., 2003; Johnson *et al*., 2012). The products were selectively released from the cells and dissolved in the solvents or adsorbents.

5. Effect of permeabilization on secondary metabolite accumulation

Cell permeabilization means cell cracking. In most cases of secondary plant product accumulation, they are stored in the vacuoles. This problem of product storage within plant cells has led to the development of techniques for plant cell permeabilization. In order to release the products from vacuoles of plant cells, two membrane barriers-tonoplast and plasma membrane have to be penetrated. Cell permeabilization depends on the formation of pores in one or more of the membrane systems of the plant cell, enabling the passage of various molecules into and out of the cell. Several popular agents have been used in cultured cells for accomplishing this feat. Agents such as dimethyl sulfoxide (DMSO) and tolerance of cells to such agents are important. Cells appear to survive its application and treated plant cells maintain a relatively high level of enzyme activity in many cases (Komaraiah *et al*., 2002, 2003). Besides DMSO, enzymes like cellulose and pectinase can also be used for permeabilization of plant cells. Other agents such as chitosan can also permeabilize cells due to its interaction with cell membrane. Chitosan binds to polygalacturonate, a plant cell wall component, and induces leakage of low molecular weight compounds as well as some proteins. This is important for the industry since the compound/s of interest can be easily isolated and the cells can be reused. Therefore, down-stream processing becomes easy. Attempts have been made to permeabilize the plant cells transiently, in order to maintain the cell viability and to have short time periods of increased mass transfer of substrate and metabolites to and from the cell. Other permeabilizing methods (other than chitosan, DMSO, and enzymes) include ultrasonication (Komaraiah *et al*., 2005), electroporation, and ionophoretic release, in which the cells are subjected to a low current in a specially designed device (Brodelius, 1988). Over and above, using high electric field pulses and ultrahigh pressure has been reported for the recovery of secondary plant products. Majerus and Parilleus (1986) observed a sharp increase of the excretion of alkaloids by *Catharanthus roseus* when the pH of the culture medium was changed from 9 to 4.3. By using pluronic F-68 at 2%, Basetti *et al*. (1995) reported long-term non-lethal release of anthraquinones from suspensions of *Morinda citrifolia*. Table 3 shows the list (not a complete list) of some of the secondary metabolites that have been enhanced *in vitro* from different plant species. Besides the above factors, environmental factors like media optimization, choice of culture system, plant growth regulators, light, pH, temperature and the gaseous environment on the accumulation of secondary plant products has been studied (Johnson *et al*., 2012). Also, biological factors like growth of callus and suspension cultures, morphological differentiation of cultures into roots and shoot buds or somatic embryos, variation in biosynthetic activity and cell line selection, precursor feeding influence the accumulation of secondary products considerably and has been described earlier (Johnson *et al*., 2012). Further, the effects of combined elicitors on several plant secondary products and metabolic profiling of plumbagin and tanshinone has been studied both in suspensions and hairy roots respectively (Komaraiah *et al*., 2002, 2003; Cheng *et al*., 2013). Therefore, it is not discussed in this chapter.

Table 2: Effect of immobilization on the accumulation of secondary metabolites *in vitro*

Name of plant species	Secondary metabolite	Immobilizing material	Reference
Gossypium arboreum L.	Gossypol	Cotton matrix entrapment	Hojoon *et al*., 1995
Plumbago rosea L.	Plumbagin	Calcium alginate gel beads	Komaraiah *et al*., 2003
Andrographis paniculata Nees.	Andrographolide	Calcium alginate gel beads	Chauhan *et al*., 2010
Catharanthus roseus (L) G. Don	Serpentine	Nonwoven short-fiber polyester material A07	Archambault *et al*., 1990
Lithospermum erythrorhizon Siebold & Zucc.	Shikonine	Polyurethane Foam Matrices	Young *et al*., 1990
Rubia tinctoram L.	Alizarin and purpurin	Lignocellulosic materials	Nartop *et al*., 2012
Nicotiana tabacum L.	Scopolin	Calcium alginate gel beads	Gillet *et al*., 2000
Tinospora cordifolia (Willd) Miers	Arabinogalactan	Calcium alginate gel beads	Roja *et al*., 2005

Table 3: Effect of permeabilization on the secondary metabolite accumulation in cultured cells

Name of plant species	Secondary metabolite	Permeabilizing agent	Reference
Thalictrum rugosum Tourn. ex L.	Berberine	Dimethylsulfoxide	Brodelius, 1988
Chenopodium rubrum L.	Betanin	Dimethylsulfoxide	Brodelius, 1988
Catharanthus roseus (L) G. Don	Ajmalicine	Triton –X 100 and n-Hexadecane	Thakore *et al*., 2013
Azadirachta indica A. Juss.	Azadirachtin	n-Hexadecane	Prakash and Srivastava, 2011
Plumbago rosea L.	Plumbagin	Amberlite XAD-7	Komaraiah *et al*., 2003
Cinchona ledgeriana Moens	Anthraquinone	Amberlite XAD-7	Richard *et al*., 1986
Cichorium intybus L.	Coumarin	Dimethylsulfoxide	Bias *et al*., 2001
Tagete spatula L.	Thiophene	Amberlite XAD-7	Buitelaar *et al*., 1993

6. Genetic engineering of plant secondary metabolism

The biosynthetic pathways involved in secondary plant product formation are either complex or very poorly characterized. Consequently, the genes that are associated with the biosynthetic pathways have not been isolated till recently for genetic manipulation studies in medicinal plants. But metabolic engineering is needed to enhance the accumulation of medicinally important compounds from plants. But, genome sequencing of many plants including *Arabidopsis* and medicinal plants has helped us to annotate genes and also for transcript profiling using micro array technology. Also, validation of functional genes will help to create a metabolic network. Therefore, integrated functional genomics is necessary to link genes and metabolites at a large scale (Zhao *et al*., 2014). Such a goal of integrated functional genomics will ultimately assist us about the function/s of genes on a large scale. Both transcript profiling and proteome analysis of many medicinal plants will lead to the identification of candidate genes or proteins associated with secondary product biosynthetic pathway. Though there is enormous interest in identifying the candidate genes, there have been several lacunae in our understanding of the secondary product biosynthesis and their accumulation (Takemura *et al*., 2010). The overall interest in the genetic engineering of the medicinal plants is to produce plants with new chemical traits, enhance the accumulation of the compounds, thereby, improving the plant defense against pests, improved aroma and nutrition (Pichersky and Dudareva, 2007; Nishihara and Nakatsuka, 2010; Sugiyama *et al*., 2011). But, unless key genes involved in the biosynthetic pathway are identified, genetic engineering will not be possible. With the identification of such candidate genes involved in biosynthesis, overexpression of these genes is possible for increased production. Further, identification of gene specific promoters is important for such overexpression studies instead of the use of universal promoters like CaMV35S (Aharoni *et al*., 2003, 2006; Dixon, 2005; Dudareva and Pichersky, 2008).

6.1 Genetic engineering of flavonoids and anthocyanins

Both flavonoids and anthocyanins have antioxidant properties. Therefore, lot of interest has been attached for the manipulation of this pathway. Flavonoid and anthocyanin biosynthetic pathway has been well studied and is the first target for genetic engineering. Overexpression biosynthetic pathway genes have been carried out to manipulate flower colour. Chalcone synthase has been found to be the key enzyme to enhance flavonoid production. Overexpression of *Petuniachalcone synthase* gene led to a 78-fold increase of flavonoid levels in the tomato peels (Muir *et al*., 2001). But, down-regulation or RNA interference silencing of chalcone synthase, the first step in the flavonoid biosynthesis pathway lead to parthenocarpic development of fruit in tomato (Schijlen *et al*., 2007).

6.2 Genetic engineering for indole alkaloid production

It is not only addition of novel genes that can improve the secondary plant product accumulation, certain times, decrease in the production of unwanted compounds is also necessary. For example, reducing the level of the corresponding mRNA *via* antisense or co-suppression or RNA interference technologies is also possible.

The best studied pathway at the genetic level is the pathway associated with the formation of flavonoids and anthocyanins. Fortunately, most of the genes in the anthocyanin biosynthetic pathway have been cloned. Here are few examples where genetic engineering has led to the more accumulation of secondary plant products. Large number of genes encoding monoterpenoid indole alkaloid biosynthetic pathway enzymes (including strictosidine synthase, tryptophan decarboxylase, geraniol 10-hydroxylase, scopaloganin synthase) have been cloned, characterized and overexpressed in hairy roots of *Catharanthus roseus* (Huges *et al*., 2004; Zarate and Verpoorte, 2007; Kumar *et al*., 2011; Jaggi *et al*., 2011). Strictosidine synthase (Str) gene has been cloned and its overexpression has yielded 200 mg/l terpenoid indole alkaloid in *Catharanthus roseus* (Huges *et al*., 2004; Zarate and Verpoorte, 2007; Kumar *et al*., 2011; Jaggi *et al*., 2011). ORCA3 and G10H genes in *Catharanthus roseus* plants regulated alkaloid biosynthesis and the metabolism as revealed by NMR metabolomics (Pan *et al*., 2012).

6.3 Genetic engineering for tropane alkaloids

The tropane alkaloid scopolamine is synthesized in the pericycle of branch roots in certain species of the Solanaceae. The enzyme responsible for the synthesis of scopolamine from hyoscyamine has been identified and it is hyoscyamine 6-beta-hydroxylase (H6H). H6H was isolated from the plant *Hyoscyamus niger* (Kanegae *et al*., 1994). Overexpression of 6β-hydroxylase led to the production of 17 mg/ml of scopolamine which was about 100-times more than the wild-type clones (Zhang *et al*., 2004).

6.4 Genetic engineering for benzylisoquinoline alkaloid production

Both morphine and codeine are two important isoquinoline alkaloids produced in *Papaver somniferum*. Yamada and co-workers (Sato *et al*., 2001) hypothesized that overexpression of an enzyme at a branchpoint in a pathway should lead to an increased flux through the affected branch. In the biosynthesis of berberine, the enzyme (*S*)-scoulerine 9-O-methyltransferase (SMT) is important for the controlling the ratio of coptisine:berberine plus columbamine in *Coptis japonica* cells (Sato *et al*., 2001). Overexpression of this gene resulted in an increase ofberberine and columbamine from 79% of the total alkaloid content in wild-type cells to 91% intransgenic cells. Similarly, overexpression of (S)-*scoulerine 9-O-methyltransferase* (*SMT*) gene in cell cultures of *Escholzia californica*, a plant lacking this enzyme, resulted in the production of columbamine.Similarly, opiates and related molecules are now being produced in yeast. Yeast strains were genetically modified to express heterologous genes from *Papaver somniferum* and bacterium *Psedumonas putida* M10, which converted thebaine to codeine, morphine and hydromorphone, hydrocodone and oxycodone (Thodey *et al*., 2014). They discovered a new biosynthetic branch to neopine and neomorphine which diverted pathway flux from morphine and other target products. Their strategies yielded 131 mg/l of opioid titers which is important step towards the biosynthesis of benzylisoquinoline alkaloid drug molecules.

6.5 Genetic engineering for monoterpene and sesquiterpene-production

Terpenoids are the most structurally varied compounds in plants.It has been proposed that in nature they play a vital role in plant-plant communication and plant-insect and plant-animal interactions (Pichersky and Gershenzon, 2002).Therefore, altering terpenoid production can serve as a tool for improving disease resistance, increased value of ornamentals and fruit (fragrance and flavour) and improved pollination by altering scent profiles. Following the discovery of the role of the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in the biosynthesis of plastidial terpenoids, such as the carotenoids, monoterpenes and diterpenes, several genes of this pathway have been cloned. Silencing of geranylger any ldiphosphate synthase in tobacco dramatically impaired resistance to tobacco hornworm (Jassabi *et al*., 2008). Incorporating *FaNES1* (*Fragaria ananassa* nerolidol synthase I) gene into *Arabidopsis* enhanced the level of linlool (Aharoni *et al*., 2003). Thus, it is feasible to engineer genetically the terpenoid biosynthetic pathway genes which can influence the insect behaviour (Aharoni *et al*., 2006).

6.6 Genetic engineering of plant volatiles

Genetic engineering of the volatile substances offers great potential for improving the plants. Many genes and enzymes associated with the biosynthesis of volatile compounds have been discovered. Again genetic engineering of these genes has improved plant defence, scent and aroma quality of flowers and fruits (Dudareva and Pichersky, 2008).

6.7 Production of plant terpenoids in yeast

In yeast, mevalonic acid pathway operates for the biosynthesis of ergosterol as the major end product. But, genetic engineering in yeast has resulted in both mono- and sesquiterpene production. Oswald *et al*. (2007) genetically modified the yeast system (*Saccharomyces cerevisiae*) to produce monoterpenoids by overexpressing *linalool synthase* and *geraniol synthase* genes. Ro *et al*. (2007) used the yeast to produce high amounts of artemisinic acid by engineering mevalonic acid pathway genes like *amorphadiene synthase* and a novel cytochrome *P450 monooxygenase* isolated from *Artemisia annua*. But, yeast is not able to produce carotenoids when engineered with the biosynthetic pathway genes (Gunnel *et al*., 2006).

7. Future perspectives

It appears that plant cell cultures are able to produce many secondary plant products with the manipulation of nutrient media composition, plant growth regulator levels, carbohydrate quantity and quality, pH, light, elicitation, permeabilization and immobilization. But, the rate of success is very limited to shikonin, berberine and few other products which have been taken to the commercial level. In future, metabolic engineering and biotechnological approaches need to be employed for the production of medicinally important compounds either *in vitro* or *in vivo*. There are three aspects of genetic modifications that require our attention. We should concentrate on the expression of developmentally regulated genes such as tyrosine/DOPA decarboxylase, and overexpress the pathway enzymes that help in the accumulation of specific secondary compounds. Likewise, down-regulation of specific genes using antisense or SiRNA techniques is also vital for achieving the goals. It is also important to identify new genes that are associated in the biosynthetic pathway of secondary metabolites so that progress can be made in this area at a rapid rate.

Conclusion

Elevated levels of many secondary plant products have been reported earlier. But, the underlying mechanisms are poorly understood. Further, genes which are associated with the biosynthetic pathway need to be identified immediately and their regulation must be found out so that we can enhance the concentrations of important secondary metabolites both *in vivo* and *in vitro*.

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Conflict of interest

We declare that we have no conflict of interest.

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