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REVIEW

Secondary metabolism of pharmaceuticals in the plant in vitro cultures: strategies, approaches, and limitations to achieving higher yield

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Abstract Biotechnology is playing a vital alternative role in the production of pharmaceutical plant secondary metabolites to support industrial production and mitigate over-exploitation of natural sources. High-value pharmaceuticals that include alkaloids, flavonoids, terpenes, steroids, among others, are biosynthesized as a defensive strategy by plants in response to perturbations under natural environmental conditions. However, they can also be produced using plant cell, tissue, and organ culture techniques through the application of various in vitro approaches and strategies. In the past decades, efforts were on the clonal propagation, biomass and secondary metabolites production in the in vitro cultures of medicinally important plants that produce these molecules. In recent years, the effort has shifted towards optimizing culture conditions for their production through the application of cell line selection, elicitation, precursor feeding, two-phase co-culture among cell, tissue, and organ culture approaches. The efforts are made with the possibility to scale-up the production, meet pharmaceutical industry demand and conserve natural sources of the molecules. Applications of metabolic engineering and production from endophytes are also getting increasing attention but, the approaches are far from practical application in their industrial production.

Keywords Medicinal plants · Plant secondary metabolism · Pharmaceuticals · Natural products · Bioactive compounds · Elicitation

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Introduction

The use of phytochemicals in treatment of diseases has been known since time immemorial and developed through herbal medical practice into modern drugs produced by the pharmaceutical industry using plant raw materials (Park and Paek 2014; Isah 2016). Plants accumulate the high-value metabolites in specific tissues and structures such as vacuoles, specialized glands, trichomes, and sometimes only during certain stage of development. Their biosynthesis occurs through phenylpropanoid, mevalonate, 2-C-methyl-D-erythritol-4-phosphate, amino acid, glucose, acetate-malonate or combined pathways (e.g., Fig. 1). It is affected by genotype, plant physiology, climate, environmental conditions, and pathogens among others. Plant Cell Tissue and Organ Culture (PCTOC) techniques permit manipulation of growth and production of the phytoconstituents in the microenvironment of in vitro cultures (Gaosheng and Jingming 2012; Isah 2015b). This can be achieved through alteration of various growth parameters and factors to optimize production independent of geographical or seasonal variation. Its application in their production arose due to the increasing awareness about threat posed by overexploitation of plant sources of the molecules to meet industrial need for the manufacture of drugs (Yue et al. 2016; Isah 2016). The application in

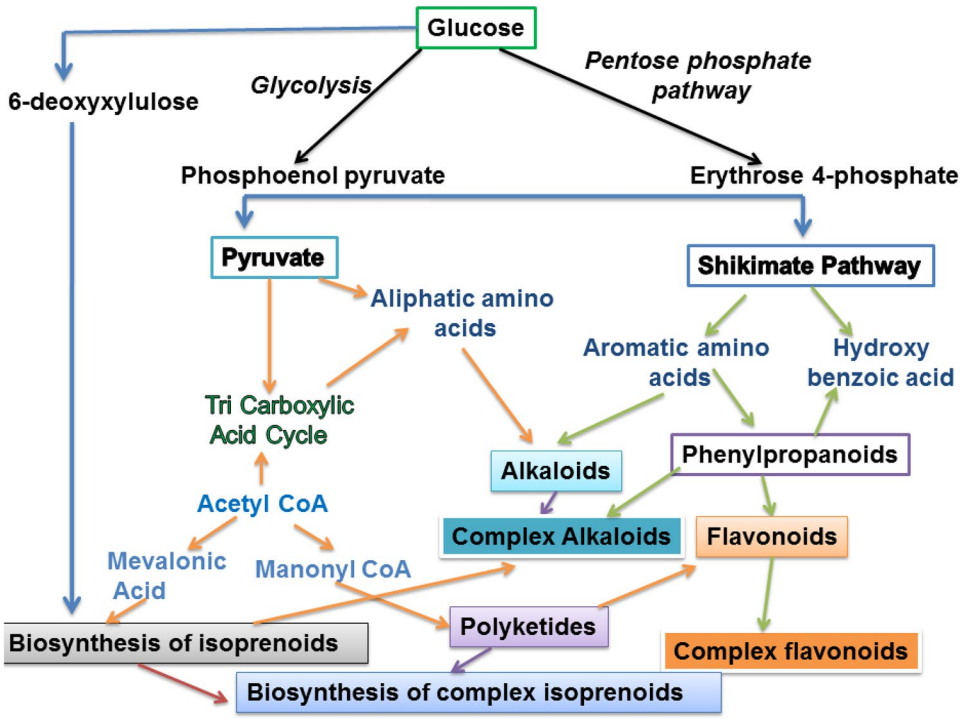

Fig. 1 Biosynthetic pathway of certain classes of plant secondary metabolites from glucose in plant cells

mass propagation has made it an effective technique for large-scale production to overcome the effect of interfering compounds that could affect productivity of the molecules in field grown plants, and provide an efficient year-round system without seasonal constraints. The unique opportunity to profile phytochemicals that are produced by plants in the microenvironment of culture vessel and simplicity in extraction of the phytochemicals from in vitro-raised tissue make it easier for commercial application (DiCosmo and Misawa 1995; Kolewe 2011). However, impediments in the plant cell culture systems that includes physiological heterogeneity, the slow growth of in vitro cultures, genetic instability and product secretion that could lead to low production of the molecules need to be addressed for efficient production. The requirement of aseptic condition, shear mixing and sensitivity, wall adhesion and light requirements are operational challenges that need to be overcomed in scale-up production systems (Smetanska 2008).

Besides recognition of the techniques as an alternative approach for producing the metabolites, endophytic production is additional source (Petrovska 2012; Suryanarayanan et al. 2017). The technologies when harnessed could provide appropriate solution to exploitation of plant sources of the molecules for drug(s) production by the pharmaceutical industry. Although few of the isolated plant natural products have found use as drugs, several of the pharmaceutically active compounds are serving as lead for semi-synthetic and synthetic drugs (Table S1). Chemical synthesis of many of the metabolites, particularly alkaloids, is limited by the high cost of production, complex structure and stereochemistry of the molecules (Fig. 2a–e) that makes production by cell culture a better alternative to harness for mitigating the over-exploitation of plant sources (Croteau et al. 2006; Reina and Gonzalez-Coloma 2007; Greger 2017). Certainly, application of in vitro technology through the PCTOC techniques could offer better alternative for efficient production system of the high-value pharmaceuticals over chemical synthesis.

Since the recognition of PCTOC in the production of pharmaceutical plant secondary metabolites in the 1960s, considerable progress have been made on production using callus cultures and differentiated tissues such as somatic embryos, adventitious root and shoot cultures (Bourgaud et al. 2001; Park et al. 2002; Murthy et al. 2014a; Park and Paek 2014; Isah 2015c; Ali et al. 2016). However, greater of the studies were carried out using undifferentiated cells with few on organ cultures in bioreactor (Yue et al. 2016). The most studied class of the plant secondary metabolites using PCTOC production systems are alkaloids which about 12,000 are known whose production is in most of the reported cases restricted to certain plant families (Facchini and De Luca 2008; Newman and Cragg 2016). Few of the metabolites such as paclitaxel, vinca alkaloids, ginsenoside, saponins, protoberberines, scopolamine, echinacea's polysaccharides, many flavonoids, steroids, and shikonin have reached worldwide market commercial production success. However, limitations in their production to the certain developmental stage and low yield from many of the plant's constraints production. For instance, taxol production in

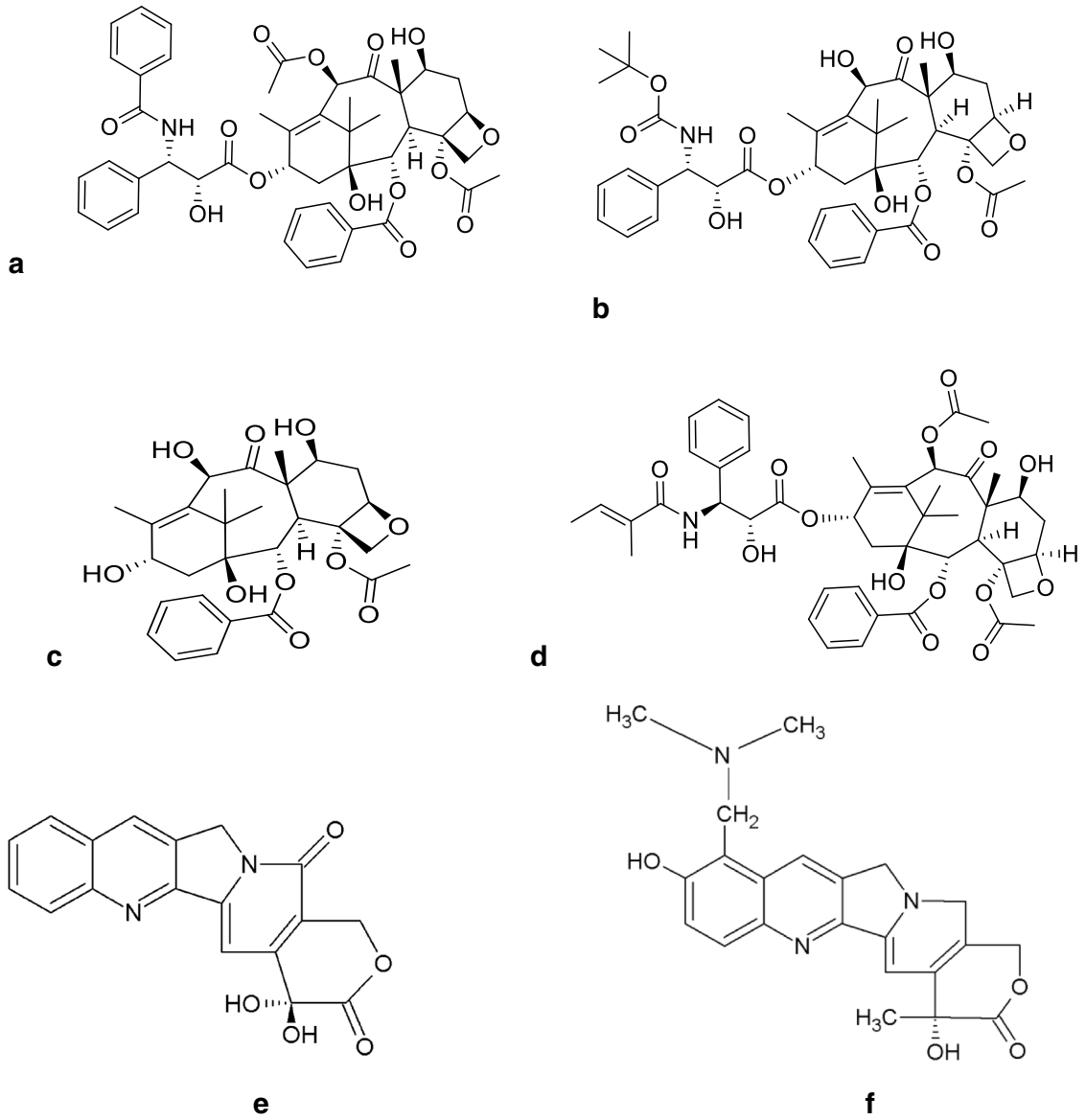

Fig. 2 Chemical structure of some anti-cancer alkaloids isolated from higher plants; **a** paclitaxel, **b** docetaxel, **c** 10-deacetylbaccatin III, **d** cephalomannine, **e** camptothecin, **f** topotecan (camptothecin analog drug)

Taxus spp is only achieved at higher yield only after the tree reaches 60 years age (Bedi et al. 1996) while ginsenoside produced by *Panax ginseng* need the plants attain about 6 years age before harvested in vivo (Bonfill et al. 2002).

Despite limited commercial success achieved in the production of the pharmaceuticals through application of PCTOC, production at very low yield, the difficulty in scaling-up production continues to prove a hindrance in exploiting maximum commercial gains, to large extent, due to limited knowledge about biosynthetic pathway(s) of the molecules. Exploiting DNA technology thus, emerged as an alternative that needs to be harnessed to improve production efficiency by engineering biosynthetic pathway(s) of the molecules in plant cells (Yue et al. 2016). Many of the plant sources (higher plants in particular) are also proving valuable sources (Table S1) leading to increasing the search for them in plant kingdom for sustainable utilization of the biota (Ruan et al. 2014; Isah 2016; Newman and Cragg 2016). Natural products that include alkaloids, terpenes, flavonoids, and glycosides (Table S1) among others, can be produced by the application of PCTOC to produce biomass and secondary metabolites in similar or near to the in vivo grown plants using various in vitro technology approaches and strategies (Dandin and Murthy 2012; Jimenez-Garcia et al. 2013; Murthy et al. 2014a). The approaches may include culture media optimization and environment, enhancing productivity of

the in vitro cultures using the optimized culture media by elicitation, permeabilization, biosynthetic precursors feeding, immobilization of the cultivated cells, tissues or organs and biotransformation methods (Hussain et al. 2012). The major impediments to application of these strategies in producing the metabolites are addressed through efficient use of PCTOC techniques to harness productivity gains in the in vitro cultures. The employed strategies may involve multi-steps for improving production that among others include (1) elucidating the signal transduction pathways involved in a given production strategy for enhanced biomass and biosynthesis of the molecules (2) regulation of the controlling elements and mechanisms of production that involves gene manipulation to improve production (3) cloning of genes involved in the biosynthesis and their modification to engineer metabolic flux of the targeted molecules (4) profiling of the metabolic flux and intermediates for understanding their biosynthetic pathway(s) and regulation(s). When these are comprehensively known, exploiting the possibility of commercial production and in-depth study of biochemical and metabolic pathways for their production in the micro-environment of PCTOC could solve production problems in the cell cultures.

Production of the pharmaceuticals in the plants in vitro cultures may occur in multi-steps involving growth and metabolite production stages. High productivity may be accompanied by low production of the metabolites during early stages of the cultivation. This may, in turn, be followed by low biomass production at later stage with higher metabolite production (Bhadra and Shanks 1997; Isah 2017). Certainly, PCTOC could be an alternative tool for producing the pharmaceuticals due to the advantage it offers as reliable and simpler predictable method for isolating the phytochemicals at high efficiency within a short time when compared to the extraction from wild plant populations (Zhong 2001; Verpoorte et al. 2002). Despite decades of concerted efforts on the production of the pharmaceuticals, regarded as natural products more studied, production of only a few mentioned above have reached commercial scale production success. Slow-growing trees and shrubs produce most of the high value and highly demanded pharmaceuticals such as taxol, Camptothecin (CPT), shikonin, berberine, and at very low level in the in vivo and in vitro cultures (Isah 2016). Therefore, promising strategies and approaches that boost production need to be identified, harnessed and employed to achieve the desired goal of higher production in the cell, tissue and organ cultures (Ramachandra and Ravishankar 2002).

Production of the phytochemicals could be accomplished using undifferentiated calli cells, cell suspension cultures or organized structures such as shoot, roots or somatic embryos with variable yield of the molecules. In some cases, the metabolites are produced only in organized structures while for the unorganized structures as callus; the production is at very low level in most of the reported cases. Additionally, a certain degree of differentiation may be needed for the biosynthesis to occur in some plants (Karuppusamy 2009). While production of the metabolites in differentiated tissues such as root cultures, somatic embryos and shoot cultures are better alternative approaches for higher yield/productivity in the majority of plant sources of the metabolites, practical and economic feasibility continues to hamper scale-up exploitation (Flores 1987; Ahmad et al. 2013; Murthy et al. 2014a). The limitations of shear damage or formation of some abnormalities (Fig. 3) that are induced by high orbital shaking cultivation conditions to differentiated tissues compared to the undifferentiated callus cultures in liquid media is another impediment to the application. Pharmaceutically relevant natural products such as taxol, CPT, podophyllotoxin, vincristine, and vinblastine that have found use in the production of anti-cancer drugs are the most widely explored metabolites in the past decades using PCTOC techniques, possibly due to greater attention the disease has received. Therefore, cultivation media optimization would allow their scale-up production to meet industrial demand, especially when combined with the application of various approaches, strategies, and techniques for enhanced productivity. In this review, in vitro approaches and strategies employed in the

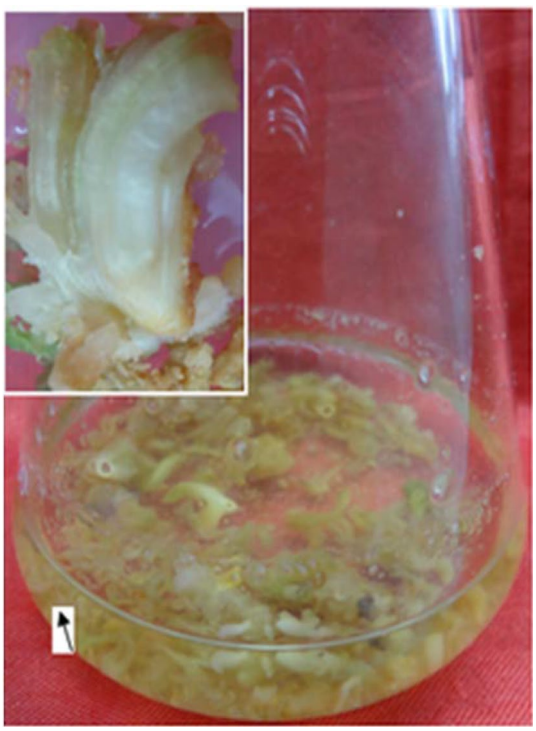

Fig. 3 Mature somatic embryos of *Catharanthus roseus* (inset) treated with 50 µM Methyl Jasmonate elicitor showing some embryos with shear damage (arrow head) induced by high orbital shaker speed conditions

production of pharmaceutical plant secondary metabolites will be discussed. The limitations in their successful use to produce the pharmaceutical bioactive molecules through the in vitro plant cell, tissue and organ cultures achievements are highlighted and assessed.

Culture media optimization

The ultimate goal of PCTOC application in the production of pharmaceutical plant secondary metabolites is in the scale-up production systems using bioreactor to exploit the commercial gains and meet the industrial need of raw materials for drug(s) production. This can be achieved via adapting suitable biomass and secondary compounds production through studies on growth, biosynthesis kinetics and excretion of the metabolite(s) into cultivation media for possible recovery from the in vitro cultures. Achieving the desired productivity of the plant in vitro cultures needs appropriate culture media selection, optimization of parameters and factors as variation in any of the conditions could affect biomass and metabolites productivity of the cultures.

Because of the differences in the composition of various PCTOC media formulations, its selection is a major step in optimizing in vitro production of biomass and pharmaceutical plant secondary metabolites. The various PCTOC media formulations devised offer alternatives for efficient clonal mass production of plants, some of which are better suited to certain plant groups, based on the nutritional composition and physiological requirement of the plants. For instance, proliferation of Protocorm-Like Bodies (PLB) in the *Phalaenopsis* in vitro cultures was influenced by the type of culture media used; explants percentage survival and PLB survival was high when MS, VW, and Hypomex media were used but, poor with the KC and LM. However, modified hypomex medium produced optimal explant survival with average of 17.4 PLBs (Park et al. 2002). Parameter and factors among others include media pH, Plant Growth Regulators (PGRs) type and concentration amended into the culture medium and external environment. Hence, developing an optimal cultural condition for producing a given pharmaceutical plant secondary metabolite is of prime importance to improving growth and biosynthetic capacity of the in vitro cultivated plant cells, tissues or organs. This is so because change in the culture media condition(s) could result in considerable variation in biomass and metabolites biosynthetic outputs (Trejo-Tapia et al. 2003). In *Gloriosa superba* for instance, sulfate and ammonium nitrate ions promoted production of colchicine from its callus cultures while higher concentration of phosphate and calcium inhibited alkaloid biosynthesis (Bharathi and Philomina 2010), emphasizing the importance of media nutrients optimization for achieving greater productivity in the in vitro cultures.

Production of the pharmaceuticals in the cultivated cells is in most of the reported cases low during the early stage of growth due to high carbon utilization that is evoked by higher primary metabolism. However, it is high at late stage when carbon is less needed by the cells for primary metabolism. The most widely used carbon source is sucrose due to the better enhancing effect it has on secondary metabolites production over maltose, glucose, and other carbon sources used in most PCTOC systems (Karwasara and Dixit 2013; Murthy et al. 2014a). The selected carbon source that is amended in the culture media plays significant roles in the signal transduction systems through regulating gene expression and developmental processes in cultivated cells, tissues and organ cultures. This is achieved through playing a role in defense response(s) that induces biosynthesis of the metabolites, and optimizing type and concentration of a given carbon source is therefore of prime importance for the optimal production of a pharmaceutical plant secondary metabolite in cell cultures (Rolland et al. 2002; Gibson 2005). For example, in the cell suspension cultures of *Nothapodytes nimmoniana*, sucrose was more efficient in enhancing growth and CPT production over glucose, maltose, and fructose. It's feeding using the optimized concentration in the culture medium resulted in greater biomass and CPT production (Karwasara and Dixit 2013). Similarly, sucrose was found optimal at 2–5% concentration for enhanced biomass and ginsenoside production in the cell suspension cultures of *Panax vietnamensis*. Higher levels of 6–7% inhibited ginsenoside accumulation (Thanh et al. 2007).

Plant Growth Regulators (PGRs) type and concentrations amended in the culture media have profound effect on cellular differentiation, biomass and pharmaceutical plant secondary metabolites production in cell, tissue and organ cultures (Bienaime et al. 2015; Raj et al. 2015a). Formation of embryogenic callus and shoot, their proliferation using petal explants and bioactive compounds production in *Rosa rugosa* in vitro cultures were influenced by PGRs type, concentration, the nitrogen source, ratio amended and bioreactor system (Jang et al. 2016a, b). In *Chonemorpha fragrance*, the amount of CPT synthesized was influenced by the PGRs type and concentrations, and higher levels inhibited alkaloid production (Isah unpublished

). The size of inoculums also affects biomass and metabolites production in the plant in vitro cultures, and for a species, inoculums density for maximum production of the biomass and secondary metabolites is variable. Thus, it becomes a major step in establishing production system in given cell, tissue and organ cultures. This is so due to the effect inoculums density has on primary and secondary productivity of *in vitro* cultivated cells through the induction of enzymes involved in phenylpropanoid metabolism during cultivation of the cells in fresh medium. Culture media pH also has profound effect on the productivity of cultivated

cells as primary metabolism, and biosynthetic pathway enzymes are affected by culture media pH. By changing pH of the culture media between 4.3 and 9.0, a sharp increase in the production and excretion of alkaloid can be achieved in the cell suspension cultures of *Catharanthus roseus* (Majerus and Pareilleus 1986). High and low media pH could not stimulate biomass and withanolide A production in *Withania somnifera* cell cultures (Nagella and Murthy 2010) but, in the shoot cultures initial media pH of 4.5 was optimal for production of biomass and Bacoside A (Naik et al. 2010). Therefore, pH of the in vitro cultures depends on substrate use, mixing and circulation in bioreactor (in the case of scale-up production); improper mixing may cause spatial variation within the bioreactor, leading to heterogeneity that could create temperature and pH gradients. Thus, in turn, cause variation in the physiological performance of the cultivated cells, tissues and organs with overall effect in accumulation of the metabolites. Because agitating cultures enhances aeration with concomitant influence on nutrients uptake, cell growth, and metabolites production, the agitation speed must be at low level as high shear rate and time accompanies proper mixing with profound effect on reduction of cell aggregate size. This could adversely affect viability of the in vitro cultivated plant cells, tissues or organs. For example, in *Rosa rugosa* shoot cultures, agitation and aeration significantly influenced biomass production when solid, liquid culture, temporary or continuous immersion systems were used (Jang et al. 2016b). Production of phenolics in the cultures was higher using solid over liquid media but, highest when CIB cultivation system was employed, and was enhanced by nitrogen source amended in the culture media.

Strategies for optimizing and improving biomass and secondary metabolites production

Culture media optimization for enhanced productivity of the plants in vitro cultured cells, tissues or organs exploits maximum productivity gains by the application of many approaches and techniques for enhanced biomass and pharmaceutical secondary metabolites production in a system. Media that supports biomass production may not necessarily support metabolites production at high levels in the in vitro cultures and may show variability with the culture duration. For example, in the shoot cultures of *Hypericum perforatum*, low biomass and hypericin production was observed during 20–30 days cultivation. However, the yield of hypericin was higher at later stage of cultivation, suggesting the use of energy for growth and multiplication during the early stage of cultures growth (Figueiro et al. 2010). In transgenic tobacco cell suspension cultures, carbon source played crucial role in the biosynthesis of geraniol, based on filling volume, light, and inoculums size. Sucrose filling volume and inoculums size promoted Geraniol production by improving cultures growth but, illumination supported biosynthesis. Shaking frequency using conditioned medium had less effect on growth and metabolites production, suggesting the importance of optimizing cultures growth parameters to boost production in the in vitro cultures (Vasilev et al. 2014). Formation of PLB was influenced by light intensity and overall explants survival. Low intensity of 10 μ mol m^{-2} s^{-1} was best for the survival of explants and formation of PLB while increased intensity up to 30 and 60 μ mol m^{-2} s^{-1} affected the survival (Park et al. 2002). Therefore, application of strategies that involve high-yielding cell lines selection, maximizing the productivity of the in vitro cultured cells through two-phase co-culture, absorption techniques, metabolic engineering among others are promising approaches for optimal production of pharmaceutical plant secondary metabolites in cell, tissue, and organ culture systems.

Because the site of their synthesis and storage varies in the cultivated plant cells, the low productivity of the compounds in cell cultures may occur due to feedback inhibition, enzymatic or non-enzymatic degradation of the synthesized product in culture media or may be attributable to volatility of the produced metabolites. Thus, the addition of artificial production site of accumulation as the use of liquid or second solid phase into liquid cultivation media could increase net productivity (Rao and Ravishankar 2002a; Murthy et al. 2014a). Two-phase co-culture that exploits the use of partitioning system to redistribute secreted product in the cultivation media into second nonpolar phase that effectively avoids feedback inhibition effects have important application in optimizing production in the cell, tissue and organ cultures (Malik et al. 2013). This may involve the use of nutrient or aqueous phase followed by liquid or solid material(s) that could retain the synthesized secondary metabolite without loss through degradation, volatilization, etc, similar to the biological system obtainable in plants wherein the metabolites are systematically synthesized and stored in specialized structures. Because production of most of the metabolites is not associated with cell growth and occurs more during the end of growth cycle (Dixon 2001), it is possible to employ the system by culturing cells using nutrient-rich media that promotes cell growth. This could be succeeded by cultivation using metabolites production medium augmented with precursors or elicitors (Malik et al. 2013). The nutrient-rich media that support cell growth is usually in aqueous phase while the second media could be in solid or liquid form—choice of the second media is typically dependent upon substrate to be recovered and compatibility of the phase with the substrate (Malik et al. 2013). For example, by developing a two-stage culture system that uses growth medium for cell biomass and naphthoquinone pigment production in the first stage, a dark condition at room temperature with alkaline

pH in the latter stage, it was possible to enhance biomass production by six-fold and optimize metabolite production in the cell suspension cultures of *Arnebia* sp. (Gupta et al. 2014). Culture process that involves cultivating cells in alkaloid production and growth medium could be used to optimize production of indole alkaloids in shake flask suspension cultures of *Catharanthus roseus*. The cultures produced up to 20 g DW L^{-1} of biomass but, 2-stage culture technique produced more active cell biomass with ten-times higher indole alkaloids production over the 1-stage with significant product release into the culture media in both systems (Tom et al. 1991). Thus, enabling designing a model for establishing scale-up production of the pharmaceutical plant secondary metabolites through the studies on scale-up production kinetics in bioreactor (Tziampazis and Sambanis 1994; Guardiola et al. 1995; Fazal et al. 2014; Gupta et al. 2014). This system was exploited in the production of taxol in the cell suspension cultures of *Taxus baccata* (Malik et al. 2011a), and improved the production of paclitaxel and baccatin III in *T. media* (Cusido et al. 2002, 2014). In the latter, two-stage culture of cell suspension using 5 L-capacity stirred bioreactor operated for 30 days with elicitor and two putative biosynthetic precursors amended in the culture media aided achievement of 21.12 mg L^{-1} paclitaxel and 56.03 mg L^{-1} baccatin III production after 8 days cultivation (Cusido et al. 2002). Two-phase co-culture may also involve co-cultivating cells or tissues of different species for enhanced metabolites production. The co-culture of *Panax ginseng* and *Echiancea purpurea* adventitious root at various proportions in 5 L-capacity airlift bioreactors using Murashige and Skoog (1962) medium added with Indole-3-butyric acid (25 µM) and sucrose (50 g L^{-1}) maintained at 25 °C in the dark condition for 40 days, and under elicitation with methyl jasmonate (200 µM) for 30 days promoted production of ginsenosides and caffeic acid derivatives when the inoculum density of ginseng to echinacea was in higher proportion (Wu et al. 2008a). Two-phase culture system have application in simplifying purification of the synthesized plant secondary metabolites from the culture media for recovery. For instance, production of paclitaxel in the cell suspension cultures of *Taxus chinensis* was enhanced by six-fold when aqueous-organic two-phase culture systems with sucrose feeding was employed, and 63% of the products were released into the culture media for recovery (Wang et al. 2001).

Feeding nutrients into the culture media is another promising strategy for optimizing production of biomass and pharmaceutical plant secondary metabolites in many cell, tissue and organ culture systems. However, maintaining continuity of the system is a challenge that many operational procedures and systems need to be developed. This may include the use of fed-batch operation that employs addition of nutrients at the start of cultures or along the process. By selective feeding of 60 mM nitrogen sources and 2% sucrose to the added 3% in the culture medium, it was possible to boost biomass production and CPT accumulation by 2.4-fold in the cell suspension cultures of *N. nimmoniana* (Karwasara and Dixit 2013). Perfusion cultivation that employs continuous feeding of fresh media into cultivation system accompanied by removal of media devoid of cells is promising in scaling-up the production of pharmaceutical plant secondary metabolites using plant cell, tissue and organ cultures considering the advantage it offers on biomass retention in system with high productivity of the metabolites. By using the system in the batch cultures, it is possible to overcome nutrients depletion and accumulation of growth inhibitors in the culture media to promote biomass and secondary metabolites production. Semi-continuous perfusion of *Anchusa officinalis* cultures in the shake flask with manual exchange of media promoted more than two-fold enhanced cell density and rosmarinic acid production over batch cultures (Su and Humphrey 1990). This was subsequently automated in prototype perfusion bioreactor to optimize productivity of the cultures (Su and Humphrey 2001). If an intermittent medium exchange is employed by two-stage perfusion involving batch mode operation with growth and production stages, highest success in the production of rosmarinic acid (about 4 g L^{-1}) with maximum cell biomass dry weight (35 g L^{-1}) can be achieved in the cultures. This is possible when 10 days cultivation using B5 medium added with sucrose (3%) and Naphthalene Acetic Acid (NAA) (0.25 mg L^{-1}) is accompanied by perfusion using media added with sucrose (6%) at constant perfusion rate of 0.1/day (Su et al. 1993). Perfusion cultivation method was employed to overcome browning effect in the culture media using batch mode operation of *Glycyrrhiza inflata* cell cultures in stirred-tank bioreactor having maximum cell concentration productivity up to 26 g L^{-1} dry weight (Wang and Qi 2009) and the growth kinetics optimized in bioreactor (Wang et al. 2010a).

Therefore, continuous cultivation systems described above that uses many variants may or may not lack feedback control. In some culture systems, simultaneous use of strategies for enhanced productivity may be employed. For example, by using combinations of cell immobilization, permeabilization, and elicitation, it was possible to boost production of gossypol, overcome slow metabolic rate, low production, and sensitivity of *Gossypium arboreum* cell cultures when cultivated using immobilized cell reactor operated on batch and continuous operation. Permeabilization increased the release of gossypol by 30% while elicitation boosted productivity by eight-fold but, combined treatment enhanced over 20-fold productivity over batch cultures (Choi et al. 1995). Recent efforts are towards understanding gene expression, enzymes activity, and signal transductions that are linked to the biosynthesis of pharmaceutical plant

secondary metabolites in the plant in vitro cultures. Thus, the first step in optimizing production in a given plant cell, tissue and organ culture systems involves identifying optimal media conditions and factors, followed by selecting high yielding cell lines. It could be followed by multi-step process of advancing culture media optimization in the established cultures for higher biomass and secondary metabolites accumulation through application of various in vitro techniques for enhanced productivity.

High yielding cell lines selection

Selecting high biomass and metabolite(s) producing cell lines is of utmost importance in optimizing the productivity of in vitro cultivated plant cells, tissues and organs as yield of the molecules may vary, in many cases within variety, genotype or population of plant species (Padmanabha et al. 2006; Suhas et al. 2007; Revadigar et al. 2008; Ramesha et al. 2008; Isah and Mujib 2015a, b; Jang et al. 2016a). This may be succeeded by identification of growth and production media step-by-step, involving identification and establishment of high producing and fast-growing in vitro cultures, particularly when the selected cell line can produce a molecule at or greater levels than the in vivo grown plants (DiCosmo and Misawa 1995; Dandin and Murthy 2012; Deepthi and Satheeshkumar 2016). A typical case of this scenario is in *N. nimmoniana* where the yield of CPT varies among individuals of its population (Padmanabha et al. 2006; Suhas et al. 2007; Ramesha et al. 2008). The yield of the alkaloid in the in vitro cultures of the tree varies with explants used to establish cultures—leaves, shoot, roots and embryo axes, making selecting higher yielding cell lines an essential step for optimizing production of CPT from the species (Isah and Mujib 2015a, b; Isah 2017). Similarly, shoot cultures of the tree produced biomass and CPT higher than the mother plants, thus, further highlighting the essentiality of cell lines selection before and during establishing high production system in the in vitro cultures (Dandin and Murthy 2012). In this case, high yielding cell lines are identified by metabolite profiling using various qualitative and quantitative analytical instrumentation techniques. However, limitation in the use of most qualitative and quantitative techniques to profile and evaluate the yield of a pharmaceutical plant secondary metabolite from plant tissue extract is the spatial and temporal changes that may occur during the process. The biosynthesis may have become repressed before the analysis as well as loss during the process, based on working downstream processing conditions. Hence, analysis of a given tissue extract may not necessarily indicate the actual yield of a pharmaceutical plant secondary metabolite from a species. Selection of the high-yielding lines may also be achieved via efficient way of mutants selection through identification of cell line of interest to maximize production of a metabolite in cell cultures. It may involve protoplast fusion or engineering genes encoding key enzymes involved in the biosynthetic pathway into a cell. The selection of mutants may involve exposing the population of cells to toxic inhibitors, biosynthetic precursor or culture environmental stress induction. This may be followed by selecting cells that show effective resistance in selection procedure for higher production. By using phenylalanine analogs, it was possible to select high rosmarinic acid yielding cell lines in the cell cultures of *Lavandula vera* (Georgiev et al. 2006). *p*-Fluorophenylalanine was used to select high phenolics yielding cell lines of *Capsicum annuum* (Ravishankar and Rao 2000). The cell line aggregate cloning method facilitated identification of high biomass and CPT-producing lines in the cell suspension cultures of *Opphiorhiza mungos*. By adjusting the cultivation media parameters, this strategy aided optimization of culture media for maximum CPT production in the cell suspension cultures of the species (Deepthi and Satheeshkumar 2016). For the differentiated tissues such as somatic embryos, the embryogenic capacity of cultures/explants may be evaluated by the cell lines to be used. For instance, the embryogenic capacity of explants was influenced by the levels of endogenous hormones, DNA content, the proliferation of embryogenic callus and SE; based on the genotypes of *Kalopanax septemlobus* studied. The embryogenic state was influenced by the genotype and cytokinin/abscisic acid ratio added to the culture media. It decreased with the age of the culture with marginal reduction in DNA content, suggesting that younger cultures are better for the formation of SE over long-term cultivated calli (Park et al. 2011).

Because production of the metabolites at higher yield is the primary target, application of cell lines selection using visual screening and studying growth kinetics of in vitro cultures are essential with regards to their application in enhancing production of the molecules, particularly when growth kinetics can be evaluated (Murthy et al. 2014a; Yue et al. 2016). Therefore, changing composition of the culture media to enhance production efficiency of the selected cell line is a promising approach to maximize productivity in scale-up production system. Although following the steps optimizes production, long-term cultivation of the cultures could lead to reduction in yield. This was observed in the shoot cultures of St. Johnson′s Wort where cultivation on Murashige and Skoog′s (1962) medium amended with Benzyladenine (BA) or in combination with the NAA for up to 100 days affected production of biomas, phenolics and hypericin without effect on flavonoids accumulation while 30 days cultivation did not show effect (Figueiro et al. 2010). It may have occurred due to somaclonal variation that was induced by the long-term cultivation of the cultures (Bairu et al. 2011). This case has also been encountered in the explants and somatic embryo-regenerated *Doritaenopsis*

plantlets and long-term cultures of *Phalaenopsis* 'Spring Dancer' where floral morphology of petals diameter and organs thickness showed variability through endoreduplication, and were linked to the overexpression of MADS-box transcription factor (MADS4) in the flowers over leaves (Park and Paek 2006; Lee et al. 2016).

Stability of the cultures is recognized when cultivated cell lines show efficiency in given growth parameter index over extended subculture cycles under stable condition (Bouque et al. 1998). This may involve evaluating metabolite production by the cell lines in the intracellular and extracellular along with biomass production. For example, application of molecular markers were used to establish higher genetic diversity existence among 18 *Guizotia abyssinica* clustered into early and late maturing cultivars by the use of Random Amplified Polymorphic DNA (RAPD) analysis (Nagella et al. 2008) and genetic fidelity of in vitro propagated *Nothapodytes nimmoniana* in relation to chemical profile stability of the regenerates (Prakash et al. 2016). Once high yielding lines are selected, many alternatives in vitro production strategies could be employed to harness productivity to meet the primary target of higher pharmaceutical secondary metabolites production. This may involve precursor feeding, permeabilization, immobilization of the cells, elicitation or even metabolic engineering (among others) to maximize and scale-up the production.

Elicitation as an effective strategy to enhance productivity of in vitro cultures

Plant secondary metabolites are produced by plant cells in response to environmental perturbation during in vivo growth and as defensive strategy triggered against invading pathogen elicitors. Hence, there is increasing interest to use compounds that trigger the defense response by the cultivated plant cells, tissues or organs for an improved productivity of bioactive compounds in the in vitro cultures (Zhao et al. 2005). The agents could be biotic or abiotic and may include signaling molecules (e.g., methyl jasmonate, salicylic acid), microbial cell wall extracts (e.g., yeast extract, chitosan), inorganic salts, heavy metals or even physical agents such as UV radiation among others (Ramirez-Estrada et al. 2016). This could provide an alternative avenue to improve production in the plant in vitro cultures and has achieved success with many cell, tissue and organ culture systems (Karuppusamy 2009; Giri and Zaheer 2016; Ramirez-Estrada et al. 2016).

Elicitation strategy have application in understanding the regulation of plant secondary metabolism pathways through studies on differential gene expression changes in the in vitro cultures that accumulated low or high secondary metabolites, particularly when associating genotype of plant species to yield of the metabolites. The elicitation of plants in vitro cultures is regarded most effective biotechnological strategy for the production of pharmaceutical plant secondary metabolites given that the compounds are produced as defensive response to perturbations under in vivo or in vitro conditions. Elicitors are biotic or abiotic substances that even at added low concentration into the culture media can induce production and accumulation of targeted phytopharmaceutical secondary metabolite in the in vitro cultures (Angelova et al. 2006). Most of the biotic elicitors that are used to elicit their production are either of exogenous or endogenous microbial origins while abiotic are heavy metals. Their principal mechanism of action involves recognition by the cell plasma membrane and cytosol signal transduction system and its ability to stimulate changes in cells that lead to enhancement effect on biosynthesis of the plant secondary metabolites. This, in turn, depends on genetic and physiological state of the cultivated plant cells, tissues or organs (Baenas et al. 2014). For example, production of paclitaxel in the cell suspension cultures of *Taxus* sp. showed variation in levels based on passages, aggregate size, ploidy and extracellular sugar levels, and cell cycle distribution (Patil et al. 2013a). Elicitation of five high and low taxol-producing *Taxus* sp. cell lines cultures showed alternating state of low and high taxol and biomass production, based on the culture age and physiology. The growth of high-producing cultures was inhibited upon subcultures while those of non-producing showed less growth inhibition upon elicitation. The increased inhibition occurred due to taxanes accumulation and subculture but, cell line mixing of the cultures resulted in sustained higher production of taxol (Kim et al. 2004). Therefore, by using elicitation strategy, it is possible to enhance productivity of the cultivated plant cells, tissues or organs through improving biomass and secondary metabolites production at higher yield, and has shown remarkable promise in many plants in vitro culture systems (Zhao et al. 2005; Giri and Zaheer 2016; Ramirez-Estrada et al. 2016).

Parameters such as elicitor concentration and selectivity, cultures age, exposure duration, cultivated cell line, nutrients composition of the culture media, growth regulation and quality of cell wall material are of crucial importance in the application of elicitation strategies (Smetanska 2008). For instance in *T. baccata*, cell mixing due to the physiological culture components induced changes among cell lines with overall effect on paclitaxel production (Bonfill et al. 2006). Production of thiorubrine A in the hairy root cultures of *Ambrosia artemisiifolia* treated with elicitors was dependent on cultures age, elicitor concentration and exposure time. Maximum of eight-fold thiorubrine A production was achieved when 16-day-old cultures were elicited with 50 mg L^{-1} vanadyl sulfate elicitor for 72 h while exposure to 50 mM autoclaved *Penicillium gravidus* for 48 h produced three-fold enhanced production (Bhagwath

and Hjortso 2000). Elicitation strategy could be of application in the scale-up production of pharmaceutical plant secondary metabolites in cell cultures at pre-set time. The product may be excreted into cultivation media for recovery in many of the reported cases (Cai et al. 2012a, b; Isah 2017). Thus, enabling recycling of cultivated cells and re-elicitation. For instance, elicitation of *Eleutherococcus senticosus* embryogenic cultures with up to 200 µM methyl jasmonate enhanced production of eleutherosides by 7.3-fold and chlorogenic acid by 3.9-fold when bioreactor system was used but, strongly inhibited biomass fresh and dry weight with the higher concentrations (Shohael et al. 2007a). However, limitations on the specificity of elicitors to activation of specific metabolite production or biosynthetic pathway in specific plant species remains an impediment to exploiting maximum commercial application. In some instances, may not necessarily lead to desired production by molecular expression profile of an evaluated sample (Yamazaki et al. 2003; Angelova et al. 2006). Most of the progresses made on the elicitation of plant in vitro culture systems for production of the pharmaceuticals are on experimental manipulation of culture media nutrients and conditions for enhanced productivity. Limited studies have been carried out on the signal transduction pathways, gene expression and cellular, molecular responses that are evoked by the introduction of elicitors into cultivated plant cells, tissues and organ cultures. In recent years, metabolomics, proteomic and transcriptomic approaches are emerging as powerful tools to explore on the differential gene expression induced by the introduction of elicitors to the in vitro cultivated plant cells, tissues, and organs that lead to enhanced production of the metabolites. The approaches when adequately explored could certainly facilitate understanding biosynthetic pathways with prospects in the application of metabolic engineering of intermediates for enhanced productivity in the plant cell, tissue, and organ culture systems.

Precursor feeding

The strategy evolved based on the premise that biosynthetic intermediates of bioactive molecules when added at beginning or during the culture period could serve as an additional substrate for improving high production of the metabolites in cultivated plant cells, tissues or organ cultures. Thus, exogenous or endogenous compounds that are converted by the cultivated plant cells into secondary metabolites through biosynthetic pathways are referred to as precursors. Their amount in callus and cell suspension cultures is usually lower than in differentiated tissues, explaining the association of cellular differentiation to higher yield of pharmaceutical plant secondary metabolites in many plants tissue and organ in vitro culture systems (Constabel and Kurz 1999). Feeding these substances into the culture media is a promising approach with capacity to boost production of the molecules in cell cultures, especially when transgenic cell lines are used. The strategy was found effective in enhancing the production of many pharmaceutical secondary metabolites in the in vitro plant cell cultures (Namdeo et al. 2007). Factors to consider in precursor selection and feeding into the systems includes timing for the addition, the concentration of precursor molecule to be added and its entry into targeted biosynthetic pathway (Jackson and Attalla 2010). However, timing of the addition into the culture media is critical for optimal production, and feedback inhibition must be monitored when evaluating optimal amount to be amended in the culture media of plant cells, tissues, and organ cultures. A precursor such as L-Alanine needed for the biosynthesis of plumbagin in *Plumbago indica* promoted 14-fold enhanced production when it was added to the root cultures at 14th day of cultivation along with sequential addition of Diaion HP-20 36 h after it was fed (Jaisi and Panichayupakaranant 2016).

The pioneer works on feeding biosynthetic precursor to promote pharmaceutical plant secondary metabolites production in the plant cell cultures were on alkaloids (Chan and Staba 1965). A production media was developed by Zenk et al. (1977) and strategies for feeding biosynthetic intermediated developed in many cell culture systems (Namdeo et al. 2007). Experiments on precursor feeding can be exploited to elucidate biosynthetic pathway of a plant secondary metabolite. By isotope labeling the precursor and its feeding into the media of cultivation, uptake is enabled for the biosynthesis in growing cultures. Thus the isotope-labelled secondary compound is biosynthesized and intermediate incorporated at different positions during biosynthesis of the targeted metabolite. When in vitro cultures are harvested, the metabolite(s) can be purified, its chemical structure(s) elucidated and biosynthetic origin identified. The approach though classic and having challenges is efficient in elucidating the biosynthetic origin of many pharmaceutical plant secondary metabolites (Herbert 1989; Dieuaide-Noubhani et al. 2007; Chokkathukalam et al. 2014).

Before a precursor molecule is selected for enhanced production of a pharmaceutical, there is need to establish relationship kinetics of the biosynthetic precursor and production. For instance, synergistic and antagonistic relationship was established on the production of vindoline, catharanthine, vincristine and vinblastine when biosynthetic precursor was added to the cell aggregate of *Catharanthus roseus* cultures, based on the precursor introduction duration (Pandiangan et al. 2013). Precursors such as L-phenylalanine needed for the biosynthesis of silymarin (a flavonolignan) could be used to enhance its production in the in vitro cultures when supplemented in the cultivation media of the hairy roots at 30th day of culture, and 72 h after 100 µM

concentration was added to *Silymarin marianum* in vitro cultures (Rahimi et al. 2011). It is also a precursor in the biosynthesis of podophyllotoxin in the cell suspension cultures of *Podophyllum hexandrium* (Tumova et al. 2006). The addition of organic compounds such as vitamins and amino acids into the culture medium of plant cells, tissues or organs, by type and concentration, have found application in enhancing in vitro production of many pharmaceutical plant secondary compounds. Amino acids' feeding enhanced production of triterpenes in the callus and cell suspension cultures of *Centella asiatica*, and manifold enhanced production of asiaticoside was achieved when leucine was added into the cultivation medium (Kiong et al. 2005). Shoot cultures of *Hyraceum perforatum* cultivated in media enriched with valine and isoleucine showed enhanced production of adhyperforin by 3–7 folds while the addition of 3 mM threonine enhanced productivity by two-fold (Karppinen et al. 2007). Sometimes feeding a biosynthetic precursor into the cultivation media may promote metabolite production without effect on biomass accumulation. In some instances, application of biosynthetic precursor may not yield the desired productivity in the plant in vitro cultures but, combination with enhancement strategies could overcome the obstacle. This may be due to the limited supply of the biosynthetic precursor or limitations in its uptake from the culture media for incorporation into metabolic pathway. Thus, application of other enhancement strategies becomes necessary to achieve desired productivity. By using combination of elicitation with precursor feeding, it was possible to enhance biosynthesis of sennoside A and B in the leaf-derived callus cultures of *Cassia augustifolia* (Chetri et al. 2016). The combination of elicitation with precursor feeding increased productivity of L-Dopa by several folds in the cell suspension cultures of *Mucuna pruriens* but, precursor feeding was efficient in the enhanced production over elicitation when separately assayed (Raghavendra et al. 2011). Also, in some instances, a precursor may have an effect on the production of more than one secondary metabolite, based on the biosynthetic pathways involved as many of them are cross-linked in cells (e.g., Fig. 1). Casein hydrolysate and L-Phenylalanine addition in the culture media of *Spilanthes acmella* promoted biomass and scopoletin production when supplemented at an average concentration. However, the latter was more efficient with 4.51-fold enhanced production (Abyari et al. 2016). Feeding squalene into the cultivation media of *Centella asiatica* calli promoted production of madecassoside and asiaticoside (Kiong et al. 2005). In *Solanum lyratum* cell cultures, the addition of exogenous sterols such as cholesterol, stigmasterol or mixed sterols promoted the production of solasodine, solasonidine, and solanine without effect on biomass accumulation (Lee et al. 2007a). Although successes had been achieved in enhancing the productivity of many plants in vitro cultures through the use of precursor feeding, some limitations continue to hinder commercial exploitation of the approach in many developed systems, and overcoming them will provide an avenue for the application. Critical among them includes developing protocols that optimize feeding a given precursor that proves the cheapest byproduct of other processes for conversion of particular plant secondary metabolite produced in a cell culture system.

Permeabilization

The ultimate goal of applying in vitro technology in the production of plant pharmaceutical secondary metabolites using cell, tissue and organ cultures is to achieve industrial production through the large-scale production system to meet industrial demand for raw materials supply in drugs production, thus, conserve natural sources of the molecules. This needs immobilization of the cultivated cells for continuous operation, separating growth and production stages, reuse and simplified separation of biocatalysts from cultivation media to enable optimizing product-oriented media of culture at reduced cultivation time (Dornenburg and Knorr 1995). Immobilization strategy provides an avenue for exploiting such productivity gains in plant cell, tissue and organ cultures. However, choice of suitable system based on factors such as high cell density and arresting potentials of matrices, enzymes activity of selected biocatalyst, among others, need to be diminished by the working process, and manipulation of the formed biocatalyst should be simplified (Dornenburg and Knorr 1995). Achievements in the scale-up production and immobilization techniques are playing a leading role in the application of cell cultures for the production of many pharmaceuticals isolated from plants (Table S1). By using the technique of permeabilization, it was possible to enhance production of many pharmaceutical plant secondary metabolites, as for instance, the use of an immobilizing agents such as calcium alginate in the production of taxol (Bentebibel et al. 2005), capsaicin, vanillin, and cymalicine in *Capsicum frutescens* (Rao and Ravishankar 2002a, b). Cell suspension cultures have found wide application in scaling-up the production through optimizing culture condition for improved productivity using the strategy; agitation, aeration and other physical conditions of cultivation of the plants provides reliable production alternative.

Because the release of product from biomass into cultivation media permits establishing continuous system that improves productivity about standard batch growth, excretion can be forced using permeabilization agents. Several attempts have been made to permeabilize plant cells using strategies that retain cell viability with enhanced substrate and metabolites transfer (Choi et al. 1995; Cai et al. 2012a). This among others includes the use of treatments such as pH shock and addition of stressors when cell viability can be

preserved (Brodelius 1988; Mukundan et al. 1998). Because permeabilization of plant cells depends on the formation of pore in plasma membrane to enable passage of molecules into and out of the cultivated cells, and that synthesized secondary metabolites in plant in vitro cultures are stored in vacuoles (in the case of undifferentiated cells cultures) or specialized structures (for organized structures), manipulating cell membrane permeability to stably secrete out a pharmaceutical secondary metabolite into cultivation media for possible recovery is a good alternative strategy. This has application in scaling-up the production, particularly when the metabolite can be stably secreted and recovered from cultivation media. Many permeabilization agents have found application in cell cultures by promoting enzyme(s) access with enhanced effect on product release into the extracellular for recovery. Organic solvents such as dimethyl sulfoxide (DMSO), polysaccharides like chitosan and isopropanol are common permeabilization agents currently in use. By permeabilizing transformed hairy root cultures of *Datura stramonium* with Tween 20, it was possible to achieve at least 25% hyoscyamine release from the cells and maintain cultures alive (Khelifi et al. 2011). In *Catharanthus roseus* hairy root cultures, permeabilization using Triton X-100 (0.1%) and n-hexadecane (2%) stimulated 98% increase in ajmalicine production. Treatment of the cultures with DMSO and Triton X-100 facilitated efflux of the metabolite into the cultivation medium with 12 and 16-folds enhancement, respectively, suggesting application in scale-up production (Thakore et al. 2013).

Ultrasonication, electroporation and iontophoretic release are permeabilization techniques that have found application in the production of pharmaceutical plant secondary metabolites. The enhancing effect of ultrasonication to production of the metabolites in plant cells, to a large extent, can be attributed to physiological effect on treated cells than mass transfer that is ascribed for other biological systems (Wang et al. 2006; Cai et al. 2012a). Production of valepotriate was doubled in *Valeriana glechomifolia* in the liquid culture when sonicated with ultrasound for up to 2.5–5 min but, no effect on biomass production was observed (Russowski et al. 2013). Combined treatment of *Morinda citrifolia* suspension cultures with elicitor, ultrasonication and sucrose feeding boosted in vitro cultures productivity. Treating the cultures with short pulses of ultrasonication enhanced accumulation of anthraquinones by up to 2.5-folds (Komaraiah et al. 2005).

Gel entrapment and surface immobilization are other promising techniques employed in the production of pharmaceutical secondary metabolites in plant cell cultures. However, cells entrapment in specific gels or their combinations is the most widely employed approach. Matrices used for the entrapment must not be toxic to the cultivated cells, economically feasible and have excellent polymerization property. Another immobilization strategy is surface immobilization that exploits the ability of cultivated cells to adhere to an inert surface when immersed in liquid media of culture (Murthy et al. 2014a). Immobilization techniques that confine cells in proper matrix support allow flux of substrate and product, thus, extending the viability of cells in production stage (stationary phase of growth), and may enable prolonged biomass production over time. Certainly, immobilization system that maintains viable cells over extended duration with the release of products in bulk into the culture media and in stable form could reduce the cost of pharmaceutical plant secondary metabolites production in cell, tissue and organ cultures. However, application of the system is constrained by limited production coupled with the biomass production, extracellular secretion and degradation, and the use of gel matrix for maximum recovery effects on diffusion barriers (Oksman-Caldentey and Inze 2004). Although immobilization techniques are promising and successfully employed in the scale-up production of some high-value secondary metabolites such as taxol and rosmarinic acid (Ramawat and Merillon 2008). The critical limiting factors as compound-specific production in growth and differentiation of cultures, the physiological effect of immobilization on biosynthesis of the molecules, elicitation strategy employed and cost of scaling-up the production are proving a hindrance to exploiting maximum commercial gains with other pharmaceuticals.

Application of root cultures

Production of pharmaceutical secondary metabolites is in most of the reported cases higher in the in vivo grown plant roots than other parts, possibly due to variety of environmental stresses they are exposed given that production of the compounds is induced by environmental stresses (Filova 2014). Similarly, in most plants that produce the metabolites, the yield may be higher in adventitious or hairy root produced in the in vitro cultures (Doran 2002; Rudrappa et al. 2005) or may even produce metabolites that the mother plant lack biosynthetic capacity (Veerasham 2004). Thus, making the technology of root culture attractive for higher production of the metabolites through the transformed or untransformed root cultures with possibility to exploit in the commercial application. However, challenges in the application of the root cultures technology is the cells, tissues or organ specificity of the metabolites biosynthesis with some plants. In many of the plants, the metabolites are synthesized in certain specialized cells and translocated to others or organs for storage. If this organs are the roots, they could be of application in production of the pharmaceutical but, in the absence of such, the application can not be feasible. For example, genes for the biosynthesis of tropane alkaloids are expressed only in the root cells of *Atropha* species but, not

in other organs. In this case root cultures will have application in their production. For many such examples, readers is referred to Sharma et al. (2013).

In broad sense, production of the root cultures may involve: (1) Induction of the roots from selected explant (2) culture of the established root cultures in liquid medium contained in shake-flask or bioreactor (3) establishing the cultures growth kinetics through suitable culture medium components adjustments for higher biomass and metabolites production (4) developing strategies that promotes biomass and metabolites production (5) large-scale production of the cultures in bioreactor for up-scaled production and (6) downstream processing of the produced biomass for biosynthesized metabolite(s) recovery from the root cultures (Murthy et al. 2008a). Because of the highly differentiated nature and productivity of the root cultures, it offers tendency for production of the metabolites with genetic stability and retained production over longer time in plant in vitro culture system. For example, root culture of *Cayratia trifolia* synthesized stilbenes at higher levels over the intact roots. Application of elicitation strategy along with precursor feeding improved productivity of polyphenol in the cultures by up to 12-folds over the culture period (Arora et al. 2009). However, the yield was low in the absence of high sucrose concentration in the culture media (Roat and Ramawat 2009).

Untransformed root cultures can be established using various plant tissue culture media types by amending auxins such as IBA, NAA or IAA in the selected culture medium (in order of priority by efficiency) and sometimes in combination with the cytokinins. For instance, adventitious root cultures of *Polygonum multiflorum* cultivated in an air-lift bioreactor using MS medium added with IBA (9.84 µM) and 5% sucrose produced higher biomass and bioactive compounds over other treatments (Lee et al. 2015). Similarly, by using combination of NAA and BA, it was possible to induce and proliferate untransformed root cultures from zygotic embryo explant with higher productivity of CPT and its derivative in *N. nimmoniana* when MS medium was used. The combination of NAA with BA favored greater productivity over that with the Kin (Fulzele et al. 2002). Greater of the studies on the root cultures is on transformed hairy root due in most cases to its higher productivity, the fast growth rate and genetic stability (Wysokinska and Chmiel 2006; Cui et al. 2010). Many studies have compared these parameters on the overall productivity of the two root culture systems for possible scale-up production in bioreactor; the transformed hairy root cultures have proved of better application in production of the pharmaceuticals (Baiza et al. 1998; Andarwulan and Shetty 1999; Aryanti et al. 2001; Hu and Du 2006; Cui et al. 2010, 2011). However, in some instances, untransformed root cultures have proved of higher productivity over the transformed ones as in *Coluria geoides* (Olszowska et al. 1996) and *Silybum marianum* (Alikaridis et al. 2000).

Hairy root technology has found application in the scale-up production of many pharmaceutical plant secondary metabolites such as ginsenoside and some alkaloids, and is increasingly getting considerable attention. The hairy root cultures in many cases show rapid and plagiotropic growth with branching on phytohormone-free medium. It has been extensively studied in the root nodules formation research (Hu and Du 2006) and found extensive application in studies of pharmaceutical plant secondary metabolites production in many species. This can be attributed to their ability to produce the compounds over successive generations without loss of biosynthetic capacity or genetic stability (Giri and Narasu 2000a, b). The genotypic and biochemical stability, ability to grow on PGRs-free cultivation media, cytodifferentiation, fast growth and low doubling time, the ease at which established hairy root cultures could be maintained and ability to biosynthesize range of compounds are among advantages offered by the technology (Bourgaud et al. 2001; Abraham and Thomas 2017).

Transformed hairy root cultures can be developed by inoculating plant cell, tissue or organ cultures with *Agrobacterium rhizogenes* strain (Palazon et al. 1997). The root cultures can be established on aseptically wounded plant part when inoculated with the selected *A. rhizogenes* strain. However, the time taken for the emergence may vary with plant species used. Root tip or other explant/cultures could be cultured in PGRs-free media followed by selecting and characterizing root clone by capacity for growth and secondary metabolites production. This may be followed by identifying cultures growth conditions via more productive clones selection and testing of ionic concentration, carbon sources, and pH of the cultivation media. For establishing the cultures, key decision and conditions such as the suitable bacterial strain of *A. rhizogene* needed, the explant, antibiotic selection for co-cultivation and culture media are critical. After explant selection and wounding, cocultivation with the *A. rhizogenes* strain and optimizing the composition of the culture media nutrients for formation and proliferation of the induced hairy roots for higher productivity of plant secondary metabolites is essential. Factors such as carbon source and concentration, media pH, ionic concentration, PGRs amended into the culture media, physical factors, and inoculum are then optimized for production of the metabolites (Smetanska 2008).

Transformed hairy root cultures have been obtained from many monocots (albeit with difficulty) and dicotyledonous plants, and in many instances produce the metabolites at similar or higher levels to the in vivo grown roots. Thus, providing sustainable alternative for large-scale production of the metabolites (Srivastava and Srivastava 2007). Among the unique advantage of growing the transformed hairy root

cultures are the fast growth and hormonal-independence that lack geotropism, lateral branching, and genetic stability and that the secondary metabolites could be produced at equal or near to the level produced in the intact root of plant (Sevon and Oksman-Candentey 2002). Thus enabling genetic stability with rapid growth in simple culture media that lack phytohormones, a feature that makes them suitable for studying biochemical changes in the growth of root cultures compared to the intact plant roots. Hence, genetic transformation using *A. rhizogenes* is an alternative way to optimize production of pharmaceuticals in cell, tissue and organ cultures. It have been employed in the production of hairy root cultures of many plant species for production of the metabolites at an improved rate. Because only part of T-DNA region in the Ri plasmid of the bacterium is needed for successful transfer of foreign DNA into nuclear genome of host plant and stably maintained in cell, the strategy is effective in developing large-scale pharmaceuticals producing in vitro root culture systems (Abraham and Thomas 2017). The transformation method offers advantage of transferring foreign genes encoding pharmaceutical plant secondary metabolite pathway step enzymes into a vector for transformation of the hairy root clones and, thus, enhance production of the metabolites. This was successfully achieved with *Hyocyamus muticus* and *Atropa belladona* decades ago by the use of binary vector system of *A. rhizogenes* (Hashimoto et al. 1993) and later with several plant species (Srivastava and Srivastava 2007; Sharma et al. 2013). Hairy root cultures have found application in studying the biology of chemical bioactive compounds production and engineering because it could be used as an experimental model to study root-specific metabolism and regulation, as well as metabolic engineering for potential production of biopharmaceuticals.

The first report on the successful application of scale-up production for adventitious root cultures technology in the large-scale industrial production set up of *Panax ginseng* was achieved by Choi et al. (2000) whom reported 150-fold enhanced growth of ginseng adventitious roots that were grown in 500 L-capacity balloon-type bubble bioreactor over 7 weeks. In the study, production of biomass was higher with callus and hairy root cultures in liquid media compared to the earlier reports. Since then, many successes had been reported using the technology, suggesting its application as the most suitable strategy in the industrial production of the pharmaceutical through scale-up production. The limitations associated with the application of root cultures technology to scale-up production of the metabolites includes oxygen and nutrients mass transfer that accompany increase in root cultures biomass during cultivation of the cultures. This may lead to cell death and necrosis at the expense of biomass and secondary metabolites production in an inadequately designed production systems (Jeong et al. 2002; Suresh et al. 2005). It may occur due to deficiencies in oxygen transfer from the air bubbles in bioreactor to the cultured roots through the culture medium of bioreactor systems (Neelwarne and Thimmaraju 2009). However, enhanced mass transfer can be facilitated when vigorous mixing is employed but, has limitation of inducing possible reduction in viability of the hairy root cultures produced through increased hydrodynamic shear stress (Hitaka et al. 1997; Steingroewer et al. 2013).

When designing bioreactor system for scale-up production of the hairy roots to produce biomass and secondary metabolite, factors such as morphological characteristics of the hairy root during proliferation, medium nutrients requirements and composition, availability and rates of utilization, mass transfer, method for providing support, mechanical properties, protecting shear damage of the cultured roots, density of the inoculum and distribution, and chances of restricted flow of nutrients due to the high tangled state of root masses are put into consideration (Mishra and Ranjan 2008; Wilson and Roberts 2012; Steingroewer et al. 2013; Georgiev and Weber 2014). Currently, the most successful commercial application of the root cultures technology for the large-scale production of pharmaceuticals was established by Green2Chem in Belgium (http://www.green2chem.com/) and Root lines technology in France (http://www.rootlines-tech.com/). The companies' uses transformed hairy root cultures in the large scale production of pharmaceutical plant secondary metabolites for use in the industrial manufacture of drugs using bioreactors of various shapes and sizes.

Scale-up production

There is increasing interest to evaluate the feasibility of pharmaceutical plant secondary metabolites production at industrial scale through the use of bioreactors of varying sizes and features (Georgiev and Weber 2014; Park and Paek 2014). Modern studies on the scale-up production focus on the cell suspension cultures with fewer using differentiated tissues such as somatic embryos and hairy roots (Ziv 2000; Ferrie 2010). This could be attributed to the advantage of simplicity, predictability and high efficiency of ease at which the metabolites can be isolated from biomass or cultivation media in the cell suspension cultures over tissue and organ cultures (Park and Paek 2014). Scaling up the production using somatic embryos is promising alternative strategy due to the advantage it offers in higher yield, particularly when the gains of studying morphological and physiological process associated with biosynthesis of the metabolites can be comprehensively studied (Tejavathi et al. 2007). Successful bioreactor operating system provides optimal biomass and metabolite production by ensuring low-stress to the cultivated plant cell, tissue or organ through efficient oxygen and nutrients supply and homogenous distribution of cultivated

biomass (Steingroewer et al. 2013). However, the correlation between metabolites production and morphological differentiation reported with most plant species must be exploited to achieve higher yield of the metabolites in cell, tissues or organ cultures for a designed scale-up production system to be of success (Rao and Ravishankar 2002a; Steingroewer et al. 2013). The application of PCTOC technology through the scale-up production has facilitated production supply of some of the expensive pharmaceuticals that are produced at very low yield in the in vitro cultures or in vivo grown plants. For others, low or lack of production in the cultures continues to be an impediment to the application (Verpoorte et al. 2002).

Among the great achievements in the employed scale-up production system was with taxol for which Phyton Biotech (Germany) achieved its use to produce quantities demanded by Bristol-Meyers Squibb Co. (USA) through biotechnological application of plant cell culture during the year 2002 (Kintzios 2008; Phyton Biotech 2014). Shikonin, berberine, and ginsenosides are other pharmaceutical plant secondary metabolites that are currently produced at commercial scale using large-scale production systems through the use of bioreactors of varying sizes, shapes, and properties. The earliest remarkable achievement in the scale-up production of Shikonin was achieved by Tabata and Fujita (1985) and provided platform for the subsequent achievements in its production by the use of scale-up production systems for commercial exploitation. Production of ginseng in plant cell cultures through the scale-up production system is another milestone in the application of PCTOC in production of biopharmaceuticals (Hibino and Ushiyama 1999). The production using bioreactor to scale-up their production is increasingly receiving recognition given the promise it offers for producing the molecules in plant cell cultures, especially when cell suspension cultures are employed. However, the physical and chemical, environmental factors that modulate biomass production and secondary metabolites accumulation need to be addressed in designed scale-up production systems. Challenges in scaling up the production using bioreactors, besides culture conditions optimization, include biomass production measurement (especially with tissue and organ cultures) given the uneven and non-homogenous nature of its output in bioreactors of varying sizes and shapes (Ruffoni et al. 2010; Steingroewer et al. 2013). Genetic instability encountered with many in vitro culture systems that are induced by submergence could have effect in biomass production and metabolites yield. Therefore key factor in the successful application of a selected bioreactor system is the achievement of targeted productivity, based on the employed monitoring system and parameters. This may involve the use of analytical technologies applied during development and production of a biopharmaceutical to achieve maximum production efficiency of the developed bioprocess system. It may involve understanding the process to be optimized at an accelerated timeline, characterization and validation for understanding essential control limits, the set points and critical parameters that influence performance and product quality. Controlling the process is crucial at set point in the operating ranges and is usually defined while developing and characterizing a process developed for achievement of desired quality in an operating process with validation needed from authorities. For instance, Process Analytical Technology (PAT) that was prepared by the US FDA in the year 2004 contained description of mechanisms for designing, controlling and analyzing manufacturing process of pharmaceuticals through the measurement of parameters that have an effect on quality attributes of products (Pohlscheidt et al. 2013). However, limitations on the knowledge of biosynthetic pathways, instability of productive cell lines in the case of cell suspension cultures and slow growth are challenges that need to be addressed for an efficient productivity of a developed bioprocess system. Automation of bioprocess that employs sensors and monitoring systems based on the attributes characterized by pharmaceutical in production may involve monitoring physical, chemical and biological parameters (Pohlscheidt et al. 2013). The attributes of a system such as pH measurement, viscosity, temperature, pressure, redox potential, osmolarity, oxygen supply and carbon dioxide, weight and liquid levels, flow measurement and control of liquids and gas among others are closely monitored.

The main stages in the bioprocess automation involve biomass production, product synthesis and secretion of targeted metabolite into culture medium for downstream processing and recovery. From practical point of view, demarcation does not exist between the biomass and product synthesis stages in most cell, tissue and organ culture systems (Steingroewer et al. 2013). Various models for estimating biomass and metabolite production therefore have to be developed, based on culture type, scale-up conditions, and strategies employed in the working production systems so as to overcome challenges encountered in a scale-up production system (Georgiev and Weber 2014; Pohlscheidt et al. 2013; Xu and Zhang 2014). The model(s) can be developed for a designed system, putting into consideration possible operational cost and labor of production that may include culture media, growth measurement, the size of inoculums, the effect of physical parameters on cultures growth and product recovery—inter and intracellular, for commercial application (Srivastava and Srivastava 2007; Pohlscheidt et al. 2013). Productivity of the systems is evaluated as the overall biomass and metabolite production efficiency by analytical strategies used for the evaluation. This may involve measurements and downstream quantification of a biopharmaceutical yield from generated biomass using select technique(s) to employ and cultures growth kinetics can then be established

by utilizing mathematical models (Pohlscheidt et al. 2013). For instance, development of proper mixing and aeration provided by airlift bioreactors permits high-density cultivation of cell suspension cultures. However, the shear sensitivity and rapid setting or cell aggregate with tendency for floating of cultures need to be addressed during designing bioreactor for scale-up production of a given pharmaceutical plant secondary metabolite. This depends on the size of the designed bioreactor system. Currently, Phyton Biotech in Germany owns and operates the worlds' largest cGMP plant cell culture facility designed for large scale production of Taxanes in its 75,000 L-size bioreactors that run upto 880.000 L per year (Yesil-Celiktas et al. 2010; Phyton Biotech 2014).

Endophytic production

The discovery of bioactive molecules production in endophytes offered an alternative biota sources but, till date there is no reported commercial exploitation of the endophytic production of pharmaceutical secondary metabolites. Microbial world have contributed to the production of plant secondary metabolites quite reasonably at academic scale through the provision of models that could be applied in the elucidation of biosynthetic route of many phytochemicals. The ability of the endophytes to biotransform non-native molecules and detoxify host plant defensive compounds for their survival confers on them advantage(s) on biosynthesis of secondary metabolites that are produced by their host plants. Majority of the published works on the endophytic production of pharmaceuticals are from fungal and few bacterial endophytes. There have been wide argument concerning the evolutionary origin of secondary metabolism in the endophytes (Kusari et al. 2012; Sudhakar et al. 2013; Kumara et al. 2014). Early in the discovery, it was hypothesized that the biosynthetic genes evolved through horizontal gene transfer between host plants and endophytes, co-evolved in the two parties or either endophyte or the host plants/endophytes evolved the genes and transferred them to the other (Stierle et al. 1993; Kusari et al. 2014). The horizontal gene transfer from the host plant as the source of secondary metabolism genes in endophytes carried more weight and was investigated and debated for decades (Kusari et al. 2012). However, recent studies are giving some insight into the origin of these genes (Heinig et al. 2013; Yang et al. 2014). The case of taxol production in endophytes was the most extensively investigated and debated, and currently, it is believed that taxol-producing endophytes might have evolved the genes from the host plants. However, the biosynthesis is independent of the host plant, suggesting the independent production of taxol by endophytes in cultures with regards to the host plants. This was supported by many reported productions of taxol in endophytes isolated from non-taxol-producing host plants (Yang et al. 2014; Isah 2015a).

Challenges in the production of pharmaceutical secondary metabolites by the endophytes includes attenuation, defined as decline in yield of the molecules that has been reported with many microbes, more particular with the fungal cultures (Kusari et al. 2009a, b; Gurudatt et al. 2010). Endophytic fungi were hypothesized to posses gene clusters that encode unobserved secondary metabolites and whose activation or attenuation determines production from the endophytes (Shwab et al. 2007; Wang et al. 2010b; Beau et al. 2012). Although how the attenuation occurs is yet unclear, it was hypothesized to be due to the lack of host-specific stimuli in the case of fungal cultures or silencing the expression of essential genes by axenic microbial cultures. It's reversing through media supplementation with the host tissue extract could be promising in overcoming the limitation (Priti et al. 2009). The attenuation in most of the reported cases occurs due to increase in subcultures and in some instances is irreversible. Strategies that involve passing the endophyte into the host tissue or treatment with DNA methyltransferase inhibitor were reported to reverse the attenuation process in CPT production, indicating its association with epigenetic control and host tissue environment (Vasanthakumari et al. 2015). For instance, production of CPT by the cultures of *Fusarium solani* INFU/Ca/KF/3 was reported to show attenuation in productivity. Reconstitution into the host or supplementing plant tissue extract into the cultures could not restore the productivity after seven subculture generations. This was attributed to the random mutation in the cultures coupled with immediate degradation of alkaloid biosynthetic machinery in the fungus (Kusari et al. 2011a). However, in some culture systems, certain endophytic strains sustained production of CPT for several generations without attenuation in the production (Pu et al. 2013).

Many theories advanced have attempted explaining the attenuation process in the production of secondary metabolites by endophytes upon subcultures (Kumara et al. 2014; Sachin et al. 2013). For example, Sachin et al. (2013) proposed three possible alternative ways that explained production of CPT by endophytes and suggested potential applicability in taxol and other secondary metabolites production. According to their hypothesis, the attenuation may occur due to (1) Replacement of strictosidine synthase gene in the plants CPT biosynthetic pathway with a new strictosidine synthase-like gene or completely different protein and silencing of the gene through differential methylation over the subculture period, leading to attenuation in production of the metabolites. (2) The endophytes carries a critical gene cluster that is needed for the biosynthesis in extra chromosomal plastids and the genes possibly evolved independently or through acquisition by the host tissue through horizontal

genes transfer or (3) The endophytes harbour plasmids that bears gene clusters in the endo hyphal bacteria, based on the existence of some endo-hyphal bacteria in some endophytes (Bianciotto et al. 1996; Bertaux et al. 2005; Hoffman and Arnold 2010; Kumara et al. 2014). According to these hypotheses, second and third concur with the attenuation process, as observed with filamentous fungi and plasmid in bacteria upon subcultures (Sachin et al. 2013). Indepth studies on the endophytes-host plant relationship as it relates to production of the metabolites could open up insight into the attenuation process and the regulatory mechanism. It is likely that secondary metabolites produced by the host tissues influences production in microbial endophytes.

Similar to the plant cell cultures, strategies that involve media manipulation to enhance products yield can be applied to the endophytic cultures. For instance, by manipulating media nutrients composition and precursor feeding, it was possible to enhance the yield of CPT by 2.5-folds in the *Entrophospora infrequens* cultures (Amna et al. 2012). Co-cultivation of endophyte strain or species is another approach that can be employed to augment higher metabolites yield from the endophytic cultures. Co-cultivation of *Colletotrichum fructicola* and *Corynespora cassiicola* through the use of submerged culture system significantly enhanced higher yield of CPT compared to their culture separately (Bhalkar et al. 2016). Strain improvement that involves repetitive cycles of metagenomics at random is another strategy for enhancing secondary metabolites production in endophytes. This could be achieved through mutation, genetic recombination or selection. However, indepth knowledge of the physiology, biosynthetic pathway control and regulations, and creative screening approach are needed. By using protoplast mutation of UV radiation and diethyl sulfate, a mutant of *Fusarium maire* K178 with higher production of paclitaxel from 20 to 225.2 μg L^{-1} taxol yield can be developed (Xu et al. 2006). Elicitation of endophytic cultures is another strategy for enhancing production of the secondary metabolites yield. Addition of pure and ethanolic extract to the cultures of *F. solani* enhanced CPT production by 15.5 and 10.6-fold respectively (Venugopalan and Srivastava 2015).

Metabolic engineering for the production of pharmaceuticals in microbial endophytes has industrial application and could be of implementation in overcoming the attenuation process. This may involve cumbersome steps of using mutants to identify enzymes and genes involved in certain steps of the biosynthetic pathway, involving large number of mutants grown in cultures. It may be followed by analysis for their production of intermediates involving various selection markers, based on the microbe and secondary metabolite investigated. This may involve the use of biosynthetic pathway intermediates or confirmation of enzyme activity. Recently, analysis of transcriptome, proteome, and metabolome are recognized as screening approach for comparing producing or non-producing cells or cell lines (Giri and Zaheer 2016; Paz et al. 2017). However, the limitation of the missing immediate logical link between metabolome and proteomes is an impediment to the application. This is so because correlating proteomic profile with the metabolomic data is hard to obtain with endophytes. However, combining transcriptomic data with the metabolomic could provide complete and deep view of pharmaceutical plant secondary metabolism in endophytes. Despite the significant progress made in the microbial production of secondary metabolites through the endophytes research, enhancing their production continues to pose challenge due to the complexities involved. A number of challenges that includes pathways elucidation by the application of "Omics" approaches, improvement in the expression of secondary metabolism in endophytes, regulatory finetuning of the metabolic pathways using synthetic biology tools and applications continue to pose challenges. Heterologous expression of biosynthetic pathway of a given pharmaceuical secondary metabolite in host microbe is a challenging task given the possibility of missing link in the biosynthetic route through linking enzymes activity of the genes participating in a pathway that is unidentified or cloned. The participation of many enzymes in the biosynthetic pathway, with each having its properties and kinetics, makes linear function and cloning of many genes and their co-expression difficult in microbial host. Recent understandings in the field showed that expression of transcription factors offers application in achieving global upregulating in secondary metabolism of endophytes through genetic modification of biosynthetic routes with potentials in overriding endogenous regulatory controls. Engineering 'hybrid pathways' through combining endophyte genes encoding biosynthetic pathway step of a bioactive compound will be of use in enhancing pharmaceutical secondary metabolites production in microbes.

Role of metabolic engineering

Metabolic engineering that permits altering biosynthetic pathways offers new perspective on understanding the expression of genes involved in the biosynthesis of many plant pharmaceutical secondary metabolites through overexpression studies (Verpoorte and Alfermann 2000; O'Connor 2015). It exploits the understandings to (or "intending to") harnessing the productivity of in vitro cultures for higher yield. However, this needs an in-depth study of enzymatic reactions and biosynthetic processes at gene, transcriptomic and proteomic levels with emphasis on the regulation at various steps of production (Farre et al. 2014; Lu et al. 2016). One limitation is that overexpression of certain genes participating in a biosynthetic pathway may not necessarily improve production, as observed with some alkaloids (Oksman-Caldentey and Arroo 2000; Verpoorte

et al. 2002). Metabolic engineering also exploits the overexpression of genes that encode rate-limiting steps enzymes or overcoming the effect of feedback inhibition to enhance production (Oksman-Caldentey and Arroo 2000). Engineering the biosynthetic pathways enables modification of secondary metabolite structure to make novel molecules having improved therapeutic biological properties through bioactivity studies. By overexpressing genes encoding regulatory enzymes involved in a biosynthetic pathway(s), it is possible to improve productivity of the in vitro plant cell cultures (Verpoorte et al. 2002). Genes participating in the biosynthetic pathways of important alkaloids such as nicotine, scopolamine and berberine have been cloned, opening new way for their production through the application of metabolic engineering (Sato et al. 2001). However, complex physiological factors that are involved in biosynthesis of the metabolites through the target genes identification from experimental point view in application of metabolic pathways engineering and through reactions involved in their production needs elaborate and meticulate study for success to be achieved in their higher yield/production in plants in vitro cultures.

Metabolic engineering approach also uses inhibition of competitive pathways to increase metabolic flux of targeted biosynthetic pathway intermediates for higher production through variety of approaches. For instance, by blocking competitive branch of a monoterpene metabolic network for the conversion of pulegone to the menthofuran, an increased production of menthol could be achieved through developing an antisense gene for menthofuran synthase (Mahmoud and Croteau 2001). Certain metabolic steps in the biosynthetic pathway could be inhibited to facilitate accumulation of preceding intermediates. For instance, by reducing the expression of codeinone reductase through gene silencing and the use of chimeric small hairpin RNA construct, it was possible to facilitate accumulation of (s)-reticuline in transgenic *Papaver somniferum* at the expense of cordeine, morphine, thebaine and oripavine thus, increase production of the alkaloids (Allen et al. 2004). Similarly, production of a secondary metabolite can be increased by engineering its biosynthetic regulatory mechanism. By silencing *DET1* regulatory gene through the RNAi, it was possible to enhance apocarotenoid and flavonoids production (Davuluri et al. 2005). In *Catharanthus roseous*, upregulated expression of *ORCA3* resulted in an upregulation in the expression of genes participating in terpenoids indole alkaloids pathway with resultant three-fold enhanced accumulation of the alkaloids (van der Fits and Memelink 2000).

Recent advances in bioinformatic techniques offer approaches for application in unraveling molecular information about biosynthetic route of many plant secondary metabolites with possible insight about the most suitable strategies that could be employed to harness productivity of in vitro plant cell cultures (Gaosheng and Jingming 2012; Sharma and Shrivastava 2016). The most successful modern application was on the understandings of the general phenylpropanoyl biosynthetic pathway involved in the biosynthesis of many plant secondary metabolites, especially across different biota (Poulter et al. 1981; Rohmer 1999; Dudareva et al. 2005; Nanda et al. 2016). Application of biochemical molecular studies of metabolic pathways of phytopharmaceuticals through the use of molecular biology techniques such as Polymerase Chain Reaction, cDNA cloning and expression analysis of genes are regarded to have widen the scope of metabolic engineering into the pre-genomic era (Sharma and Shrivastava 2016) while analysis of response by the cultured cells to biosynthesis of the metabolites through proteomic studies and expressed sequence tags by bioinformatics analysis and application to the post-genomic era (Gandhi et al. 2015; Sharma and Shrivastava 2016; Paz et al. 2017).

Although metabolic engineering is regarded an alternative with possible practical application in producing the molecules as metabolic pathway can be targetted for engineering to enhance production, limitations as an understanding of the pathways is still a barrier to the achievement (Verpoorte et al. 1999; Oksman-Caldentey and Arroo 2000). Functional analysis of genes and their engineering to enhance productivity of in vitro plant cell cultures in response to application of the strategies is the current approach harnessed for successful application with view to understanding biosynthetic pathways of phytopharmaceuticals to (or "intending to") harnessing productivity. This may involve combining metabolome profiling with the genomic expression data evaluation to gain insight into biosynthetic regulation of the pharmaceuticals in the plants in vitro culture systems. For example, application of proteomic studies revealed the effectiveness of elicitation strategy in enhancing the biosynthesis of secondary metabolism in the cell cultures of *Maytenus ilicifolia* (Paz et al. 2017). High-value anticancer alkaloid CPT, though, having many natural sources (Malik and Laura 2014) is currently supplied to the pharmaceutical industry via exploitation of natural plant sources. Only few of the enzymes involved in its biosynthesis have been characterized (Tryptophan decarboxylase, geraniol 10-hydroxylase, secologanin synthase and strictosidine synthase), making application of metabolic engineering a distant reality. However, combination of metabolome and "omics" expression profile of the few known biosynthetic pathway enzymes could further our understandings about the regulation in plants *in vitro* cultures to (or "intending to") enhance production. For instance, comparison of transcriptomic data and metabolome analysis of *Ophiorrhiza pumila* hairy root and cell suspension cultures showed correlated metabolite profile. Transcriptomic profile revealed 3649 unique preferentially expressed unigenes in the hairy root with

an accumulation of most metabolic intermediates of CPT biosynthesis, chlorogenic and anthraquinones pathways in the metabolome data (Yamazaki et al. 2013). The approach seems interesting in identifying unique transcripts expressed during CPT biosynthesis in the plant in vitro cultures when combined with the metabolome profile (Higashi and Saito 2013). Therefore, in-depth studies of the biosynthetic pathways to identify rate-limiting steps and regulation along with bottlenecks on lack of clarity of the biosynthetic pathway of most economically important pharmaceutical plant secondary molecules that need to be produced at large-scale to meet industry demand in the present decade continues to hinder successful application of metabolic engineering. The development of transgenics is a promising step towards establishing high-yielding cell lines that could be upscaled for production of the pharmaceuticals in the plants *in vitro* cultures of many endangered sources (Yano and Takekoshi 2004; Thomas et al. 2011; Yao et al. 2015). Because plant secondary metabolites are produced as a defensive strategy and in response to stressful condition as the one imposed by the in vitro culture condition to cultivated plant cells, it will be interesting to study how cultures respond through genomic expression and regulation at transcriptomic and proteomic levels of the genes participating in metabolic pathway(s), especially in response to various strategies applied for enhancing yield that is getting attention in the recent. This could facilitate practical application of metabolic engineering for optimized production of the pharmaceuticals in the plants in vitro cultures as accumulation of a bioactive compound can be established by genome-wide analysis, especially when combined with transcriptomic, proteomic studies or other "omic" approaches during biosynthesis of the metabolites.

Case study of camptothecin production

Camptothecin is a monoterpene indole anti-cancer alkaloid that was first isolated in *Camptotheca acuminata* and later in many plants (mostly woody) and endophytes with greater of them from India (Puri et al. 2005; Rehman et al. 2008; Amna et al. 2006, 2012; Ramesha et al. 2013; Malik and Laura 2014). CPT and other terpenoid indole alkaloids are derived from their precursor strictosidine which is formed via condensation of secologanin and indole tryptamine in reaction catalyzed by strictosidine synthase (Kutchan 1995; Lu et al. 2009). Tryptamine is synthesized through the shikimate pathway while secologanin from the terpene biosynthetic pathway (Yamazaki et al. 2004; Kai et al. 2014). The MVA and MEP pathways provide common terpene precursor for the biosynthesis of the alkaloid (Kai et al. 2014) and strictosidine is transformed into strictosamide. Remaining steps involved in the CPT biosynthetic pathway are not yet understood.

Camptothecin annual demand was reported to have reached US$ 2.2 billion in the year 2008 with the increase projected to be higher in the future (Sankar 2010). The threatened status of the natural sources and difficulty in the chemical synthesis of the alkaloid and its derivatives coupled with raising plantations are other challenges in its production. Due to its cytotoxicity and poor natural solubility, many of the semi-synthetic analog drugs developed through structural modification of CPT overcame these limitations in the human body. Currently, pharmaceutical industrial production of CPT analogs relie on sourcing of the alkaloid from field-grown plants. Production by the application of biotechnological strategies and approaches is yet to reach commercial exploitation but, in vitro clonal production for mass propagation and using biosynthesis enhancement strategies offer alternatives in production of the alkaloid. Although metabolic engineering offers an alternative to engineering biosynthetic pathway of CPT in plants and endophytes, limitations on its poor understanding is an impediment to the application (Kai et al. 2015). Thus, PCTOC technique is the only biotechnological strategy that could be explored to produce CPT and mitigate overexploitation of the natural sources (Malik and Laura 2014; Isah and Mujib 2015a, b). Production of the alkaloid from the in vitro cultures of natural plant sources have been reported from differentiated and undifferentiated tissues (Kai et al. 2015) with *N. nimmoniana* regarded most convenient for large-scale production of the alkaloid (Isah and Mujib 2015a, b). Among plant sources of CPT, the most successful production in the plant in vitro cultures was in *Ophiorrhiza pumila* where scale-up using hairy root cultures was achieved in 3L-capacity bioreactor with productivity of 8.7 ± 1.3 mg CPT L^{-1} (Sudo et al. 2002). Since *N. nimmoniana* is regarded most convenient natural source for large-scale isolation of the alkaloid, effort towards bioprospecting and production in the in vitro cultures was achieved using differentiated, undifferentiated tissues and root cultures of the species (Padmanabha et al. 2006; Suhas et al. 2007; Ramesha et al. 2008; Isah and Mujib 2015a, b) and few of the strategies discussed in this review applied (Karwasara and Dixit 2013; Fulzele et al. 2015; Isah 2017). A study on the application of elicitation strategy in the production of CPT using yeast extract and vanadyl sulfate elicitors by the use of solid and liquid media yielded maximum four-fold enhanced productivity in the elicited callus cultures (Isah 2017). The yield of the alkaloid varied among cultivated cell lines, based on the culture duration, and cell suspension cultures proved better in the overall productivity. Media browning and alkaloid leaching into the culture media were the common problems that need to be overcomed when developing

scale-up production system of CPT in bioreactor by the application of the discussed strategies in this review, so as to achieve optimal biomass and CPT production in *N. nimmoniana* and other natural plant and endophyte sources of the alkaloid.

Conclusion and prospects

The discovery of most plant pharmaceutical natural products that have found application in drugs production (e.g., Taxol and Camptothecin) coincided with the 'birth' of PCTOC techniques, and soon potential application was recognized. Application of the technique played historical landmark role in the anti-cancer drugs development from taxol that is isolated from Pacific yew trees. However, successful large-scale production using the technique to produce the taxol and other pharmaceuticals was not realized until decades later. The realization of the potentials and possible achievements when PCTOC technology is employed in their production lead to development and application of in vitro production strategies to boost productivity of cultures to a higher yield and ameliorate overharvest of endangered plant sources of the molecules. However, challenges in the application of PCTOC techniques to produce the metabolites includes species-dependent yield that in turn depends on in vitro culture condition(s), cultivation factors (internal and external) and the strategies employed. Thus, for a species, many years of labor may be needed to make any achievement possible. This is the basis through which current understandings of plant secondary metabolism of pharmaceuticals in the plant in vitro cultures evolved. Studies on the productivity response regarding the production of biomass and secondary metabolites in response to application of these strategies are still needed, especially gene expression changes, given the poor understanding of the biosynthetic pathways gene expression and regulation about production in the in vitro plant cell, tissue and organ cultures. New strategies that facilitate rapid understanding of metabolic processes in the cultures are also needed so as to exploit the maximum productivity gains given the fast rate at which plant biota is disappearing due to overexploitation for the molecules.

Although metabolic engineering (less exploited compared to other strategies), production from endophytes and PCTOC are employed and seems promising in large-scale production of the pharmaceuticals to meet industrial need for the manufacture of drugs, limitations in understanding the biosynthetic culture condition regulators and pathways is an impediment to exploiting maximum productivity gains in the cell, tissue and organ cultures of most plant sources of the pharmaceuticals. Recent interest in the genomic expression regulation in the plant in vitro cultures (epigenetic modifications in particular induced by the culture conditions) and application of various strategies on the biosynthesis of the pharmaceuticals seems interesting in understanding biosynthetic expression of phytopharmaceuticals but, are poorly explored. Application of metagenomics, transcriptomics, proteomics and phenomics are crucial in the identification of genes, enzymes and metabolites of secondary metabolism pathways to facilitate engineering for higher yield of the pharmaceuticals in the plants in vitro cultures. Advances in modern molecular biology techniques and tools when exploited could provide an avenue for exploring maximal application of metabolic engineering of biosynthetic intermediates with possibility to develop high-yielding cell lines and their application in the cell, tissue and organ cultures scale-up cultivation for industrial application in producing biomass and the pharmaceuticals, especially the costly anti-cancer agents.

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Compliance with ethical standards

Conflict of interest The authors declare that no conflict of interest exists in the manuscript contents.

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