17 Biotechnological Application of *Meta*-topolins as Highly Active Aromatic Cytokinins in Micropropagation of Medicinal Plants

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Abstract

Medicinal herbs and plants are the major reservoirs for new drug formulations. Owing to pharmaceutical demands and limited wild resources, it is vital to conserve genetic diversity, which is continuously under the risk of extinction due to its overexploitation which resulted in natural habitat abolition and unmonitored medicinal plant trade. To deal with this major havoc, plant biotechnological techniques and approaches such as plant tissue culture and micropropagation

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hold promise for conservation and rapid mass multiplication to obtain genetically elite (true to type) populations under in vitro conditions. The most critical factor that has an effect on in vitro plant propagation success is the choice and selection of plant growth regulators (i.e., auxins and cytokinins). Plant growth regulators are often supplemented in the nutrient medium to harness the totipotent potential such as development of shoots, roots and whole plants. Most commonly used cytokinin in plant tissue culture micropropagation protocols is N^6-benzyladenine (BA), but nowadays *meta*-topolin as highly active aromatic cytokinin along with its derivatives have been largely investigated and proved as an effective alternative to BA in micropropagation protocols of medicinal plants. This chapter addresses the biotechnological application of *meta*-topolins such as their effect on in vitro plant regeneration, micropropagation and hardening, countering micropropagation troubles and its influences on secondary metabolite production.

Keywords

Plant growth regulators · Micropropagation · Secondary metabolites · Cytokinins · Topolins · Metabolism

17.1 Introduction

Secondary metabolites/bioactive compounds present in medicinal plants are currently being widely expoited commercially at large scale and play vital role in high-value product development in the pharmaceutical industry. Due to continuous demand and the over-exploitation of medicinal plants/herbs in general, the Himalayan plants/high-altitude medicinal plants are facing threat of extinction with a reduced distribution range. In addition, various biotic and abiotic stress factors, loss in seed viability, poor seed germination and survival percentage also affect the overall growth and development of these plants (Jan and Abbas 2018; Kumar et al. 2020). Biotechnological applications such as plant tissue culture (conservation and propagation of valuable, rare and endangered plants), cell culture and tissue engineering (mass production of bioactive constituents via callus or suspension cultures), genetic engineering and pathway and metabolic engineering (for genetic makeup modification for higher biomass production) is playing a significant role in high quality plant material supply and value addition to harness potential applications.

For the cultivation of medicinal plants comprehensive understanding of the species biology is required. For in vitro plant propagation, detailed standardization and optimization procedures (phytohormones, pH, temperature, aeration, agitation, light, etc.) are required for culture initiation, growth, multiplication and acclimatization (Briskin 2000; Harsh et al. 2017; Kumar et al. 2017; Jan and Abbas 2018). Once the reliable, genetically stable and highly efficient in vitro plant regeneration protocol has been optimized, the crucial step is successful

acclimatization and hardening of tissue culture raised plants. Hardening of in vitro raised culture depends on optimization of humidity, light, temperature and other environmental conditions approximating the natural environment of the cultured plant species. Re-introduction of in vitro raised plants in their natural habitat is the most critical step in plant conservation strategies and dependes on a successful acclimatization procedure (Chaturvedi et al. 2007). The process however, is time-consuming, tedious and requires extensive effort. These factors are a major obstacle in large-scale quality plant production (Harsh et al. 2017; Kumar et al. 2017).

Micropropagation is an important plant biotechnological process in which excised plant parts (explant) such as cells, tissues or organs from a particular plant, are surface sterilized and cultured on an appropriate nutrient medium to produce a large number of clonal plantlets under in vitro conditions (Kumar and Srivastava 2016). This is a valuable tool for selecting, multiplying and screening promising genotypes of Himalayan medicinal plants. Development of an efficient protocol for in vitro culture establishment is a vital procedure in plant biotechnology xhich exploits the totipotent nature of plant cells. Among the various cytokinins used in plant tissue culture protocol optimization, N^6-benzyladenine (BA) is the most commonly used. However, use of topolins and their derivatives as an alternative to BA N^6-shows efficacy in standardizing high frequency shoot and root regeneration protocols and countering various micropropagation problems arising under in vitro conditions (Werbrouck et al. 1996).

Meta-topolins (*m*T) is a hydroxylated BA analogue with the following chemical formula: 6-(3-hydroxybenzylamino)purine. It is a relatively new cytokinin with an aromatic ring but similar to other cytokinins in its metabolism. The N^6-benzyl ring of *meta*-topolins have a hydroxyl group which can lead in O-glucoside metabolite formation similar to zeatins. Although topolins and their derivatives have been identified and distinguished in various plant species, their main source is poplar leave. From the discovery of cytokinins plant tissue culture grew rapidly and exponentially in the field of their relevance. Cytokinins are indispensable group of plant growth regulators (PGRs) undeviating physiological processes, which have a distinctive attribute for promoting cell division during micropropagation (Miller et al. 1955; Heyl and Schmulling 2003) during micropropagation. This group of PGRs affects various developmental and physiological processes such as the regulation of root and shoot growth, chloroplast development, stress response, pathogen resistance and leaf senescence. Cytokinins are mainly classified into two major groups that is, naturally occurring cytokinin and synthetic ones. The former are mainly adenine derivatives and further classified on the basis of adenine N^6-side chain with an aliphatic side chain (isoprenoid group) and aromatic side chain (aromatic group) (Strnad 1997). Synthetic cytokinins have a phenylurea group such as thidiazuron (N'-phenyl-N'-l, 2,3-thiadiazol-5-yl urea) and CPPU (*N*-(2-chloro-4-pyridyl)-N'-phenylurea) (Mok and Mok 2001). The goal of micropropagation is the mass multiplication of homogeneous, true to type nature (regenerants) and healthy plantlets development. However, a large number of physiological disorders and in vitro abnormalities have been encountered during micropropagation protocol optimization such as low shoot multiplication rate,

hyperhydricity, poor root regeneration, high production cost, underdeveloped growth and epigenetic and somaclonal variation (Smulders and de Klerk 2011). These are some of the factors that challenge the micropropagation of most plants, and for these reasons, commercial application of plant tissue culture in many plant species is also reduced. These problems can be alleviated by optimizing hormonal regulation (auxin to cytokinin ratio), using appropriate plant growth regulator (type and concentration) and by standardizing various factors which affect the in vitro morphogenic potential during efficient micropropagation protocol development. To solve this, the efficacy of topolins as naturally occurring highly active aromatic cytokinins has been highlighted in plant tissue culture systems as it has emerged as a viable choice to other cytokinins used such as N^6-benzyladenine, kinetin, and *trans*-zeatin in plant tissue culture.

Some studies have also described topolins and their substitute's role either alone or in combination with auxins in comparison to BA. *Meta*-topolin (*m*T) was reported to be very effective in high-frequency shoot regeneration and secondary metabolite production compared to BA in the case of *Huernia hystrix*, a medicinal plant. *m*T also helped solve the problem of vitrification when media was supplemented with *m*T (2.5 mg/L) and NAA (0.1 mg/L) and adenine sulfate (50 mg/L) in *Withania coagulans* (Stocks) Dunal, medicinally important plant of Solanaceae family (Joshi et al. 2016). Therefore, it can be concluded that using *m*T as an alternative to other cytokinins possibly will go ahead for remarkable change in the growth and developmental control of micropropagated plants.

17.2 *Meta*-topolins' Effect on In Vitro Plant Regeneration and Hardening During Micropropagation

Cytokinins promote plant cell division and cell differentiation processes and influence physiological aspects such as leaf senescence and chlorophyll accumulation; thus shoot formation and quality of the explant obtained with in vitro culture are highly dependent on type and concentration of cytokinins used (Mok and Mok 2001; Haberer and Kieber 2002; Sakakibara 2006). Zeatin is a naturally occurring cytokinin, but BA is cheaper than others and the most effective cytokinin in promoting in vitro shoot regeneration and proliferation. For this reason, it is currently used in commercial laboratories. However, it was also discovered that this cytokinin can also induce physiological disorders in some plant species (Amoo et al. 2011; Werbrouck 2010). In order to resolve these issue, new investigations should be done to find alternative potent cytokinins, i.e., alternative to BA, most widely used cytokinin in plant tissue culture, which could be able to maintain efficient shoot multiplication rates and production of high-quality planting material. Use of *m*T is gaining interest day by day because it leads to efficient micropropagation (Bairu et al. 2007), improved biochemical and physiological traits (Mala et al. 2013), efficient vitro root regeneration, successful acclimatization and hardening (Aremu et al. 2012a).

The main prerequisite of micropropagation/in vitro plant regeneration is to have a reliable, efficient, and high-frequency regeneration protocol, i.e., efficient healthy shoots and root regeneration and successful acclimatization and hardening in the field. Topolins have been used as promising alternatives to other cytokinins in in vitro regeneration and micropropagation. There are reports suggesting *meta*-topolin (*m*T) is nearly twice as effective as BA in multiple shoot induction in a number of plant species. Use of topolins in medicinal plant micropropagation has been increasing at a rapid rate (Werbrouck 2010; Aremu et al. 2012a). Werbrouck et al. (1996) observed healthy shoot and root regeneration response when medium was supplemented with equimolar concentration (10.0μM) of *m*T and BA during *Spathiphyllum floribundum* micropropagation. In earlier studies, comparable effects of *m*T cytokinins on efficient in vitro regeneration of sugar beet (Kubalaková and Strnad 1992), *Curcuma longa* (Salvi et al. 2002), *Aloe polyphylla* (Bairu et al. 2007), and banana cv. Williams (Bairu et al. 2008) have been investigated. There is a number of reports which showed that when *mT* is used in place of BA, there was remarkable improvement in the mass in vitro propagation (Kubalaková and Strnad 1992; Strnad et al. 1997). Bairu et al. (2007) reported that *meta*-topolins resulted in a greater number of shoots in *Aloe polyphylla* micropropagation compared with other cytokinins such as BA and *trans*-zeatin at different concentrations. Use of kinetin riboside (KR), N^6-isopentenyladenine (iP) or *meta*-topolins (*m*T) in plant micropropagation, i.e., *Curcuma longa* revealed a maximum in vitro shoot regeneration response compared to other cytokinins used such as BA (Salvi et al. 2002). Different concentrations of aromatic cytokinins such as *meta*-topolin riboside (7.5μM), *meta*-methoxytopolin (15μM), and *meta*-methoxytopolin riboside (30μM) showed maximum shoot proliferation rates versus BA in banana cv. Williams (Bairu et al. 2008). From the available literature it is reported that *m*T was found more effective than other cytokinins such as BA for efficient shoot regeneration and proliferation in longa (Salvi et al. 2002), *Musa* spp. (Escalona et al. 2003; Bairu et al. 2007); *Curcuma* and *Aloe* spp. (Bairu et al. 2008), *Pelargonium* cultivars (Wojtania 2010), and *Huernia hystrix* (Amoo et al. 2013). However several adverse effects on shoot multiplication were also observed in a number of plant species such as *Vaccinium corymbosum* (Meiners et al. 2007), wild service tree (*Sorbus torminalis* L. Crantz) (Malá et al. 2009), and in *Citrus* hybrid (Niedz and Evens 2010).

Bairu et al. (2007) described *m*T as a more potent cytokinin over BA for efficient, high frequency in vitro shoot as well as root regeneration. It also resulted in reduced hyperhydricity with improved ex vitro acclimatization of *Aloe polyphylla*. These results are in accordance with investigation of Nacheva et al. (2017). These authors observed that substituting BA with *m*T remarkably improves the in vitro lateral bud induction and shoot multiplication and ensured commercial propagation in *Ginkgo biloba* plants. Amoo et al. (2015) investigated a novel aromatic cytokinin (CK) derivative, i.e., *meta*-topolin-tetrahydropyran-2-yl (*m*TTHP) effects for efficient micropropagation in *Merwilla plumbea*. *Merwilla plumbea* (Lindl.) Speta is an important medicinal bulbous plant belonging to the family Asparagaceae and in much demand in the medicinal plant market of South Africa (Williams et al. 2013).

Meta-topolin derivatives, i.e., *meta*-topolin riboside and *m*TTHP treatments resulted in improved shoot regeneration with highest adventitious shoot response over the control and thidiazuron growth regulator used. Maximum root regeneration response was also observed in *m*TTHP treatments. It also also resulted in significantly greater photosynthetic efficiency and increase in antioxidant enzyme activities.

Lata et al. (2016) *m*T as a potent aromatic cytokinin in *Cannabis sativa* L. micropropagation. *Cannabis sativa* is a high value medicinal plant of the Cannabaceae family having bioactive compounds, namely tetrahydrocannabinol (9-THC) and cannabidiol (CBD). Cannabidiol (non-psychoactive) and tetrahydrocannabinol (psychoactive) molecules have a number of potential medicinal applications such as epileptic seizures treatments in children (Pertwee 2014). Using *meta*-topolin (*m*T) as a potent highly active aromatic natural cytokinin, an efficient, reliable, simple, and cost effective one step regeneration system for high frequency shoot and root regeneration was optimized in *Cannabis sativa* from nodal explants. Highest shoot regeneration frequency as mean number of shoots was observed on Murashige and Skoog (MS) medium containing 2μM *m*T. Shoot multiplication and proliferation was also noted on the same regeneration medium and it was also able to induce a healthy root system within 4–6 weeks. For root induction, the medium was not supplemented with any kind of plant growth regulator. Remarkable achievement with 100% survival rate was observed during in vitro regenerated plantlets hardening and acclimatization. Genetic stability studies to ensure true type regenerants were also tested and confirmed using inter simple sequence repeat (ISSR) molecular markers. Cannabinoid profiles and content were found similar to each other in in vitro regenerants and in mother plant, when analyzed qualitatively and quantitatively by using gas chromatography-flame ionization detector (GC-FID). Finally, it was concluded that for large scale production of true to type *C. sativa* plants *m*T and its derivatives are an appropriate alternative being both highly potent and effective.

17.2.1 *Meta*-topolins Effect on In Vitro Rooting of Medical Plants

Auxins play a major role in in vitro rooting, but there is some evidence that cytokinins are also important. It has been found out that *m*T raised microshoots show higher rooting frequency than those raised on BA medium. Further, *m*T raised micro shoots were longer and healthier than BA raised plantlets. Aremu et al. (2012a, b) and Naaz et al. (2019) showed significant improvement in micropropagation of *Syzygium cumini* and their acclimatization to ex vitro conditions when supplemented with *m*T. Increase in root regeneration response in *m*T raised microshoots was obtained in *Uniola* (Aremu et al. 2012a), *Aloe polyphylla* (Bairu et al. 2007) and *Pelargonium* × *hortorum* (Mutui et al. 2012). Similarly, Bairu et al. (2007) observed efficient root regeneration response on micropropagated shoots of *Aloe polyphylla.* These effects were observed only on multiplication medium which had either *m*T or its riboside (*m*TR). In, *Solanum*

tuberosum cv. Jaerla plantlets, root regeneration was increased significantly at low concentrations (5–10μg.L^{-1}) of *m*TR in culture medium (Baroja-Fernandez et al. 2002). There are also reports which show that *m*T raised regenerants have a higher number of healthy roots compared to BA regenerants. For this reason, these plantlets were more resistant to environmental stresses in field conditions. However, an inhibitory effect on in vitro root regeneration response was also observed in *Musa* spp. (cv. Williams) as compared to BA (Bairu et al. 2008). Similarly, it has been reported that increased concentrations of BA (1.33μM) and *m*T (22.2μM) resulted in root regeneration reduction on plantain cv. CEMSA 3/4 (Escalona et al. 2003). However, the highest number of roots was found on 1.33μM *m*T. As topolins showed both positive and detrimental results on in vitro rooting, much research investigation is still needed for the study of topolin effects in root development biology.

17.3 *Meta*-topolins Influences on Secondary Metabolite Production

Secondary metabolites are organic compounds that help plants to cope with different environmental stresses, i.e., biotic and abiotic. Secondary metabolites are an economically very important class of compounds because they can be utilized as insecticides, colourants, antimicrobials, fragrances and therapeutics. The production of secondary metabolites is triggered in only specific stages of growth and development or during various stresses (biotic and abiotic) or the constraint of supplements. The extraction and purification of secondary metabolites are complex and arduous their production only occurs under certain environmental conditions and depends upon an individual species and genus. For these reasons, commercially available secondary metabolites, for example, pharmaceuticals, flavours, colours, antimicrobials, fragrances and pesticides are of higher value than primary metabolites (Nielsen et al. 2019).

Biotechnology emerges up as a new boom for secondary metabolite production by the use of in vitro regenerating cells, tissues, organs or entire organisms and use of advanced genetic engineering tools for desired modification and desired bioactive ingredients (Rao and Ravishankar 2002). Thus, in vitro regeneration has enormous potential in the control of plant bioactive molecule production. Different plant tissue culture approaches including cell and tissue culture, organ, callus, suspension, and hairy root cultures are used for secondary metabolite production. The application of an in vitro cell culture systems for the production of therapeutic compounds has made conceivable the manufacturing of a wide range of pharmaceuticals, such as amino acids, phenolics, flavonoids, terpenoids, saponins, alkaloids and steroids (Paric et al. 2017).

The biosynthesis of secondary metabolites through plant tissue culture depends upon various factors such as plant growth regulators, type of cell line and explants, environmental factors (temperature, humidity) and elicitors. Out of all these, plant growth regulators play vital role in growth and secondary metabolite biosynthesis. The optimum concentrations and combinations of plant growth regulators like

auxins and cytokinins must be used in nutrient media to enhance the development as well as regulation of cell metabolism (Filova 2014). The exogenous application of different types and concentrations of aromatic cytokinins during plant tissue culture especially impacts the in vitro generation of bioactive secondary compounds. Cytokinins act as enhancers for the metabolism of secondary compounds and play a vital role in cyto-differentiation and sub-cellular differentiation (Jain et al. 2012). They also assist cell division and initiation of callus growth and development. Cytokinins have diverse impact on secondary metabolite production and this depends on type of metabolites and species used. For example, kinetin triggered anthocyanin production in *Haplopappus gracilis* whereas it inhibits anthocyanin synthesis in *Populus* cell cultures. Total phenolic and flavonoid content was increased in *Thymus vulgaris* and *Origanum vulgare* using BA and indole-3-butyric acid (IBA), however, it was decreased in *Ocimum basilicum* (Karalija et al. 2016). Similarly, in *Mentha piperita* us of BA alone resulted in increased essential oil yields as well as its components as menthone, menthol, pulegone and menthofuran, whereas BA in combination with IBA had no significant effect on production of essential oils (Santoro et al. 2013). Cytokinins also stimulate alkaloid biosynthesis in some tumor cell line (Rhodes et al. 1994).

In recent decades, topolins, especially *m*T have emerged as one of the important cytokinins in various micropropagation protocols. They have demonstrated promising outcomes regarding growth and development and for overcoming various physiological disorders that have been encountered under in vitro system. Normally, *m*T invigorated higher secondary metabolite production compared to other cytokinins. *m*T even at very low concentration resulted in higher production of secondary metabolites in some plant species. The use of *m*T resulted in higher proliferation of callus and shoots which was further linked with increased bioactive compound accumulation. The foremost reason for the prevalence of *m*T over other cytokinins such as BA has been due to its confined accumulation in plant tissues and due to its faster transformation rate (Kaminek et al. 1997). This aside, the metabolic final product is effectually degradable and *O*-glucoside metabolites are considered as the cytokinin storage form, which is stable under specific conditions. However, this is rapidly converted into active cytokinin bases when needed as a result of hydrolyzation of glucose in the *m*T side chain (Bairu et al. 2009). The reversible sequestration of the *O*-glucosides allows for unlimited accessibility of cytokinins over a longer period at a physiologically active level resulting in a higher rate of shoot multiplication in plant tissue cultures (Strnad 1997).

In some plant species, a higher callus growth rate and adventitious shoot regeneration within subsequently increased secondary compound production were observed using mT over other cytokinins. For example, the topolins, especially *m*T and *meta*-methoxytopolin 9-tetrahydropyran-2-yl increased the total phenolics, total flavonoids and proanthocyanidin content in subsurface and aerial parts of micropropagated banana plantlets compared to BA (Adeyemi et al. 2012). In general, secondary metabolites such as phenolics and flavonoids play a fundamental role during plant micropropagation. The increased flavonoids content revealed improved ex vitro root regeneration in differentiated shoots. A significantly higher

level of total phenolics, flavonoid, iridoid and antioxidant activity was observed in *Aloe arborescens* shoots regenerated using *m*TR containing medium compared to media containing other growth regulators (Amoo et al. 2013). Thus, the utilization of topolins may be a viable approach for stimulating the biosynthesis of important secondary metabolites of value to the pharmaceutical, food and agrochemical industry.

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