# Use of *Meta*-topolin in Somatic Embryogenesis

14

Carolina Sánchez-Romero

## Contents

14.1 Introduction .... 188
14.2 Somatic Embryogenesis .... 189
14.2.1 Phases of Somatic Embryogenesis .... 189
14.2.2 Applications of Somatic Embryogenesis .... 191
14.3 *Meta*-topolin and Somatic Embryogenesis .... 193
14.3.1 Embryogenic Cultures Initiation .... 193
14.3.2 Proliferation of Embryogenic Cultures .... 194
14.3.3 Somatic Embryo Development and Maturation .... 196
14.3.4 Embryo Germination .... 198
14.4 Concluding Remarks and Future Prospects .... 199
References .... 200

**Abstract**

Somatic embryogenesis is the developmental process through which a somatic cell or group of somatic cells give rise to an embryo, capable of developing into a whole plant. Somatic embryogenesis is a powerful in vitro technique, with multiple applications, including plant breeding by conventional or biotechnological means. Technologically, it is carried out through a sequence of in vitro culture steps. Although different physical and chemical factors influence the process, plant growth regulators play an essential role.

Cytokinins are one of the most important hormones regulating somatic embryogenesis, and, although hormone requirements vary greatly in different experimental systems, cytokinins are frequently included in different phases of this developmental process. Since their discovery as naturally occurring

C. Sánchez-Romero (✉)
Departamento de Botánica y Fisiología Vegetal, Universidad de Málaga, Málaga, Spain
e-mail: c.sanchez@uma.es

© Springer Nature Singapore Pte Ltd. 2021



N. Ahmad, M. Strnad (eds.), *Meta-topolin: A Growth Regulator for Plant Biotechnology and Agriculture*, https://doi.org/10.1007/978-981-15-9046-7_14

cytokinins, *meta*-topolin and some of its derivatives have been increasingly used in plant tissue culture. Their use was initially limited to shoot multiplication, rooting, and seed germination, and only recently, this plant growth regulator has been applied to somatic embryogenesis protocols.

In the present chapter, the use of *meta*-topolin and its derivatives in somatic embryogenesis is revised. I examine their effects in the different phases of the somatic embryogenesis process and compare the results with those of other cytokinins.

**Keywords**

Biotechnology · Cytokinin · *Meta*-topolin · Plant growth regulators · Regeneration · Somatic embryogenesis

## 14.1 Introduction

Cytokinins are a group of plant hormones with an essential role in promoting cell division, a central process in plant growth and development. They are also involved in other physiological and developmental processes, including leaf senescence, apical dominance, formation and activity of apical meristems, promotion of sink activity, vascular development, and breaking of bud dormancy (Taiz et al. 2015).

Structurally, cytokinins can be divided into two main groups: adenine-type and phenylurea cytokinins. Adenine-type cytokinins can in turn be classified as aromatic or isoprenoid cytokinins, according to the nature of their $N^6$-side chain (Beyl 2011).

All the natural cytokinins are adenine derivatives, while phenylurea cytokinins are synthetic compounds exhibiting cytokinin activity, which have not been identified in plants (Bogaert et al. 2006; Taiz and Zeiger 2010).

*Meta*-topolin (*m*T) ($N^6$-(*meta*-hydroxybenzyl)adenine) is a naturally occurring aromatic cytokinin, which has been identified in different plant species (Strnad 1997; Beyl 2011). Chemically, *m*T is a hydroxylated $N^6$-benzyladenine (BA) analog, differing from BA only by the presence of an extra OH group in the *meta*-position on the aromatic ring of BA (Werbrouck 2010). A number of *m*T derivatives have also been identified in different plants species: 6-(3-methoxybenzylamino)purine (*meta*-methoxytopolin, Me*m*T), 6-(3-methoxybenzylamino)purine-9-riboside (Me*m*TR), 6-(3-fluorobenzylamino)purine (3FBA), and 6-(3-fluorobenzylamino) purine-9-riboside (3FBAR) (Bogaert et al. 2006).

As reported by Aremu et al. (2012), since the discovery of *m*T and its derivatives as natural aromatic cytokinins, their use in plant tissue culture has increased rapidly. Positive results have been obtained in different in vitro culture processes, such as shoot multiplication, rooting, and seed germination, improving shoot quality, shoot length, number of leaves, shoot dry weight, shoot/root ratio, and acclimatization competence. Interestingly, multiple investigations reported on a corrective role in some physiological disorders including abnormality reduction, hyperhydricity and shoot tip necrosis alleviation and delayed senescence (Aremu et al. 2012). In line with this, Solórzano-Cascante et al. (2018) reported that *m*T has less negative

carryover effects, i.e., residual effects of the compound beyond the time period in which it was present.

Thus, *m*T has proved to be valuable in micropropagation systems and can be considered a suitable alternative to cytokinins traditionally used for this purpose. However, few investigations have described the use of this plant growth regulator (PGR) in somatic embryogenesis (SE). In the review of Aremu et al. (2012) on the application of topolins in plant tissue culture, no information is included on the utilization of *m*T in SE. However, this plant hormone has subsequently been repeatedly utilized in studies aiming to optimize SE protocols.

In the present chapter, the use of *m*T in SE is examined. The investigations in which this PGR or some of its derivatives have been used in some SE phases are revised, and their effects are compared with those of other cytokinins.

## 14.2 Somatic Embryogenesis

Somatic embryogenesis is the process through which differentiated somatic cells, single or in small groups, change their developmental program, giving rise to embryogenic cells. These embryogenic cells then follow a differentiation pathway to generate an embryo, which can further develop into a whole plant (Zimmerman 1993; Guan et al. 2016).

### 14.2.1 Phases of Somatic Embryogenesis

Although the initial morphological patterns of somatic embryo formation can be quite different from those of zygotic embryos and difficult to categorize (Elhiti and Stasolla 2016), subsequent steps followed in somatic embryo development are similar to those of zygotic embryos. Thus, in dicots, further development occurs through the typical consecutive stages, namely, globular, heart, torpedo, and cotyledonary (Winkelmann 2016).

In practice, SE is executed through a sequence of in vitro culture steps. Usually it includes induction of embryogenic cultures, proliferation, development and maturation of somatic embryos, and germination.

Induction of embryogenic cultures refers to all events that reprogram a differentiated somatic cell into an embryogenic cell (Winkelmann 2016). This process, which has recently been divided into different phases, i.e., dedifferentiation, acquisition of totipotency and commitment into embryogenic cells (Elhiti et al. 2013), implies a complete reorganization of the cellular state, including physiology, metabolism, and gene expression (Fehér et al. 2002).

Induction of embryogenic cultures can be accomplished through two pathways: direct SE, when somatic embryos arise directly from the initial explant, and indirect SE, when embryogenic differentiation is preceded by a phase of unorganized growth (Bhojwani and Razdan 1996).

Once established, embryogenic cells can be maintained in the embryogenic state under adequate culture conditions (Bhojwani and Razdan 1996). Proliferation of embryogenic cultures by repetitive subcultures allows multiplication of the original plant material (Lelu-Walter et al. 2013), an important quality of SE.

Embryogenic culture proliferation can occur by formation of proembryogenic masses, i.e., localized groups of meristematic cells (Bhojwani and Razdan 1996), or by secondary embryogenesis, a recurrent process in which new somatic embryos develop from previously existing embryos.

Maintenance of embryogenic cultures can be performed in both solid and liquid medium. Culture in liquid medium enables culture synchronization and multiplication at higher rates than on solid medium (von Arnold et al. 2002). It also permits scaling up in bioreactors and automation of the process (Egertsdotter et al. 2019).

Development and maturation of somatic embryos are usually induced in a separate culture phase by appropriate modifications of culture conditions. During this step, the developmental program switches from a proliferative pathway to a highly organized phase, in which somatic embryos arise and advance through successive developmental stages. Both events occurring during this phase, histodifferentiation and storage product accumulation, largely determine the quality of the obtained embryos and, consequently, the final performance of the SE process.

Finally, somatic embryos are induced to germinate, normally under culture conditions similar to those used for conversion of zygotic embryos. Development of root and shoot gives rise to a somatic plantlet that may exhibit the same characteristics as seedlings (Lelu-Walter et al. 2013).

To execute this developmental pathway efficiently, a number of critical physical and chemical treatments should be applied timely (von Arnold et al. 2005). Although hormones are not the only factors controlling SE, as in other morphogenic events in vitro, addition of PGRs to culture medium is the preferred way to manipulate SE (Jiménez 2005). According to Loyola-Vargas and Ochoa-Alejo (2016), these compounds regulate multiple genes temporally and spatially, which cause the changes in the genetic program of somatic cells and regulate the transition between each embryonic developmental stage.

Although there is no single mechanism for executing each SE step and large variability is found, some generalizations can be made. Thus, it is well established that auxin plays an important role in SE, both in induction of embryogenic cultures and in the subsequent elaboration of the proper morphogenetic events during embryo development (Karami et al. 2009). However, there are also species in which cytokinins, alone or in combination with auxins, induce SE (Altamura et al. 2016). According to Jiménez (2005), the importance of both auxin and cytokinin in the determination of embryogenic response can be explained by their determinant participation in cell division and cell cycle regulation. Nevertheless, in some species, other PGRs such as abscisic acid (ABA), gibberellins, or ethylene have been found to induce SE (Jiménez 2005) or SE has been initiated without hormone supplement.

Proliferation of embryogenic cultures is usually performed in culture media similar to those used for SE initiation (von Arnold 2008), although hormones are generally added at lower concentrations. Auxin is the main factor associated with

proliferation and plays an important role in inhibiting development of proembryogenic masses into somatic embryos (von Arnold 2008). Nevertheless, cytokinins and auxin-cytokinin combinations have also been utilized in multiple cases (Jiménez 2005).

According to the role of auxin inhibiting somatic embryo development, development and maturation of these structures have commonly been addressed by reducing or removing this hormone from culture medium. In fact, this phase is normally induced in culture media lacking PGRs. Nevertheless, addition of a cytokinin, alone or in combination with an auxin, has been found to be beneficial for embryo development in some species (Jiménez 2005). In conifers, ABA is used to promote somatic embryo maturation, but its role on this phase is not clear in other plant groups (von Arnold et al. 2002).

Somatic embryo germination is usually induced in culture media without hormonal supplement, although auxins and cytokinins can promote this process (von Arnold 2008). In some cases, gibberellins, alone or in combination with cytokinins, have also been added to convert somatic embryos into plants (Jiménez 2005).

### 14.2.2 Applications of Somatic Embryogenesis

SE has multiple applications in different fields, such as basic research, genetic improvement, and commercial plant production.

As previously indicated, although the initial steps of somatic and zygotic embryogenesis present important differences, developing somatic embryos pass through stages similar to those described in zygotic embryos (Elhiti and Stasolla 2016). These similarities allow the utilization of SE as a model system to investigate the morphological, physiological, and molecular events occurring during plant embryogenesis (Elhiti and Stasolla 2016).

SE has important advantages in embryological studies as in vitro culture allows the targeted manipulation of culture conditions, which is difficult, if not impossible to perform in vivo. The selective addition or removal of specific chemicals to particular developmental stages is often used as a strategy to investigate the nature of the inductive conditions for the proper development of embryos (Elhiti and Stasolla 2016). Thus, SE may help us understand differentiation, as well as the biochemical and genetic mechanisms involved in the transition from one developmental stage to another.

Once embryogenic cultures have been established, they can produce a theoretically unlimited number of exact copies. This property makes SE an in vitro technique appropriate for large scale production of clonal plants. High proliferation rate, singulation (in most cases embryos can be separately handled), and the bipolar nature of embryos (simultaneous development of root and shoot meristem, which allows conversion into plants in a single step) are some of the advantages of SE over other clonal propagation methods (Janick 1993; Guan et al. 2016). This aside, the possibility of scaling up and automating in bioreactors contributes to reducing labor

and costs and increases the reliability of the production process (Egertsdotter et al. 2019).

As pointed out by Murashige (1977), encapsulation of somatic embryos inside an artificial layer creating a capsule makes possible the production of synthetic seeds. The encapsulation technology provides to somatic embryos protection from mechanical damage and a supply of nutrients during germination. Synthetic seeds can be easily handled for storage, transport, and sowing, similar to their zygotic counterparts (Rai et al. 2009); and, therefore, they can be considered delivery systems for somatic embryos used as a means of clonal propagation (Janick 1993).

Cryopreservation, i.e., conservation in liquid nitrogen (−196 °C), is the only safe and cost-effective option for long-term conservation of plant material (Engelmann 2004).

Embryogenic cultures are composed of small, actively dividing cells, with few small vacuoles and a high nucleus-cytoplasmic ratio. These characteristics make them more able to withstand cryopreservation than large, highly vacuolated and differentiated cells (Engelmann 2004). In fact, embryogenic cultures are in general considered amenable tissues to cryopreservation (Bradaï et al. 2017) and have been repeatedly cryopreserved by one-step freezing or slow cooling techniques.

The joint use of SE and cryopreservation enables conservation of genetic resources, elite germoplasm, clonally propagated species, and biotechnological products, while in-field testing of the regenerated plants and other analyses are being carried out (Dunstan et al. 1995; Altamura et al. 2016).

However, the greatest potential use of SE is as regeneration method associated with biotechnological techniques for crop improvement (Janick 1993). In many biotechnological tools, plant improvement is achieved through manipulations at the cellular level, and, consequently, their applicability is only possible if a whole plant can be regenerated from a single cell (Bhojwani and Razdan 1996). Hence, the availability of an efficient regeneration protocol is a requisite for biotechnology exploitation, and, therefore, SE is at the base of some biotechnological applications, such as (1) production of transgenic plants; (2) generation of variant plants through somaclonal variation or by using mutagenic agents; (3) production of haploid and double-haploid plants, by induction of embryogenesis from microspores and subsequent chromosome doubling (Janick 1993); (4) production of somatic hybrids by protoplast fusion from intra- or intergeneric sources (Loyola-Vargas and Ochoa-Alejo 2016); and (5) cell selection against biotic or abiotic stressing agents (Janick 1993).

## 14.3 *Meta*-topolin and Somatic Embryogenesis

### 14.3.1 Embryogenic Cultures Initiation

*Meta*-topolin was first used for induction of SE by Lai et al. (2014). Using zygotic embryos and coleoptile segments from in vitro germinated embryos of *Mesomelaena pseudostygia*, these authors investigated the influence of different auxins and cytokinins on this process.

In a first experiment, Lai et al. (2014) cultured zygotic embryos on half-strength MS medium (Murashige and Skoog 1962) solidified with 6 g $L^{-1}$ agar and supplemented with different auxins at various concentrations and several combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), the auxin inducing higher callusing response, with different cytokinins. Specifically, they tested PGR-free medium, 1, 2, 5, or 10μM 2,4-D or α-naphthaleneacetic acid (NAA), 1, 10, or 40μM picloram; 2μM 2,4-D plus 0.5 or 1μM BA; 2μM 2,4-D plus 1, 2, or 5μM *m*T; 2μM 2,4-D plus 1, 5, or 10μM thidiazuron (TDZ); and 5μM 2,4-D plus 0.5 or 1μM BA.

Treatments in which basal medium was supplemented with 2μM 2,4-D and 2 or 5μM *m*T resulted in 10% callus formation, while no callus development was observed with 1μM *m*T. The results from other hormone treatments ranged from 0 to 20% callusing. The callus obtained was rarely friable, exhibiting in most cases a compact appearance and limited growth.

Due to limited callus material availability, the authors combined all calli obtained from the 2,4-D treatments and cultured them in PGR-free basal medium or supplemented with 1μM kinetin (KIN) or TDZ, to induce somatic embryo development and conversion into plants. A small proportion of calli transferred to medium lacking hormones gave rise to embryo-like structures. These were observed 2 weeks after calli transference to the medium and small plantlets developed 2 weeks later. No somatic embryos were observed in calli subcultured in cytokinin-containing media, although shoots and roots developed during the culture period. However, calli pooling makes it difficult to draw conclusions on the effect of *m*T on SE induction in *Mesomelaena pseudostygia*.

Callus was also initiated from coleoptiles 5–10 mm long excised from in vitro germinated embryos. Coleoptile segments were cultured on half-strength MS medium with different treatments of auxins and cytokinins, alone or in combination. Thus, PGR-free medium was compared with 1, 2, 5, or 10μM 2,4-D; 1, 5, or 20μM BA; 1, 2, or 5μM *m*T; 1μM NAA; 0.1, 0.5, 1, 2, or 10μM TDZ; 2μM 2,4-D plus 0.5 or 1μM BA; 5μM 2,4-D plus 0.5 or 1μM BA; 2μM 2,4-D plus 1, 2, or 5μM *m*T; and 2μM 2,4-D plus 1μM TDZ. Callus development was only observed in 2,4-D-supplemented media. The calli obtained were occasionally organogenic, but embryogenic characteristics were never evident.

Baskaran et al. (2015a) investigated the influence of *meta*-topolin riboside (*m*TR, 6-(3-hydroxybenzylamino)-9-ß-D-ribofuranosylpurine) on initiation of SE in an optimization experiment.

Expanding young leaves excised from in vivo grown plants of *Mondia whitei* were cultured on MS medium with 8 g $L^{-1}$ agar and different concentrations of sucrose and PGR treatments. In the first experiment, the authors tested the effect of sucrose concentration (30, 35, 40, and 50 g $L^{-1}$) and the auxins 2,4-D and picloram (10, 15, 20 and 25μM). Second, various cytokinins (BA, *m*TR, KIN, or TDZ) at 1μM were combined with 20μM 2,4-D or picloram in MS medium containing 35 or 40 g $L^{-1}$ sucrose. After 8 weeks, the calli obtained in the different treatments were transferred to MS medium with 20 g $L^{-1}$ sucrose and 8 g $L^{-1}$ agar to promote somatic embryo maturation and conversion into plants. Friable embryogenic callus developed over 2 weeks, and, 6 weeks later, differentiation of somatic embryos at different developmental stages could be observed in all treatments, except for the control, which lacked PGRs. Nevertheless, somatic embryo development and plantlet formation improved after transference to MS medium with 20 g $L^{-1}$ sucrose and 8 g $L^{-1}$ agar.

Although SE was induced at a high rate and acceptable numbers of somatic embryos at the different developmental stages were obtained with only auxins, the second experiment revealed that combination of 2,4-D or picloram with cytokinins was more effective in the production of somatic embryos at advanced stages and plantlets. Culture medium containing 40 g $L^{-1}$ sucrose plus 20μM 2,4-D and 1μM TDZ gave rise to the highest production of somatic embryos at early stages and plant regeneration; however, higher frequency of embryogenesis (98.0 $\pm$ 0.20%) and enhanced development of cotyledonary-stage embryos were achieved when 20μM picloram was added jointly with 1μM *m*TR.

Following a similar experimental design, in which different sucrose concentrations and hormone combinations were tested, Baskaran et al. (2015b) initiated embryogenic cultures in *Aloe pruinosa*. Leaf explants excised from 20-day-old in vitro germinated seedlings were inoculated in solid (8 g $L^{-1}$ agar) MS medium with 30–50 g $L^{-1}$ sucrose, 20μM 2,4-D or picloram, and 20μM picloram plus 5μM BA, *m*TR, or zeatin (*t*Z). Although 6 weeks after culture initiation, embryogenic calli were observed in all PGR-containing treatments, the primary role in the production of friable embryogenic callus was only attributed to picloram, BA, and *t*Z.

### 14.3.2 Proliferation of Embryogenic Cultures

Baskaran et al. (2015b) also investigated the influence of different auxins and cytokinins on proliferation of embryogenic cultures and shoot regeneration of *Aloe pruinosa*. For this purpose, friable embryogenic callus obtained in the induction treatments previously indicated were transferred to embryogenic callus proliferation medium, consisting of solidified (8 g $L^{-1}$ agar) MS medium with 30–50 g $L^{-1}$ sucrose and a reduced auxin concentration (5μM), alone or combined with 5–15μM BA, *m*TR, *t*Z or TDZ. Twenty five μM phloroglucinol, a flavonoid used as PGR, was also tested in this phase.

Four weeks after culture initiation, the results revealed that BA and *t*Z were the most effective regulators for embryogenic callus proliferation and maturation, not being evident a relevant effect of *m*TR. Browning and necrosis of callus were a serious problem observed during this phase, conditioning embryo germination and conversion into plants. Interestingly, *m*TR and phloroglucinol appeared to be effective controlling phenolic activities, as both components delayed callus necrosis.

Saeed and Shahzad (2015) optimized a protocol for proliferation of *Albizia lebbeck* via secondary embryogenesis, utilizing *m*T as a cytokinin supplement. Optimization of secondary embryogenesis was addressed through a two-step culture sequence: firstly inducing maturation of the somatic embryoids in the embryogenic callus and later promoting the formation of secondary embryos through adventitious budding from primary embryos at advanced developmental stages.

As initial material, Saeed and Shahzad (2015) used embryogenic callus with somatic embryoids, initiated from shoot tips of an adult tree in WPM medium (Lloyd and Mc Cown 1981) solidified with 8 g $L^{-1}$ agar and supplemented with 12.5μM KIN. In order to induce primary somatic embryo maturation, 100 mg of embryogenic tissues were cultured in solid (8 g $L^{-1}$ agar) MS medium supplemented with different *m*T concentrations (2.5, 5.0 and 7.5μM), 5μM *m*T combined with various NAA concentrations (1.0, 2.5 and 5.0μM) or 5μM *m*T plus 2.5μM NAA, and 50, 75, or 100μM glutamine. Higher maturation rates, both in terms of maturation percentage and number of mature embryos per 100 mg embryogenic tissue, were achieved when 5μM *m*T was combined with 2.5 NAA and 75μM glutamine.

In a second experiment, approximately 50 mg of embryogenic tissues, mainly containing embryos at the cotyledonary stage, were cultured in solid MS with 5.0μM *m*T alone or in combination with NAA (1.0, 2.5 and 5.0μM) and glutamine (50, 75 and 100μM). Optimum results were newly achieved in MS medium with 5.0μM *m*T, 2.5μM NAA and 75μM glutamine. Under these conditions, 85.70 $\pm$ 0.67% secondary embryogenesis induction was accomplished, and 100 $\pm$ 1.15 secondary embryos were produced per 50 mg of primary embryogenic tissue.

Hence, for long-term maintenance, approximately 50 mg of embryogenic cultures of *Albizia lebbeck* were subcultured at 4-week intervals in MS medium plus 5.0μM *m*TR, 2.5μM NAA, and 75μM glutamine. Under these conditions, the embryogenic competence was maintained for at least 3 years (Saeed and Shahzad 2015).

Histological analysis revealed that development of somatic embryos occurred 2 weeks after transfer, from the peripheral region of preexisting embryos, at the base of their adaxial surface. Higher secondary embryogenesis was observed in embryos at the cotyledonary stage. Although a broad connection between the primary and the newly formed embryos was evident at the beginning of the secondary embryo development, these progressively separated becoming individualized as their development advanced. Secondary embryo development was similar to that observed in primary somatic embryos. Saeed and Shahzad (2015) identified six different stages: globular, elongated, early heart-shaped, heart-shaped, torpedo-shaped, and cotyledonary.

### 14.3.3 Somatic Embryo Development and Maturation

As previously indicated (Sect. 14.3.2), Saeed and Shahzad (2015) optimized maturation of primary somatic embryos as a first step to induce a cyclic SE system by secondary embryogenesis in *Albizia lebbeck*, obtaining the best results in MS medium with 5μM *m*TR, 2.5μM NAA, and 100μM glutamine. Interestingly, better germination (involving expansion of the two cotyledons and root formation) and conversion (involving differentiation of shoots and leaves and a developed root system) rates were achieved from secondary than from primary somatic embryos. Under optimum germination conditions (MS medium at half strength with 1.0μM gibberellic acid ($GA_3$)), 26.6 ± 0.88% of primary embryos and 46.7 ± 0.88% of secondary somatic embryos germinated, and 23.3 ± 0.88% and 41.7 ± 0.88% converted into plants, respectively. Saeed and Shahzad (2015) surmised that the positive role of *m*TR on the maturation of *Albizia lebbeck* somatic embryos may contribute to the development of a more efficient protocol for SE in this species.

Baskaran et al. (2015a) tested different PGR combinations for inducing somatic embryo development and conversion in *Mondia whitei*. Embryogenic callus initiated from leaf explants in solid (8 g $L^{-1}$ agar) MS medium with 40 g $L^{-1}$ sucrose, 20μM 2,4-D, and 1μM TDZ were transferred to solid MS medium with 20 g $L^{-1}$ sucrose and 0.5μM of different cytokinins (BA, *m*TR, TDZ, and KIN), alone or in combination with 0.25μM indole-3-acetic acid (IAA) or NAA. Twelve weeks after culture initiation, somatic embryo development and plantlet formation were observed in all treatments. The best results were obtained in MS medium supplemented with 0.5μM *m*TR and 0.25μM IAA, with higher production of somatic embryos at heart, torpedo, and cotyledonary stages. More plantlets were also obtained in this culture medium, 20.40 ± 2.65 versus 9.00 ± 2.34–15.20 ± 2.59, in the rest of treatments. Regenerated plants exhibited good quality (2–3 cm shoot and 5–6 cm radicle) and were successfully acclimatized to ex vitro conditions, with a survival rate of 90%. According to the results, the combination of auxin and cytokinin, IAA and *m*TR in this case, proved to be essential to improve SE in this species.

Although embryogenic suspensions are highly productive, the development and maturation of somatic embryos have proven to be more problematic in liquid media (Timmis 1998; Gupta and Timmis 2005; Salaj et al. 2007). However, Baskaran et al. (2015b, 2017) tested the effect of *m*TR using cell suspension cultures for these purposes. Baskaran et al. (2015b) analyzed the effect of different PGRs, including *m*TR, on embryogenic suspension culture of *Aloe pruinosa*. Following the protocol of Baskaran and Van Staden (2012), suspension cultures were initiated by inoculating approximately 500 mg fresh weight of 3-week-old friable embryogenic callus selected from different proliferation media. Embryogenic tissues were cultured in 100 mL Erlenmeyer flasks containing 20 mL of liquid MS medium lacking hormones or supplemented with 0.5μM 2,4-D or picloram and 1–2μM BA or 1μM *t*Z, *m*TR, or TDZ. All culture media contained 30 g $L^{-1}$ sucrose, and most PGR-containing treatments were supplemented with 10μM phloroglucinol. Four weeks later, significant differences were found in terms of somatic embryo development. The best results (38.7 ± 1.42 somatic embryos per settled cell volume at

globular, club, and torpedo stages and 26.2 ± 0.87 at the cotyledonary stage) were obtained in liquid MS medium supplemented with 0.5μM picloram, 1μM TDZ, and 10μM phloroglucinol. Embryogenic tissue used in this case had been induced in MS medium with 40 g $L^{-1}$ sucrose, 20μM picloram, and 5μM *tZ* and subsequently cultured in MS medium with 40 g $L^{-1}$ sucrose, 5μM picloram, 5μM *tZ*, and 25μM phloroglucinol. Embryogenic callus derived from the same induction and proliferation culture conditions had significantly fewer embryos when suspension culture medium was supplemented with *m*TR: 11.4 ± 1.05 somatic embryos at early developmental stages and 4.7 ± 0.28 somatic embryos at late stages were obtained in liquid MS plus 0.5μM 2,4-D, 1μM *m*TR and 10μM phloroglucinol, and 16.9 ± 0.94 and 7.2 ± 0.43 embryos, respectively, in liquid MS plus 0.5μM picloram, 1μM *m*TR, and 10μM phloroglucinol. No germination was observed in suspension cultures or germination medium. Baskaran et al. (2015b) attributed this issue to oxidation of polyphenols, which play an inhibitory role in somatic embryo growth and germination.

As the frequency of SE and the number of somatic embryos obtained in *Mondia whitei* with their previous protocol (Baskaran et al. 2015a) was low, Baskaran et al. (2017) developed a new SE system, in which somatic embryo development and maturation were performed using cell suspension culture. In an investigation similar to that performed in *Aloe pruinosa* (Baskaran et al. 2015b), Baskaran et al. (2017) tested the effect of different auxin and cytokinin combinations on somatic embryo development in liquid medium. For this purpose, Baskaran et al. (2017) utilized 3-week-old friable embryogenic callus induced from expanding young leaves but with two different origins: initiation in MS medium with 35 g $L^{-1}$ sucrose and 15μM 2,4-D and maintenance in MS medium supplemented with 5μM 2,4-D and 0.5μM TDZ and initiation in MS medium with 35 g $L^{-1}$ sucrose and 15μM picloram and maintenance in MS medium supplemented with 5μM picloram and 0.5μM BA.

Somatic embryo development was performed by inoculating approximately 500 mg fresh weight of embryogenic callus in 20 mL liquid medium contained in 100 mL Erlenmeyer flasks. Liquid MS medium with 30 g $L^{-1}$ sucrose was supplemented with different PGR combinations depending on culture origin. Thus, whereas embryogenic cultures derived from induction and proliferation media containing 2,4-D were transferred to liquid medium supplemented with 0.5μM 2,4-D and 1μM BA, TDZ, *m*TR, 6-(γ,γ-dimethylallylamino)purine (iP), or KIN, embryogenic cultures obtained from induction and proliferation media containing picloram were cultured in liquid medium supplemented with 0.5μM picloram and 1–2μM BA, TDZ, *m*TR, iP, or KIN as well as 0.5μM NAA and 1μM BA, TDZ, or *m*TR. One week after suspension culture initiation, 10 mL of culture medium was replaced by the same volume of freshly prepared medium, and 1 week later, embryogenic tissue was filtered through 200μm meshes and transferred to 250 mL Erlenmeyer flasks containing 30 mL of fresh medium. Incubation was carried out on an orbital shaker at 180 rpm. The effect of the different treatments was assessed by recording the number of somatic embryos at different developmental stages per settled cell volume, 3 weeks after culture initiation in embryo development medium. Production of somatic embryos at advanced developmental stages was significantly

improved in *m*TR-containing media. Thus, 0.5μM picloram plus 1μM *m*TR produced the highest number of heart-shaped embryos, and 0.5 NAA plus 1μM *m*TR gave rise to the highest number of embryos at late developmental stages (torpedo and cotyledonary). However, higher globular-shaped embryo production was achieved in MS medium supplemented with 0.5μM 2,4-D and 1μM TDZ.

The germination capacity of embryos regenerated under optimum developmental conditions (0.5μM 2,4-D plus 1μM TDZ, 0.5μM picloram plus 1μM *m*TR, and 0.5μM NAA plus 1μM *m*TR) was subsequently evaluated by culturing them in solid (8 g $L^{-1}$ agar) MS medium with different sucrose concentrations, alone or supplemented with 2–4μM NAA, and liquid MS medium at half strength with 0.5μM NAA. The best germination rate (98.3%) was achieved with somatic embryos developed in embryo development medium supplemented with 0.5 NAA and 1μM *m*TR and germinated in liquid MS medium at half strength with 0.5μM NAA. Therefore, the results obtained in *Mondia whitei* revealed a primary effect of *m*TR on somatic embryo development using cell suspension culture.

### 14.3.4 Embryo Germination

*Meta*-topolin has also been utilized in the germination phase. Solórzano-Cascante et al. (2018) compared the efficiency of BA and *m*T on shoot development in somatic embryos of *Carica papaya*. Somatic embryos used in this investigation had been regenerated from embryogenic cultures initiated from half-cut seeds cultured in half-strength MS medium supplemented with different 2,4-D concentrations (9.0, 18.0, 27.1, 36.2, or 45.2μM). Embryogenic cultures derived from the different 2,4-D treatments were combined and subsequently multiplied in solid (2.8 g $L^{-1}$ phytagel) and liquid MS medium supplemented with 9μM 2,4-D. Somatic embryos obtained from both treatments were then used, without distinction, for analyzing the effect of BA and *m*T on germination. The influence of both cytokinins was tested in two independent experiments. In both cases, basal medium consisted of the MS formulation solidified with 9 g $L^{-1}$ agar.

In the first trial, Solórzano-Cascante et al. (2018) used 0.0, 0.9, 1.8, 2.7, or 3.6μM BA to germinate somatic embryos derived from 2 months culture in solid medium with 9μM 2,4-D. However, the effect of different *m*T concentrations (0.0, 5.0, 10.0, 15.0, or 20.0μM) was tested using somatic embryos selected from 12-month-old cultures. Initial explants used in each case were slightly different too. While in the BA experiment, clusters of 5–10 somatic embryos at the cotyledonary stage were utilized; in the *m*T study, treatments were applied to callus sections, with approximately 20 somatic embryos per section. The effect of the different treatments was assessed 2 months after culture initiation, taking data of somatic embryo sprouting (epicotyl growth) and germination (radicle and epicotyl growth). Both BA and *m*T positively affected somatic embryo sprouting. Statistical analysis of this variable as a logistic regression revealed a significant second-order polynomial trend for both cytokinins. However, although similar maximum sprouting rates were achieved for BA (40%) and *m*T (44%), significant differences were observed in the hormone

concentrations required for achieving these percentages, 1.8μM for BA versus 10μM for *m*T.

Although the effect of BA and *m*T on somatic embryo sprouting is analyzed in these experiments, it is not easy to draw clear conclusions about their comparative effect. As previously indicated, initial explants used for each experiment were different (clusters of somatic embryos and callus sections), and different numbers of somatic embryos were cultured per treatment replication (5–10 somatic embryos per cluster versus about 20 embryos per callus section). Additionally, the range of concentrations tested was very different (0.9, 1.8, 2.7, and 3.6μM for BA and 5.0, 10.0, 15.0, and 20.0μM for *m*T), and no coinciding treatments were included. Radicle development was rarely observed in these germination conditions. Thus, no rooting was evident in *m*T treatments, and less than 5% of the somatic embryos cultured in BA-containing media developed roots. Interestingly, significant differences were found in explant browning. Oxidation symptoms were observed in a considerable proportion of the somatic embryos germinated in BA-containing media (13–45%), while they were very scarce in those cultured in *m*T media (0–6%).

## 14.4 Concluding Remarks and Future Prospects

*Meta*-topolin and its riboside *m*TR have been used in the different phases of the SE process, i.e., induction of embryogenic cultures, proliferation, somatic embryos development and maturation, and embryo germination. The results have not been positive in all cases, with evident and important differences related to the species, culture conditions, and developmental stage.

In relation to the initiation step, no beneficial influence of *m*T or *m*TR was observed when they were compared to other cytokinins commonly used for this purpose, such as BA, KIN, *t*Z, or TDZ. However, in *Mondia whitei*, an improvement in the subsequent development of somatic embryos at cotyledonary stage was observed when embryogenic cultures induced in culture medium containing 40 g $L^{-1}$ sucrose, 20μM picloram, and 1μM *m*TR were transferred to maturation conditions (Baskaran et al. 2015a).

In the proliferation phase, a positive role of *m*T on cyclic SE was reported in *Albizia lebbeck* (Saeed and Shahzad 2015). However, this hormone was not compared with other cytokinins, and there was no control without *m*T addition. In *Aloe pruinosa*, Baskaran et al. (2015b) found that BA and *t*Z were more effective than the other cytokinins tested, including *m*TR. Nevertheless, *m*TR showed a positive influence reducing the impact of polyphenol exudation, thus protecting tissue from oxidative stress.

Both *m*T and *m*TR have been used to promote development and maturation of somatic embryos. A positive effect of *m*T was observed in *Albizia lebbeck*, as higher maturation percentages and production of mature embryos were observed in *m*T-supplemented media, compared to control PGR-free medium. These parameters greatly improved when NAA and glutamine were added to culture medium containing 5μM *m*T (Saeed and Shahzad 2015). In relation to *m*TR, this *m*T

derivative produced different effects depending on the species. In *Aloe pruinosa*, Baskaran et al. (2015b) reported negative effects when *m*TR was added to suspension cultures to promote somatic embryo development and maturation. In contrast, positive results were achieved in *Mondia whitei* using both solid and liquid culture media. Baskaran et al. (2015a) reported optimum production of plants and somatic embryos at advanced developmental stages in solid MS medium supplemented with 0.5μM *m*TR and 0.25μM IAA. Using cell suspension cultures, Baskaran et al. (2017) also found that production of somatic embryos at advanced developmental stages was significantly improved in *m*TR-containing media.

In relation to germination of somatic embryos, the only study concerning to this phase reported similar sprouting and rooting rates for the two cytokinins tested, BA and *m*T (Solórzano-Cascante et al. 2018).

As previously indicated (Sect. 14.1), a corrective role of some physiological disorders has been attributed to *m*T (Aremu et al. 2012). In different phases of SE, *m*T and *m*TR exhibited an important role in mitigating browning and necrosis, harmful processes in in vitro culture (Baskaran et al. 2015b; Solórzano-Cascante et al. 2018). According to Baskaran et al. (2015b), this quality could be used to improve future in vitro programs.

Therefore, *m*T and their derivatives can be considered suitable substitutes of the cytokinins traditionally used in SE protocols. Although its utilization in this developmental process is relatively recent, some of the results are promising, and an increasing use of this PGR can be expected. Nevertheless, negative results have also been obtained. Hence, as usually occurring in plant tissue culture, until a more complete knowledge of the structure-activity relationships of these PGRs is available, their adequacy should be investigated for each specific circumstance, by trial and error experimentation.

## References


Altamura MM, Della Rovere F, Fattorini L, D'Angeli S, Falasca G (2016) Recent advances on genetic and physiological bases of *in vitro* somatic embryo formation. In: Germanà MA, Lambardi M (eds) *In vitro* embryogenesis in higher plants. Humana Press, New York, pp 47–86

Aremu AO, Bairu MW, Doležal K, Finnie JF, Van Staden J (2012) Topolins: a panacea to plant tissue culture challenges? Plant Cell Tissue Organ Cult 108:1–16

Baskaran P, Van Staden J (2012) Somatic embryogenesis of *Merwilla plumbea* (Lindl.) Speta. Plant Cell Tissue Organ Cult 109:517–524

Baskaran P, Kumari A, Van Staden J (2015a) Embryogenesis and synthetic seed production in *Mondia whitei*. Plant Cell Tissue Organ Cult 121:205–214

Baskaran P, Kumari A, Naidoo D, Van Staden J (2015b) *In vitro* propagation and biochemical changes in *Aloe pruinosa*. Ind Crop Prod 77:51–58

Baskaran P, Kumari A, Van Staden J (2017) Rapid propagation of *Mondia whitei* by embryonic cell suspension culture *in vitro*. S Afr J Bot 108:281–286

Beyl CA (2011) PGRs and their use in micropropagation. In: Trigiano RN, Gray DJ (eds) Plant tissue culture, development, and biotechnology. CRC Press, Boca Raton, pp 33–56

Bhojwani SS, Razdan MK (1996) Plant tissue culture: theory and practice, a revised edition. Elsevier, Amsterdam

Bogaert I, Van Cauter S, Werbrouck SPO, Doležal K (2006) New aromatic cytokinins can make the difference. Acta Hortic 725:265–270

Bradaï F, Almagro-Bastante J, Sánchez-Romero C (2017) Cryopreservation of olive somatic embryos using the droplet-vitrification method: the importance of explant culture conditions. Sci Hortic 218:14–22

Dunstan DI, Tautorus TE, Thorpe TE (1995) Somatic embryogenesis in woody plants. In: Thorpe TA (ed) *In vitro* embryogenesis in plant. Kluwer Academic Publishers, Dordrecht, pp 471–538

Egertsdotter U, Ahmad I, Clapham D (2019) Automation and scale up of somatic embryogenesis for commercial plant production, with emphasis on conifers. Front Plant Sci 10:109

Elhiti M, Stasolla C (2016) Somatic embryogenesis: the molecular network regulating embryo formation. In: Mujib A (ed) Somatic embryogenesis in ornamentals and its applications. Springer, New Delhi, pp 217–230

Elhiti M, Stasolla C, Wang A (2013) Molecular regulation of plant somatic embryogenesis. In Vitro Cell Dev Biol Plant 49:631–642

Engelmann F (2004) Plant cryopreservation: progress and prospects. In Vitro Cell Dev Biol Plant 40:427–433

Fehér A, Pasternak T, Ötvös K, Miskolczi P, Dudits D (2002) Induction of embryogenic competence in somatic plant cells: a review. Biologia 57:5–12

Guan Y, Li SG, Fan XF, Su ZH (2016) Application of somatic embryogenesis in woody plants. Front Plant Sci 7:938

Gupta PK, Timmis R (2005) Mass propagation of conifer trees in liquid cultures - progress towards commercialization. Plant Cell Tissue Organ Cult 81:339–346

Janick J (1993) Agricultural uses of somatic embryos. Acta Hortic 336:207–213

Jiménez VM (2005) Involvement of plant hormones and plant growth regulators on *in vitro* somatic embryogenesis. Plant Growth Regul 47:91–110

Karami O, Aghavaisi B, Mahmoudi Pour A (2009) Molecular aspects of somatic-to-embryogenic transition in plants. J Chem Biol 2(4):177–190

Lai S, Menon A, Turner S, Kodym A, Bunn E (2014) Development of an *in vitro* protocol for a difficult to-propagate endemic Australian dryland sedge species *Mesomelaena pseudostygia* (Cyperaceae). In Vitro Cell Dev Biol Plant 50:99–109

Lelu-Walter MA, Thompson D, Harvengt L, Sanchez L, Toribio M, Pâques LE (2013) Somatic embryogenesis in forestry with focus on Europe: state-of-the-art, benefits, challenges and future direction. Tree Genet Genomes 9(4):883–899

Lloyd G, Mc Cown B (1981) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Int Plant Prop Soc Proc 30:421–427

Loyola-Vargas VM, Ochoa-Alejo N (2016) Somatic embryogenesis. An overview. In: Loyola-Vargas VM, Ochoa-Alejo N (eds) Somatic embryogenesis: fundamental aspects and applications. Springer, Basel, pp 1–8

Murashige T (1977) Plant cell and organ cultures as horticultural practices. Acta Hortic 78:17–30

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Rai MK, Asthana P, Singh SK, Jaiswal VS, Jaiswal U (2009) The encapsulation technology in fruit plants—a review. Biotechnol Adv 27:671–679

Saeed T, Shahzad A (2015) High frequency plant regeneration in Indian Siris via cyclic somatic embryogenesis with biochemical, histological and SEM investigations. Ind Crop Prod 76:623–637

Salaj T, Blehová A, Salaj J (2007) Embryogenic suspension cultures of *Pinus nigra* Arn.: growth parameters and maturation ability. Acta Physiol Plant 29:225–231

Solórzano-Cascante P, Sánchez-Chiang N, Jiménez VM (2018) Explant type, culture system, 6-benzyladenine, meta-topolin and encapsulation affect indirect somatic embryogenesis and regeneration in *Carica papaya* L. Front Plant Sci 9:1769

Strnad M (1997) The aromatic cytokinins. Physiol Plantarum 101:674–688

Taiz L, Zeiger E (2010) Plant physiology, 5th edn. Sinauer Associates, Sunderland

Taiz L, Zeiger E, Møller IM, Murphy A (2015) Plant physiology and development, 6th edn. Sinauer Associates, Sunderland

Timmis R (1998) Bioprocessing for tree production in the forest industry: conifer somatic embryogenesis. Biotechnol Prog 14:156–166

von Arnold S (2008) Somatic embryogenesis. In: George EF, Hall MA, De Klerk GJ (eds) Plant propagation by tissue culture, 3rd edn. Springer, Dordrecht, pp 335–354

von Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Developmental pathways of somatic embryogenesis. Plant Cell Tissue Organ Cult 69:233–249

von Arnold S, Bozhkov P, Clapham D, Dyachok J, Filonova L, Högberg KA, Ingouff M, Wiweger M (2005) Propagation of Norway spruce via somatic embryogenesis. Plant Cell Tissue Organ Cult 81:323–329

Werbrouck SPO (2010) Merits and drawbacks of new aromatic cytokinins in plant tissue culture. Acta Hortic 865:103–107

Winkelmann T (2016) Somatic versus zygotic embryogenesis: learning from seeds. In: Germanà MA, Lambardi M (eds) *In vitro* embryogenesis in higher plants. Humana Press, New York, pp 25–46

Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. Plant Cell 5(10):1411–1423