Meta-topolins: In Vitro Responses and Applications in Large-Scale Micropropagation of Horticultural Crops

15

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Contents

Abstract

Large-scale micropropagation is one of the key techniques in the production of vegetatively propagated horticultural crops, allowing the expansion of cultivated areas and high-quality, disease-free plantlets. This is due, among other things, to highly controlled environmental conditions, from culture medium to the source and type of light. In this controlled environment, cytokinins (CK) are the main plant growth regulators used in different phases of micropropagation in order to obtain the high-multiplication rates needed for the commercial production of millions of clonal plantlets of horticultural crops per year. However, the widely employed CKs, such as N^6-benzyladenine (BA), can lead to somaclonal variations and negative and undesired physiological effects in different stages of micropropagation. The more recently discovered natural BA derivatives,

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topolins, are promising alternatives for micropropagation of horticultural crops with reduced disadvantages of the standard cytokinins. The main effects of these BA derivatives in different micropropagated horticultural crops as well as their strengths and limitations compared with other CKs widely used in micropropagation are discussed in this book chapter.

Keywords

Meta-topolins · Horticulture · Plant tissue culture · Cytokinins · Shoot proliferation · Efficiency · Physiological responses

15.1 Horticulture, Propagation Systems, and the Importance of Commercial Micropropagation

Horticulture, the production of fruit, vegetables, and ornamental and medicinal plants, is a sector of great socioeconomic importance in world agriculture. The color, texture, size, and flavor of horticultural products are the predominant qualities needed for successful commercial activity in this area. Product nutraceutical value is also a quality that plays an important role.

Fruit, vegetables, and ornamental and medicinal plants can be propagated sexually, conventional vegetatively, and through micropropagation. In those propagated by seeds, the seedlings are produced commercially and used for direct (field) or indirect seeding, by transplanting pre-germinated seedlings under substrate and greenhouse conditions. In the seed propagated group, we can divide the cultivated species into two. The first group is self-propagating, for example, in lettuce cultivars (*Lactuca sativa*). In this case, after the development of the new cultivar, seed production is naturally obtained by natural self-fertilization. The other group is equivalent to F1 hybrid seeds, where heterozygous plants are used to obtain homozygous ones, naturally (tomato) or induced by controlled manual pollination, in order to obtain pure lineages (highly homozygous). Specific selected lines are crossed with each other to obtain the F1 hybrids, highly heterozygous, genetically uniform, and with characteristics of commercial interest (RHS 2019). In both cases, seed propagation reduces phytosanitary problems, when due care is taken to produce high-quality seeds.

The other group which uses conventional vegetative propagation refers to those species propagated by stem cutting (guava, chrysanthemum, and sweet potato), grafting (apple, citrus, mango, and grape), air layering (litchi), and the use of stolons (strawberries), rhizomes (banana, ginger, turmeric), divisions (banana, pineapple), and tubers (potatoes), among others. Although vegetative propagation maintains the genetic characteristics of new plantlets obtained, the methods of vegetative propagation have resulted in the dissemination and increase in the frequency of serious crop-specific diseases, especially those of viral or bacterial origin, resulting in a significant reduction in horticultural productivity.

A third group is micropropagated plants obtained under in vitro conditions, using plant tissue culture techniques. The need for micropropagated plants was based on

issues found especially in vegetatively propagated plants. These included inefficient multiplication rate, failure to obtain a large number of shoots from a mother plant throughout the year, and the occurrence of diseases that were serious in these species. The latter resulted in the accumulation of contamination in plants and significant and gradate reduction in productivity along cycles of propagation (Cardoso et al. 2018), for example, garlic (*Allium sativum*) crop cultivation in Brazil, where conventional vegetative propagation through bulbils leads to the accumulation of viruses of the genera *Allexivirus*, *Carlavirus*, and *Potyvirus*, and significant reduction in bulb productivity with the advancement of propagation cycles and production of bulbs (Fernandes 2012). In different garlic cultivars that have undergone micropropagation, including clonal virus-free programs using shoot tip/meristem culture, the increased productivity can reach up to 141% in the first cycle of bulb production, compared to plants without clonal virus-free programs, with productivity up to 49% higher until the fifth cycle of bulb production, even after virus re-infection in this period (Melo Filho et al. 2006). This demonstrated a marked difference in micropropagation methods, in relation to those conventionally used, especially when micropropagation was the only method producing commercially virus-free clonal plantlets.

15.2 The Success of Commercial Micropropagation with the Discovery of Cytokinins

The historical success of plant tissue culture came about with advances in knowledge of plant hormones or plant growth regulators (PGRs), especially cytokinins. One of the oldest trials demonstrating the key role of cytokinins in the success of in vitro development was reported with tomato plants, where it was possible to obtain unlimited growth of roots in a culture medium containing minerals, sucrose, and vitamins, while the shoot tips maintained limited growth under the same conditions, without the addition of PGRs or even with the addition of only auxins to the culture media. Only when the adventitious root was induced, the restarting of growth on in vitro shoots was reported, demonstrating that an unknown diffusible factor from roots was capable of sustaining cell division (White 1934). Before that, Haberlandt (1913) had reported the presence of a diffusible substance in vascular tissue capable of stimulating cell division. However, the discovery was made of the first cytokinin, kinetin (6-furfurylaminopurine), a synthetic molecule found in autoclaved herring sperm DNA, which enabled cell proliferation of tobacco pulp cells (Miller et al. 1955). By means of this molecule and in search of similar molecules, the first natural cytokinin, zeatin, found in immature endosperms of maize (*Zea mays*) seeds was reported (Miller 1961; Letham 1963). The characterization of a cytokinin was due to its physiological effects in promoting cell division and other regulatory functions similar to kinetin (Skoog and Armstrong 1970), associated with molecules with similar molecular structure (Strnad 1997; Frébort et al. 2011).

Other synthetic molecules with cytokinin effect were also obtained with the ability to promote effects very similar to zeatin in vegetables. Of these,

N^6-benzyladenine (BA) is one of the most used and most effective synthetic PGRs for use in plant micropropagation, especially for the large-scale production of micropropagated horticultural plantlets (Oliveira et al. 2009; Silva et al. 2012; Ahmadian et al. 2017). It promotes cell division, dedifferentiation, adventitious regeneration, and the induction of multiple sprouts in individualized explants, by stimulating the cellular division of meristems located in the axillary buds of these shoots and breaking the apical dominance exerted by auxins (Müller and Leyser 2011).

In vitro cytokinins have made plant micropropagation a highly profitable commercial activity, in particular by promoting the formation of a large number of shoots from individual explants in a short period of time, making large-scale micropropagation an alternative for many species which have limited propagation by seed or even conventional vegetative propagation, such as low yield of new plantlets and important phytosanitary problems.

In addition to the isoprenoid-based cytokinins (ISCK) (Strnad 1997), some phenylureas also have cytokinin activity (Bruce and Zwar 1966), such as forchlorofenuron (CPPU; Kapchina-Toteva et al. 2000) and thidiazuron (TDZ; Sunagawa et al. 2007).

The discovery of the natural *meta*-hydroxylated analogues of BA called topolins (Strnad 1997), such as 6-(2-hydroxybenzylamino)purine and 6-(3-hydroxybenzylamino)purine also called *ortho*- and *meta*-topolin (*o*T, *m*T), was made more recently. These aromatic cytokinins were proven as efficient as isoprenoid (ISCK) and phenylurea types. Topolins have since been used in the multiplication/sprouting/shoot proliferation of different species, such as sugarcane (Vinayak et al. 2009), *Pelargonium* × *hortorum* and *P.* × *hederaefolium* (Wojtania 2010), the hybrid C35 of *Citrus sinensis* × *Poncirus trifoliata* (Chiancone et al. 2016), and *Prunus* spp. (Monticelli et al. 2017), among others.

15.3 The Importance of N^6-Benzyladenine in Commercial Micropropagation

The most widely used cytokinins for micropropagation are BA and TDZ, especially in the in vitro establishment phases, helping to maintain and stimulate cell division of shoot tips taken from donor plants and inoculated in vitro as reported in *Aloe vera* (Lavakumaran and Seran 2014) and even promoting adventitious organogenesis in non-meristem tissues, in order to obtain adventitious shoots from leaf segments of different commercial cultivars of *Anthurium andraeanum* (Cardoso and Habermann 2014). Cytokinins have also been reported to induce somatic embryogenesis, such as in *Dendrobium* 'Chengmai Pink' (Chung et al. 2005); however, the embryogenic response of somatic tissues is controlled by a complex mechanism involving hormones including cytokinins, auxins, abscisic acid, and ethylene and stress-related responses (Fehér 2015).

In in vitro establishment of culture medium, cytokinins, such as BA and TDZ, are used for the regeneration and multiplication phase, stimulating the morphogenetic

response and development of multiple axillary or adventitious shoots in explants from different plant species, making micropropagation an efficient tool for clonal plant production.

In conclusion, especially for the cytokinin BA, its use in micropropagation protocols was a trigger for the development of commercial techniques, allowing regulation of in vitro regeneration processes, as well as the development of practically all existing commercial micropropagation protocols for different species used in horticulture and other agricultural applications. BA is the most widely used cytokinin in the micropropagation plant industry because of its efficiency, availability (Bairu et al. 2007), and effective response in different plant species, including herbaceous and woody plants used in horticulture.

For example, in *Prunus avium* cv. Lapins, BA was the only cytokinin capable of inducing shoot multiplication, resulting in a multiplication rate of up to 2.83 in culture medium containing 5 μM of this cytokinin and 0.5 μM indole-3-butyric acid (IBA), compared to other cytokinins such as TDZ {maximum multiplication index (MMI)—1.4)}, N^6-isopentenyladenine (iP) (MMI—1.17), and kinetin (KIN) (MMI—1.61) (Ruzic and Vujovic 2008).

Using the *Prunus* 'Flordaguard' rootstock, the addition of BA to the culture medium increased the percentage of explants with shoots (80%), and up to four shoots per explants were obtained using concentrations close to 4.0 mg L^{-1} (Radmann et al. 2011).

Similar results were obtained in a comparative study using BA and KIN cytokinins in the in vitro cultivation of gerbera, *Philodendron*, *Spathiphyllum*, and *Musa* cultivars (Vardja and Vardja 2001).

15.4 BA Is Not Effective for All Micropropagated Species

The question about testing these new cytokinins, the topolins, was needed. This was due to the fact that BA also leads to undesirable effects in micropropagated plantlets. Vardja and Vardja (2001) micropropagated different ornamental species and noted that for *Cordyline*, *Dracaena*, and *Dieffenbachia* genera, although the cytokinin BA increased the multiplication rate, there were undesirable effects, such as vitrification (hyperhydricity) and reduction in rooting and elongation, in the subsequent phases of in vitro cultivation. At the Laboratory of Plant Physiology and Tissue Culture (UFSCar), our research group observed different symptoms of BA in plant tissues of different horticultural species: in tea tree (*Melaleuca alternifolia*), we found marked reduction in size of shoots (Fig. 15.1a, b, 1.0–3.0 mg L^{-1}) and early leaf necrosis in explants (Fig. 15.1b, 3.0 mg L^{-1}) compared with normal development in BA-free culture medium (study of Carla Midori Iiyama); in strawberry (*Fragaria* × *ananassa*), some cultivars showed as symptoms severe reduction of petiole size, yellowing of leaves, and callus production (instead of shoots) using 0.5 mg L^{-1} BA, and reduction of concentration to 0.25 mg L^{-1} resulted in good shoot proliferation with no symptomatic shoots (Fig. 15.1c; study of Camila Y. Nishimura Saziki); in the medicinal plant *Phyllanthus amarus*, the main

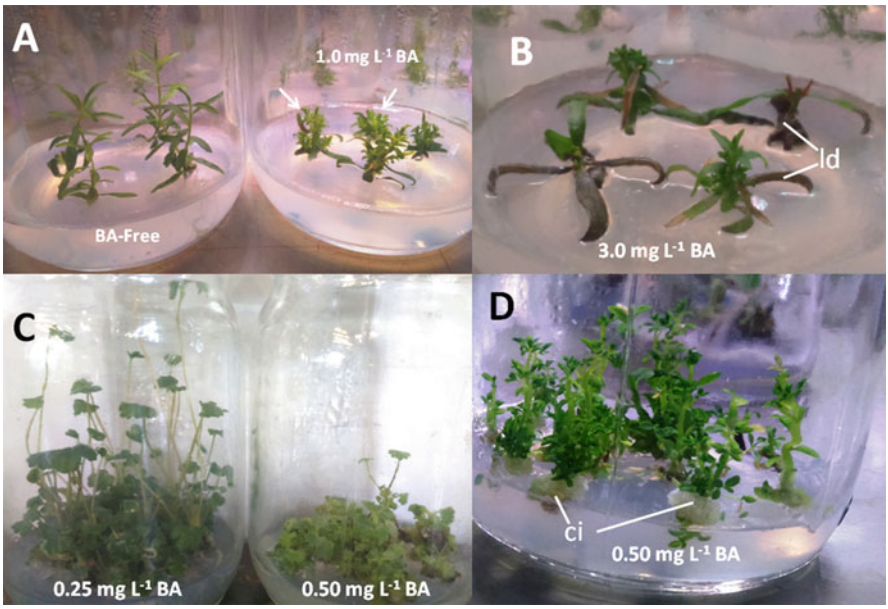

Fig. 15.1 Different symptoms observed in horticultural species micropropagated in culture medium containing N^6-benzyladenine (BA): (**a**) multiple shoot induction (arrows) in explants of tea tree (*Melaleuca alternifolia*); (**b**) symptoms of reduction of size and leaf necrosis (ln) caused by BA in high concentration; (**c**) symptoms of size reduction and leaf yellowing in strawberry (*Fragaria* × *ananassa*); (**d**) hyperhydricity in shoots and callus development (cd) in basal region of shoots of the medicinal plant *Phyllanthus amarus*

symptoms observed using 0.5 mg L^{-1} BA were callus development in the basal region of shoots and hyperhydricity in leaves and stems (Fig. 15.1d, study of Maria Eduarda Barboza Souza de Oliveira).

There are published reports for different micropropagated species in which BA is a potent inhibitor of adventitious rooting on in vitro shoots, such as those observed in the medicinal plant *Achyrocline satureioides*, in which concentrations of BA above 2.5 μM completely inhibited the formation of roots in in vitro shoots cultivated in Woody Plant Medium (Guariniello et al. 2018). Vitrification or hyperhydricity is another physiological abnormality observed in plants cultured in vitro normally caused by the addition of cytokinins, such as BA and TDZ, into the culture medium. The type and concentration of these cytokinins as well as the type of gelling agent and the physical state of the culture medium affect this undesirable response in some micropropagated plants (Ivanova and Van Staden 2011).

For these reasons, in the micropropagation industry, studies using the alternative cytokinins, topolins, for micropropagation of horticultural plant species are mostly concentrated on comparisons of efficiency with the cytokinin BA, e.g., *m*T, compared to BA added to the culture medium for in vitro evaluations of multiplication or shoot proliferation efficiency and shoot/plantlet development (Table 15.1).

Table 15.1 The use of topolins on micropropagation of horticultural species and its effects on shoot proliferation, in vitro development and quality of plantlets

Species/hybrids/cultivars	Application of species	Culture medium	*m*T concentration used	Multiplication index or rate using *m*T	Other reported effects compared to other cytokinins	Reference
Aloe polyphylla	Me; Or	MS	5 μM *m*T	Total (near 12:1) and bigger shoots (near 8:1)	Bigger shoots; spontaneously rooting in multiplication medium; 91% successful acclimatization	Bairu et al. (2007)
Ansellia africana (Orchidaceae)	Me; Or	MS	10 μM *m*T + 5 μM NAA	9.3 ± 0.4a shoots/explant and 83% PLB formation	Increases protocorm-like bodies (PLB) formation; higher genetic stability	Battacharyya et al. (2017)
Barleria greenii	Or	MS	7 μM Me*m*TR	5.04 ± 0.62 shoots/explant	Higher multiplication rate and higher adventitious shoot length	Amoo et al. (2011)
Dendrobium nobile (Orchidaceae)	Me; Or	MS	1 mg L^{-1} *m*T + 0.5 mg L^{-1} NAA	9.2 shoots/explant	Higher and better frequency in PLB formation; genetic stability of derived plants	Battacharyya et al. (2016)
Huernia hystrix	Me	MS	20 μM *m*T + 10 μM NAA	Near 12 shoots/explant (only 4 shoots/explant using *m*T isolately)	Drastic reduction in rooting, compared to BA-treated; increased total phenolics and flavonoids	Amoo and van Staden (2013)

(continued)

Table 15.1 (continued)

Species/hybrids/cultivars	Application of species	Culture medium	*m*T concentration used	Multiplication index or rate using *m*T	Other reported effects compared to other cytokinins	Reference
Pelargonium × *hederaefolium* and *P.* × *hortorum*	Or	MS	1 mg L^{-1} *m*T	3–5 depending on cultivar used	Increased regeneration frequency at establishment; increased success propagation of all cultivars using *m*T; increased rooting percentage of shoots	Wojtania (2010)
Turmeric (*Curcuma longa*) *cv. Elite*	Me; Ar	MS medium with B5 vitamins	10 μM *m*T	6.3 shoots/explant	Shortest shoot and root length and very reduced number of roots per shoot, compared with other eight cytokinins, including BA	Salvi et al. (2002)
Musa spp. AAA 'Williams' and 'Grand Naine'	Fruit	Modified MS	15 μM *m*T or *m*TR	6.1 for 'Williams' and near of 7.0 for 'Grand Naine'	Root inhibition caused by *m*T and *m*TR; somaclonal variation does not differ among BA, *m*T, and *m*TR treatments	Bairu et al. (2008)
Pineapple 'Patawai'	Fruit	MS liquid under 100 rpm	2.5 μM *m*T	15.75	Non-reported; authors compared three in vitro systems,	Teklehayamanot et al. (2010)

		orbital shaker			TIB, semi-solid, and liquid shaking	
Prunus rootstocks	Fruit	QL macrosalts and MS microsalts	2.1 μM *m*T	2.4 for 'Ferdor' and 4.0 for 'Torinel', equal to BA treatments	Null hyperhydric shoots; higher shoot quality, chlorophyll contents, and rooting percentage	Gentile et al. (2014)
Plaintain (*Musa* AAB)	Fruit	MS liquid under bioreactor (TIB)	4.4 μM *m*T	10.7	Higher multiplication rate; non-morphologically aberrant (TDZ); effective for successive multiplication	Roels et al. (2005)
Corylus colurna	Nut	Basal medium described in the paper	8.2 μM *m*T	3.8 ± 0.14	Increased shoot length, shoot fresh and dry weight, and leaf width; higher percentages of root induction and increased survival in acclimatization	Gentile et al. (2017)
Pistachio (*Pistacia vera*)	Nut	MS medium with B5 vitamins	2.0 mg L^{-1} *m*T	4.5 ± 2.4 shoots/explant	Increased number of regenerated shoots and usable shoots	Benmahioul et al. (2012)

(continued)

Table 15.1 (continued)

Species/hybrids/cultivars	Application of species	Culture medium	*m*T concentration used	Multiplication index or rate using *m*T	Other reported effects compared to other cytokinins	Reference
Black walnut (*Juglans nigra*)	Nut	Modified liquid DKW	8.9 μM BA + 4.1 μM *m*T	6.4 (Genotype no. 55) and 5.1 (Genotype no. 189) nodes	Combinations of BA or Zea with *m*T improved percentage of shoot elongated and health of mature nodal explants	Stevens and Pijut (2018)
Coleonema album	Me/Or	MS, 3% sucrose	5.0 μM *m*T + (1 μM NAA or 2.0 μM IBA)	14.5 ± 1.76 adventitious shoots/explant	Increased length and fresh weight of shoots	Fajinmi et al. (2014)

In this sense, an advantage in the use of topolins, compared to BA, would be in maintaining or increasing the positive effects of BA (Werbrouck et al. 1996), especially for efficient induction of multiple shoots that enable the commercial micropropagation of different horticultural species (Cardoso 2018; Cardoso et al. 2018). *m*T application is usually associated with a reduction of undesirable effects reported in plants cultivated with BA or TDZ cytokinins in culture medium, such as hyperhydricity and inhibition of rooting (Bairu et al. 2007).

For this reason, and due to the similar effects to BA, topolins are used especially in the induction of multiple shoots in the multiplication phase of micropropagation (Table 15.1).

15.5 Topolins Have Effects Not Only on the Multiplication Phase

The use of topolins may also have other effects on later phases of multiplication and micropropagation, such as rooting and acclimatization (Werbrouck et al. 1996). In the micropropagation of potato (*Solanum tuberosum*) cv. Jaerla, an increase in the dry mass and survival percentage of acclimatized plantlets was observed (91.8–94.7% survival rate) by adding 0.005–0.01 mg L^{-1} of the *meta*-topolin riboside (*m*TR) compared to untreated plants (70.9% survival). This response was attributed to an increase in endogenous cytokinin content in shoots under acclimatization conditions, promoted by the application of *m*TR, which resulted in lower plant losses in this phase (Baroja-Fernandéz et al. 2002).

In banana 'Williams', the application of specific topolins, such as *meta*-methoxytopolin (Me*m*T) and *meta*-methoxytopolin-9-tetrahydropyran-2-yl (Me*m*TTHP), promoted an increase of in vitro shoots and root length under acclimatization, while other more common topolins such as *m*T or *m*TR did not have the same effect (Aremu et al. 2012). The studies done demonstrated the diversity of responses to different types of topolins, according to the species used. Interestingly, Aremu et al. (2012) observed a reduction in the levels of chlorophylls *a*, *b*, and *a* + *b* with the use of most of the topolins, compared to the control.

The use of *m*T improves the late phases of somatic embryogenesis, for example, in hybrid papaya, *m*T applied in the concentration of 10 μM stimulated sprouting in 40–45% of somatic embryos, with similar effects to BA at 1.8 μM (Solórzano-Cascante et al. 2018). Similar results were obtained by Bhattacharyya et al. (2018a) who reported increases in percentage of shoot emergence of encapsulated PLBs of *Ansellia africana* orchid by using 5.0–10.0 μM of *Mem*TTHP topolin. In *Dendrobium aphyllum* orchids, the use of *m*T up to a concentration of 15 μM increases the percentage of transverse-Thin Cell Layers (tTCLs) that proliferated shoots (79.43±0.12) and number of shoots per tTCL (11.22±0.39) (Bhattacharyya et al. 2018b).

Another positive effect of the use of *m*T was reported for *Corylus avellana* micrografting on *Corylus colurna* rootstocks, in which up to 70% survival of micrografts was reported with the shoots obtained using *m*T treatment and only 30% in the treatment with BA (Gentile et al. 2017).

The different types of topolins may also result in effects on metabolism, affecting, for example, in vitro secondary metabolite production. In *Aloe arborescens*, Amoo et al. (2012) observed significant effects of different types and concentrations of topolins on the production of in vitro secondary metabolites, such as iridoids, total phenolics, flavonoids, and condensed tannins. Similar results demonstrated that the use of *m*T at 20 μM alone in the culture medium promoted increase in total phenolic and flavonoid content in the medicinal plant *Huernia hystrix*, whereas the combination of this topolin with 10 μM NAA resulted in increase in shoot proliferation (3×) but had negative effects on the levels of these same secondary metabolites (Amoo and van Staden 2013).

15.6 Topolins Show Similar Negative Effects to BA Depending on Species, But Could Be an Important Tool for Commercial Micropropagation

Despite the positive responses cited in most studies using topolins as cytokinins, some studies have also reported non-significant or even negative effects of topolins, compared with BA used in culture medium. As examples, in 'Williams' and 'Grand Naine' bananas, the rate of abnormalities obtained with *m*T was similar to that obtained with the use of BA, both up to 15 μM, but treatments with topolins *m*T (3.3 roots) and *m*TR (0.4 roots) at 22.2 μM resulted in a drastic reduction in root numbers, whereas cytokinin BA maintained the number of roots (10.4 roots) compared to control (11.8 roots) (Bairu et al. 2008). In the same study, the authors also observed a dwarf mutant plant in the seventh cycle of in vitro multiplication, identified in the treatment with topolins and using a specific molecular marker. Drastic reductions in rooting percentage of micropropagated shoots were also observed in the medicinal plant *Huernia hystrix* using 20–25 μM *m*T (<10% rooting) compared to the use of BA at the same concentration (>90% rooting).

Thus, as observed in previous studies with different classes of plant hormones or different molecules of the same class, the cytokinins show different responses according to species and genotypes (genotype-dependent responses). Thus, it is expected that the responses to topolin-type cytokinins will also be genotype-dependent. This means that topolins have advantages in the micropropagation of some species over other cytokinins, such as BA and TDZ, while in other species BA, the most widely used cytokinin in micropropagation, remains the mainstay of efficient micropropagation protocols.

In this case, the most reasonable proposal is that topolin-type cytokinins are an important and additional tool in the micropropagation of species used in horticulture, especially those with in vitro cultivation issues (Werbrouck 2010; Amoo et al. 2011; Bandaralaje et al. 2015) and for which the most available cytokinins on the market do not result in efficient protocols for commercial micropropagation.

For example, in the micropropagation of the medicinal species *Harpagophytum procumbens*, excessive callus production at the base of the explants and shoot apical necrosis were reported as physiological disorders caused by BA addition to the

culture medium (Bairu et al. 2011). These same symptoms were reported as a problem in the micropropagation of other species, such as in walnut *Corylus colurna* (Gentile et al. 2017). In both cases, the replacement of BA with *m*T resulted in resolution of the problem, and in the latter case, the use of *m*T also led to a significant increase in the percentage of acclimatized plants (78–84% survival in acclimatization), compared to symptomatic plants micropropagated using BA (24–30% survival only).

Similarly, in vitro micropropagation issues, such as early leaf senescence and loss of bud regeneration capacity in different *Pelargonium* cultivars, were overcome with *m*T at 0.5–1.0 mg L^{-1}. This resulted in increased multiplication rates of six different cultivars, including those showing symptoms of vitrification, shoot deformities, excessive callus formation, and death (67% of all cultivars) in the presence of cytokinin BA at 0.5 mg L^{-1} in the culture medium. Using *m*T as a cytokinin in the culture medium, the authors reported shoot proliferation during five subcultures in all six *Pelargonium* cultivars tested, with higher multiplication rates and none of the negative symptoms reported for BA treatments (Wojtania 2010).

15.7 Some Remarks About Differential Responses to Topolins and BA

The mechanisms underlying species peculiar responses to the various types of cytokinins are not yet elucidated. Possibly, the differences in species or cultivar responses to treatment with different types of cytokinins are associated with the fact that they represent a different stimulus for the natural biosynthesis of individual cytokinin types in the plants in question (Aremu et al. 2014) and thus lead to different responses regarding proliferation, undesirable symptoms, and subsequent effects on rooting and acclimatization of the sprouts and plantlets obtained.

In many studies, the lower toxicity of topolins, compared to BA and TDZ, is mentioned because the external application of cytokinins leads to natural CK biosynthesis in plants. The final metabolites generated by topolins are more easily degradable, especially *O*-glucosides, a readily storable form of cytokinins which are rapidly converted into plant-active cytokinin bases (Bairu et al. 2009; Strnad 1997). However, this explanation cannot be applied to all species, since undesirable effects such as inhibition of in vitro sprouting, callus production, and rooting inhibition have also been reported in plant species using some topolins, similar to those reported for the cytokinins BA and TDZ (Table 15.1).

One hypothesis that could explain the heterogeneity of responses to cytokinins among species, especially the differences between BA and topolins, may be related to the type of proliferation of new shoots under in vitro conditions in the production of commercially micropropagated plantlets. Although most species used in horticulture are micropropagated using the multiple shoot induction technique (Cardoso 2018; Cardoso et al. 2019), in some species this is precluded for a variety of reasons, such as negative effects of cytokinins on the in vitro culture environment (Duarte et al. 2019). Micropropagation using the micro-cutting technique, in which the nodes

and internodes are segmented and induced to sprout new axillary shoots has been demonstrated is a viable and efficient alternative to multiplying shoots of some horticultural crops (Cardoso and Teixeira da Silva 2013; Duarte et al. 2019).

For example, in the propagation of Krymsk®5 rootstock (*Prunus fruticosa* × *Prunus lannesiana*), a higher number of shoots/explants (2.23) using BA instead of *m*T (0.57) are reported, but the use of *m*T resulted in a higher number of nodes per explant and shoot length than those cultivated with other cytokinins, including BA (Tsafouros and Roussos 2019). In this case, if each node were segmented and considered as a source of explants (microcuttings) to maintain sprout proliferation, it is possible that the multiplication rate using *m*T would increase significantly.

15.8 Conclusions

In conclusion, the cytokinins topolins discovered recently are being used as an important tool in the micropropagation of species of great importance for horticulture. In particular, the use of topolins in many cases has addressed the micropropagation and proliferation of high-quality shoots of species or genotypes in which other cytokinins were not efficient for shoot proliferation or resulted in undesirable effects, commonly caused by cytokinins BA and TDZ. However, it is important to note that the use of topolins is subject to the same symptoms caused by other cytokinins at high concentrations or even in species in which BA is still the most efficient for micropropagation. For this reason, studies that consider in particular evaluation of the genetic stability of micropropagated plants with these cytokinins are necessary because they should increase the use of topolins on a commercial scale, aiming at the production of horticultural species. In addition, topolins could increase efficiency of micropropagation in some species. The high costs of are among the major current challenges in large-scale micropropagation (Cardoso et al. 2018), and the increased efficiency of micropropagation is a preponderant factor for effective cost reduction (Chen 2016). The combination of techniques such as temporary immersion bioreactors, light-emitting diodes (LEDs), and photoautotrophic cultivation, among others, commercially emergent with the use of topolins added to the culture medium as well as the effects of topolins in tissue culture applied to plant breeding could also be considered and studied. As example, the use of topolins in the substitution of other cytokinins was also used for inducing gametic embryogenesis in microspores isolated from recalcitrant *Citrus* genotypes (Chiancone et al. 2015).

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References

Ahmadian M, Babaei A, Shokri S et al (2017) Micropropagation of carnation (Dianthus caryophyllus L.) in liquid medium by temporary immersion bioreactor in comparison with solid culture. J Genet Eng Biotech 15(2):309–315

Amoo SO, Aremu AO, Van Staden J (2012) *In vitro* plant regeneration, secondary metabolite production and antioxidant activity of micropropagated *Aloe arborescens* Mill. Plant Cell Tissue Organ Cult 111(3):345–358

Amoo SO, Finnie JF, Van Staden J (2011) The role of *meta*-topolins in alleviating micropropagation problems. Plant Growth Regul 63:197–206

Amoo SO, Van Staden J (2013) Influence of plant growth regulators on shoot proliferation and secondary metabolite production in micropropagated *Huernia hystrix*. Plant Cell Tissue Organ Cult 11(2):249–256

Aremu AO, Bairu MW, Szüčová L et al (2012) Assessment of the role of *meta*-topolins on in vitro produced phenolics and acclimatization competence of micropropagated 'Williams' banana. Acta Physiol Plant 34:2265–2273

Aremu AO, Plačková L, Bairu MW et al (2014) How does exogenously applied cytokinin type affect growth and endogenous cytokinins in micropropagated *Merwilla plumbea*? Plant Cell Tissue Organ Cult 118:245

Bairu MW, Jain N, Stirk WA et al (2009) Solving the problem of shoot-tip necrosis in *Harpagophytum procumbens* by changing the cytokinin types, calcium and boron concentrations in the medium. S Afr J Bot 75:122–127

Bairu MW, Novak O, Doležal K et al (2011) Changes in endogenous cytokinin profiles in micropropagated *Harpagophytum procumbens* in relation to shoot-tip necrosis and cytokinin treatments. Plant Growth Regul 63(2):105–114

Bairu MW, Stirk WA, Dolezal K et al (2007) Optimizing the micropropagation protocol for the endangered *Aloe polyphylla*: can *meta*-topolin and its derivatives serve as replacement for benzyladenine and zeatin? Plant Cell Tissue Organ Cult 90:15–23

Bairu MW, Stirk W, Dolezal K et al (2008) The role of topolins in micropropagation and somaclonal variation of banana cultivars 'Williams' and 'Grand Naine'. Plant Cell Tissue Organ Cult 95(3):373–379

Bandaralaje JCAH, Hayward A, O'Brien C et al (2015) Gibberellin and cytokinin in synergy for a rapid nodal multiplication system of avocado. In: VIII Congresso Mundial de la Palta: genetic resources and nursery management (proceedings), Peru, pp 95–103

Baroja-Fernandéz E, Aguirreolea J, Martínkova H et al (2002) Aromatic cytokinins in micropropagated potato plants. Plant Physiol Biochem 40(3):217–224

Battacharyya P, Kumaria S, Tandon P (2016) High frequency regeneration protocol for *Dendrobium nobile*: a model tissue culture approach for propagation of medicinally important orchid species. S Afr J Bot 104:232–243

Battacharyya P, Kumar V, van Staden J (2017) Assessment of genetic stability amongst micropropagated *Ansellia Africana*, a vulnerable medicinal orchid species of Africa using SCoT markers. S Afr J Bot 108:294–302

Battacharyya P, Kumar V, van Staden J (2018) In vitro encapsulation based short term storage and assessment of genetic homogeneity in regenerated Ansellia Africana (Leopard orchid) using gene targeted molecular markers. Plant Cell Tissue Organ Cult 133(2):299–310

Bhattacharyya P, Kumar V, Van Staden J (2018a) In vitro encapsulation based short term storage and assessment of genetic homogeneity in regenerated *Ansellia africana* (Leopard orchid) using gene targeted molecular markers. Plant Cell Tissue Org Cult 133(2):299–310

Bhattacharyya P, Paul P, Kumari S, Tandon P (2018b) Transverse thin cell layer (t-TCL)-mediated improvised micropropagation protocol for endangered medicinal orchid *Dendrobium aphyllum* Roxb: an integrated phytomolecular approach. Acta Physiol Plant 40:137. https://doi.org/10.1007/s11738-018-2703-y

Benmahioul B, Dorion N, Kaid-Harche M et al (2012) Micropropagation and ex vitro rooting of pistachio (*Pistacia vera L.*). Plant Cell Tissue Organ Cult 108:353–358

Bruce MI, Zwar JA (1966) Cytokinin activity of some substituted ureas and thioureas. Proc R Soc B 165:245–265

Cardoso JC, Habermann G (2014) Adventitious shoot induction from leaf segments in Anthurium andreanum is affected by age of explant, leaf orientation and plant growth regulator. Hortic Environ Biotech 55:56–62

Cardoso JC, Teixeira da Silva JA (2013) Micropropagation of *Zeyheria montana* Mart. (Bignoniaceae), and endagered endemic medicinal species from the Brazilian Cerrado Biome. In vitro Cell Dev Biol Plant 49:710–716

Cardoso JC, Sheng Gerald LT, Teixeira da Silva JA (2018) Micropropagation in twenty-first century. Methods Mol Biol 1815:17–46

Cardoso JC (2018) *In vitro* responses of gerbera (*Gerbera jamesonii*) cultivars multiplied under different photoperiods. Adv Hortic Sci 32(4):557–561

Cardoso JC, Oliveira MEBS, Cardos FCI (2019) Advances and challenges on the in vitro production of secondary metabolites from medicinal plants. Hort Brasil 37:124–132. http://dx.doi.org/10.1590/S0102-053620190201

Chen C (2016) Cost analysis of plant micropropagation of *Phalaenopsis*. Plant Cell Tissue Organ Cult 126:167–175

Chiancone B, Karasawa MMG, Gianguzzi V et al (2015) Early embryo achievement through isolated microspore culture in *Citrus clementina* Hort. ex Tan., cvs. 'Monreal Rosso' and 'Nules'. Front Plant Sci 6:Article 413

Chiancone B, Martorana L, Casales F et al (2016) The effects of benzyladenine and meta-topolin on in vitro sprouting and regrowth after encapsulation of C35 citrange [*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf] microcuttings. Acta Hortic 1113:99–106

Chung H-H, Chen J-T, Chang W-C (2005) Cytokinins induced direct somatic embryogenesis of *Dendrobium chiengmai* pink and subsequent plant regeneration. In Vitro Cell Dev Biol Plant 41 (6):765–769

Duarte WN, Zanello CA, Cardoso JC (2019) Efficient and easy micropropagation of Morus nigra and the influence of natural light on acclimatization. Adv Hortic Sci 33(3):433–439

Fajinmi OO, Amoo SO, Finnie JF, Van Staden J (2014) Optimization of in vitro propagation of *Coleonema album*, a highly utilized medicinal and ornamental plant. S Afr J Bot 94:9–13

Fehér A (2015) Somatic embryogenesis – stress induced remodeling of plant cell fate. Biochim Biophys Acta 1849(4):385–402

Fernandes FR (2012) Medidas gerais de controle de viroses em alho. Nosso Alho. Disponível em: https://ainfo.cnptia.embrapa.br/digital/bitstream/item/74810/1/Nosso-alho.pdf. Acessado em 25 de Abril de 2019 (text in portuguese)

Frébort I, Kowalska M, Hluska T et al (2011) Evolution of cytokinin biosynthesis and degradation. J Exp Bot 62:2431–2452

Gentile A, Frattarelli A, Nota P et al (2017) The aromatic cytokinin *meta*-topolin promotes in vitro propagation, shoot quality and micrografting in *Corylus colurna* L. Plant Cell Tissue Organ Cult 128:693

Gentile A, Jàquez Gutiérrez M, Martinez J et al (2014) Effect of *meta*-topolin on micropropagation and adventitious shoot regeneration in *Prunus* rootstocks. Plant Cell Tissue Organ Cult 118:373–381

Guariniello J, Iannicelli J, Peralta PA, Escandón AS (2018) In vivo and in vitro propagation of "macela": a medicinal-aromatic native plant with ornamental potential. Orn Hort 24(4):361–370

Haberlandt G (1913) Zur physiologie der zellteilung. Sitzungsber Akad Wiss Berlin Phys Math C l:318–345

Ivanova M, Van Staden J (2011) Influence of gelling agent and cytokinins on the control of hyperhydricity in *Aloe polyphylla*. Plant Cell Tissue Organ Cult 104:13–21

Kapchina-Toteva VV, van Telgen HJ, Yakimova E (2000) Role of phenylurea cytokinin CPPU in apical dominance release in in vitro cultured *Rosa* hybrida L. J Plant Growth Regul 19:232–237

Lavakumaran L, Seran TH (2014) Effect of 6-benzylaminopurine and thidiazuron on in vitro shoot organogenesis of *Aloe vera* (L.) Burm. Chilean J Agric Res 74:497–501

Letham DS (1963) Zeatin, a factor inducing cell division isolated from zea mays. Life Sci 2:569–573

Monticelli S, Gentile A, Frattarelli A et al (2017) Effects of the natural cytokinin *meta*-topolin on in vitro shoot proliferation and acclimatization of *Prunus* spp. Acta Hortic 1155:375–380
Melo Filho PA, Resende RO, Cordeiro CMT et al (2006) Viral reinfection affecting bulb production in garlic after seven years of cultivation under field conditions. Europ J Plant Pathol 116:95–101
Miller CO, Skoog F, Von Saltza MH, Strong F (1955) Kinetin, a cell division factor from deoxyribonucleic acid. J Am Chem Soc 77:1392
Miller CO (1961) A kinetin-like compound in maize. Proc Natl Acad Sci USA 47:170–174
Müller D, Leyser O (2011) Auxin, cytokinin and the control of shoot branching. Ann Bot 107 (7):1203–1212
Oliveira ET, Crocomo OJ, Farinha TB, Gallo LA (2009) Large-scale micropropagation of *Aloe vera*. HortScience 44(6):1675–1678
Radmann EB, Bianchi VJ, Fachinello JC et al (2011) In vitro multiplication of 'Flordaguard' rootstock: cytokinin source and concentration effects, explants orientation and period of permanence in the culture medium. Braz Arch Biol Technol 54(1):25–34
RHS (Royal Horticultural Society) (2019) F_1 hybrids. Disposable. https://www.rhs.org.uk/advice/profile?pid=710. Accessed 14 Oct 2019
Roels S, Escalona M, Cejas I et al (2005) Optimization of plaintain (*Musa* AAB) micropropagation by temporary immersion system. Plant Cell Tissue Organ Cult 82:57–66
Ruzic DV, Vujovic TI (2008) The effects of cytokinin types and their concentration on in vitro multiplication of sweet cherry cv. Lapins (*Prunus avium* L.). Hort Sci (Prague) 35:12–21
Sunagawa H, Agarie S, Umemoto M et al (2007) Effect of urea-type cytokinins on the adventitious shoots regeneration from cotyledonary node explant in the common ice plant, *Mesembryanthemum crystallinum*. Plant Prod Sci 10:47–56
Salvi ND, George L, Eapen S (2002) Micropropagation and field evaluation of micropropagated plants of turmeric. Plant Cell Tissue Organ Cult 68:143–151
Silva RC, Luis ZG, Scherwinski-Pereira JE (2012) Short-term storage in vitro and large-scale propagation of grapevine genotypes. Pesq Agropec Bras 47(3):344–350
Skoog F, Armstrong DJ (1970) Cytokinins. Annu Rev Plant Physiol 21:359–384
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
Stevens ME, Pijut PM (2018) Rapid in vitro shoot multiplication of the recalcitrant species *Juglans nigra* L. In Vitro Cell Dev Biol Plant 54:309–317
Strnad M (1997) The aromatic cytokinins. Physiol Plant 101:674–688
Teklehayamanot T, Wannakrairoj S, Pipattanawong N (2010) Meta-topolin for pineapple shoot multiplication under three in vitro systems. Am Eur J Agric Environ Sci 7(2):157–162
Tsafouros A, Roussos PA (2019) First report of Krymsk®5 (cv. VSL2) cherry rootstock in vitro propagation: studying the effect of cytokinins, auxins and endogenous sugars. Notulae Bot Hort Agrobot Cluj Napoca 47(1):152–161
Vardja R, Vardja T (2001) The effect of cytokinin type and concentration and the number of subcultures on the multiplication rate of some decorative plants. Proc Estonian Acad Sci Biol Ecol 50(1):22–32
Vinayak V, Dhavan A, Gupta VK (2009) Efficacy of non-purine and purine cytokinins on shoot regeneration in vitro in sugarcane. Indian J Biotechnol 8:227–231
Werbrouck SPO (2010) Merits and drawbacks of new aromatic cytokinins in plant tissue culture. Acta Hortic 865:103–107
Werbrouck SPO, Strnad M, van Onckelen HA et al (1996) Meta-topolin, an alternative to benzyladenine in tissue culture? Physiol Plant 98(2):291–297
Wojtania A (2010) Effect of meta-topolin on in vitro propagation of *Pelargonium x Hortorum* and *Pelargonium x Hederaefolium* cultivars. Acta Soc Bot Pol 79(2):101–106
White PR (1934) Potentially unlimited growth of excised tomato root tips in a liquid medium. Plant Physiol 9:585–600