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REVIEW

# Topolins: A panacea to plant tissue culture challenges?

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**Abstract** Since the discovery of topolins as naturally occurring aromatic cytokinins (CKs), they have emerged as genuine alternatives to the long serving CKs such as benzyladenine, zeatin and kinetin in plant tissue culture (PTC). Globally, the past 15 years has witnessed a surge in the use of topolins and their derivatives in research laboratories. Topolins, especially the *meta*-topolin and its derivatives have been employed for culture initiation, protocol optimization and for counteracting various in vitro induced physiological disorders in many species. Evidence from various studies indicate the rising popularity and advantages (although not universal for all species) of topolins compared to other CKs. In this review, we assess the use of topolins in PTC with emphasis on their metabolism, structure–activity relations and effect on morphogenesis in vitro. In addition, the review provides a detailed list of species that have been used to study the effect of topolins in comparison with other CKs, the growth parameters affected and recommended concentrations are also provided.

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**Abbreviations**

| | |
|---|---|
| Ad | Adenine |
| AdS | Adenine sulphate |
| BA | $N^6$ benzyladenine |
| BA9G | $N^6$-benzyladenine-9-glucoside |
| BAR | $N^6$-benzyladenine-9-riboside |
| BPA | 6-Benzyl-9-(2-tetrahydropyranylamino)purine |
| CK | Cytokinin |
| CPPU | 6-(2-Chloro-4-pyridyl)-*N*′-phenylurea |
| DHZ | Dihydrozeatin |
| FmT | *meta*-Flourotopolin |
| FmTR | *meta*-Flourotopolin riboside |
| IAA | Indole-3-acetic acid |
| IBA | Indole-3-butyric acid |
| iP | $N^6$-(2-isopentenyl)adenine |
| KIN | Kinetin |
| KINR | Kinetin riboside |
| Me*m*T | *meta*-Methoxy topolin |
| Me*m*TR | *meta*-Methoxy topolin riboside |
| Me*o*T | *ortho*-Methoxy topolin |
| Me*o*TR | *ortho*-Methoxy topolin riboside |
| MS | Murashige and Skoog medium |
| *m*T | *meta*-Topolin |
| *m*TR | *meta*-Topolin riboside |
| *o*T | *ortho*-Topolin |
| *PAS* | *Pasticcino* |
| PGR | Plant growth regulator |
| PTC | Plant tissue culture |
| STN | Shoot-tip necrosis |
| TDZ | Thidiazuron |
| Z | Zeatin |

ZOG1 Zeatin *O*-glucosyltransferase
ZR Zeatin riboside

## Introduction

After the discovery of cytokinins (CKs) (Hall and De Ropp 1955; Miller et al. 1955; Letham 1963) the science of plant tissue culture (PTC) grew rapidly and exponentially with an equally large expansion in the field of their application (Vasil 2008). In addition to the valuable contributions of PTC to plant propagation, improvement and conservation (Castelblanque et al. 2010; Faizal et al. 2011; Parimalan et al. 2011), it has become a fundamental tool to understand various physiological processes which were once explained using speculative hypotheses and growth parameters such as number of regenerated shoots and roots as well as their survival ex vitro. The ultimate goal of PTC protocols is the rapid multiplication of uniform and healthy plantlets. During micropropagation of many plant species however, physiological disorders such as stunted growth, epigenetic and somaclonal variation are often encountered (Bairu et al. 2011a; Smulders and De Klerk 2011). These problems reduce the commercial application of PTC protocols in several plant species. Therefore, plant growth regulators (PGRs) especially CK, often implicated in the occurrence of these challenges, warrant more stringent studies. Moreover, the choice of CK remain critical to the success or failure of any micropropagation endeavour (Werbrouck 2010; Amoo et al. 2011).

Presently, information on the structure–activity relationship of CKs and its effect on plant physiology is having great significance in understanding the growth requirements of plant species in vitro (Sakakibara 2006). Most plant species exhibit inherent variation which is translated to their varied responses to the different CKs. As a result, it becomes pertinent to optimize PTC protocols for individual species. Researchers are continuously searching for new as well as superior CKs. Topolins in general and *meta*-topolin (*m*T) in particular are products of such endeavours. Since the discovery of *m*T and its derivatives as naturally occurring aromatic CKs in several plant species (Jones et al. 1996; Strnad et al. 1997; Tarkowská et al. 2003), their use in PTC increased rapidly (Table 1). Positive reports on important PTC parameters such as shoot multiplication rate, alleviating physiological disorders, better acclimatization and rooting are making topolins popular amongst plant tissue culturists. Despite the use of topolins for more than a decade, a comprehensive and detailed review on the various research outputs is still lacking. Werbrouck (2010) briefly reviewed the merits and drawbacks of the new aromatic CKs in PTC. The chemistry and synthesis as well as natural occurrence of topolins in plants has also been examined (Subbaraj 2011). This author also reviewed the structure–activity relationships and diverse applications of topolins in agriculture as well as the effect on abiotic stress management. Nevertheless, other important aspects of current research outputs on topolins especially the practical applications remain to be fully exploited. Since the discovery of topolins, our research groups (South Africa and Czech Republic) have tested these compounds on a number of species ranging from food crops, medicinal plants to plantation trees. Here, we compile (Table 1), discuss and summarize the various reports on the use of topolins in PTC when compared to other commonly used CKs.

The science of PTC in general and CKs in particular are amongst the most reviewed subjects. This review aims at providing background reading for the tissue culture community to help them augment and evaluate the various scientific reports on PTC and the use of topolins. The review therefore, discusses aspects of CK metabolism, structure–activity relationships as well as associated problems of plant growth and development, from a tissue culturist's perspective.

## Historical background

Following the discovery of kinetin (KIN) (Hall and De Ropp 1955; Miller et al. 1955) and later zeatin (Z) (Letham 1963), many other compounds with CK-like activity have been discovered (Schmülling 2004; Sakakibara 2006). At present, there are hundreds of natural and synthetic CKs. Based on their side chain configuration, the naturally occurring CKs are classified as either isoprenoid or aromatic forms. Urea based synthetic CKs such as 6-(2-chloro-4-pyridyl)-*N*′-phenylurea (CPPU) and thidiazuron (TDZ) are often recognized as a third group of CKs (Schmülling 2004). For a long time, the isoprenoid CKs have received more detailed attention than the aromatic ones which is probably due to their relative higher abundance in higher plants, better knowledge of their biosynthesis and metabolism and the notion that aromatic CKs such as benzyladenine (BA) were synthetic (Van Staden and Crouch 1996). In terms of their functions, Holub et al. (1998) postulated that the isoprenoid CKs have a more potent effect on growth processes involving continuation of the cell cycle while aromatic CKs exert a greater influence on developmental processes, especially those involving morphogenesis and senescence. Cytokinins are usually present in low concentrations (micro molar) and exist endogenously in the form of free bases, nucleosides, glucosides and nucleotides in plants (Schmülling 2004; Doležal et al. 2007). In other words, CKs form reversible conjugates with sugars and amino acids (Bajguz and Piotrowska 2009). These conjugates act as storage, transport or biologically inert forms of CKs which are responsible for the physiological and developmental plasticity observed in

**Table 1** The influence of topolins in comparison to other cytokinins on various growth parameters and physiological disorders

| Species | Cytokinin(s) tested | Preferred cytokinin(s) | Optimum concentration | Growth parameter(s)/physiological disorder(s) affected | Reference |
|---|---|---|---|---|---|
| *Agave cupreata* | BA, iP, KIN, *m*T, TDZ | BA | 6.7 μM | Shoot multiplication | Rosales et al. (2008) |
| *Agave difformis* | BA, iP, KIN, *m*T, TDZ | TDZ | 0.9 μM | Shoot multiplication | Rosales et al. (2008) |
| *Agave karwinskii* | BA, iP, KIN, *m*T, TDZ | BA | 4.4 μM | Shoot multiplication | Rosales et al. (2008) |
| *Agave obscura* | BA, iP, KIN, *m*T, TDZ | TDZ | 0.9 μM | Shoot multiplication | Rosales et al. (2008) |
| *Agave potatorum* | BA, iP, KIN, *m*T, TDZ | KIN | 13.9 μM | Shoot multiplication | Rosales et al. (2008) |
| *Albuca bracteata* | BA, *m*T | BA | 0.4 μM | Bulb induction, multiplication rate, sizes and masses | Ascough and Van Staden (2010) |
| *Aloe ferox* | BA, *m*T, *m*TR | *m*T, *m*TR | 5.0 μM | Shoot multiplication, abnormality reduction | Bairu et al. (2009b) |
| *Aloe polyphylla* | BA, *m*T, *m*TR, Me*m*T, Me*m*TR, Z | *m*T | 5.0 μM | Shoot multiplication, rooting, acclimatization competence, hyperhydricity alleviation | Bairu et al. (2007) |
| *Ananas comosus* cv. 'Pattawia' | *m*T | *m*T | 2.5 μM | Shoot multiplication | Teklehaymanot et al. (2010) |
| *Ansellia africana* | BA, *m*TR, TDZ, Z | *m*TR | 5.0 μM | Shoot length, number of leaves | Vasudevan and Van Staden (2011) |
| *Barleria greenii* | BA, KIN, *m*T, *m*TR, Me*m*TR | Me*m*TR | 7.0 μM | Shoot multiplication, abnormality reduction | Amoo et al. (2011) |
| *Beta vulgaris* | BA, *m*T, *o*T, Z | *m*T | n/s | Shoot multiplication, rooting | Kubalákova and Strnad (1992) |
| *Beta vulgaris* | *m*TR | *m*TR | n/s | Delayed senescence, increased yield | Čatský et al. (1996) |
| *Citrus reticulate* × *Poncirus trifolia* | BA, *m*T | BA | 1.0 μM | Shoot multiplication, length and quality | Niedz and Evens (2010) |
| | | *m*T | 13.25 μM | | |
| *Curcuma longa* cv. 'elite' | Ad, AdS, BA, BAR, iP, KIN, KINR, *m*T, Z | KINR | 10.0 μM | Shoot multiplication, quality of plantlets | Salvi et al. (2002) |
| | | KIN | 10.0 μM | Rooting | |
| *Dierama erectum* | BA, KIN, *m*T, Z | Z | 1.0 μM | Shoot multiplication | Koetle et al. (2010) |
| *Eucomis zambesiaca* | BA, iP, *m*T, Z | BA | 22.19 μM | Bulb induction, multiplication rate, sizes and masses | Cheesman et al. (2010) |
| *Harpagophytum procumbens* | BA, *m*T, *m*TR | *m*TR | 5.0 μM | Acclimatization competence, shoot-tip necrosis alleviation | Bairu et al. (2009a) |
| *Hibiscus sabdariffa* | BA, *m*T, TDZ | BA | 17.74 μM | Shoot multiplication, rooting | Gomez-Leyva et al. (2008) |
| *Hordeum vulgare* | BA, *m*T | *m*T | 1.0 μM | Seed germination, rooting | Huyluoglu et al. (2008) |
| *Hydrangea macrophylla* | BA, *m*T, TDZ | BA | 8.9 μM | Shoot multiplication | Doil et al. (2008) |
| *Hypericum* L. H2003-004-016 | BA, *m*T | *m*T | 5.0 μM | Shoot quality | Meyer et al. (2009) |
| *Lycaste armomatica* | BA, KIN, *m*T, TDZ | TDZ | 4.4 μM | Shoot multiplication | Mata-Rosas et al. (2010) |
| *Malus domestica* 'M.26' | BA, BAR, KIN, KINR, *m*T, *m*TR, TDZ, Z, ZR | BAR | 18.20 μM | Shoot multiplication | Dobránszki et al. (2004) and Dobránszki et al. (2006) |
| *Malus domestica* 'Red Fuji' | BA, BAR, *m*T, BA + KIN | *m*T | 20.7 μM | Rooting | Magyar-Tábori et al. (2001) |
| | | BA + KIN | (4.4 + 7.0) μM | | |
| *Malus domestica* 'Royal Gala' | BA, BAR, *m*TR, TDZ | BAR | 14.0 μM | Rooting, acclimatization competence | Magyar-Tábori et al. (2011) |
| *Malus domestica* 'Royal Gala' | BA, BAR, KIN, KINR, *m*T, *m*TR, TDZ, Z, ZR | TDZ | 2.27 μM | Shoot multiplication | Dobránszki et al. (2004) and Dobránszki et al. (2006) |

**Table 1** continued

| Species | Cytokinin(s) tested | Preferred cytokinin(s) | Optimum concentration | Growth parameter(s)/physiological disorder(s) affected | Reference |
|---|---|---|---|---|---|
| *Malus domestica* cv. 'Royal Gala' | BA, *m*T | *m*T | 2.1–6.2 μM | Shoot multiplication, hyperhydricity alleviation | Dobránszki et al. (2002) |
| *Malus domestica* cv. 'Royal Gala' | BA, KIN, *m*T, BA + KIN, BA + *m*T | *m*T | 2.1 μM | Shoot multiplication | Dobránszki et al. (2005) |
| | | *m*T | 6.2 μM | Hyperhydricity alleviation | |
| *Malus domestica* cv. Jonagold | BA, BAR, *m*T | *m*T | 20.7 μM | Shoot multiplication | Magyar-Tábori et al. (2002) |
| *Musa* spp. (AAA) | BA, *m*T, *m*TR, Me*m*T, Me*m*TR | *m*T, *m*TR | 22.2 μM | Shoot multiplication, plantlet quality, abnormality index | Bairu et al. (2008) |
| *Musa* spp. (AAB) cv. 'CEMSA 3/4' | BA, *m*T | *m*T | 4.4 μM | Shoot multiplication, length, rooting | Escalona et al. (2003) |
| *Musa* spp. (AAB) cv. 'CEMSA 3/4' | BA, *m*T, TDZ | *m*T | 4.4 μM | Shoot multiplication | Roels et al. (2005) |
| *Pelargonium* × *hederaefolium* 'Bonete' | BA, *m*T | BA | 2.2 μM | Shoot multiplication and quality | Wojtania and Gabryszewska (2001) |
| | | *m*T | 4.1 μM | | |
| *Pelargonium* × *hortorum* 'Bergpalais' | BA, *m*T | *m*T | 4.1 μM | Shoot multiplication and quality | Wojtania and Gabryszewska (2001) |
| *Pelargonium* × *hederaefolium* cv. 'Beach' | BA, *m*T | *m*T | 4.1 μM | Shoot multiplication, quality, rooting, delayed senescence | Wojtania (2010) |
| *Pelargonium* × *hederaefolium* cv. 'Luna' | BA, *m*T | *m*T | 2.1 μM | Shoot multiplication, quality, rooting, delayed senescence | Wojtania (2010) |
| *Pelargonium* × *hederaefolium* cv. 'Sofie Cascade' | BA, *m*T | *m*T | 4.1 μM | Shoot multiplication, quality, rooting, delayed senescence | Wojtania (2010) |
| *Pelargonium* × *hortorum* cv. 'Bergpalais' | BA, *m*T | *m*T | 4.1 μM | Shoot multiplication, quality, rooting, delayed senescence | Wojtania (2010) |
| *Pelargonium* × *hortorum* cv. 'Grand Prix' | BA, *m*T | *m*T | 4.1 μM | Shoot multiplication, quality, rooting, delayed senescence | Wojtania (2010) |
| *Pelargonium* × *hortorum* cv. 'Jazz Rocky Mountain' | BA, *m*T | *m*T | 4.1 μM | Shoot multiplication, quality, rooting, delayed senescence | Wojtania (2010) |
| *Pelargonium* × *hortorum* cv. 'White Rocky Mountain' | BA, *m*T | *m*T | 4.1 μM | Shoot multiplication, quality, rooting, delayed senescence | Wojtania (2010) |
| *Petunia hybrida* | BA, Me*m*TR | Me*m*TR | 2.0 μM | Histogenic stability | Bogaert et al. (2006) |
| *Pinus pinaster* | BA, *m*T, TDZ, Z | BA, Z, *m*T | 25 μM | Shoot multiplication | De Diego et al. (2010) |
| *Pinus pinea* | BA, BPA, *m*T, TDZ | TDZ | 2.5 μM | Shoot multiplication | Cortizo et al. (2009) |
| *Pinus sylvestris* | BA, *m*T, TDZ, Z | *m*T | 25 μM | Shoot multiplication | De Diego et al. (2010) |
| *Prunus avium* | BA, CPPU, *m*T, TDZ | *m*T | 4.1 mM | Fruit weight | Zhang and Whiting (2011) |
| *Prunus microcarpa* | BA, *m*T, TDZ | BA | 2.5 μM | Shoot multiplication | Nas et al. (2010) |
| *Raphanus sativus* | *m*T | *m*T | 1.0 mM | Cotyledon growth, chlorophyll content and peroxidase activity | Palavan-Ünsal et al. (2002a) |

**Table 1** continued

| Species | Cytokinin(s) tested | Preferred cytokinin(s) | Optimum concentration | Growth parameter(s)/physiological disorder(s) affected | Reference |
|---|---|---|---|---|---|
| *Rosa hybrida* cv. 'Païline' | BA, F*m*T, F*m*TR, *m*T, Me*m*T, Me*m*TR | F*m*TR | 2.5 μM | Shoot multiplication | Bogaert et al. (2006) |
| | | Me*m*TR | 2.5 μM | Delayed senescence | |
| *Sacchrum officinarum* var. clone 'Gungera' | CPPU, *m*T, TDZ | *m*T | 10.0 μM | Shoot multiplication | Vinayak et al. (2009) |
| *Sacchrum officinarum* var. Co 1148 | CPPU, *m*T, TDZ | TDZ | 0.01 μM | Shoot multiplication | Vinayak et al. (2009) |
| *Sacchrum officinarum* var. CoS 8436 | CPPU, *m*T, TDZ | CPPU | 5.0 μM | Shoot multiplication | Vinayak et al. (2009) |
| *Sclerocarya birrea* | BA, *m*T, *m*TR, Me*m*TR | *m*T | 8.0 μM | Shoot multiplication, quality and length | Moyo et al. (2011) |
| *Sisyrinchium laxum* | BA, *m*T | *m*T | 20.7 μM | Shoot multiplication | Ascough et al. (2011) |
| *Solanum tuberosum* cv. Jaerla | *m*TR | *m*TR | 11.3 nM | Improved root/shoot ratio, acclimatization competence | Baroja-Fernández et al. (2002) |
| *Sorbus torminalis* | BA, *m*T, Me*m*TR | BA | 0.9 μM | Shoot multiplication | Malá et al. (2009) |
| | | Me*m*TR | 0.8 μM | Rooting | |
| *Spathiphyllum floribundum* cv. 'Petite' | BA, BAR, BPA, *m*T | *m*T | 10.0 μM | Rooting, acclimatization competence | Werbrouck et al. (1996) |
| *Triticum aestivum* | *m*T | *m*T | 1.0 mM | Delayed senescence | Palavan-Ünsal et al. (2002b) |
| *Uniola paniculata* EK 16-3 | BA, *m*T | *m*T | 20.0 μM | Shoot multiplication, shoot dry weight | Valero-Aracama et al. (2010) |
| | | | 2.2 μM | Rooting, acclimatization competence | |
| *Uniola paniculata* EK 11-1 | BA, *m*T | *m*T | 10.0 μM | Shoot multiplication, shoot dry weight | Valero-Aracama et al. (2010) |
| | | | 2.2 μM | Rooting, acclimatization competence | |
| *Vaccinium corymbosum* cv. Highbush blue berry | *m*T, TDZ, Z | Z | 20.0 μM | Shoot multiplication | Meiners et al. (2007) |
| *Vaccinium vitis-idaea* cv. Lingonberry | *m*T, TDZ, Z | Z | 20.0 μM | Shoot multiplication | Meiners et al. (2007) |

*n/s* not stated

plants (Letham and Palni 1983; Bajguz and Piotrowska 2009). Although the adenine-based forms of CKs are mostly preferred in PTC, the synthetic CKs such as TDZ and CPPU are also useful, especially in developing protocols for recalcitrant genotypes (Murthy et al. 1998). The effectiveness of these synthetic CKs has been demonstrated in micropropagation of apple (Fasolo et al. 1989), banana (Makara et al. 2010), sugar cane (Vinayak et al. 2009) and orchids (Mata-Rosas et al. 2010).

Despite the presence of numerous natural and synthetic CKs for use in PTC, the search for new CKs has remained active although not many laboratories are committed to it. The major advances in chromatographic and spectrometric techniques has aided the search (Tarkowski et al. 2009). Most importantly, the ever growing application of plant hormones in plant growth and improvement as well as the physiological and economic (to a lesser extent) limitation of the existing CKs have spurred the active search for new CKs (Kamínek 1992; Strnad 1997; Werbrouck 2010). Consequently, a new class of aromatic CKs, the topolins were discovered and identified. In the topolin basic structure, the presence of an hydroxyl group on the benzyl ring differentiates it from BA. Although the first isolation and identification of hydroxylated BA (*o*T) from *Populus robusta* dates back to the early 1970s (Horgan et al. 1973; Horgan et al. 1975), Strnad et al. (1997) are credited with the isolation of *m*T [6-(3-hydroxybenzylamino)purine], the highly active hydroxylated BA derivative from mature leaves of *Populus* × *canadensis*. Thereafter, four new methoxy derivatives of the topolins identified as 6-(2-methoxybenzylamino)purine (*ortho*-methoxytopolin, Me*o*T), 6-(3-methoxybenzylamino) purine (*meta*-methoxytopolin, Me*m*T) and their 9-$\beta$-D-ribofuranosyl derivatives (Me*o*TR and Me*m*TR) were isolated from *Arabidopsis thaliana* and *Populus* × *canadensis* (Tarkowská et al. 2003). A recent review has subsequently listed different topolins from a few other plant species (Subbaraj 2011). These major breakthroughs coupled with favourable results from the activity evaluation experiments using various bioassays and PTC systems have stimulated the increased use of these new CKs.

Countless PTC protocols have been developed and optimized for many plant species. These protocols are developed and optimized based on quantitative and qualitative evaluation of growth parameters such as shoot multiplication rate, rooting, plant appearance and acclimatization competence. The choice of medium type such as Murashige and Skoog (MS), gelling agent as well as type and concentration of PGRs are based on these essential growth parameters. Growth, by and large, is a function of cell division and it is the factors controlling this process that receive major emphasis when developing a PTC protocol. With the exception of a few laboratories worldwide, PTC experiments aimed at an in-depth understanding of the physiological and biochemical mechanisms by which PGRs bring about the changes are considered only when things do not work and/or when tissue abnormalities are encountered. As a result, many scientific reports on PTC lack basic metabolic and physiological explanation of growth. Recently, we proposed an improved or alternative approach whereby the type and concentrations of PGRs (and some other components) used in the cultivation media are optimized not only based on essential growth and acclimatization parameters but also correlated with optimum levels of active endogenous PGRs as well as minimization of inactive toxic metabolites (Malá et al. 2009; Bairu et al. 2011b). We believe that plant tissue culturists need to have a basic understanding of plant hormone physiology in relation to PTC. Hence, brief overviews on the CK biosynthesis, metabolism and structure–activity relationships are discussed.

## CK biosynthesis and metabolism in relation to plant tissue culture

Plant tissue culture entails the growth of plants in artificial environments involving the use of externally applied PGRs such as CKs and auxins. This process interferes with, or influences the natural process of CK biosynthesis, a process little understood despite considerable attention and work on plant hormone physiology (Hutchison and Kieber 2002; Pan et al. 2008; Jiang and Guo 2010). Taylor et al. (2003) identified factors such as the extremely low levels of endogenous CKs, the central role of the likely precursors in cellular metabolism, the existence of numerous native substances with more or less pronounced CK-like activity and the reliance on incorrect or only partially correct assumptions about CK biosynthesis, as obstacles to the understanding of the biosynthetic pathways.

Recent findings on CK analysis of various in vitro grown species such as *Watsonia lepida* (Ascough et al. 2009) and *Harpagophytum procumbens* (Bairu et al. 2011b) indicated variation in type and composition of CK metabolites suggesting that plants vary in their CK metabolism patterns. According to Palmer et al. (1981) and Van Staden and Crouch (1996), the variation in the metabolism of applied CKs may be attributed to differences in the stages of development, physiological condition of the plant, organ type, plant species analyzed, concentration of applied PGRs and methods of application. Variation in the composition of CK metabolites of normal and necrotic shoots of in vitro grown *Harpagophytum procumbens* is a notable example of the effect of physiological condition on CK metabolism (Bairu et al. 2011b). This variation therefore makes it difficult to draw a general conclusion about the role of CK metabolites in plant growth and development in vitro. The variation in the composition of CK metabolites may also indicate that it is

unlikely for all the CKs to be converted to a common metabolite responsible for the growth responses observed.

Plant tissues metabolize exogenous CKs to different types of metabolites such as products of ring substitution (ribosides, nucleotides, N-glucosides) and products of side chain substitution (*O*-glucosides) or cleavage (adenine, adenosine, adenosine-5′-monophosphate) (Letham and Palni 1983; Van Staden and Crouch 1996). Although the functional significance of these metabolites is still not well understood (Wagner and Beck 1993; Van Staden and Crouch 1996), long standing suggestions by Letham and Palni (1983) indicate that these compounds could be: active forms of CK, i.e. the molecular form that induces growth or physiological response; translocation forms; storage forms which would release free (active) CKs when required; detoxification products formed due to the application of exogenous CK at toxic levels; deactivation products formed to lower endogenous (active CK) levels; and post-activation products, the formation of which is coupled with CK action.

The structural variation among CKs is another aspect contributing to the difference in CK metabolism. Van Staden and Crouch (1996) emphasized the absence of a common metabolic pattern for aromatic CKs compared to the knowledge on isoprenoid CK metabolism. The need for better understanding of the pattern of CK metabolism triggered the study of CK action at molecular and cellular levels with emphasis on signal perception, transduction and response (Kamada-Nobusada and Sakakibara 2009). Initially, these studies were mainly done by searching for proteins which may serve as signal receiving molecules followed by studying the signal transduction pattern(s) (Strnad 1997). Although very little is known about the molecular mechanism by which target cells for PGRs translate the signals to specific responses, advances in molecular genetics are making such studies at protein and gene level possible (Kamada-Nobusada and Sakakibara 2009). Earlier studies by Libbenga and Mennes (1995) indicated that in a hormonal system, cells of different tissues and organs not only transmit signals, but are also capable of detecting signals which they receive from other parts and respond to those signals in their own characteristic way.

Strnad (1997) discussed the specificity and complexity of CK binding. The existence of two related groups of CK binding proteins (CBPs), one with low affinity to Z but with strong affinity to BA and the other with an opposite character, serves as an indication that there is a distinction between aromatic and isoprenoid CKs with respect to nature of binding and receptor response. In line with the proposal by Iwamura et al. (1980), the differences found in the electron structure and hydrophobicity of the $N^6$-substituent are probably related to the CK binding site; the role of modified adenines in CK-binding interaction has been elucidated using X-ray crystallographic structural studies (Strnad 1997).

Mok et al. (2005) reported that topolins and hydroxylated TDZ derivatives are substrates for CK *O*-glucosyltransferase with position specificity related to receptor recognition. It was shown that *m*T and *o*T derivatives are preferred substrates of zeatin *O*-glucosyltransferase (ZOG1; enzyme encoded by *Phaseolus lunatus*) and *cis*-ZOG1 (enzyme encoded by *Zea mays*), respectively. The authors reported a correlation between the activity of the CKs and their ability to serve as a substrate for glucosyltransferase, thereby suggesting a similarity between CK-binding sites on the enzyme and CK receptors. In addition, they found support for their interpretation from CK recognition studies involving the *Arabidopsis* CRE1/WOL/AHK4 and *Zea mays* ZmHK1 receptors. The AHK4 receptor responded to *trans*-Z and *m*T while the ZmHK1 receptors responded to *cis*-Z and *o*T.

Cytokinins, in combination with auxins, are known to affect the basic mechanisms of cell proliferation and differentiation (Faure et al. 1998). Harrar et al. (2003) demonstrated that this hormonal control of cell proliferation and differentiation requires PASTICCINO (*PAS*) genes. The authors determined the role of the *PAS* gene by analysing the expression profiles of several genes involved in cell division and meristem functioning and found that differentiated and meristematic cells of the *PAS* mutants were more competent for ectopic cell division, and were especially enhanced by CKs. They further demonstrated that disorganised cell divisions were associated with the deregulation of cell cycle marker genes like cyclin-dependent kinase A (CDKA) and cyclin B1 (CYCB1).

These variations in CK metabolism, binding and signal perception could possibly explain the inherent variation in response among plants in PTC systems. In addition, the structural variation among CKs is undoubtedly another avenue worth investigating because the literature indicates that a slight variation in structure results in a major change in activity in tissue culture systems. Hence, factors affecting CK structure–activity relationships are discussed below.

## CK structure–activity relationships

The concept of CK structure–activity relationship has attracted the attention of researchers for more than five decades. Today, research interest and focus on the same topic is as topical as previously, due to the growing application of our knowledge of PGRs. It is well documented that the activity of CKs is highly affected by their structure; with the structure activity relationship of CKs being affected in a number of different ways (Haberer and

Kieber 2002). The influence of structural modifications on the activity of natural and synthetic CKs has been extensively studied. Different factors such as using: an intact adenine moiety with an $N^6$-substituent of moderate molecular length (Skoog and Armstrong 1970), an intact purine ring, unsubstituted 1- and 3-positions, optimum side chain length of five carbon atoms, unsaturation in the side-chain, a substituent which is planar and hydrophobic (Hecht et al. 1970; Nishikawa et al. 1986), an electron rich nitrogen group located opposite to the substituent and linkage atoms or a group that connects the purine ring with the side-chain and restrict the molecular configuration (Nishikawa et al. 1986), 4-hydroxylation of the *iso*-pentenyl side chain (Matsubara 1980) have been mentioned as a structural requirement for high CK activity. Alternatively, Chen and Kristopeit (1981) suggested that activity depends on the interconversion between the free bases and their ribosides. Matsubara (1980) attributed the structure activity relationship of CKs to several factors which could be placed in one or more of the above mentioned categories. General structural and functional requirements for CK activity are discussed briefly.

## The general effect of ring substitution of aminopurines

Most of the pioneering research on the effect of ring substitution on CK activity dates back to the 1950s, 1960s and 1970s. A detailed and comprehensive review of this effect was done by Matsubara (1980). Replacing the furfuryl group of KIN by a wide range of other side-chains was possible without any considerable loss of CK activity. BA, arguably the most utilized CK in PTC owes its discovery to this form of ring substitution—replacing the furfuryl group by a benzyl group. BA was found to be more active than KIN in the tobacco callus assay. The activity of ring substituted aminopurines is also affected by the degree of saturation. Generally, aminopurines with an unsaturated ring are more active than the saturated ones (Skoog et al. 1967; Skoog and Armstrong 1970; Matsubara 1980).

## The effect of the hydroxyl group on the side chain

Hydroxylation of the *trans* methyl group in the $N^6$ side-chain of $N^6$-($\Delta^2$-*iso*-pentenyl) adenosine increased its biological activity but the activity was not affected or decreased when the *cis* methyl group was hydroxylated. This targeted hydroxylation of aromatic CKs is believed to regulate their activity (Kamínek et al. 1987). This form of hydroxylation is likely to have an effect on the dihedral angle and the formation of an hydrogen bond with the nitrogen at $N^1$ position—both known to affect the activity of aromatic CKs depending on the position of the hydroxyl group (Trávníček et al. 1997; Nirmalram et al. 2011).

The position of the hydroxyl group on the side-chain has a significant effect on the biological activity of the parent CKs. Substitution on the phenyl ring enhanced the activity in the order of *meta* > *ortho* > *para* in most bioassays for purinyl and urea-type CKs (Kamínek et al. 1987). Horgan et al. (1975) noted a decrease in activity of $N^6$-benzyladenosine after hydroxylation of the phenyl ring at the *ortho* position. Mok and Mok (1985) also demonstrated that activity was in order of *meta* > *ortho* > *para* in the *Phaseolus lunatus* callus assay for the hydroxylated derivatives of TDZ. The positive reports on the role of *m*T in PTC (see Table 1 for references) could therefore be attributed to these structural changes.

## The effect of a double bond on the side chain

Hecht et al. (1970) highlighted that the presence of a double bond on the side chain is one of the structural requirements for high CK activity. A high CK activity can also be achieved or maintained by keeping side-chain planarity. Furthermore, the authors postulated that the addition of a substituent to the double bond disturbed the side-chain planarity, thereby reducing CK activity. The position of the double bond also affects CK activity. Leonard et al. (1968) demonstrated that the 2,3-position of the side-chain as in 6-(3-methyl-2-butenylamino)purine enhanced activity while shifting the double bond to the 3,4-position as in 6-(3-methyl-3-butenylamino)purine reduced the activity in the tobacco callus bioassay. By synthesizing and testing the biological activity of two 6-substituted purines, one with an $\acute{\alpha}$-double bond and one without, Nishikawa et al. (1986) revealed that the compound with the $\acute{\alpha}$-double bond was twice as active as the one without, indicating the importance of the double bond in CK activity.

## The effect of the methyl and amino groups on the side chain

Cytokinin activity is affected by the methyl group(s) and their position on the side-chain of $N^6$-substituted adenines. Lengthening the bridge or removal of the methyl group between the ring and the amino group on the 6-position of the purine ring in ring-substituted adenines decreases CK activity (Matsubara 1980). Alternatively (at least in the case of aromatic CK), high ability of these anilinopurines to inhibit CK oxidase-dehydrogenase, the key enzyme of CK catabolism, and thereby to increase levels of endogenous active CKs in the treated plant tissues has been recently shown (Zatloukal et al. 2008). Di-substitution with two methyl groups at position 3- produced a highly active 6-($\gamma$,$\gamma$-dimethylallylamino)purine whereas addition of a methyl group to the 1, 2, or 3-positions of the carbon atom on the side-chain did not affect the activity. However,

putting these two methyl groups at position 1- of the side-chain reduced the activity a 100 fold. Shifting the methyl group from position 3- to 2- of the side-chain of *cis* and *trans*-Z or removing it from *cis*-Z resulted in a significant decrease in activity (Skoog et al. 1967; Matsubara 1980). The replacement of the amino group of KIN by a sulphur atom resulted in a considerable decrease in activity suggesting the importance of the amino group on the $N^6$-position for CK activity (Matsubara 1980).

### The effect of side chain configuration (geometrical and optical isomers)

Studies have shown that the side-chain configuration affects CK activity. For example, *trans*-Z was more active than the *cis* isomer in stimulation of cotyledon expansion, retention of chlorophyll in detached leaf pieces, induction and stimulation of chlorophyll synthesis in *Cucumis sativus* and betacyanin synthesis in *Amaranthus caudatus* seedlings grown in the dark (Kamínek et al. 1979). The difference in the side-chain configuration of geometrical isomers affects the side-chain planarity, which in turn affects activity (Hecht et al. 1970). The interaction between the CK molecule and its receptor site is also influenced by the absolute configuration around the asymmetric carbon (Matsubara 1980).

### The effect of position of the substituent on the purine ring

The position of the substituents in the purine ring affects CK activity and gives the CKs their respective identities. For a CK to remain active, the reactive $N^1$ position must remain free (Matsubara 1980). This concept elaborates the fact that *o*T is less active than *m*T due to the formation of the hydrogen bond with the nitrogen at $N^1$ position (Holub et al. 1998).

The effect of substitution at $N^2$ varied depending on the type of assay and the nature of the substituents. An example of a compound with substitution at $N^3$ position is triacanthine—a naturally occurring adenine derivative. Similar to the isomers with $N^1$ substitution, triacanthine was active only following autoclaving leading to the postulation of producing $N^6$-substituted adenine (Leonard and Henderson 1975). These authors demonstrated that when autoclaved, 3-substituted adenines undergo rearrangement and conversion in low quantity to $N^6$-substituted adenines. The $N^6$ substituted adenines are the most active of all N-substituted adenines and generally, the mono-substituted ones are more active than the corresponding di-substituted ones (Skoog et al. 1967). In addition, substitutions at positions $N^7$, $N^8$ and $N^9$ produce inactive or slightly active products depending on the nature of the substituent molecules (Matsubara 1980).

### The effect of an halogen substituent

The activity enhancing effect of halogen substitution on isoprenoid (Clemenceau et al. 1996; Haidoune et al. 1998) and aromatic (Bogaert et al. 2006; Doležal et al. 2006; Doležal et al. 2007) CKs are well documented. Different authors explained the activity enhancing effect of halogen substitution differently, for example: the high electronegativity and small size of the halogens (Crocker et al. 2007), the ability of organo-halogen compounds to form structural motifs via inter-molecular interactions (Emmerling et al. 2007), decreased sensitivity of the compounds to CK oxidase (Clemenceau et al. 1996; Haidoune et al. 1998), and ability to form an hydrogen bond with electron donors of a CK receptor (Doležal et al. 2007) are proposed to be the contributing factors.

The structural variability of organic compounds in general and CKs in particular offered a wide range of possibilities to modify their structure and enhance their application spectrum. The variations in the biological activity of the same CKs in various bioassay systems are logical indications of the diverse recognition patterns and/or signalling mechanisms that may operate in CK-dependent physiological responses. It also highlights the possibility of designing specific compounds to modulate a particular CK-dependent response (Doležal et al. 2006; Doležal et al. 2007). This makes interpretation of biological activity test results to be dependent on the nature of the assay and plant species used.

While the above structure–activity relationships and considerations provide an insight on the factors that affect CK activity, these also probably help us understand and/or explain some of the growth related observations in PTC systems. Therefore, we examine the role of topolins in PTC systems in comparison to other CKs.

## Effect of topolins on shoot multiplication and elongation

Shoot regeneration and multiplication during micropropagation is affected by the type and concentration of applied PGRs, especially the CKs due to their importance in cell division and organogenesis (Howell et al. 2003). Benzyladenine remains one of the most effective CK that promotes in vitro shoot regeneration and multiplication of plant species. In an attempt to address various physiological and developmental problems associated with CKs in general and BA in particular however, other CKs are being tested.

As shown in Table 1, various studies on a wide range of plant species highlighted the potential of topolins as a substitute to the commonly used CKs. Using equimolar concentration (10.0 μM) of either *m*T or BA, Werbrouck et al. (1996) observed better shoot–root balance with *m*T-treated *Spathiphyllum floribundum* plantlets. Similarly, *m*T at various concentrations produced more shoots compared to either BA or Z during the micropropagation of *Aloe polyphylla* (Bairu et al. 2007). In banana cv. 'Williams', the use of topolins (*m*T, *m*TR, Me*m*T, Me*m*TR) at 7.5, 15 and 30 μM had higher shoot multiplication rates than BA (Bairu et al. 2008). Studying the effect of nine CKs on shoots production in micropropagated *Curcuma longa*, Salvi et al. (2002) obtained more shoots with the use of kinetin ribosides (KINR), iP or *m*T (no significant difference) compared to either BA or other CKs used. However, regenerated shoots from *m*T treatment were greener and stouter than KINR and iP-treated one.

Nevertheless, unfavourable responses from the use of topolins for shoot regeneration and multiplication rate have also been observed in certain plant species. In *Rosa hybrida* for example, Bogaert et al. (2006) reported that Me*m*TR-treated explants had a lower multiplication rate than the BA treatment. A number of similar responses (where other CKs were more effective) have been documented by several authors (Table 1). There was no definite pattern in the plant species responses to the different CKs; an indication of the interaction of several factors during shoot regeneration and multiplication phase. Factors such as genotype, CK concentration used and medium type (Dobránszki et al. 2004; Vinayak et al. 2009; Magyar-Tábori et al. 2010; Wojtania 2010) remain vital and need to be taken into consideration while optimizing PTC protocols.

## Effect on rooting and ex vitro acclimatization

Lack or inadequate rooting is among the major problems in PTC. In micropropagated plants generally, there is a positive correlation between good rooting (mainly stimulated by auxins) and their ability to acclimatize faster (Werbrouck et al. 1996; Koetle et al. 2010). Acclimatization of in vitro grown plants to natural conditions is a vital step in the micropropagation of many species especially for large scale application of in vitro techniques As opposed to the ex vitro conditions, during in vitro micropropagation, plants grow under constant temperature, high relative humidity, low irradiance, sugars as carbon source, PGRs in nutrient medium, variable and often insufficient $CO_2$ concentrations (Pospíšilová et al. 2007). These differences result in variation in factors such as leaf structure, water relations and photosynthetic parameters which are critical for the successful acclimatization of in vitro plants (Pospíšilová et al. 1999). In addition, the effect of the applied PGRs such as CKs, auxins and abscisic acid are crucial in the successful and improved acclimatization of in vitro plants. Several studies on different species have reported that the type and concentration of CKs have a profound effect on in vitro plant acclimatization competence (Moncaleán et al. 2001; Bairu et al. 2008; Valero-Aracama et al. 2010). In addition, some authors have reported that CKs generally have inhibitory effects on rooting, resulting in poor acclimatization rates afterwards (Werbrouck et al. 1995; Bairu et al. 2008).

Although application of exogenous CKs enhances shoot formation during micropropagation, the CK BA is often associated with negative side-effects during acclimatization of some plant species. According to Werbrouck et al. (1995), these harmful effects are partly due to the formation of N-glucosides or alanine conjugation, which are biologically inactive (characterize by slow release of active CK free bases) and chemically stable metabolites. More importantly, BA-treated plantlets have the tendency of accumulating these toxic BA metabolites in their basal (rooting zone) portions. Consequently, these metabolites interfere with rooting and acclimatization competence in micropropagated plantlets (Werbrouck et al. 1995). As demonstrated by Valero-Aracama et al. (2010), BA treatment caused detrimental biochemical, physiological and developmental effects in *Uniola paniculata* cultures that resulted into reduced acclimatization competence. The authors discovered that similar problems were absent in *m*T treatments; which is a further indication of the relative lower toxicity of *m*T over BA and its analogues. Concomitantly, Werbrouck et al. (1996) postulated that *m*T metabolites are less stable and produce reversibly sequestrated metabolites. The presence of an hydroxyl group in topolins give them a structural advantage over BA in that they can undergo *O*-glucosylation to form storage forms (Bairu et al. 2011b).

In contrast to BA, *m*T stimulated in vitro rooting activity in *Spathiphyllum floribundum* (Werbrouck et al. 1996). Similarly, Bairu et al. (2007) observed that both *m*T and *m*TR promoted the rooting of *Aloe polyphylla* shoots in the multiplication medium and approximately 90% of plantlets treated with *m*T acclimatized successfully compared to a 65% survival rate recorded with BA-treated plantlets. Low concentrations (0.005–0.010 mg $l^{-1}$) of *m*TR significantly increased the rooting and survival of *Solanum tuberosum* cv. Jaerla plantlets (Baroja-Fernández et al. 2002). However, the authors observed a decrease in the survival rate as the concentration of the *m*TR was increased.

The positive effect of topolins on rooting, however, is not universal. *Meta*-topolin and *m*TR at 22.2 μM had an inhibitory effect on rooting of *Musa* spp. (cv. Williams) compared to BA (Bairu et al. 2008). Valero-Aracama et al.

(2010) observed that *m*T at 10 μM or higher had inhibitory effects on the in vitro rooting of two *Uniola paniculata* genotypes. Similarly, Escalona et al. (2003) in a study on plantain cv. 'CEMSA 3/4' reported a progressive reduction in root production as the concentrations of BA and *m*T were increased (1.33–22.2 μM), however, 1.33 μM *m*T gave the highest number of roots. The general trend indicates that higher concentrations of most topolins (as is the case with most CKs) were detrimental to the rooting and acclimatization competence of micropropagated plants. Hence, a wide range of concentrations should be incorporated while investigating the use of these topolins. The presence of well-developed roots in multiplication media and inhibition of rooting in some species warrant detailed investigation on the effect of topolins in the physiology of root development.

The interaction of CKs with auxins has always been an important consideration in PTC. Very little published work on topolins is available on this aspect. Recently, we observed the differential effect of the role of CK-auxin interaction on endogenous CK levels in relation to shoot-tip necrosis (STN) (Bairu et al. 2011b). It was noted that the presence of the auxin, indole-3-acetic acid (IAA) in the culture medium enhanced the formation of 9-glucosides in BA-treated cultures but reduced it in topolin-treated cultures. Better *O*-glucosylation was also observed in topolin-treated cultures when IAA was omitted. Similarly, Malá et al. (2009) measured lower levels of IAA in BA-treated explants of *Sorbus torminalis* compared to *m*T—an explanation to reduced rooting by BA-treated cultures. Although the structural differences will have a role to play, the physiological and biochemical events leading to these effects are yet to be fully understood.

## Effect of topolins on countering in vitro abnormalities

### Effect on hyperhydricity

Hyperhydricity is a morphological and physiological disorder observed in micropropagated plants (Debergh et al. 1992). It results from either a passive diffusion of water into the tissue or an active phenomenon related to strong metabolic disturbances (Pâques 1991). Hyperhydricity is a common problem that has been observed in micropropagated plants and hinders the potential of in vitro techniques for mass propagation of plant species (Kevers et al. 1984).

Plant growth regulators, the type and concentration of the gelling agents, presence of large quantities of mineral nutrient (mainly $NH_4^+$ and $Cl^-$) ions in the medium and high relative humidity in the culture vessels are among the factors causing hyperhydricity (Pâques and Boxus 1987; Franck et al. 2004). Although the mechanism and interactions of these factors are still not well understood, the effect of PGRs in general and CKs in particular are well documented.

Bairu et al. (2007) investigated the effect of topolins on hyperhydricity in *Aloe polyphylla*. At an optimum concentration of 5.0 μM, there were no hyperhydric shoots in topolin (*m*T and Me*m*T)-treated plants. Although all the CK treatments caused hyperhydricity at higher concentrations, it was most severe with BA treatments. Similarly, drastic alleviation of hyperhydricity was also reported with the use of a low concentration of *m*T for *Beta vulgaris* (Kubalákova and Strnad 1992) and for several *Malus* × *domestica* cultivars (Dobránszki et al. 2002; Dobránszki et al. 2004; Dobránszki et al. 2005).

### Effect on shoot-tip necrosis (STN)

Shoot-tip necrosis (shoot die-back) is a common physiological disorder in micropropagated plants (Bairu et al. 2009c). The abnormality is often accompanied by browning of buds and leaves eventually leading to plant death. The symptoms result from the senescence and death of tissues in the apical bud which subsequently proceed basipetally (Kataeva et al. 1991). Shoot-tip necrosis is a consequence of a complex set of factors (Bairu et al. 2009c), the effect of PGRs especially CKs however, remains dominant (Il'ina et al. 2006). Although the rate of STN increased with increasing CK concentration, Bairu et al. (2009a) observed significantly lower STN in micropropagated *Harpagophytum procumbens* supplemented with *m*TR compared to BA treatment. Similarly, the use of Me*m*TR alleviated STN associated with micropropagation of *Barleria greenii* (Amoo et al. 2011).

### Effect on early senescence

Senescence is a genetically programmed process that involves a general termination of cellular structures, followed by mobilization of the degradation products to other parts of the plant (Woo et al. 2004). Leaf senescence is a critical process for the fitness of plants and plays a major role in evolution of plants (Nam 1997). Generally, it is controlled by many internal and external factors (Noodén 1988). Plant growth regulators such as CKs, abscisic acid, auxin and ethylene do play some roles in regulation of leaf senescence. It is understood that CKs are one of the main regulators of senescence in plants (Van Staden et al. 1988). Cytokinins are known to delay aging in many plant organs through protein breakdown inhibition as well as the stimulation of RNA and protein synthesis (Palavan-Ünsal et al. 2002b). Molecular studies have provided evidence on the effect of CKs on senescence (Gan and Amasino 1996). Yet, the molecular mechanisms of CK action as well as the

molecular basis of plant senescence generally are poorly understood.

Early senescence is a common problem in PTC of many plant species, for example, Mithila et al. (2001) highlighted this problem as hindering the micropropagation of many *Pelargonium* cultivars. Subsequently, Wojtania (2010) discovered that *m*T was better than BA in the inhibition of early senescence in seven *Pelargonium* cultivars. *Meta*-topolin-treated *Pelargonium* plantlets had higher chlorophyll content. Likewise, the application of *m*T to leaf segments of *Triticum aestivum* retarded senescence by decreasing protease activity and chlorophyll loss (Palavan-Ünsal et al. 2002b). Further studies also established that both nitrogen and polyamine contents increased due to application of *m*T. As a result, the authors suggested that increased peroxidase activity, polyamines and nitrogen contents in *m*T-treated excised *Triticum aestivum* was a contributing factor to the overall anti-senescence activity of *m*T (Palavan-Ünsal et al. 2004). Using *Rosa hybrida* as a model plant, Bogaert et al. (2006) investigated the anti-senescence effect of BA and topolins on micropropagated plants. Although no CK treatment prevented the older (lower) leaves from senescing after 6 weeks, Me*m*TR treatment showed the most noteworthy anti-senescing activity. Furthermore, after 18 weeks, 50% of Me*m*TR-treated plants were alive and the next active CK, 6-(3-fluorobenzylamino)purine-9-riboside (F*m*TR) had only a 14% survival rate.

Foliar application of *m*TR to field grown sugar beet delayed senescence with increased content of natural cytokinins as well as higher yield compared to the untreated control (Čatský et al. 1996). These effects were associated with the reduced respiration and/or stimulated membrane transport processes in the *m*TR-treated plants (Kotyk et al. 1996). In view of the importance of early senescence inhibition, molecular approaches have been suggested for better understanding of the process (Gan and Amasino 1997). In addition, we recommend both in vitro and ex vitro application of these topolins, to evaluate their effect on retarding senescence on various plant species.

### Effect on histogenic stability

Histogenic stability is an important factor in commercial micropropagation of plants for two contrasting purposes, namely preserving pre-existing variation (chimeras) and preventing unnecessary (somaclonal) variation, see Bairu et al. (2011a) for a detailed review. In addition, incidence of somaclonal variation serve as a potential avenue for generating new variants in ornamental potted plants such as *Anthurium* (Winarto et al. 2011).

During micropropagation of ornamentals, it is essential to maintain the pre-existing genetic composition of chimeric plants for its commercial value. Presently, KIN is the only tool to slowly and safely regenerate these valuable ornamentals (Bogaert et al. 2006). However, KIN being a weak CK is often associated with lower shoot multiplication (Amoo et al. 2011). These drawbacks probably stimulated the application of topolins in the micropropagation of chimeras. Maintaining the histogenic stability of *Petunia* meristem was improved using Me*m*TR when compared to BA. Furthermore, application of Me*m*TR resulted in a superior visual quality of the axillary shoots (Bogaert et al. 2006).

An increase in BA concentration increased the rate of variation in Cavendish banana cv. 'Zelig' (Bairu et al. 2006). In another study, Bairu et al. (2008) reported the absence of significant differences in the occurrence of somaclonal variation when BA, *m*T and *m*TR were compared for 'Williams' banana. The authors postulated that the results obtained in their investigation could have been influenced by carry-over effects of BA from the initial cultures due to its effect on somaclonal variation (Bairu et al. 2006). A similar scenario was observed during the micropropagation of *Barleria greenii* that was initially maintained on BA-supplemented media (Amoo et al. 2011). The authors observed that the abnormality index of *m*TR and Me*m*TR treatments were lower than the control treatment. Thus, it was suggested that the observed abnormality index in *m*TR and Me*m*TR treatments was a carry-over effect from BA. Presently, stringent studies to investigate the role of topolins on somaclonal variation by minimizing the influence of BA as a confounding factor on the genetic stability of 'Williams' bananas are on-going in our laboratories.

## Concluding remarks and future considerations

The choice of CK is amongst the most critical factors in developing a successful PTC protocol. Numerous examples from Table 1 have shown that topolins can successfully replace the commonly used CKs in many PTC protocols. They can also play corrective roles on some physiological disorders. It should however, be noted that there are species that respond better to CKs other than the topolins; hence topolins should not be taken as a panacea and must pass through the routine process of selection. While it is essential to optimize efficient PTC protocols through stringent choice of CKs, tissue culturists should also give emphasis to the associated physiological and metabolic events taking place in culture during the optimization process so that they contribute towards better understanding of the mode of action of these molecules. Such an approach will help us solve associated physiological and developmental problems in vitro. Since many tissue culture

laboratories are not well equipped for physiological experiments, collaborative efforts could help bridge the gap.

We recommend that fresh cultures are started to investigate the role of topolins to avoid possible carry-over effects of other CKs and culture additives thereby eliminating reaching incorrect conclusions. To elucidate the broad spectrum of action of topolins, our research group at the Research Centre for Plant Growth and Development, University of KwaZulu-Natal in South Africa in collaboration with the Laboratory of Growth Regulators in the Czech Republic are testing these CKs on a wide range of species.

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## References

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

Ascough GD, Van Staden J (2010) Micropropagation of *Albuca bracteata* and *A. nelsonii*—indigenous ornamentals with medicinal value. S Afr J Bot 76:579–584

Ascough GD, Novák O, Pěnčík A, Rolčík J, Strnad M, Erwin JE, Van Staden J (2009) Hormonal and cell division analyses in *Watsonia lepida* seedlings. J Plant Physiol 166:1497–1507

Ascough GD, Swart PA, Finnie JF, Van Staden J (2011) Micropropagation of *Romulea minutiflora*, *Sisyrinchium laxum* and *Tritonia gladiolaris*—Iridaceae with ornamental potential. S Afr J Bot 77:216–221

Bairu MW, Fennell CW, Van Staden J (2006) The effect of plant growth regulators on somaclonal variation in Cavendish banana (*Musa* AAA cv. 'Zelig'). Sci Hortic 108:347–351

Bairu MW, Stirk WA, Doležal K, Van Staden J (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 WA, Doležal K, Van Staden J (2008) The role of topolins in micropropagation and somaclonal variation of banana cultivars 'Williams' and 'Grand Naine' (*Musa* spp. AAA). Plant Cell Tissue Organ Cult 95:373–379

Bairu MW, Jain N, Stirk WA, Doležal K, Van Staden J (2009a) 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, Kulkarni MG, Street RA, Mulaudzi RB, Van Staden J (2009b) Studies on seed germination, seedling growth, and in vitro shoot induction of *Aloe ferox* Mill., a commercially important species. HortScience 44:751–756

Bairu MW, Stirk WA, Van Staden J (2009c) Factors contributing to in vitro shoot-tip necrosis and their physiological interactions. Plant Cell Tissue Organ Cult 98:239–248

Bairu MW, Aremu AO, Van Staden J (2011a) Somaclonal variation in plants: causes and detection methods. Plant Growth Regul 63:147–173

Bairu MW, Novák O, Doležal K, Van Staden J (2011b) Changes in endogenous cytokinin profiles in micropropagated *Harpagophytum procumbens* in relation to shoot-tip necrosis and cytokinin treatments. Plant Growth Regul 63:105–114

Bajguz A, Piotrowska A (2009) Conjugates of auxin and cytokinin. Phytochemistry 70:957–969

Baroja-Fernández E, Aguirreolea J, Martínková H, Hanus J, Strnad M (2002) Aromatic cytokinins in micropropagated potato plants. Plant Physiol Biochem 40:217–224

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

Castelblanque L, García-Sogo B, Pineda B, Moreno V (2010) Efficient plant regeneration from protoplasts of *Kalanchoe blossfeldiana* via organogenesis. Plant Cell Tissue Organ Cult 100:107–112

Čatský J, Pospíšilová J, Kamínek M, Gaudinová A, Pulkrábek J, Zahradníček J (1996) Seasonal changes in sugar beet photosynthesis as affected by exogenous cytokinin $N^6$-(*m*-hydroxybenzyl)adenosine. Biol Plantarum 38:511–518

Cheesman L, Finnie JF, Van Staden J (2010) *Eucomis zambesiaca* baker: factors affecting in vitro bulblet induction. S Afr J Bot 76:543–549

Chen C-M, Kristopeit SM (1981) Metabolism of cytokinin: deribosylation of cytokinin ribonucleoside by adenosine nucleosidase from wheat germ cells. Plant Physiol 68:1020–1023

Clemenceau D, Cousseau J, Martin V, Molines H, Wakselman C, Mornet R, Nogue F, Laloue M (1996) Synthesis and cytokinin activity of two fluoro derivatives of $N^6$-Isopentenyladenine. J Agr Food Chem 44:320–323

Cortizo M, de Diego N, Moncaleán P, Ordás R (2009) Micropropagation of adult Stone Pine (*Pinus pinea* L.). Trees Struct Funct 23:835–842

Crocker PJ, Mahadevan A, Wiley JL, Martin BR, Razdan RK (2007) The role of fluorine substitution in the structure-activity relationships (SAR) of classical cannabinoids. Bioorg Med Chem Lett 17:1504–1507

De Diego N, Montalbán IA, Moncaleán P (2010) In vitro regeneration of *Pinus* spp. adult trees: new method for obtaining clonal plants. Acta Hortic 865:361–366

Debergh P, Aitken-Christie J, Cohen D, Grout B, Arnold S, Zimmerman R, Ziv M (1992) Reconsideration of the term 'vitrification' as used in micropropagation. Plant Cell Tissue Organ Cult 30:135–140

Dobránszki J, Magyar-Tábori K, Jámbor-Benczúr E, Kiss E, Lazányi J, Bubán T (2002) Effect of conditioning apple shoots with *meta*-topolin on the morphogenic activity of in vitro leaves. Acta Agron Hung 50:117–126

Dobránszki J, Hudak I, Magyar-Tábori K, Jámbor-Benczúr E, Galli Z, Kiss E (2004) Effects of different cytokinins on the shoot regeneration from apple leaves of 'Royal Gala' and 'M.26'. Int J Hort Sci 10:69–75

Dobránszki J, Jámbor-Benczúr E, Reményi ML, Magyar-Tábori K, Hudák I, Kiss E, Galli Z (2005) Effects of aromatic cytokinins on structural characteristics of leaves and their post-effects on subsequent shoot regeneration from in vitro apple leaves of 'Royal Gala'. Int J Hort Sci 1:41–46

Dobránszki J, Hudak I, Magyar-Tábori K, Jámbor-Benczúr E, Galli Z, Kiss E (2006) How can different cytokinins influence the process of shoot regeneration from apple leaves in 'Royal Gala' and 'M.26'. Acta Hortic 725:191–196

Doil A, Zhang R, Schum A, Serek M, Winkelmann T (2008) In vitro propagation and regeneration of *Hydrangea macrophylla* Thunb. 'Nachtigall'. Propag Ornam Plants 8:151–153

Doležal K, Popa I, Kryštof V, Spíchal L, Fojtíková M, Holub J, Lenobel R, Schmülling T, Strnad M (2006) Preparation and

biological activity of 6-benzylaminopurine derivatives in plants and human cancer cells. Bioorg Med Chem 14:875–884
Doležal K, Popa I, Hauserová E, Spíchal L, Chakrabarty K, Novák O, Kryštof V, Voller J, Holub J, Strnad M (2007) Preparation, biological activity and endogenous occurrence of $N^6$-benzyladenosines. Bioorg Med Chem 15:3737–3747
Emmerling F, Orgzall I, Dietzel B, Schulz BW, Reck G, Schulz B (2007) Structural studies on trifluoromethyl substituted 2, 5-diphenyl-1, 3, 4-oxadiazoles. J Mol Struct 832:124–131
Escalona M, Cejas I, González-Olmedo J, Capote I, Roels S, Cañal MJ, Rodríguez R, Sandoval J, Debergh P (2003) The effect of *meta*-topolin on plantain propagation using a temporary immersion bioreactor. InfoMusa 12:28–30
Faizal A, Lambert E, Foubert K, Apers S, Geelen D (2011) In vitro propagation of four saponin producing *Maesa* species. Plant Cell Tissue Organ Cult. doi:10.1007/s11240-010-9909-z
Fasolo F, Zimmerman RH, Fordham I (1989) Adventitions shoot formation on excised leaves of in vitro grown shoots of apple cultivars. Plant Cell Tissue Organ Cult 16:75–87
Faure JD, Vittorioso P, Santoni V, Fraisier V, Prinsen E, Barlier I, Van Onckelen H, Caboche M, Bellini C (1998) The PASTICCINO genes of *Arabidopsis thaliana* are involved in the control of cell division and differentiation. Development 125:909–918
Franck T, Kevers C, Gaspar T, Dommes J, Deby C, Greimers R, Serteyn D, Deby-Dupont G (2004) Hyperhydricity of *Prunus avium* shoots cultured on gelrite: a controlled stress response. Plant Physiol Biochem 42:519–527
Gan S, Amasino RM (1996) Cytokinins in plant senescence: from spray and pray to clone and play. BioEssays 18:557–565
Gan S, Amasino RM (1997) Making sense of senescence. Molecular genetic regulation and manipulation of leaf senescence. Plant Physiol 113:313–319
Gomez-Leyva JF, Martinez Acosta LA, Lopez Muraira IG, Silos Espino H, Ramirez-Cervantes F, Andrade-Gonzalez I (2008) Multiple shoot regeneration of roselle (*Hibiscus sabdariffa* L.) from a shoot apex culture system. Int J Bot 4:326–330
Haberer G, Kieber JJ (2002) Cytokinins. New insights into a classic phytohormone. Plant Physiol 128:354–362
Haidoune MB, Raynaud I, O'Connor N, Richomme P, Mornet R, Laloue M (1998) Synthesis and cytokinin activity of new zeatin derivatives. J Agric Food Chem 46:1577–1582
Hall RH, De Ropp RS (1955) Formation of 6-furfurylaminopurine from DNA breakdown products. J Am Chem Soc 77:6400
Harrar Y, Bellec Y, Bellini C, Faure J-D (2003) Hormonal control of cell proliferation requires PASTICCINO genes. Plant Physiol 132:1217–1227
Hecht SM, Leonard NJ, Schmitz RY, Skoog F (1970) Cytokinins: influence of side-chain planarity of $N^6$-substituted adenines and adenosines on their activity in promoting cell growth. Phytochemistry 9:1907–1913
Holub J, Hanuš J, Hanke DE, Strnad M (1998) Biological activity of cytokinins derived from *ortho*- and *meta*-hydroxybenzyladenine. Plant Growth Regul 26:109–115
Horgan R, Hewett EW, Purse JG, Wareing PF (1973) A new cytokinin from *Populus robusta*. Tetrahedron Lett 14:2827–2828
Horgan R, Hewett EW, Horgan JM, Purse J, Wareing PF (1975) A new cytokinin from Populus × robusta. Phytochemistry 14:1005–1008
Howell SH, Lall S, Che P (2003) Cytokinins and shoot development. Trends Plant Sci 8:453–459
Hutchison CE, Kieber JJ (2002) Cytokinin signaling in arabidopsis. Plant Cell 14:S47–S59
Huyluoglu Z, Unal M, Palavan-Unsal N (2008) Cytological evidences of the role of *meta*-topolin and benzyladenine in barley root tips. Adv Mol Biol 1:31–37
Il'ina E, Dodueva I, Ivanova N, Lutova L (2006) The effect of cytokinins on in vitro cultured inbred lines of *Raphanus sativus* var. radicula Pers. with genetically determined tumorigenesis. Russ J Plant Physiol 53:514–522
Iwamura H, Fujita T, Koyama S, Koshimizu K, Kumazawa Z (1980) Quantitative structure-activity relationship of cytokinin-active adenine and urea derivatives. Phytochemistry 19:1309–1319
Jiang ZQ, Guo HW (2010) A comparative genomic analysis of plant hormone related genes in different species. J Genet Genomics 37:219–230
Jones L, Martinková H, Strnad M, Hanke D (1996) Occurrence of aromatic cytokinins in oil palm (*Elaeis guineensis* Jacq.). J Plant Growth Regul 15:39–49
Kamada-Nobusada T, Sakakibara H (2009) Molecular basis for cytokinin biosynthesis. Phytochemistry 70:444–449
Kamínek M (1992) Progress in cytokinin research. Trends Biotechnol 10:159–164
Kamínek M, Pačes V, Corse J, Challice JS (1979) Effect of stereospecific hydroxylation of N6-(Δ2-Isopentenyl)adenosine on cytokinin activity. Planta 145:239–243
Kamínek M, Vaněk T, Motyka V (1987) Cytokinin activities of $N^6$-benzyladenosine derivatives hydroxylated on the side-chain phenyl ring. J Plant Growth Regul 6:113–120
Kataeva NV, Alexandrova IG, Butenko RG, Dragavtceva EV (1991) Effect of applied and internal hormones on vitrification and apical necrosis of different plants cultured in vitro. Plant Cell Tissue Organ Cult 27:149–154
Kevers C, Coumans M, Coumans-Gillès M-F, Caspar T (1984) Physiological and biochemical events leading to vitrification of plants cultured in vitro. Physiol Plantarum 61:69–74
Koetle MJ, Finnie JF, Van Staden J (2010) In vitro regeneration in *Dierama erectum* Hilliard. Plant Cell Tissue Organ Cult 103:23–31
Kotyk A, Kamínek M, Pulkrábek J, Zahradníček J (1996) Effect of in vivo and in vitro application of the cytokinin $N^6$-(*m*-hydroxybenzyl)adenosine on respiration and membrane transport processes in sugar beet. Biol Plantarum 38:363–368
Kubalákova M, Strnad M (1992) The effects of aromatic cytokinins (populins) on micropropagation and regeneration of sugar beet in vitro. Biol Plantarum 34:578–579
Leonard NJ, Henderson TR (1975) Purine ring rearrangements leading to the development of cytokinin activity. Mechanism of the rearrangement of 3-benzyladenine to $N^6$-benzyladenine. J Am Chem Soc 97:4990–4999
Leonard NJ, Hecht SM, Skoog F, Schmitz RY (1968) Cytokinins: synthesis of 6-(3-methyl-3-butenylamino)-9-β-d-ribofuranosylpurine (3iPA) and the effect of side-chain unsaturation on the biological activity of isopentenylaminopurines and their ribosides. Proc Natl Acad Sci USA 59:15–21
Letham DS (1963) Zeatin, a factor inducing cell division isolated from *Zea mays*. Life Sci 2:569–573
Letham DS, Palni LMS (1983) The biosynthesis and metabolism of cytokinins. Annu Rev Plant Physiol 34:163–197
Libbenga KR, Mennes AM (1995) Hormone binding and signal transduction. In: Davies PJ (ed) Plant hormones: physiology. biochemistry and molecular biology. Kluwer Academic Publishers, The Netherlands, pp 272–297
Magyar-Tábori K, Dobránszki J, Jámbor-Benczúr E, Bubán T, Lazányi J, Szalai J, Ferenczy A (2001) Post-effects of cytokinins and auxin levels of proliferation media on rooting ability of in vitro apple shoots (*Malus domestica* Borkh.) 'Red Fuji'. Int J Hort Sci 7:26–29
Magyar-Tábori K, Dobránszki J, Jámbor-Benczúr E (2002) High in vitro shoot proliferation in the apple cultivar Jonagold induced by benzyladenine analogues. Acta Agron Hung 50:191–195
Magyar-Tábori K, Dobránszki J, Teixeira da Silva J, Bulley S, Hudák I (2010) The role of cytokinins in shoot organogenesis in apple. Plant Cell Tissue Organ Cult 101:251–267

Magyar-Tábori K, Dobránszki J, Hudák I (2011) Effect of cytokinin content of the regeneration media on in vitro rooting ability of adventitious apple shoots. Sci Hortic. doi:10.1016/j.scienta.2011.05.011

Makara AM, Rubaihayo PR, Magambo MJS (2010) Carry-over effect of thidiazuron on banana in vitro proliferation at different culture cycles and light incubation conditions. Afr J Biotechnol 9:3079–3085

Malá J, Máchová P, Cvrčková H, Karady M, Novák O, Mikulík J, Hauserová E, Greplová J, Strnad M, Doležal K (2009) Micropropagation of wild service tree *Sorbus torminalis* [L.] Crantz): the regulative role of different aromatic cytokinins during organogenesis. J Plant Growth Regul 28:341–348

Mata-Rosas M, Baltazar-García R, Moon P, Hietz P, Luna-Monterrojo V (2010) In vitro regeneration of *Lycaste aromatica* (Graham ex Hook) Lindl. (Orchidaceae) from pseudobulb sections. Plant Biotechnol Rep 4:157–163

Matsubara S (1980) Structure-activity relationships of cytokinins. Phytochemistry 19:2239–2253

Meiners J, Schwab M, Szankowski I (2007) Efficient in vitro regeneration systems for *Vaccinium* species. Plant Cell Tissue Organ Cult 89:169–176

Meyer EM, Touchell DH, Ranney TG (2009) In vitro shoot regeneration and polyploid induction from leaves of *Hypericum* species. HortScience 44:1957–1961

Miller CO, Skoog F, Von Saltza MH, Strong FM (1955) Kinetin, a cell division factor from deoxyribonucleic acid. J Am Chem Soc 77:1392–1393

Mithila J, Murch SJ, KrishnaRaj S, Saxena PK (2001) Recent advances in *Pelargonium* in vitro regeneration systems. Plant Cell Tissue Organ Cult 67:1–9

Mok MC, Mok DWS (1985) The metabolism of [$^{14}$C]-thidiazuron in callus tissues of *Phaseolus lunatus*. Physiol Plantarum 65:427–432

Mok MC, Martin RC, Dobrev PI, Vanková R, Shing Ho P, Yonekura-Sakakibara K, Sakakibara H, Mok DWS (2005) Topolins and hydroxylated thidiazuron derivatives are substrates of cytokinin *O*-glucosyltransferase with position specificity related to receptor recognition. Plant Physiol 137:1057–1066

Moncaleán P, Rodríguez A, Fernández B (2001) In vitro response of *Actinidia deliciosa* explants to different BA incubation periods. Plant Cell Tissue Organ Cult 67:257–266

Moyo M, Finnie JF, Van Staden J (2011) Recalcitrant effects associated with the development of basal callus-like tissue on caulogenesis and rhizogenesis in *Sclerocarya birrea*. Plant Growth Regul 63:187–195

Murthy B, Murch S, Saxena P (1998) Thidiazuron: a potent regulator of in vitro plant morphogenesis. In Vitro Cell Dev Biol Plant 34:267–275

Nam HG (1997) The molecular genetic analysis of leaf senescence. Curr Opin Biotechnol 8:200–207

Nas MN, Bolek Y, Sevgin N (2010) The effects of explant and cytokinin type on regeneration of *Prunus microcarpa*. Sci Hortic 126:88–94

Niedz RP, Evens TJ (2010) The effects of benzyladenine and *meta*-topolin on in vitro shoot regeneration of a *Citrus citrandarin* rootstock. Res J Agric Biol Sci 6:45–53

Nirmalram J, Tamilselvi D, Muthiah P (2011) Hydrogen bonded supramolecular assembly in $N^6$-benzyladeninium nitrate and $N^6$-benzyladeninium 3-hydroxy picolinate: a synthetic cytokinin. J Chem Crystallogr 41:864–867

Nishikawa S, Kumazawa Z, Mizutani H, Kashimura N (1986) Substituent-directing effect on cytokinin activity of the α-double bond in the 6-substituent of purines. Agric Biol Chem 50:1089–1091

Noodén LD (1988) The phenomena of senescence and aging. In: Noodén LD, Leopold AC (eds) Senescence and Aging in Plants. Academic Press, Inc., San Diego, California, USA, pp 1–50

Palavan-Ünsal N, Çağ S, Çetin E (2002a) Growth responses of excised radish cotyledons to *meta*-topolin. Can J Plant Sci 82:191–194

Palavan-Ünsal N, Çağ S, Çetin E, Büyüktunçer D (2002b) Retardation of senescence by *meta*-topolin in wheat leaves. J Cell Mol Biol 1:101–108

Palavan-Ünsal N, Çağ S, Çetin E (2004) The role of *meta*-topolin in senescence of wheat leaf segments. J Cell Mol Biol 3:23–31

Palmer MV, Horgan R, Wareing PF (1981) Cytokinin metabolism in *Phaseolus vulgaris* L.: I: variations in cytokinin levels in leaves of decapitated plants in relation to lateral bud outgrowth. J Exp Bot 32:1231–1241

Pan X, Welti R, Wang X (2008) Simultaneous quantification of major phytohormones and related compounds in crude plant extracts by liquid chromatography-electrospray tandem mass spectrometry. Phytochemistry 69:1773–1781

Pâques M (1991) Vitrification and micropropagation: causes, remedies and prospects. Acta Hortic 289:283–290

Pâques M, Boxus P (1987) Vitrification: review of literture. Acta Hortic 212:155–166

Parimalan R, Giridhar P, Ravishankar G (2011) Enhanced shoot organogenesis in *Bixa orellana* L. in the presence of putrescine and silver nitrate. Plant Cell Tissue Organ Cult 105:285–290

Pospíšilová J, Synková H, Haisel D, Čatský J, Wilhelmová N, Šrámek F (1999) Effect of elevated $CO_2$ concentration on acclimation of tobacco plantlets to ex vitro conditions. J Exp Bot 50:119–126

Pospíšilová J, Synková H, Haisel D, Semorádová S (2007) Acclimation of plantlets to ex vitro conditions: effects of air humidity, irradiance, $CO_2$ concentration and abscisic acid (a review). Acta Hortic 748:29–38

Roels S, Escalona M, Cejas I, Noceda C, Rodriguez R, Canal MJ, Sandoval J, Debergh P (2005) Optimization of plantain (*Musa* AAB) micropropagation by temporary immersion system. Plant Cell Tissue Organ Cult 82:57–66

Rosales MSD, Solís ÁGA, Méndezy NLV, Balch EPM (2008) Effect of cytokinins on the in vitro propagation of Mexican *Agaves*. Rev Fitotec Mex 31:317–322

Sakakibara H (2006) Cytokinins: activity, biosynthesis, and translocation. Annu Rev Plant Biol 57:431–449

Salvi ND, George L, Eapen S (2002) Micropropagation and field evaluation of micropropagated plants of turmeric. Plant Cell Tissue Organ Cult 68:143–151

Schmülling T (2004) Cytokinin. In: Lennarz WJ, Lane MD (eds) Encyclopedia of Biological Chemistry. Academic Press/Elsevier Science, New York, USA, pp 1–6

Skoog F, Armstrong DJ (1970) Cytokinins. Annu Rev Plant Physiol 21:359–384

Skoog F, Hamzi HQ, Szweykowska AM, Leonard NJ, Carraway KL, Fujii T, Helgeson JP, Loeppky RN (1967) Cytokinins: structure/activity relationships. Phytochemistry 6:1169–1192

Smulders M, de Klerk G (2011) Epigenetics in plant tissue culture. Plant Growth Regul 63:137–146

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

Strnad M, Hanuš J, Vanek T, Kamínek M, Ballantine JA, Fussell B, Hanke DE (1997) Meta-topolin, a highly active aromatic cytokinin from poplar leaves (*Populus* × *canadensis* Moench., cv. *Robusta*). Phytochemistry 45:213–218

Subbaraj AK (2011) Topolins: current research status and applications. Int J Biores Stress Manag 2:10–25

Tarkowská D, Doležal K, Tarkowski P, Åstot C, Holub J, Fuksová K, Schmülling T, Sandberg G, Strnad M (2003) Identification of new aromatic cytokinins in *Arabidopsis thaliana* and *Populus* × *canadensis* leaves by LC-(+)ESI-MS and capillary liquid chromatography/frit-fast atom bombardment mass spectrometry. Physiol Plantarum 117:579–590

Tarkowski P, Ge L, Yong JWH, Tan SN (2009) Analytical methods for cytokinins. Trends Anal Chem 28:323–335

Taylor NJ, Stirk WA, Van Staden J (2003) The elusive cytokinin biosynthetic pathway. S Afr J Bot 69:269–281

Teklehaymanot T, Wannakrairoj S, Pipattanawong N (2010) Meta-topolin for pineapple shoot multiplication under three in vitro systems. Am Euras J Agr Environ Sci 7:157–162

Trávníček Z, Marek J, Doležal K, Strnad M (1997) The structure of 6-(2-hydroxybenzylamino)purine acetic acid solvate. Z Kristallogr 212:538–541

Valero-Aracama C, Kane M, Wilson S, Philman N (2010) Substitution of benzyladenine with *meta*-topolin during shoot multiplication increases acclimatization of difficult- and easy-to-acclimatize sea oats (*Uniola paniculata* L.) genotypes. Plant Growth Regul 60:43–49

Van Staden J, Crouch NR (1996) Benzyladenine and derivatives—their significance and interconversion in plants. Plant Growth Regul 19:153–175

Van Staden J, Cook EL, Noodén LD (1988) Cytokinins and senescence. In: Noodén LD, Leopold AC (eds) Senescence and Aging in Plants. Academic Press, Inc., San Diego, California, USA, pp 281–328

Vasil I (2008) A history of plant biotechnology: from the cell theory of Schleiden and Schwann to biotech crops. Plant Cell Rep 27:1423–1440

Vasudevan R, Van Staden J (2011) Cytokinin and explant types influence in vitro plant regeneration of Leopard Orchid (*Ansellia africana* Lindl.). Plant Cell Tissue Organ Cult. doi: 10.1007/s11240-011-9964-0

Vinayak V, Dhawan AK, Gupta VK (2009) Efficacy of non-purine and purine cytokinins on shoot regeneration in vitro in sugarcane. Indian J Biotechnol 8:227–231

Wagner BM, Beck E (1993) Cytokinins in the perennial herb *Urtica dioica* L. as influenced by its nitrogen status. Planta 190:511–518

Werbrouck SPO (2010) Merits and drawbacks of new aromatic cytokinins in plant tissue culture. Acta Hortic 865:103–108

Werbrouck S, Van der Jeugt B, Dewitte W, Prinsen E, Van Onckelen H, Debergh P (1995) The metabolism of benzyladenine in *Spathiphyllum floribundum* 'Schott Petite' in relation to acclimatisation problems. Plant Cell Rep 14:662–665

Werbrouck SPO, Strnad M, Van Onckelen HA, Debergh PC (1996) *Meta*-topolin, an alternative to benzyladenine in tissue culture? Physiol Plantarum 98:291–297

Winarto B, Rachmawati F, Pramanik D, Teixeira da Silva J (2011) Morphological and cytological diversity of regenerants derived from half-anther cultures of anthurium. Plant Cell Tissue Organ Cult 105:363–374

Wojtania A (2010) Effect of meta-topolin on in vitro propagation of Pelargonium × hortorum and Pelargonium × hederaefolium cultivars. Acta Soc Bot Pol 79:101–106

Wojtania A, Gabryszewska E (2001) Effect of cytokinins and amino acids on multiplication of *Pelargonium* cultivars. Acta Soc Bot Pol 70:203–207

Woo HR, Kim JH, Nam HG, Lim PO (2004) The delayed leaf senescence mutants of *Arabidopsis*, *ore1*, *ore3*, and *ore9* are tolerant to oxidative stress. Plant Cell Physiol 45:923–932

Zatloukal M, Gemrotová M, Doležal K, Havlíček L, Spíchal L, Strnad M (2008) Novel potent inhibitors of *A. thaliana* cytokinin oxidase/dehydrogenase. Bioorg Med Chem 16:9268–9275

Zhang C, Whiting MD (2011) Improving 'Bing' sweet cherry fruit quality with plant growth regulators. Sci Hortic 127:341–346