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ORIGINAL PAPER

Assessment of the role of *meta*-topolins on in vitro produced phenolics and acclimatization competence of micropropagated 'Williams' banana

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Abstract The effects of five topolins (*meta*-Topolin, *m*T; *meta*-Topolin riboside, *m*TR; *meta*-Methoxy topolin, Me*m*T; *meta*-Methoxy topolin riboside, Me*m*TR and *meta*-Methoxy topolin 9-tetrahydropyran-2-yl, Me*m*TTHP) on the phenolic content and subsequent acclimatization potential of micropropagated 'Williams' bananas were compared to benzyladenine (BA). Sterile shoot-tip explants were cultured on modified Murashige and Skoog (MS) media containing 10, 20 or 30 μM of the above aromatic cytokinins (CKs) for 42 days. Phenolic contents were quantified spectrophotometrically. Growth parameters and photosynthetic pigments of the greenhouse-acclimatized plants were determined after 5 months. Total phenolic levels were highest in 10 μM *m*T-treated plantlets within the aerial parts and 30 μM Me*m*TTHP for the underground parts. In the underground parts, 10 μM *m*T resulted in the production of the highest amount of proanthocyanidins which was approximately five-fold higher than in the control plants. Furthermore, 10 μM Me*m*TTHP-treated plantlets had significantly higher total flavonoids (30.1 ± 0.24 mg CE/g DW) within the aerial parts. Plantlets regenerated using Me*m*T, Me*m*TR and Me*m*TTHP had significantly longer roots and better shoot/root ratios compared to the control and BA-treated plants. In terms of root fresh weight, it was significantly higher in Me*m*T-treated plantlets than in the control and BA treatments. Chlorophyll *a*/*b* ratio was significantly improved with the use of Me*m*T, *m*TR and *m*T compared to control. Current findings indicate the potential of topolins in stimulating the accumulation of phenolic compounds in micropropagated plantlets. In view of the importance of plant secondary metabolites, their substantial accumulation probably enhanced the acclimatization and subsequent ex vitro survival of the micropropagated plantlets. Topolins, particularly, the new derivative Me*m*TTHP could be an alternative CK for the micropropagation of plant species based on their stimulatory effect on ex vitro rooting that inevitably enhances acclimatization competence. Furthermore, topolins are demonstrated as potential elicitors in micropropagation.

Keywords Cytokinins · Chlorophyll · Phenolics · Micropropagation · *Musa* spp

Abbreviations

BA	N^6-Benzyladenine
CCE	Cyanidin chloride equivalents
CE	Catechin equivalents
CK	Cytokinin
DMRT	Duncan's multiple range test
DW	Dry weight
Folin-C	Folin-Ciocalteu
FW	Fresh weight
GAE	Gallic acid equivalents
Me*m*T	*meta*-Methoxy topolin
Me*m*TR	*meta*-Methoxy topolin riboside

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Me*m*TTHP	*meta*- Methoxy topolin 9-tetrahydropyran-2-yl
MS	Murashige and Skoog basal medium
*m*T	*meta*-Topolin
*m*TR	*meta*-Topolin riboside
PPFD	Photosynthetic photon flux density
PTC	Plant tissue culture

Introduction

As the fifth most important staple crop in the world, the significance of bananas for food security cannot be overemphasized (Jain 2004). To sustain the world's rising population, the application of plant tissue culture (PTC) techniques is a viable means of increasing food production (Caponetti et al. 2005). Indeed, micropropagation (PTC) has played a major role in the mass production of clonal material, conservation and breeding of bananas (Vuylsteke 1998; Jain 2004). Despite the success achieved on a global level with the use of PTC, some challenges are still encountered. These include low multiplication rates, rooting inhibition and somaclonal variations amongst others (Bairu et al. 2011a; Aremu et al. 2012). In addition, improved acclimatization and successful establishment in the field remain problematic, especially for large-scale application of PTC techniques (Pospíšilová et al. 1999). Often, acclimatization failures are due to poor development of photosynthetic capacity in vitro, coupled with the induced stress from the artificial environment (Valero-Aracama et al. 2006; Pospíšilová et al. 2007).

In contrast to primary metabolites which are required for basic life processes, plants synthesize and accumulate secondary metabolites in response to ecological and biochemical differentiation (Lewinsohn and Gijzen 2009). Secondary metabolites are useful for plant interaction and survival in their environment (Namdeo 2007). The important roles of secondary metabolites, especially the phenolic compounds in PTC as well as subsequent survival in the ex vitro environment, are well known (Curir et al. 1990; Wuyts et al. 2005; Wu et al. 2007; Buer et al. 2010). Studies have also indicated that CKs regulate the quantity of some secondary metabolites in micropropagated plants (Ramachandra Rao and Ravishankar 2002; Quiala et al. 2012).

In the past few decades, the use of topolins, especially the *meta*-form in several PTC protocols, has shown promising results in terms of growth as well as minimizing various physiological disorders (Aremu et al. 2012). However, reports on their effects on secondary metabolites are scanty. Findings from such investigations could provide clues and may elucidate additional physiological evidence to support the superiority of topolins over BA in micropropagation of some species. In this study, we investigated the effect of five topolins on the accumulation of the phenolic compounds and acclimatization competence in micropropagated 'Williams' bananas.

Materials and methods

Cytokinins

The aromatic cytokinins tested were benzyladenine (BA) and five topolins namely: *meta*-Topolin (*m*T), *meta*-Topolin riboside (*m*TR), *meta*-Methoxy topolin (Me*m*T), *meta*-Methoxy topolin riboside (Me*m*TR), and *meta*-Methoxy topolin 9-tetrahydropyran-2-yl (Me*m*TTHP). All the topolins were obtained from the Laboratory of Growth Regulators, Palacký University and Institute of Experimental Botany, Academy of Sciences of the Czech Republic while BA was purchased from Sigma, USA.

Culture conditions and preparation of plant material

Aseptically maintained, in vitro banana plantlets (*Musa* spp. AAA cultivar 'Williams') regularly sub-cultured at 6-weekly intervals were used. Originally, the shoot-tip explants were initiated by surface sterilizing greenhouse-established banana plantlets purchased from Du Roi Laboratory, South Africa. Sterile plantlets at the fourth multiplication cycle were used for the experiment. Murashige and Skoog (MS) inorganic salts (Murashige and Skoog 1962) as modified for bananas by Vuylsteke (1998) were used. The medium was supplemented with varying concentrations of aromatic CKs (10, 20 and 30 μM) and 30 g l^{-1} sucrose. Thereafter, the medium was adjusted to pH 5.8 with either 0.1 M KOH or HCl prior to addition of 3 g l^{-1} gelrite (Labretoria, Pretoria, South Africa) and then autoclaved at 103 kPa and 121 °C for 20 min. Filter sterilized ascorbic acid (0.180 g l^{-1}) was aseptically added to the medium before solidification (ca 50 °C). Shoot-tip explants (10 mm) were cut in half longitudinally and inoculated in culture tubes (100 × 25 mm, 40 ml volume) containing 12 ml of growth medium. Cultures were incubated in a growth room under 16 h light/8 h dark conditions and photosynthetic photon flux density (PPFD) of 45 μmol m^{-2} s^{-1} at 25 ± 2 °C.

Acclimatization competence experiment

In vitro plantlets derived from the 10 μM CK treatments, due to ease of rooting, were washed and potted in 12.5 cm

pots containing sand, soil, vermiculite (1:1:1, v/v) treated with 1 % Benlate® (Du Pont de Nemours Int., South Africa). The plantlets were maintained for 3 months in the mist-house with day/night temperatures of 30/12 °C, relative humidity between 80 and 90 %, a misting interval of 15 min for 10-s duration. Ten well-established plantlets for the six CK treatments as well as the control were transferred to a greenhouse with 30–40 % relative humidity, day/night temperatures of 30/15 °C with an average PPFD of 450 μmol m^{-2} s^{-1}. During the experiment, photoperiod was that of prevailing natural conditions during summer (12 h). After 2 months, plantlets were harvested and data on various quantitative parameters such as plant height, root length and number of leaves were collected.

Sample preparation for secondary metabolite quantification

After 6-week incubation, regenerated plantlets from the CK treatments were separated into aerial and underground parts. The plant materials were oven-dried at 50 ± 2 °C for 7 days and milled into powder form. Ground plant samples were extracted in 50 % methanol (MeOH) at 0.1 g/10 ml in an ultrasonic sonicator (Julabo GmbH, West Germany), that contained ice, for 20 min. The mixture was separated using a Benchtop centrifuge (Hettich Universal, Tuttlingen, Germany) to obtain the supernatant required for the phytochemical quantification.

Total phenolic, flavonoid and proanthocyanidin contents

Total phenolic content was evaluated using the Folin-Ciocalteu (Folin-C) assay as outlined by Makkar et al. (2007). Absorbance at 725 nm was measured using a UV–visible spectrophotometer (Varian Cary 50, Australia) against a blank consisting of 50 % MeOH. Total phenolic content was expressed in mg gallic acid (Sigma-Aldrich, USA) equivalents (GAE) per g dry weight (DW).

Total flavonoid content was determined by the aluminium chloride colorimetric assay (Zhishen et al. 1999) with slight modifications (Marinova et al. 2005). The absorbance at 510 nm was measured using a UV–visible spectrophotometer against a blank consisting of 50 % MeOH. Total flavonoid content was expressed in mg catechin (Sigma-Aldrich, Germany) equivalents (CE) per g DW.

The HCl/Butan-1-ol assay adapted from Porter et al. (1985) and described by Makkar et al. (2007) was used to quantify the proanthocyanidin content. The absorbance at 550 nm was measured using a UV–visible spectrophotometer against a blank consisting of 50 % MeOH. The proanthocyanidins content was expressed in μg cyanidin chloride (Carl Roth, Germany) equivalents (CCE) per g DW.

Data collection and analysis

After 2 months, the greenhouse-acclimatized plantlets were harvested, washed and air dried. The vegetative growth, such as number (leaf, shoot and root), length (shoot and root) and fresh weights (shoot and root), was measured. The shoot/root ratio was calculated as the ratio of the dry weights of the aerial part over that of the root. The leaf area was determined using an L1-3100 area meter (Li-Cor Inc., Lincoln, Nebraska, USA). Dry weights of the harvested plantlets were determined after the fresh materials were oven-dried at 50 ± 2 °C for 7 days.

Photosynthetic pigments of the acclimatized plantlets were measured spectrophotometrically. Fresh leaf samples (0.1 g) were homogenized in 5 ml acetone with addition of a few pinches of acid-washed sand (BDH Chemicals, England). The mixture was filtered through Whatman No. 1 filter paper and separated using a Benchtop centrifuge at 5,000 rpm for 5 min at room temperature. The absorbance of the resultant filtrate was measured at 470, 645, and 662 nm. The pigment content expressed as μg per g fresh weight (FW) was calculated based on the formulae of Lichtenthaler (1987).

$$\text{Chlorophyll } a\,(C_a) = 11.24\text{A}_{662} - 2.04\text{A}_{645}$$

$$\text{Chlorophyll } b(C_b) = 20.13\text{A}_{645} - 4.19\text{A}_{662}$$

$$\text{Total chlorophylls} = 7.05\text{A}_{662} + 18.09\text{A}_{645}$$

$$\text{Total carotenoids} = (1,000\text{A}_{470} - 1.90C_a - 63.14C_b)/214$$

Data were subjected to one-way analysis of variance (ANOVA) using SPSS software package for Windows (SPSS®, version 10.0, Chicago, USA). Where there was statistical significance ($P = 0.05$), the mean values were further separated using the Duncan's multiple range test (DMRT).

Results

Effect of the cytokinins on phenolic contents

Overall, total phenolics and flavonoids were higher in the aerial parts compared to underground parts (Table 1). However, total phenolic content was higher in the underground part in 30 μM Me*m*TTHP treatment. In terms of the proanthocyanidin levels, the underground parts generally had an higher content with the exception of 20 and 30 μM *m*TR regenerants. Treatments with *m*T and Me*m*TTHP indicated that increasing the concentration of these CKs decreased the quantity of total phenolic and flavonoid contents in aerial parts. On the other hand, an increase in quantity was observed with higher concentrations of

Table 1 Effect of aromatic cytokinins on the accumulation of secondary metabolites in micropropagated 'Williams' bananas

Cytokinin treatment	Total phenolics (mg GAE/g DW)		Total flavonoids (mg CE/g DW)		Proanthocyanidins (μg CCE/g DW)	
	Aerial part	Underground part	Aerial part	Underground part	Aerial part	Underground part
0						
Control	25.9 ± 0.73 de	7.5 ± 0.54 i	25.5 ± 0.18 d	5.67 ± 0.55 l	448.0 ± 21.56 def	617.3 ± 41.87 fg
10 μM						
*m*T	33.7 ± 2.10 a	16.6 ± 0.53 e	25.4 ± 0.53 d	12.9 ± 0.18 g	643.8 ± 21.12 abcd	3,363.5 ± 13.38 a
*m*TR	28.5 ± 1.01 c	11.4 ± 0.18 g	24.8 ± 0.20 d	8.0 ± 0.08 j	495.6 ± 12.81 bcdef	711.2 ± 11.13 efg
Me*m*T	14.4 ± 0.61 i	10.6 ± 0.20 gh	12.1 ± 0.18 k	8.1 ± 0.07 j	335.5 ± 32.34 ef	707.2 ± 13.52 efg
Me*m*TR	17.5 ± 0.40 gh	14.4 ± 0.55 f	13.9 ± 0.14 j	11.7 ± 0.14 h	603.1 ± 10.20 bcd	947.1 ± 39.34 def
BA	31.7 ± 0.64 ab	17.7 ± 1.25 de	29.07 ± 0.22 b	15.1 ± 0.04 ef	484.7 ± 89.53 cdef	1,269.0 ± 273.99 cd
Me*m*TTHP	33.4 ± 1.01 a	21.7 ± 1.36 b	30.1 ± 0.24 a	18.8 ± 0.39 b	555.0 ± 15.56 bcdef	1,827.4 ± 156.13 b
20 μM						
*m*T	27.0 ± 0.37 cd	12.3 ± 0.24 fg	22.8 ± 0.34 e	9.0 ± 0.05 i	656.86 ± 67.19 abcd	745.7 ± 13.38 efg
*m*TR	16.5 ± 0.55 hi	12.2 ± 0.23 fg	11.2 ± 0.07 l	9.5 ± 0.10 i	478.6 ± 25.19 cdef	384.2 ± 21.61 g
Me*m*T	20.7 ± 0.56 f	17.9 ± 0.19 de	15.5 ± 0.68 i	14.9 ± 0.08 f	702.68 ± 86.83 abc	781.3 ± 11.44 efg
Me*m*TR	23.6 ± 0.35 e	19.7 ± 0.74 bcd	20.3 ± 0.26 f	15.9 ± 0.08 d	425.5 ± 1.50 def	659.7 ± 49.91 efg
BA	24.2 ± 0.97 e	8.0 ± 0.58 i	20.68 ± 0.10 f	4.0 ± 0.05 m	327.58 ± 3.39 f	562.4 ± 7.22 fg
Me*m*TTHP	30.9 ± 0.13 b	11.8 ± 0.61 g	26.6 ± 0.45 c	7.4 ± 0.10 k	832.8 ± 12.6 a	1,028.6 ± 39.77 def
30 μM						
*m*T	19.4 ± 1.16 fg	18.9 ± 0.24 cde	16.7 ± 0.16 h	14.7 ± 0.14 f	360.4 ± 4.83 ef	1,119.1 ± 47.05 cde
*m*TR	19.2 ± 0.18 fg	7.7 ± 0.20 i	14.7 ± 0.04 ij	4.5 ± 0.05 m	617.25 ± 21.74 abcd	576.5 ± 23.28 fg
Me*m*T	11.8 ± 0.27 j	8.5 ± 0.39 hi	9.25 ± 0.20 m	5.2 ± 0.12 l	722.49 ± 240.55 ab	1,537.8 ± 510.55 bc
Me*m*TR	24.3 ± 1.43 e	21.2 ± 0.26 bc	20.26 ± 0.38 f	17.2 ± 0.15 c	549.36 ± 6.30 bcdef	807.9 ± 10.23 efg
BA	25.1 ± 0.39 de	19.7 ± 0.50 bcd	17.9 ± 0.42 g	15.5 ± 0.13 de	563.5 ± 6.86 bcde	1,271.85 ± 41.59 cd
Me*m*TTHP	24.8 ± 0.46 de	27.9 ± 2.14 a	22.4 ± 0.04 e	21.6 ± 0.14 a	648.9 ± 81.60 abcd	772.27 ± 55.22 efg

Mean values ± standard error (n = 6) in the same column with different letter(s) are significantly different (P = 0.05) based on Duncan's multiple range test

Cytokinin treatment: *m*T, *meta*-Topolin; *m*TR, *meta*-Topolin riboside; Me*m*T, *meta*-Methoxy topolin; Me*m*TR, *meta*-Methoxy topolin riboside; *BA* benzyladenine; Me*m*TTHP, *meta*-Methoxy topolin 9-tetrahydropyran-2-yl; *GAE* gallic acid equivalents, *CE* catechin equivalents, *CCE* cyanidin chloride equivalents

Me*m*TR for total phenolics and flavonoids (aerial and underground parts) as well as Me*m*T for proanthocyanidin (underground part) content.

Generally, *m*T and Me*m*TTHP stimulated higher phytochemical production compared to BA-treated and control plants. Notably, *m*T and Me*m*TTHP at 10 μM increased the proanthocyanidin content in the underground part by five- and threefold, respectively, compared to the controls. Furthermore, the same concentration of BA yielded approximately 2.7-fold lower content compared to *m*T. Similarly, Me*m*TTHP plantlets had 1.4- and 3.7-fold more total phenolics than the corresponding BA and control ones, respectively, in the underground parts.

Effect of cytokinins on the growth of greenhouse-acclimatized banana plants

At the end of the experiment, the greenhouse-acclimatized plantlets from the six CK treatments are shown in Fig. 1a, b. Overall, the acclimatization was above 80 % in all the treatments (data not shown). The effects of the CKs were more pronounced in the underground compared to the aerial parts. Plantlets regenerated from Me*m*TTHP were significantly better than the control and BA treatments, in terms of the length of the roots (Fig. 2a) as well as shoot/root ratio and plant height (Fig. 2f, g). In most of these parameters, however, Me*m*TR treatment produced similar effects. The highest number of leaves was recorded in Me*m*T-treated plants (Fig. 2d). On the other hand, similar responses were observed across all the treatments as well as the control plants for shoot length (Fig. 2c), number of new shoots (Fig. 2e) and total leaf area of individual plants (Fig. 2h).

Biomass accumulation for the various CK treatments is depicted in Fig. 3. Even though the shoot fresh weight had a wide range, from around 13 g in the control to 18 g in Me*m*T regenerants, there was no statistical difference among the treatments (Fig. 3a). Root fresh weight was highest in Me*m*T and lowest in *m*TR plants (Fig. 3b). Similarly, Me*m*T-treated plants had higher dry weight than

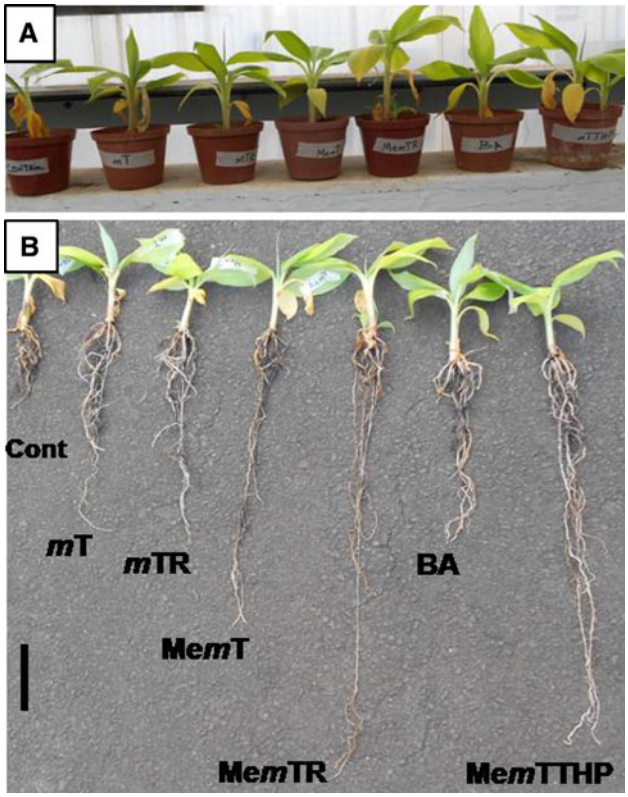

Fig. 1 Effect of six aromatic cytokinins on the morphology of greenhouse-acclimatized 'Williams' bananas. **a** aerial and **b** underground parts. *Cont,* control; *m*T, *meta*-Topolin; *m*TR, *meta*-Topolin riboside; Me*m*T, *meta*-Methoxy topolin; Me*m*TR, *meta*-Methoxy topolin riboside; *BA* benzyladenine; Me*m*TTHP, *meta*-Methoxy topolin 9-tetrahydropyran-2-yl. *Bar* 10 cm

either the control or other CK treatments (Fig. 3c). In terms of root dry weighs, *m*T and Me*m*TTHP were the most desired CKs as indicated in Fig. 3d.

Effect of cytokinins on photosynthetic pigment content of the greenhouse-acclimatized banana plants

The total carotenoids and chlorophyll (chlorophyll *a* and *b*) in the acclimatized banana plants are presented in Table 2. Generally, the lowest photosynthetic pigments were observed in *m*T treatments. The highest total chlorophyll and carotenoid contents were detected in control, *m*TR and Me*m*T-treated plantlets, in a slightly decreasing pattern. Conversely, chlorophyll *a*/*b* ratio in both the control and *m*TR regenerated plants were significantly lower than Me*m*T which had the optimal ratio. In fact, the control plants exhibited the lowest chlorophyll *a*/*b* ratio. The highest total chlorophyll/carotenoid ratio was observed with *m*TR while BA-regenerated plants had the lowest ratio.

Discussion

The regulatory role of CKs on different physiological and developmental processes is well-documented (Werner et al. 2001; Criado et al. 2007). Studies have shown that PGR's signaling pathways are not isolated, they are interconnected with a complex regulatory network involving various defence signaling pathways and developmental processes (Bari and Jones 2009). In recent times, there has been numerous evidence of the increasing superiority of topolins over the commonly used CKs such as BA and kinetin in PTC (Aremu et al. 2012). For example, the advantages of the use of topolins over BA were clearly observed in the micropropagation of species such as *Aloe polyphylla* (Bairu et al. 2007), *Spathiphyllum floribundum* (Werbrouck et al. 1996) and *Musa* spp. (Escalona et al. 2003; Bairu et al. 2008). In addition, this study demonstrated the better stimulatory effects of topolins (*m*T and Me*m*TTHP) on phenolic content in 'Williams' banana compared to BA. Generally, secondary metabolites such as phenolics and flavonoids play vital roles during the micropropagation of several species. The diverse functions of these compounds have been investigated in detail by several researchers in PTC (Wu et al. 2007; Buer et al. 2010; De Klerk et al. 2011). As postulated by Curir et al. (1990) and Buer et al. (2010), rooting is enhanced in the presence of certain types of flavonoids. The high accumulation of flavonoids could have accounted for the observed better ex vitro rooting in *m*T and Me*m*TTHP regenerated plantlets. This result also shows the advantage of Me*m*TTHP over BA, in that the formation of the metabolite 6-benzylamino-9-β-D-glucopyranosylpurine, known for inhibition of rooting and associated abnormalities (Bairu et al. 2011b), is blocked due to the conjugation of this molecule at the N^9 position (Szücová et al. 2009). In the current study, BA negatively affected rooting as indicated in the number and length of the roots compared to the topolins. The detrimental effects of BA during the acclimatization of micropropagated plants are well-documented (Werbrouck et al. 1995; Baroja-Fernández et al. 2002; Valero-Aracama et al. 2010; Bairu et al. 2011b). Accumulation of the more stable and toxic metabolite 6-benzylamino-9-β-D-glucopyranosylpurine has been implicated in these acclimatization problems. Among the tested CKs, Me*m*TTHP consistently remained the most promising by stimulating better acclimatization in the micropropagated 'Williams' bananas. Szücová et al. (2009) synthesised Me*m*TTHP in an attempt to improve the biological activity of the parent structure (*m*T). Indeed, the compound maintained or improved CK activity when tested in the three classical CK bioassays. As postulated by the authors, the improved Me*m*TTHP activity was due to higher resistance to enzymatic breakdown by CK oxidase. Hence, the stimulatory effect of the CK is probably due to

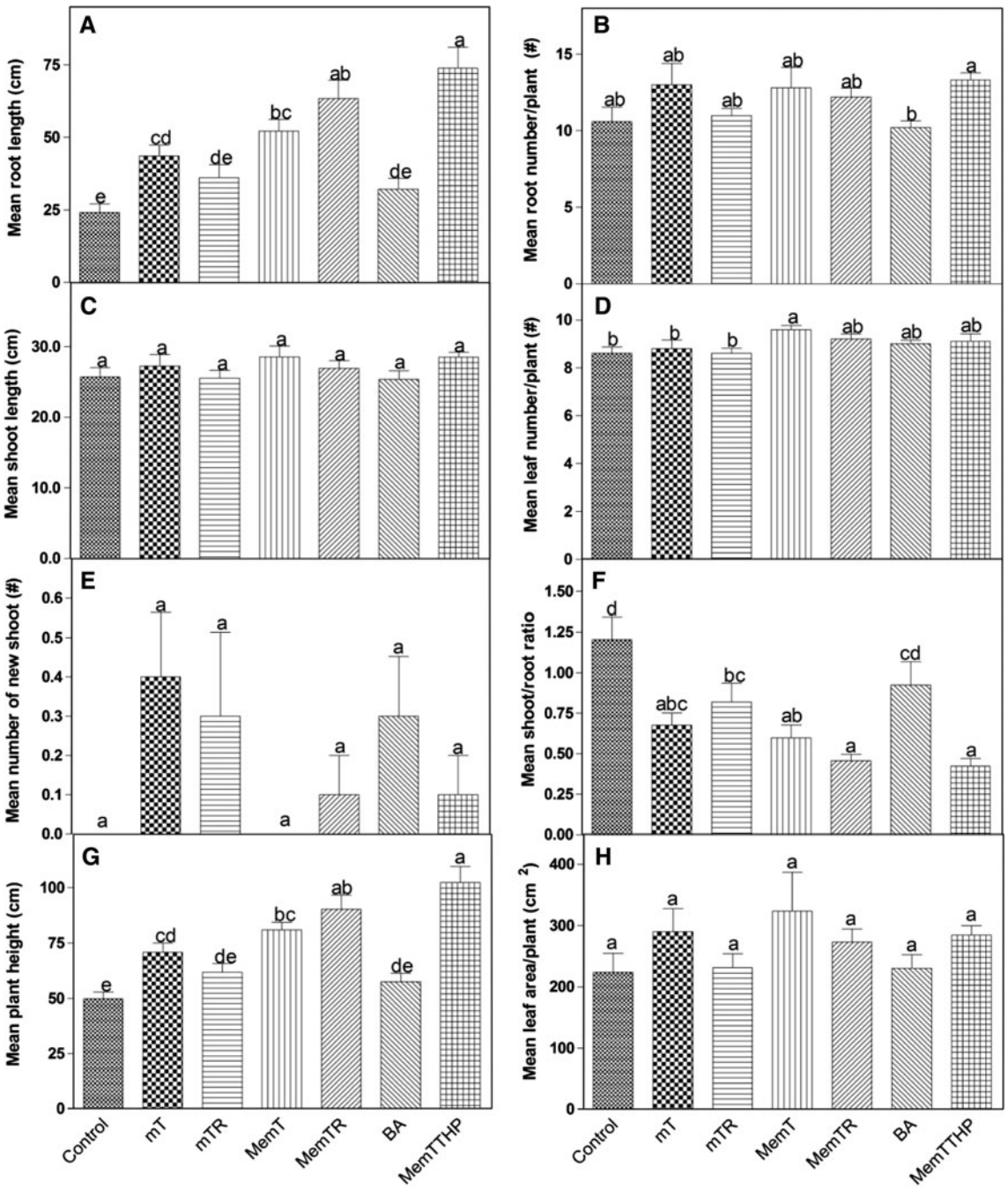

Fig. 2 Effects of six aromatic cytokinins on the growth of 'Williams' bananas. **a** root length; **b** number of root; **c** shoot length; **d** number of leaves; **e** number of new shoot produced; **f** shoot/root ratio; **g** plant height and **h** leaf area. In each graph, *bars* with *different letter*(*s*) are significantly different ($P = 0.05$) based on Duncan's multiple range test. *m*T, *meta*-Topolin; *m*TR, *meta*-Topolin riboside; Me*m*T, *meta*-Methoxy topolin; Me*m*TR, *meta*-Methoxy topolin riboside; *BA* benzyladenine; Me*m*TTHP, *meta*-Methoxy topolin 9-tetrahydropyran-2-yl

their longer lifespan as well as availability in the plant. If confirmed with more similar studies on different species, Me*m*TTHP would be an ideal CK for other plants with rooting and acclimatization problems.

The numerous advantages associated with the use of PTC for production of secondary metabolites remain of major interest to researchers (Ramachandra Rao and Ravishankar 2002). These authors highlighted the regulatory role of different CKs in the accumulation of secondary metabolites. The type and concentration of CKs affect the quantity of secondary metabolites produced. In *Hypericum perforatum,* e.g., BA-treated plantlets accumulated more hypericins compared to zeatin-treated ones (Liu et al. 2007). There was a reduction in the phenolic content of

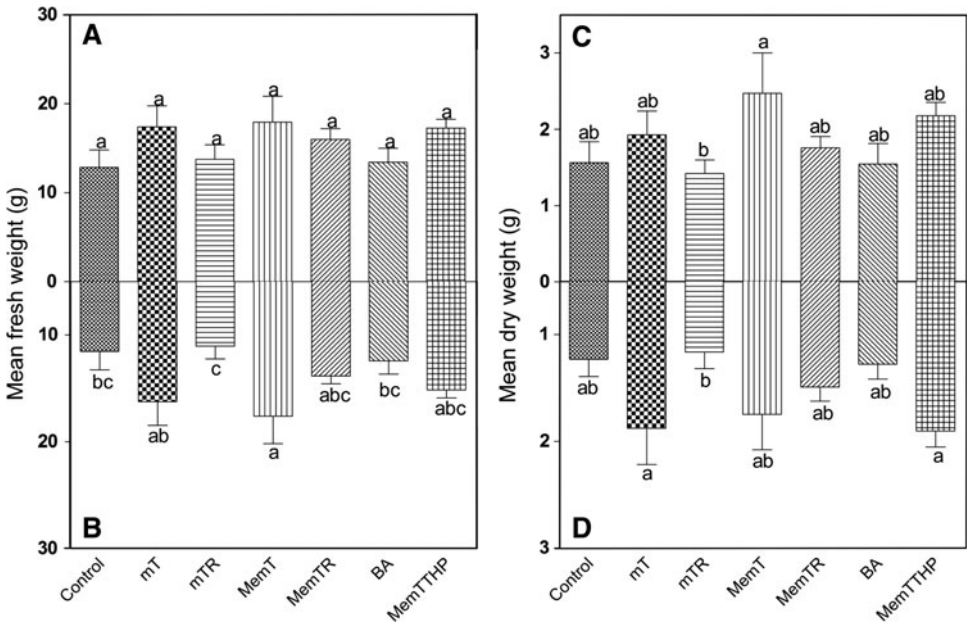

Fig. 3 Effects of six aromatic cytokinins on biomass of 'Williams' bananas. **a** Shoot fresh weight; **b** root fresh weight; **c** shoot dry weight and **d** root dry weight. In each graph, *bars* with *different letter*(*s*) are significantly different ($P = 0.05$) based on Duncan's multiple range test. *m*T, *meta*-Topolin; *m*TR, *meta*-Topolin riboside; Me*m*T, *meta*-Methoxy topolin; Me*m*TR, *meta*-Methoxy topolin riboside; *BA* benzyladenine; Me*m*TTHP, *meta*-Methoxy topolin 9-tetrahydropyran-2-yl

Table 2 Effect of cytokinin on the photosynthetic pigment contents of greenhouse-acclimatized 'Williams' bananas

Cytokinin treatment	Chlorophyll *a* (μg/g FW)	Chlorophyll *b* (μg/g FW)	Total chlorophyll (μg/g FW)	Total carotenoid (μg/g FW)	Chlorophyll *a*/*b* ratio	Total chlorophyll/carotenoid ratio
Control	527.8 ± 39.48 a	208.5 ± 16.85 a	736.4 ± 56.25 a	170.4 ± 11.05 a	2.56 ± 0.030 c	4.27 ± 0.063 abc
*m*T	417.0 ± 12.20 c	157.9 ± 4.11 c	574.8 ± 16.26 c	133.6 ± 3.75 c	2.64 ± 0.020 b	4.30 ± 0.038 ab
*m*TR	530.3 ± 14.59 a	200.1 ± 5.59 a	730.4 ± 20.16 a	168.9 ± 4.71 a	2.65 ± 0.007 b	4.33 ± 0.026 a
Me*m*T	500.6 ± 13.84 ab	185.2 ± 5.46 ab	685.7 ± 19.20 ab	160.0 ± 4.14 ab	2.71 ± 0.018 a	4.28 ± 0.028 abc
Me*m*TR	453.3 ± 14.83 bc	174.2 ± 6.15 bc	627.5 ± 20.90 bc	149.9 ± 4.00 bc	2.61 ± 0.020 bc	4.17 ± 0.044 cd
BA	450.9 ± 15.59 bc	172.4 ± 6.10 bc	623.3 ± 21.63 bc	153.3 ± 5.41 ab	2.62 ± 0.014 bc	4.07 ± 0.027 d
Me*m*TTHP	441.2 ± 17.58 bc	169.6 ± 7.00 bc	610.8 ± 24.54 bc	145.8 ± 6.07 bc	2.60 ± 0.015 bc	4.19 ± 0.023 bc

Mean values ± standard error($n = 20$) in the same column with different letter(s) are significantly different ($P = 0.05$) based on Duncan's multiple range test

Cytokinin treatment: *m*T, *meta*-Topolin; *m*TR, *meta*-Topolin riboside; Me*m*T, *meta*-Methoxy topolin; Me*m*TR, *meta*-Methoxy topolin riboside; BA, benzyladenine; Me*m*TTHP, *meta*-Methoxy topolin 9-tetrahydropyran-2-yl

micropropagated *Tectona grandis* with an increase in BA concentrations (Quiala et al. 2012). The current findings demonstrate varying levels of the quantified phenolic compounds due to different CK types as well as concentration in micropropagated bananas. The topolins, particularly, *m*T and Me*m*TTHP increased total phenolics, flavonoids and proanthocyanidins in both underground and aerial parts of regenerated banana plantlets. Consequently, the use of topolins could be an alternative method for the production of high-value secondary metabolites required for the pharmaceutical, agrochemical, flavor, fragrance, and food industries.

Plant survival depends on their interaction with the environment aided by their secondary metabolite content. During stress, the phenylpropanoid pathway is of critical importance as its products (phenolic compounds) protect the plant against abiotic and biotic factors (Dixon and Paiva 1995). Evidence has shown that phenylpropanoid-based polymers, such as lignin, and proanthocyanidin, contribute substantially to the stability and robustness of higher plants towards mechanical or environmental damage (Vogt 2010). Generally, phenolic compounds are essential in UV protection and modulating the levels of reactive oxygen species in plants (Peer and Murphy 2007; Buer et al. 2010). Particularly, flavonoids have been shown to be polar auxin transport modulators via different regulatory pathways (Peer and Murphy 2007). Recently, De Klerk et al. (2011) also reported that the presence of certain phenolic compounds protects auxins from decarboxylation which inevitably stimulated better rooting in *Malus* spp. Banana being a large herbaceous plant requires firm attachment thereby necessitating the development of a good root system. Plantlets regenerated from Me*m*TTHP had the best shoot/root ratio, an indication of the plantlet

survival potential during unfavorable conditions such as drought (Bernier et al. 1995). Furthermore, a positive correlation has been established between host resistance and products (mainly phenolic compounds) of the phenylpropanoid pathway (Binks et al. 1997; Collingborn et al. 2000). The biochemical basis for secondary metabolites and degree of resistance is not fully elucidated (Wuyts et al. 2005, 2007). In addition, different PGRs play positive or negative roles against various biotrophic and necrotrophic pathogens depending on the type of plant–pathogen interactions (Bari and Jones 2009). Unfortunately, most of the underlying molecular mechanisms involved in these interactions remain poorly understood.

Another important function of CK is the delay of senescence by accumulating and maintaining photosynthetic pigments in plants (Van Staden et al. 1988). The exogenous application of topolins such as *m*T and *m*TR delayed chlorophyll degradation in *Pelargonium* (Mutui et al. 2012), *Triticum aestivum* (Palavan-Ünsal et al. 2004) and *Beta vulgaris* (Čatský et al. 1996). In vitro studies have, however, demonstrated both stimulatory and inhibitory effects of different CKs on photosynthetic pigment content in micropropagated species, such as *Phaseolus vulgaris* (Adedipe et al. 1971; Yokoyama et al. 1980) and *Dianthus caryophyllus* (Genkov et al. 1997). In this study, the topolins generally had higher chlorophyll *a*/*b* ratios compared to the control or BA treatments. The higher ratio is an indication of the topolin-stimulated adaptive response required for effective photosynthesis by the acclimatized plants (Chow et al. 1990). High total chlorophyll/carotenoid ratio is related to the presence and maintenance of substantial total chlorophyll content in plants (Lichtenthaler 1987), and all the topolin treatments (except Me*m*TR) had significantly higher total chlorophyll/carotenoid ratios and were greener than the BA-treated banana plants.

In conclusion, the importance of the right choice of CK has been further demonstrated in this study. Besides the obvious effect during the micropropagation phase, the CKs generally have other carry-over attributes which could either enhance or hinder the acclimatization of in vitro regenerated plantlets. In vitro treatment with *m*TR and Me*m*T enhanced the photosynthetic pigments in the greenhouse-acclimatized banana plants. In addition to *m*T and Me*m*TTHP increasing the phenolic compounds in micropropagated bananas, plantlets regenerated from most of the topolins were better acclimatized than the BA treatment. Clearly, Me*m*TTHP, which is a new topolin derivative, has great promise as a CK which could be useful to circumvent rooting inhibition and acclimatization failure in PTC. In addition to the investigation of the effect of Me*m*TTHP on other micropropagated plant species, we are also studying the effect of these topolins on the endogenous CK profiles of micropropagated 'Williams' bananas.

Author contribution A.O. Aremu, M.W. Bairu, J.F. Finnie and J. Van Staden were responsible for the experimental design and conducted the experiment. L. Szüčová and K. Doležal were responsible for the synthesis of the topolins. A.O. Aremu drafted the manuscript and all authors read and approved the final manuscript.

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References

Adedipe NO, Hunt LA, Fletcher RA (1971) Effects of benzyladenine on photosynthesis, growth and senescence of the bean plant. Physiol Plant 25:151–153

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

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, 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

Bari R, Jones JDG (2009) Role of plant hormones in plant defence responses. Plant Mol Biol 69:473–488

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

Bernier PY, Lamhamedi MS, Simpson DG (1995) Shoot:root ratio is of limited use in evaluating the quality of container conifer stock. Tree Planters Notes 46:102–106

Binks RH, Greenham JR, Luis JG, Gowen SR (1997) A phytoalexin from roots of *Musa acuminata* var. *Pisang sipulu*. Phytochemistry 45:47–49

Buer CS, Imin N, Djordjevic MA (2010) Flavonoids: new roles for old molecules. J Integrat Plant Biol 52:98–111

Caponetti JD, Gray DJ, Trigiano RN (2005) History of plant tissue and cell culture. In: Trigiano RN, Gray DJ (eds) Plant development and biotechnology. CRC Press, Florida, pp 9–15

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 Plant 38:511–518

Chow WS, Anderson JM, Melis A (1990) The photosystem stoichiometry in thylakoids of some Australian shade-adapted plant species. Aust J Plant Physiol 17:665–674

Collingborn FMB, Gowen SR, Mueller-Harvey I (2000) Investigations into the biochemical basis for nematode resistance in roots of three *Musa* cultivars in response to *Radopholus similis* infection. J Agric Food Chem 48:5297–5301

Criado M, Roberts I, Echeverria M, Barneix A (2007) Plant growth regulators and induction of leaf senescence in nitrogen-deprived wheat plants. J Plant Growth Regul 26:301–307

Curir P, VanSumere CF, Termini A, Barthe P, Marchesini A, Dolci M (1990) Flavonoid accumulation is correlated with adventitious roots formation in *Eucalyptus gunnii* Hook micropropagated through axillary bud stimulation. Plant Physiol 92:1148–1153

De Klerk G-J, Guan H, Huisman P, Marinova S (2011) Effects of phenolic compounds on adventitious root formation and oxidative decarboxylation of applied indoleacetic acid in *Malus* 'Jork 9'. Plant Growth Regul 63:175–185

Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell 7:1085–1097

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

Genkov T, Tsoneva P, Ivanova I (1997) Effect of cytokinins on photosynthetic pigments and chlorophyllase activity in in vitro cultures of axillary buds of *Dianthus caryophyllus* L. J Plant Growth Regul 16:169–172

Jain SM (2004) Introduction: cellular biology and biotechnology including mutation techniques for creation of new useful banana genotypes. In: Jain SM, Swennen R (eds) Banana improvement: cellular, molecular biology, and induced mutations. Science Publishers, Inc., Enfield, pp xv–xvi

Lewinsohn E, Gijzen M (2009) Phytochemical diversity: the sounds of silent metabolism. Plant Sci 176:161–169

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. In: Douce R, Packer L (eds) Methods in enzymology, vol 148., Academic Press New York, USA, pp 350–382

Liu X-N, Zhang X-Q, Sun J-S (2007) Effects of cytokinins and elicitors on the production of hypericins and hyperforin metabolites in *Hypericum sampsonii* and *Hypericum perforatum*. Plant Growth Regul 53:207–214

Makkar HPS, Sidhuraju P, Becker K (2007) Plant secondary metabolites. Humana Press Inc., New Jersey

Marinova D, Ribarova F, Atanassova M (2005) Total phenolics and total flavonoids in Bulgarian fruits and vegetables. J Univ Cheml Technol Metal 40:255–260

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

Mutui TM, Mibus H, Serek M (2012) Effect of *meta*-topolin on leaf senescence and rooting in *Pelargonium* × *hortorum* cuttings. Postharvest Biol Technol 63:107–110

Namdeo AG (2007) Plant cell elicitation for production of secondary metabolites: a review. Pharmacogn Rev 1:69–79

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

Peer WA, Murphy AS (2007) Flavonoids and auxin transport: modulators or regulators? Trends Plant Sci 12:556–563

Porter LJ, Hrstich LN, Chan BG (1985) The conversion of proanthocyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry 25:223–230

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

Quiala E, Cañal M-J, Meijón M, Rodríguez R, Chávez M, Valledor L, de Feria M, Barbón R (2012) Morphological and physiological responses of proliferating shoots of teak to temporary immersion and BA treatments. Plant Cell Tissue Organ Cult. 109:223–234

Ramachandra Rao S, Ravishankar GA (2002) Plant cell cultures: chemical factories of secondary metabolites. Biotechnol Adv 20:101–153

Szücová L, Spíchal L, Dolezal K, Zatloukal M, Greplová J, Galuszka P, Krystof V, Voller J, Popa I, Massino FJ, Jørgensen J-E, Strnad M (2009) Synthesis, characterization and biological activity of ring-substituted 6-benzylamino-9-tetrahydropyran-2-yl and 9-tetrahydrofuran-2-ylpurine derivatives. Bioorg Med Chem 17:1938–1947

Valero-Aracama C, Kane M, Wilson S, Vu J, Anderson J, Philman N (2006) Photosynthetic and carbohydrate status of easy-and difficult-to-acclimatize sea oats (*Uniola paniculata* L.) genotypes during in vitro culture and ex vitro acclimatization. In Vitro Cell Dev Biol Plant 42:572–583

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, Cook EL, Noodén LD (1988) Cytokinins and senescence. In: Noodén LD, Leopold AC (eds) Senescence and aging in plants. Academic Press, Inc., San Diego, pp 281–328

Vogt T (2010) Phenylpropanoid biosynthesis. Mol Plant 3:2–20

Vuylsteke DR (1998) Shoot-tip culture for the propagation, conservation, and distribution of *Musa* germplasm. International Institute of Tropical Agriculture, Ibadan

Werbrouck SPO, Van der Jeugt B, Dewitte W, Prinsen E, Van Onckelen HA, Debergh PC (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 Plant 98:291–297

Werner T, Motyka V, Strnad M, Schmülling T (2001) Regulation of plant growth by cytokinin. Proc Natl Acad Sci 98:10487–10492

Wu HC, du Toit ES, Reinhardt CF, Rimando AM, van der Kooy F, Meyer JJM (2007) The phenolic, 3,4-dihydroxybenzoic acid, is an endogenous regulator of rooting in *Protea cynaroides*. Plant Growth Regul 52:207–215

Wuyts N, Lognay G, Sági L, De Waele D, Swennen R (2005) Secondary metabolites in roots and implications for nematode resistance in banana (*Musa* spp.). In: Turner DW, Rosales FE (eds) Banana root system: towards a better understanding for its productive management. Proceedings of an international symposium. The International Network for the Improvement of Banana and Plantain, Montpellier, pp 238–246

Wuyts N, Lognay G, Verscheure M, Marlier M, De Waele D, Swennen R (2007) Potential physical and chemical barriers to infection by the burrowing nematode *Radopholus similis* in roots of susceptible and resistant banana (*Musa* spp.). Plant Pathol 56:878–890

Yokoyama M, Naito K, Suzuki H (1980) Effects of benzyladenine on chlorophyll, DNA, RNA and protein content of attached young bean (*Phaseolus vulgaris* L.) leaves. Ann Bot 45:649–653

Zhishen J, Mengcheng T, Jianming W (1999) The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem 64:555–559