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ORIGINAL PAPER

Physiological and biochemical effects of a tetrahydropyranyl-substituted *meta*-topolin in micropropagated *Merwilla plumbea*

Stephen O. Amoo · Adeyemi O. Aremu · Mack Moyo · Taofik O. Sunmonu · Lucie Plíhalová · Karel Doležal · Johannes Van Staden

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Abstract In comparison to the most active cytokinins (CKs) previously reported for the micropropagation of *Merwilla plumbea*, we examined the effect of *meta*-topolin tetrahydropyran-2-yl (*m*TTHP—a novel aromatic CK derivative) on in vitro adventitious shoot production, rooting and photosynthetic pigment content of regenerated plants. Its carry-over effect on ex vitro growth, photosynthetic performance and antioxidant enzyme system of this bulbous medicinal plant was also investigated. The treatments with *m*TTHP and *meta*-topolin riboside (*m*TR) gave the highest number of adventitious shoots when compared to thidiazuron (TDZ) application and the control. The highest rooting frequency was observed in *m*TTHP treatments. Unlike in *m*TTHP treatments, an increase in *m*TR or TDZ concentration beyond 0.5 μM resulted in a significant decrease in the concentrations of all the photosynthetic pigments quantified. After 6 months of ex vitro growth, regenerated plants from 0.5 μM *m*TTHP treatment had the highest significant total leaf area, total leaf fresh weight and bulb size compared to all *m*TR and TDZ-treated plants. Plants regenerated from *m*TTHP or *m*TR treatments demonstrated a high capacity for energy dissipation in comparison to TDZ-regenerated plants with low photochemical quenching, PSII quantum efficiency and non-photochemical quenching. Despite a significant increase in the antioxidant enzyme activities, malondialdehyde concentration was significantly high in the leaves of TDZ-regenerated plants compared to other CK treatments. This finding indicated a high production of reactive oxygen species beyond the scavenging efficiency of the antioxidant enzymes leading to oxidative stress and subsequent low biomass accumulation in TDZ-derived plants.

Keywords Antioxidant enzymes · Chlorophyll fluorescence · Medicinal plants · Thidiazuron · Topolins

Abbreviations

CAT	Catalase
CKs	Cytokinins
ETR	Relative electron transport rate
Fv/Fm	Maximum photochemical efficiency of PSII
MDA	Malondialdehyde
Me*m*TTHP	*meta*-Methoxytopolin 9-tetrahydropyran-2-yl or 6-(3-methoxybenzylamino-9-tetrahydropyran-2ylpurine
MS	Murashige and Skoog
*m*TR	*meta*-Topolin riboside
*m*TTHP	*meta*-Topolin tetrahydropyran-2-yl or 6-(3-hydroxylbenzylamino)-9-tetrahydropyran-2-ylpurine
NPQ	Non-photochemical quenching
PGR	Plant growth regulator
POD	Peroxidase

S. O. Amoo · A. O. Aremu · M. Moyo · T. O. Sunmonu · J. Van Staden (✉)
Research Centre for Plant Growth and Development, School of Life Sciences, University of KwaZulu-Natal Pietermaritzburg, Private Bag X01, Scottsville 3209, South Africa
e-mail: rcpgd@ukzn.ac.za

Present Address:
S. O. Amoo
Agricultural Research Council, Roodeplaat Vegetable and Ornamental Plant Institute, Private Bag X293, Pretoria 0001, South Africa

L. Plíhalová · K. Doležal
Department of Chemical Biology and Genetics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Institute of Experimental Botany, Academy of Sciences of Czech Republic, Šlechtitelů 11, 783 71 Olomouc-Holice, Czech Republic

PPF	Photosynthetic photon flux
PSII	Photosystem II
PTC	Plant tissue culture
*q*P	Photochemical quenching
SOD	Superoxide dismutase
TDZ	Thidiazuron
THP	Tetrahydropyranyl
Φ_{PSII}	Actual quantum yield of PSII

Introduction

Cytokinins (CKs) are ubiquitous plant growth regulators (PGRs) in plants where they control different aspects of plant growth and development. Among others, they are known to control cell division, morphogenesis, retard senescence as well as modulate the activities of different antioxidant enzymes (Synková et al. 2006; Werner and Schmülling 2009). They are an essential component of growth media used in plant tissue culture (PTC), including micropropagation. The exogenous application of different CKs can however, have differential effect on photosynthetic pigment content in micropropagated plants (Adedipe et al. 1971; Genkov et al. 1997; Yokoyama et al. 1980). CKs are also known to modulate the activities of antioxidant enzymes involved in scavenging reactive oxygen species generated during normal plant metabolism (Davey et al. 2005; Petit-Paly et al. 1999; Synková et al. 2006). The over-production of reactive oxygen species beyond their scavenging mechanism often results in oxidative damage to biomolecules such as proteins, carbohydrates and lipids. The accumulation of malondialdehyde (MDA), an end-product of lipid peroxidation, is thus widely used as a biomarker or an indicator of oxidative stress in plants.

Recent research indicating the superiority of aromatic CKs, particularly the *meta*-hydroxylated analogues in PTC (see the review by Aremu et al. 2012a) have led to the synthesis of an array of *meta*-topolin derivatives substituted at N^9 position with the objective of improving CK activity (Szüčová et al. 2009). One such new derivative, 6-(3-hydroxylbenzylamino)-9-tetrahydropyran-2-ylpurine (otherwise known as *meta*-topolin tetrahydropyran-2-yl, *m*TTHP) had the advantage of inducing rooting at a low concentration in the micropropagation of *Aloe arborescens* and *Harpagophytum procumbens* (Amoo et al. 2014). However, there is a dearth of information on the physiological and biochemical potential of this novel CK in the micropropagation of different plant species. Studies have indicated that CK application during in vitro stages affects acclimatization competence, secondary metabolite production and pharmacological activity of micropropagated plants (Baskaran et al. 2014; Magyar-Tábori et al. 2001; Szopa and Ekiert 2012). Although the carry-over effect of *m*TTHP on secondary metabolite production and antioxidant activity has recently been demonstrated (Aremu et al. 2014), its carry-over effect (if any) on ex vitro growth as well as the underlying physiological and/or biochemical processes has not been evaluated.

Merwilla plumbea (Lindl.) Speta (family: Asparagaceae) is a bulbous plant, widely used in traditional South African medicine. An estimated 95.5 t equivalent to about 432,000 bulbs was reported to be traded annually in one South African medicinal plant market (Williams et al. 2008). The destructive harvesting of its bulbs for use in traditional medicine is certainly unsustainable, leading to its 'near threatened' conservation status (Raimondo et al. 2009). Considering that a micropropagation protocol has already been developed for *M. plumbea* (Baskaran et al. 2012), we used this plant species as a model plant for the current investigation. The objective of this study was to evaluate the potential of this novel CK as a viable alternative in PTC. Its effect on in vitro shoot production, rooting and photosynthetic pigment content of regenerated *M. plumbea* plants was evaluated in comparison to the previously reported most active CKs for the micropropagation of this species (Baskaran et al. 2012). Its carry-over effect (if any) on ex vitro growth (including bulb production), photosynthetic performance and antioxidant enzyme system was also investigated.

Materials and methods

Shoot proliferation experiment

Leaf explants (measuring approximately 1 cm × 1 cm) obtained from in vitro cultured *M. plumbea* shoots, which were maintained on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 30 g l^{-1} sucrose and 0.1 g l^{-1} myo-inositol without any PGR were used in the present study. Based on previous findings (Baskaran et al. 2012) reporting efficient shoot proliferation using thidiazuron (TDZ) and *meta*-topolin riboside (*m*TR), these CKs were selected and compared with a new *meta*-topolin derivative, *m*TTHP. Four different concentrations (0.25, 0.5, 1.0 and 2.0 µM) were used in each case while media without PGR served as a control. In each case, the pH of the media was adjusted to 5.8 using 1 N KOH or 1 N HCl before solidifying with 8.0 g l^{-1} agar (Bacteriological agar-Oxoid Ltd., Basingstoke, Hampshire, England) and autoclaving at 121 °C and 103 kPa for 20 min. There were 44 leaf explants per treatment. Cultures were incubated in a growth room with 16 h light/8 h dark conditions (40–45 µmol m^{-2} s^{-1} photosynthetic photon flux, PPF) at 25 ± 2 °C. After 12 weeks of culture, the number of shoots produced per explant, number of transplantable

shoots (shoots >2 cm) and frequencies of shoot regeneration and root production were recorded.

Quantification of photosynthetic pigments

After 12 weeks of culturing as indicated above, the photosynthetic pigment quantification in regenerated shoots from each treatment was done using a colorimetric method by Lichtenthaler (1987) with slight modifications as detailed by Aremu et al. (2012b). The pigment content was expressed in µg per g fresh weight.

Acclimatization and ex vitro growth

In vitro regenerated plants carefully washed free of agar residue were individually transferred to pots (12.5 cm diameter) containing soil:vermiculite (1:1, v/v) mixture, treated with 1 % Benlate®. The potted plants were placed in a mist house with about 90 % relative humidity for 3 months. They were subsequently transferred to a greenhouse with a day/night temperature of approximately 30/15 °C, average PPF of 450 µmol m^{-2} s^{-1} and 30–40 % relative humidity under natural photoperiod conditions. After an additional 3 months of growth in the greenhouse, the number of leaves per plant, leaf area, leaf fresh weight, bulb diameter, bulb fresh weight, number of roots per plant and length of longest root of 20 randomly selected plants from each CK treatment (as detailed above) were recorded.

Measurement of chlorophyll fluorescence

Chlorophyll fluorescence was measured in the leaves of different plants from the CK treatments detailed above after 6 months of ex vitro growth using a FMS 2 modulated fluorometer (Hansatech Instruments, King's Lynn, UK) as detailed by Beckett et al. (2005). Measurements were recorded from 10 leaves of 10 randomly selected intact plants per CK treatment. Each replicate was dark-incubated for a minimum of 10 min before any measurement. Measurements were taken under four different PPF, which were 264, 488, 800 and 1,200 µmol m^{-2} s^{-1}. The Fv/Fm value which is the maximum photochemical efficiency of dark-adapted photosystem II (PSII) was calculated as described by Maxwell and Johnson (2000). At each light intensity, the following fluorescence parameters were calculated for each CK-regenerated plants and the control as described by Maxwell and Johnson (2000): actual quantum yield of PSII (Φ_{PSII}), relative electron transport rate (ETR), photochemical quenching (*q*P) and non-photochemical quenching (NPQ).

Antioxidant enzyme assay and malondialdehyde (MDA) quantification

Fresh leaf and bulb materials (1 g each) from harvested plants were homogenized separately with 4 ml of 0.1 M Tris–HCl buffer (pH 7.8) under chilled conditions using a pestle and mortar. The homogenate was carefully filtered and the resultant supernatant was retained for MDA quantification and antioxidant enzyme assay. The antioxidant enzymes assayed were superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and peroxidase (POD, EC 1.11.1.7). Details of assay procedure used for these enzymes and MDA quantification have been documented (Sunmonu and Van Staden 2014). The experiment was conducted in triplicate.

Table 1 Effect of cytokinins on adventitious shoot production and rooting of *M. plumbea* after 12 weeks of culture

Cytokinin treatment (µM)	Rooting frequency (%)	Shoot regeneration frequency (%)	Total no of shoots	No of shoots with length >2 cm
0.25 *m*TR	88.6	91.7	2.5 ± 0.28 cd	2.0 ± 0.25 bc
0.5 *m*TR	75	90.9	3.9 ± 0.33 a	2.8 ± 0.36 a
1.0 *m*TR	54.5	93.6	4.1 ± 0.30 a	2.8 ± 0.34 a
2.0 *m*TR	41.5	85.4	3.3 ± 0.25 abc	1.27 ± 0.22 d
0.25 *m*TTHP	82.9	81.4	2.7 ± 0.23 bcd	2.3 ± 0.28 abc
0.5 *m*TTHP	94.1	85	3.4 ± 0.29 ab	2.8 ± 0.25 a
1.0 *m*TTHP	61	89.1	3.3 ± 0.25 abc	2.5 ± 0.19 ab
2.0 *m*TTHP	61	85.4	2.8 ± 0.28 bcd	0.37 ± 0.12 e
0.25 TDZ	62.2	82.2	2.7 ± 0.23 bcd	0.1 ± 0.06 e
0.5 TDZ	20	69.8	2.7 ± 0.29 bcd	0 e
1.0 TDZ	0	77.1	2.4 ± 0.22 d	0 e
2.0 TDZ	0	78.6	2.3 ± 0.20 d	0 e
Control	94.9	88.6	2.1 ± 0.15 d	1.7 ± 0.16 cd

Data are mean ± SE (n = 44)

*m*TR = *meta*-topolin riboside; *m*TTHP = *meta*-topolin tetrahydropyran-2-yl; TDZ = thidiazuron

Mean values in the same column followed by different letters indicate significant differences ($P \leq 0.05$) based on Duncan's multiple range test

Data analysis

Data were subjected to analysis of variance using SPSS software version 16.0. Where there was statistical significance ($P \leq 0.05$), the separation of mean values was done using Duncan's multiple range test.

Results and discussion

In vitro shoot production and rooting

Table 1 shows the effect of applied CKs on in vitro adventitious shoot production and rooting frequency after

Table 2 Effect of cytokinins on photosynthetic pigment contents of in vitro regenerated *M. plumbea* after 12 weeks of culture

Cytokinin treatment (μM)	Total carotenoid (μg/g FW)	Chlorophyll a (μg/g FW)	Chlorophyll b (μg/g FW)	Chlorophyll a/b ratio	Total chlorophyll (μg/g FW)	Total chlorophyll/ carotenoid ratio
0.25 *m*TR	177.3 ± 4.77 b	662.3 ± 17.45 bc	290.3 ± 21.78 b	2.3 ± 0.11 ab	952.6 ± 36.22 bc	5.4 ± 0.09 ab
0.5 *m*TR	196.0 ± 7.45 ab	730.0 ± 23.87 ab	302.3 ± 9.52 ab	2.4 ± 0.02 a	1,032.4 ± 33.20 ab	5.3 ± 0.03 bc
1.0 *m*TR	173.1 ± 14.57 bc	614.7 ± 52.47 cd	278.6 ± 25.28 bc	2.2 ± 0.05 bc	893.3 ± 77.26 cd	5.2 ± 0.08 cdef
2.0 *m*TR	124.8 ± 8.61 e	426.1 ± 31.51 e	212.3 ± 14.54 d	2.0 ± 0.01 de	638.4 ± 46.03 e	5.1 ± 0.06 def
0.25 *m*TTHP	140.8 ± 10.41 de	511.4 ± 36.84 e	236.1 ± 16.00 cd	2.2 ± 0.02 c	747.5 ± 52.80 e	5.3 ± 0.02 abc
0.5 *m*TTHP	132.8 ± 4.69 de	475.3 ± 20.03 e	221.4 ± 8.86 d	2.1 ± 0.01 c	696.8 ± 28.87 e	5.2 ± 0.05 bcde
1.0 *m*TTHP	149.9 ± 2.69 cde	519.9 ± 8.68 de	243.5 ± 2.23 cd	2.1 ± 0.02 c	763.4 ± 10.69 de	5.1 ± 0.02 ef
2.0 *m*TTHP	152.1 ± 3.51 cd	524.1 ± 19.72 de	244.0 ± 7.13 cd	2.1 ± 0.02 c	768.1 ± 26.80 de	5.0 ± 0.07 f
0.25 TDZ	144.4 ± 14.43 de	519.1 ± 52.32 de	239.0 ± 20.71 cd	2.2 ± 0.04 c	758.1 ± 73.00 de	5.3 ± 0.02 bcd
0.5 TDZ	151.8 ± 3.40 cd	521.4 ± 14.56 de	246.3 ± 4.13 cd	2.1 ± 0.02 cd	767.7 ± 18.69 de	5.1 ± 0.03 f
1.0 TDZ	128.4 ± 10.47 de	449.4 ± 43.66 e	202.3 ± 17.64 d	2.2 ± 0.02 bc	651.7 ± 61.30 e	5.1 ± 0.06 f
2.0 TDZ	80.1 ± 4.51 f	264.5 ± 17.60 f	138.9 ± 6.64 e	1.9 ± 0.04 e	403.5 ± 24.17 f	5.0 ± 0.02 f
Control	206.6 ± 5.55 a	786.3 ± 16.44 a	337.3 ± 12.28 a	2.3 ± 0.05 ab	1,123.6 ± 28.07 a	5.4 ± 0.04 a

Data are mean ± SE ($n = 6$)

*m*TR = *meta*-topolin riboside; *m*TTHP = *meta*-topolin tetrahydropyran-2-yl; TDZ = thidiazuron

Mean values in the same column followed by different letters indicate significant differences ($P \leq 0.05$) based on Duncan's multiple range test

Table 3 Effect of cytokinins applied during in vitro shoot proliferation of *M. plumbea* after 6 months of ex vitro growth

Cytokinin treatment (μM)	No of leaves per plant	Total leaf area per plant (cm^2)	Total leaf fresh weight per plant (g)	Bulb diameter (mm)	Bulb fresh weight (g)	No of roots per plant	Length of longest root (cm)
0.25 *m*TR	4.1 ± 0.15 ab	26.5 ± 2.15 bc	1.5 ± 0.12 bc	11.4 ± 0.34 ab	1.7 ± 0.13 b	6.6 ± 0.29 a	17.9 ± 1.32 bc
0.5 *m*TR	4.2 ± 0.15 ab	26.9 ± 2.58 bc	1.4 ± 0.11 cd	11.3 ± 0.34 ab	1.7 ± 0.14 b	5.8 ± 0.22 abcd	17.0 ± 1.13 bcd
1.0 *m*TR	4.2 ± 0.25 ab	19.0 ± 2.13 bc	0.9 ± 0.10 cde	9.6 ± 0.35 cd	1.1 ± 0.09 cd	5.6 ± 0.21 bcd	17.5 ± 1.10 bcd
2.0 *m*TR	3.9 ± 0.21 ab	15.0 ± 2.39 c	0.7 ± 0.10 e	8.7 ± 0.43 d	0.9 ± 0.11 d	5.4 ± 0.24 cd	14.1 ± 0.92 de
0.25 *m*TTHP	4.1 ± 0.23 ab	23.1 ± 2.90 bc	1.2 ± 0.15 cde	10.3 ± 0.40 bc	1.4 ± 0.12 bc	5.9 ± 0.23 abcd	16.2 ± 1.02 cd
0.5 *m*TTHP	4.4 ± 0.22 a	47.7 ± 7.27 a	2.3 ± 0.34 a	12.6 ± 0.50 a	2.3 ± 0.24 a	6.2 ± 0.24 ab	20.1 ± 1.20 ab
1.0 *m*TTHP	4.2 ± 0.14 ab	26.7 ± 3.09 bc	1.3 ± 0.16 cde	10.4 ± 0.41 bc	1.3 ± 0.13 bcd	5.7 ± 0.15 bcd	16.8 ± 1.05 bcd
2.0 *m*TTHP	4.4 ± 0.27 a	41.0 ± 4.10 a	1.9 ± 0.22 ab	10.9 ± 0.43 bc	1.6 ± 0.15 b	6.0 ± 0.26 abc	14.8 ± 0.89 cd
0.25 TDZ	3.9 ± 0.21 ab	28.5 ± 3.40 b	1.3 ± 0.16 cd	10.3 ± 0.44 bc	1.3 ± 0.12 bcd	5.4 ± 0.23 cd	14.8 ± 1.17 cd
0.5 TDZ	3.5 ± 0.23 b	18.5 ± 2.80 bc	0.8 ± 0.13 de	8.4 ± 0.54 d	0.9 ± 0.13 d	5.2 ± 0.26 d	11.1 ± 0.78 e
Control	4.5 ± 0.20 a	46.6 ± 4.85 a	2.3 ± 0.23 a	12.4 ± 0.43 a	2.1 ± 0.17 a	6.0 ± 0.31 abc	21.7 ± 1.36 a

Data are mean ± SE ($n = 20$)

The carry-over effect of 1.0 and 2.0 μM TDZ treatments on ex vitro growth was not evaluated due to complete root inhibition observed during the in vitro stage

*m*TR = *meta*-topolin riboside; *m*TTHP = *meta*-topolin tetrahydropyran-2-yl; TDZ = thidiazuron

Mean values in the same column followed by different letters indicate significant differences ($P \leq 0.05$) based on Duncan's multiple range test

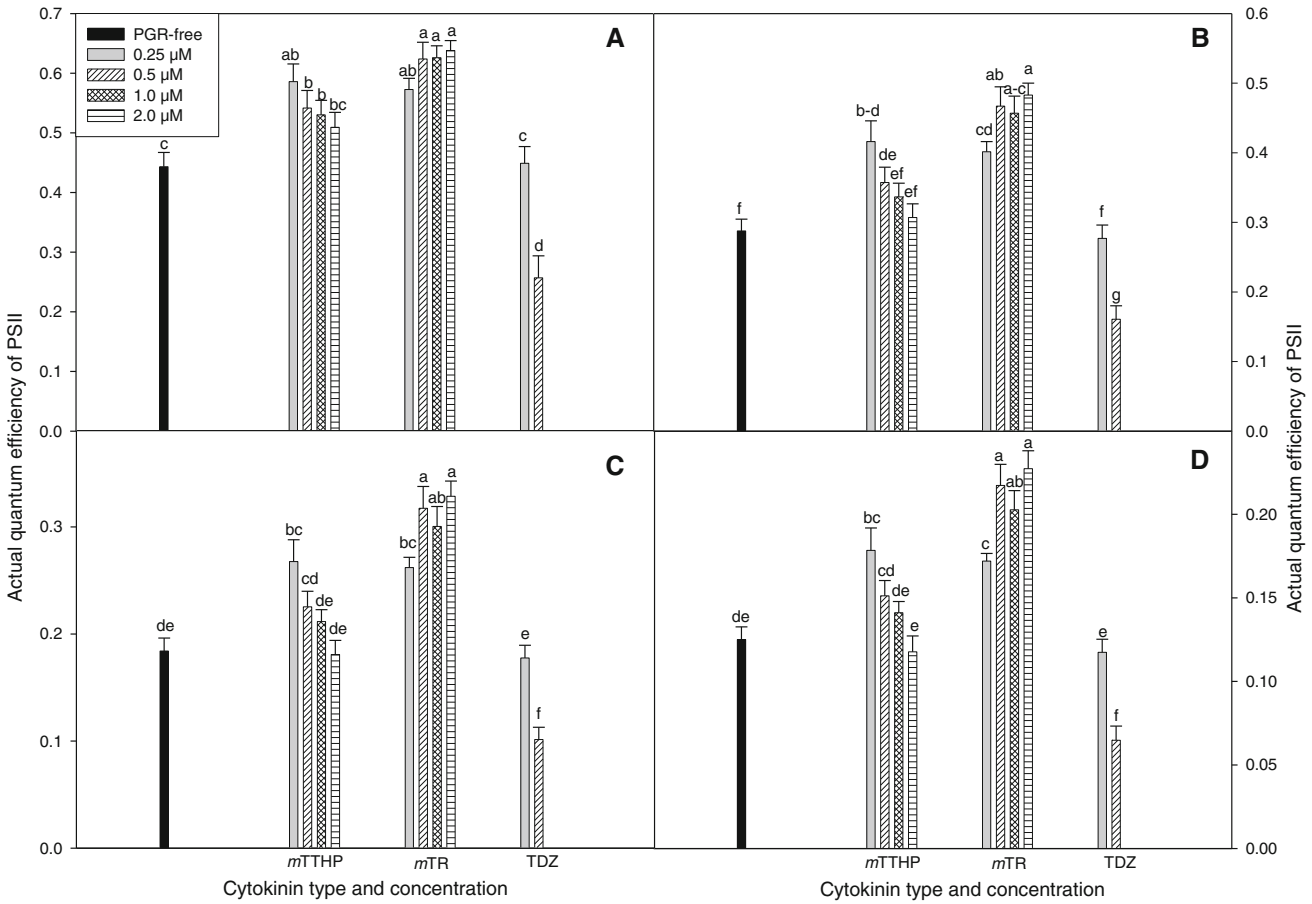

Fig. 1 Carry-over effect of cytokinins (CKs) applied during in vitro shoot proliferation of *M. plumbea* on actual PSII quantum efficiency (Φ_{PSII}), measured at **a** 264 μmol m^{-2} s^{-1} photosynthetic photon flux, **b** 488 μmol m^{-2} s^{-1} photosynthetic photon flux, **c** 800 μmol m^{-2} s^{-1} photosynthetic photon flux, and **d** 1,200 μmol m^{-2} s^{-1} photosynthetic photon flux in the leaves of different CK-regenerated plants after 6 months of ex vitro growth. *m*TR = *meta*-topolin riboside; *m*TTHP = *meta*-topolin tetrahydropyran-2-yl; TDZ = thidiazuron; PGR = plant growth regulator. *Bars with different letters* in each graph are significantly different ($P \leq 0.05$) based on Duncan's multiple range test. Data are mean ± SE ($n = 10$). Due to complete root inhibition observed during the in vitro stage, the carry-over effect of 1.0 and 2.0 μM TDZ treatments was not evaluated

12 weeks of culture. The highest number of shoots was recorded in treatment with 1.0 μM *m*TR, although not significantly different from some *m*TR (0.5 and 2.0 μM) and *m*TTHP (0.5 and 1.0 μM) treatments. Unlike the treatments with *m*TR and *m*TTHP, the application of TDZ resulted largely in the production of small shoots. There was an inverse relationship between rooting frequency and increase in CK concentration. Besides the control, the highest rooting frequency was observed in the treatment with 0.5 μM *m*TTHP. Generally, the application of TDZ resulted in a reduction in rooting frequency when compared to other CKs at equimolar concentration. At 1.0 and 2.0 μM TDZ, rooting was completely inhibited. Dobránszki et al. (2000) and Malá et al. (2009) also demonstrated that the rooting ability of a plant species can be differentially affected by the application of different CK types and concentration and their metabolism. The high rooting frequency observed in treatments with *m*TTHP when compared to other CKs used in the present study is consistent with a previous report on the rooting stimulatory activity of this new aromatic CK (Amoo et al. 2014). This rooting advantage could be due to the enhancement effect of tetrahydropyranyl (THP) substitution at N^9 position of a CK purine ring on acropetal CK transport, leading to less accumulation of non-active CK metabolites that could hamper rooting (Podlešáková et al. 2012).

Photosynthetic pigments

Cytokinins play a vital role in photosynthetic pigment production, among other functions (Chernyad'ev 2009). The effect of applied CKs on photosynthetic pigments of regenerated shoots is presented in Table 2. Overall, the control and the treatment with 0.5 μM *m*TR gave highest significant pigment levels. Unlike in *m*TTHP treatments, an increase (beyond 0.5 μM concentration) in *m*TR or TDZ

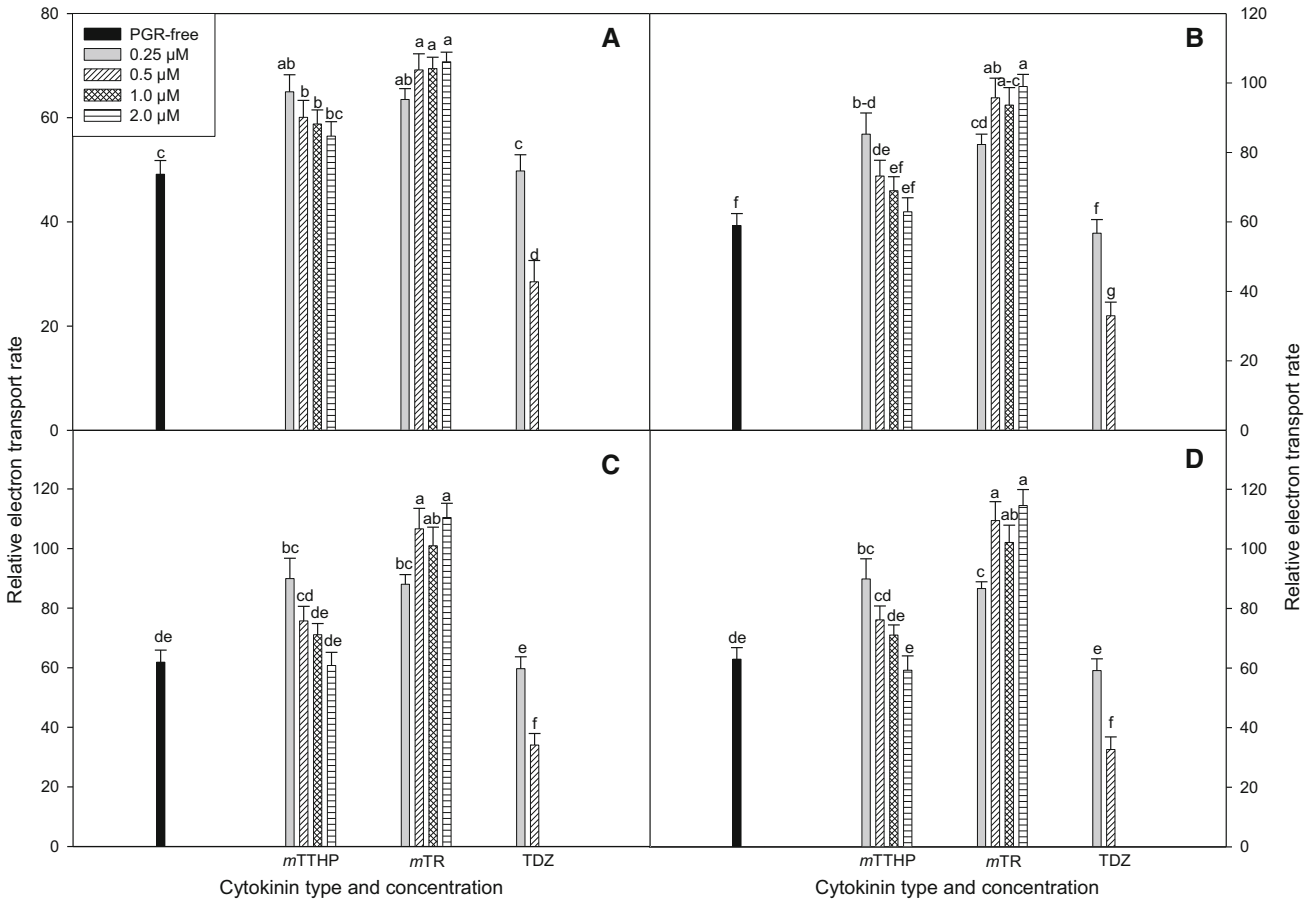

Fig. 2 Carry-over effect of cytokinins (CKs) applied during in vitro shoot proliferation of *M. plumbea* on photochemical electron transport rate (ETR), measured at **a** 264 µmol m^{-2} s^{-1} photosynthetic photon flux, **b** 488 µmol m^{-2} s^{-1} photosynthetic photon flux, **c** 800 µmol m^{-2} s^{-1} photosynthetic photon flux, and **d** 1,200 µmol m^{-2} s^{-1} photosynthetic photon flux in the leaves of different CK-regenerated plants after 6 months of ex vitro growth. *m*TR = *meta*-topolin riboside; *m*TTHP = *meta*-topolin tetrahydropyran-2-yl; TDZ = thidiazuron; PGR = plant growth regulator. *Bars with different letters* in each graph are significantly different ($P \leq 0.05$) based on Duncan's multiple range test. Data are mean ± SE ($n = 10$). Due to complete root inhibition observed during the in vitro stage, the carry-over effect of 1.0 and 2.0 µM TDZ treatments was not evaluated

concentration resulted in a significant decrease in the levels of total carotenoid, chlorophyll a, chlorophyll b and total chlorophyll. A similar decrease in photosynthetic pigments with an increase in CK concentration was reported in *A. arborescens* (Amoo et al. 2014). In most cases, CK application resulted in a reduced photosynthetic pigment concentration when compared to PGR-free plants in the present study. In contrast to the well-known anti-senescence effect of CKs, previous study has indicated that CKs can differentially induce programmed cell death by accelerating senescence depending on their catabolism by CK oxidase/dehydrogenase (Carimi et al. 2004).

Ex vitro growth

Table 3 shows the carry-over effect of CKs applied during in vitro shoot regeneration phase on different growth parameters after 6 months of ex vitro growth. In general, none of the CK-derived plants significantly outperformed the PGR-free-derived plants in terms of the measured growth parameters. However, among the CK-derived plants, those regenerated from medium containing 0.5 µM TDZ had a significant less number of leaves recorded per plant. Regenerated plants from the treatment with 0.5 µM *m*TTHP had the highest total leaf area and total leaf fresh weight of 47.68 cm^2 and 2.3 g, respectively. These values were significantly different compared to all *m*TR and TDZ-treated plants. With an increase in CK concentration, a general decrease in bulb diameter, bulb fresh weight, number of roots produced per plant and length of longest root was observed in plants regenerated from *m*TR and TDZ treatments. Overall, shoots originally regenerated from medium containing 0.5 µM *m*TTHP gave the highest yield in terms of bulb production as indicated by the bulb size. The length of longest root produced by plants with this particular treatment was significantly high when

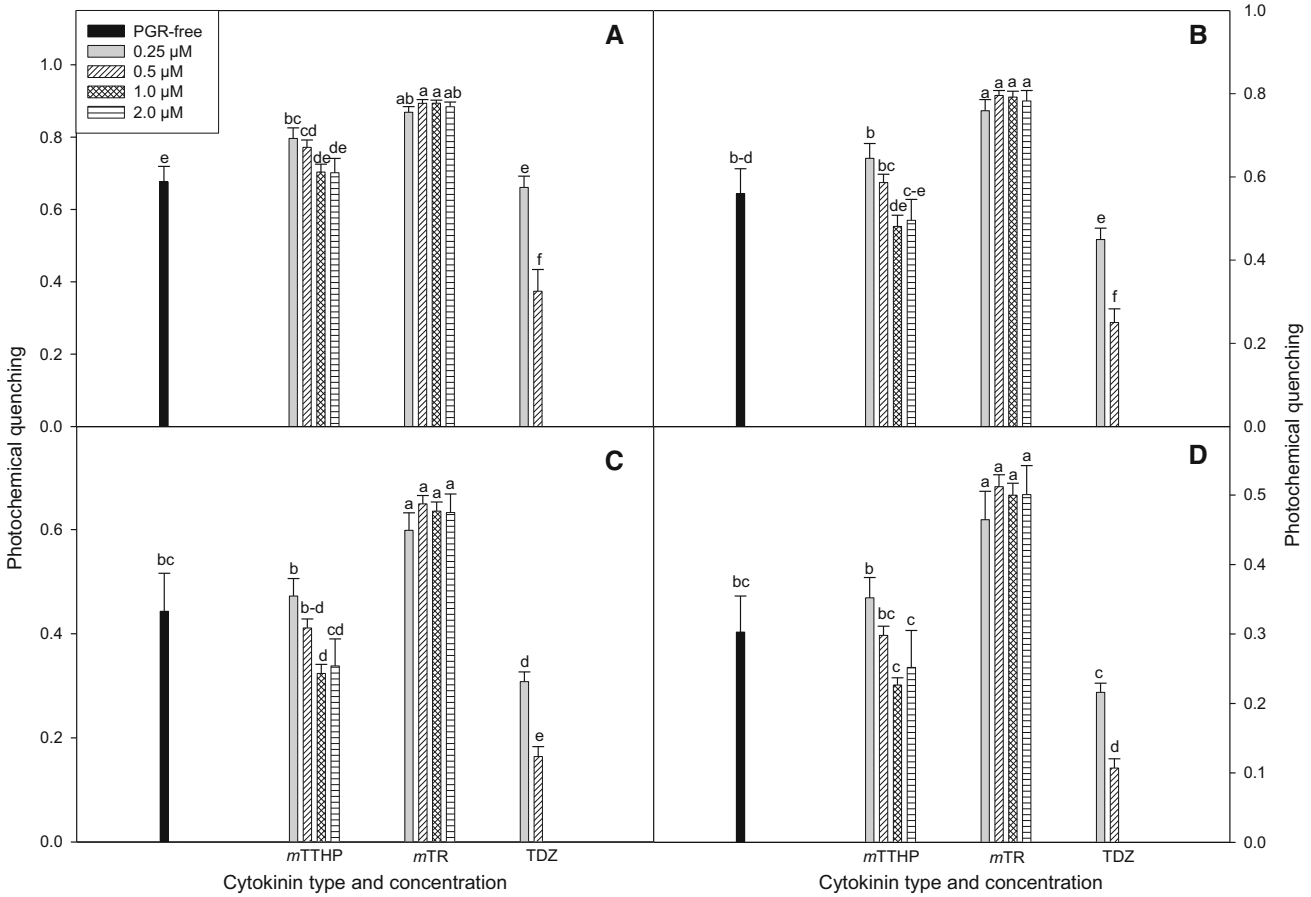

Fig. 3 Carry-over effect of cytokinins (CKs) applied during in vitro shoot proliferation of *M. plumbea* on photochemical quenching (*q*P), measured at **a** 264 µmol m^{-2} s^{-1} photosynthetic photon flux, **b** 488 µmol m^{-2} s^{-1} photosynthetic photon flux, **c** 800 µmol m^{-2} s^{-1} photosynthetic photon flux, and **d** 1,200 µmol m^{-2} s^{-1} photosynthetic photon flux in the leaves of different CK-regenerated plants after 6 months of ex vitro growth. *m*TR = *meta*-topolin riboside; *m*TTHP = *meta*-topolin tetrahydropyran-2-yl; TDZ = thidiazuron; PGR = plant growth regulator. *Bars with different letters* in each graph are significantly different ($P \leq 0.05$) based on Duncan's multiple range test. Data are mean ± SE ($n = 10$). Due to complete root inhibition observed during the in vitro stage, the carry-over effect of 1.0 and 2.0 µM TDZ treatments was not evaluated

compared to the TDZ-derived plants (Table 3). Aremu et al. (2012b) similarly reported the production of significantly longer roots with the application of *meta*-methoxytopolin 9-tetrahydropyran-2-yl (Me*m*TTHP, another THP-substituted CK) in micropropagated 'Williams' banana after 2 months of ex vitro growth.

Chlorophyll fluorescence

There was no significant difference in the potential quantum efficiency or the maximum photochemical efficiency (Fv/Fm) of dark-adapted PSII in the leaves of the different CK-regenerated plants. The values obtained ranged from 0.82 (in plants originally treated with 0.5 µM TDZ) to 0.84 (in PGR-free and *m*TTHP-treated plants). This is an indication that the leaves were healthy with an efficient PSII light energy conversion. It also indicates that the CK treatments did not cause any non-reversible photoinhibitory damage to the reaction centres at PSII (Björkman and Demmig 1987; Martins et al. 2013). The actual PSII quantum efficiency (Φ_{PSII}) was, however, affected significantly by CK type and concentration at low, medium and high PPF (Fig. 1). In particular, the Φ_{PSII} consistently decreased with an increase in concentration of *m*TTHP or TDZ while 0.5 µM TDZ treatment gave the significantly lowest value. The influence of CK treatment as observed on Φ_{PSII} was also noticeable on the ETR at different PPF (Fig. 2) since the measurements were done each time under constant PPF.

Photochemical quenching (*q*P) which indicates the proportion of open PSII reaction centres can provide information about the process responsible for changes in Φ_{PSII} (Maxwell and Johnson 2000). As stated by the authors, an increase in the proportion of closed reaction centres leads to a decrease in photochemical efficiency. In the current study, the recorded *q*P seems to 'correlate' directly to the effect of applied CKs on Φ_{PSII}. As with Φ_{PSII},

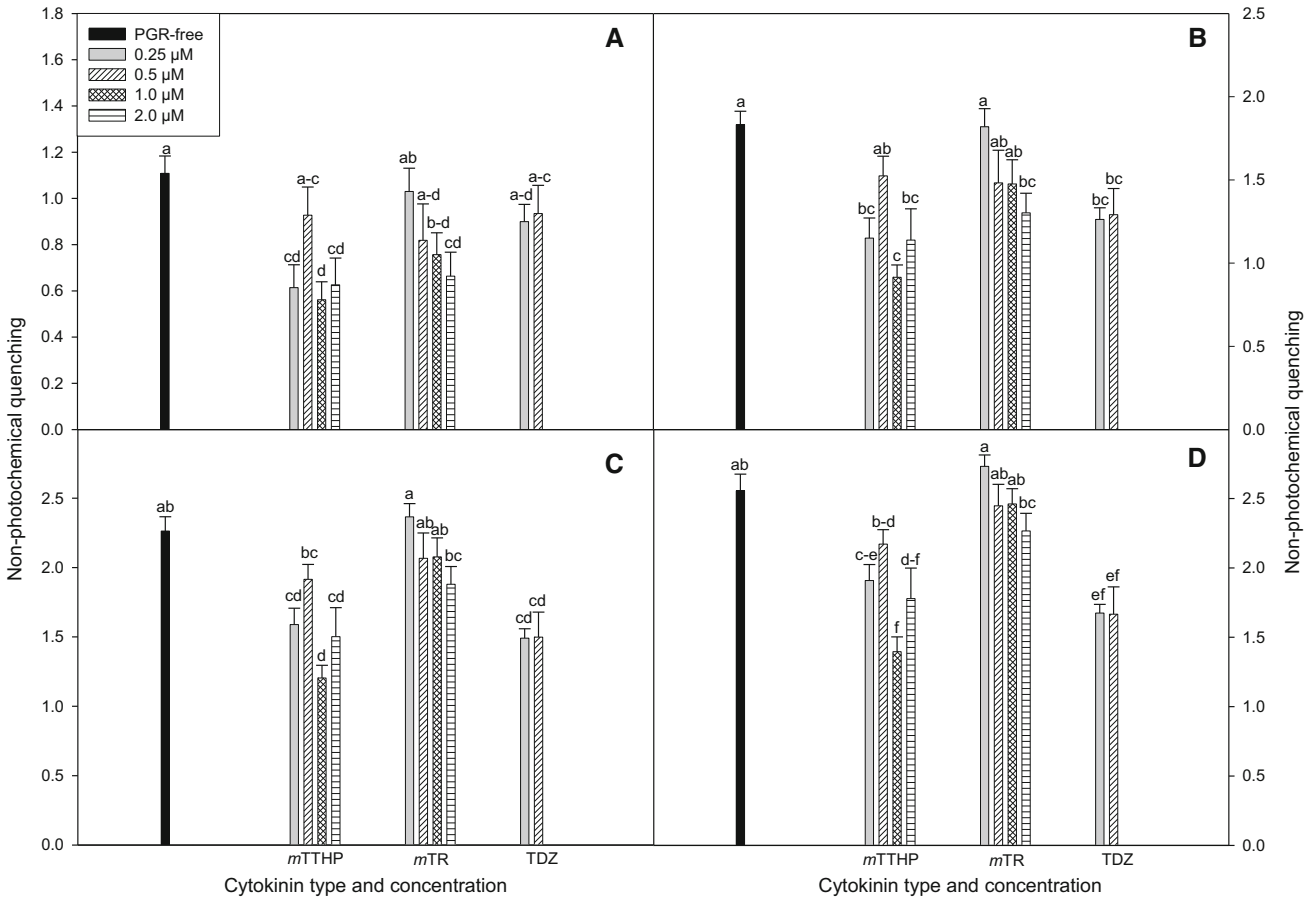

Fig. 4 Carry-over effect of cytokinins (CKs) applied during in vitro shoot proliferation of *M. plumbea* on non-photochemical quenching (NPQ), measured at **a** 264 μmol m^{-2} s^{-1} photosynthetic photon flux, **b** 488 μmol m^{-2} s^{-1} photosynthetic photon flux, **c** 800 μmol m^{-2} s^{-1} photosynthetic photon flux, and **d** 1,200 μmol m^{-2} s^{-1} photosynthetic photon flux in the leaves of different CK-regenerated plants after 6 months of ex vitro growth. *m*TR = *meta*-topolin riboside; *m*TTHP = *meta*-topolin tetrahydropyran-2-yl; TDZ = thidiazuron; PGR = plant growth regulator. *Bars with different letters* in each graph are significantly different ($P \leq 0.05$) based on Duncan's multiple range test. Data are mean ± SE ($n = 10$). Due to complete root inhibition observed during the in vitro stage, the carry-over effect of 1.0 and 2.0 μM TDZ treatments was not evaluated

there was a decrease in *q*P with an increase in concentration of *m*TTHP or TDZ at all PPF (Fig. 3). Plants regenerated with 0.5 μM TDZ had the lowest significant *q*P (with values close to zero at all PPF), indicating that the majority of their PSII reaction centres are closed and that the primary quinone electron acceptors of PSII are in a reduced state (Kalaji et al. 2014). Thus, the influence of applied CK on Φ_{PSII} appeared to be largely due to its direct or indirect regulatory role on PSII reaction centres.

According to Chernyad'ev (2009), CKs are known to selectively influence the expression of certain genes which induce the synthesis of proteins associated with the electron-transport chain, photosynthetic chlorophyll-protein complexes and ribulose bisphosphate carboxylase/oxygenase. In the present study, it was observed that as PPF increased, *q*P and consequently Φ_{PSII} decreased irrespective of the CK types and concentration (Figs. 1, 3). Prokopová et al. (2010) similarly observed that an increase in PPF enhanced the impairment of PSII function caused by exogenous application of CKs in *Lactuca sativa*. On the other hand, NPQ increased as PPF increased (Fig. 4). NPQ is an energy dissipation mechanism in PSII that protects plants against photo-oxidative damage by reducing the production of very reactive oxygen species in the PSII antenna (Müller et al. 2001). At the highest PPF (comparable to the greenhouse conditions) though, plants regenerated with 0.5 μM TDZ had a significantly low NPQ compared with plants regenerated with 0.5 μM *m*TTHP, *m*TR or without any CK (Fig. 4d). The low Φ_{PSII}, NPQ and *q*P values recorded in TDZ-regenerated plants (especially the 0.5 μM treatment) at high PPF would suggest that both photochemical and non-photochemical energy conversion pathways are insufficient to protect these plants against excess light, making the plants more vulnerable to oxidative stress (Osório et al.

Table 4 Effect of cytokinins applied during in vitro shoot proliferation of *M. plumbea* on antioxidant enzyme activities after 6 months of ex vitro growth

Cytokinin treatment (μM)	Superoxide dismutase (SOD) activity (μmol/min/g FW)		Catalase (CAT) activity (μmol/min/g FW)		Peroxidase (POD) activity (mmol/min/g FW)	
	Bulb	Leaf	Bulb	Leaf	Bulb	Leaf
0.25 *m*TR	56.8 ± 3.30 e	53.4 ± 0.66 g	0.7 ± 0.00 f	0.2 ± 0.00 g	1.7 ± 0.02 ij	1.0 ± 0.08 hi
0.5 *m*TR	117.7 ± 1.09 d	55.7 ± 2.1 g	0.7 ± 0.00 f	0.3 ± 0.01 fg	2.2 ± 0.13 hi	1.3 ± 0.05 h
1.0 *m*TR	121.4 ± 1.18 c	60.1 ± 0.69 g	0.7 ± 0.00 f	0.4 ± 0.01 f	2.8 ± 0.04 gh	1.9 ± 0.09 g
2.0 *m*TR	130.3 ± 0.21 b	68.1 ± 3.09 f	0.7 ± 0.01 f	0.5 ± 0.03 e	3.1 ± 0.03 fg	3.3 ± 0.02 f
0.25 *m*TTHP	131.7 ± 0.11 b	79.5 ± 2.47 e	0.8 ± 0.02 e	0.6 ± 0.01 d	3.7 ± 0.24 f	4.2 ± 0.05 e
0.5 *m*TTHP	132.5 ± 0.31 b	87.6 ± 1.29 d	1.0 ± 0.01 d	0.7 ± 0.00 d	6.2 ± 0.18 e	7.4 ± 0.03 d
1.0 *m*TTHP	133.6 ± 0.22 b	98.9 ± 3.98 c	1.1 ± 0.03 c	0.9 ± 0.02 c	7.1 ± 0.26 d	8.5 ± 0.02 c
2.0 *m*TTHP	138.3 ± 0.34 a	104.9 ± 0.46 bc	1.2 ± 0.03 bc	1.0 ± 0.06 b	8.1 ± 0.03 c	8.4 ± 0.26 c
0.25 TDZ	140.2 ± 0.38 a	110.5 ± 1.50 b	1.2 ± 0.02 b	1.1 ± 0.05 ab	11.7 ± 0.24 b	9.8 ± 0.04 b
0.5 TDZ	141.6 ± 0.28 a	122.9 ± 3.69 a	1.7 ± 0.00 a	1.1 ± 0.01 a	18.8 ± 0.76 a	11.5 ± 0.34 a
Control	44.9 ± 0.07 f	46.9 ± 0.15 h	0.3 ± 0.00 g	0.1 ± 0.00 h	1.0 ± 0.0 3 j	0.9 ± 0.03 i

Data are mean ± SE ($n = 3$)

Due to complete root inhibition observed during the in vitro stage, the carry-over effect of 1.0 and 2.0 μM TDZ treatments was not evaluated

*m*TR = *meta*-topolin riboside; *m*TTHP = *meta*-topolin tetrahydropyran-2-yl; TDZ = thidiazuron

Mean values in the same column followed by different letters indicate significant differences ($P \leq 0.05$) based on Duncan's multiple range test

2013). In other words, plants derived from media containing *m*TTHP or *m*TR had a high capacity for energy dissipation in comparison to TDZ-regenerated plants. Dobránszki and Mendler-Drienyovszki (2014) recently indicated that CK application affected the performance of photosynthetic apparatus in 3-week-old apple cultures. However, to the best of our knowledge, the current study is the first available report on the post-flask (ex vitro) effect of CK application on the functionality of photosynthetic apparatus (particularly the PSII system) in micropropagated plants.

Antioxidant enzyme activities and malondialdehyde production

Another photo-protective mechanism used by plants to dissipate excitation energy in the electron transport chain is through the Mehler reaction (Gong et al. 2013; Makino et al. 2002; Müller et al. 2001; Veljović-Jovanović 1998). As stated by the authors, this often involves the photo-reduction of oxygen at photosystem I (PSI) to superoxide anion radicals ($O_2^{\cdot-}$) which, by disproportionation, become converted to hydrogen peroxide (H_2O_2) and oxygen through the action of SOD enzyme. The resulting H_2O_2 is then detoxified to water and oxygen by the activities of CAT or POD enzyme (Asada 2000). Table 4 shows the effect of CK application during in vitro shoot proliferation on antioxidant enzyme activities after 6 months of ex vitro plant growth. A significantly high antioxidant enzyme activity was recorded in CK-derived plants when compared to the control (plants regenerated without PGR). In most cases, the activities of the antioxidant enzymes increased with increased CK concentrations. An increased antioxidant enzyme activity with increased CK content was also reported in transgenic *Pssu-ipt* tobacco plants (Synková et al. 2006). Díaz-Vivancos et al. (2011) similarly observed the stimulatory effect of CKs on SOD and POD activities in *Crocus sativus*, a perennial bulbous plant.

Furthermore, the highest antioxidant enzyme activity was recorded with SOD, followed by the POD enzyme. This enhancement of SOD activity, which is the first line of defence against reactive oxygen species in the detoxification process (Alscher et al. 2002), particularly indicates a high production of superoxide anion radicals possibly due to oxygen photo-reduction by electron leakage at PSI in the leaves. Synková et al. (2006) specifically reported an increase in the activities of POD, ascorbate peroxidase, glutathione reductase and monodehydroascorbate reductase enzymes with the application of TDZ compared to other CKs in *C. sativus*. Similarly, in the present study, there was a significant increase in the activities of CAT and POD enzymes concomitantly with SOD in TDZ-regenerated plants when compared to other CK treatments. This suggests oxidative stress in TDZ-derived plants. This stress is most likely due to the production and/or accumulation of reactive oxygen species in an attempt by the plants to control excess excitation energy. Excess excitation energy (an environmental or abiotic stress) can increase the generation of reactive oxygen species during electron transport processes such as photosynthesis (Neill et al. 2002). The over-production of reactive oxygen species (greater than

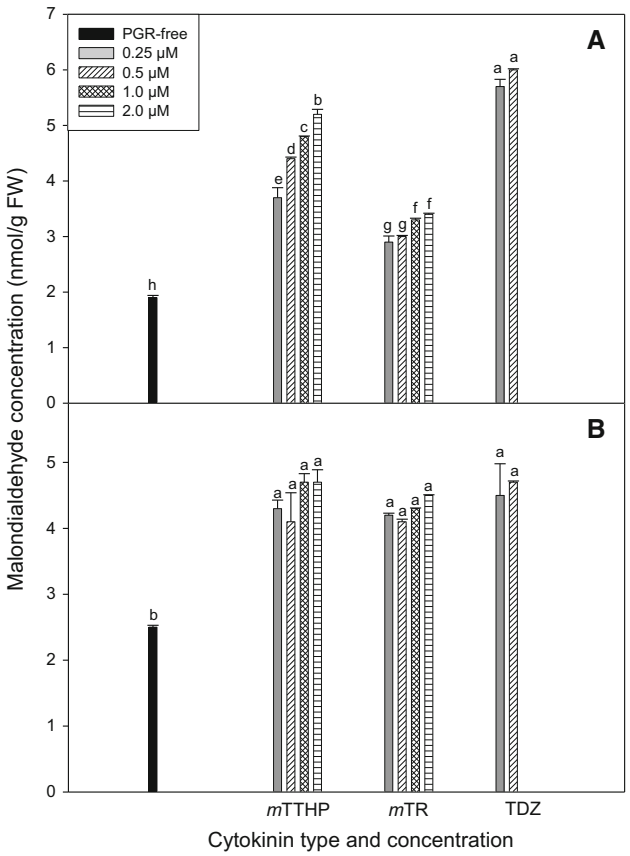

Fig. 5 Carry-over effect of cytokinins (CKs) applied during in vitro shoot proliferation of *M. plumbea* on malondialdehyde (MDA) production in the **a** leaves and **b** bulbs of different CK-regenerated plants after 6 months of ex vitro growth. *m*TR = *meta*-topolin riboside; *m*TTHP = *meta*-topolin tetrahydropyran-2-yl; TDZ = thidiazuron; PGR = plant growth regulator. *Bars with different letters* in each graph are significantly different ($P \leq 0.05$) based on Duncan's multiple range test. Data are mean ± SE ($n = 3$). Due to complete root inhibition observed during the in vitro stage, the carry-over effect of 1.0 and 2.0 μM TDZ treatments was not evaluated

their metabolism) in turn, can result in an oxidative stress (Baťková et al. 2008).

A commonly used biomarker of oxidative stress in plants is the production or accumulation of MDA, an end-product of membrane lipid peroxidation due to oxidative damage (Davey et al. 2005; Dewir et al. 2006). Figure 5 presents the effect of CKs applied during in vitro shoot proliferation on MDA production in the leaves and bulbs of different CK-regenerated plants after 6 months of ex vitro growth. MDA concentration significantly increased in both the leaf and bulb of CK-derived plants when compared to the control (PGR-free regenerated plants). In *m*TTHP- and *m*TR-regenerated plants, the leaf MDA level increased significantly with an increase in CK concentration. Although there was no significant difference in leaf MDA level between the two TDZ concentrations evaluated, the highest leaf MDA content was recorded in plants regenerated from TDZ-containing medium.

The significant increase in MDA level in the leaves of TDZ-regenerated plants despite increased antioxidant enzyme activities indicates a high production of reactive oxygen species beyond the scavenging efficiency of the antioxidant enzymes, leading to oxidative stress. Although reactive oxygen species can serve as signalling molecules in plants, an overproduction can be highly destructive to lipids, proteins and nucleic acids (Gaspar et al. 2002). Lipid peroxidation due to oxygen radicals can lead to an increased membrane permeability and subsequent low NPQ (Kalaji et al. 2014). Stressful conditions can in turn, affect carbohydrate storage, translocation and metabolism (Mishra and Dubey 2008) which may lead to a reduced biomass accumulation (Gong et al. 2013). Thus, the decreased leaf and bulb biomass recorded with TDZ-derived plants (especially at 0.5 μM concentration) might be associated with low photochemical efficiency coupled with a relatively high oxidative stress in the leaves of these plants when compared to other CK treatments.

Conclusions

The present study indicates the potential of this novel CK (*m*TTHP) as a viable alternative in PTC. Its ability to improve shoot production without compromising rooting capacity as well as its positive carry-over effect on ex vitro growth in comparison to other CKs used was demonstrated in this medicinal plant species. The possible mechanism underlying ex vitro growth heterogeneity as a result of CKs applied during in vitro stage was elucidated. Unlike in *m*TTHP- and *m*TR-derived plants, photosynthetic machinery such as photochemical efficiency, actual PSII quantum efficiency and the protective capacity by NPQ were suppressed in TDZ-derived plants. This possibly led to a relatively high oxidative stress (evident in high leaf MDA concentration in spite significantly increased antioxidant enzyme activities) and subsequent biomass reduction in TDZ-derived plants.

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