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ORIGINAL PAPER

How does exogenously applied cytokinin type affect growth and endogenous cytokinins in micropropagated *Merwilla plumbea*?

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Abstract *Merwilla plumbea* (Lindl.) Speta is a popular and highly sought after South African medicinal plant with diverse therapeutic values. Using Ultra performance liquid chromatography (UPLC), the effect of five cytokinins (CKs) [either isoprenoid = N^6-isopentenyladenine (iP) or aromatic = benzyladenine, *meta*-topolin (*m*T), *meta*-topolin riboside (*m*TR), and 6-(3-methoxybenzylamino)-9-tetrahydropyran-2-ylpurine] Me*m*TTHP on growth and level of endogenous CKs during micropropagation and acclimatization stages was evaluated. Aromatic CK (*m*T/*m*TR) elicited the highest shoot proliferation (7–8 shoots per explant) during in vitro culture. Following acclimatization, iP-treated and the control plants were healthier with longer leaves, roots and higher fresh weight when compared to aromatic CKs. A total of 37 (22 isoprenoid and 15 aromatic) CK variants were quantified in both in vitro and acclimatized plants. Based on their metabolic function, these were grouped into five types including free bases, ribosides, ribotides, *O*- and 9-glucosides. In addition to enhancing our understanding of the hormone physiology in *M. plumbea*, the current findings are discussed in line with the effect of the exogenously applied CK on the observed differences in growth before and after the important stage of acclimatization. The observed dynamics in endogenous CK provide an avenue to manipulate in vitro growth and development of investigated species.

Keywords Conservation · Medicinal plant · Micropropagation · Phytohormones · Plant growth regulators

Abbreviations

ANOVA	Analysis of variance
BA	N^6-Benzyladenine
BA9G	N^6-Benzyladenine-9-glucoside
BAR	N^6-Benzyladenine riboside
BAR5′MP	N^6-Benzyladenosine-5′-monophosphate
CK	Cytokinin
*c*Z	*cis*-Zeatin
*c*Z9G	*cis*-Zeatin-9-glucoside
*c*Z*O*G	*cis*-Zeatin-*O*-glucoside
*c*ZR	*cis*-Zeatin riboside
*c*ZR5′MP	*cis*-Zeatin riboside-5′-monophosphate
*c*ZR*O*G	*cis*-Zeatin-*O*-glucoside riboside
DHZ	Dihydrozeatin
DHZ9G	Dihydrozeatin-9-glucoside
DHZ*O*G	Dihydrozeatin-*O*-glucoside
DHZR	Dihydrozeatin riboside
DHZR5′MP	Dihydrozeatin riboside-5′-monophosphate
DHZR*O*G	Dihydrozeatin-*O*-glucoside riboside

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iP	N^6-Isopentenyladenine
iP9G	N^6-Isopentenyladenine-9-glucoside
iPR	N^6-Isopentenyladenosine
iPR5′MP	N^6-Isopentenyladenosine-5′-monophosphate
KIN	Kinetin
KIN9G	Kinetin-9-glucoside
KINR	Kinetin riboside
Me*m*T	*meta*-Methoxy topolin
Me*m*TTHP	6-(3-Methoxybenzylamino)-9-tetrahydropyran-2-ylpurine
MS	Murashige and Skoog medium
*m*T	*meta*-Topolin
*m*T9G	*meta*-Topolin-9-glucoside
*m*TR	*meta*-Topolin riboside
*o*T	*ortho*-Topolin
*o*T*O*G	*ortho*-Topolin-*O*-glucoside
*o*TR	*ortho*-Topolin riboside
PGR	Plant growth regulator
PPF	Photosynthetic photon flux density
*p*T	*para*-Topolin
*p*T*O*G	*para*-Topolin-*O*-glucoside
*t*Z	*trans*-Zeatin
*t*Z9G	*trans*-Zeatin-9-glucoside
*t*Z*O*G	*trans*-Zeatin-*O*-glucoside
*t*ZR	*trans*-Zeatin riboside
*t*ZR5′MP	*trans*-Zeatin riboside-5′-monophosphate
*t*ZR*O*G	*trans*-Zeatin-*O*-glucoside riboside
UPLC	Ultra performance liquid chromatography

Introduction

Plant growth regulators (PGRs) are ubiquitous in plants and regulate various physiological and developmental processes. Despite their presence at low concentrations, they are essential for vital events during all phases of a plant's life cycle (Gaba 2005). During micropropagation, growth of plants in an artificial environment is stimulated by externally applied PGRs. One of the most important groups of PGRs are the cytokinins (CKs) which play a crucial role in regeneration and proliferation of shoots (Krikorian 1995). As such, CKs are often manipulated in micropropagation protocols to enhance shoot production (Aremu et al. 2012a). The interaction between the exogenous and endogenous PGRs influences the intrinsic natural process of CK biosynthesis/accumulation. Thus, CK levels in plant cells depend on their rate of de novo biosynthesis and/or uptake from extracellular sources, metabolic inter-conversions, inactivation and degradation (Kamínek et al. 1997; Sakakibara 2006). At any particular time, CK homeostasis is an essential factor that determines the physiological activities including the growth and development of plants (Cuesta et al. 2012; Klemš et al. 2011; Montalbán et al. 2013).

In micropropagation, the primary objective remains the production of a maximum number of genetically identical shoots which can be easily rooted, acclimatized and successfully established in the field (Aremu et al. 2012a). Most plant species exhibit inherent variation which is evident in their diverse responses to different CKs. As a result, it becomes pertinent to optimize micropropagation protocols for individual species (Krikorian 1995). However, most micropropagation protocols lack the basic metabolic and physiological explanations for the resultant growth. It is important that the type and concentration of CKs 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 CKs as well as minimum levels of inactive toxic metabolites (Bairu et al. 2011; Malá et al. 2009). Information on the metabolic pathway of CKs and its effect on plant physiology are valuable as it allows for a better understanding of the growth requirements of plant species in vitro. The relative abundance of different CKs varies among plant species, genotypes, tissues and developmental stages (Bairu et al. 2011; Cuesta et al. 2012; Frébort et al. 2011; Montalbán et al. 2013). Better understanding of the CK-induced physiological processes related to growth would allow for more controlled growth manipulation. This is important in medicinal plant research whereby regulation of specific metabolic pathways can influence the overall physiological and biochemical status of the regenerants.

With a conservation status of 'near threatened', *Merwilla plumbea* (Lindl.) Speta (previously known as *Scilla natalensis*) is a popular and highly sought-after South African medicinal plant with numerous therapeutic uses. The pharmacological potential have been substantiated (Amoo et al. 2012) and some important phytochemicals have been detected (Aremu et al. 2013). Despite the numerous conservation approaches via the development of micropropagation protocols for *M. plumbea* (Baskaran et al. 2012; McCartan and Van Staden 1998), there is little (if any) information on the hormone physiology of the species to date. The dynamics of endogenous CK levels mediated via the exogenously applied CK type may be a useful tool to understand and manipulate in vitro growth. Topolins as a new class of CKs are known to produce less toxic metabolites and are gaining increasing popularity in micropropagation. The current study evaluated the effect of different (isoprenoid and aromatic) CKs on growth and endogenous CK profiles of *M. plumbea* during micropropagation and greenhouse acclimatization. The use of a single (low) concentration was based on previous finding (Baskaran et al. 2012) and application of five different CK

types was expected to provide overall possible outcome when the different CK types are tested on this species.

Materials and methods

Chemicals

Benlate® (antifungal compound) and bacteriological agar (gelling agent) were purchased from Du Pont de Nemours Int., South Africa and Oxoid Ltd., Basingstoke, Hampshire, England, respectively. Sigma-Aldrich (Steinheim, Germany) supplied the benzyladenine (BA) and N^6 isopentenyladenine (iP) while the topolins [*meta*-Topolin (*m*T), 6-(3-Methoxybenzylamino)-9-tetrahydropyran-2-ylpurine (Me*m*TTHP)] were prepared as published previously (Doležal et al. 2006, 2007; Szücová et al. 2009). The 23 deuterium-labelled CK internal standards were obtained from Olchemim Ltd (Olomouc, Czech Republic). All chemicals used in the study were of analytical grade.

Culture initiation, medium composition and tissue culture conditions

Merwilla plumbea seeds were obtained from the University of KwaZulu-Natal Botanical Garden (Pietermaritzburg, South Africa). Details of the decontamination and culture initiation have been outlined earlier (Aremu et al. 2013; Baskaran et al. 2012). Leaf explants (1 × 1 cm^2) were obtained from plantlets maintained on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 30 g l^{-1} sucrose, 0.1 g l^{-1} myo-inositol without any PGR. The regenerants were regularly subcultured at 3 months interval and served as source materials for the current study. Apart from the control medium which had no CK, 1 µM of five different CKs (iP, BA, *m*T, *m*TR, Me*m*TTHP) were tested for the current experiment. A single (low) concentration of CKs were applied based on initial trials and previous findings (Baskaran et al. 2012). Cultures were incubated in a growth room at 25 ± 2 °C with 16 h light/8 h dark conditions and photosynthetic photon flux density (PPFD) of 40–45 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes (Osram® L58 W/640, Germany). The experiment was repeated twice with 25 explants per treatment/control each time. After 12 weeks culture, plantlets from each treatment including the control was divided into two groups; the first group was harvested and separated into aerial (leaves) and underground (bulbs and roots) sections for CK analysis while the second group was acclimatized ex vitro.

Acclimatization conditions

For the acclimatization experiment, 15–20 rooted tissue culture regenerants (control and CK-treatments) with average leaf (≈20 mm) and root (≈10 mm) size were selected due to the better tendency to properly establish. These plantlets were washed and potted in 15 × 25 cm rectangle-shaped plastic trays containing sand:soil:vermiculite (1:1:1, v/v/v) treated with 1 % benlate. The plants were maintained for 1 month in a mist house with day/night temperatures of 30/12 °C, relative humidity between 80 and 90 %, and a 10 s misting at 15 min interval. The plants 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 3 months, the greenhouse acclimatized plants were harvested.

Preparation of plant materials

Both tissue culture and acclimatized (pooled from at least 10 plants) plant materials were quickly cut into aerial and underground parts, freeze-dried and lyophilized to minimize any metabolic process that may degrade the CKs. Subsequently, three samples (0.1 g FW) were extracted in 15 mL Bieleski buffer (60 % methanol, 25 % $CHCl_3$, 10 % HCOOH and 5 % H_2O). Deuterium-labeled CK internal standards (1 pmol) were added to samples to check recoveries during purification and to validate the results. The resultant supernatants were cleaned using an SCX cationic exchange column (Varian Inc., CA, USA). Thereafter, two purified CK fractions (ribotides and other bases as well as ribosides and glycosides) were obtained using coupled Sephadex (Sigma-Aldrich, St. Louis, MA, USA) and reverse-phase (C18) columns (Waters, Milford, MA, USA). The obtained eluates were evaporated to dryness.

Endogenous cytokinin quantification

For the qualitative and quantitative phytohormone analysis, the eluates from the two CK fractions were analyzed using the method of Svacinova et al. (2012). This was achieved by using an Acquity ultra-performance liquid chromatograph (UPLC®) equipped with a reverse-phase BEH C18 column (1.7 µm, 2.1 mm × 150 mm) (Milford, MA, USA), linked to a Xevo™ TQ MS triple quadrupole mass spectrometer with an electrospray interface (Waters MS Technologies, Manchester, UK). The column was held at 40 °C and the mobile phase used for chromatography consisted of a 30-min linear gradient of 10–50 % methanol with 15 mM ammonium formate (pH 4). The compounds,

which had retention times ranging from 11.0 to 24.5 min, were monitored as they eluted from the instrument using an ultraviolet-diode array detector (scanning range 210–400 nm, resolution 1.2 nm, sampling rate 10 spectra s^{-1}) and the tandem mass spectrometer. Analytes were quantified by multiple reaction monitoring of their $[M + H]^+$ and appropriate product ions, using optimized cone voltages and collision energies for diagnosis of each CK (Novák et al. 2008). Quantification was performed with Masslynx software using a standard isotope dilution method. Subsequently, the ratio of endogenous CK to appropriate labelled standard was determined and used to quantify the level of endogenous CKs in the original plant extract, based on the known concentration of internal standard added (Novák et al. 2003).

Data analysis

Growth data were subjected to one-way analysis of variance (ANOVA) using SPSS for Windows (SPSS®, version 10.0 Chicago, USA). Where there was statistical significance ($p \leq 0.05$), the mean values were further separated using Duncan's multiple range test. Results of the CK analysis are presented as mean ± SD pmol g^{-1} FW.

Results and discussion

Effect of exogenously applied cytokinins (CKs) on growth of micropropagated *Merwilla plumbea*

The importance of supplementing media with CK(s) during micropropagation is well established. One of the reasons is that the absence of roots which are one of the main sites of CKs synthesis makes it necessary to add exogenous CK to the culture media (Arigita et al. 2005). Despite the production of multiple shoots in PGR-free medium, the presence of PGRs including CKs has been documented to increase the shoot proliferation in *M. plumbea* (Baskaran et al. 2012; McCartan and Van Staden 1998). In the current study, all the explants in both the control and CK treatments responded positively (Fig. 1). Based on the number of regenerated shoots, *m*TR (aromatic) was the best CK as it generated approximately two-fold more shoots when compared to the control (Table 1). At the tested concentration, all the aromatic CKs stimulated production of more shoots compared to the isoprenoid treatments. Despite the differences in structures and metabolic products in isoprenoid and aromatic CKs, evidence of overlap in biological activity to a certain extent still exists (Strnad 1997). However, aromatic CKs wield a greater influence on developmental processes such as morphogenesis and senescence whereas the isoprenoid CKs mainly impact on cell division and continuation of the cell cycle (Holub et al. 1998). While all the CK-treated and control plants had similar numbers of leaves, the length of the regenerated leaf in the controls was significantly ($p \leq 0.05$) larger when compared to the CK treatments. Fresh weight was lowest in iP and highest in *m*TR regenerants.

Effect of exogenously applied cytokinins (CKs) on growth of acclimatized *Merwilla plumbea*

At the final stage of micropropagation, proper acclimatization and successful establishment of in vitro derived plants in the field remain crucial. A number of factors including the type of CK applied during micropropagation influence acclimatization of in vitro regenerants (Aremu et al. 2012a; Baroja-Fernández et al. 2002). For instance, some CKs have been reported to inhibit rooting which results in the failure of the acclimatized plants to establish or survive (Aremu et al. 2012a). The acclimatized plants (control and CK treatments) had no new/additional shoot in the current study (data not shown). Overall, the iP-derived plants were generally better compared to the aromatic CK-derived ones (Table 2). The evaluated morphological traits such as number of leaves and roots as well as fresh weights were higher in iP and control plants. These aforementioned aspects especially the root morphology, plays a very crucial role during acclimatization (Aremu et al. 2012a; Hazarika 2006). Thus, the ability of isoprenoid CKs to enhance these parameters and subsequent survival of regenerated *M. plumbea* evidently justifies their use during micropropagation. Caution must be exercised to prevent any physiological disturbance in the optimum required endogenous level of isoprenoids in micropropagated plants.

Among the tested aromatic CKs, there was higher survival with *m*TR- and Me*m*TTHP-derived plants (Table 2). There was also evidence of better morphological development (based on plant appearance and vigour) in the *m*TR- and Me*m*TTHP-treated plants (Fig. 2). In terms of acclimatization competence, the superiority of topolin (for e.g. *m*T) over BA have been demonstrated in several micropropagated species such as *Uniola paniculata* (Valero-Aracama et al. 2010) and 'Williams' bananas (Aremu et al. 2012b). In the current study however, *M. plumbea* plants with BA and *m*T treatment showed comparably poor acclimatization competence compared to other aromatic CKs. Thus, the often reported advantages of *m*T are clearly related to the plant species investigated. In fact, the likelihood of the same species of different genotypes subjected to similar type and concentration of CKs exhibiting divergent response is relatively high (Valero-Aracama et al. 2010). In addition, proper acclimatization of micropropagated plants is also influenced by several abiotic and biotic factors which either singly or in various combination

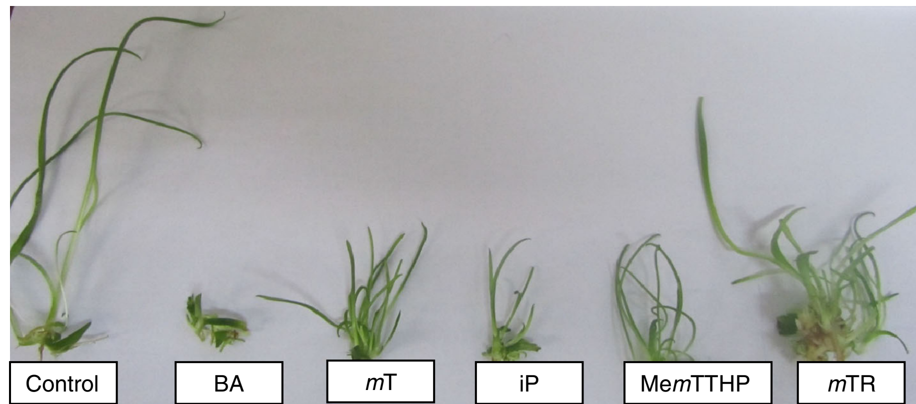

Fig. 1 In vitro tissue cultured *Merwilla plumbea* plantlets derived from different cytokinins at 1 µM. iP = Isopentenyladenine; BA = Benzyladenine; *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Mem*T*THP = 6-(3-Methoxybenzylamino)-9-tetrahydropyran-2-ylpurine

Table 1 Effect of different exogenously applied cytokinins on the growth of micropropagated *Merwilla plumbea*

Treatment	Shoot number (#)	Leaf number (#)	Leaf length (mm)	Root number (#)	Root length (mm)	Plant FW(mg)	Plant DW (mg)
Control	4.4 ± 0.44 c	17.7 ± 1.38 ab	61.5 ± 3.64 a	6.0 ± 0.83 b	10.7 ± 1.22 b	219.8 ± 33.40 a	18.96 ± 3.581 abc
iP	4.0 ± 0.54 c	13.6 ± 1.37 b	20.0 ± 1.25 d	9.7 ± 0.62 a	16.7 ± 1.22 a	91.2 ± 12.70 b	12.35 ± 2.184 bcd
BA	5.5 ± 0.67 c	15.9 ± 1.42 ab	18.6 ± 1.17 d	6.0 ± 0.61 b	7.4 ± 0.72 b	120.7 ± 17.50 b	11.31 ± 1.592 cd
*m*T	7.7 ± 0.78 ab	19.6 ± 2.41 a	29.2 ± 2.65 c	7.3 ± 0.75 b	16.7 ± 1.71 a	228.3 ± 53.40 a	20.69 ± 4.487 ab
*m*TR	8.0 ± 0.73 a	20.4 ± 1.34 a	37.0 ± 2.41 b	7.4 ± 0.85 b	15.5 ± 1.3 a	262.8 ± 42.80 a	21.35 ± 3.167 a
Mem*T*THP	5.9 ± 0.65 bc	14.7 ± 1.37 b	23.4 ± 1.79 cd	2.9 ± 0.66 c	9.0 ± 1.42 b	94.8 ± 11.90 b	8.58 ± 0.977 d

Mean value (±SE and n = 25) in same column with different letter(s) indicate significant differences ($p \leq 0.05$) based on Duncan's Multiple Range Test. iP = Isopentenyladenine; BA = Benzyladenine; *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Mem*T*THP = 6-(3-Methoxybenzylamino)-9-tetrahydropyran-2-ylpurine. FW = fresh weight, DW = dry weight

Table 2 Effect of different exogenously applied cytokinins during micropropagation on the growth of acclimatized *Merwilla plumbea*

Treatment	Leaf number (#)	Leaf length (cm)	Root number (#)	Root length (cm)	Fresh weight (g)	Survival rate (%)
Control	4.2 ± 0.21 ab	8.8 ± 0.40 a	3.9 ± 0.14 b	9.4 ± 0.38 a	1.27 ± 0.105 a	95
iP	4.5 ± 0.21 a	8.4 ± 0.56 a	4.4 ± 0.15 a	9.1 ± 0.49 ab	1.00 ± 0.089 b	95
BA	3.6 ± 0.20 c	4.9 ± 0.25 c	2.9 ± 0.15 d	8.0 ± 0.56 bc	0.37 ± 0.032 d	70
*m*T	3.4 ± 0.17 c	4.0 ± 0.27 c	2.5 ± 0.11 d	4.1 ± 0.36 d	0.18 ± 0.030 d	80
*m*TR	4.3 ± 0.16 ab	6.9 ± 0.47 b	4.1 ± 0.17 ab	8.5 ± 0.36 abc	0.78 ± 0.101 c	90
Mem*T*THP	3.7 ± 0.23 bc	5.1 ± 0.33 c	3.3 ± 0.13 c	7.3 ± 0.50 c	0.39 ± 0.079 d	90

Mean value [±SE and n = 15 (BA) or 20 (for other cytokinins and control)] in same column with different letter(s) indicate significant differences ($p \leq 0.05$) based on Duncan's Multiple Range Test. iP = Isopentenyladenine; BA = Benzyladenine; *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Mem*T*THP = 6-(3-Methoxybenzylamino)-9-tetrahydropyran-2-ylpurine

with CK are of paramount importance to their survival (Hazarika 2006).

Effect of exogenously applied cytokinins (CKs) on total quantified endogenous CK content

Explants metabolize exogenously applied CKs (mainly free bases) which are then expressed as products of substitution on purine moiety (ribosides, nucleotides, *N*-glucosides), side chain substitution (*O*-glucosides) or cleavage (adenine, adenosine, adenosine-5′-monophosphate) in the tissues of the regenerants (Letham and Palni 1983; Van Staden and Crouch 1996). These metabolic products act as storage, transport or biologically inert forms of CKs which are responsible for the physiological and developmental plasticity observed in plants (Kamínek et al. 1997; Letham and Palni 1983). The quality and quantity of these aforementioned metabolites depend on several factors including stages of development (Arnau et al. 1999; Stirk et al. 2008), physiological condition of the plant (Ivanova et al. 2006; Lexa et al. 2003; Sáenz et al. 2010), organ type

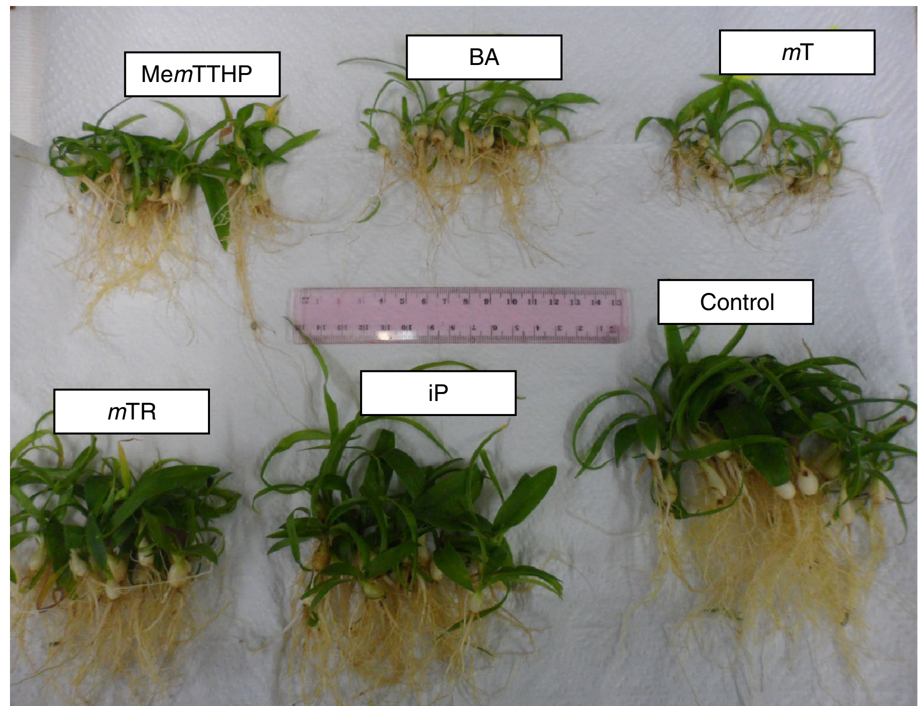

Fig. 2 *Merwilla plumbea* plants derived from different cytokinins after 3 month greenhouse acclimatization. iP = Isopentenyladenine; BA = Benzyladenine; *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*TTHP = 6-(3-Methoxybenzylamino)-9-tetrahydropyran-2-ylpurine

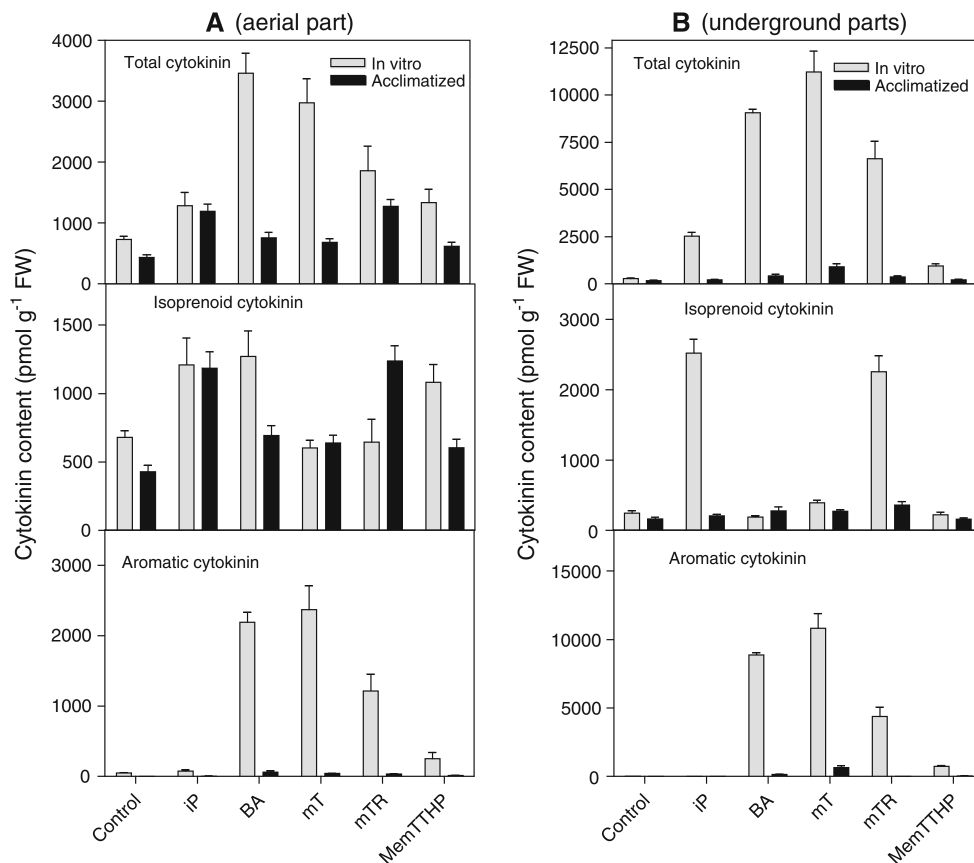

Fig. 3 Effect of different exogenous cytokinins on the endogenous cytokinin (pmol g^{-1} FW) pool in **a** aerial and **b** underground parts of *Merwilla plumbea*. iP = Isopentenyladenine; BA = Benzyladenine; *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*TTHP = 6-(3-Methoxybenzylamino)-9-tetrahydropyran-2-ylpurine

Table 3 Endogenous concentrations (pmol g^{-1} FW) of different types of isoprenoid cytokinins (CKs) in *Merwilla plumbea*

Isoprenoid CK quantified	Applied CK	Tissue cultured			Acclimatized		
		Aerial	Underground	Total	Aerial	Underground	Total
trans-Zeatin	Control	54.7 ± 19.40	2.1 ± 0.08	56.8 ± 19.48	41.7 ± 9.19	25.4 ± 2.33	67.2 ± 11.52
	iP	42.2 ± 3.67	1060.3 ± 56.46	1102.5 ± 60.13	0.9 ± 0.48	6.1 ± 1.18	6.9 ± 1.66
	BA	0.0 ± 0.00	1.4 ± 0.01	1.4 ± 0.01	32.7 ± 6.62	26.2 ± 9.86	58.9 ± 16.49
	*m*T	33.0 ± 10.79	214.1 ± 27.75	247.0 ± 38.55	29.5 ± 5.35	127.8 ± 4.21	157.3 ± 9.56
	*m*TR	52.6 ± 5.96	667.4 ± 49.68	719.9 ± 55.64	40.8 ± 9.37	16.9 ± 4.92	57.7 ± 14.29
	Me*m* TTHP	2.1 ± 0.07	26.1 ± 9.58	28.2 ± 9.65	37.5 ± 7.10	7.0 ± 1.80	44.6 ± 8.90
cis-Zeatin	Control	353.5 ± 12.43	183.6 ± 28.19	537.1 ± 40.62	112.4 ± 8.53	83.6 ± 19.88	196.0 ± 28.41
	iP	553.4 ± 127.23	150.9 ± 5.98	704.2 ± 133.20	500.7 ± 60.85	125.9 ± 12.58	626.6 ± 73.43
	BA	848.6 ± 139.99	98.4 ± 13.60	947.0 ± 153.58	117.3 ± 12.99	142.6 ± 29.22	260.0 ± 42.21
	*m*T	275.7 ± 10.41	126.4 ± 4.18	402.1 ± 14.59	163.9 ± 14.84	86.3 ± 12.89	250.1 ± 27.73
	*m*TR	259.8 ± 95.48	117.6 ± 19.60	377.3 ± 115.08	325.4 ± 35.46	250.6 ± 36.24	576.1 ± 71.69
	Me*m*TTHP	417.7 ± 54.84	136.9 ± 13.50	554.6 ± 68.33	161.5 ± 20.98	138.4 ± 12.12	299.9 ± 33.11
Dihydrozeatin	Control	0.0 ± 0.00	0.3 ± 0.03	0.3 ± 0.03	0.7 ± 0.21	3.3 ± 0.59	4.0 ± 0.80
	iP	29.0 ± 2.95	68.5 ± 3.76	97.6 ± 6.71	19.7 ± 4.64	2.1 ± 0.22	21.8 ± 4.86
	BA	0.3 ± 0.09	0.0 ± 0.00	0.3 ± 0.09	0.3 ± 0.04	29.0 ± 10.79	29.3 ± 10.83
	*m*T	1.8 ± 0.36	0.9 ± 0.06	2.7 ± 0.42	0.0 ± 0.00	2.8 ± 0.46	2.8 ± 0.46
	*m*TR	0.6 ± 0.24	202.9 ± 15.15	203.5 ± 15.39	10.9 ± 2.17	17.7 ± 2.69	28.6 ± 4.86
	Me*m*TTHP	0.6 ± 0.13	0.0 ± 0.00	0.6 ± 0.13	0.4 ± 0.14	0.8 ± 0.05	1.2 ± 0.19
Isopentenyladenine	Control	271.9 ± 16.33	59.1 ± 7.86	331.0 ± 24.19	275.1 ± 28.76	53.0 ± 2.21	328.1 ± 30.97
	iP	585.1 ± 62.46	1238.8 ± 131.75	1823.9 ± 194.20	665.0 ± 51.76	73.7 ± 6.42	738.7 ± 58.17
	BA	422.6 ± 46.06	91.6 ± 6.06	514.2 ± 52.13	543.6 ± 52.68	79.4 ± 7.90	622.9 ± 60.58
	*m*T	291.6 ± 35.96	50.7 ± 7.35	342.3 ± 43.31	446.3 ± 37.15	56.0 ± 1.57	502.3 ± 38.71
	*m*TR	332.9 ± 64.72	1268.4 ± 138.53	1601.3 ± 203.25	860.7 ± 63.96	75.7 ± 4.79	936.4 ± 68.75
	Me*m*TTHP	661.3 ± 75.64	60.4 ± 14.74	721.7 ± 90.38	404.5 ± 35.01	15.6 ± 2.21	420.1 ± 37.22

Value are presented as mean ± SD, n = 3. iP = Isopentenyladenine; BA = Benzyladenine; *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*TTHP = 6-(3-Methoxybenzylamino)-9-tetrahydropyran-2-ylpurine

(Aremu et al. 2014), type and concentration of applied PGRs (Malá et al. 2009).

In the current study, the total quantified endogenous CK ranged from 1 to 14 nmol g^{-1} FW (control and *m*T) in in vitro regenerants and 0.6–1.6 nmol g^{-1} FW (control and *m*TR) in acclimatized plants (supplementary data). Although *m*TR (aerial parts) and BA (underground parts) treatments had more isoprenoid-type CK after acclimatization, there was generally a drastic reduction in CKs in the acclimatized plants when compared to the corresponding in vitro regenerants. In terms of distribution between aerial and underground regions, the bulk of the quantified aromatic endogenous CKs were detected in the underground parts of the in vitro cultures and acclimatized plants in majority of the treatments (Fig. 3a, b). As such, the underground organs are possibly the main site of CK biosynthesis in *M. plumbea*. These observations are in agreement with the widely-held assumption as previously documented (Chen et al. 1985; Letham and Palni 1983; Stirk and Van Staden 2010). However, the presence of high level of CKs does not necessary prove that a tissue is a site of biosynthesis as CKs may have been translocated/transported from other plant parts (Stirk and Van Staden 2010). In addition, recent evidence on spatial distribution of CK metabolism have indicated their production within the aerial parts of plants (Kudo et al. 2010).

Levels of endogenous cytokinins (CKs) based on side chain configuration of parent compounds

A total of 37 CKs identified in both in vitro and acclimatized plants were classified as either isoprenoid (Table 3) or aromatic (Table 4) CKs depending on the side chain structure. The 15 isoprenoid CKs were divided into four types including *t*Z (*t*Z, *t*ZOG, *t*ZR, *t*ZROG, *t*Z9G, *t*ZR5′MP), *c*Z (*c*Z, *c*ZOG, *c*ZR, *c*ZROG, *c*Z9G, *c*ZR5′MP), DHZ (DHZ, DHZOG, DHZR, DHZROG, DHZ9G, DHZR5′MP) and iP (iP, iPR, iP9G, iPR5′MP). As indicated in Table 3, iP was the most dominant in both the control and CK-treated acclimatized plants. Similarly, iP

Table 4 Endogenous concentrations (pmol g^{-1} FW) of different types of aromatic cytokinins (CKs) in *Merwilla plumbea*

Aromatic CK quantified	Applied CK	Tissue cultured			Acclimatized		
		Aerial	Underground	Total	Aerial	Underground	Total
Benzyladenine	Control	0.0 ± 0.00	5.6 ± 0.30	5.6 ± 0.30	0.0 ± 0.00	0.5 ± 0.12	0.5 ± 0.12
	iP	68.8 ± 20.93	1.9 ± 0.33	70.7 ± 21.26	0.6 ± 0.38	0.8 ± 0.10	1.4 ± 0.49
	BA	2086.8 ± 115.66	8708.9 ± 132.81	10795.7 ± 248.46	0.0 ± 0.00	0.8 ± 0.13	0.8 ± 0.13
	*m*T	97.1 ± 24.09	35.4 ± 2.83	132.5 ± 26.92	13.6 ± 0.33	0.3 ± 0.12	13.9 ± 0.45
	*m*TR	0.0 ± 0.00	27.4 ± 3.32	27.4 ± 3.32	18.3 ± 0.43	4.8 ± 0.87	23.1 ± 1.29
	Me*m* TTHP	0.0 ± 0.00	8.7 ± 0.52	8.7 ± 0.52	0.0 ± 0.00	0.2 ± 0.07	0.2 ± 0.07
meta-Topolin	Control	4.3 ± 0.38	8.1 ± 0.63	12.4 ± 1.01	1.0 ± 0.50	1.1 ± 0.09	2.1 ± 0.58
	iP	4.1 ± 0.38	0.7 ± 0.31	4.8 ± 0.69	2.2 ± 1.24	1.0 ± 0.50	3.1 ± 1.74
	BA	14.9 ± 1.53	28.1 ± 1.56	43.0 ± 3.09	59.8 ± 18.94	138.2 ± 28.33	197.9 ± 47.27
	*m*T	2269.4 ± 314.08	10784.7 ± 1066.34	13054.1 ± 1380.43	25.4 ± 2.59	635.6 ± 133.68	661.0 ± 136.28
	*m*TR	1203.7 ± 236.39	4346.1 ± 689.29	5549.8 ± 925.67	5.9 ± 0.93	0.8 ± 0.42	6.8 ± 1.36
	Me*m*TTHP	251.5 ± 87.49	719.5 ± 64.51	971.0 ± 152.01	3.2 ± 0.46	39.7 ± 12.03	42.9 ± 12.48
ortho-Topolin	Control	0.0 ± 0.00	10.9 ± 1.43	10.9 ± 1.43	0.0 ± 0.00	2.5 ± 0.39	2.5 ± 0.39
	iP	0.0 ± 0.00	6.2 ± 2.00	6.2 ± 2.00	0.0 ± 0.00	1.7 ± 0.50	1.7 ± 0.50
	BA	14.7 ± 5.41	49.4 ± 5.22	64.1 ± 10.63	0.0 ± 0.00	2.2 ± 0.82	2.2 ± 0.82
	*m*T	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	3.4 ± 1.30	3.4 ± 1.30
	*m*TR	5.7 ± 0.67	0.0 ± 0.00	5.7 ± 0.67	9.0 ± 0.68	0.0 ± 0.00	9.0 ± 0.68
	Me*m*TTHP	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	7.9 ± 0.45	3.0 ± 2.35	11.0 ± 2.79
para-Topolin	Control	0.0 ± 0.00	1.4 ± 0.35	1.4 ± 0.35	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
	iP	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	1.6 ± 0.43	0.0 ± 0.00	1.6 ± 0.43
	BA	70.7 ± 20.65	89.9 ± 27.44	160.6 ± 48.09	0.0 ± 0.00	0.2 ± 0.11	0.2 ± 0.11
	*m*T	0.0 ± 0.00	3.3 ± 1.45	3.3 ± 1.45	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
	*m*TR	2.1 ± 0.70	1.7 ± 1.59	3.8 ± 2.29	0.0 ± 0.00	3.6 ± 1.20	3.6 ± 1.20
	Me*m*TTHP	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	1.5 ± 0.18	1.5 ± 0.18
Kinetin	Control	43.7 ± 5.38	0.0 ± 0.00	43.7 ± 5.38	0.7 ± 0.12	0.3 ± 0.13	0.9 ± 0.25
	iP	0.5 ± 0.09	0.6 ± 0.10	1.1 ± 0.19	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
	BA	0.8 ± 0.05	1.0 ± 0.07	1.8 ± 0.12	1.2 ± 0.04	2.6 ± 0.46	3.9 ± 0.51
	*m*T	2.3 ± 0.33	0.8 ± 0.24	3.1 ± 0.56	2.5 ± 0.72	0.4 ± 0.18	2.9 ± 0.91
	*m*TR	0.3 ± 0.23	0.0 ± 0.00	0.3 ± 0.23	0.0 ± 0.00	0.6 ± 0.27	0.6 ± 0.27
	Me*m*TTHP	1.5 ± 0.09	0.6 ± 0.17	2.1 ± 0.26	2.4 ± 1.12	0.0 ± 0.00	2.4 ± 1.12

Value are presented as mean ± SD, n = 3. iP = Isopentenyladenine; *m*TR = *meta*-Topolin riboside; Me*m*TTHP = 6-(3-Methoxy-benzylamino)-9-tetrahydropyran-2-ylpurin

was the most abundant isoprenoid CK-type in in vitro plants supplemented with iP, *m*TR and Me*m*TTHP. As postulated by Sakakibara (2006), iPR (which constituted approximately 60 % of total iP content) is a precursor of the other isoprenoid CKs via the de novo biosynthetic CK iPRMP-dependent pathway thereby accounting for their high abundance in the current study.

Generally, high quantities of *c*Z were detected in both tissue cultured and acclimatized *M. plumbea* (Table 3). Despite the absence of the precise role of *c*Z cytokinins (Gajdošová et al. 2011), there are increasing reports on their possible importance based on their frequent and high abundance in plants (Aremu et al. 2014; Stirk et al. 2008; Stirk and Van Staden 2010; Tarkowská et al. 2012). Based on evolutionary trends, iP- and *c*Z-type CKs are predominantly associated with lower plants while *t*Z- and DHZ-type CKs are more prevalent in angiosperms (Auer 1997; von Schwartzenberg et al. 2007). Conversely, we observed that with the exception of the iP treatment where *t*Z was the least common isoprenoid in the acclimatized plants, among the four quantified endogenous isoprenoid CK-types, the levels of DHZ were the lowest in both the in vitro and acclimatized plants in other treatments.

The 15 endogenous aromatic CKs were grouped into five different groups (Table 4). These included: BA (BA, BAR, BA9G, BAR5′MP), *m*T (*m*T, *m*TR, *m*T9G), *o*T (*o*T, *o*TOG, *o*TR), *p*T (*p*T, *p*TOG) and KIN (KIN, KINR, KIN9G). Even though the biosynthetic pathways for these aromatic CKs are not fully elucidated, their natural occurrence in plants have been unequivocally established (Strnad 1997). In the current study, BA-type was dominant in iP- and BA-treated cultures, *m*T-type was the major aromatic CK in *m*T, *m*TR and Me*m*TTHP treatments. In the absence of exogenous CK (control) in the medium, endogenous KIN was the major CK-type and mainly located in the aerial parts of the in vitro plantlets. Despite being initially discovered and isolated as a breakdown product from the DNA molecule, evidence of the natural occurrence of KIN have been documented in some plant species (Barciszewski et al. 2007). In several micropropagation protocols, KIN exhibited a relatively weaker CK ability when compared to BA or topolins (Aremu et al. 2012a). Nevertheless, the high abundance observed in the current study (controls) possibly demonstrate the (metabolic) importance of KIN, thus requires further stringent investigation. On the other hand, the high level of endogenous *m*T and BA as main CK-types in topolin and BA treatments respectively, has also been documented in other species (Aremu et al. 2014; Malá et al. 2009; Montalbán et al. 2013). These observations have been postulated to be due to the existence of a unidirectional enzymatic pathway in aromatic CKs which allows for accumulation of more aromatic CK metabolites in the presence of (excess) exogenous applied aromatic CK (Montalbán et al. 2013). After acclimatization, *m*T-type CK was generally the most abundant aromatic CKs in most treatments. In addition, *o*T remained the main aromatic CK in acclimatized plants derived from CK-free (control) media. These observations are further evidence on the potential importance of *o*T in plants.

Levels of endogenous cytokinins (CKs) based on function and physiological importance

Although the precise role and contribution of individual CKs and associated metabolic products in the various growth processes is not fully understood, circumstantial evidence on their physiological importance is available (Brzobohatý et al. 1994; Letham and Palni 1983). These products could be active forms of CK (free bases) that induces growth or physiological responses; translocation or storage (ribosides, ribotides and *O*-glucosides) forms which would release free (active) CKs when required; detoxification products formed due to the application of exogenous CK at toxic levels; deactivation products (9-glucosides) formed to lower endogenous (active CK) levels; and post-activation products. Thus, the levels of quantified endogenous CKs in *M. plumbea* were divided into free bases, ribosides, ribotides, *O*- and 9-glucosides (Fig. 4). In most cases, higher levels of these products were observed in aromatic treatments when compared to isoprenoids. Particularly, there was a remarkably high level of free bases in *m*T and BA treatments. To a certain extent, the concentration of *O*-glucosides especially in the underground part of micropropagated plants correlated with the shoot proliferation in *M. plumbea* (Fig. 4b). Apart from the fact that *O*-glucoside conjugates are resistant to CK oxidases, they are readily converted into the active free bases and ribosides by the highly substrate specific β-glucosidase when necessary (Brzobohatý et al. 1994; Spíchal et al. 2004; Werbrouck et al. 1996). Thus, they are considered as inactive but stable forms of excess CKs that play an important role in balancing CK levels (Sakakibara 2006). The presence of these form of metabolites via reverse sequestration are partly associated with various physiological and developmental processes in micropropagation (Aremu et al. 2012a; Bairu et al. 2011; Klemš et al. 2011).

As postulated by Klemš et al. (2011), it was generally difficult to fully elucidate how the shifts in levels of CKs and their metabolic forms contribute to shoot proliferation. The current study establishes that at equimolar concentration aromatic CKs result in the formation of more 9-glucosides compared to the isoprenoid types. Similarly, increased levels of exogenous aromatic CK (BA) were associated with elevated accumulation of 9-glucosides during the micropropagation of *Pinus radiate* (Montalbán

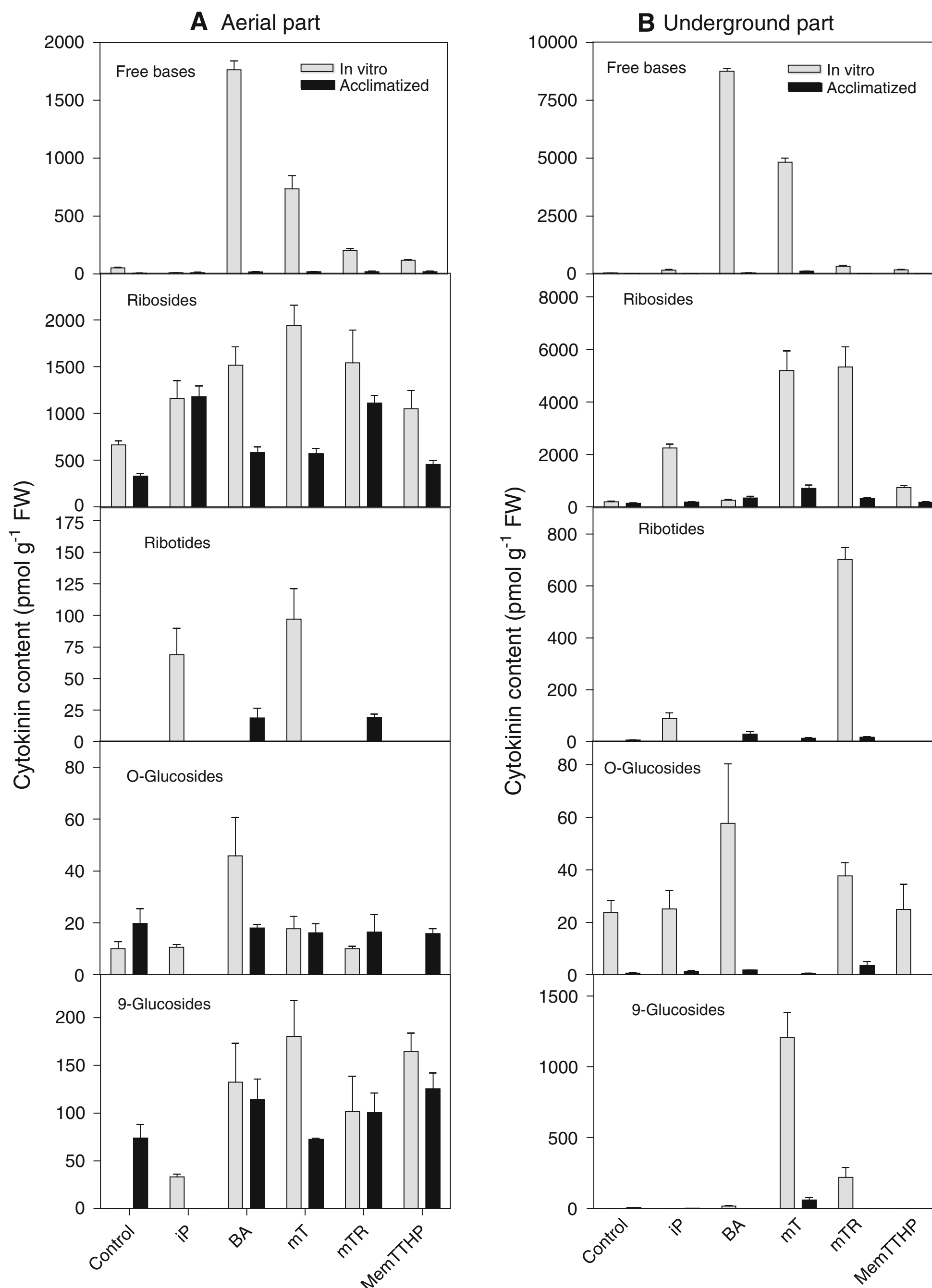

Fig. 4 Effect of different exogenous cytokinins on the level (pmol g^{-1} FW) of different structural and functional endogenous cytokinin forms in **a** aerial and **b** underground parts of *Merwilla plumbea*. iP = Isopentenyladenine; BA = Benzyladenine; *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Mem*T*THP = 6-(3-Methoxybenzylamino)-9-tetrahydropyran-2-ylpurine

et al. 2013). Klemš et al. (2011) also reported accumulation of 9-glucosides during micropropagation of *Nicotiana tabacum* in the presence of exogenous *t*Z and DHZ. Generally, it is believed that *N*-glucosylation provides a primary mechanism of detoxification in plants (Sakakibara 2006).

Conclusions

During micropropagation, the regulation of organogenesis is associated with an endogenous PGR balance in combination with the interaction of exogenously supplied ones. Despite the better shoot proliferation with the use of

aromatic (*m*TR) over isoprenoid (iP) CK during micropropagation, the number of roots was generally higher in isoprenoid-derived plants when compared to aromatic treatments in the acclimatized plants. The presence of more roots allows for proper establishment and stability thereby enhancing survival. For the first time, varying concentrations of endogenous CKs were quantified in both tissue culture and acclimatized plants in this species. We demonstrated the importance of *O*-glycosides as storage forms with potential regulatory functions in *M. plumbea*. Overall, the current findings are a useful addition to the increasing knowledge of plant hormone physiology during and after micropropagation. Nevertheless, further experiments focusing on the enzymes involved in CK metabolism and CK receptors as well as interactions with other PGRs especially auxins, will be valuable towards complete elucidation of the events occurring during growth and development in *M. plumbea*. In addition, the high abundance of KIN mainly located in the aerial parts of the in vitro plantlets warrants further study.

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