Plant Cell Tiss Organ Cult (2007) 90:15–23
DOI 10.1007/s11240-007-9233-4

ORIGINAL PAPER

Optimizing the micropropagation protocol for the endangered *Aloe polyphylla*: can *meta*-topolin and its derivatives serve as replacement for benzyladenine and zeatin?

Michael W. Bairu · Wendy A. Stirk · Karel Dolezal · Johannes Van Staden

Received: 8 June 2006 / Accepted: 18 April 2007 / Published online: 4 July 2007

Abstract Benzyladenine (BA) is the most widely used cytokinin in the micropropagation industry due to its effectiveness and affordability. It, however, has disadvantages such as genetic alteration and abnormal growth in some plants. Naturally occurring zeatin on the other hand is not as widely used as BA and is far more expensive. The use of *meta*-topolin and its derivatives as alternatives to BA and zeatin, both of which frequently have negative effects in tissue culture was investigated. In vitro grown *Aloe polyphylla* (an endangered medicinal and ornamental aloe) were cultured on full strength Murashige and Skoog basal medium with different concentrations of cytokinins and solidified with 1% Bacteriological Agar (Oxoid No. 1). *m*T was the preferred cytokinin both in terms of multiplication rate and rooting. The optimum concentration that induced regeneration and rooting is 5.0 μM. The problem of hyperhydricity was totally controlled. Plants rooted spontaneously in multiplication medium, thus avoiding the extra rooting step of the protocol. More than 91% of the plants transferred to ex vitro conditions were successfully acclimatized.

Keywords Aromatic cytokinins · Conservation · Hyperhydricity · Metabolites · Multiplication rate · Plant growth regulators · Root and shoot growth

Abbreviations

[9G]BA	[6-benzylamino-9-β-D-glucopyranosylpurine]
BA	[6-benzylaminopurine]
IBA	indole butyric acid
MS	Murashige and Skoog (1962) medium
Me*m*T	[6-(3-methoxybenzylamino)purine]
Me*m*TR	[6-(3-methoxybenzylamino)-9-β-D-ribofuranosylpurine]
*m*T	[6-(3-hydroxybenzylamino)purine]
*m*TR	[6-(3-hydroxybenzylamino)-9-β-D-ribofuranosylpurine]
*o*T	[6-(2-hydroxybenzylamino)purine]
PGR	plant growth regulators
*p*T	[6-(4-hydroxybenzylamino)purine]

Introduction

Aloe polyphylla (commonly called spiral aloe), a member of the Asphodelaceae, is a beautiful endangered plant native to the Maluti Mountains in Lesotho

M. W. Bairu · W. A. Stirk · J. Van Staden (✉)
Research Centre for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal Pietermaritzburg, Private Bag X01, Scottsville 3209, South Africa
e-mail: rcpgd@ukzn.ac.za

K. Dolezal
Laboratory of Growth Regulators, Palacky University and Institute of Experimental Botany AS CR, 783 71 Olomouc-Holice, Czech Republic

(southern Africa). It grows in basalt rock crevices on high altitude slopes (2230–2720 m) and is the only alpine member of the genus *Aloe*. It thrives well in loose rocks that facilitate good drainage (Aubrey 2002; Chukwujechwu et al. 2002). This plant is grown mainly for horticultural (ornamental) and medicinal purposes.

Populations of *Aloe polyphylla* in its wild habitat have diminished mainly due to threats to its specific habitat requirement, overgrazing (associated with change in water regime), unsustainable harvesting for horticultural and medicinal purposes and increasing rarity of its pollinator, the Malachite Sunbird. As a result, this plant is currently registered in the Red Data List of endangered species by SABONET (Southern African Biodiversity Network). Cooperative efforts between conservationists and nurseries to propagate the plant for commercial trade is having some success in reducing the number of spiral aloes collected from its wild habitat (Aubrey 2002). To ensure conservation of this endangered species a micropropagation protocol has been developed (Abrie and van Staden 2001; Chukwujechwu et al. 2002). Incidence of hyperhydricity, the need for an additional rooting step and the use of zeatin (a very expensive cytokinin) are limitations of this protocol.

The search for new plant growth regulatory molecules or compounds is continuing owing to the limitations of the existing phytohormones. This search along with a study of identity, effect and mechanism of action of hormones will greatly depend on in vitro techniques and their applications. In vitro culture of plants has been used as closely linked tools in identification and characterization of the role of plant growth regulators in plant growth regulation (Krikorian 1995). Since the discovery of cytokinins, most cytokinin research has been concentrated on members of the isoprenoid cytokinins represented by zeatin, isopentenyladenine, and related compounds (Strnad 1997). The aromatic cytokinin BA and its derivatives were considered synthetic until the discovery of cytokinins with an aromatic side chain. Horgan et al. (1975) isolated, for the first time, the aromatic cytokinin 6-(2-hydroxybenzylamino)-9-β-D-ribofuranosylpurine from poplar leaves. Jones et al. (1996) reported the occurrence of aromatic cytokinins, BA, *meta*-topolins (*m*T) and *ortho*-topolins (*o*T) in various tissues of oil palm (*Elaeis guineensis* Jacq.). Strnad et al. (1997) isolated N^6- (*meta*-hydroxybenzyl) adenine, a highly active aromatic cytokinin, from poplar leaves (*Populus* × *canadensis* Moench, cv. *robusta*) and proposed the trivial name '*meta*-topolin'. Recently, monomethoxy derivatives of 6-benzyladenine and 6-benzyladenosine were also isolated and identified from several different plant sources and their high cytokinin activity has been confirmed (Tarkowská et al. 2003).

Currently BA is the most widely used cytokinin in the micropropagation industry due to its effectiveness and affordability. It, however, has disadvantages in some crops. Werbrouck et al. (1995) reported that in micropropagated *Spathiphylum floribundum* [9G]BA accumulated at the base of the plant. A slow release of this BA derivative caused heterogeneity in growth and inhibition of rooting during acclimatization. BA is also reported to cause hyperhydricity in many species (Leshem and Sachs 1985; Leshem et al. 1988; Teramoto et al. 1993). Therefore, it is imperative to try and find an alternative to BA while maintaining reasonable multiplication rates and acceptable plant quality.

Werbrouck et al. (1996) compared the types and effects of the derivatives of BA and *m*T in tissue culture of *S. flouribundum.* They found that the main metabolite of BA, [9G]BA, was more stable but had a negative impact on rooting and acclimatization when compared with the main metabolite of *m*T, the O-glucoside, which was degraded easily during acclimatization. They also compared the post vitro effect of different concentrations of BA and *m*T on rooting after an acclimatization period of four weeks. Their results revealed that plants treated with *m*T produced a significantly higher number and greater length of roots than those treated with BA. Baroja-Fernandez et al. (2002) also reported that addition of the aromatic cytokinin *m*TR to the culture medium significantly improved survival in cultures of potato. Kamínek et al. (1987a) compared the activities of BA and *m*T in induction of growth of lateral buds in *Poinsettia* and gerbera daisy and found the later to be more active. These results indicate that the slight structural difference between BA and *m*T could have a profound impact on plants during micropropagation.

The objectives of this investigation were to optimize the micropropagation protocol for *Aloe polyphylla*, to assess the effect of topolins on hyperhydricity, and to evaluate *meta*-topolin and its derivatives as a potential replacement for BA and zeatin.

Materials and methods

Soybean callus bioassay

*m*T, *o*T, *p*T, *m*TR, Me*m*T, and Me*m*TR were produced by the Laboratory of Growth Regulators, Palacky University and Institute of Experimental Botany AS CR, Czech Republic. BA, kinetin and zeatin were purchased from SIGMA. The soybean callus bioassay developed by Miller (1965), as used by Van Staden and Drewes (1991), was employed to determine the biological activity of the different cytokinins. Four 25 ml Erlenmeyer flasks each containing 15 ml of media solidified with 1% agar (Oxoid No. 1) were used per cytokinin per concentration (5, 0.5, 0.05, 0.005 and 0.0005 μM). Three pieces of soybean callus were then transferred to each flask aseptically. Cultures were maintained for 28 days at 25 ± 2°C under a low light intensity of 0.5 μmol m^{-2} s^{-1}. Data were collected as mass of callus per flask and subjected to general analysis of variance (GenStat 7th edition). Activity was expressed as mean mass of callus per treatment per concentration with Least Significant Difference ($P = 0.05$). The experiment was repeated twice.

Source material and bulking of explants

In vitro grown cultures of *A. polyphylla*, initiated from seeds, were obtained from the Research Centre for Plant Growth and Development, University of KwaZulu–Natal Pietermaritzburg, South Africa. Bulking of stock material was done using full strength MS media (Murashige and Skoog 1962) supplemented with 30 gl^{-1} sucrose, 0.1 g l^{-1} myo-inositol, 0.5 mg l^{-1} IBA, 1.0 mg l^{-1} zeatin and solidified with 1% agar (Bacteriological agar-Oxoid Ltd., Basingstoke, Hampshire, England). Cultures were incubated in a growth room with continuous cool fluorescent tubes (Osram L75 W/20X) light intensity of 45 μmol m^{-2} s^{-1} and a temperature of 24 ± 1°C for 9 weeks (Chukwujechwu et al. 2002).

Shoot multiplication

After bulking sufficient explants, experiments to investigate the effect of *meta*-topolins on shoot multiplication were designed. The *meta*-topolin derivatives *m*T, Me*m*T and Me*m*TR along with BA and zeatin were investigated. The same type of growth media used for bulking was used for the multiplication experiments. Concentrations of 0.0, 0.5, 2.5, 5.0, 7.5 and 15.0 μM were tested with five replicates. Five shoot tip explants with 2–4 young leaves attached were cultured in screw cap jars (300 ml) containing 50 ml of media. A total of 25 explants per treatment were used. Cultures were then incubated under the same growth conditions mentioned above. After nine weeks growth parameters including total number of shoots, multiplication rate, multiplication rate for shoots >1.5 cm, number of hyperhydric shoots, number of normal shoots, number of shoots >1.5 cm, number of shoots <1.5 cm, fresh weight, rooting ability (scored as 0 = absence, 1 = moderate rooting and 2 = excessive rooting) and leaf growth (scored as 1 = reduced leaf growth, 2 = average leaf growth and 3 = excessive leaf growth) were measured. The experiment was repeated three times.

Acclimatization

On the basis of results of the multiplication experiments, only those treatments that gave acceptable multiplication rates and the optimum concentration that induced better morphogenesis were considered for the acclimatization experiments. Plants treated with 2.5 μM and 5.0 μM of *m*T and zeatin and 2.5 μM BA were considered. BA totally failed to produce healthy shoots but was only considered for comparison purposes as it is the most widely used cytokinin in tissue culture. Shoot clusters were carefully separated from each other in such a way that all had at least one functional root. Individual plants were then washed thoroughly and extra large roots cut back. Plants were planted in potting mixture with a 1:1:1 ratio of soil, sand and vermiculite, treated with fungicide (benlate, 0.01%) and transferred to a mist house with 90% relative humidity. After four weeks in the mist house, plants were transferred to a greenhouse in the same potting mix (Chukwujechwu et al. 2002). After two months of growth in the greenhouse, fresh weight of shoots and roots was analyzed.

Results and discussion

Soybean callus bioassay

The results for the soybean callus bioassay are presented in Table 1. The effect of position

Table 1 Relative biological activity of different cytokinins as indicated by the mean callus yield per flask

Treatments	Mean mass of callus (g) per treatment				
	5.0 μM	0.5 μM	0.05 μM	0.005 μM	0.0005 μM
Control	0.016 ± 0.001	0.017 ± 0.002	0.012 ± 0.0003	0.013 ± 0.001	0.013 ± 0.003
BA	0.718 ± 0.06	**2.016** ± 0.14	1.501 ± 0.25	0.492 ± 0.21	0.041 ± 0.006
*m*T	**1.818** ± 0.33	1.735 ± 0.08	0.761 ± 0.23	0.299 ± 0.1	0.185 ± 0.11
Me*m*T	**1.314** ± 0.17	0.488 ± 0.105	0.448 ± 0.07	0.215 ± 0.13	0.380 ± 0.13
Me*m*TR	0.807 ± 0.23	**1.269** ± 0.24	0.49 ± 0.16	0.739 ± 0.56	0.052 ± 0.02
Zeatin	**1.156** ± 0.06	0.928 ± 0.31	0.953 ± 0.20	0.084 ± 0.032	0.090 ± 0.06
Kinetin	**1.887** ± 0.18	1.316 ± 0.16	0.686 ± 0.21	0.220 ± 0.06	0.188 ± 0.09
*m*TR	**1.101** ± 0.26	0.749 ± 0.32	0.472 ± 0.21	0.062 ± 0.017	0.037 ± 0.008
*o*T	**1.525** ± 0.22	1.296 ± 0.23	0.583 ± 0.43	0.047 ± 0.013	0.039 ± 0.008
*p*T	**0.153** ± 0.06	0.019 ± 0.003	0.051 ± 0.008	0.028 ± 0.004	0.034 ± 0.008
LSD	0.541	0.543	0.563	ns	0.159
F-Probability	<0.001	<0.001	<0.001	0.139	0.002

The statistical test was done at 5% probability level

ns – Indicates that there is no significant difference between the treatment means

Please note that results of this table are for additional information on the activity of the CKs as there is a need to test a higher concentration to find an optimum curve

specificity in cytokinin activity has long been understood. Significant effects on the activity of cytokinins has been observed by altering the position of the hydroxyl group on the side chain (Kamínek et al. 1979; Iwamura et al. 1980). Kamínek et al. (1979) demonstrated that the hydroxylation of the *trans* methyl group in the N^6 side chain of N^6-(Δ^2-isopentenyl) adenosine increased the biological activity and this activity either decreased or was not significantly affected when the *cis* methyl group was hydroxylated. The soybean callus bioassay undertaken in this study showed that hydroxylation of BA at the *ortho* and *para* positions (as in *o*T and *p*T) decreased activity, however, activity increased when the hydroxylation was on the *meta* position (as in *m*T) compared to their free base (BA) (Table 1). This result is similar to the report by Kamínek et al. (1987b). They tested cytokinin activity of BA and its derivatives hydroxylated on the side chain phenyl ring at the *ortho*, *meta* and *para* positions in four bioassays (excluding the soybean callus assay) and found that

Table 2 Statistical table showing the effect of treatments on overall shoot and root growth

Treatments (μM concentration)	Shoot growth		Root growth	
	Pearson chi-square value	Probability level	Pearson chi-square value	Probability level
Control	–	–	–	–
0.5	31.50	<0.001	17.53	0.063[ns]
2.5	35.56	<0.001	24.00	0.008
5.0	30.77	<0.001	34.29	<0.001
7.5	19.44	0.035	40.80	<0.001
15.0	28.26	0.002	43.20	<0.001

Observations for the control had no variation among replicates and hence the statistical test was not applied

[ns] indicates non-significant treatment effect

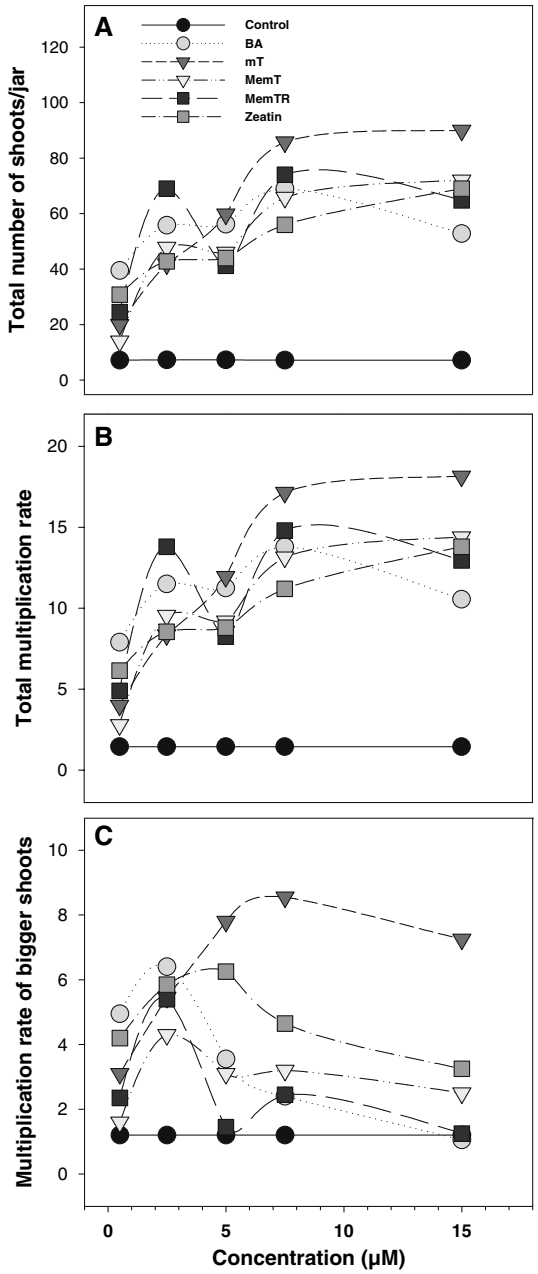

Fig. 1 The effect of the type and concentration of cytokinin on shoot multiplication (**A**), total multiplication rate (**B**) and multiplication rate of shoots greater than 1.5 cm in length (**C**). Note that *meta* topolin gave more shoots per jar and a larger number of shoots big enough for acclimatization. The 5 μM concentration was selected as an optimum concentration due to the good balance between shoot and root growth

activity was decreased in the *ortho* and *para* positions but increased in the *meta* position compared to BA.

Effect of meta-topolin on shoot multiplication

The choice of cytokinin to use in tissue culture is determined by its cumulative efficiency in inducing an acceptable rate of shoot multiplication, normal shoots and roots and the eventual ability of plants to acclimatize easily. The shoot and root growth responses and variations observed and analyzed in this study are due to treatment effects as indicated in Table 2. Means of total number of shoots showed significant differences among the different cytokinins and concentrations tested. At lower concentrations, BA produced more shoots. As the concentration increased larger numbers of shoots were recorded with the *meta* topolin treatments (Fig. 1A–C). Both total multiplication rate (Fig. 1B) and multiplication rate of shoots greater than 1.5 cm in length (Fig. 1C) were recorded and analyzed separately. Results of the analysis showed that treatment means were significantly higher than the control for most cytokinin levels tested. Multiplication rates increased with an increase in concentration upto 15.0 μM, where a decline in multiplication rate and excessive abnormal growth were observed. A better multiplication rate, spontaneous rooting and healthy explants were found at 5.0 μM level for the *m*T treatment (the quality not found in any of the other treatments), hence selected as optimum cytokinin level. At this level multiplication rate of eight shoots (shoots greater than 1.5 cm in length) per explant was found with the *m*T treatment. This rate is significantly higher than for the rest of the treatments and better than previous reports of a total of seven shoots per explant (Chukwujechwu et al. 2002).

A few reports on the use of topolins indicate that this group of cytokinins could be a new source of cytokinins with high morphogenetic activity. Kamínek et al. (1987a) found that *m*T was nearly twice as effective as BA in the induction of shoot growth of cuttings. Kubalakova and Strnad (1992) compared the effects of aromatic and isoprenoid (zeatin) cytokinins on micropropagation and organogenesis of sugar beet culture and found higher activity (greater number of shoots per explant) with *m*T. They also observed abnormal growth in BA-treated plants during subculturing, but not with *m*T. The use of *m*TR for improving survival of potato cultures has been reported (Baroja-Fernandez et al. 2002). We have also noticed that *m*TR has a comparable effect

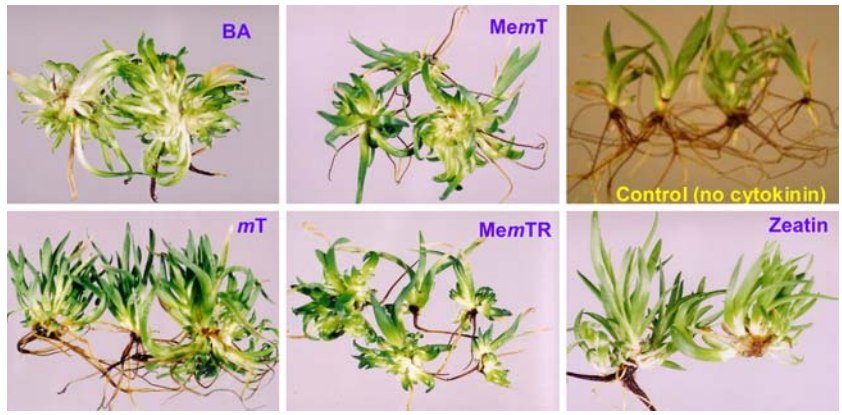

Fig. 2 Effect of the selected optimum concentration (5 μM) on shoot and root growth and incidence of abnormality of *A. polyphylla*. Note the-*m*T treated plants with healthy shoot growth and numerous roots as opposed to the abnormal growth (BA) and failed rooting (zeatin)

Table 3 Effect of different types and concentrations of cytokinins on percentage of hyperhydric shoots (HS) and total number of shoots (TNS)

Treatments	Cytokinin concentration (μM)									
	0.5		2.5		5.0		7.5		15.0	
	TNS	%HS	TNS	%HS	TNS	%HS	TNS	%HS	TNS	%HS
Control	7.3 ± 1.31	0	7.3 ± 1.31	0	7.3 ± 1.31	0	7.3 ± 1.31	0	7.3 ± 1.31	0
BA	39.5 ± 2.83	2.53	55.8 ± 4.06	36.74	56.2 ± 4.6	21.8	69.0 ± 13.8	80	52.8 ± 7.87	85.23
*m*T	20.0 ± 4.34	0	41.8 ± 2.15	0	59.8 ± 2.43	0	85.8 ± 7.92	25.64	90.8 ± 10.4	58.6
Me*m*T	14.0 ± 5.8	5.35	47.8 ± 4.11	0	46.0 ± 7.83	0	65.8 ± 8.84	13.22	72.0 ± 13.45	33.33
Me*m*TR	24.5 ± 6.79	1.02	69.0 ± 7.25	0	41.2 ± 6.56	3.64	74.0 ± 12.9	5	64.8 ± 6.45	37.35
Zeatin	30.8 ± 2.32	7.31	42.8 ± 6.15	7.01	44.0 ± 1.15	5.11	56.0 ± 7.82	41.43	69.0 ± 4.08	56.52
LSD (5%)	11.35		15.00		14.92		26.69		27.02	
F-Probability	<0.001		<0.001		<0.001		<0.001		<0.001	

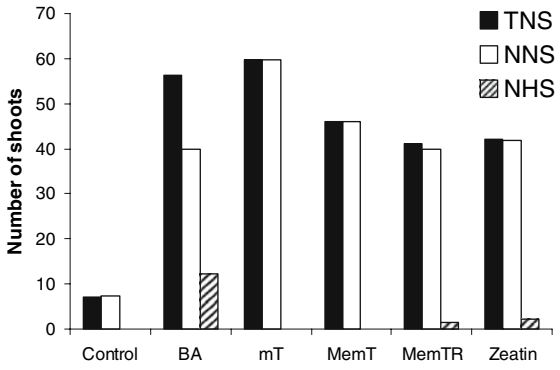

Fig. 3 The effect of the selected optimum cytokinin concentration (5 μM) on hyperhydricity. TNS = total number of shoots; NNS = number of normal shoots; NHS = number of hyperhydric shoots. Note that hyperhydricity was totally controlled using *m*T. See Table 1 for statistical details

with that of mT when applied at higher concentrations than the optimum level of *mT* (5 μM). Between 5 and 15 μM concentrations *m*TR produced a comparable number of shoots without affecting the rooting ability of the plants unlike *m*T, which caused inhibition of rooting and abnormal growth at concentrations greater than 5 μM (results not presented).

Werbrouck et al. (1996) demonstrated that the slight structural difference between BA and *m*T had a notable effect on cultures of *S. flouribundum.* We also found that plants treated with *m*T were superior in quality and quantity compared to plants treated with BA (Fig. 2). *m*T apparently would be a good replacement for zeatin in the tissue culture of *A. polyphylla*. Given the structural difference between zeatin and *m*T, one way of explaining this result would be looking at the pattern of receptor

Table 4 Effect of type and concentration of cytokinin on mean fresh weight (g)

Treatments	Cytokinin concentration (μM)				
	0.5	2.5	5.0	7.5	15
Control	4.00 ± 0.46	4.00 ± 0.46	4.00 ± 0.46	4.00 ± .46	4.00 ± .46
BA	17.52 ± 0.84	18.85 ± 1.95	15.27 ± 1.4	11.86 ± 1.62	14.98 ± 1.33
*m*T	11.16 ± 2.20	15.6 ± 1.66	17.75 ± 2.04	20.58 ± 0.62	22.96 ± 1.08
Me*m*T	9.11 ± 2.16	10.77 ± 0.46	8.88 ± 1.17	9.84 ± 1.85	14.55 ± 1.60
Me*m*TR	12.10 ± 1.86	11.84 ± 1.76	7.54 ± 1.48	9.12 ± 1.86	11.37 ± 1.81
Zeatin	16.85 ± 2.32	18.76 ± 1.2	22.06 ± 1.05	16.71 ± 1.25	16.55 ± .026
LSD (5%)	3.564	4.410	3.729	4.281	3.334
F-Probability	<0.001	<0.001	<0.001	<0.001	<0.001

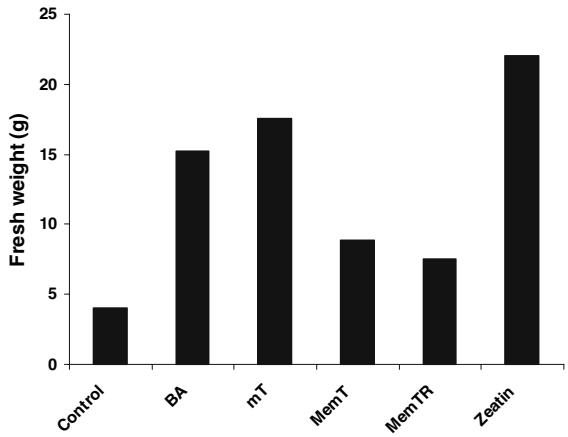

Fig. 4 Effect of the selected optimum cytokinin concentration on fresh weight. See Table 4 for statistical details

recognition. Mok et al. (2005) reported that position specificity is related to receptor recognition. In their cytokinin recognition study involving the *Arabidopsis* CRE1, WOL, AHK4 and maize ZmHK1 receptors, they found that AHK4 responded to *trans*-zeatin and *m*T while ZmHK1 responded to *cis*-zeatin and *o*T. This similar affinity in receptor recognition could be the possible reason why *m*T and zeatin had comparable effects in tissue culture of *A. polyphylla*. Spontaneous rooting of plants, a better multiplication rate and cost however, make *m*T the preferred cytokinin.

Effect on hyperhydricity

Generally, the incidence of hyperhydricity increased with an increase in the concentration of cytokinins (Table 3, Fig. 3), a fact well documented in the literature. At an optimum concentration (5.0 μM) no hyperhydric shoots were recorded for the *m*T and Me*m*T treatments. Hyperhydricity was most severe with BA treatment although all treatments caused hyperhydricity at higher concentrations. Although there was not a significant difference among the treatments (except for BA), the higher multiplication rate and good rooting in a multiplication media makes *m*T the preferred treatment. Apart from

Table 5 Effect of the different cytokinins tested on ex vitro growth

Fresh weight of ten (random) fully acclimatized (2-month-old) plants per treatment was used for this analysis. Only those treatments which produced plantlets with both shoots and roots were considered

Cytokinin concentration (μM)	Mean fresh weight (g)	
	Shoot	Root
2.5 BA	5.9 ± 1.37	0.56 ± 0.12
2.5 *m*T	19.3 ± 2.83	1.75 ± 0.26
2.5 zeatin	13.2 ± 2.23	1.00 ± 0.14
5.0 *m*T	23.6 ± 7.03	1.66 ± 0.44
5.0 zeatin	14.9 ± 2.65	0.82 ± 0.17
LSD (5%)	10.87	0.726
F-Probability	0.028	0.006

hyperhydricity, BA-treated plants failed to root, were yellowish in color and had excessive abnormal shoot growth (Fig. 2).

Effect on fresh weight (FW)

Total fresh mass per jar was measured and analyzed. There was a strong treatment effect at all concentrations for all the cytokinins tested. Mean mass of treatments were significantly higher than the control. At lower concentrations BA and zeatin gave higher FW. With an increase in concentration, a consistent increase of FW was observed with *m*T treatment unlike other treatments where a decline was observed at higher concentrations (Table 4). At optimum concentration zeatin gave significantly higher FW but had lower multiplication rates compared to *m*T (Fig. 4). This indicates that FW was not a reliable measure of multiplication and normal growth as it was affected more by growth of individual plants than by multiplication rate. It has also been observed that hyperhydricity and an undifferentiated mass of tissue due to abnormal growth contributed to an increase in FW.

At higher cytokinin concentrations, plants treated with BA and zeatin had lower multiplication rates, more pronounced abnormal growth and smaller mass of fresh weight compared to plants treated with *m*T (Tables 3 and 4). This could lead to the conclusion that at higher equimolar concentration *m*T is less toxic to *A. polyphylla* compared to BA and zeatin. Kamínek et al. (1987a) indicated that *m*T promoted shoot formation of stem cuttings at higher concentration of 10^{-4} mol L^{-1}; at this concentration BA inhibited shoot formation. They explained that this effect could be due to faster translocation of *m*T in plant tissues which prevents its localized accumulation.

Effect on acclimatization ex vitro

More than 90% of plants treated with *m*T acclimatized successfully compared to 65% survival rate recorded with BA treated plants (results not presented). Results ex vitro showed that the effect of *m*T is not limited to in vitro growth. Significant treatment effects were noted during the acclimatization process. Plants treated with *m*T showed faster growth and superior rooting compared to BA and zeatin-treated plants as indicated by mean fresh weight of roots and shoots (Table 5). Under normal conditions of growth, root and shoot growth complement one another. The poor ex vitro growth observed with plants treated with BA and zeatin (although statistically not significant when zeatin was compared to *m*T) could therefore be attributed to a treatment effect. The negative effect of BA on ex vitro growth could be due to the accumulation of the more stable derivative, [9G]BA. Werbrouck et al. (1995) reported that the accumulation on the basal portion of the plant of this derivative could result in slow release of BA during acclimatization, which can cause different problems such as heterogeneity in growth and inhibition of rooting. They also noted that the concentration of [9G]BA was about ten times more than the BA concentration in the medium after just two weeks of growth. Apart from its high concentration, this derivative is produced and stored at the plant base and apparent failure of being transported (Werbrouck et al. 1995) could have contributed to its negative effect.

Acknowledgement We acknowledge the financial support of the National Research Foundation (NRF), Pretoria. This work was also supported by the Grant Agency of the Czech Republic (GA 522/06/0108).

References

Abrie AL, Van Staden J (2001) Micropropagation of the endangered *Aloe polyphylla*. Plant Growth Regul 33:19–23

Aubrey A (2002) *Aloe polyphylla*. Available online at http://www.plantzafrica.com/plantab/aloepoly.htm Cited 11 Sept 2005

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

Chukwujechwu JC, Fennell CW, van Staden J (2002) Optimisation of the tissue culture protocol for the endangered *Aloe polyphylla*. S Afr J Bot 68:424–429

Horgan R, Hewett EW, Horgan JM, Purse JG, Wareing PF (1975) A new cytokinin from *Populus* × *robusta*. Phytochemistry 19:1005–1008

Iwamura H, Fujiita T, Koyama S, Koshimizu K, Kumazawa Z (1980) Quantitative structure-activity relationship of cytokinin-active adenine and urea derivatives. Phytochemistry 19:1309–1319

Jones LH, Martinková H, Strnad M, Hanke DE (1996) Occurrence of aromatic cytokinins in oil palm (*Elaeis guineensis* Jacq.). J Plant Growth Regul 15:39–49

Kamínek M, Paces V, Corse J, Challice JS (1979) Effect of stereospecific hydroxylation of N^6-(Δ^2-Isopentenyl)adenosine on cytokinin activity. Planta 145:239–243

Kamínek M, Vanék T, Kalendova A, Pilar J (1987a) Effect of two cytokinins on production of stem cuttings by stock plants of *Euphorbia pulcherrima* Wild. and *Gerbera jamesonii* Hook. Sci Hortic 33:281–289

Kamínek M, Vanék T, Motyka V (1987b) Cytokinin activities of N^6-benzyladenosine derivatives hydroxylated on the side-chain phenyl ring. J Plant Growth Regul 6:113–120

Krikorian AD (1995) Hormones in tissue culture and micropropagation. In: Davies PJ (eds) Plant hormones: physiology, biochemistry and molecular biology. Academic Publishers, The Netherlands, pp 774–796

Kubalakova M, Strnad M (1992) The effect of aromatic cytokinins (populins) on micropropagation and regeneration in vitro. Biol Plant 34:578–579

Leshem B, Sachs T (1985) 'Vitrified' *Dianthus*- teratomata in vitro due to growth factor imbalance. Ann Bot 56:613–617

Leshem B, Shaley DP, Izhar S (1988) Cytokinin as an inducer of hyperhydricity in melon. Ann Bot 61:255–260

Miller CO (1965) Evidence for the natural occurrence of zeatin and derivatives: compounds from maize which promote cell division. Proc Natl Acad Sci USA 54:1052–1058

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

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

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

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

Tarkowská D, Doležal K, Tarkowski P, Åstot C, Holub J, Fuksová K, Schmülling T, Sandberg G, Strnad M (2003) Identification of new aromatic cytokinins in Arabidopsis thaliana and Populus × canadensis leaves by LC-(+)ESI-MS and capillary liquid chromatography frit-fast atom bombardment mass spectrometry. Physiol Plant 117(4):579–590

Teramoto H, Momotani E, Tsuji H (1993) Benzyladenine-induced changes in the translatable mRNA population in excised cucumber cotyledons. Physiol Plant 87:584–591

Van Staden J, Drewes FE (1991) The biological activity of cytokinin derivatives in soybean callus bioassay. Plant Growth Regul 10:109–115

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

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 acclimatization problems. Plant Cell Rep 14:662–665