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ORIGINAL ARTICLE

Effect of cytokinins and sucrose concentration on the efficiency of micropropagation of 'Zes006' *Actinidia chinensis* var. *chinensis*, a red-fleshed kiwifruit cultivar

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Abstract

The effect of N^6(3-hydroxybenzyl)adenine (*meta*-Topolin—*m*T) was compared with that of N^6-benzylaminopurine (BAP) and zeatin at the proliferation stage of micropropagation of red-fleshed *Actinidia chinensis* var. *chinensis* 'Zes006' in two separate experiments. Shoot number, shoot weight, leaf number and leaf area were significantly higher in *m*T-supplemented media compared with BAP or zeatin. When transferred to rooting media, plantlets that were propagated in *m*T-supplemented media readily produced roots, enabling easy acclimation to the greenhouse, whereas none of the plantlets propagated in BAP- or zeatin-supplemented media produced roots. Using 12 pairs of Simple Sequence Repeat primers designed for *A. chinensis* var. *chinensis*, a very low rate of somaclonal variation was detected at some loci in plantlets produced in zeatin- (1.04%), BAP- (0.4%) as well as in *m*T- (0.2%) supplemented media. Overall, *m*T in equimolar concentrations was the better cytokinin for tissue culture of 'Zes006' kiwifruit and may well be applicable to many other kiwifruit genotypes.

Key message

Supplementation of media with meta-Topolin for in vitro propagation of the red-fleshed kiwifruit cultivar 'Zes006' enhanced better shoot proliferation, giving healthy plantlets that were easier to acclimatize to the greenhouse environment compared with media supplemented with 6-benzylaminopurine or zeatin. It also induced a lower rate of somaclonal variation, as detected by SSR markers.

Keywords Shoot proliferation · Acclimation · Meta-Topolin · Somaclonal variation · SSR markers · In vitro culture · *O*-glucosylation

Introduction

Exogenous cytokinins applied at the proliferation stage of plant micropropagation play a critical role in the shoot multiplication rate as well as subsequent rooting and acclimation of plantlets and their final quality (Gentile et al. 2014; Lata et al. 2016). The widely used synthetic cytokinin N^6-benzylaminopurine (BAP) is readily available, effective and affordable (Ivanova and van Staden 2008). It is often used in kiwifruit tissue culture (Bachiri et al. 2001; Tyson et al. 2018) However, it can induce hyperhydricity and an increased incidence of somaclonal variation in tissue culture (Biswas et al. 2009; Ivanova and van Staden 2008). To overcome the negative effects of exogenous BAP in tissue culture, use of zeatin has been suggested, including kiwifruit tissue culture (Mathew et al. 2018; Prado et al. 2007;

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Takahashi et al. 2004), but this cytokinin can also result in hyperhydricity of tissue (Ivanova and van Staden 2008). A combination of BAP and zeatin has also been used in regeneration of *Actinidia* spp. (Han et al. 2010; Takahashi et al 2004). The choice of a cytokinin for the proliferation stage during plant micropropagation is also determined by the ability of plantlets to produce roots in its absence (Lata et al. 2016). High multiplication rates, normal shoot and root development, and easy acclimation to the greenhouse conditions are important criteria for successful micropropagation, and cytokinins play a critical role in these processes (Ivanova and van Staden 2008).

Topolins are a relatively new group of cytokinins first extracted from poplar leaves (Strnad et al. 1997). They are aromatic cytokinins, differing from isoprenoid cytokinins such as zeatin in their biochemistry and biological activity, and N^6(3-hydroxybenzyl)adenine (*meta*-Topolin – *m*T) is the most biologically active topolin (Strnad et al. 1997). It is a hydroxylated form of BAP which produces an *O*-glucoside that has greater activity in comparison with other derivatives, reduces hyperhydricity and rooting problems, and consequently improves acclimation of tissue-cultured plantlets (Lata et al. 2016; Werbrouck et al. 1996). Replacement of BAP with *meta*-Topolin (*m*T) at the multiplication stage of tissue culture has resulted in better rooting and acclimation of plantlets in *Spathiphyllum floribundum* (Werbrouck et al. 1996), *Eucalyptus* spp. (Westhuizen 2014), *Corylus colurna* (Gentile et al. 2017), cannabis (Lata et al. 2016), cassava (Chauhan et al. 2018) and pineapple (Teklehaymanot et al. 2010). Replacement of BAP with *m*T reduced hyperhydricity, increased multiplication rate and resulted in spontaneous rooting in banana (Bairu et al. 2008), cannabis (Lata etal. 2016) and *Eucalyptus* spp. (Westhuizen 2014). In another study on *Prunus* rootstock micropropagation, plantlets grown in tissue culture media containing *m*T exhibited improved growth and shoot quality and acclimated better than shoots produced in BAP-supplemented media (Gentile et al. 2014). However, there are no reports on the use of *m*T in tissue culture of kiwifruit.

Over 500 different accessions of kiwifruit are held in an *in vitro* repository at The New Zealand Institute of Plant and Food Research Limited (PFR), New Zealand. Most accessions are amenable to propagation using BAP and indole-3-butryic acid (IBA) (Debenham et al. 2016). Methodologies for in vitro mutagenesis (Pathirana et al. 2016), transformation (Han et al.; Wang et al. 2006), shoot tip cryopreservation (Mathew et al. 2018) and polyploidy induction (Wu et al. 2011) have been developed for several *Actinidia* species using BAP and/or zeatin. However, there is still a challenge in some genotypes with respect to the type of cytokinin as well as the cytokinin/auxin balance required to achieve high production rates of good-quality plantlets cost effectively. The aim of the current study was to compare the effect of BAP and zeatin with *m*T on in vitro growth during the proliferation step of micropropagation and subsequent rooting and acclimation to the greenhouse in a red-fleshed kiwifruit genotype that is typically difficult to propagate in tissue culture, particularly at the rooting stage. We also compared the degree of somaclonal variation among plants propagated using different cytokinins as it is important to ensure genotypic integrity in in vitro germplasm collections and during micropropagation.

Materials and methods

Plant material

A. chinensis var. *chinensis* 'Zes006' plantlets used in the experiments were sourced from *in vitro* collection held in cold storage at PFR in Palmerston North, New Zealand (Debenham et al. 2016). The cultures were stored at 5 ± 1 °C for 32 weeks in a basal medium containing half-strength Murashige and Skoog (1962) (MS) macro salts, full-strength MS micro salts, B5 vitamins (Gamborg et al. 1968) with 3% (w/v) sucrose solidified using 7.5 g/L agar (Coast Biologicals, Opotiki, New Zealand). The plantlets were maintained in 30 mL screw-cap vials under low light intensity (1–3 µmol m^{-2} s^{-1}) with a 16 h photoperiod (Debenham et al. 2016). These were propagated in plant growth regulator (PGR) free MS (half-strength macro salts) medium over a period of 5 months for establishing the proliferation and rooting experiments described below.

Media preparation and culture conditions

All chemicals for tissue culture media preparation were supplied by Sigma Aldrich®, New Zealand unless otherwise stated. For all media, the pH was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. For plant proliferation, growth and rooting experiments 290 mL clear, wide-mouth vessels, with snap-on lids, containing approximately 50 mL of medium were used. Cultures were maintained in a growth room under light (35–45 µmol m^{-2} s^{-1} at shelf level provided by Philips® cool white fluorescent tubes) with a 16 h photoperiod at 24 ± 2 °C.

Shoot induction and proliferation

Two kiwifruit proliferation media routinely used in our laboratory, one containing BAP (1.33 µM) with 3% sucrose (Medium 1) (Debenham et al. 2016) and the other containing zeatin (0.9 µM) with 2% sucrose (Medium 2) (Mathew et al. 2018) were used as control media for the two experiments (Table 1). For the experiment to compare the effect of BAP and *m*T, BAP in Medium 1 was replaced with 1.32 and

Table 1 Two tissue culture media used as control and the modified media with cytokinins replaced with *meta*-Topolin, used in the proliferation stage, and the two rooting media tested

Media component	Proliferation medium 1[a]	Modified from medium 1	Proliferation medium 2[b]	Modified from Medium 2	Rooting medium 1	Rooting medium 2
Macro salts	MS	MS	MS	MS	1/2MS	MS
Micro salts	MS	MS	MS	MS	MS	MS
Vitamins	B5	B5	B5	B5	B5	B5
BAP	1.33				0.44	
Zeatin, µM			0.9			
meta-Topolin, µM		1.32, 2.64		0.90, 1.84, 2.70		
IBA, µM	0.246	0.246	0.492	0.492	1.48	
GA_3, µM	0.289	0.289				
Sucrose (%)	3	2, 3	2	2, 3	3	2

*BAP N*6-benzylaminopurine, *IBA* indole-3-butyric acid, GA_3 gibberellic acid

[a]Debenham et al. (2016)

[b]Mathew et al. (2018), MS (Murashige and Skoog 1962), B5 (Gamborg et al. 1968)

2.64 µM *m*T (Duchefa Farma B.V., Netherlands) (Table 1). To compare the effect of zeatin and *m*T, zeatin in Medium 2 was replaced with 0.9, 1.8, or 2.7 µM *m*T (Table 1). These combinations with two concentrations of sucrose (2 and 3%) were tested in two separate experiments. Zeatin and *m*T were filter sterilized and added after autoclaving; BAP was added to the media before autoclaving.

For assessing effect of cytokinins on growth of plantlets, shoots with two nodes were used to establish the cultures in a factorial design with three replicates per treatment. Nodal pieces were selected randomly from plant material grown on MS media (half-strength macro with no PGR). A replicate comprised one tissue culture vessel with five plantlets. Shoot number, shoot weight, leaf number, leaf area and chlorophyll content were recorded after 12 weeks with one subculture at 6 weeks. Images of the leaves flattened on a glass surface were captured with a digital camera, then ImageJ software (Schneider et al. 2012) was used to calculate leaf area. Chlorophyll was extracted using 80% acetone, absorption recorded at 663 and 645 nm and concentration calculated according to Arnon (1949). Data were statistically analyzed by analysis of variance using SAS/STAT® software (Version 9, 2014).

Rooting and greenhouse acclimation

Based on the results of the two proliferation experiments, the medium with optimum *m*T concentration was selected to establish the rooting experiment. In the experiment with BAP it was 2% sucrose and 2.64 µM *m*T, and in the zeatin experiment it was 2% sucrose and 1.84 µM *m*T. Randomly selected two-nodal sections from plantlets grown on MS media (half-strength macro with no PGR) were used to establish this experiment. The plantlets in the four media were grown for 12 weeks for assessing rooting, with one subculture at 6 weeks.

The plantlets from these four media were then transferred onto two kiwifruit root initiation media routinely used in our laboratory: (a) MS salts with macro elements in half-strength, Linsmaier and Skoog (1965) vitamins supplemented with 0.44 µM BAP, 14.8 µM indole-3-butyric acid (IBA) with 3% sucrose (Debenham et al. 2016), or (b) MS salts with 2% sucrose (Table 1). After 6 weeks, the number and size of roots (0–5 scale with 0 - no roots, 5 - thick roots > 0.5 mm diameter) were assessed, and rooted plantlets were deflasked by carefully washing roots to remove agar and placing in trays of a soil-free medium (bark:pumice 50:50). The plantlets were placed in a fog tent within a greenhouse without supplementary lighting for 1 week before transfer to an open greenhouse bench. On the open bench they were misted for 5 s every 20 min for 1 week. The bases of the fog tent and the greenhouse bench were heated to 28 °C. Finally the plants were transferred to a greenhouse bench with capillary watering for another week before transfer to a greenhouse bench with overhead watering. The greenhouse was heated from 15 °C and vented from 26 °C. Acclimated plants were transferred into individual pots after about three months in the greenhouse.

Assessment of somaclonal variation using SSR markers

To detect somaclonal variation in regenerated plants, leaf samples were collected at the deflasking step from kiwifruit plantlets grown in proliferation media containing BAP, zeatin and *m*T (used only 2.64 µM *m*T treatment). Leaves

of 20 independent random plantlets were sampled from each treatment and 10 × 7 mm pieces were used for DNA extraction using a modified CTAB method (Doyle and Doyle 1987). DNA concentration was between 10 and 23.5 ng/ul in extracted solutions. Three SSR markers, Ke116, Ke150 and Ke209 (Fraser et al. 2009) and nine new SSR primers developed at PFR (Table 2) were used to detect potential variation in genomic DNA. PCR amplification was carried out using a modified version of the fluorescent M13 universal primer system (Schuelke 2000) and a touchdown PCR program with annealing temperature 60–55 °C (94 °C/2 min 45 s; 10 cycles: 94 °C/55 s, 60 °C/55 s (−0.5 °C per cycle); 72 °C/1 min 30 s; 30 cycles: 94 °C/55 s, 55 °C/55 s, 72°C/1 min 30 s; 72 °C/10 min). The fragments were separated using a 3500 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA) and their size analyzed with GeneMarker® v 2.2.0 software (© SoftGenetics, LLC.). The degree of genetic variation was calculated as the percentage of alleles modified per number of alleles x number of plants sampled.

Results

Significantly higher shoot number and shoot weight were recorded in *m*T-supplemented media when compared with BAP (Experiment 1) and zeatin (Experiment 2) supplementation (Table 3; Figs. 1, 2). In the experiment where the effect of BAP was compared with *m*T, both concentrations of *m*T tested resulted in significantly higher shoot weight and shoot number; however, shoots in 2.64 µM *m*T showed slightly better growth than those in the lower concentration of 1.32 µM (Fig. 1). In the experiment where the effect of zeatin was compared with *m*T, the latter resulted in higher shoot numbers and shoot weight, with 1.8 µM *m*T in both sucrose concentrations promoting better growth than the lower or higher *m*T concentrations (Fig. 2). Overall, plants propagated in *m*T had significantly increased shoot proliferation from axillary buds in comparison with both BAP and zeatin media (Figs. 1, 2). Variance analysis showed there

Table 3 Analysis of variance of *Actinidia chinensis* var. *chinensis* 'Zes006' tissue culture growth factors when BAP or zeatin were replaced with *meta*-Topolin

Growth factor	F value BAP medium	F value Zeatin medium
Shoot weight	4.72 *	2.9**
Fold increase in weight	3.59*	1.69
Shoot number	5.57**	6.89**
Total chlorophyll	1.77	1.43
Leaf number	1.52*	1.6
Total Leaf Area	2.09**	0.17
Average Leaf Area	2.19*	0.4

*,** indicate significant differences at $P < 0.05$ and 0.01 respectively, degrees of freedom for BAP tests 3 and 11; for zeatin 4 and 11

Table 2 Primer sequences, repeat motif, and PCR product sizes of nine SSR primers designed from kiwifruit *Actinia chinensis* var. *chinensis* 'Zes006' genome assembly version PS1.1.68.5 used to detect somaclonal variation

Locus code	Primer sequences (5′ →3′)	Repeat motif	Length(bp)[a]
Hy_sc585_17_9642687	5′ GGGATGTTTCTCAAGCCGTA	(GT)6	182; 186
	5′ TGGCAATACGATCTGATGGA		
PS1_sc109-2333432_5_3212854	5′ AAAACATTCATCACCCATGC	(CT)11(AT)7	242/246
	5′ CCTCGATTTTCCTTCCATCA		
PS1_sc578_599507_1_2162428	5′ TGAAGTAAGTTTGAAGTTTTTGCTG	(TA)7	345
	5′ GGTGAATGCTACGGTGTGTG		
PS1_sc39_447216_25_16003325	5′ ACCTTTTGAGGTTGCAGACG	(GA)13	293/295
	5′ GGTATCCCTCCGATAAGAATAAA		
PS1_sc29_804855_9_12622265	5′ TGACCCTTTCTTTATGACTCCA	(CT)17	164; 184/186
	5′ GCATCTTCTGGTGCAACCTA		
PS1_sc14_2378780_24_12972021	5′ CCGGAACAAATCGAGGTT	(CT)26	209/212
	5′ TTGCTGAAGTAGAAGTGGAAAGC		
PS1_sc444_15_14575338	5′ TGGGAGAACCAGTGCTTTTA	(CT)9	327/331
	5′ TTGGTTTGGGTTAGGGTTTG		
PS1_sc138_1058880M13_25_19079309	5′ CGCTCATTGTTTGGCTGTT	(CT)13	302/304
	5′ TGGATGGTACACCGGAGAAT		
PS1_sc165_172318_27_3494743	5′ AACGCAGTAGTTGGGGAAAG	(GA)16	176
	5′ GAATGACCAATGCAAACATCA		

[a]Calculated with GeneScan™ 500 LIZ™ dye size standard and GeneMarker® v 2.2.0 software (© SoftGenetics, LLC.)

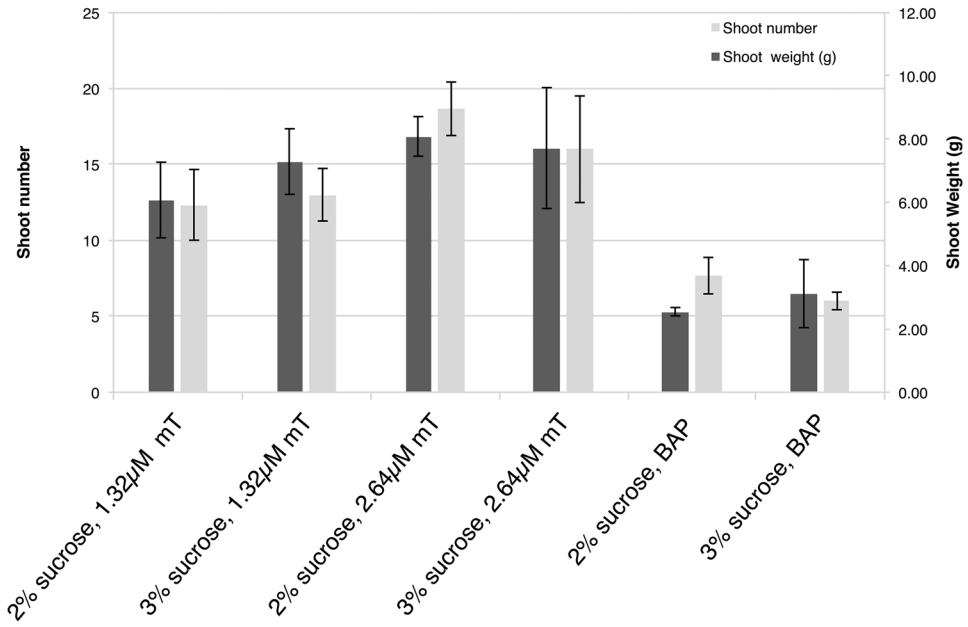

Fig. 1 Comparison of shoot weight and shoot number per *Actinidia chinensis* var. *chinensis* 'Zes006' plantlet after 12 weeks in culture in *meta*-Topolin (*m*T)- and 1.33 µM 6-benzylaminopurine (BAP)-supplemented media using two sucrose concentrations. Error bars indicate standard error of the mean (n = 3)

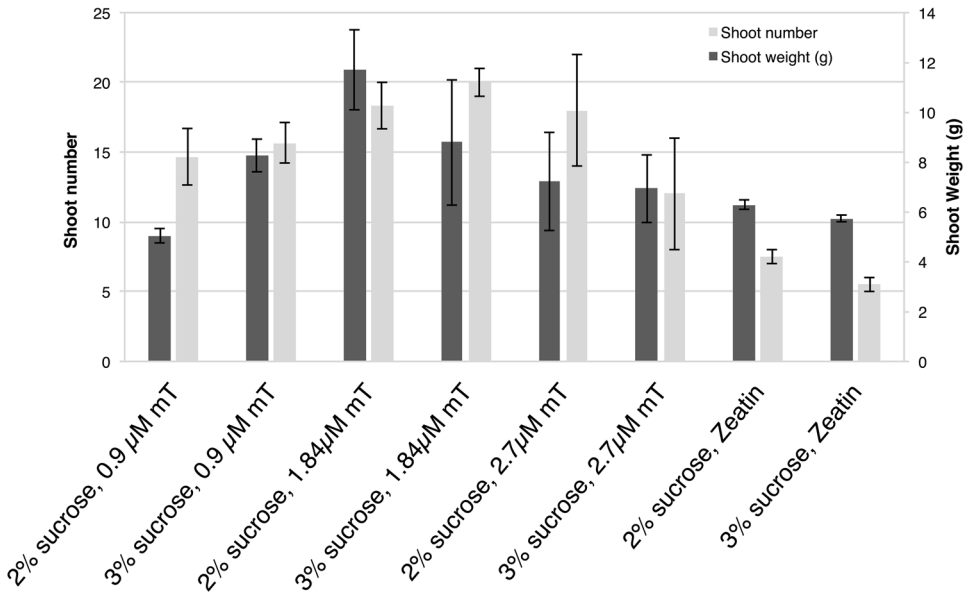

Fig. 2 Comparison of shoot weight and shoot number per *Actinidia chinensis* var. *chinensis* 'Zes006' plantlet after 12 weeks in *meta*-Topolin (mT)- and 0.91 µM zeatin-supplemented media using two sucrose concentrations. Error bars indicate standard error of the mean (n = 3)

was no significant difference between sucrose concentrations of 2 and 3% for these parameters (Supplementary Table 1).

Significantly increased leaf number and total leaf area were also recorded in mT-supplemented media compared with BAP (Table 3; Fig. 3). The lower sucrose concentration of 2% gave rise to a significantly higher leaf number with both *m*T concentrations tested. The higher *m*T concentration (2.64 µM) induced the highest leaf number (Fig. 3). Analysis of variance of leaf number and total leaf area showed no significant differences between zeatin- and *m*T-supplemented media (Table 3), but the total leaf area tended to be greater in *m*T-supplemented media, achieved through a higher number of leaves (Fig. 3). The chlorophyll content did not show significant differences among treatments (Table 3); however, 1.32 µM *m*T with 2% sucrose in the medium resulted in an elevated chlorophyll concentration compared with BAP and zeatin (Supplementary Fig. 1).

Root initiation of plantlets following propagation in BAP-, zeatin- or *m*T-supplemented media was tested in two rooting media: one with BAP and IBA, and the other with no PGR. For plantlets produced in *m*T media, root initiation and root growth was observed in 58 and 80% of plantlets respectively, after 45 days in these two rooting media. Plantlets produced in media containing BAP or zeatin had not

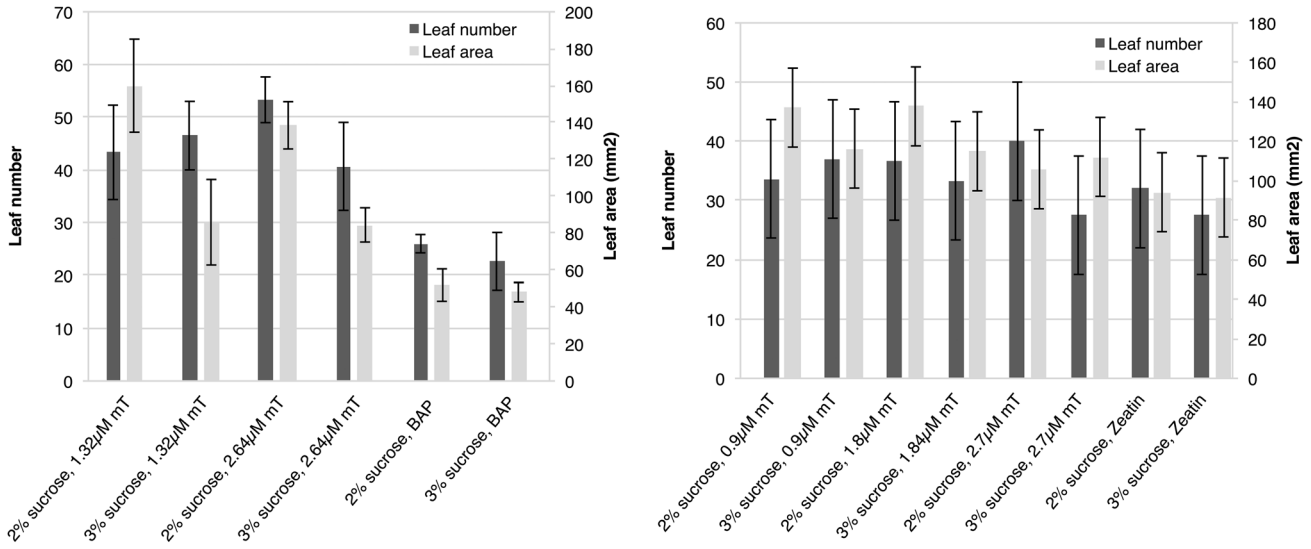

Fig. 3 Comparison of total leaf area and leaf number per tissue culture vessel in *Actinidia chinensis* var. *chinensis* 'Zes006' grown in *meta*-Topolin (mT)- supplemented media with those grown in 1.33 µM 6-benzylaminopurine (BAP)-supplemented media or 0.91 µM zeatin-supplemented media under two sucrose concentrations. Error bars indicate standard error of the mean (n = 3)

produced roots by 45 days in either of the two rooting media evaluated. Therefore root production was not included in the statistical analysis. Following transfer to rooting medium without PGR, plantlets propagated on *m*T media produced longer and thinner roots with extended branches compared with plantlets propagated on rooting media containing 0.44 µM BAP and 1.48 µM IBA. Plantlets on the rooting media with PGR had a 50- to 70-mm wide callus tissue at the base (Fig. 4). Acclimation success for plantlets from *m*T media was 80% for rooting medium with PGR and 95% for PGR-free rooting medium.

SSR analysis of 20 plantlets per treatment indicated a low rate of genetic variation, exhibited as the deletion of a locus, among plants grown in different media (Fig. 5). Plantlets proliferated in BAP-supplemented media had a variation of 0.4% at a given locus, while zeatin-supplemented media induced the highest variation, at 1% for a given locus. Plantlets sampled from *m*T-supplemented media exhibited least genetic variation, of 0.2%. Figure 5 shows an output from GeneMarker® v 2.2.0 software, displaying results of one plantlet per treatment as an example, exhibiting identical profiles within their treatment: SSR PS1_sc138_1058880_25_19079309 exhibited allele 302/304 bp from a plantlet raised in zeatin-supplemented medium, *m*T media as well as in the control 'Zes006' kiwifruit plantlet from the in vitro genebank (Fig. 5a). However, this allele was not exhibited by plantlets raised in BAP media, possibly because of a deletion. The results from SSR PS1_sc444_15_14575338 indicate that this was not due to a technical error (such as failed PCR), because fragments at 327 and 331 bp from this SSR were amplified in the same

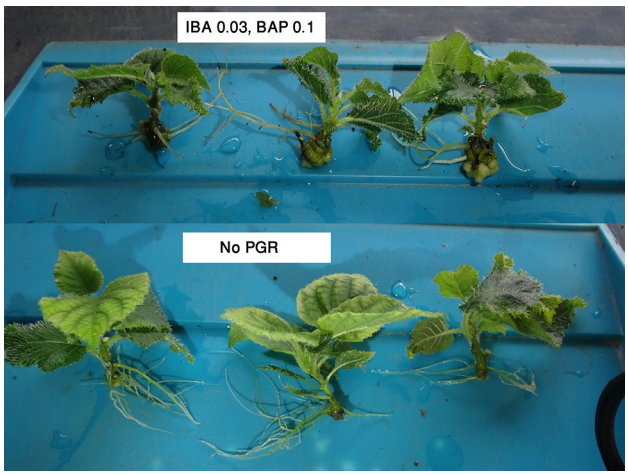

Fig. 4 Comparison of rooting in *Actinidia chinensis* var. *chinensis* 'Zes006' plantlets grown on rooting media with basal medium supplemented with 0.44 µM BAP and 1.48 µM IBA (above) and with no PGR (below) after 6 weeks. The plantlets were grown in *meta*-Topolin-supplemented media for 12 weeks before transfer to rooting media. Plantlets raised in 6-benzylaminopurine- and zeatin-supplemented media did not produce any roots in either of the rooting media

DNA sample. Another example of genetic variation among plantlets was found in medium supplemented with zeatin for PS1_sc444_15_14575338 (Fig. 5b), which exhibited alleles 327 bp and 331 bp in plantlets raised in BAP media, *m*T media as well as in a control 'Zes006' kiwifruit plantlet, but were absent in plantlet from zeatin-supplemented medium. Here the DNA quality control in SSRPS1_sc138_1058880_25_19079309 (Fig. 5a) amplified alleles

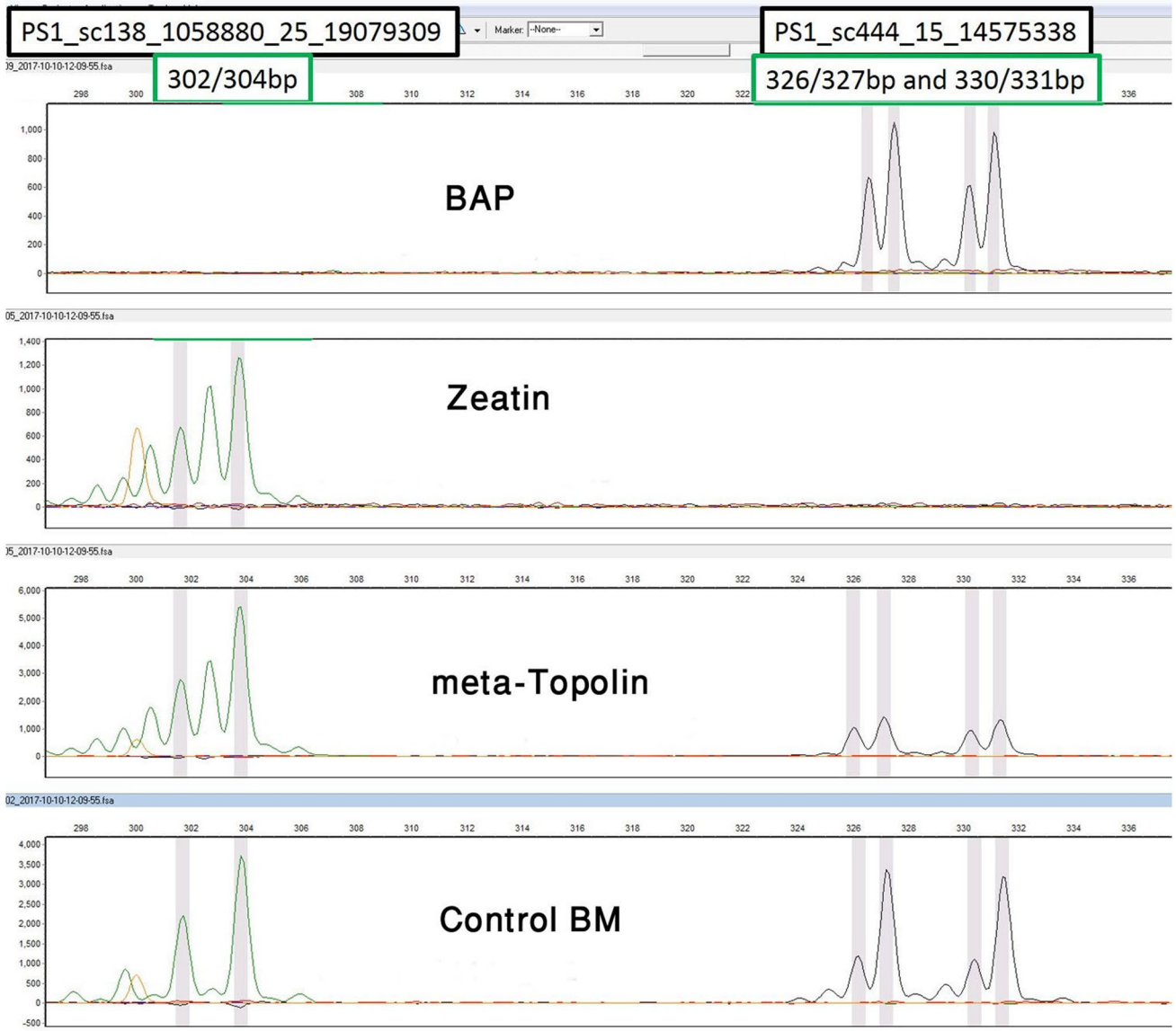

Fig. 5 Comparison of single sequence repeat fragment analysis results for *Actinidia chinensis* var. *chinensis* 'Zes006' plantlets grown in 1.33 µM 6-benzylaminopurine (BAP)-, 0.91 µM zeatin- and 2.64 µM *meta*-Topolin-supplemented media and control plants grown in basal media (control BM) without cytokinin, using primers PS1_SC138_1058880_25_19079309 (5a) and PS1_sc444_15_14575338 (5b). X-axis shows fragment size in base pairs (bp) and Y-axis shows intensity of dye absorption for each fragment. PS1_SC138_1058880_25_19079309 (5a) exhibits allele at 302/304 bp in zeatin-, *meta*-Topolin-supplemented media and control plants grown in plant growth regulator-free media, but absent in BAP-supplemented media. PS1_sc444_15_14575338 (5b) exhibits allele at 327 bp and 331 bp in BAP-, meta-Topolin-supplemented media and control plants, but absent in zeatin-supplemented media

302/304 bp in zeatin-supplemented medium. In addition, SSR Ke116 exhibited a deletion of an allele at 210 bp in a plantlet grown in zeatin-supplemented medium, which was present in plantlets grown in media supplemented with BAP and *m*T as well as in the control. All other SSRs screened (Ke150, Ke209, Hy_sc585_17_9642687, PS1_sc109-2333432_5_3212854, PS1_sc578_599507_1_2162428, PS1_sc39_447216_25_16003325, PS1_sc29_804855_9_12622265, PS1_sc14_2378780_24_12972021 and PS1_sc165_172318_27_3494743; Table 2) displayed consistent allele size among the three treatments and controls.

Discussion

We have clearly demonstrated that *m*T is a better alternative to BAP or zeatin for the micropropagation of a recalcitrant accession of *A. chinensis* var. *chinensis*. In tissue culture, shoot formation and quality of plantlets are highly dependent

on PGR, particularly the type of cytokinin used and its concentration (Amoo and Van Staden 2013). The antagonistic role of cytokinins and auxins in shoot regeneration is the key for higher shoot multiplication rates when cytokinins are used in high concentrations, or in more active forms such as *m*T. More active forms of cytokinin negate the auxin effect on apical dominance and enhance induction of axillary bud growth and proliferation (Shimizu-Sato et al. 2009) in a similar way to apical bud decapitation and subsequent removal of apical dormancy in plantlets (Cline 1991). The positive effect of *m*T in comparison with BAP or other cytokinins has been attributed to its higher activity because of the presence of a hydroxyl group in the N9-position and formation of *O*-glucoside conjugate N^6(3-O-β-D-glucopyranosyl) benzyladenine-9-glucoside (Aremu et al. 2012a; Werbrouck et al. 1996). This structure promotes translocation to avoid local accumulation, as well as ready conversion to the active form, when required (Amoo et al. 2011). In our experiments, all tested concentrations of *m*T increased shoot formation in kiwifruit significantly. Similar results were reported by other researchers in a range of horticultural species (Aremu et al. 2012b; Bairu et al. 2007, 2008; Gentile et al. 2014; Teklehaymanot et al. 2010; Westhuizen 2014). Cytokinins also influence biological processes such as chlorophyll accumulation (Sakakibara 2006). Aremu et al. (2012c) attributed the better acclimation capability of *m*T-raised banana compared with other cytokinins to improved photosynthetic parameters. Although statistically not significant, highest chlorophyll content in our research was also recorded in *m*T-supplemented media.

In our research it was noted that abnormalities in leaf shape and shoots were fewer in plantlets grown in *m*T than in plantlets raised using equimolar concentrations of BAP and zeatin. This may indicate lower toxicity of *m*T and its products, as reported by Werbrouck et al. (1996). Plantlets grown in *m*T media readily acclimated to the greenhouse and commenced normal growth because of efficient root formation in rooting media prior to deflasking. Similar results on acclimation have been reported in other species when BAP was replaced with *m*T (Amoo et al. 2015, 2014, 2012; Aremu et al. 2012b; Gentile et al. 2014; Lata et al. 2016; Naidoo et al. 2017). Plantlets grown in *m*T-supplemented media appeared normal, without abnormal leaf shape and chlorotic effects.

Somaclonal variation can be a major drawback in micropropagation (Smulders and De Klerk 2011), *in vitro* conservation and cryopreservation (Harding et al. 2009). In tissue culture, the occurrence of somaclonal variation has been attributed to several factors that are largely related to stress conditions, including wounding, imbalanced media in culture, and high concentrations of plant growth regulators, mainly cytokinins (Sato et al. 2011; Skirvin et al. 1994; Smulders and De Klerk 2011). Changes detected in the present study on kiwifruit are minor. Although previous research has shown SSR markers to be useful for early detection of somaclonal variation in micropropagated horticultural crops (Gao et al. 2009; Palombi and Damiano 2002; Pandey et al. 2012), more effective methods need to be employed for more precise estimation. In fact Palombi and Damiano (2002), who compared SSR and Randomly Amplified Polymorphic DNA (RAPD) markers for detecting undesirable genetic variation in micropropagated *Actinidia deliciosa* 'Tomuri' using BAP as the cytokinin, suggested the use of more than one DNA approach. One proliferation medium tested in our study (Medium 1 in Table 1) is used for tissue culture of kiwifruit in general across several species (Debenham et al. 2016). The plants produced in this medium are cultured on basal MS media with half-strength macro elements (with no PGR) for maintaining a large *in vitro* collection of kiwifruit accessions at PFR. The other proliferation medium (Medium 2 – Table 1) that was tested in our studies is used specifically for *A. chinensis* var. *chinensis* 'Hort16A' (Mathew et al. 2018; Pathirana et al. 2016), to produce healthy and uniform plantlets. The relatively low rate of somaclonal variation detected in 'Zes006' by SSR makers further confirms the suitability of these media as well as the new medium supplemented with *m*T for tissue culture of 'Zes006'. Although some differences in the rate of somaclonal variation were observed among plantlets produced in three different media, with zeatin giving rise to the highest rate and mT the lowest, these changes can be considered minor. Bairu et al. (2008) also reported no significant differences between somaclonal variation in banana plants propagated on BAP and *m*T.

Conclusion

Our research showed that supplementation of media with *m*T for micropropagation of the *A. chinensis* var. *chinensis* kiwifruit cultivar 'Zes006' enhanced shoot proliferation, giving healthy plantlets that were easier to acclimatize to the greenhouse environment, when compared with BAP and zeatin. We did not detect a significant effect of sucrose on any of the growth parameters studied. *m*T also induced a lower rate of somaclonal variation, as detected by SSR markers for the *A. chinensis* var. *chinensis* genotype studied. Future work will include screening other kiwifruit genotypes and different *Actinidia* species to test if *m*T is a better alternative cytokinin in kiwifruit tissue culture in general.

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Author Contributions RP, HS and CW conceptualised the research and designed the experiments. HS, CW and RP conducted the research. HBB designed the primers described in Table 1. RP managed the project. HS, RP, CW and MM wrote the manuscript. HS conducted the statistical analysis.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest with the contents of this article.

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