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Full Length Research Paper

The properties and interaction of auxins and cytokinins influence rooting of shoot cultures of *Eucalyptus*

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Success in *Eucalyptus* micropropagation varies with genotype. Although some protocols have proven suitable for suites of clones, many genotypes are recalcitrant to rooting. Their micropropagation is addressed empirically through the manipulation of auxins and cytokinins, which work antagonistically to produce roots and shoots, respectively. Rooting success of three genotypes with 0.1 mg/l indole-3-butyric acid (IBA) was initially recorded as 87, 45 and 41% for clones 1, 2 and 3, respectively. Further studies using the auxin signal transduction inhibitor ρ-chlorophenoxyisobutyric acid (PCIB) or the auxin conjugation inhibitor dihydroxyacetophenone (DHAP) indicated that the poor rooting response of clone 2 was not due to deficient auxin signal perception or auxin conjugation. Omitting kinetin during elongation, followed by auxin-free rooting, significantly increased root production in clone 2 (from 45 to 80.3%), but had no effect on clone 1. Gas chromatography-mass spectrometry (GC-MS) analysis of auxins and kinetin of shoots, prior to rooting, revealed a strong relationship (R^2 = 0.943) between rootability and the shoot kinetin:auxin. Replacing kinetin with the less stable *trans*-zeatin significantly increased rooting of clone 2 (from 19 to 45%) and clone 3 (31 to 52%). It is suggested that root induction in poor-rooters is dependent on exogenous cytokinin depletion from *in vitro* shoots.

Key words: *Eucalyptus*, phytohormone interaction, root quality.

INTRODUCTION

Eucalyptus forestry programs have proven invaluable in their contribution to meeting timber demands worldwide. Profitable plantations have been established in more than 70 countries, and the products of these establishments continue to serve the wood, paper, pulp, and charcoal industries, among others (Eldridge et al., 1994; Turnbull, 1999; Watt et al., 2003).In order to remain competitive, forestry programs need to seek and maintain superior genotypes that confer traits of interest (for example, preferred timber properties), allow for genotype-to-site matching and other strategies to increase production yields. In the pursuit of this, superior eucalypt hybrids have found favour, and their selection programmes often seek to combine stress tolerance with superior wood characteristics in a sustainable, cost effective manner that meets industrial requirements (Watt et al., 2003; de Assis et al., 2004).

The traditional eucalypt propagatory method, that is, through seedlings, is often not possible for hybrids (Denison and Kietzka, 1993b). Consequently, vegetative propagation, for example, through macro-, mini- or micro-cuttings is often the only option, and it has the benefit of ensuring the maintenance and continuity of value-added

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Abbreviations: DHAP, Dihydroxyacetophenone; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **GC-MS,** gas chromatography-mass spectrometry; **NAA,** α-naphthalene acetic acid; **PCIB,** ρ-chlorophenoxyisobutyric acid.

traits (Denison and Kietzka, 1993a, b). Even for pure species, vegetative propagation has many advantages over seedling propagation, most notably the increase in yield and the maintenance and conservation of superior genotypes (Eldridge et al., 1994). However, propagation through macro- and mini-cuttings has its limitations, in that the rooting ability amongst clones is variable and is known to decrease with the age of the parent plants (Eldridge et al., 1994; de Assis et al., 2004).

Micropropagation potentially addresses such shortcomings by providing a highly controlled environment that yields high shoot proliferation rates (Le Roux and van Staden, 1991), improved potential, speed and quality of rooting (de Assis et al., 2004), and is often the only viable method of propagating difficult-to-root clones (Mokotedi et al., 2000; Watt et al., 2003; Yasodha et al., 2004).

A number of studies have investigated and developed micropropagation protocols for commercially important pure and hybrid eucalypt clones (Le Roux and van Staden, 1991; Jones and van Staden, 1997; Watt et al., 2003, Aggarwal et al., 2010; Hung and Trueman, 2011). These are based on the empirical manipulation of key plant growth regulators -notably auxins and cytokinins, to achieve the desired morphogenesis in each of the *in vitro* culture stages.

In general, the initial stages of bud induction from minicuttings (taken from the parent plant) and subsequent shoot proliferation are achieved using either a single cytokinin type or a combination of cytokinins. Shoot elongation is then stimulated by a combination of auxins and cytokinins, and lastly rooting is accomplished using one or more types of auxins at various concentrations, depending on the clone in question (Jones and van Staden, 1997; George et al., 2008). Since each of the established protocols empirically addressed the micropropagation needs of a specific clone, their interclonal application often leads to large variations in propagation and rooting success.

As core to the success of a micropropagation protocol is the ability of the shoots to produce roots (de Assis et al., 2004), there is a large body of published reports on the rooting efficiencies of eucalypts in response to the various auxin types and concentrations, on an empirical and clone-specific basis (Jones and van Staden, 1997; Watt et al., 2003). However, little attention has been paid to the antagonistic effects of the auxin and cytokinin types on morphogenesis, in particular the inhibitory effect that persistent cytokinins may exert on root induction of eucalypt shoots *in vitro*.

Both auxins and cytokinins are recognised as the key signalling molecules in plant development (Moubayidin et al., 2009). It is accepted that elevated cytokinin content favours shoot development, elevated auxin content favours root development, while equal concentrations of both results in callus formation (George et al., 2008). While this general model dictates the use of these phytohormones during the various stages in *in vitro* protocols, the properties of the various cytokinins and auxins used should also be considered.

Indole-3-acetic acid (IAA) is the most frequently encountered natural auxin, and is known to be more easily oxidated than the other natural auxin indole-3-butyric acid (IBA), or the synthetic auxin α-naphthaleneacetic acid (NAA) (George et al., 2008; De Klerk et al., 1999). The differences in the stabilities of IBA and IAA and their effect on root production in eucalypts have been investigated previously (Nakhooda et al., 2011). Similarly, the properties of the various cytokinins differ, with the synthetic cytokinin kinetin (6-furfuryl aminopurine) been shown to persist longer (George et al., 2008) than the natural cytokinin compounds such as *trans*-zeatin, which is rapidly degraded by the enzyme cytokinin-oxidase (Mok and Mok, 2001; Haberer and Kieber, 2002; George et al., 2008).

An enzyme other than cytokinin oxidase is thought to be involved in kinetin degradation in some plant species (Forsyth and van Staden, 1987). Given that the most commonly-used auxins and cytokinins in eucalypt culture vary in their stabilities, their interaction at each of the culture stages, and subsequent effect on rooting, need further elucidation.

In the present study, the apparent inability of two eucalypt clones to root ‘with ease’, *in vitro*, was investigated in relation to the perception and conjugation of the most commonly-used auxins in eucalypt culture. The tested hypothesis was that the inhibition of root induction in some difficult-to-root clones is due to cytokinin persistence from the pre-rooting culture stages, resulting in a supra-optimal cytokinin:auxin ratio in shoots prior to rooting. The aim was to optimise *in vitro* protocols for eucalypt culture, to maximise yields of both easy- and difficult-to-propagate clones.

MATERIALS AND METHODS

Decontamination and culture initiation

The eucalypt clones used in the present study were a *Eucalyptus grandis* (clone 1) and an *E. grandis* × *E. nitens* hybrid (clone 2). A further *E. grandis* × *nitens* natural hybrid (that is, not a result of a forestry breeding programme) (clone 3) was later used to confirm observations. All clones were obtained from Mondi Business Paper, Hilton, KwaZulu-Natal. They were chosen on the basis of their rooting performance as mini-cuttings in the Mondi nursery (unpublished) that is, as examples of a ‘good rooter’ (easy-to-root, > 75% rooting) (clone 1) and of two ‘poor rooters’ (difficult-to-root, < 50% rooting) (2 and 3). Cuttings of the parent plants were surface

Table 1. Concentration and composition of plant growth regulators (PGRs) in the eight different elongation media (E1 to 8) used in this study.

PGR (mg/l)/(µM)	Media							
	E1	E2	E3	E4	E5	E6	E7	E8
0.2/0.93 Kinetin	Y	Y	Y	Y	N	Y	N	N
0.3/1.6 NAA	Y	Y	N	N	Y	N	N	N
0.05/0.25 IBA	Y	N	Y	N	Y	N	N	N
0.37/2.1 IAA	N	N	N	Y	N	N	N	Y
0.2/0.91 *trans*-zeatin	N	N	N	N	N	N	N	Y

N = Absent; Y = present.

decontaminated in 0.02% (w/v) $HgCl_2$ with a drop of Tween® -20 for 10 min, followed by 1% (w/v) calcium hypochlorite for 10 min. After several rinses in sterilised distilled water, they were cut into nodal segments and placed on 10 ml bud induction medium in 50 ml culture tubes with snap-on lids containing MS nutrients (Murashige and Skoog, 1962), 0.1 mg/l biotin, 0.1 mg/l calcium pantothenate, 0.04 mg/l (0.21 µM) NAA, 0.1 mg/l (0.44 µM) 6-benzylaminopurine (BAP), 0.05 mg/l (0.23 µM) kinetin, 20 g/l sucrose and 4 g/l Gelrite® for 2 weeks.

Micropropagation protocol

The shoot proliferation and elongation media were as for bud induction, except that elongation media contained different combinations of plant growth regulators (PGRs) (Table 1). The elongation treatment E1 represents the standard elongation medium used for eucalypt micropropagation in our laboratory. Treatments E2 to E8 represent various combinations (or lack thereof) of PGRs used in an attempt to establish the PGR requirements necessary for root induction in the eucalypt clones investigated presently. Shoots were maintained in culture bottles on 20 ml of medium during the shoot proliferation and elongation stages, which typically lasted 3 and 4 weeks, respectively.

Upon reaching a height of at least 1.5 cm, shoots were transferred to 10 ml of rooting medium (¼ MS nutrients, 0.1 mg/l biotin, 0.1 mg/l calcium pantothenate, 15 g/l sucrose and 4 g/l Gelrite®), in 50 ml culture tubes. Manipulations of the rooting media (where indicated) included the addition of the auxins IAA, IBA, or NAA at 0.1 mg/l (0.57, 0.49 and 0.54 µM, respectively) or at 0.5 mg/l (2.85, 2.46, and 2.7 µM, respectively). Two studies were undertaken with clone 2 (poor rooter), where the following were added to the rooting medium: 50 µM of the inhibitor of auxin signal transduction ρ-chlorophenoxyisobutyric acid (PCIB) (to test its auxin-responsive ability) and 2 mM of the auxin conjugation inhibitor 1,6-dihydroxyacetophenone (DHAP) (to test the effects of auxin conjugation on root induction).

All media were adjusted to a pH of 5.6 to 5.8 prior to autoclaving at 121°C and 1 kPa for 20 min, with phytohormones added as per suppliers' (Sigma-Aldrich) instructions. Maintenance of cultures was under a 16 h light (200 µmol m^{-2} s^{-1})/8 h dark photoperiod at 25 and 23°C, respectively, as is established practice for eucalypt culture in our laboratory (Mokotedi et al., 2000). Mean rooting times were evaluated according to the method reported by Fett-Neto et al. (2001).

Sample preparation for phytohormone analysis

Concentrations of auxins and cytokinins within whole shoots, just prior to rooting, were evaluated using gas chromatography-mass spectrometry (GC-MS). Freeze-dried shoots were homogenised and suspended in 500 µl sodium phosphate buffer (pH 7) and incubated for 1 h at 4°C. The pH was then reduced with HCl to 2.6, and compound absorption was carried out, facilitated by the addition of Amberlite® XAD-7 (Sigma-Aldrich). The solution was incubated for a further hour at 4°C, followed by two washes with 500 µl of 1% (v/v) acetic acid and dichloromethane. Samples were then dried down, and after the addition of 50 µl of 2 M trimethylsilyl-diazomethane, were incubated for 30 min at room temperature. This was then quenched with the addition of acetic acid (1% v/v) and dried down overnight, followed by heptane additions and sample analysis, using the GCT Premier™ benchtop orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer, Waters, USA.

Statistical analysis

All statistical analyses were carried out using PAST, version 2.01 (Hammer et al., 2001). Experiments were repeated at least 3 times, with sample sizes of at least 25 for rooting studies, and at least 3 for phytohormone analysis. Where necessary, data were analysed using one-way analysis of variance followed by Fisher's least significant difference.

RESULTS

The *in vitro* rooting responses of the tested clones recorded in this study (Figure 1A) reflected their reported behaviour as minicuttings in the nursery (Mondi staff, pers. comm.). After exposure to standard shoot proliferation and elongation conditions (E1) (Table 1), and 30 days in rooting medium containing 0.1 mg/l IBA, the percent rooting of the clones were 85% for clone 1 (good rooter), 45% for clone 2 (poor rooter) and 41% for clone 3 (poor rooter) (Figure 1A), with mean rooting times of 12, 5.9 and 4.6 days, respectively. The high *in vitro* rooting efficiency of clone 1 has been established previously (Nakhooda et al., 2011) and together with clone 3, were

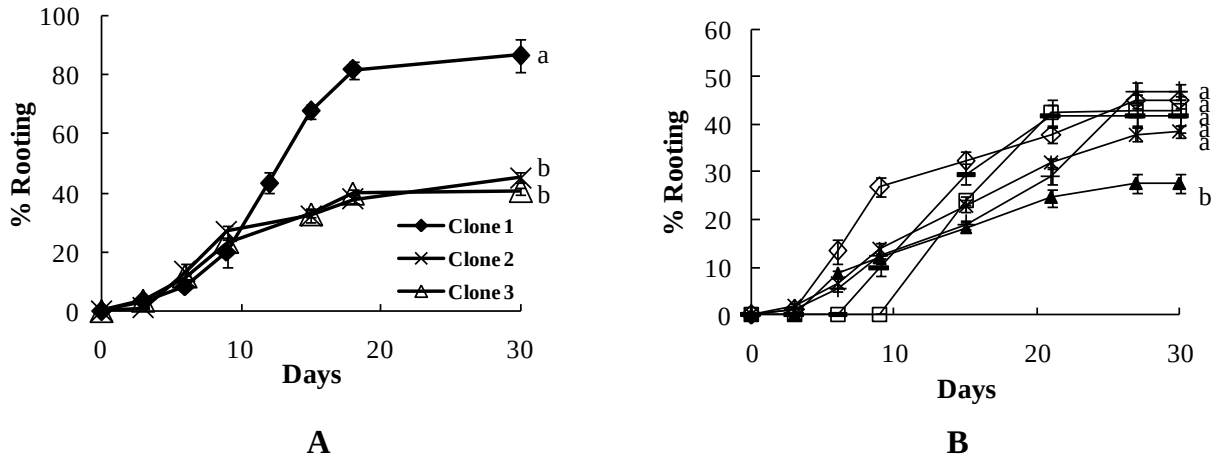

Figure 1. (A) Percentage rooting of shoots of clones 1, 2 and 3, over 30 days on rooting medium containing 0.1 mg/l IBA. Shoots were produced on standard shoot proliferation and elongation media (E1). The values are the mean ± SE (n = 30), different letters denote significant differences as determined by students t-test ($P < 0.05$); (B) percentage rooting of clone 2 shoots over 30 days on rooting medium containing 0.1 or 0.5 mg/l IBA (◊, □), IAA (▲, |) or NAA (X, —). Shoots were produced on standard shoot proliferation and elongation media (E1). The values are the mean ± SE (n = 30). Different letters indicate significant differences ($P < 0.05$).

Figure 2. (A) Typical basal callus formation in response to a 0.5 mg/l IBA or NAA, or (B) 0.5 mg/l IAA.

used in some studies for comparative purposes against clone 2, the focus of subsequent investigations.

To further characterise the poor-rooting clone 2, its rooting response in the presence of different auxin analogues was assessed. The response of its shoots to 0.1 and 0.5 mg/l IBA, IAA and NAA, following elongation on the standard medium (E1), indicated that none of the tested auxins yielded greater than 50% rooting (Figure 1B). Mean rooting times for each auxin analogue (0.1 and 0.5 mg/l) were recorded as IBA = 5.9 and 7.6 days; IAA = 3.9 and 8.9 days; and NAA = 6.1 and 6.4 days. The 0.1 mg/l IAA treatment resulted in the least efficient root production (30%) (Figure 1B). Furthermore, upon these treatments, basal callus was observed in all the shoots, with callus production being greatest at 0.5 mg/l, regardless of the type of auxin used (Figure 2). At

Table 2. Percentage rooting of clone 2 shoots cultured on rooting media containing 0.5 mg/l indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) or α-napthalene acetic acid (NAA), together with the auxin signal transduction inhibitor ρ-chlorophenoxyisobutyric acid (PCIB, 50 µM).

Time (days)	% Rooting		
	IAA	IBA	NAA
5	0	0	0
15	6.3±5.5	0	0
30	6.3±5.5	6±5.7	5.3±5.5

The values are the mean ± SE (n = 30). All treatments were found to be statistically similar (P > 0.05 for all statistical comparisons).

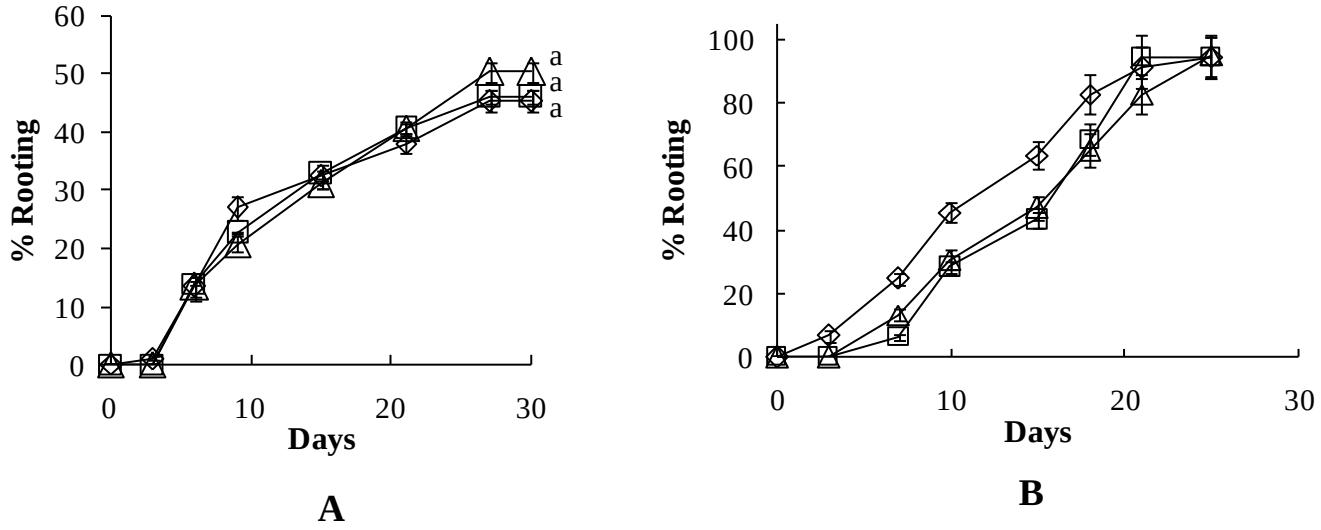

Figure 3. (A) Percentage rooting of clone 2 shoots, over 30 days on rooting medium containing 0.1 mg/l IBA alone (◊), and 0.1 mg/l IBA with either 1 mM (□) or 2 mM 1,6-dihydroxyacetophenone (DHAP) (Δ). Shoots were produced on standard shoot proliferation and elongation media. The values are the mean ± SE (n = 30). The treatments were found to be statistically similar, as indicated by common letters (P > 0.05); (B) percentage rooting of shoots of clone 2, elongated on E7 (Table 1) and transferred to rooting media containing 0.1 mg/l IAA (◊), IBA (□) or NAA (Δ). The values are the mean ± SE (n = 30). The data were found to be statistically similar (P > 0.05).

this concentration, IBA and NAA resulted in larger basal callus formation than IAA (Figure 2).

The extent to which the shoots of clone 2 were able to perceive exogenous auxin was then tested by including the inhibitor of auxin signal transduction, PCIB (Oono et al., 2003) in the rooting medium, which was also supplemented with 0.5 mg/l IBA, IAA or NAA (Table 2). In the presence of 50 µM PCIB, none of the auxin treatments were able to induce root production significantly by day 30 (Table 2).

In order to test the hypothesis that rooting efficiency is dependent on a clone's ability to hydrolyse auxin conjugates (van der Krieken et al., 1992; Epstein and Ludwig-Müller, 1993; Epstein et al., 1993; George et al., 2008), the rooting ability of clone 2 was assessed by including the auxin conjugation inhibitor DHAP, together with 0.1 mg/l IBA in the rooting medium (Figure 3A). By the end of the 30 day culture period, no significant increase in rootability was observed, with rooting still below 55% in the presence of 2 mM DHAP (Figure 3A). Mean rooting time for the 0.1 mg/l IBA (without DHAP) was 5.9 days, 6.5 days for the 0.1 mg/l + 1 mM DHAP, and 7.7 days for the 0.1 mg/l + 2 mM DHAP treatments.

Having established that clone 2 was able to perceive exogenous auxin (Table 2) and inhibition of exogenous auxin conjugation did not markedly increase its rooting efficiency (Figure 3A), the effect of plant growth regulators (PGRs) supplied during the elongation stage

Table 3. Percentage rooting of shoots of clone 1 and clone 2 after 30 days, following elongation on different media (E1 to E7), and rooted in the absence of exogenous auxins. E1 = standard protocol (Table 1).

Elongation media	PGR (mg/l)	% Rooting	
		Clone 1	Clone 2
E1	0.3 NAA, 0.2 kinetin, 0.05 IBA	100[a]	10±2[d]
E2	0.3 NAA, 0.2 kinetin	68.3±2.9[b]	6±5.3[d]
E3	0.05 IBA, 0.2 kinetin	29±3.6[c]	18±2[e]
E4	0.37 IAA, 0.2 kinetin	31.3±5.5[c]	19±2.6[e]
E5	0.3 NAA, 0.05 IBA	95±8.6[a]	80.3±4.5[f]
E6	0.2 kinetin	37.3±2.5[c]	21.3±4.2[e]
E7	None	91.7±7.6[a]	9.3±1.1[d]

The values are the mean ± SE (n = 30). Values that do not share letters indicate treatments that were significantly different ($P < 0.05$) from each other.

on rooting was tested. Shoots of clone 1 (good rooter) and clone 2 (poor rooter) were transferred from shoot proliferation medium onto seven elongation media, each with different combinations of auxin and cytokinin analogues (variation on the standard elongation medium, E1) (Table 1). Following these treatments, the shoots were transferred to rooting medium lacking auxin. The results show that the levels and combinations of the PGRs used during shoot elongation significantly influenced the rooting ability of the shoots of both tested clones (Table 3). Clone 1 rooted best (100%) when elongated on the standard medium (E1, containing kinetin, NAA and IBA), on the medium lacking kinetin (E5, containing NAA and IBA) and on the medium devoid of PGRs (E7) (Table 3). However, its percentage rooting was significantly inhibited when kinetin was supplied alone (E6), or in conjunction with the unstable auxin IAA (E4), or with a low concentration of the stable auxin IBA (E3) (Table 3).

While a similar trend in rooting ability in response to the auxins and cytokinin present in the elongation media was observed for shoots of clone 2, it was apparent that this clone was more dependent on exogenous auxin to counteract the inhibitory effects of kinetin on root production than clone 1 (Table 3). In the presence of kinetin, root production was inhibited in relation to the concentration and stability of the auxins used in the elongation stage. This was indicated by the percentage rooting of clone 2 shoots following elongation on media E2, E3 and E4 (Table 3). While no significant difference in rooting was observed between E1 and E2 (containing the relatively stable, but synthetic NAA), a significant increase in rooting resulted following elongation on media containing kinetin and either a low concentration of the relatively stable IBA (E3), or a higher concentration of the less stable IAA (E4) (Table 3). The percentage rooting of clone 2 shoots elongated with kinetin alone was not significantly different from that obtained, following elongation on E3 or E4, eluding to the possible effects of endogenous auxins on rootability (Table 3).

As the rooting results of clone 2 from the E1 and E7 treatments show, the absence of exogenous phytohormones from the elongation stage did not significantly affect root production (Table 3). However, rooting was significantly enhanced (80.3%) by removing the kinetin while retaining the auxins NAA and IBA (E5) during shoot elongation, prior to rooting on an auxin-free rooting medium. The rooting ability of this clone was restored (100% rooting) with the addition of 0.1 mg/l IAA, IBA or NAA to the rooting medium (Figure 3B), with mean rooting times of 12.3, 14.9 and 15.2 days for the IAA, IBA and NAA treatments, respectively.

The inability of clone 2 shoots to produce roots when subjected to the standard protocol (E1), and the restoration of rootability by removing kinetin from the elongation medium suggested that exogenous cytokinin during the pre-rooting culture stages inhibited root production, either directly or through the alteration of endogenous phytohormone levels in the shoots. To test this, the endogenous levels of kinetin, IAA, IBA and NAA of shoots of clones 1 and 2, cultured on selected elongation media (E1, E5, E6 and E7) were determined using GC-MS (Table 4). As expected, shoots produced on media containing no phytohormones (E7) had the lowest levels of these compounds and those produced on media containing kinetin (E1 and E6) had the highest synthetic cytokinin content, irrespective of the clone identity. The addition of the auxin analogues IBA and NAA (E1 and E6) resulted in an increase in endogenous IAA content to levels comparable or in excess of those of the auxin analogues themselves (Table 4). After elongation on E1, the ratio of kinetin to auxin was four times higher in clone 2 (poor rooter) than in clone 1 (good rooter) (2.6 and 0.58, respectively). However, when

Table 4. Average concentrations (± standard error of the mean) of IAA, IBA, NAA and kinetin in elongated shoots of the tested clones, prior to rooting in an auxin-free medium, following each elongation treatment.

Clone	Elongation medium	PGR (µmol/g DW)				Total cytokinin/total auxin
		IAA	IBA	NAA	Kinetin	
1	E1	0.33±0.08	0.33±0.02	0.15±0.04	0.47±0.2	0.58
	E5	0.13±0.015	0.02±0.007	0.22±0.13	0.12±0.03	0.2
	E6	0.12±0.1	0.04±0.03	0.07±0.03	0.51±0.2	2.2
	E7	0.08±0.04	0.02±0.01	0.014±0.01	0.09±0.07	0.8
2	E1	0.2±0.05	0.05±0.02	0.14±0.09	1.01±0.2	2.6
	E5	0.18±0.06	0.03±0.008	0.63±0.3	0.33±0.04	0.39
	E6	0.17±0.08	0.09±0.06	0.02±0.013	0.68±0.35	2.42
	E7	0.06±0.04	0.02±0.004	0.007	0.28±0.16	3

Refer to Table 3 for % rooting for each treatment. The ratio of kinetin: auxin is indicated.

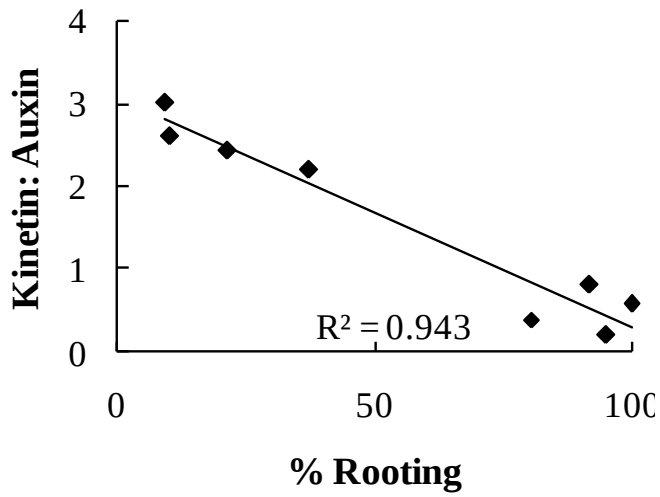

Figure 4. Linear regression of rootability (percentage rooting) to endogenous cytokinin:auxin ratios during the elongation culture stage.

shoots of clone 2 were elongated on E5 (excluding kinetin, but containing NAA and IBA), kinetin:auxin was reduced to 0.39 (Table 4) and rooting increased to 80% (Table 3). A strong relationship between the kinetin:auxin ratio and percent rooting (R^2=0.943) of shoots of both tested clones following elongation on selected media, indicated the inhibitory effect of high kinetin:auxin on root induction (Figure 4).

Since kinetin appeared to accumulate to higher amounts in the shoots of clone 2 than in clone 1 and subsequently inhibited rooting, an additional elongation medium was tested (E8). It contained *trans*-zeatin, a cytokinin less stable than kinetin (George et al., 2008), and was selected in an attempt to reduce the inhibitory effect of cytokinin accumulation on root induction. This *trans*-zeatin-containing medium (E8) was comparable to E4, except that kinetin was replaced with 0.91 µM *trans*-zeatin (Table 1). Following elongation, shoots were transferred to rooting media containing 0.1 mg/l IAA. Under these conditions, percentage rooting was significantly higher in the *trans*-zeatin (E8) than in kinetin-containing (E4) medium, albeit lower than on the medium devoid of cytokinins (E5) (Figure 5A).

Mean rooting times for shoots on these treatments (Figure 5A) were 12.3, 8.11 and 2.9 days for cytokinin-free, *trans*-zeatin and kinetin-containing media,

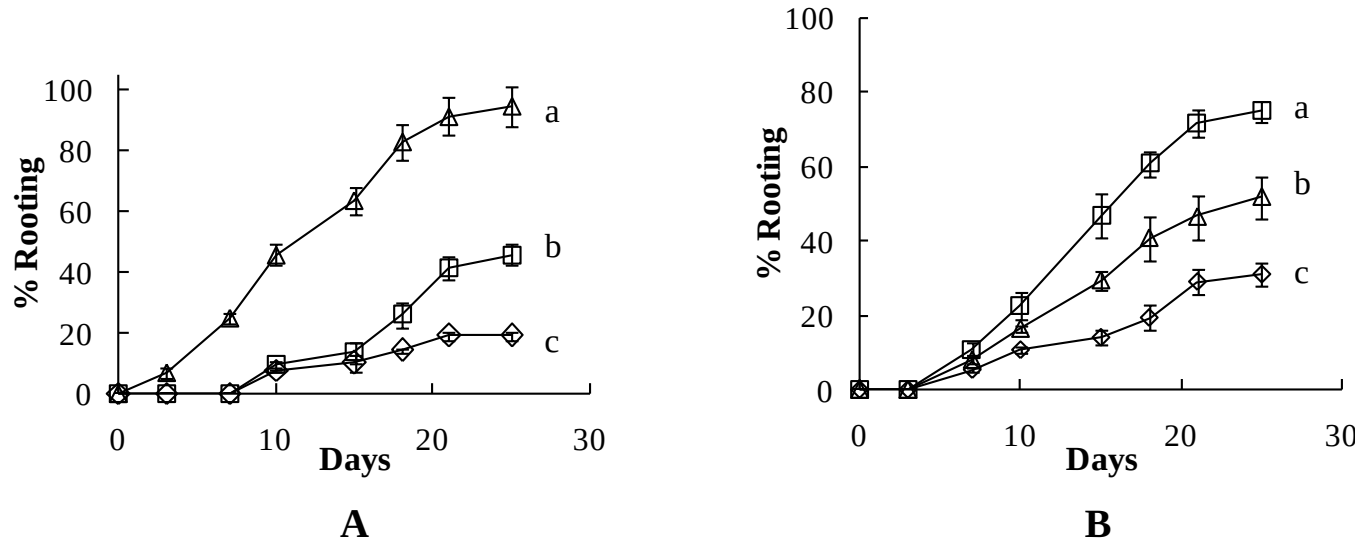

Figure 5. (A) Percentage rooting of shoots of clone 2, elongated on either E4 (kinetin-containing medium) (◊), E7 (no cytokinins) (Δ), or E8 (*trans*-zeatin-containing medium) (□), followed by rooting on medium containing 0.1 mg/l IAA. The values are the mean ± SE (n = 30). Different letters denote significant differences (P < 0.05); (B) percentage rooting of shoots of clone 3, elongated on either E4 (kinetin-containing medium) (◊), E7 (no cytokinins) (□), or E8 (*trans*-zeatin-containing medium) (Δ), followed by rooting on media containing 0.1 mg/l IAA. The values are the mean ± SE (n = 30). Different letters denote significant differences between treatments (P < 0.05).

respectively. The observed inhibitory effect of kinetin and *trans*-zeatin supply during shoot elongation on subsequent rooting was then tested with clone 3, another poor rooting clone (Figure 1A). Following elongation on E4 (kinetin-containing), E5 (cytokinin-free), and E8 (*trans*-zeatin-containing) (Figure 5B), it became apparent that the rooting ability of the two tested poor-rooters could be significantly improved by omitting or modifying the cytokinin type used in the elongation medium. Recorded mean rooting times were 11.2, 7.9 and 4.9 days for the cytokinin-free, *trans*-zeatin and kinetin treatments, respectively.

DISCUSSION

Successful root induction is a critical step in vegetative propagation programs, and different species and genotypes within a species are known to vary greatly in this regard (Eldridge et al., 1994; Jones and van Staden, 1997; George et al., 2008). Such variation has been attributed to a range of biotic and abiotic factors (Geiss et al., 2009), most notably the availability of PGRs, particularly auxins and cytokinins (George et al., 2008).

The present study was aimed at elucidating the effects of auxins and cytokinins on root induction in three eucalypt clones of varying rooting ability (Figure 1A), using an *in vitro* approach. It was initially found that the popular auxins employed in *Eucalyptus* sp. micropropagation, that is IBA, IAA and NAA, did not increase the rooting ability of clone 2 (poor-rooter) (Figure 1B), none of which were able to produce over 50% rooted shoots, even at 0.5 mg/l (Figure 1B). Predictably, given that IAA is the least stable of the tested auxins (George et al., 2008), treatment of clone 2 shoots with 0.1 mg/l IAA resulted in the lowest rooting potential. While callus production was visible at the base of all shoots, those treated with 0.5 mg/l IAA developed the least basal callus, in keeping with the higher stabilities of IBA and NAA in plant tissues (George et al., 2008). The lack of any significant rooting response from shoots of clone 2 in the presence of PCIB, a known inhibitor of auxin signal transduction (Oono et al., 2003), indicated that it was able to perceive the exogenous auxin (Table 2). This, together with shoot basal callus formation in the presence of exogenous auxin in the rooting stage (without PCIB), suggested that in this poor-rooter (clone 2), the supplied auxin was directed towards callus formation, rather than rhizogenesis.

Studies into auxin metabolism have shown that auxins are rapidly taken up by cells either through influx carrier proteins or through passive diffusion (Leyser, 1999; Muday and DeLong, 2001). Further, they are rapidly conjugated or oxidated to inactive forms through enzymatic action within the cell (De Klerk et al., 1999; George et al., 2008), which results in only a small portion

of the supplied auxin occurring in the free form (De Klerk et al., 1999). This has led to the suggestion that a clone's ability to produce roots depends on the ease and timing at which it can hydrolyse these conjugated auxins to free auxin forms (van der Krieken et al., 1992; Epstein and Ludwig-Müller, 1993; Epstein et al., 1993; George et al., 2008). In support of this, work conducted by Epstein et al. (1993) on cuttings of sweet cherry, showed that an easy-to-root cultivar metabolised IBA conjugates slower than a difficult-to-root cultivar, leaving free IBA available for a longer period within the shoots. Such conjugates have been suggested to serve as a sustainable source of auxin (Wiesman et al., 1989).

The addition of the auxin conjugation inhibitor DHAP significantly improved the percentage rooting in difficult-to-root cuttings in that study (Epstein et al., 1993). However, such a response was not obtained in the present investigation with the addition of 2 mM DHAP. This did not significantly increase the rootability of clone 2 shoots, indicating that this clone's poor rooting ability could not be attributed solely to its inability to hydrolyse auxin conjugates (Figure 3A). Auxin metabolism is not autonomous, and a complex interaction exists between auxin and a number of other plant growth regulators. Of interest to the present study, was the interaction between auxins and cytokinins as they are the main PGRs used in *in vitro* regeneration protocols (George et al., 2008). The general model of organogenesis states that a high auxin to low cytokinin ratio favours root formation, while a high cytokinin to low auxin ratio favours shoot proliferation (Skoog and Miller, 1957; George et al., 2008), a principle applied in micropropagation protocols. Both auxins and cytokinins are essential in the regulation of the cell cycle (Mok and Mok, 1994) and they have been shown to work antagonistically within root tissues (Brault and Maldiney, 1999; George et al., 2008; Kuderová and Hejátko, 2009). Cytokinins have also been found to modulate auxin-induced organogenic processes through the regulation of auxin efflux (Pernisová et al., 2009).

Apart from research based on establishing effective (largely clone-specific) *in vitro* protocols by Le Roux and van Staden (1991), Jones and van Staden (1997), Gomes and Canhoto (2003) and Arya et al. (2009), relatively few studies have documented the interaction between auxins and cytokinins on the rootability through micro- or macro-propagation of commercially important eucalypt clones, or the reasons for poor rooting percentages achieved with many of these clones.

On the hypothesis that the plant growth regulators used during the pre-rooting culture stages affected the tested clones' rootability, both clones were elongated on a range of media (Table 1) and subsequently transferred to rooting medium without exogenous auxins. Compared with clone 2 (poor rooter), shoots of clone 1 (good rooter) displayed a greater ease of rooting relative to the exogenous auxin type and concentration supplied in the elongation treatments (Table 3). The more stable auxins, IBA and NAA, in elongation yielded higher rooting percentages for clone 1 than the elongation medium with IAA (except in E5, in which the IBA concentration was minimal). Shoots of clone 2 (poor rooter) on the other hand, were not able to achieve more than 22% rooting when elongated on media containing kinetin, either with or without auxins. Eliminating kinetin from the elongation medium (E7) of clone 2, did however resulted in at least 80% rooting in auxin-free rooting medium and 100% rooting in media containing 0.1 mg/l auxin (either as IAA, IBA or NAA) (Figure 3B). This indicates that the poor rootability of some eucalypt clones may be due to excess cytokinin supplied and stored during the pre-rooting culture stages. This proposal was further supported by GC-MS analysis of IAA, IBA, NAA and kinetin in the shoots of both tested clones, following elongation on selected media (Table 4), but prior to rooting. From these results, it was deduced that the exogenous kinetin in the elongation stage heavily influenced the cytokinin:auxin ratio, thereby inhibiting root induction in clone 2 (Figure 4).

Cytokinins themselves have complex metabolic pathways which include conjugation and degradation reactions (Mok and Mok, 1994; van Staden and Crouch, 1996; Haberer and Kieber, 2002; George et al., 2008). Natural cytokinins (such as *trans*-zeatin and isopentenyladenine) are degraded by the naturally-occuring enzyme cytokinin oxidase (Mok and Mok, 2001; Haberer and Kieber, 2002; George et al., 2008). The rapid enzymatic breakdown of some natural cytokinins has been regarded as the reason for their ineffectiveness in many culture protocols. In this context, it has been suggested that cytokinins that are not substrates of the cytokinin oxidase enzyme (such as kinetin), may last longer in plant tissues (George et al., 2008).

In the present study, the synthetic cytokinin kinetin would therefore have likely persisted within shoots of the two poor-rooting clones. Since cytokinins generally delay or even inhibit root formation (Brault and Maldiney, 1999; George et al. 2008; Kuderová and Hejátko, 2009), the percentage rooting of clones 2 and 3, following elongation with either kinetin or *trans*-zeatin (Figure 5A and B), reflected the relative persistence of these cytokinins in shoots. These results suggest that a relationship exists between cytokinin depletion and root formation in the tested poor-rooting eucalypt clones, regardless of genotype. The use of *trans*-zeatin instead of kinetin during elongation did significantly increase the rooting ability of clones 2 (Figure 5A) and 3 (Figure 5B), while complete cytokinin omission during elongation resulted in even higher rooting percentages in both

clones 2 and 3 (Figure 5A and B).

Improving eucalypt micropropagation protocols to increase the yield of difficult-to-propagate clones (such as in the present study), is an essential tool in tree improvement programs, which are highly beneficial to the forestry industry (de Assis et al., 2004). Collectively, the data indicate that in the development of micropropagation protocols for specific clones, the complex interactions that exist between the two main phytohormone groups; auxins and cytokinins, and in particular, their stabilities and metabolic requirements within plant tissues, need to be considered in order to achieve the objective of each culture stage and hence, increased plantlet yields.

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