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The influence of ammonium nitrate, pH and indole butyric acid on root induction and survival in soil of micropropagated *Eucalyptus globulus*

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Abstract

Rooting of *Eucalyptus globulus* shoots was influenced by the concentration of the indole butyric acid (IBA) and NH_4^+ in the root-induction medium. Optimum plantlet vigor and survival were achieved using low concentrations (1 - 2.5 μM) of IBA and when NH_4NO_3 was removed. Removal of NH_4^+ also had a significant effect on medium pH, its presence caused a decrease in pH as the culture period proceeded. When different nitrate compounds (excluding NH_4NO_3) were used as the nitrogen source, the medium pH was more stable and this was associated with higher root production. The higher root production, in association with appropriate IBA concentrations, produced plantlets with higher survival and better growth on transfer to soil.

Additional key words: acclimatization, *in vitro* rooting, micropropagation, Tasmanian bluegum.

Introduction

Important features in the successful micropropagation of eucalypts are culture stabilisation through continuous subculture (McCown and McCown 1987, McComb *et al.* 1996, Trindade and Pais 1997, McCown 2000), within and between species variation (Le Roux and Van Staden 1991, McComb *et al.* 1996) and media composition and culture protocol (Curir *et al.* 1990, Bennett *et al.* 1994). While successful shoot multiplication is readily achievable for most species, there are often problems with root production, particularly when mature explants are used.

Some eucalypts are particularly recalcitrant *in vitro* (Le Roux and Van Staden 1991, McComb *et al.* 1996). These include economically important species such as *E. grandis* (Lakshmi Sita and Shoba Rani 1985, Mac Rae and van Staden 1990), *E. regnans* (Blomstedt 1991) and *E. globulus* (Hartney and Barker 1983, Bennett *et al.* 1994, Trindade and Pais 1997). Recent studies have shown that adjustments to media composition and culture conditions can produce significant improvements in responses obtained. For example, rooting can be improved by alteration to the composition of the multiplication media, an approach which has been successful for *E. gunnii* (Curir *et al.* 1990) and *E. globulus* (Bennett *et al.* 1994).

The presence of auxin and medium nutrient composition have been shown to influence root production in many woody species (Sriskandarajah *et al.* 1990, Orlikowska 1992). The most significant nutrient is usually nitrogen. We report here the improvement in adventitious root production and subsequent survival in soil of *E. globulus*, resulting from adjustments to nutrient and auxin content of the root induction medium. We also illustrate some chemical changes that can occur by varying media components.

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Abbreviations: EDTA - ethylenediaminetetraacetate; IBA - indole butyric acid; MS medium - Murashige and Skoog (1962) medium.

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Materials and methods

Shoot multiplication: Shoot cultures were initiated from coppice of 4 to 5-year-old *E. globulus* trees selected for high growth rates and superior pulping quality. Shoots used in all rooting experiments were multiplied using the protocol reported by Bennett *et al.* (1994) for at least 18 months. This involved alternating the cytokinin in the multiplication medium at each subculture. The multiplication medium contained Murashige and Skoog (1962) (MS medium) nutrients and organics, 2 % sucrose, either 2.5 μM benzyl-aminopurine or 2.5 μM kinetin, 2.5 g dm^{-3} agar, 2.5 g dm^{-3} phytogel and the pH adjusted to 5.8 prior to autoclaving. Shoots were taken from medium containing 2.5 μM kinetin prior to root induction. Clone numbers correspond to those used by Bennett *et al.* (1994). Shoot cultures were maintained in a temperature of 25 °C and a 16 h photoperiod with an irradiance of 90 μmol $m^{-2} s^{-1}$.

Basal rooting medium: This contained 1/2 strength MS medium macronutrients, full strength micronutrients, 1/2 strength FeNaEDTA, 2 % sucrose, indole butyric acid (IBA) at various concentrations, 2.5 g dm^{-3} agar, 2.5 g dm^{-3} phytogel, and the pH was adjusted to 5.5 before autoclaving (McComb and Bennett 1982). When shoots were placed on rooting media they were kept in complete darkness for 7 d then transferred to the same environmental conditions as the shoot cultures. For rooting experiments, five shoots were placed in each culture vessel (250 cm^3 polycarbonate with 50 cm^3 of medium), at least 25 shoots were used for each treatment.

Nutrients: Shoots of clone 9 were transferred to rooting media containing 10 μM IBA with: *1*) 0, 1/4, 1/2, and full strength of MS medium macronutrients, *2*) different concentrations of NH_4NO_3 (0 and 1/4 strength), KNO_3 (1/4, 1/2, and full strength) and FeNaEDTA (1/4 and 1/2 strength), *3*) different concentrations of NH_4NO_3 (0, 1/4, and 1/2 strength), KNO_3 (1/4 and 1/2 strength) and FeNaEDTA (1/4 and 1/2 strength), and *4*) 5 or 10 mM KNO_3, in combination with one of the following: 2.5 mM $Ca(NO_3)_2$, 5 mM NH_4NO_3, 2.5 mM $(NH_4)_2SO_4$, 1.25 mM $(NH_4)_2SO_4$, or 0.625 mM $(NH_4)_2SO_4$. The pH of these media was measured at regular intervals up to 28 d after shoots were transferred to rooting media.

pH: Shoots (from clone 9) were transferred to basal rooting medium with 5 mM KNO_3 and 10 μM IBA (without NH_4NO_3). The pH was adjusted prior to autoclaving to 6.0, 5.5, 5.0, 4.5, 4.0 and a medium with 5 mM NH_4NO_3 and pH 5.5 was also included. Rooting and medium pH in each culture vessel was measured after 4 weeks.

IBA concentration: To determine the optimum IBA concentration for root induction, shoots of *E. globulus* (clone 9) were transferred to rooting media containing 0, 1.0, 2.5, 5.0 or 10.0 μM IBA in combination with 5.0 mM NH_4NO_3, 2.5 mM $(NH_4)_2SO_4$ or no NH_4^+ compound.

Effect of rooting medium on survival of plantlets in soil: To determine survival of plantlets in soil, plantlets were produced on rooting medium containing 1.0, 5.0 or 10 μM IBA and no NH_4^+. Plantlets from the medium with 1.0 or 10.0 μM IBA media were sorted into categories according to the number of roots per shoot (1, 2-3, 4-5 and 6+) and then transferred to soil. Plantlets were transferred to small pots (*ca.* 85 cm^3) containing steam pasteurised soil (coarse sand:peat:perlite 2:1:2) and maintained under a wet tent (Drew *et al.* 1991) for two weeks. Relative humidity was maintained at 87 - 100 % with a temperature range of 28 - 29 °C bottom heat and 20 - 28 °C air temperature. Plantlets were planted in randomised blocks with, in each block, 5 plantlets from each treatment. The plants were then placed under shadecloth (50 % light penetration) on a glasshouse bench for 1 week before survival and height were recorded.

Culture assessment and statistical analysis: Rooting response was scored after 28 d on rooting media by calculating the percentage rooting in each culture container and counting the number of roots produced by each shoot. Shoots were characterised by chlorophyll content (Bennett *et al.* 1994) measured according to Moran and Porath (1980). Medium pH was measured using a surface sterilised *IJ42* pH probe (*Ionode Pty. Ltd.*, Queensland, Australia) inserted into the medium approximately 1 - 2 mm from the cut end of the shoots.

Pearson's correlation coefficient was used to examine relationships between pH and rooting and pH and shoot chlorophyll content. One way analysis of variance was performed on percentage rooting and the mean number of roots produced per shoot for each experiment. If data were not homogeneous according to Levene's test, either a $\ln(x+1)$ transformation or an arcsin transformation (for percentages) was performed. Where there was a difference due to treatment, Tukey's multiple range test was applied to compare means. Results were considered significant when they were within 95 % confidence intervals.

Results

Effects of nutrients and pH: It was found that a decrease in rooting resulted when KNO_3 or $MgSO_4$ concentrations were reduced to zero, but an increase was obtained when NH_4NO_3 concentration was reduced to zero (data not shown). Varying the concentrations of KNO_3, NH_4NO_3 and FeNaEDTA suggested that optimum rooting, both in terms of percentage rooting and mean number of roots per shoot, was obtained when NH_4^+ was removed from the medium. The 1/4 and 1/2 strength concentrations of KNO_3^- and FeNaEDTA tested did not alter rooting (Table 1).

Different sources and concentrations of nitrogen produced a similar result with higher rooting occurring when concentrations of NH_4^+ were reduced or no NH_4^+ was present. The rooting response was similar whether NO_3^- was supplied as $Ca(NO_3)_2$ or KNO_3 (Table 2).

Table 1. Rooting of shoots from *E. globulus* clone 9 on root induction media containing different concentrations of NH_4NO_3, KNO_3 and FeNaEDTA (proportion of MS medium). Different superscripts indicate significant differences to other treatments.

NH_4NO_3	KNO_3	FeNaEDTA	Rooting [%]	Number of roots [shoot^{-1}]
0	¼	¼	94 ± 3^a	5.9 ± 0.6^a
¼	¼	¼	62 ± 6^b	4.2 ± 0.7^b
½	¼	¼	62 ± 9^b	3.0 ± 0.5^b
0	½	¼	90 ± 3^a	7.0 ± 0.7^a
¼	½	¼	64 ± 6^b	4.1 ± 0.7^b
½	½	¼	49 ± 4^b	3.1 ± 0.6^b
¼	¼	½	60 ± 9^b	3.4 ± 0.5^b

Table 2. Rooting of shoots of *E. globulus* clone 9 on media containing different sources of nitrogen. Different superscripts indicate significant differences to other treatments.

Medium	Number of roots [shoot^{-1}]
5 mM KNO_3	2.5 ± 0.4^a
10 mM KNO_3	2.5 ± 0.4^a
5 mM KNO_3 + 2.5 mM $Ca(NO_3)_2$	2.4 ± 0.3^a
5 mM KNO_3 + 5.0 mM NH_4NO_3	1.5 ± 0.3^{ab}
5 mM KNO_3 + 2.5 mM $(NH_4)_2SO_4$	0.9 ± 0.2^b
5 mM KNO_3 + 1.2 mM $(NH_4)_2SO_4$	1.5 ± 0.4^{ab}
5 mM KNO_3 + 0.6 mM $(NH_4)_2SO_4$	2.2 ± 0.4^a

All media containing NH_4^+ showed a rapid decrease in pH over the first 7 d, with values leveling off to between 4.0 and 4.5. Media without NH_4^+ showed less of an initial decrease in pH and after 7 d pH values rose and stabilised at values between 5.5 and 6.0 (Fig. 1).

The initial pH of the medium did not affect the number of roots produced from the shoots. As in the previous experiment, the final pH was related to medium nutrient content, and not the initial medium pH. Media that contained no NH_4^+ had a final mean pH between 5.64 and 5.82 while the medium with NH_4^+ had a final mean pH 4.20

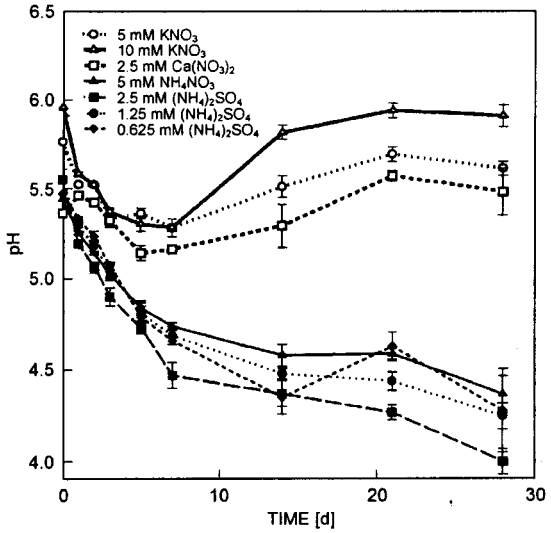

Fig. 1. Change in pH of medium containing *E. globulus* shoots. Shoots were grown in media containing various nitrogen sources over 28 d. The *vertical bars* represent standard errors of the mean of 5 replicates.

Table 3. Rooting of shoots of *E. globulus* clone 9 on media containing no NH_4^+ and differing initial pH values. Different superscripts indicate significant differences to other treatments. * - medium with 5 mM NH_4NO_3.

Medium pH	pH after 4 weeks	Number of roots [shoot^{-1}]
6.0	5.98 ± 0.15^a	2.6 ± 0.3^a
5.5	5.64 ± 0.11^a	2.1 ± 0.4^a
5.0	5.75 ± 0.10^a	1.8 ± 0.3^a
4.5	5.82 ± 0.07^a	2.3 ± 0.4^a
4.0	5.82 ± 0.15^a	2.0 ± 0.4^a
5.5*	4.20 ± 0.10^b	1.3 ± 0.3^b

Table 4. Rooting and chlorophyll content of shoots of clone 9 on root induction medium with different concentrations of IBA. Different superscripts indicate significant differences to other treatments.

IBA [μM]	Number of roots [shoot^{-1}]	Chlorophyll content [μg g^{-1}(f.m.)]
0	0	828 ± 31^a
1	3.6 ± 0.5^b	1124 ± 62^b
5	5.1 ± 0.8^b	814 ± 53^a
10	4.1 ± 0.8^b	669 ± 56^b

(Table 3). Again a lower number of roots per shoot were produced on the medium with NH_4^+.

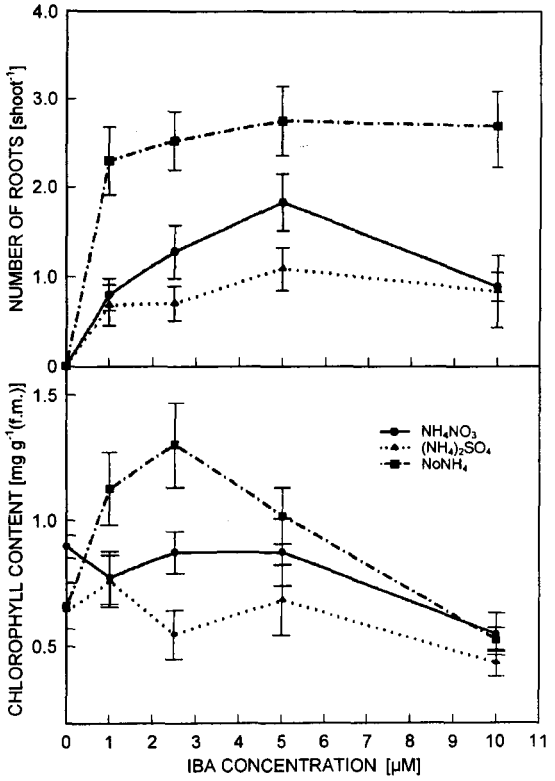

Fig. 2. Responses of *E. globulus* (clone 9) shoots to variations in auxin and NH_4^+ content. Mean numbers of roots per shoot were recorded from 50 replicates for each IBA treatment, and mean chlorophyll content from 10 shoots of each treatment. *Vertical bars* represent standard errors of the mean ($NoNH_4$ - medium without NH_4^+).

Effects of auxin: Shoots on media containing no IBA produced very few roots but there was no difference in the number of rooted shoots or mean number of roots per shoot on media containing IBA (1 - 10 µM). However, the chlorophyll content of the shoots was optimal at 1 µM IBA (Table 4). Using different sources of NH_4^+ in the rooting medium and increasing the range of IBA concentrations produced a similar result with the optimum chlorophyll content obtained on media containing 1-5 µM IBA without NH_4^+ (Fig. 2). There was no correlation between the number of roots on a shoot and its chlorophyll content.

Transfer of plantlets to soil: Survival in soil increased when plantlets had higher numbers of roots when potted out. When plantlets with the same number of roots were compared from media with 1 or 10 µM IBA, those originating from media with the lowest auxin, showed highest survival (Table 5). The same response pattern was observed with shoot heights after 3 weeks. Plants with higher numbers of roots were taller, and those from the medium with 1 µM IBA were taller than those from 10 µM IBA medium (Table 5).

Table 5. Survival and shoot height 3 weeks after transfer to soil of plantlets of clone 9 taken from rooting medium containing 1 or 10 µM IBA. Different superscripts indicate significant differences to other treatments.

Roots	Survival [%]		Shoot height [mm]	
	1 µM	10 µM	1 µM	10 µM
1	67 ± 10^a	24 ± 13^a	37 ± 5^a	26 ± 5^a
2 - 31	82 ± 6^{ab}	44 ± 7^a	54 ± 3^b	27 ± 4^a
4 - 5	92 ± 8^{bc}	56 ± 7^a	58 ± 5^b	34 ± 4^a
> 6	100 ± 0^c	75 ± 5^a	80 ± 4^c	36 ± 4^a

Discussion

For a number of species, improvement in rooting has been shown to be a consequence of varying NH_4^+:NO_3^- ratios in the medium (Hyndman *et al.* 1982, Sriskandarajah *et al.* 1990). For clone 9 of *E. globulus* the removal of NH_4^+ from the root induction medium clearly improved the rooting response. This, in combination with use of an appropriate IBA concentration, substantially increased the growth and survival of rooted plantlets. When these modifications were applied to three other clones, all showed improved rooting.

The change in medium pH, as the culture period progressed, has been reported previously, but this has not necessarily been linked to NH_4^+ content (Williams *et al.* 1985, 1990). George (1993) discussed how NH_4^+:NO_3^- ratios can buffer a medium, however, the responses reported were variable. We found no buffering effect by altering NH_4^+:NO_3^- ratios in media containing NH_4^+, while the pH in media with NO_3^- as its sole source of nitrogen remained relatively constant. The large changes in pH in media containing NH_4^+ may be partly due to low levels of other macronutrients in our basal medium (*i.e.* 1/4 strength MS medium). When full strength MS medium macronutrients are used, compounds such as KH_2PO_4 may have a substantial buffering effect.

The mechanism of the effects of pH on plant responses *in vitro* is uncertain (Williams *et al.* 1985, 1990, Williams 1993). It has been suggested that it may assist in auxin action or that auxin action is optimal at a particular pH (Selby and Harvey 1990). It is difficult to disentangle the influence of NH_4^+ and NO_3^- ratios and medium pH because in most of the work reported, pH has not been measured (*e.g.* Chaillou *et al.* 1991, Grimes and Hodges 1990). There is no indication from our work that lowering the pH or

changing NH_4^+:NO_3^- ratios, enhances the auxin effect. In addition, it is not possible to determine whether the effects obtained were due to varying nitrogen sources, variations in pH or a combination of both. It is possible that the change in pH influences nitrogen availability with a subsequent effect on root production. Investigation into the appropriate use of buffers (*e.g.* 2-(N-morpholino) ethanesulfonic acid) may help separate the influences of these two variables.

Although an increase in root number might be expected to result in higher survival and better growth in the glasshouse, we showed that root number alone is not sufficient to achieve this. The higher concentrations of IBA produced similar numbers of roots per shoot, but a better plantlet, in terms of growth and survival, was obtained from media with lower IBA. We have shown that even minor alterations of medium components can increase the proportion of plantlets in the rooting categories required for high survival in soil (*i.e.* from a mean of 1 root/shoot to 2 or more roots per shoot).

The alteration of medium components other than auxin can result in a significant improvement on the number of plants that are readily produced from this micropropagation protocol. In our situation, by removing the NH_4^+ a greater proportion of plantlets are produced with more than two roots per shoot, resulting in higher survival in soil provided the appropriate concentration of auxin is used in the root induction medium. This improvement takes the micropropagation protocol for *E. globulus* one step closer to commercialisation.

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