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Activated Charcoal Improves Rooting in *in Vitro*-Derived *Acacia leucophloea* Shoots

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ABSTRACT

An improved method of root induction in *in vitro*-derived *Acacia leucophloea* shoots was developed. Roots were induced on Murashige and Skoog medium containing half-strength nutrients, 3.0% sucrose, 1.0 mg l^{-1} indole-3-butyric acid and 200 mg l^{-1} activated charcoal (AC). Incorporation of AC could induce roots in more than 88% of shoots. The rooted plantlets were free from any callus and therefore showed better survival during hardening and acclimatization. The role of AC in obtaining callus-free rooting was highly beneficial.

Keywords: acclimatization, clonal propagation, hardening, *in vitro*, pulse treatment
Abbreviations: BA, 6-benzyl adenine; **CAZRI**, Central Arid Zone Research Institute; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **Kn**, kinetin; **MS**, Murashige and Skoog medium; **PGR**, plant growth regulator

INTRODUCTION

Natural regeneration in most forest tree species is very slow mainly on account of poor rate of seed germination and high rate of seedling mortality in natural habitats (Dewan *et al*. 1992). Emergence of newer tools of plant cell culture has opened up possibilities for rapid multiplication of such trees. The application of tissue culture methods like clonal propagation has gained momentum to meet the growing demands for biomass and forest products. However, commercialization of micropropagation technology has been hampered by poor rate of survival of *in vitro* raised plantlets during acclimatization (Kozai *et al.* 1997; Joshi *et al.* 2009). For many plant species, a high percentage of losses of *in vitro* propagated plantlets is accrued when transferring them *ex vitro* (Pospíšilová *et al*. 1999).

The micropropagation system for any plant species requires not only stable shoot multiplication but also successful rooting for *ex vitro* establishment of plantlets (De Klerk 2002). Good quality propagules with well-developed roots and leaves are easy to acclimatize to the *ex vitro* conditions (Nowak and Pruski 2004). Therefore, quality of tissue culture plantlets is an important factor for success during the transition to *ex vitro* conditions (Ray and Bhattacharya 2008). *De novo* formation of root meristem involves complex changes in the metabolism and it is evident that endogenous factors interact in the developmental shift leading to adventitious root formation both at the biochemical and molecular level (Caboni *et al.* 1997). Rooting of *in vitro* produced shoots of tree species is a complex phenomenon (Nandwani *et al.* 2004). The method of auxin treatment to the tissue culture-derived shoots is crucial since *in vitro*-raised plantlets may produce unwanted callus at the root shoot junction showing poor survival during soil transfer (Nagori *et al.* 2008). Callus formation with roots may also reduce the survival rate of *in vitro*-raised plantlets (Nandwani *et al.* 2004).

The accumulation of inhibitory substances (phenolic compounds) in the growth medium is a major problem more frequently associated with micropropagation and root formation in woody perennials (Vengadeshan *et al*. 2002). Activated charcoal (AC) promotes or inhibits *in vitro* growth, depending on the species and tissues used. Several strategies such as optimization of auxin types and concentrations (De Klerk *et al.* 1997), two-step rooting using pulse treatment of auxins (Gray and Benton 1991), reduction in concentration of salts and sucrose (Cheng *et al.* 1992), use of AC (Thomas 2008) and *ex vitro* rooting, combining both the rooting and hardening phase (Nowak and Pruski 2004) have been demonstrated in rooting of tissue culture propagules in order to improve rooting quality.

To promote *ex vitro* survival and physiological competence, especially to protect them against water stress and to encourage autotrophy a transitional environment, is usually provided for the acclimation interval of one to several weeks (Fabbri *et al.* 1986).

In the last decade, *in vitro* protocols to regenerate several woody species have been developed. Species of *Acacia* have been given due importance in tree tissue culture owing to their ecological and economic significance (Vengadesan *et al*. 2002). The present study was carried out to determine optimum conditions of rooting for *in vitro*-derived shoots of *Acacia leucophloea* for better field performance.

MATERIALS AND METHODS

The plant growth regulators i.e. 6-benzyl adenine (BA), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and kinetin (Kn) were purchased from Sigma-Aldrich, St. Louis, MI, USA and were plant cell culture tested. Mercuric chloride and activated charcoal (AC) GR grade were obtained from E-Merck (Worli, Mumbai, India). Mature and dry pods of *A. leucophloea* were collected from identified superior trees during the period of April-May from Badi village (15 km from Udaipur). Seeds were also obtained from CAZRI (Central Arid Zone Research Institute), Jodhpur.

Seeds were separated, washed thoroughly with tap water, kept in warm water at 70°C for 1 h and soaked overnight. To obtain seedlings, seeds were surface sterilized under aseptic conditions with autoclaved 0.1% mercuric chloride ($HgCl_2$) for 10 min and rinsed thoroughly with autoclaved double distilled water. Seeds were inoculated aseptically on plant growth regulator free Murashige and Skoog medium (Murashige and Skoog 1962) for germination. Cotyledonary nodes (1.5 cm) obtained from 20-25-days-old *in vitro* raised seedlings were cultured on MS basal medium

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(BMS) supplemented with 0.5 mgl^{-1} BA, 2.0 mgl^{-1} Kn and 0.05 mgl^{-1} IAA for both initiation and multiplication of shoot cultures (Sharma 1996). First subculture was done eight weeks after initial establishment of shoot cultures on MS medium supplemented with 0.5 mgl^{-1} BA, 2.0 mgl^{-1} Kn and 0.05 mgl^{-1} IAA. For subculture either the proliferating shoot culture was transferred to fresh medium along with mother explant or the proliferated shoots were clipped-off (diverted to rooting experiments) and the remaining stump with very small primordia was divided into two pieces and subcultured on same fresh medium. Successive subcultures were done at four weeks interval on the same fresh medium.

Rooting of *in vitro* elongated shoots was achieved on 1.0 mgl^{-1} IBA. However, such roots were associated with callus. To produce callus-free rooting elongated shoots were inoculated in MS medium containing reduced salt concentration (½, ¾ and ¼) and varying sucrose levels (0-5%). In dip treatment of elongated shoots, for root induction the cut end was dipped in pre-autoclaved aqueous solution of IBA (100, 500 and 1000 mgl^{-1}) for 10 min and inoculated on PGR-free ½ MS medium. Effect of addition of AC (50-500 mgl^{-1}) in the rooting medium was also studied. Each experiment was repeated three times with a minimum of 12 replicates each time.

The pH of the medium containing sucrose and 0.8% agar was adjusted to 5.8 prior to autoclaving at 1.06 kg cm^{-2} (121°C) for 15 min. All cultures were kept under controlled conditions of temperature (28±2°C), light (45 µmol m^{-2} s^{-1} in a 16-h photoperiod provided by cool fluorescent tubes) and 60-70% relative humidity.

For hardening and acclimatization, 15-days-old rooted plantlets of *A. leucophloea* were transferred to culture bottles containing autoclaved Soilrite™ after removing adhered medium. These plants were irrigated with ¼-strength MS liquid nutrient solution without sucrose for further growth. After 15 days the caps were loosened and finally opened and gradually exposed to the external environment for 2-3 days before they were transplanted to pots under mist house conditions. These pots were kept in locally fabricated polychambers for hardening. Humidity of 70-90% was maintained by regular spray misting of water. Plants were kept for 15 days in this chamber and gradually they were shifted to greenhouse conditions.

The experiments were set up in a randomized block design. Data were analysed in SPSS® 11.0 (SPSS Inc.) using one-way analysis of variance (ANOVA) to detect significant differences among means, expressed as the mean±standard deviation (SD). Tukey's test was used to determine significant differences between means.

RESULTS

In the present studies maximum rooting response of 80% was observed with 1.0 mgl^{-1} IBA with an average 3.4 roots per shoot with an average length 2.5 cm. Roots were produced within 15 days of inoculation. However, callus formation of different degrees was associated with all concentrations of IBA. Reduction of MS salt concentration to ½ supplemented with 1.0 mgl^{-1} IBA resulted in maximum rooting response of 85% with an average 4.6 roots per shoot with mean length of 3.5 cm (**Table 1**). The rooting was observed after 10 days of inoculation. Still there was little callusing associated with such rooted shoots. No rooting was observed in ¼ MS medium and water agar. Standard sucrose concentration of 3% was optimum for maximum rooting response (85%). Variation in sucrose concentration on either side did not improve the rooting response (**Table 2**). Shoots which were pulse treated with different concentration of IBA (100-1000 mgl^{-1}) for 10 min produced roots with different percentage. The best rooting response (70%) with an average of 2.5 roots per shoot of 2.5 cm mean length was observed when elongated shoots were pulse treated with 100 mgl^{-1} IBA for 10 min and subsequently inoculated on ½ MS medium with 3% sucrose and 0.8% agar (**Table 3**). Pulse treatment with higher concentration of IBA reduced the per cent rooting but also the root length. However, dip treatment was not found suitable for rooting due to callus formation at all the concentrations. It was therefore concluded that pulse treatment was not suitable for callus-free rooting. Addition of AC (200 mgl^{-1}) in the rooting medium (½ MS + 1.0 mgl^{-1} IBA) improved rooting response (88%) and eliminated the problem of callusing

Table 1 Effect of salt concentration of MS medium supplemented with 1.0 mg l^{-1} IBA on rooting in shoots of *A. leucophloea* obtained from seedling derived cotyledonary node segments.

MS salt concentration	Per cent rooting	Mean No. of roots ± S.D	SE	Mean length of roots ± S.D (in cm)	SE	Callus Intensity
BMS (control)	80.0	3.40 ± 0.55	0.24	2.50 ± 0.79	0.35	+
¾ MS	83.0	4.00 ± 1.00	0.45	3.10 ± 0.74	0.33	+
½ MS	85.0	4.60 ± 1.14	0.51	3.50 ± 1.06	0.47	+
¼ MS	-	-		-		–
W.A.	-	-		-		–
		2.077^{NS}		1.652^{NS}		

NS = non significant

Table 2 Effect of sucrose concentration on rooting of shoots obtained from seedling nodes of *A. leucophloea* and implanted on ½ strength MS medium containing 1.0 mg l^{-1} IBA.

Sucrose concentration (g l^{-1})	Per cent rooting	Mean No. of roots ± S.D.	SE	Mean length of roots ± S.D. (cm)	SE	Callus intensity
0	00.0	--	-	-	-	-
10	25.0	1.20 ± 0.45	0.20	1.50 ± 0.35	0.16	+
20	61.9	1.80 ± 0.84	0.37	2.40 ± 0.42	0.19	+
30	85.0	4.60 ± 1.52	0.68	3.50 ± 1.06	0.47	+
40	75.0	3.20 ± 0.84	0.37	2.00 ± 0.50	0.22	+
50	36.0	2.20 ± 0.84	0.37	2.20 ± 0.47	0.21	+
		9.674^{***}		7.216^{***}		

*** = Significant at 0.1% ($P < 0.001$)

Table 3 Effect of pulse treatment of pre-autoclaved IBA (10 min) on root induction in shoots of *A. leucophloea* implanted on MS medium.

IBA concentration (mg l^{-1})	Per cent rooting	Mean No. of roots ± S.D.	SE	Mean length of roots ± S.D. (cm)	SE	Callus intensity
100	70	2.50 ± 0.50	0.22	2.50 ± 1.06	0.47	+
500	39	3.80 ± 0.76	0.34	1.80 ± 0.84	0.37	++
1000	34	5.00 ± 1.00	0.45	1.20 ± 0.57	0.25	++
		12.849^{**}		2.953^{NS}		

** = significant at 1% ($P < 0.01$)
NS = non significant

Table 4 Effect of activated charcoal (AC) incorporated in rooting medium (1/2 MS + 1.0 mg l^{-1} IBA) on rooting of shoots from seedling cotyledonary nodes of *A. leucophloea*.

AC concentration (mg l^{-1})	Per cent rooting	Mean No. of roots± S.D.	SE	Mean length of roots ± S.D. (cm)	SE	Callus intensity
50	79.0	1.80 ± 0.84	0.37	2.70 ± 0.57	0.25	+
100	80.0	2.80 ± 0.84	0.37	3.50 ± 0.79	0.35	+
200	88.0	4.60 ± 0.89	0.40	3.50 ± 0.87	0.39	-
500	83.0	1.00 ± 0.00	0.00	1.80 ± 0.57	0.25	-
		21.909***		6.477**		

*** = Significant at 0.1% ($P < 0.001$); ** = Significant at 1% ($P < 0.01$)

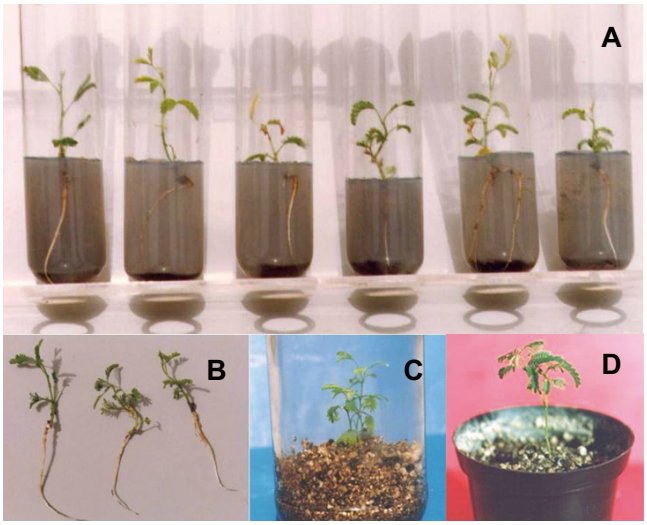

Fig. 1 (**A, B**) Callus-free rooting in shoots of *Acacia luecophloea* on ½ MS medium + 1.0 mgl^{-1} IBA + 3.0% sucrose and 200 mgl^{-1} activated charcoal. (**C**) Ten days old plantlet of *A. luecophloea* in culture bottle containing Soilrite™ irrigated with 1/4th MS liquid nutrient solution undergoing *in vitro* hardening. (**D**) An *A. luecophloea* plant in pot containing soil: Soilrite™ (1:1) mixture.

completely (**Table 4**). The root induction was observed within 3-4 days. Increase in concentration of AC than above decreased rooting percentage, root number as well as root length. Plantlets when directly transferred from culture vessels to pots desiccated rapidly. However, the plantlets transferred for *in vitro* hardening developed good root system in Soilrite™. In 15 days they attained full growth up to neck of bottles. Mixture of soil-Soilrite™ was found to be the most suitable for successful transplantation of plants. Potted plants initially showed new growth showing their successful transplantation (**Fig. 1D**).

DISCUSSION

For the present studies shoots derived from *in vitro* regeneration of cotyledonary nodes of *A. leucophloea* were used. *In vitro* regeneration from cotyledonary nodes has also been achieved in *Acacia auriculiformis* (Mittal *et al*. 1989), *Acacia nilotica* (Dewan *et al*. 1992), *Acacia tortilis* (Nangia and Singh 1996), *Acacia mangium* (Douglas and McNamara 2000), *Acacia sinuata* (Vengadesan *et al*. 2000), *Acacia chundra* (Rout *et al*. 2008) and *Acacia senegal* (Khalafalla and Daffalla 2008). *In vitro* propagation via cotyledonary node explants has also been achieved in *Pterocarpus santalinus* (Kalimuthu *et al*. 2010) and *Bauhinia racemosa* (Rajanna *et al*. 2011). *In vitro* propagation of tropical hardwood tree species has been reviewed by Pijut *et al.* (2012).

Root formation is an energy demanding process and therefore an exogenous supply of carbohydrate is required. However, this being the last stage of *in vitro* culture it is important to transform plant from heterotrophic mode of nutrition to autotrophic mode (Serret *et al*. 1997). Basic events in developmental process in roots can be severely affected by high or low sugar concentration in the media (Kerbauy 1993). Pre-conditioning with different concentrations of sugar influences the quality of plants produced *in vitro* (Morini and Melai 2003/4). In the present investigation 1/2 MS medium supplemented with 3.0% sucrose, 0.8% and 1.0 mg l^{-1} IBA produced the best rooting response in terms of number of roots produced. However, reduction in sucrose concentration has proved useful in rooting of *Acacia mangium* and *Acacia chundra* (Nanda *et al*. 2004; Rout *et al.* 2008). Reduction in salt concentration was useful in obtaining callus-free rooting in *Acacia* (Vengadeshan *et al*. 2002; Nanda *et al*. 2004; Khalafalla and Daffalla 2008; Rout *et al*. 2008; Dhabai *et al*. 2010). Rooting on reduced salt and different concentration and types of sugar in the medium to some extent causes conditioning for better performance prior to transplanting (Nowak and Shulaev 2003; Hazarika 2006).

In the present studies rooting by pulse treatment did not produce callus-free roots. However, pulse treatment has been successfully applied in woody legumes (Dhar and Upreti 1999; Hussain *et al*. 2008). In the present studies addition of AC (200 mg l^{-1}) in the rooting media produced callus-free rooting. Dark treatment has been reported to produce callus-free rooting in *A. mangium* (Monteuuis 2004). Addition of AC to promote callus-free rooting has also been reported in other legumes (Selva *et al*. 1989; Abdelwahd *et al*. 2010). The beneficial effects of AC on morphogenesis may be mainly due to its irreversible adsorption of inhibitory compounds in the culture medium and substantially decreasing the toxic metabolites, phenol exudation and brown exudates accumulation. In addition to this AC is involved in a number of stimulatory and inhibitory activities including the release of substances naturally present in AC which promote growth, alteration and darkening of culture media, and adsorption of vitamins, metal ions and plant growth regulators, including abscisic acid and gaseous ethylene. AC may gradually release certain adsorbed products, such as nutrients and growth regulators which become available to plants (Thomas 2008).

Thus it was concluded that, the best rooting media was ½ MS salts with 3.0% sucrose, 0.8% agar supplemented with 1.0 mg l^{-1} IBA and 200 mg l^{-1} AC.

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