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In vitro plant regeneration from apical buds of *Albizzia odoratissima* (L.f.) Benth

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ABSTRACT

A study was initiated to explore the possibility for in vitro propagation of the economically important leguminous tree, Albizzia odoratissima due to the lack of a proper conventional method of propagation. The apical buds from 7-days-old in vitro seedlings of A. odoratissima were evaluated for direct shoot regeneration on Murashige and Skoog's (MS) basal medium fortified with BAP (6–benzylaminopurine) and kinetin singly or in combinations of both at different concentrations. The highest percentage of response was obtained on MS media supplemented with 0.75 mg/l BAP in which 91.67% of the explants responded producing an average of 10 shoots per explants and shoot length of 4.86 ± 0.22 cms. Kinetin was found to be incapable of multiple shoot proliferation whether incorporated alone or in combination with BAP. In vitro rooting of the microshoots was induced in growth regulator-free as well as indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) supplemented half strength MS medium. The best rooting response (53.33%) was observed in growth regulator-free MS medium. The highest response for acclimatization and pot establishment of the rooted plantlets was obtained in soilrite (40%).

Keywords: leguminous tree, seedlings, kinetin, shoot proliferation, acclimatization.

Abbreviations: *MS-Murashige and Skoog; BAP-6- Benzylaminopurine; IAA- Indole-3-acetic acid; IBA- Indole-3-butyric acid; 2-iP- N^6-2-isopentenyladenine; NAA- Naphthalene-3-acetic acid; PAR-Photosynthetically Active Radiation; ANOVA- Analysis of variance; CRD-Completely randomized block design; LSD- Least significant difference test*

INTRODUCTION

The leguminous tree, *Albizzia odoratissima* is a common constituent of mixed deciduous forests. It is a large, woody, fast-growing tree reaching 15-25 m in height. The tree occurs naturally in humid tropical countries like Bangladesh, China, India, Myanmar, Nepal, Pakistan and Sri Lanka. It is widely used as a shade tree in coffee and tea plantations as it improves fertility of the soil and decreases soil erosion [1]. The tree is a good source of fuelwood, green manure, cattle

fodder and produces premium quality timber [2]. Moreover, herbal medicines are now increasingly being used for treatment against different human disorders [3] and *A. odoratissima* also plays an important role as a medicinal plant. The bark of this tree is used for the treatment of ulcers, leprosy, skin diseases, cough, bronchitis, diabetes and burning sensation [4]. Therefore, the tree enjoys considerable importance in social and agroforestry programs on farm lands. In the recent years, this tree has suffered genetic degeneration due to extensive felling of phenotypically superior trees for various commercial purposes [1]. Hence, there is an urgent need to develop conservation stands to protect the genetic resource of this species [5]. Production of trees with desired characteristics would reduce the pressure to cut quality trees from natural forests. So far, a conventional method of vegetative propagation using stem and root cuttings has not been possible for *A. odoratissima* for clonal propagation of selected genotypes with superior canopy characteristics and disease and pest resistance [6; 7]. The only method available at present for propagation of *A. odoratissima* is through seeds. However, the seeds are commonly faced with the problem of being frequently damaged by Bruchidius bilineatopygus (bruchid) larvae. *In vitro* techniques could be viable alternatives to conventional vegetative propagation method for mass propagation [8] and gene conservation. Moreover, the *in vitro* regeneration systems comprising of cell cultures or micropropagated tissues are a reliable source of genetically homogenous material and are able to regenerate shoots which can be rapidly propagated either by organogenesis or somatic embryogenesis. Genetic transformation is a viable technique for elucidating gene function and for making targeted single trait improvement in clonally propagated plants [9]. The regeneration of plants from cells or tissues is the first step for introduction of genetic variation by genetic transformation techniques for development of cultivars with improved characteristics.

In leguminous trees, direct *in vitro* shoot regeneration without an intervening callus phase has been reported in leguminous trees like *Albizzia odoratissima* [10], *Albizzia chinensis* [11], *Bauhinia variegata* and *Parkinsonia aculeata* [12], *Albizzia falcataria* [13], *Dalbergia sissoo* [14], *Acacia seyal* [15], *Pterocarpus marsupium* [16] and *Acacia chundra* [17].

In the first trial for *in vitro* shoot regeneration of *A. odoratissima* [10], field establishment of complete plantlets could not be achieved and therefore, the technique could not be used for commercial exploitation. Later, *in vitro* plantlet regeneration of *A. odoratissima* was reported from nodal regions of cotyledons and leaves [1]. Induction of callus and successful regeneration of plantlets has also been obtained from epicotyl, petiole and cotyledon explants [18]. So far as *in vitro* propagation is concerned, shoot tips or apical buds are usually preferred because of their strong growth potential and ability to produce virus-free plants [19]. However, among the tree legumes, successful *in vitro* plant regeneration from apical buds has so far been reported in only a few species like *Albizzia lebbeck* [20], *Acacia seyal* [15] and *Acacia chundra* [17].

The objective of the present study is to investigate whether the apical buds from *in vitro* grown seedlings of *A. odoratissima* can be utilized as reliable sources for mass multiplication of this species in order to increase its availability and also for commercialization of selected genotypes. The present study is the first report on regeneration of complete plantlets from apical buds of *A. odoratissima* and successful establishment of the plantlets in soil.

MATERIALS AND METHODS

Source of plant material and explant preparation

Dried and mature seeds were collected from the tea gardens of Barbheta Division of Tea Research Association. The seeds were washed with running tap water and few drops of a

commercial detergent 'Nocidet' (National Organic Chemical Industries Limited, Mumbai, Maharashtra, India) for 5 min and then subsequently rinsed three times with distilled water. This was followed by surface sterilization of the seeds with 0.1% (w/v) solution of mercuric chloride for 10 min with gentle stirring. The seeds were finally rinsed three times with sterile distilled water and germinated under aseptic conditions. For multiple shoot proliferation, the apical buds (3-4mm) from 7-d-old *in vitro* grown seedlings were excised and cultured horizontally on shoot proliferation medium.

Basal nutrient medium for germination of seeds and optimal proliferation of shoots

The basal nutrient medium used in this study consisted of Murashige and Skoog (MS) medium [21]. Germination of seeds was carried out on plant growth regulator-free MS basal medium containing half strengths of the MS macro and micro salts. The vitamins, sucrose and myoinositol were as per the original compositions of MS media.

Effect of cytokinins and their concentrations on in vitro multiple shoot proliferation

For multiple shoot proliferation, MS media fortified with various concentrations of either BAP (0.25, 0.5, 0.75, 1.0, 1.25 mg/L) or kinetin at the same concentrations solely or in combinations of both were tried. MS medium without any growth regulator was used as the control. The pH of the medium was adjusted to 5.6 with 1N NaOH or 1N HCl and the medium was solidified with 0.8% agar (Himedia, Mumbai, India). The media were then sterilized by autoclaving for 20 min at 121ºC. Each of the treatments was tried with 20 explants and repeated three times. Shoot proliferation was evaluated 30 days after the beginning of the experiment and the percentage of explants responding to shoot proliferation, the number of shoots per explant and length of shoots were recorded.

Culture conditions

The cultures were grown at 25 ± 2°C under 16 h light photoperiod with a photosynthetic photon flux density of 37.40 µmol m^{-2} sec^{-1} provided by Photosynthetically Active Radiation (PAR) sources and cool white fluorescent tubes. Subculturing of the cultures to fresh medium was carried out at every 4 weeks interval.

Effect of auxins and their concentrations on in vitro rooting of the microshoots

Healthy and actively growing shoots with 3-4 leaves were used for root induction of the microshoots. The rooting medium consisted of half strength MS medium with and without the incorporation of auxins like IAA or IBA at different concentrations of 1.25, 1 and 0.75 mg/L. Rooting was also tried in one-fourth strength MS medium with and without the incorporation of rooting hormones. Medium sterilization and culture conditions were carried out as described previously for shoot proliferation experiments. Twenty microshoots were used for each treatment. After 30 days of culture, the percentage of microshoots forming roots, number of roots per microshoot and length of the roots were recorded.

Hardening and pot establishment of the plantlets

Plantlets with well developed shoot and root systems were transferred to a hormone free minimal media of pH 5.2 incorporated with sucrose 7.5 g/L and solidified with 10 g/l agar [22]. The flasks with rooted plantlets were kept outside the culture chamber and under room temperature (30°C) for one week. The plantlets were then removed from the culture flasks, washed with tap water to remove all agar adhering to the roots and transferred to small earthen pots filled with soil, soilrite (Allied Scientific Products, Kolkata, West Bengal, India) or mixtures of sand: soil (1:1 and 1:2). The pots were covered with transparent polythene bags to keep moist conditions in the air surrounding the plantlets. The plantlets were then kept in dark conditions

under room temperature. After 10 days, the plantlets were exposed to diffused light conditions. The plantlets were irrigated regularly with tap water and the polyethene bags were removed gradually.

Experimental design and statistical analysis

All the experiments were conducted in a completely randomized block design (CRD) with twenty explants per treatment and each treatment was repeated three times. Data on percentage of explants producing multiple shoots, number of shoots per explants, and length of the shoots were recorded after 30 days in shoot regeneration experiments. Rooting experiments were also statistically evaluated after 30 days interval and data were collected on number of rooted microshoots, the number of roots per shoot and length of the roots. Hardening of plantlets was evaluated after 30 days of transfer to pots and percentage of survival of plantlets was recorded for different potting mixtures. All the data were subjected to one-way analysis of variance (ANOVA) with 5% significance level to analyze the influence of different treatments. The mean comparisons were carried out by Duncan's Multiple Range Test [23]. The Least significant difference test (LSD) was used to study differences between different treatments.

RESULTS AND DISCUSSION

In vitro germination of seeds and effect of cytokinin concentration for in vitro shoot regeneration

The seeds showed a germination percentage of 95% after 2 days of culture on half strength MS medium. The seedlings obtained a height of 8-9 cms after 7 days with 2 to 4 leaves (Figure 1a). The apical buds were then excised from the 7 day old seedlings and cultured vertically on MS media supplemented with various concentrations of BAP and kinetin.

After 15 days of culture, the emergence of several shoot bud primordia was observed from the basal region of the apical bud without the induction of an intervening callus phase. The productivity of shoots was significantly affected by the concentrations of cytokinins in the media (Table 1).

Table 1. Effect of cytokinins on shoot multiplication from apical buds of *Albizzia odoratissima* after 30 days of culture

Growth regulators (mg/l)		MS		
BAP	**Kinetin**	**Percentage of explants regenerating shoots (mean ± SE)**	**No. of shoots per explants (mean ± SE)**	**Length of shoots (cm) (mean ± SE)**
-	-	-	-	-
1.00	-	71.67 ± 2.89^{b}	7.0 ± 2.0^{ab}	4.82 ± 0.16^{a}
0.75	-	91.67 ± 2.89^{a}	10.0 ± 4.0^{a}	4.86 ± 0.22^{a}
0.50	-	58.33 ± 7.64^{c}	7.0 ± 1.0^{ab}	4.01 ± 0.47^{bc}
0.25	-	45.00 ± 5.00^{d}	5.0 ± 1.0^{bc}	1.70 ± 0.16^{e}
1.00	1.00	45.00 ± 5.00^{d}	3.0 ± 1.0^{c}	2.50 ± 0.45^{d}
1.00	0.75	48.33 ± 2.87^{d}	4.0 ± 1.0^{bc}	3.37 ± 0.90^{c}
1.00	0.50	50.00 ± 0.00^{d}	6.0 ± 1.0^{bc}	3.73 ± 0.14^{bc}
1.00	0.25	51.67 ± 2.87^{cd}	7.0 ± 2.0^{ab}	4.38 ± 0.36^{ab}

The values represented within the columns are the mean ± SE of three repeated experiments with 20 explants per treatment. The values followed by different letters are significantly different at P<0.05.

MS medium fortified with 0.75 mg/l BAP was found to be the best for multiplication of shoots (P<0.05) in which 91.67% of the explants responded producing 6-14 shoots per shoot (Figure 1b). The length of the proliferated shoots ranged from 5.08 to 4.64 cms. The highest frequency of shoot regeneration (82.5%) with a maximum of only 6.9 shoots per explant was reported earlier

from cotyledonary node explants of *A. odoratissima* cultured on a MS medium containing both 10µM BAP and 10µM 2-iP [1] An average of 15 shoots was achieved from epicotyl explants of *A. odoratissima* on MS medium supplemented with 2.5 µM BAP and 0.5 µM NAA but the percentage of explants producing shoots was found to be very less (29%) [18]. Kinetin was found to be ineffective in shoot bud induction whether incorporated alone or in combination with BAP.

Media composition used in tissue culture studies accounts to a large proportion of any *in vitro* mass propagation protocol [24]. The use of BAP in the media for *in vitro* propagation of leguminous trees has been of profound importance. The addition of BAP in the nutrient medium has been found to be necessary to induce multiple shoot proliferation from apical buds of other leguminous trees like *Albizzia lebbeck* [20], *Acacia seyal* [15] and *Acacia chundra* [17]. In the present study, a decline in rate of shoot proliferation was observed at increased concentration of BAP. An inhibitory effect on shoot multiplication at higher concentrations of BAP has also been observed by other workers [25; 26; 27; 28] which are in agreement with our observations. In our studies, a decrease in the shoot regeneration frequency was also recorded at lower concentrations of BAP. No multiple shoot proliferation could be obtained in MS medium supplemented with only kinetin. When BAP 1 mg/l was combined with different concentrations of kinetin, no enhancement in shoot proliferation was achieved. Stunted growth of shoots was also observed with kinetin supplemented media. Thus, kinetin was found to be antagonistic to shoot proliferation in our studies. The inefficacy of kinetin for shoot proliferation rate was also observed in *Petroselinum, crispum* [29], banana [30] and *Aloe vera* [31] which are in agreement with our findings.

The apical buds from the microshoots when isolated and subcultured in 1 mg/l BAP added MS medium, proliferated in the same manner like the mother apical bud producing an average of 6-14 shoots per apical bud within 30 days. Thus it was possible regenerate an average of 36-196 shoots from one mother apical bud within a period of 60 days.

Rooting of the in vitro regenerated shoots

The regenerated microshoots (5-6 cm), with 2-3 leaves, when isolated and cultured in one-fourth strength MS medium failed to survive after 10 days of transfer to rooting medium. However, plant growth regulator free half strength MS medium as well as medium supplemented with IBA and IAA promoted induction of rooting (Figure 1c). Root induction was observed from the base of the microshoots after 15 days of culture.

Table 2. Effect of half strength MS media supplemented with and without auxins on root induction from microshoots of *A. odoratissima* after 30 days of culture

Auxins		Percentage of rooting (%) (mean ± SE)	No. of roots per explants (mean ± SE)	Length of roots (cm)(mean ± SE)
IAA	IBA			
-	-	53.33 ± 5.77[a]	2 ± 1.0 [a]	3.69 ± 0.74[a]
-	1.0	30.0 ± 10.0[b]	2 ± 0.0 [a]	3.48 ± 1.36[a]
1.0	-	40.0 ± 10.0[ab]	2 ± 0.0 [a]	3.59 ± 1.33[a]

The values represented within the columns are the mean ± SE of three repeated experiments with 20 explants per treatment. The values followed by different letters are significantly different at P<0.05.

The ANOVA showed that the highest percentage of rooting (53.33%) with an average of 1-3 roots per microshoot which could be obtained in hormone-free half strength MS medium ($P<0.05$) (Table 2). The average length of the roots ranged from 2.95 to 4.43 cms. A reduction in the percentage of rooting was observed with the incorporation of auxins in the nutrient medium.

The earlier reports suggest the presence of an auxin in the basal for induction of *in vitro* rooting of *A. odoratissima* [1; 18] which do not agree with our findings. *In vitro* rooting success in MS medium without any growth regulators was also reported earlier in *Cordia verbenacea* [32] and dumb cane [33] in which are in agreement with our results.

Hardening of the rooted plantlets

The percentage of hardening success of the plantlets was highest in soilrite (40%), as compared to the other potting mixtures ($P<0.05$) (Table 3). The plantlets exhibited normal growth and did not show any phenotypic abnormalities (Figure 1d) as compared to the *in vivo* grown seedlings.

Table 3. Hardening success of *in vitro* derived plantlets of *A. odoratissima* after 30 days of transfer to potting mixtures

Potting mixture	Response (%)
Soil	1.67 ± 2.89^{b}
Sand:soil (1:1)	6.67 ± 2.89^{b}
Sand:soil (2:1)	8.33 ± 2.89^{b}
Soilrite	40.00 ± 10.00^{a}

The values represented within the columns are the mean ±SE of three repeated experiments with 20 plants per treatment. The values followed by different letters are significantly different at $P<0.05$.

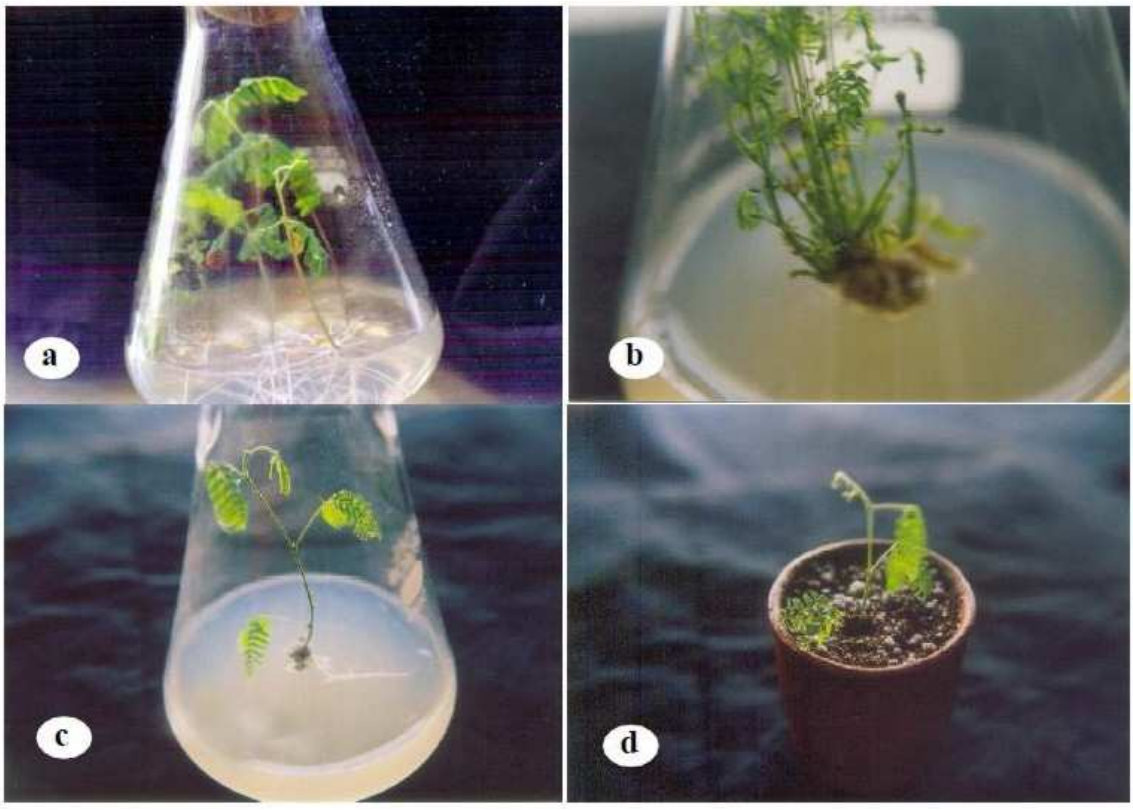

Fig-1. *In vitro* regeneration of shoots from apical buds of *A. odoratissima*

(a) *In vitro* grown seedling
(b) *In vitro* regenerated shoots from apical buds after 30 days of culture
(c) *In vitro* rooting of the microshoots
(d) Plant established in pot

CONCLUSION

The protocol established in this study for *in vitro* propagation of *A. odoratissima* from apical buds was highly efficient, rapid and reproducible. Mass propagation of plants obtained by this

method would definitely ease the pressure to cut down trees from the forests. The *in vitro* derived plants would be an ideal source of aseptic and homogenous material in transformation experiments. The *in vitro* propagation method developed in the current study would be a promising step in developing technologies for the clonal propagation and genetic transformation of this species to generate plants with desirable traits like uniform shade canopy, disease and pest resistance, etc. The protocol was optimized by manipulating different concentrations of BAP and kinetin for shoot regeneration. BAP was indispensable and highly efficient in promoting multiple shoot proliferation. Kinetin was found to be ineffective in multiplication of shoots. In the rooting stage, media without the incorporation of plant growth regulators produced the best results for induction of *in vitro* rooting. Rooted plantlets were best acclimatized in soilrite. However, there is enough scope in improvement of the technique for better multiplication of shoots and acclimatization of the *in vitro* derived plantlets.

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