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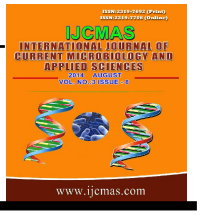

Original Research Article

Enhanced *in vitro* regeneration from seedling explants of a medicinally important leguminous tree (*Albizia lebbeck* Benth.)

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Keywords

Albizia lebbeck, seedling explants, N^6-Benzylamino purine, callus induction, multiple shoots

A B S T R A C T

An efficient protocol for *in vitro* callus induction and multiple shoot regeneration from leaf pinnae, cotyledonary leaf and root explants derived from 10 to 14-day-old seedlings of *Albizia lebbeck* has been developed. Of the two different cytokinins tested, N^6-Benzylaminopurine (BAP) at a concentration of 3.0 mg/l along with 0.8 mg/l of 3-Indoleacetic acid (IAA) resulted in high frequency of callus induction from cotyledonary leaf (95.50%), leaf pinnae (91.06%) and root (59.96%) explants respectively. A maximum number (20.50%) of multiple shoots were obtained upon culturing the cotyledonary leaf derived calli on MS medium containing BAP (1.0 mg/l) along with 1-Naphthaleneacetic acid (NAA) (0.125 mg/l). A maximum frequency (69.96%) of rooting was observed in the elongated shoots when cultured on the half-strength MS medium containing Indole-3-butyric acid (IBA). Regenerated plants could survive up to 95 to 100% under the poly house conditions. Following this procedure, hardened plants were obtained within a period of 12 to 14 weeks as compared to the prolonged culture periods reported earlier. Tissue culture raised plants are similar to that of control plants obtained through seed germination.

Introduction

Albizia lebbeck (Mimosaceae), originated from Africa is widely distributed in India, South Africa and Australia comprises about 150 species (Shirisha et al., 2013; Paula *et al.,* 2001). *A. lebbeck* is a fast growing, moderate to large deciduous tree, much tolerant to dry weather. This tree is planted as a shade tree in tea and coffee plantations to decrease soil desiccation, to suppress weed growth and to protect plants from hail and rain storms. Besides being a great nitrogen fixer *A. lebbeck* produces wood for manufacturing of paper and provides fodder for livestock (Mamun et al., 2004). *A. lebbeck* is a valuable medicinal tree with every part being a rich source of medicinally important compounds. The plant has been reported to possess immumomodulatory effect (Chaudhary et al., 2012), anti-tumor activity (Haque et al., 2000), insecticidal

activity (Sharma et al., 2012), anti-anaphylactic activity (Tripathi and Das, 1977), anti-allergic (Venkatesh et al., 2010), anti-inflammatory (Paramanic et al., 2005), anti-histaminic (Kumar et al., 2010), anti-tussive (Yadav et al., 2010), anti-oxidant (Resmi, 2006), anti-convulsant (Kasture et al., 1996) and anti-spermatogenic effects (Gupta et al., 2004), apart from mast cell stabilizing activity (Shashidhara et al., 2008). Flowers are used in treatment of opthalmia, night blindness, psychological disorders, insomnia and warts (Kasture et al., 2000). Decoction of the leaves and bark is protective against bronchial asthma and other allergic disorders (Saha and Ahmed, 2009). Flowers are applied as cataplasm on furuncles and used for retention of seminal fluid (Asif et al., 1986). Extracts of *A. lebbeck* have shown significant inhibitory effects against pathogens such as *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Staphylococus aureus* (Bobby et al., 2012).

Utility of this multipurpose tree can be enhanced many folds if it is genetically enriched either by breeding or by genetic engineering. The high degree of complexicity associated with the genetic enrichment of trees through breeding procedures can be successfully overcome by the employment of biotechnological interventions. Genetic engineering approaches to boost the medicinal value of this tree by expressing different candidate genes could be one of the areas of interest and could prove to be a commercially advantageous approach. For effective utilization of biotechnological tools *viz*., biolistics or *Agrobacterium*-mediated gene transfer procedures, reproducible regeneration protocols enabling the capturing of all possible transformation events and successful regeneration of transgenic plants from the transformed tissues are essential (Chakravarthy et al., 2014). Earlier reports describing regeneration of *A. lebbeck* using different explants like internodes (Mamun et al., 2004; Yadav and Singh, 2011), root (Perveen et al., 2011), stem and petiole (Gharyal and Maheshwari, 1990), anthers (Gharyal et al., 1983) and apical meristems (Borthakur *et al.,* 2011) have been reported.

However, most of these reports are aimed towards mass multiplication of shoots from preexisting meristems, which are mostly applicable for mass cultivation and clonal propagation. For genetic transformation and successful production of germline transformants, callus based regeneration is preferred over direct organogenesis, to avoid the problems arising due to frequent occurrence of chimeras, where the inheritance of the transgenes to the progenies may not be achieved. Although, there are some reports of regenerating *A. lebbeck* via callus phase are available, these procedures required prolonged culture periods and relatively less number of shoots are produced, low frequency of rooting and hardening of the plants. Moreover, somaclonal variations arising due to prolonged culture periods are undesirable when a true-to-type transgenic plant is the ultimate objective. In this study, we report the development of an efficient protocol for rapid production and elongation of multiple shoots from seedling explants of *A. lebbeck*. A high frequency of rooted shoots survived and developed into mature plants under poly house conditions.

Materials and Methods

Plant material

For germination, healthy seeds were chosen

and surface sterilized with 0.1 % (w/v) aqueous mercuric chloride solution for 10 to 12 min, followed by 3 rinses in sterile distilled water for 5 min duration. The sterilized seeds were then allowed to imbibe in sterile distilled water for overnight in dark at 37°C. Later, seeds with emerging radicals were transferred to the culture tubes containing half-strength MS (Murashige and Skoog, 1962) basal medium for seedling development.

Media preparation and culture conditions

Media used for seed germination contained half-strength MS salt formulation with 1.5% sucrose. The media used for callus and multiple shoot induction was prepared by adding full-strength MS salt formulation with 3% sucrose and the media used for root induction contained half-strength MS salt formulation with 2% sucrose. The pH of the media was adjusted to 5.8 before adding 0.8% agar and sterilized by autoclaving at a pressure of 1.1 kg cm^{-2} (121°C) for 20 min. All plant growth regulators used in this study were added prior to autoclaving. The cultures were maintained in the culture room at 25±2°C under cool white fluorescent light intensity of 3000 lux.

Callus induction and shoot differentiation

Cotyledonary leaf (5–8 mm^2), leaf pinnae (5–8 mm^2) and root (8–10 mm) explants obtained from 10–14 day-old *in vitro* germinated seedlings were used as explants for callus induction. The callus induction ability of the explants was evaluated by culturing on MS medium supplemented with different concentrations of N-6-Benzylaminopurine (BAP) (0.5–3 mg/l) and 6-Furfurylaminopurine (KN) (0.5–3.0 mg/l) in combination with 3-Indoleacetic acid (IAA) (0.8 mg/l). After five weeks, the callus was sub cultured to MS medium containing BAP (0.2–1.0 mg/l) and 1-Naphthaleneacetic acid (NAA) (0.125 mg/l) for shoot induction and elongation.

Rooting of *in vitro* shoots and hardening of rooted plantlets

To identify the optimal auxin concentration required for induction of early and healthy root system, the elongated (4 to 5 cm) shoots were excised from the calli and individually transferred onto half-strength MS medium containing 2% maltose and 0.50 to 3.0 mg/l of Indole-3-butyric acid (IBA) or NAA or IAA. A control experiment without any plant growth regulator (MS0) was also conducted by parallel. Data were recorded after four-weeks. Plantlets with well developed root and shoot systems were carefully removed from the culture tubes and washed with tap water to remove the adhering medium and transferred to plastic cups containing equal ratio of autoclaved oak forest humus, sand and soil. After two weeks of acclimatization, the plants were transferred to pots containing sand and garden soil in 1:3 ratio and maintained under the poly house conditions, before transferring to field.

Statistical analysis

Experiments for callus induction, multiple shoot and root induction were carried out in 30 replicates per treatment in three repeated experiments. Mean and standard error were computed with the help of sigma plot, programmed for statistical calculations.

Results and Discussion

An efficient protocol for multiple shoot regeneration is pre-requisite for genetic manipulation of plants. Though there is a large body of literature available on regeneration in *A. lebbeck*, efforts on the

establishment of procedures for induction and simultaneous elongation of multiple shoots in short period were rare.

Poor and non-uniform germination was a problem at the beginning of the experiment because of the variability in water absorption and possibly, seed age and vigor. This problem was overcome by imbibing the sterilized seeds in sterile distilled water overnight in dark at 37°C and aseptic removal of the seed coat prior to transfer to the half-strength MS medium for germination. The germination of pre-imbibed seeds with emerging radicals resulted in significant increase in the seed germination of up to 90 to 95% in comparison with the poor (25 to 30%) and non-uniform germination observed with the un-imbibed seeds. Salmasi (2008) reported morpho-physiological dormancy in seeds can be broken by maintaining appropriate humidity and temperature.

The effect of varying concentrations of BAP and KN along with IAA on callus induction from different explants is presented in Table 1. Callus formation was observed within a period of 9–11 days from the cut surfaces of all three types of explants tested. However, callusing rate was significantly varied by the type of explant and growth regulator used (cotyledonary leaf > leaf pinnae > root). An early callus induction with in a period of one-week was observed in the explants cultured on MS medium containing BAP (3.0 mg/l) and IAA (0.8 mg/l). Earlier report on callus initiation from leaf explants of *A. lebbeck* took 12-15 days (Varghese and Kaur, 1988). On this medium, cotyledonary leaf explants showed the maximum response of 95.50% followed by leaf pinnae (91.06%) and root explants (59.96%) (Table 1). This kind of differential response by different explant types within the same treatment may be because of the differences in the endogenous level of plant growth hormone in different explants (Quoirin et al., 1988). Further, the calli produced on BAP medium were greenish and compact with high proliferation. Earlier, similar effects of BAP on callus induction from explants of *A. lebbeck* were reported (Varghese and Kaur, 1988). The frequency of explants responding to KN was found to be moderate at all the concentrations tested (Table 1). KN at a concentration 3 mg/l induced callusing of 82.20% of cotyledonary leaf explants, 79.96% leaf pinnae and 53.30% in root explants respectively. The callus obtained on kinetin containing medium is less compact and resulted in delayed shoot induction upon transferring to shoot induction medium.

The compact, healthy and proliferative calli upon transferring onto the medium containing reduced concentrations of BAP along with NAA at a concentration of 0.125 mg/l produced elongated multiple shoots (Table 2). An early shoot initiation in more than 70% of the cultures was observed after 15 days on medium containing BAP (1.0 mg/l) along with 0.125 mg/l of NAA. Further, this combination produced a maximum number of multiple shoots (20.50±0.24) from cotyledonary leaf derived calli, followed by calli derived from leaf pinnae (12.75±0.54) and (17.17±0.25) root explants (Fig.1a–c & Table 2). Gharyal and Maheshwari (1990) reported 50% shoot dedifferentiation from callus derived from stem explants and 36% from petioles on B5 medium containing BAP (1.0 mg/l) and NAA (0.5 mg/l). Kumar *et al*., (1998) reported 60% shoot regeneration by culturing leaflet explants on MS medium containing BAP and NAA. A further increase in the BAP concentration up to 3.0 mg/l in the culture medium did not significantly increased mean number of multiple shoots (data not shown), and also resulted in the abnormal morphology and vitrification of the tissues.

Table.1 Effects of explant type and concentration of BAP and KN along with IAA on callus induction from seedling explants of *A. lebbeck* after two weeks of culture. Data represents means from three replicates ± SE

Growth regulators (mg/l)			Callus induction (%)		
BAP	KN	IAA	Cotyledonary leaf	Leaf pinnae	Root
0.5	--	0.8	31.10±1.10	22.20±2.20	12.20±2.20
1.0	--	0.8	48.86±1.13	39.96±3.83	19.96±1.93
1.5	--	0.8	83.30±1.90	72.20±4.85	31.06±2.93
2.0	--	0.8	93.30±1.90	88.83±2.23	44.40±1.10
2.5	--	0.8	94.40±1.10	89.96±1.93	55.53±2.23
3.0	--	0.8	95.50±1.10	91.06±2.23	59.96±1.93
--	0.5	0.8	25.50±1.10	16.63±1.93	7.73±1.13
--	1.0	0.8	33.30±1.90	27.73±2.94	12.20±1.10
--	1.5	0.8	48.83±2.23	41.06±2.23	23.30±1.90
--	2.0	0.8	64.40±2.20	49.96±1.93	34.43±2.94
--	2.5	0.8	76.63±1.93	65.53±2.23	48.86±1.13
--	3.0	0.8	82.20±1.10	79.96±1.93	53.30±1.90

Table.2 Effect of different concentrations of BAP on multiple induction from cotyledonary leaf, leaf pinnae and root explants of *A. lebbeck* after four weeks of culture. Data represents means from three replicates ± SE

Growth regulators (mg/l)		Cotyledonary leaf		Leaf pinnae		Root	
BAP	NAA	% of calli producing shoots	Mean no. of shoots/calli	% of calli producing shoots	Mean no. of shoots/calli	% of calli producing shoots	Mean no. of shoots/calli
0.2	0.125	65.55±4.44	5.98±0.26	64.44±4.0	4.12±0.18	67.77±4.84	2.86±0.22
0.4	0.125	75.54±3.99	9.36±0.24	73.32±1.92	6.70±0.41	71.11±1.11	5.17±0.36
0.6	0.125	82.22±2.22	14.25±0.52	75.53±2.93	8.06±0.27	84.44±2.93	10.12±0.16
0.8	0.125	88.88±2.93	17.39±0.22	89.96±3.33	12.62±0.45	88.88±2.93	15.33±0.28
1.0	0.125	95.55±1.11	20.50±0.24	92.16±2.94	12.75±0.54	89.99±1.92	17.17±0.25

Fig.1 Plant regeneration from seedling explants of *A. lebbeck*: **a** Multiple shoot induction in the calli derived from cotyledonary leaf explant; **b** Multiple shoot induction in the calli derived from leaf pinnae explant; **c** Multiple shoot induction in the calli derived from root explant; **d** Plantlet with well developed root system on half-strength MS medium containing IBA; **e** Hardening of regenerants.

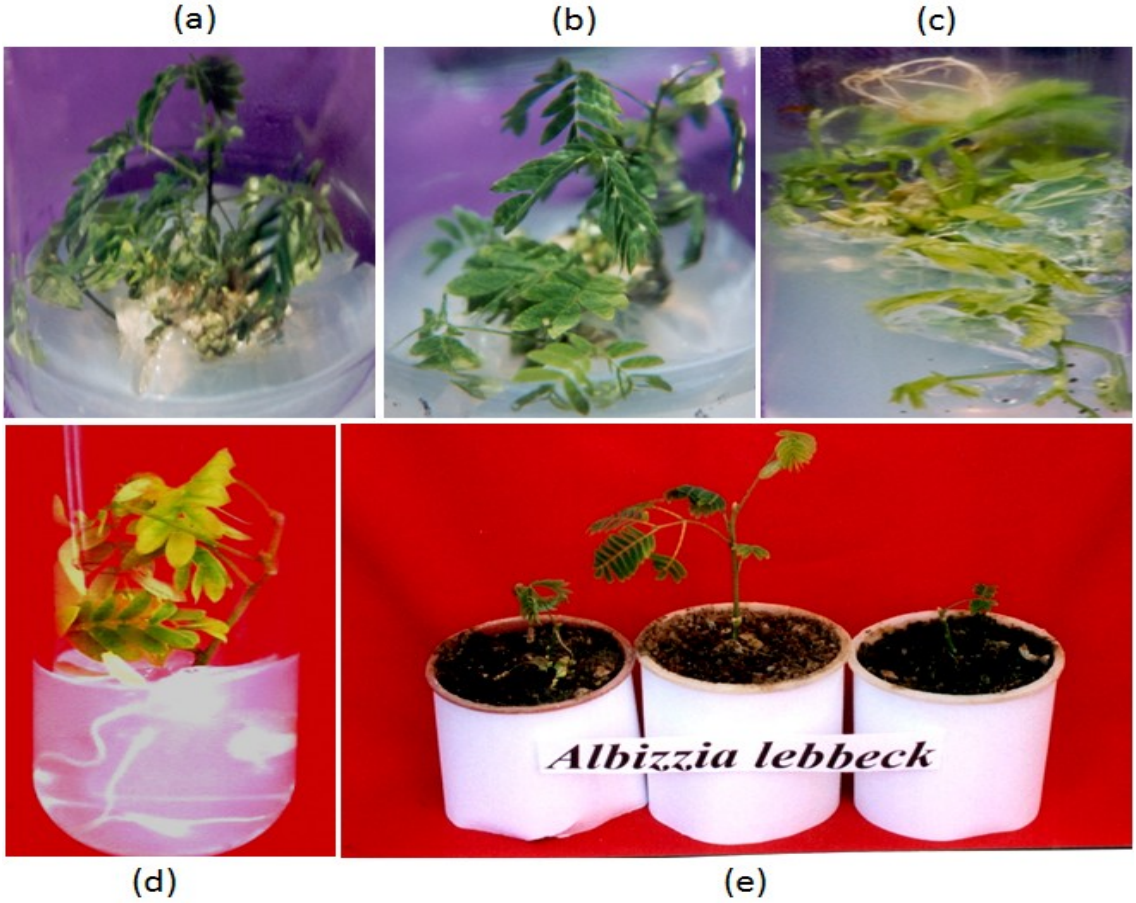
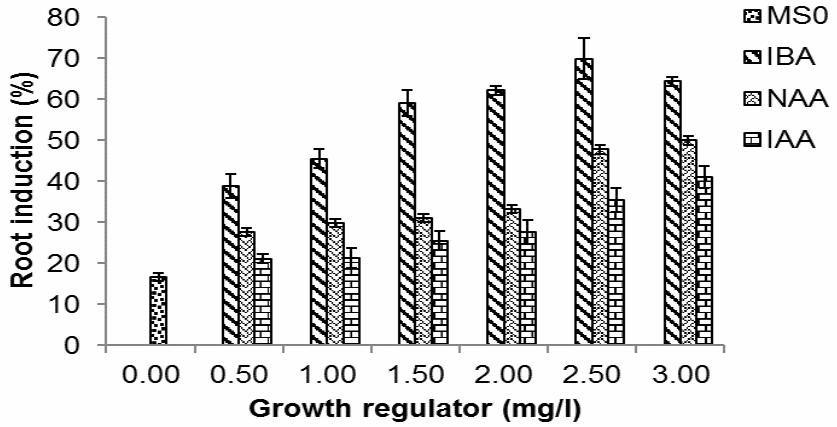


Fig.2 Effect of different concentrations of IBA, NAA and IAA on root formation from the *in vitro* derived shoots of *A. lebbeck.*



Similar observations were reported with high concentrations of BAP from the cotyledonary node explants of *Pisum sativum* (Jackson and Hobbs, 1990).

In this study, the presence of NAA (0.125 mg/l) along with BAP in the medium used for multiple shoot induction caused simultaneous elongation of multiple shoots without further sub culturing for shoot elongation. By adopting this method, *A. lebbeck* plants could be established in the poly house within 12 to 14 weeks. The decrease in the time span may be attributed to the synergistic effects of auxins and cytokinins used in the present study. Similar observations pertaining to the synergistic effects BAP and NAA or IAA were reported earlier in Cotton (Chakravarthy, 2013) and banana (Anbazhagan et al., 2014).

Shoots cultured on the growth-regulator free half-strength MS medium showed ~16% root induction after three weeks of culture. Whereas, the shoots cultured on half-strength MS medium supplemented with auxins (IBA/NAA/IAA) have shown well developed root system, and the plantlets produced were healthy (Fig.1d). The vigorous growth of plantlets could be due to optimum utilization of nutrients present in the medium by the established root system. The frequency of rooted shoots ranged from 69.96% in IBA to 41.06% in IAA containing medium (Fig.2). The variation observed in the effectiveness of different auxins may be attributed to their differential affinity to the auxin receptors involved in the rhizogenesis of the shoots. The superiority of IBA over other auxin sources for *in vitro* root formation, observed in the present investigation, is consistent with the earlier findings in cotton (Chakravarthy, 2013) and *Melia dubia* (Ram et al., 2014).

Plantlets with well developed root system were transferred to the plastic cups containing equal ratio of autoclaved oak forest humus, sand and soil (Fig.1e). After two weeks of acclimatization, the plants were transferred to the bigger pots containing soil and shifted to the poly house.

In summary, we report a fast and efficient regeneration system for multiple shoot regeneration from three different explants of *A. lebbeck*. The time taken from explanting to the establishment of plants in the poly house is reduced to about 12–14 weeks compared to the longer periods as reported in published regeneration protocols. Regenerated plants resembled the control plants obtained through seed germination for several morphological characters.

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