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# SHOOT BUD REGENERATION FROM LEAF EXPLANTS OF *ALBIZZIA AMARA* BOIV.

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## SUMMARY

**High frequency plant regeneration was achieved from the callus of leaf explants of *Albizzia amara* Boiv. Optimum callus was achieved on Murashige and Skoog medium supplemented with 3.5 mg/l of α-naphthalene acetic acid (NAA) and 0.5 mg/l of 6-benzylaminopurine (BAP). Subsequent culturing of callus on the same medium containing various concentrations and combinations of auxins and cytokinins, resulted in shoot bud induction. Maximum number of shoot buds were produced on the medium supplemented with 3.5 mg/l NAA and 1.5 mg/l BAP. Further growth and development was achieved on hormone free MS medium. The regenerated shoots were rooted on ½ strength MS medium containing 0.6 mg/l indole-3 butyric acid (IBA) and the plantlets were hardened and transferred to soil in the open. Anatomical studies revealed that shoot bud differentiation was observed from sub epidermal level as well as in the center of the callus.**

***Key words*: *Albizzia amara*, *in vitro*, leaf explants, plant production.**

## INTRODUCTION

*Albizzia amara* Boiv. (chigaraku) belonging to the family Leguminaceae, is a valuable economic medicinal and multipurpose deciduous tree commonly found in tropical dry forests of India. This plant is used for national developmental programmes such as afforestation, agroforestry, and social forestry as it is a drought tolerant species. The wood is purplish brown with lighter bands, very hard and strong used for cabinets in building and agriculture purpose (Gamble 1935). The wood is also used in the preparation tools, handles, mallet heads, brake blocks and employed for carving implements. Seeds are generally used as a remedy for piles, diarrhoea and gonorrhoea and as astringent. The flowers are externally applied to inflammations, swellings, boils and ulcers (Pezzuto *et al.* 1992). Leaves are useful in opthalmia. The powder of the leaf is also used as herbal cosmetic for hair maintenance. Tree yields gum which is used for ulcers (Kashyapa and Ramesh 1992) and Molluscicidal activity was shown by extracts of *A. amara* (Ayoub and Yankov 1986). The seed oil contains high content of linoleic, oleic and palmitic acids and low content of capric, lauric and lignoceric acids (Munir *et al.* 1995). Pezzuto *et al.* (1992) isolated 3 spermine macrocytic alkaloids (bud munchiamines A, B and C) with cytotoxic from the seeds of *A. amara* for antibacterial and other biological activities.

Vegetative propagation of this plant did not yield satisfactory results. Due to hard seed coat, germination is slow. Propagation by stem cuttings showed scanty rooting. *In vitro* multiplication of this useful economic and medicinal plant species can provide a means of disease free healthy clones for the extraction of pure drugs. In this communication we report an efficient protocol for micropropagation of *A. amara* using leaf segments of aseptic seedlings.

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## MATERIALS AND METHODS

Seeds were collected from Tirumala forest and surface sterilized with detergent for 3 min and 70% alcohol for 2 min, followed by 0.5% mercuric chloride for 5 min. They were rinsed thoroughly thrice with sterile distilled water. The seeds were placed on MS (Murashige and Skoog, 1962) medium without growth hormones and supplemented with 2% sucrose and 0.8% agar to raise aseptic seedlings. The leaf segments from the aseptic seedlings were placed on the MS medium containing different concentrations and combinations of auxins (2, 4-D, IBA and NAA) and cytokinins (BAP and Kinetin). The pH of the medium was adjusted to 5.6-5.8 before gelling with bacteriological agar. The gelled medium was dispensed into 150 mm × 25 mm rimless culture tubes (20 ml medium/culture tube) and plugged with non-absorbent cotton plugs and autoclaved at 1.06 kg/cm$^2$ pressure at ±121°C for 15 min. All the cultures were incubated under 1000 lux intensity provided by white fluorescent lamp for 16 h photoperiod at 25 ± 2°C. For each treatment 20 replicates were made and the experiment was repeated thrice.

For anatomical studies, the part of leaf at callus initiation state was fixed in FAA solution (formaldehyde, acetic acid, alcohol) for three hours and were preserved in 70% alcohol and passed through ethanol and xylene series for dehydration and infiltration followed by embedding in paraffin wax. The serial sections were cut at 10 µm and were stained with safranin and light green (Raghuramulu *et al.* 1983).

Isozyme (peroxidase) analysis was performed by macerating 1 g leaf material collected from mature parent plant and one year old tissue culture raised plants, growing in the same environment. Leaf tissue was macerated in a pre-chilled mortar by adding 3 ml of 0.1 M phosphate buffer (pH 7). Extract was centrifuged at 18,000g for 15 min at 4°C. The supernatant was used as an enzyme extract (Reddy and Garber 1971). SDS-PAGE was performed using 12% polyacrylamide gels (VanEldic *et al.* 1980). 8 µg protein was loaded in each slot. Proteins were separated by supplying a constant voltage of 200 V and 30 amp at 4°C for 5h and gels were incubated in a staining solution containing benzidine (2.08g) and acetic acid (18ml). When the blue coloured bands appeared, the reaction was stopped by immersing the gel in 300 ml of 7% acetic acid solution for 10 min.

## RESULTS AND DISCUSSION

### *Establishment of callus culture*

Eighty per cent of leaf segments callused on MS medium containing 3.5 mg/l NAA and 0.5 mg/l BAP. Callusing started from the cut ends within 6-8 days and the entire explant was covered with a callus mass in 20 days. The leaf explants were more responsive than the other explants like cotyledons, hypocotyl and epicotyl. Calli induced from leaf segments were light green and friable. Variation among explant types with respect to callus induction has been reported in several woody species such as *Albizzia lebbeck* (Lakshmana Rao and De 1987). Therefore the calli was transferred onto MS medium containing higher concentration of BAP in combination with NAA for shoot differentiation.

### *Shoot differentiation*

MS medium alone did not induce any morphogenic response from the callus and also did not promote further growth. However, the nodular structures of the light green compact callus became more prominently dark green and later developed into shoot buds in the medium supplemented with BAP (1.5 mg/1) in combination with NAA (3.5 mg/1) within 3 weeks of transfer (Plate 1A). These buds grew into long, green healthy shoots after another 2 weeks (Plate 1B). Variation in shoot differentiation ability of the light green compact callus is also reported in *Arachis* sps (Pittman *et al.* 1983). The shoot differentiation potential of light green compact callus was influenced by growth regulators (Table 1). MS medium containing only BAP induced shoots from light green compact callus, but at a low frequency and slow growth. The addition of NAA to MS medium containing BAP markedly improved overall shoot differentiation and shoot elongation. Optimum shoot differentiation resulted when light green compact callus was transferred to medium supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA. In this medium the highest percentage of calli form shoots (79%) and the highest number of shoots (6.2) were observed. Similar shoot regeneration responses for calli derived from various explants such as shoot segments for leaf discs in *Dalbergia*

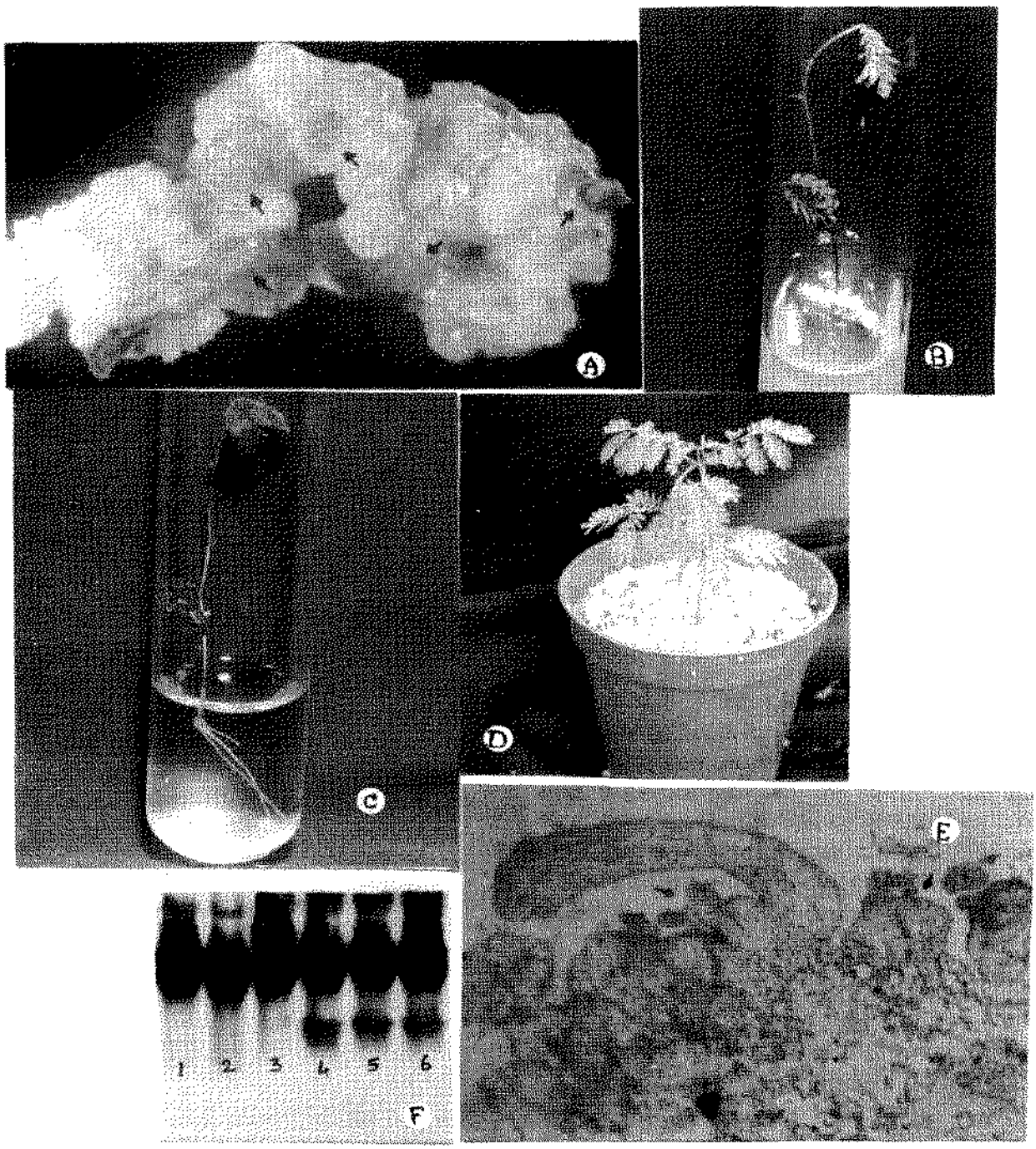


**Plate 1.**

**A. Induction of shoot buds from leaf explant on MS medium containing 1.5 mg/1 BAP and 3.5 mg/1 NAA after 2 weeks. Dark green regions marked by arrows indicates origion of shoot primordia from green and compact callus.**
**B. Elongation of regenerated shoot on the same medium after 4 weeks.**
**C. Rhizogenesis of shoots on ½ strength MS medium with 0.6 mg/1 IBA after 3 weeks.**
**D. *In vitro* regenerated hardened plant after 5 weeks.**
**E. Histology of shoot development from leaf derived callus. Arrows indicate initiation of shoots from peripheral cortical region.**
**F. Banding pattern of peroxidase isozyme in leaf tissue. 1-3 mature parent plant, 4-6 regenerated plant.**

**Table 1.** Effect of various combinations of PGR in MS medium on indirect shoot regeneration from leaf callus of *A. amara*. Mean of 20 replications.

| Growth regulators (mg/1) | | | | Frequency of shoot regeneration (%) | Mean No. of shoot/callus |
|---|---|---|---|---|---|
| NAA | IBA | BA | Kinetin | | |
| 0.5 | - | 0.5 | - | $11^{m}$ | $1.2^{k}$ |
| 1.0 | - | 0.5 | - | $19^{l}$ | $1.6^{j}$ |
| 1.5 | - | 1.0 | - | $29^{k}$ | $3.2^{ghi}$ |
| 2.0 | - | 1.0 | - | $46^{fgh}$ | $4.6^{c}$ |
| 3.0 | - | 1.5 | - | $69^{b}$ | $5.0^{b}$ |
| 3.5 | - | 1.5 | - | $79^{a}$ | $6.2^{a}$ |
| 4.0 | - | 1.5 | - | $56^{cd}$ | $4.1^{e}$ |
| - | 1.5 | 0.5 | - | $40^{ij}$ | $3.4^{g}$ |
| - | 2.0 | 1.0 | 0 | $52^{e}$ | $4.3^{e}$ |
| - | 2.0 | - | 1.0 | $59^{c}$ | $3.9^{gf}$ |
| - | 2.5 | - | 1.5 | $48^{f}$ | $4.0^{ef}$ |
| 3.0 | - | - | 1.5 | $47^{fg}$ | $4.5^{cd}$ |
| 3.5 | - | - | 1.5 | $38^{ij}$ | $3.3^{gh}$ |

Values are mean of 20 replicates.

Mean value followed by the same letter are not significantly different at $P < 0.05$.
(Student - Newman - Keuls Multiple Comparison Test).

*latifolia* have been reported (Rai and Jagadish Chandra 1988), *Butea monosperma* (Kulkarni and Souza 2000) and *Bixa orellina* (Ramamurthy *et al.* 1999).

The morphogenic potential of the suspension derived calli was influenced by the age of liquid culture. It was observed that calli derived from the second, third and fourth subculture passages retained a high regeneration potential with respect to frequency of shoot bud regeneration as well as number of shoot buds per callus. However, calli obtained from suspension older than the fourth passage showed a gradual decline in the regeneration potential.

*Rooting of shoots and establishment of plants in soil*

Half strength MS medium containing 0.6 mg/1 IBA induced rooting in 75.6% of the excised shoots within 20-25 days (Plate 1C). Each shoot developed 3-4 roots when evaluated after 20 days. The rooting percentage decreased 60% by supplementing the media with 0.8 mg/1 IBA. Rooting was induced in this medium with in 8-10 days with each shoot forming 2-3 roots in 30 days (Fig. 1). Root initiation was always accompanied by leaf drop and new leaves formed only after the roots were fully developed.

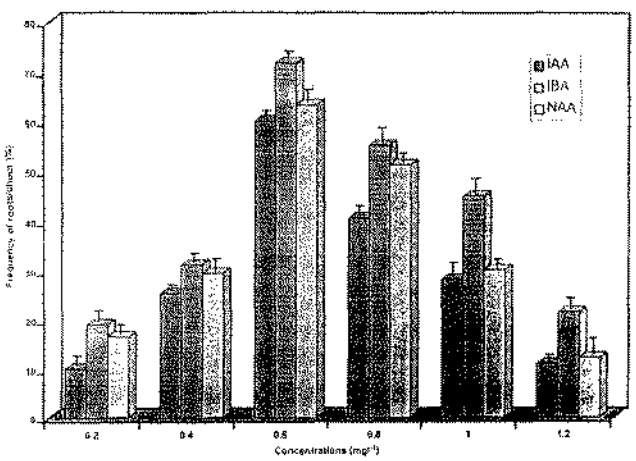


**Fig. 1. Rooting efficiency of *in vitro* raised shoots of *A. amara* on MS medium supplemented with different concentration of auxins. Observations: After 8 weeks**

Seventy one per cent plantlets survived after transfer to vermiculite (Plate 1D). Ninety five per cent of plantlets were successfully transferred to soil. The regenerated plants did not show any detectable phenotypic variation. This protocol could be useful for successful mass propagation.

The anatomical studies (Plate 1E) revealed that the formation of calli from the leaf segment and the commencement of differentiation was marked by the appearance of the distinct cells with dense cytoplasm and prominent nuclei. Generally, these cells are located at sub epidermal as well as in the center of the callus as in *Viola tricolor* (Babber and Sharma 1991). In the present study the gathering of meristematic cells, i.e., meristemoids were marked at both the depths. The upper meristemoids, i.e., cells about 3 to 4 layers possessing relatively larger size differentiated to form shoots. This type of organisation represents the exogenous nature. Similar observations were also reported in *Dalbergia latifolia* in which the shoot organization got initiated from the peripheral cortical region of the callus (Sudhadevi and Nataraja 1987).

Isozyme (peroxidase) profile showed variation between parent plants and *in vitro* raised plants (Plate 1F). Based on the results indirect shoot multiplication is not preferable for generating true-to type plants.

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