

Short Communication

Micropropagation of *Azadirachta indica* A. Juss. via cotyledonary nodes

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An efficient protocol for micropropagation of *Azadirachta indica* A. Juss., a medicinally important plant, has been standardized. Cotyledonary nodes (1 cm long) excised from 15-20 d old *in vitro*-germinated seedlings were used as explants. The seeds were germinated on half strength MS medium devoid of phytohormones. Cotyledonary nodes were cultured on MS medium supplemented with different concentrations of cytokinins (BA/TDZ/2-ip) and auxins (2,4-D and NAA). Maximum shoot proliferation from single explant was obtained on MS media incorporated with TDZ (1.5 μ M), 2,4-D (0.5 μ M), adenine sulphate (40 mg/L), glutamine (100 mg/L) and thiamine HCl (10 mg/L). *In vitro*-produced shoots were induced roots on half strength MS medium supplemented with a range of IBA concentrations (0.5-5.0 μ M). However, the highest frequency of root proliferation was observed on half strength MS medium supplemented with 2.0 μ M IBA. The regenerants were transferred to field conditions after acclimatization with a success rate of 80%.

Keywords: *Azadirachta indica*, cotyledonary node, micropropagation

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Azadirachta indica A. Juss., a member of Meliaceae family, is an evergreen tropical tree. It is popular as an avenue tree and also used for reclamation of poor soil wastelands¹. Almost all parts of the tree are used for medicinal purposes². Mainly the non-wood products of neem have great utility in daily life and serve as anti-diabetic, anti-allergic, anti-feedant, anti-fungal, anti-inflammatory, anti-pyorrhoeic, anti-scabic, anti-viral, anti-tubercular, cardiac, diuretic, insecticidal, larvicidal, nematocidal, piscidal, spermicidal, etc.^{3,4}. It has also found to be effective against cancer⁵ and AIDS⁶. Neem contains potent, biodegradable and economically safe biopesticidal compounds, such as azadirachtins⁷. The low seed viability (30%) warranted the production of *in vitro* tissue-cultured neem plants. Successful *in vitro* plantlet regeneration has been achieved from various explants, like axillary buds⁸, immature embryos⁹ inflorescences¹⁰, callus cultures⁸ through direct and indirect somatic embryogenesis and organogenesis. Present paper reports a method of rapid multiple shoot induction in *A. indica* from cotyledonary nodes.

Seeds of *A. indica* were procured from Biotechnology Research Centre, Tirupati, A.P., India. They were kept under running tap water for 5-10 min, thor-

oughly washed with teepol (0.2%) for 4-5 min and then rinsed with distilled water. Subsequently, they were surface sterilized with 0.1% mercuric chloride for 3-5 min and rinsed thoroughly with sterile distilled water. The seeds were germinated on half-strength MS media devoid of phytohormones. The pH of media was adjusted to 5.8 before autoclaving at 15 psi for 20 min. Cotyledonary node segments (1 cm) from 15-20 d old seedlings were taken and used as explants. MS medium⁶ was used for all experiments; the medium was congealed with agar (0.8%) and sucrose (2%) was used as a source of carbohydrate. Further, the medium was supplemented with cytokinins, BA and TDZ, and auxins (2,4-D and NAA) for shoot proliferation. For evaluating regeneration efficiency during subcultures, *in vitro* raised nodal explants were inoculated on MS medium supplemented with varied concentrations of TDZ (1.5-3.0 μ M) and BA (0.5-1.5). Different concentrations of additives, such as adenine sulphate, glutamine, thiamine and HCl were also added in the media. All the cultures were incubated at 25 \pm 2°C and maintained in 16 h light and 18 h dark photoperiod, having light intensity of 3000-4000 lux, with 55 \pm 5% relative humidity. Regenerated shoots were excised for rooting. They were recultured on MS medium (half strength) fortified with various auxins, viz. IAA, IBA, NAA and 2,4-D in varying concentrations (0.5-5.0 μ M). All the experiments

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were repeated thrice and had 10 replicates with single explant.

Cotyledonary nodes play an important role in shoot production from seedling explant as they supply endogenous growth regulators to the cultures². When cotyledonary node was cultured on MS medium containing different cytokinins (BA/TDZ) singly or in combination with auxins (2,4-D/NAA) varied responses were observed with respect shoot proliferation per explant. BA at 1.0 μM showed 8-9 shoots of 2.2 cm length with 60% regeneration response; whereas, increase in BA concentration (2.0 μM) reduced the number of shoots (7-8), their length (1.8 cm) and regeneration response (50%). Further, BA (1.0-2.0 μM) in combination with 2,4-D (0.5 μM) showed 10 shoots of 1.7-2.4 cm length with 45-55% response; whereas, BA combined with NAA (0.5 μM) showed 8-11 shoots of 1.7-2.8 cm length with 55-70% response. This reveals that BA in combination with 2,4-D or NAA gave more number of shoots than BA alone. On the other hand, TDZ (1.5-2.5 μM) gave per explant 14-18 shoots of 1.5-1.8 cm length with 35-65% regeneration response. However, when MS medium was supplemented with 1.5 μM TDZ + 0.5 μM 2,4-D, better results were observed in number of shoots (18-22), their-length (1.3-1.6 cm) and regeneration response (95%) after 10 d (Fig. 1A). Further, using the same concentrations of hormones gave 25-30 shoots of 1.4-1.5 cm length with 100% response after 15 d (Fig. 1B). Thus, the results show that TDZ (1.5 μM) in combination with 2,4-D (0.5 μM) proved the best combination in terms of shoot proliferation. However, high concentration of TDZ and BA hindered the shoot elongation and resulted in dwarf shoots with reduced leaves.

Nodal explants were sub-cultured for evaluation of regeneration efficiency. The results indicate that MS medium supplemented with 2.5 μM TDZ and 1.0 μM BA showed optimal response with regard to shoot multiplication (9 shoots; Table 1). However, further decrease in the concentration of TDZ and BA resulted in reduction of shoot numbers. Therefore, regular subcultures were performed on the above medium at every 3rd week. The *in vitro*-raised nodal explants were further sub-cultured to 1.5 μM TDZ and 0.5 μM BA + 0.05 μM charcoal (Fig. 1D) for shoot elongation; charcoal (0.05 μM) was used in the medium to control the browning of the medium. At the same time, incorporation of other additives, viz. adenine

sulphate (40 mg/L), glutamine (100 mg/L) and thiamine HCl (10 mg/L), were found to be most effective in shoot elongation (3-4 cm) and in acceleration of multiple shoot proliferation (Fig. 1C).

The *in vitro*-developed shoots (3-4 cm) were grown on half strength MS medium supplemented with either IAA, IBA, NAA or 2,4-D at 0.5-5.0 μM levels.

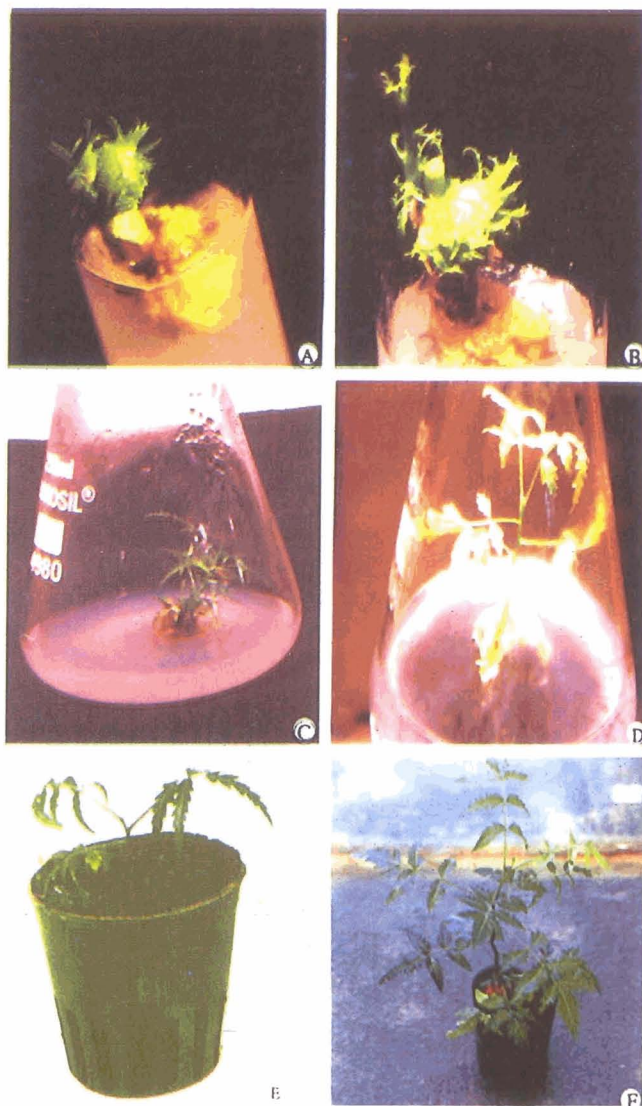


Fig. 1 (A-F)—Micropropagation of *A. indica*: A. Induction of multiple shoots on MS medium containing 1.5 μM TDZ + 0.5 μM 2,4-D from cotyledonary node after 10 d of culture; B. Induction of multiple shoots on MS medium with 1.5 μM TDZ + 0.5 μM 2,4-D after 15 d of culture; C. Regeneration of the shoot on MS medium supplemented with 2.5 μM TDZ + 1.0 μM BA from nodal explants after 20 d of culture; D. Elongation of the shoot of MS medium supplemented with 1.5 μM TDZ + 0.5 μM BA + 0.05 μM activated charcoal after 20 d of culture; E. Hardening of *in vitro* regenerated plant in poly cup containing soil with vermicompost (3:1) after 3-4 weeks; F. Ready to field transferred plant after 30 d.

Table 1—Effect of TDZ and BA in MS medium on shoot multiplication from nodal explants of *A. indica*

PGR concentration (μM)		Nodal explant			
TDZ	BA	Per cent of cultures showing response	Number of shoots/explant \pm SE	Shoot length \pm SE cm	Number of nodes/shoot \pm SE
1.5	0.5	42.6	3.25 \pm 0.08	1.70 \pm 0.10	1.40 \pm 0.16
2.0	0.5	53.4	5.10 \pm 0.11	2.30 \pm 0.07	1.90 \pm 0.01
2.5	0.5	61.2	6.05 \pm 0.17	2.90 \pm 0.01	2.30 \pm 0.12
3.0	0.5	37.3	3.70 \pm 0.12	1.40 \pm 0.08	1.10 \pm 0.16
1.5	1.0	48.2	4.00 \pm 0.09	3.60 \pm 0.18	2.80 \pm 0.17
2.0	1.0	56.3	6.80 \pm 0.16	4.10 \pm 0.12	3.10 \pm 0.14
2.5	1.0	71.4	9.10 \pm 0.13	4.20 \pm 0.16	3.60 \pm 0.08
3.0	1.0	46.3	4.10 \pm 0.17	3.60 \pm 0.12	2.90 \pm 0.07
1.5	1.5	41.6	3.80 \pm 0.02	2.50 \pm 0.06	1.60 \pm 0.12
2.0	1.5	56.6	5.20 \pm 0.16	2.75 \pm 0.01	1.70 \pm 0.14
2.5	1.5	68.4	5.90 \pm 0.06	3.10 \pm 0.08	2.60 \pm 0.17
3.0	1.5	46.3	3.20 \pm 0.06	2.40 \pm 0.07	1.30 \pm 0.07

Observations made after 20 d

Values above represented are mean of 20 replicates conducted thrice

SE=Standard error

Means having the same letter do not differ significantly at 0.001 level (one-way Anova followed by Turkey's test)

IBA induced rooting at 2.0 μM in 86.6% shoots. However, other growth hormones showed poor response as compared to IBA. The combination of IBA + IAA also showed poor rooting response as compared to individual concentrations. The promotory effect of IBA on *in vitro* rooting of shoots has also realized earlier⁷. The roots developed directly at the base of the shoots (Fig. 1D). After 3-4 weeks of culture on rooting medium, the plantlets were transferred to poly cup containing soil and vermicompost (3:1) and maintained under high humidity in the culture room (Fig. 1E). Further, the plants were transferred to the field after 30 days of acclimatization, which showed 80% of survival (Fig. 1F).

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