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In vitro clonal propagation of an adult tree of neem (*Azadirachta indica* A. Juss.) by forced axillary branching

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Abstract

In vitro clonal propagation of a 50-year-old neem tree through axillary shoot proliferation was achieved. Nodal explants collected between March and May gave the best shoot proliferation response (80% aseptic cultures). 1/2 MS (major inorganic salts reduced to half strength) medium was required initially for the establishment of nodal segment cultures. Multiple shoot formation occurred in the cultures of 1/2 MS + BAP (1 μM) + GA_3 (0.5 μM). Number of shoots enhanced further on transfer of cultures to 1/2 MS + BAP (1 μM) + CH (500 mg l^{-1}). However, both the media did not support shoot growth and the shoots remained compact and stunted. Therefore, for elongation and recurrent shoot multiplication, full MS + BAP (1 μM) + CH (250 mg l^{-1}) medium was used where the shoots elongated well, and could be multiplied through single node segment cultures at a rate of 7–8-fold every 5 weeks on the fresh medium of the same composition and this rate of shoot multiplication was maintained for almost 5 years. On 1/4 MS + IBA (0.5 μM), the shoots could be readily rooted with a frequency as high as 82%. Micropropagated plants were established in soil with >87% survival.

Keywords: Axillary-shoot-proliferation; Clonal propagation; Neem; Nodal segment culture

1. Introduction

In recent, *Azadirachta indica* A. Juss. has attracted world wide attention as a multipurpose tree. Its products are being globally used in agriculture, medicine, cosmetics and animal health care [1]. Almost every part of this tree—seeds, leaves, roots, bark, trunk and branches—has multiple uses [2]. The maximum industrial utilization of neem is for its seed-oil, which contains several active compounds. Azadirachtin is the most prominent constituent of neem kernels [3].

Neem trees show considerable variability in the azadirachtin content of their seeds, irrespective of the habitat [4,5]. Considerable improvement in azadirachtin production can be achieved by clonal propagation of elite trees. Vegetative propagation of an adult neem tree by conventional methods is difficult [6]. Natural propagation of neem occurs by seeds, and the reproductive phase normally begins after 5 years [7,8]. The seeds loose viability within 2 weeks [9]. Propagation by seeds is also undesirable because of the highly heterozygous nature of the plant due to cross pollination. Tissue culture would not only overcome these limitations but also considerably accelerate the production of clonal material for field planting.

Consequently, several investigators have attempted clonalpropagation of neem tree [10–18]. Most of these reports deal with juvenile material or material of unspecified age, which is of little significance in clonal propagation of elite trees. Most other reports lack crucial information such as the rate of multiplication in recurrent subcultures. However, the present study, reports a highly reproducible and recurrent method of clonal propagation of a 50-year-old neem tree through axillary shoot proliferation.

Abbreviations: BAP, 6-benzylamino purine; CH, casein hydrolysate; GA_3, gibberellic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid

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2. Material and methods

2.1. Plant material and initiation of aseptic cultures

Single node cuttings (2 cm) from a 50-year-old tree growing in the garden of the Botany Department, University of Delhi, were collected at monthly intervals over three consecutive years to initiate cultures. After removing leaves, the cuttings were thoroughly washed with 1% solution of savlon (Johnson and Johnson Ltd., Mumbai, India) for 20 min and rinsed twice in sterile distilled water (SDW). All subsequent operations were carried out inside a laminar air-flow cabinet (Cleanair Atlantis Appl. Engg. Pvt. Ltd., New Delhi, India). The clean cuttings were given a quick (30 s) rinse in 70% ethanol, followed by two washings in SDW. These were then surface-sterilized in 0.15% mercuric chloride ($HgCl_2$) solution for 13 min and rinsed thrice with SDW. The cuttings were slightly trimmed at both ends to expose fresh tissue before planting them on Murashige and Skoog [19] (MS) medium containing 3% sucrose and gelled with 0.8% agar (Qualigens, Mumbai, India) (basal medium).

The MS medium was supplemented with plant growth regulators and other adjuvants. After adjusting the pH to 5.8, 20 ml of medium was dispensed into each 150 × 25 mm Borosil rimless glass tube. The culture tubes were plugged with nonabsorbent cotton wrapped in cheesecloth and autoclaved at 1.06 kg cm^{-2} and 121 °C for 15 min. Thermolabile compound, such as gibberellic acid (GA_3), was filter-sterilized and added to the autoclaved medium cooled to 50 °C; medium was then dispensed into glass tubes inside a laminar air-flow cabinet. All the cultures were maintained in diffuse light (1000–2000 lx) and 16 h photoperiod at 25 ± 2 °C and 50–60% relative humidity.

In the preliminary experiments, the explants were cultured onto either full-strength Murashige & Skoog (MS) or half-strength MS (1/2 MS, major inorganic salts reduced to half-strength) basal medium. Subsequently, 1/2 MS medium was tested with BAP (1 μM) alone or in combination with GA_3 (0.5 μM). The nodal explants with clusters of shoots produced by 5-week-old primary cultures of nodal segments on 1/2 MS + BAP (1 μM) + GA_3 (0.5 μM) were transferred to 1/2 MS + BAP (1 μM) + 500 mg l^{-1} CH (Casein hydrolysate; RM013, HiMedia Laboratories Pvt. Ltd., Mumbai, India; contains Vitamin B_{12}, Pepsin, Trypsin, Papain) for further shoot multiplication.

2.2. Multiplication and rooting of shoots

Small (0.5 cm) individual shoots from 5-week-old cultures of 1/2 MS + BAP (1 μM) + CH (500 mg l^{-1}) were excised carefully and transferred to full-strength MS medium supplemented with BAP (1 μM) and lower concentration of CH (250 mg l^{-1}) for elongation of shoots. At the end of the passage, each axillary shoot, which had grown fairly long, was cut into single node segments and transferred to fresh medium for further multiplication. The number of propagules obtained at the end of a multiplication cycle was regarded as the rate of shoot multiplication.

For rooting, terminal 3 cm long portions of elongated shoots with 3–4 nodes were excised and cultured on MS, 1/2 MS (major inorganic salts reduced to 1/2 strength) or 1/4 MS (major inorganic salts reduced to 1/4 strength) medium supplemented with indole-3-butyric acid (IBA) in the range of 0.5 to 1.0 μM.

At least 24-cultures were raised for each treatment and all the experiments were repeated at least three times. Observations on number of cultures showing contamination, bud-break, shoot elongation and rooting were made at weekly intervals. Standard error of the mean was calculated and is indicated by ± sign.

2.3. Transplantation

The rooted plants were washed to remove the agar, transferred to soilrite (Chowgule Industries, India; contains peat moss with vermiculite) in hycotrays (Sigma, St. Louis, MO) and placed in a glasshouse at the Tissue Culture Pilot Plant (TCPP) of the Tata Energy Research Institute (TERI). The glasshouse is furnished with facilities to maintain a gradient of humidity by the Fan-and-Pad system and a temperature of 25 ± 2 °C. Close to the pad, which is continuously drenched with water, the relative humidity (RH) is almost 100% and it gradually decreases towards the other end of the glasshouse. Initially, the plants were placed close to the pad and covered with cling film. After 10 days the cling film was removed and after another 3 weeks the plants were transferred to soil in polythene bags and sprayed with a mixture (1:1) of 0.1% Urea (BASS, India) and Bavistin (BASS, India; an antifungal consisting of carbendazim, sodium salt of phenol, sulphonic acid condensation product, sodium salt of alkyl naphthalene and china clay). Thereafter, the plants were gradually moved away from the pad end. After another 4 weeks the plants were transferred to pots and shifted to a polyhouse (at 25 ± 2 °C). After 3 months of transplantation, the plants were shifted to a shaded area under natural conditions.

3. Results and discussion

3.1. Establishment of aseptic nodal segment cultures

Contamination of *A. indica* nodal explants was a major problem during initiation of cultures under in vitro conditions. The explants were mainly contaminated by fungus and rarely by bacteria. The rate of contamination or bud-break was highly dependent on the season during which the material was collected. By the sterilization procedure described in Section 2, the cultures initiated in March–May (during normal flowering in India) showed higher bud-break (80%) and less contamination (20%) than those raised in June–October or November–February. Since June–October

is a period coinciding with the rainy season in India, 100% explants showed contamination. By November–February the shoots became old and it was difficult to break down the mature dormant state of the buds.

Therefore, routinely, the cultures were raised in March–May because of the least contamination and best shoot growth recorded in this season. Similar seasonal effect on culture establishment has been reported for apple [20], papaya [21], sweet gum [22] and guava [23]. Sharma et al. [16] could obtain, after nine steps of rigorous surface sterilization, only 20% aseptic cultures initiated in March–April from 3-, 7-, 40-year-old trees of neem.

3.2. Axillary shoot proliferation

Axillary shoot proliferation from the nodal explants of mature tree varied considerably at different salt concentrations in the medium, and MS basal medium with half the concentration of major inorganic salts (1/2 MS) showed bud-break in 20% (1.0 nodes/shoot) of the cultures as against 5% (1.0 nodes/shoot) cultures at full concentration. Most of the nodal explants turned brown at full concentration after 2 weeks of incubation; hence for establishing nodal segment cultures 1/2 MS medium was used initially. Addition of 1 μM BAP to 1/2 MS medium enhanced the frequency of bud-break (50%). However, only a single shoot (3 cm long) developed per node. The shoots were not very healthy; yellowing and abscission of leaves occurred after 4 weeks. Incorporation of GA_3 (0.5 μM) to 1/2 MS + BAP (1 μM) medium improved the incidence of bud-break and promoted multiple shoot formation. On 1/2 MS + BAP (1 μM) + GA_3 (0.5 μM) medium bud-break occurred within 2 weeks and an average of 4 small shoots per explant were formed within 4 weeks in 70% cultures. However, the shoots remained very small (<5 mm) even after 5 weeks (Fig. 1A).

In the second passage, the explants with clusters of 3–4 shoots from 1/2 MS + BAP (1 μM) + GA_3 (0.5 μM) were transferred after 5 weeks to 1/2 MS + BAP (1 μM) + CH (500 mg l^{-1}) medium. The number of shoots enhanced further and an average of 7.5 shoots per explant were formed after 5 weeks in 91% cultures (Fig. 1B). One of the explants produced as many as 30 shoots. However, most of the shoots remained compact and stunted and did not grow beyond 0.7 cm. Shoot proliferation was associated with the proliferation of callus at the base of the explant only.

Initiation and in vitro propagation of mature trees in general is difficult due to various problems, mainly recalcitrance

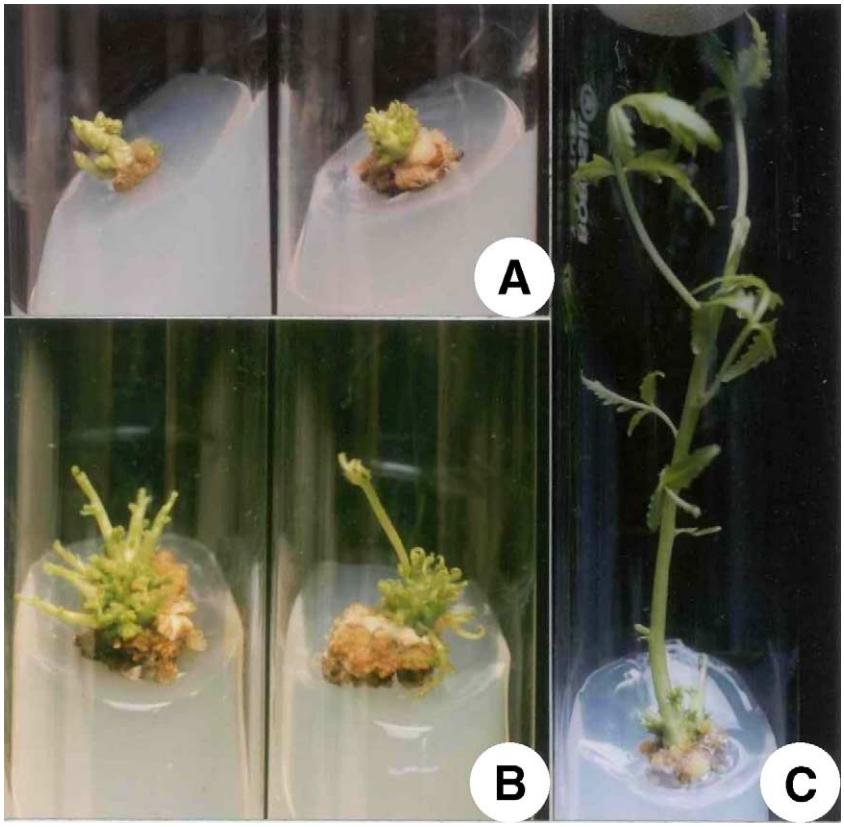

Fig. 1. (A) Five-week-old single node segment cultures on 1/2 MS + BAP (1 μM) + GA_3 (0.5 μM), showing the emergence of multiple shoots at the node and basal callusing; the shoots did not elongate (1.7×). (B) Cultures transferred from A to 1/2 MS + BAP (1 μM) + CH (500 mg l^{-1}). Some of the shoots have elongated and some new shoots have differentiated after 5 weeks (18×). (C) An excised shoot from B, 5 weeks after transfer to MS+BAP (1 μM) + CH (250 mg l^{-1}). The shoot has elongated considerably (1.6×).

of the tissue, contamination and field establishment. Joarder et al. [13] found that the pre-culture of nodal segments, taken from 30-year-old neem tree, on MS basal medium for 2 weeks followed by 4-weeks on MS + BAP (1.5 mg l^{-1}) medium was essential/ beneficial for bud-break to occur. Although the paper lacks details regarding the rate of shoot multiplication in subsequent subcultures, initially two shoots developed per node and the number of shoots increased with the increasing number of subcultures and then declined after five to six subcultures.

Joshi and Thengane [14] cultured nodal segments from 2–5-year-old juvenile trees as those from 15–20-year-old mature trees showed negligible bud-break. Sharma et al. [16] cultured nodal segments from 3, 7- and 40-year-old trees and found the explants from younger trees to be more responsive. Shoot multiplication at a rate of five-fold in 30 days was achieved, generally, after the fifth passage on MS medium supplemented with 0.25 mg l^{-1} each of BAP and IAA and 15 mg l^{-1} adenine sulphate. However, in the explants taken from 3-, 7-year-old trees, this rate of shoot multiplication was achieved even by the third subculture. Islam et al. [12] recorded 4.5 shoots per explant upto fourth subculture from nodal segments of 25-year-old tree on MS medium supplemented with 1 mg l^{-1} each of BAP and Kn.

3.3. Elongation and multiplication of shoots

In the above experiment, 1/2 MS + BAP (1 μM) + CH (500 mg l^{-1}) proved optimum for shoot multiplication in the second passage but it did not facilitate shoot growth. Therefore, 0.5 cm long individual shoots were excised carefully after 5 weeks and transferred to MS + BAP (1 μM) supplemented with 250 mg l^{-1} CH for elongation. On MS + BAP (1 μM) + CH (250 mg l^{-1}), the original shoot attained a length of 8.5 cm, with 8 nodes, after 5 weeks in 95% cultures (Fig. 1C). At the end of the passage each shoot was cut into single node segments and planted on fresh MS + BAP (1 μM) + CH (250 mg l^{-1}). Each node again produced a single multinodal shoot, which provided 7–8 cuttings after 5 weeks (Fig. 2A). Thus, eight-fold shoot multiplication every 5 weeks could be achieved on MS + BAP (1 μM) + CH (250 mg l^{-1}) by cutting the solitary shoot into single node segments and culturing them on fresh medium. This rate of shoot multiplication was maintained for almost 5 years. More than 30 times the nodal explants were taken and used for shoot multiplication. Since every time the explants were taken from freshly formed in vitro shoots, therefore, we have not observed any significant difference (variation) in the results. This was observed for 5 years and after that all the cultures were given to Tissue Culture Pilot Plant of Tata Energy Research Institute, Delhi, India.

Islam et al. [12] used a medium with a lower level of BAP (0.1 mg l^{-1}) for shoot elongation, suggesting that the shoots remained very small on the multiplication medium and required an elongation step to obtain shoots suitable for rooting.

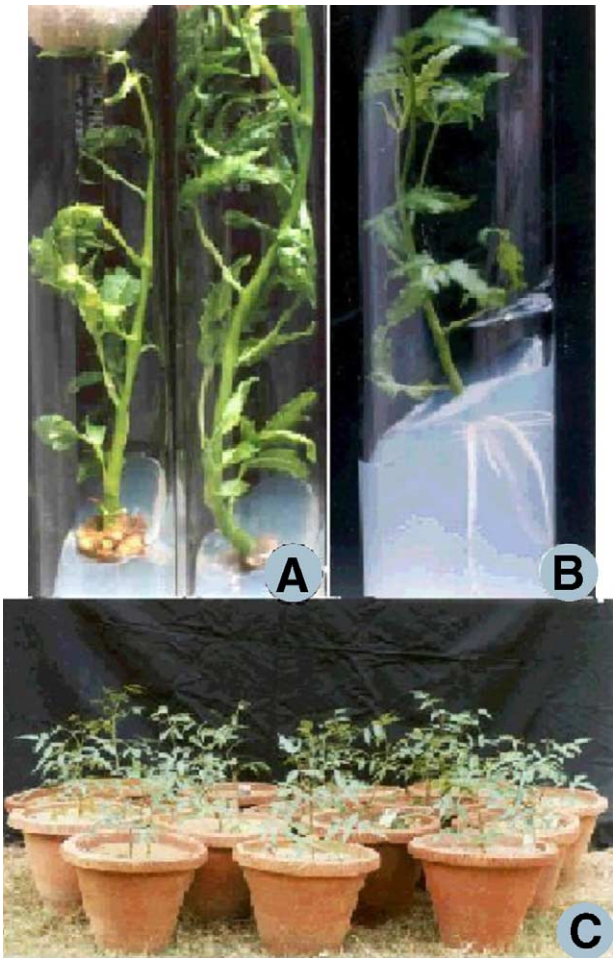

Fig. 2. (A) Single node segments from in vitro developed shoots on MS+BAP (1 μM) + CH (250 mg l^{-1}), were cultured on the same medium. The axillary bud has developed into a long, unbranched multimodal shoot after 5 week (1.3×). (B) An in vitro-developed shoot 4 weeks after transfer to 1/4 MS+IBA (0.5 μM). The shoot has developed healthy roots directly from the cut, basal end (1.3×). (C) Hardened micropropagated plants, 8 months after transfer to soil (0.1×).

3.4. Rooting of shoots

Terminal 3 cm long portions of shoots from 5-week-old cultures on MS+BAP (1 μM) + CH (250 mg l^{-1}) were used for rooting. The remaining portions of the shoots were cut into single node segments and utilized for further multiplication. For rooting, MS was tested at full (MS), half (1/2 MS) and quarter (1/4 MS) strengths of the major inorganic salts. All media were supplemented with IBA at 0.5 μM or 1.0 μM (Table 1). 1/4 MS was distinctly better than 1/2 MS or full MS in terms of the frequency of rooting. Rooting was induced directly at the base of the shoot on 0.5 μM IBA whereas it was preceded by callusing at the base of the shoot on 1 μM IBA. On 1/4 MS + 0.5 μM IBA, which proved to

Table 1
Rooting response of shoots on full MS, 1/2 MS and 1/4 MS basal media or basal media supplemented with IBA

Treatment (μM)	% Shoots rooted	No. of roots per shoot
Full MS	0	0
1/2 MS	0	0
1/4 MS	20 ± 1.0	2.0 ± 0.5
Full MS + IBA (0.5)[a]	58 ± 2.0	6.2 ± 0.5
1/2 MS + IBA (0.5)[a]	65 ± 1.0	8.2 ± 0.5
1/4 MS + IBA (0.5)[a]	82 ± 1.0	8.4 ± 0.5
Full MS + IBA (1.0)	74 ± 2.0	6.0 ± 0.5
1/2 MS + IBA (1.0)	85 ± 1.0	4.0 ± 0.5
1/4 MS + IBA (1.0)	95 ± 1.0	6.0 ± 0.5

Growth period: 4 Weeks. ±: S.E.

[a] Roots formed directly at the base of the shoot; in all other media root formation was preceded by callus formation at the base of the shoot.

be the best rooting medium, 82% shoots formed an average of 8.4 roots directly from the basal end of the shoot. Even the root length and the number of laterals were maximum on this medium (Fig. 2B). On this medium roots appeared after 3 weeks and maximum response was observed after 4 weeks.

Most of the investigators could achieve 70–100% rooting of the in vitro regenerated or multiplied shoots of neem. However, the optimum conditions described vary considerably. Generally an auxin has been necessary, and MS with reduced salts has been found to be better than full MS. Whereas Joshi and Thengane [14] and Venkateswarlu et al. [18] found IAA (2–3 mg l^{-1}) to be the best auxin for rooting of neem shoots, Joarder et al. [13] and Islam et al. [12] found IBA (0.5 mg l^{-1}) to be most effective. Gill et al. [11] and Roy et al. [15] reported rooting in the combined presence of IBA and IAA. Sharma et al. [16] found shoots multiplied from a 40-year-old tree to be recalcitrant for rooting. However, in the present study the shoots from a 50-year-old tree could be readily rooted on 1/4 MS + 0.5 μM IBA, with a frequency as high as 82%.

3.5. Transplantation

Following the protocol described under Materials and Methods, 40 plants from 1/4 MS+IBA (0.5 μM) were transferred out of culture. Of these, 35 plants survived (Fig. 2C). Thus, transplantation survival of micropropagated plants was 87.5%.

The present study, thus, demonstrated the possibility for mass clonal propagation of a 50-year-old neem tree by nodal segment cultures. MS medium supplemented with BAP (1 μM) and CH (250 mg l^{-1}) medium was used for recurrent shoot multiplication at a rate of 7–8-fold every 5 week and this rate of shoot multiplication was maintained for almost 5 years. The shoots could be readily rooted with a frequency as high as 82%. Transplantation survival of these plants was more than 87%.

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References

[1] B. Venkateswarulu, Tissue culture technology for propagation of elite neem tree, SAIC News-letter, March 1996.
[2] R. Chaturvedi, M.K. Razdan, S.S. Bhojwani, Production of haploids of neem (*Azadirachta indica* A. juss.) by anther culture, Plant Cell Rep. 21 (2003) 531–537.
[3] N. Kaushik, Determination of azadirachtin and fatty acid methyl esters of *Azadirachta indica* seeds by HPLC and GLC, Anal. Bioanal. Chem. 374 (2002) 1199–1204.
[4] K. Ermel, Azadirachtin content of neem seed kernels from different regions of the world, in: H. Schmutterer (Ed.), The Neem Tree: Source of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes, VCH, Weinheim, 1995, pp. 222–230.
[5] O.P. Sidhu, V. Kumar, H.M. Behl, Variability in neem (*Azadirachta indica*) with respect to azadirachtin content, J. Agric. Food Chem. 51 (2003) 910–915.
[6] P.D. Dogra, R.C. Thapliyal, Gene resources and breeding potential, in: N.S. Randhawa, B.S. Parmar, (Eds.), Neem, New Age International Pvt. Ltd., New Delhi, 1996, pp. 27–32.
[7] O. Koul, M.B. Islam, C.M. Ketkar, Properties and uses of neem, *Azadirachta indica*, Can. J. Bot. 68 (1990) 1–11.
[8] H. Schmutterer, The tree and its characteristics, in: H. Schmutterer (Ed.), The Neem Tree: Source of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes, VCH, Weinheim, 1995, pp. 1–29.
[9] H.Y. MohanRam, M.N.B. Nair, Botany, in: N.S. Randhawa and B.S. Parmar, (Eds.), Neem, New Age International Pvt. Ltd., New Delhi, 1996, pp. 6–26.
[10] R.A. Drew, Clonal propagation of neem by tissue culture, in: Proceedings of the World Neem Conference, Bangalore, 24–28 February 1993, pp. 999–1005.
[11] R.I.S. Gill, S.S. Gill, S.S. Gosal, Protocol for *in vitro* asexual multiplication of *A. indica*, in: A.S. Islam (Ed.), Plant Tissue Culture, Oxford and IBH, New Delhi, 1996, pp. 155–160.
[12] R. Islam, A. Hoque, M. Khalekuzzaman, O.I. Joarder, Micropropagation of *Azadirachta indica* A. Juss. from explants of mature plants, Plant Tissue Cult. 7 (1997) 41–46.
[13] O.I. Joarder, A.T.M. Naderuzzaman, R. Islam, M. Hossain, N. Joarder, B.K. Biswas, Micropropagation of neem through axillary bud culture, in: Proceedings of the World Neem Conference, 24–28 February 1993, Bangalore, pp. 961–966.
[14] M.S. Joshi, S.R. Thengane, *In vitro* propagation of *Azadirachta indica* A. Juss. (Neem) by shoot proliferation, Indian J. Exp. Biol. 34 (1996) 480–482.
[15] S.K Roy, M. Assaduzzaman, Z. Hossain, Clonal propagation of *Azadirachta indica* by *in vitro* culture, in: A.S. Islam (Ed.), Plant Tissue Culture, Oxford and IBH, New Delhi, 1996, pp. 149–154.
[16] A.K. Sharma, M. Sharma, H.C. Chaturvedi, Conservat ion of phytodiversity of *Azadirachta indica* A. Juss. through *in vitro* strategies, National Symposium on Role of Plant Tissue Culture in Biodiversity Conservation and Economic Development, 7–9 June 1999, G.B. Pant Institute of Himalayan Environment and Development, Almora, 1999, pp. 1–13.
[17] B. Venkateswarlu, K. Mukhopadhyay, Azadirachtin content in the seeds of micropropagated neem plants in relation to its mother tree, Curr. Sci. 76 (1999) 626–627.

[18] B. Venkateswarulu, J.C. Katyal, J. Choudhari, K. Mukhopadhyay, Micropropagation of plus neem (*Azadirachta indica* A. Juss.) and evaluation of field transferred plants, Indian For. 124 (1998) 537–543.
[19] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco cultures, Physiol. Plant. 15 (1962) 473–497.
[20] J.F. Hutchinson, Factors affecting shoot proliferation and root initiation in organ culture of apple 'Northern Spy', Sci. Hortic. 22 (1984) 347–358.
[21] R.E. Litz, R.A. Conover, Effect of sex type, season and other factors on *in vitro* establishment and culture of *Carica papaya* L. explants., J. Am. Soc. Hortic. Sci. 106 (1981) 792–794.
[22] E.G. Sutter, P.B. Barker, *In vitro* propagation of mature *Liquidambar styraciflua*, Plant Cell Tissue Organ Cult. 5 (1985) 13–21.
[23] M.N. Amin, V.S. Jaiswal, Rapid clonal propagation of guava through *in vitro* shoot proliferation on nodal explants of mature trees, Plant Cell Tissue Organ Cult. 9 (1987) 235–243.