Effect of casein hydrolysate and major inorganic salts on axillary-bud proliferation from nodal explants of a mature neem tree, A*zadirachta indica* A. Juss.

Srivastava Priyanka[1], Singh Mithilesh[1] and Chaturvedi Rakhi[1*]

[1*]Department of Biotechnology
Indian Institute of Technology- Guwahati
Guwahati – 781 039, Assam, India
Tel: +91-361-2582211 (off)
Fax: +91-361- 2582249; 2690762
Email: rakhi_chaturvedi@iitg.ernet.in
rakhi_chaturvedi@yahoo.co.uk

Abstract

An efficient protocol has been established to improve the incidence of bud-break and shoot-growth in single node segment cultures of a 35-year-old mature neem tree. Routinely, the cultures were raised in summer (March-May) because of the highest aseptic culture establishment and best shoot growth recorded in this season. ½ MS (major inorganic salts reduced to half strength) medium supplemented with 1µM 6-benzylaminopurine (BAP) and 500mgl^{-1}casein hydrolysate (CH) proved to be the best to initiate maximum percentage of bud-break in cultures. For further shoot multiplication, full strength MS medium containing BAP (1µM) and a low concentration of CH at 250 mgl^{-1} was found to be the best. Therefore, for recurrent shoot multiplication, single node segments from in vitro developed shoots were excised and cultured on MS + BAP (1µM) + CH (250mgl^{-1}) medium where a pre-existing axillary bud proliferated into an elongated (9.5cm long) healthy, multinodal solitary shoot (with 9 nodes/shoot), after 5 weeks. The shoot was again cut into single node segments and planted onto the fresh medium of the same composition for large scale clonal propagation. With this method, 9-fold recurrent shoot multiplication was achieved in every 5 weeks. Finally, these shoots were rooted with a frequency of 84% on ¼ MS (major inorganic salts reduced to quarter strength) medium supplemented with 0.5µM indole-3-butyric acid (IBA). Transplantation survival of micropropagated plants was 81.8%.

Keywords

Axillary-bud proliferation; *Azadirachta indica*; Casein hydrolysate; Inorganic salts; Micropropagation; Neem

Abbreviations

BAP - 6-benzylaminopurine; CH - casein hydrolysate; GA_3 - gibberellic acid, IBA - indole-3-butyric acid

1. Introduction

Azadirachta indica A. Juss. is a majestic, evergreen, tropical forest tree belonging to the family Meliaceae. Besides being a popular avenue tree with a large crown, the wood of neem has been used as timber for house building, furniture and other domestic and agricultural tools. The timber is reported to work well with hand and machine tools[36]. Its wood resembles teak wood in strength and is more resistant to shock, fungi and insect attack; it is immune to termites and durable even outdoors[37]. Neem tree is known to increase the soil fertility and its water holding capacity as the tree has a unique property of calcium mining which changes the acidic soil into neutral[2]. The tree is resistant to high temperatures and drought and has been employed for afforestation of dry localities, reforesting bare ravines and checking soil erosion[13].

Besides, various parts of the neem tree, particularly leaves, bark and seeds have been traditionally used in India in ayurvedic medicines. The seed oil has been used as antimalarial, antihelminthic, vermifuge, antiseptic, antimicrobial and is also known to cure various skin disorders. Neem owe these properties due to the presence of several bioactive compounds; the most prominent one being Azadirachtin. To meet the economic demand of the neem tree, an efficient propagation technique is required which could result in large quantities and good quality of planting materials. Vegetative propagation of an adult neem tree by conventional methods is difficult[21]. Therefore, it is normally grown from seeds but the seeds are of recalcitrant type; they loose viability within 2-3 weeks[27]. Propagation by seeds is also undesirable because of the highly heterozygous nature of the plant owing to cross-pollination and enormous heterozygosity. Moreover, the reproductive phase in neem normally begins after 5 years of seed propagation[22, 33]. So, one has to wait for a long time to obtain seeds and fruits.

In vitro micropropagation method would provide the best means for mass clonal propagation of selected elite trees in a short period of time. Plant propagation through axillary-shoot-proliferation is the choicest method for true-to-type propagation [28, 15, 16, 5]. The major advantage gained by maintaining such highly organized cultures is that the clonal fidelity is assured [28, 24]. There are a few reports on forced axillary branching and complete plantlet development in neem where the seedling material or material of unspecified age was used

as the initial explant[10, 19], which is of no significance in clonal propagation of elite trees. Two reports are available on clonal propagation of adult neem trees (25-30 years old) through axillary-shoot-proliferation, but these reports lack crucial information on rate of shoot multiplication in recurrent subcultures[18, 32]. On the contrary, few others found the explants from younger trees (2-8-year-old) are more responsive for clonal propagation of neem[14, 20, 34]. Of the above mentioned reports, though some have been successful in allowing rapid clonal propagation of young neem tress, others lack crucial information such as age of explant source and/or rate of shoot multiplication which are of immense importance in micropropagation studies. Thus, there is an urgent need to develop a protocol for large scale propagation of mature trees to supply sufficient clonal planting material. The paucity of reports on large-scale clonal propagation of many woody trees through tissue culture lies mainly in the difficulty of initiating axenic cultures and in attaining a high rooting percentage. Thus, the present study was undertaken to determine the factors that could contribute to successful initiation of cultures, continuous and rapid proliferation of axillary shoots and a high rooting rate, compatible for mass propagation of a 35-year-old field grown mature tree of neem.

2. Materials and methods

2.1. Culture establishment and induction of axillary-bud-proliferation

Single nodal segments (2.0 cm) were excised from lateral branches of the main trunk of a 35-year-old elite neem tree during three seasons, summer (March- May), rain (June-September) and winter (October-February), over three consecutive years to initiate cultures. After removing the leaves, the cuttings were thoroughly washed with 1% Savlon solution for 20 minutes and rinsed with sterile distilled water. All subsequent operations were done inside the laminar-air-flow cabinet (Saveer Biotech, India). The cuttings were given a quick rinse in 70% ethanol for 30 seconds, followed by two washings in sterile distilled water. They were then surface sterilized with 0.15% (m/v) mercuric chloride solution for 13 minutes and rinsed thrice with sterile distilled water. The cuttings were slightly trimmed at the cut ends to expose the fresh tissue before being cultured on Murashige & Skoog[29] (MS) basal medium containing 3% sucrose and gelled with 0.8% agar (HiMedia Laboratories Pvt. Ltd., India). After adjusting the pH to 5.8, 20 ml of the medium was dispensed into each 150 x 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 minutes. All

the cultures were maintained in diffuse light (1000-2000 lux) and 16 hr photoperiod at 25±2°C and 50-60% relative humidity.

The sterilized explants were initiated on either full-strength MS or half-strength MS (½ MS, major inorganic salts reduced to half-strength) basal medium. Subsequently, the cultures were tested with the medium containing 1µM 6-benzylaminopurine (BAP) alone or in combination with 500mgl^{-1} casein hydrolysate (CH; RM 013, HiMedia laboratories Pvt. Ltd., Mumbai, India; contains vitamin B12, Pepsin, Trypsin, Papain) for axillary-bud-proliferation. The axillary bud-break started from second week onwards and the shoots for in vitro multiplication became available only after the fifth week.

2. 2. Multiplication of shoots

For multiplication, single node segments from shoots induced on ½ MS + BAP (1µM) + CH (500mgl^{-1}) were excised carefully from 5-week-old cultures and transferred to full-strength MS medium supplemented with BAP (1µM) and CH in the range of 250-2000 mgl^{-1}. At the end of the passage, each axillary shoot, which had grown fairly long, was cut into single node segments of about 1 cm length and transferred to fresh medium of same composition for further multiplication. The number of propagules obtained at the end of a multiplication cycle was regarded as the rate of shoot multiplication.

2.3. Rooting of shoots

For rooting, terminal 3 cm long portions of elongated shoots with 3-4 nodes were excised, given an oblique cut at the base to increase the surface area of absorption for the nutrients from the medium and cultured on MS, ½ MS (major inorganic salts reduced to ½ strength) or ¼ MS (major inorganic salts reduced to ¼ strength) medium supplemented with 0.5 and 1.0 µM indole-3-butyric acid (IBA).

2.4. Data collection and statistical analysis

At least 24-cultures were raised for each treatment and all the experiments were repeated at least three times. Observations on the 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.

Shoot proliferation rate was evaluated by counting the number of nodes on the shoots produced, considering that every node could be used as new explant for further subcultures, and it is presented on 5 weeks base.

Rooting experiments lasted for 4 weeks and at the end of each experiment, the number of rooted shoots, number of roots per shoot, length of the longest root and number of laterals were evaluated and rooting capacity expressed as percentage.

The number of successfully acclimatized plants was recorded 3 months after transplantation and expressed as survival percentage.

2.5. Acclimatization of regenerated plants

The rooted plants were washed to remove the agar, transferred to soilrite (Chowgule Industries, India; contains peatmoss with vermiculite) in hycotrays (Sigma, St. Louis, MO) and placed in a glasshouse. The glasshouse is furnished with facilities to maintain a gradient of humidity by the microprocessor controlled overhead fogging system, with temperature of 25±2°C and a photoperiod of 1600 lux. The relative humidity (RH) of the glasshouse is almost 70%. Initially, the plants were covered with cling film to maintain humidity. 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 the 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). 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 shaded area under natural conditions.

3. Results and discussion

3.1. Culture establishment

Aseptic culture establishment and bud-break depends upon amount of contaminations and physiological status of the collected explants during various seasons of the year[31]. The explants, in the present study, were mainly contaminated by fungus and rarely by bacteria. The rate of contamination and bud-break frequency varies with the season during which material was collected. Summer (March-May) had significantly highest aseptic culture establishment (79%) and less contamination (21%). The rain (June-September) and winter seasons (October-February) recorded significantly lower aseptic culture establishment (22% and 38% respectively). Whereas, summer begins with the resumption of active vegetative growth of neem during normal flowering in India that competes with pathogens resulting in excellent culture establishment and bud-

break, by winter the shoots become old and it was difficult to break down the mature dormant state of the buds.

Therefore, routinely, the cultures were raised in summer (March-May) because of the highest aseptic culture establishment and best shoot growth recorded in this season. Similar, seasonal effect on culture establishment has been reported for papaya[23], apple[17], guava[1], mulberry[7] and bamboo[8, 25]. Sudershan and Hussain[35] attributed this difference in response to the endogenous level of growth regulators and their activity, whereas Amin and Jaiswal[1] suggested that the degree of phenolic exudation, resulting in the necrosis and death of the tissues, might be the reason for differential growth responses in vitro. The intensity of phenolic exudation is more in the explants collected from the older shoots than those from the younger ones[30].

3.2. Induction of axillary shoot proliferation

Several tree species have been reported to prefer low salt concentration for their survival and multiplication in cultures where the level of salts in the MS medium is either toxic or unnecessarily high[4]. In the present study also, the rate of bud-break was significantly affected by the strength of the major inorganic salts in the MS medium. On MS basal medium (with full strength of the major inorganic salts), 60% of the nodal segments showed bud-break but the internodes of the axillary shoots were highly condensed, and the number of nodes could not be counted. Reducing the major inorganic salt concentration to half (½ MS), promoted higher bud-break (82%) than the full-strength MS medium but the axillary shoot remained highly condensed. Addition of BAP (1µM) overcame this deficiency by considerably promoting the frequency of bud-break and the shoot elongation. On an average 70% of the cultures showed bud-break after two weeks on MS (with full strength of the major inorganic salts) + BAP (1µM) and shoots attained a length of 5.3 cm, with an average of 2.1 nodes/shoot, after 5 weeks (Fig. 1). However, on this combination shoots were not very healthy; and almost half of the cultures showed yellowing and abscission of leaves after 4 weeks. Interestingly, ½ MS (major inorganic salts reduced to half strength) + BAP (1µM) supported a fairly good percentage of bud-break and healthy shoot proliferation, where 82% cultures responded with a solitary shoot development measuring 2 cm in length, with an average of 2 nodes/shoot, after 5 weeks. Irrespective of the treatments, all the explants showed single shoot development (Fig.1). Incorporation of CH (500mgl^{-1}) to ½ MS + BAP (1µM) has further improved the incidence of bud-break and shoot-growth. ½ MS + BAP (1µM) + CH

(500mgl^{-1}) medium induced bud-break within 2 weeks and an average of 4 cm long solitary shoot, with 5 nodes/shoot, developed in 100% cultures after 5 weeks (Fig. 2). Thus, 5-fold multiplication was achieved in every 5 weeks in the initial cultures. Here, the shoot proliferation was associated with the proliferation of callus at the base of explant only (Fig. 5a).

The major problem in in vitro propagation of mature woody trees lies in their recalcitrance, contamination and establishment in field. Joarder et al.[18] cultured nodal segments from a 30-year-old tree of neem and observed maximum shoot proliferation on MS + BAP (6.6µM), where 60% of the single node cuttings exhibited bud-break and 2 shoots developed from each node. They also found that pre-culture of nodal segments on MS basal medium for 2 weeks followed by four weeks on shoot proliferation medium was essential/ beneficial for bud-break to occur. Although the details on the rate of shoot multiplication in subsequent subcultures is lacking, initially 2 shoots developed per node and the number of shoots increased with the increasing number of subcultures and then declined after 5-6 subcultures. Roy et al.[32] utilized nodal segments from 25-year-old tree and found MS + BAP (4.4 µM) + NAA (0.54 µM) to be the best medium for shoot proliferation (86% cultures with 6 shoots per explant) in the initial cultures but the report lacks details on rate of propagation in subsequent subcultures. Joshi and Thengane[20] cultured nodal segments from 2-5-year-old juvenile trees as those from 15-20-year-old adult trees showed negligible bud-break. Similarly, Sharma et al.[34], cultured nodal segments from 3, 7- and 40-year-old trees and found that the explants from the younger trees to be more responsive. Even after rigorous surface sterilization procedure, involving nine steps, only 20% aseptic cultures were obtained by them.

In the present study, higher incidence of bud-break and shoot-growth has been successfully established through single node segment cultures by altering the major inorganic salt concentration in the MS medium and with the application of BAP and casein hydrolysate. BAP is known to release the axillary buds from apical dominance[5] and the efficiency of casein hydrolysate observed in the in vitro growth of several plant cell cultures is in fact linked to its concentration of glutamine whose action would be to compensate the phosphorous deficiency of the culture medium[6]. The importance of glutamine would be greater since the MS medium is rich in nitrogen but deficient in phosphorous. Phosphorous is vital for cell division as well as for storage and transfer of energy in plants[5].

3.3. Multiplication of shoots

Although ½ MS + BAP (1µM) + CH (500mgl^{-1}) proved very good for healthy shoot proliferation, shoot elongation was not very satisfactory, maximum length being 4 cm with 5 nodes per shoot. To facilitate further shoot multiplication, single node segments were excised from shoots developed on ½ MS + BAP (1µM) + CH (500mgl^{-1}) and transferred to full-strength MS medium supplemented with BAP (1µM) and CH in the range of 250-2000 mgl^{-1} (Fig. 3). Regardless of the CH concentration, only single shoot developed in all the media. However, maximum shoot elongation was observed in 250 mgl^{-1} CH. In this case, 98% of the cultures developed a shoot that attained an average length of 9.5 cm, with 9 nodes/shoot after 5 weeks (Fig. 5b). At the end of the passage, each axillary shoot was cut into single node segments and planted on fresh MS + BAP (1µM) + CH (250mgl^{-1}). Each node again produced an elongated, healthy, multinodal, solitary shoot, which provided 9 cuttings after 5 weeks for further shoot multiplication. Thus, 9-fold recurrent shoot multiplication could be achieved in every 5-weeks on MS + BAP (1µM) + CH (250mgl^{-1}) by cutting the solitary shoot into single node segments and culturing them onto the fresh medium. This rate of shoot multiplication was maintained for almost 3 years. Since every time, the explants were taken from freshly formed in vitro shoots, therefore, any significant difference (variation) in the results were not observed.

Again the effect of CH is reflected in the results where when ½ MS was used, higher concentration of CH compensates nitrogen and phosphorus deficiency while with full strength MS even the low concentration of CH is sufficient to promote axillary bud break and development of long multinodal shoot. The higher concentrations of CH proved inhibitory. In some plants such as leguminous tree species[9], *Feijoa*[3], Japanese persimmon[12] and potato[26], it may not be possible to break apical dominance by manipulating the hormonal composition of the medium, and the bud present at priori on the initial explant grows into an unbranched shoot. The rate of shoot multiplication in such cases would depend on the number of nodal cuttings that can be excised from the shoot at the end of each passage[5].

3.4. Rooting of shoots

For rooting, terminal 3 cm long portions of shoots, from 5-week-old cultures, developed on MS + BAP (1µM) + CH (250mgl^{-1}) were used. The remaining portions of shoots were cut into single node segments and utilized for further shoot multiplication. MS basal medium was tested at full (MS), half (½ MS) and quarter (¼ MS)

strengths of the major inorganic salts. All the media were supplemented with IBA at 0.5 or 1.0µM concentration. ¼ MS was distinctly better than either ½ MS or full MS basal medium, in terms of frequency of rooting (21%) directly at the base of the shoots. MS or ½ MS basal media did not support rooting. Addition of IBA (0.5µM) to ¼ MS basal medium, drastically improved the frequency of rooting directly at the base of the shoot. Although frequency of the rooted shoots was higher with IBA (1.0µM), other parameters like number of roots per shoot, length of the longest root and number of laterals were higher with IBA (0.5µM) (Fig. 4). Moreover, IBA (0.5 µM) induced rooting directly at the base of the shoot whereas with IBA (1.0 µM) it was preceded by callusing. ¼ MS + IBA (0.5µM) proved to be the best rooting medium, where 84% shoots formed an average of 8.5 roots directly from the basal cut end of the shoot (Fig. 5c). On this medium roots appeared after 3 weeks and maximum response was observed after 4 weeks.

The optimum conditions described for rooting of in vitro regenerated shoots vary considerably. Whereas Joshi and Thengane[20] and Venkateswarlu et al.[38] found IAA (11-17 µM) to be the best auxin for rooting of neem shoots, Joarder et al.[18] found IBA (2.46 µM) to be most effective. Gill et al.[14] and Roy et al.[32] reported rooting in the combined presence of IBA and IAA.

3.5. Hardening and Transplantation

Following the protocol described in materials and methods, 22 plants from ¼ MS + IBA (0.5µM) medium were transferred out of the cultures. Of these 18 plants survived. Thus, transplantation survival of micropropagated plants was 81.8% (Fig. 5d).

Conclusions

The present study, thus, demonstrated the possibility of mass clonal propagation of a 35-year-old neem tree by forced axillary branching. Single node segment cultures could be raised during active vegetative growth season (March-May) on ½ MS + BAP (1µM) + CH (500mgl^{-1}) for high frequency bud-break. Recurrent shoot mutiplication was achieved on full strength MS medium supplemented with BAP (1µM) and CH (250mgl^{-1}), at a rate of 9-fold every 5 weeks. The shoots could be readily rooted on ¼ MS + IBA (0.5µM) with a frequency of 84%. Transplantation survival of these plants was more than 81.8%. With this effective method it is estimated that 10^{11} plants can be produced in one year, starting from a single nodal explant. Such a rate of multiplication could not be achieved by any traditional method of vegetative propagation.

Acknowledgements

Authors gratefully acknowledge the receipt of research grant from Department of Science and Technology (DST), New Delhi, India.

References

1. Amin M. and Jaiswal V.S., Rapid clonal propagation of guava through in vitro shoot proliferation on nodal explants of mature trees. *Plant Cell Tissue Organ Cult.,* **9**, 235-243 **(1987)**.

2. Anonymous, Wealth of India. Vol 1, 504-511 **(1989)**.

3. Bhojwani S.S., Mullins K. and Cohen D., Micropropagation of *Feijoa sellowiana* Berg. *Acta Hortic.*, **23,** 247-254 **(1987)**.

4. Bhojwani S.S. and Razdan M.K., Plant Tissue Culture: Theory and Practice. Amsterdam: Elsevier, 502 **(1983)**.

5. Bhojwani S.S. and Razdan M.K., Plant Tissue Culture: Theory and Practice. Amsterdam: Elsevier, 502 **(1996)**.

6. Bister-Miel F., Guignard J-L., Bury M. and Agier C., Glutamine as an active component of casein hydrolysate: its balancing effect on plant cells cultured in phosphorus deficient medium, *Plant Cell Rep.*, **4**, 161-163 **(1985)**.

7. Chitra D.S.V. and Padmaja G., Seasonal influence on axillary bud sprouting and micropropagation of elite cultivars of mulberry., *Scientia Hort.,* **92**, 55-68 **(2002)**.

8. Das M. and Pal A., In vitro regeneration of *Bambusa balcooa* Roxb.: factors affecting changes of morphogenetic competence in the axillary buds. *Plant Cell Tissue Organ Cult.,* **81**, 109-112 **(2005)**.

9. Dhawan V. and Bhojwani S.S., In vitro vegetative propagation of *Leucaena leucocephala* (Lam.) de Wit., *Plant Cell Rep.,* **4**, 315-318 **(1985)**.

10. Drew R.A., Clonal propagation of Neem by Tissue culture. In: Proc. World Neem Conference, Bangalore, 999-1005 **(1993)**.

11. Ermel K., Azadirachtin content of neem seed kernels from different regions of the world. In: Schmutterer H. (ed) The Neem Tree: Source of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes. Weinheim: VCH, 222-230 **(1995)**.

12. Fukui H., Sugiyama M. and Nakamura M., Shoot tip culture of Japanese Persimmon (*Diospyros kaki* Thunb.), *J. Jpn. Soc. Hortic. Sci.,* **58**, 43-47 **(1989)**.

13. Gill A.S. and Roy R.D., Agroforestry Uses. In: Randhawa N.S., Parmar B.S. (eds) Neem. New Age International (P) Ltd, New Delhi, 49-62 **(1996)**.

14. Gill R.I.S, Gill S.S. and Gosal S.S., Protocol for in vitro asexual multiplication of *Azadirachta indica*. In: Islam A.S. (ed) Plant Tissue Culture. Bangladesh, 155-160 **(1996)**.

15. Holdgate D.P., Propagation of Ornamentals by tissue culture. In: Reinert J., Bajaj Y.P.S. (eds) Applied and Fundamental Aspects of Plant Cell, Tissue Organ Culture Berlin: Springer-Verlag, 18-44 **(1977)**.

16. Hughes K.W., Ornamental species. In: Conger B.V. (ed) Cloning Agricultural Plants via in vitro Techniques. Boca Raton, CRC Press, 5-51 **(1981)**.

17. Hutchinson J.F., Factors affecting shoot proliferation and root initiation in organ culture of apple 'Northern Spy'. *Scientia Hort.,* **22**, 347-358 **(1984)**.

18. Joarder O.I., Naderuzzaman A.T.M., Islam R., Hossain M., Joarder N. and Biswas B.K. Micropropagation of neem through axillary bud culture. In: Proc. World Neem Conference, Bangalore, 961-966 **(1993)**.

19. Joshi M.S. and Thengane S.R., Potential application of in vitro methods for propagation of Neem (*Azadirachta indica* A. Juss.). In: Proc. World Neem Conference, Bangalore, 967-973 **(1993)**.

20. Joshi M.S. and Thengane S.R., In vitro propagation of *Azadirachta indica* A. Juss. (Neem) by shoot proliferation, *Indian J. Exp. Biol.,* **34**, 480-482 **(1996)**.

21. Kaushik N., Determination of azadirachtin and fatty acid methyl esters of *Azadirachta indica* seeds by HPLC and GLC, *Anal. Bioanal. Chem.,* **374**, 199-204 **(2002)**.

22. Koul O., Isman M.B. and Ketkar C.M., Properties and Uses of neem, *Azadirachta indica*, *Can. J. Bot.* **68**, 1-11 **(1990)**.

23. Litz R.E. and Conover R.A., Effect of sex type, season and other factors on in vitro establishment and culture of *Carica papaya* L. explants, *J. Amer. Soc. Hort. Sci.,* **106**, 792-794 **(1981)**.

24. Lutz J.D., Wang J.R., Rowe J., Tricoli D.M. and Lawrence R.H., Somatic embryogenesis for mass cloning of crop plants. In: Henke R.R., Hughes K.W., Constantin M.J., Hollaender A. (eds) Tissue Culture in Forestry and Agriculture. New York: Plenum Press, 105-117 **(1985).**

25. Mishra Y., Patel P.K., Yadav S., Shirin F. and Ansari S.A., A micropropagation system for cloning of *Bambusa tulda* Roxb., *Scientia Hort.*, **115**, 315-318 **(2008)**.

26. McCown B.H. and Joyce P.J., Automated propagation of microtubers of potato. In: Vasil I.K. (ed) Cell Culture and Somatic Cell Genetics, Vol. 8. Scale up and Automation in Plant Propagation. San Diego: Academic Press, 95-108 **(1991)**.

27. Mohan Ram H.Y. and Nair M.N.B., Botany. In: Randhawa N.S., Parmar B.S. (eds) Neem. New Age International (P) Ltd, New Delhi, 6-26 **(1996)**.

28. Murashige T., Plant propagation through tissue culture, *Ann. Rev. Plant Physiol.,* **25**, 135-166 **(1974)**.

29. Murashige T. and Skoog F., A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol. Plant.,* **15**, 473-497 **(1962)**.

30. Naik S.K., Pattnaik S. and Chand P.K., In vitro propagation of pomegranate (*Punica granatum* L. cv. Ganesh) through axillary shoot proliferation from nodal segments of mature tree, *Scientia Hort.,* **79**, 175-183 **(1999)**.

31. Ramanayake S.M.S.D. and Yakandawala K., Micropropagation of the giant bamboo (*Dendrocalamus giganteus* Munro) from nodal explants of field grown culms, *Plant Sci.,* **129**, 213–223 **(1997)**.

32. Roy S.K., Assaduzzaman M. and Hossain Z., Clonal Propagation of *Azadirachta indica* by in vitro culture, In: Islam A.S. (ed) Plant Tissue Culture. Oxford and IBH, New Delhi, 149-154 **(1996)**.

33. Schmutterer H., The tree and its characteristics. In: Schmutterer H. (ed) The Neem Tree: Source of Unique Natural Products for Integrated Pest Management, Medicine, Industry and other Purposes. Weinheim: VCH, 1-29 **(1995)**.

34. Sharma A.K., Sharma M. and Chaturvedi H.C., Conservation of phytodiversity of *Azadirachta indica* A. Juss. through in vitro strategies. In: National Symposium on Role of Plant tissue Culture in Biodiversity Conservation and Economic Development. Almora: G.B. Pant Institute of Himalayan Environment and Development, 1-13 **(1999)**.

35. Sudershan C. and Hussain J., In vitro clonal propagation of a multipurpose tree, *Ziziphus spina-christi* (L.) Desf., *Turk. J. Bot.,* **27**, 167-171 **(2003).**

36. Tewari D.N., Monograph on Neem (*Azadirachta indica* A. Juss). International Book Distributors, Dehradun, 279 **(1992).**

37. Thengane S., Joshi M. and Mascarenhas A.F., Somatic Embryogenesis in Neem (*Azadirachta indica*). In: Jain S.M., Gupta P., Newton R. (eds) Somatic Embryogenesis in Woody Plants. Vol. II. Important Selected Plants. Kluwer academic Publishers, Dordrecht, 357-374 **(1995).**

38. Venkateswarlu B., Katyal J.C., Choudhari J. and Mukhopadhyay K., Micropropagation of plus neem (*Azadirachta indica* A. Juss.) and evaluation of field transferred plants, *Indian For.,* **124**, 537-543 **(1998).**

FIGURE LEGENDS

(Line Figures)

Fig.1. Effect of basal media (full MS and ½ MS) and BAP (1µM) on bud-break and shoot growth in the cultures of single node segments of Neem. Growth Period: 5 Weeks.

Fig.2. Improvement in bud-break frequency and shoot growth upon addition of 500mgl^{-1} casein hydrolysate to ½ MS + BAP (1µM) in single node segment cultures of neem. Growth Period: 5 Weeks.

Fig.3. Comparative effect of casein hydrolysate concentrations on multiplication and shoot growth of Neem. Control: MS + BAP (1µM); Growth Period: 5 Weeks.

Fig.4. Rooting response of shoots on ¼ MS media supplemented with IBA. Growth Period: 4 Weeks.

Fig. 5. Clonal propagation of an adult plant (Photographs)

a. 5-week-old single node segment culture on ½ MS + BAP (1µM) + CH (500mgl^{-1}), showing development of a healthy, solitary axillary shoot and basal callusing. Shoot growth was not very satisfactory (x 1.9).

b. Single node segment excised from **A**, 5 weeks after transfer to MS + BAP (1µM) + CH (250mgl^{-1}). The axillary bud has developed into a long, unbranched, multinodal shoot. At the end of the passage shoot was further cut into single node segments and planted on fresh medium of the same composition for recurrent shoot multiplication (x 1.5).

c. 4-week-old culture on ¼ MS + IBA (0.5µM). The shoot has developed healthy roots directly from the cut, basal end (x 1.6).

d. Hardened micropropagated plants, 11 months after transfer to soil (x 0.1).

Fig. 1

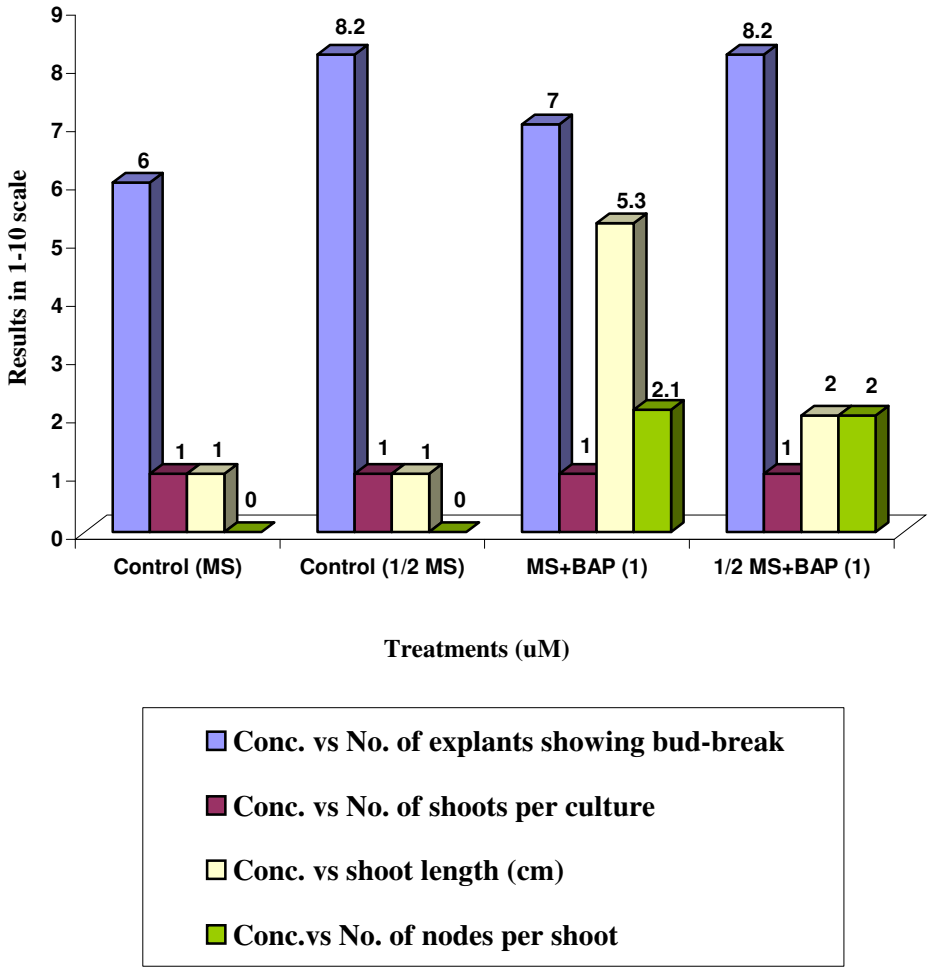
Results in 1-10 scale
9
8
7
6
5
4
3
2
1
0
6
1
1
0
8.2
1
1
0
7
1
5.3
2.1
8.2
1
2
2
Control (MS)
Control (1/2 MS)
MS+BAP (1)
1/2 MS+BAP (1)
Treatments (uM)
Conc. vs No. of explants showing bud-break
Conc. vs No. of shoots per culture
Conc. vs shoot length (cm)
Conc.vs No. of nodes per shoot

Fig. 2

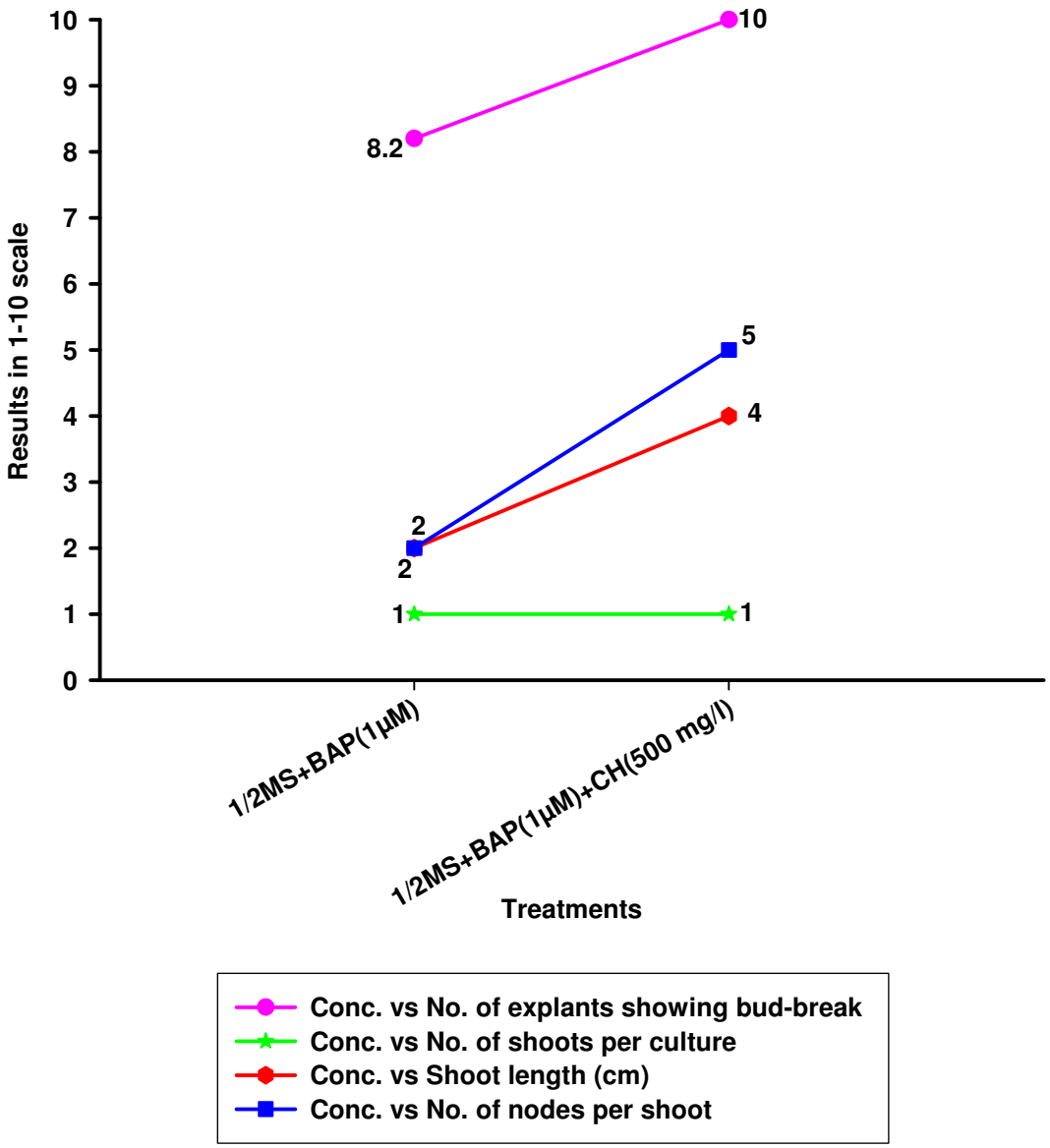
Results in 1-10 scale
10
9
8
7
6
5
4
3
2
1
0
10
8.2
5
4
2
2
1
1
1/2MS+BAP(1µM)
1/2MS+BAP(1µM)+CH(500 mg/l)
Treatments
Conc. vs No. of explants showing bud-break
Conc. vs No. of shoots per culture
Conc. vs Shoot length (cm)
Conc. vs No. of nodes per shoot

Fig. 3

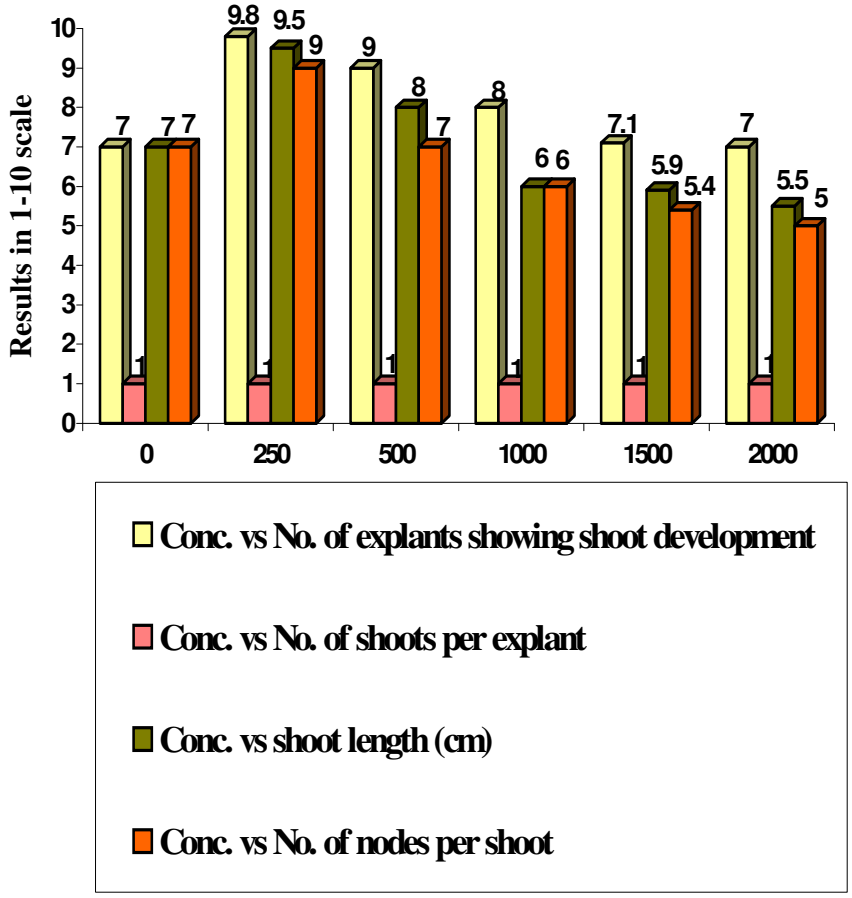
Results in 1-10 scale
10
9
8
7
6
5
4
3
2
1
0
7
7 7
9.8
9.5
9
9
8
7
8
6 6
7.1
5.9
5.4
7
5.5
5
1
0
250
500
1000
1500
2000
Conc. vs No. of explants showing shoot development
Conc. vs No. of shoots per explant
Conc. vs shoot length (cm)
Conc. vs No. of nodes per shoot

Fig. 4

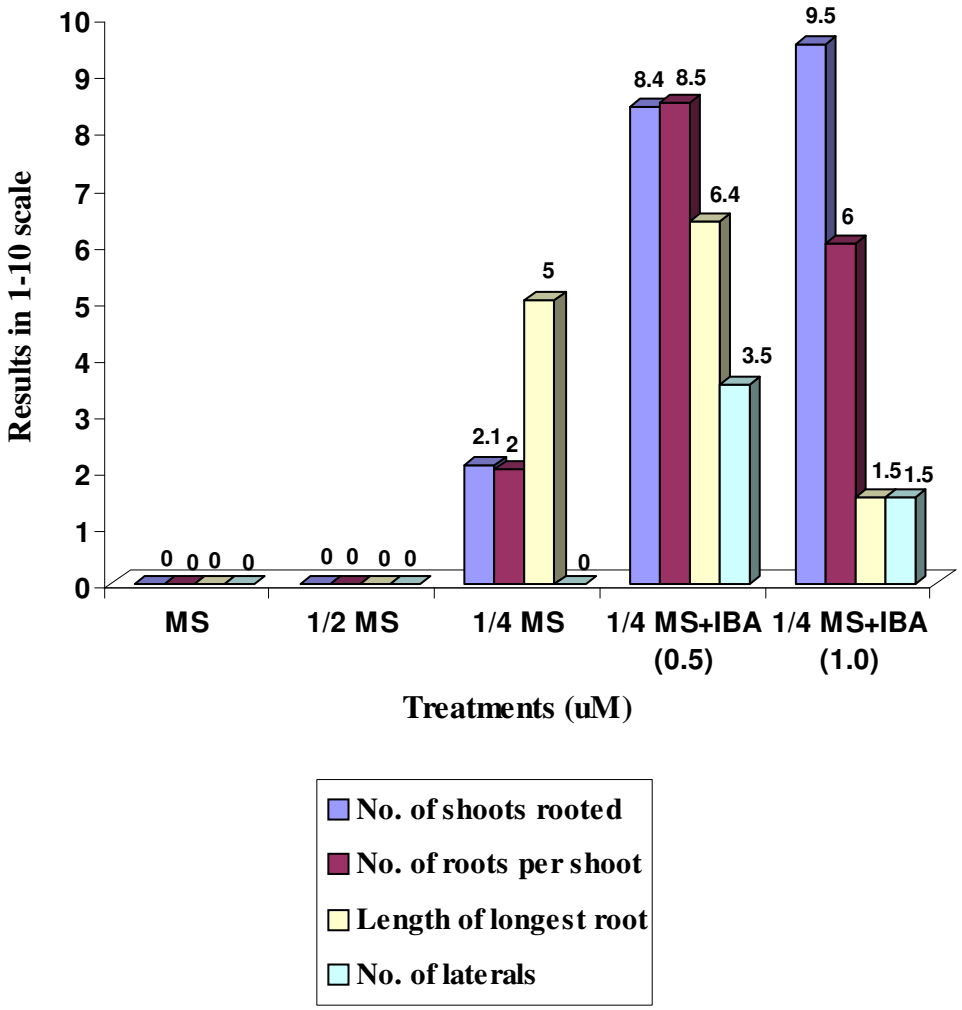
Results in 1-10 scale
10
9
8
7
6
5
4
3
2
1
0
0 0 0 0
0 0 0 0
2.1 2
5
0
8.4 8.5
6.4
3.5
9.5
6
1.5 1.5
MS
1/2 MS
1/4 MS
1/4 MS+IBA (0.5)
1/4 MS+IBA (1.0)
Treatments (uM)
No. of shoots rooted
No. of roots per shoot
Length of longest root
No. of laterals

Fig. 5