BIOLOGIA PLANTARUM 50 (2): 291-294, 2006

BRIEF COMMUNICATION

Rapid clonal propagation of *Vitex trifolia*

L.V. HIREGOUDAR*, H.N. MURTHY**[1], J.G. BHAT*, A. NAYEEM*, B.P. HEMA*, E.J. HAHN** and K.Y. PAEK**

*Plant Tissue Culture Laboratory, Department of Botany, Karnatak University, Dharwad-580003, Karnataka, India**
*Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju-361763, South Korea***

Abstract

This report describes *in vitro* shoot induction and plant regeneration from mature nodal explants of *Vitex trifolia* L. on Murashige and Skoog (MS) medium fortified with benzylaminopurine (BAP), kinetin (KN), thidiazuron (TDZ), adenine (ADE), and 2-isopentenyladenine (2-iP) (0.25 - 10.0 μM). Multiple shoots differentiated directly without callus mediation within 3 weeks when explants were cultured on medium supplemented with cytokinins. The maximum number of shoots (9 shoots per explant) was developed on a medium supplemented with 5.0 μM BAP. Shoot cultures was established repeatedly subculturing the original nodal explant on the same medium. Rooting of shoots was achieved on half strength MS medium supplemented with 0.5 μM naphthaleneacetic acid (NAA). Rooted plantlets transferred to pots containing autoclaved soil and vermiculite mixture (1:1) showed 90 % survival when transferred to outdoor.

Additional key words: auxins, cytokinins, *in vitro* culture, medicinal plant, micropropagation.

_____

Vitex trifolia L. (*Verbenaceae*) is a small tree known for its medicinal property. Conventional propagation of *Vitex* trees is through vegetative cuttings and suckers (Chadha 1976) and it is slow growing, age and season dependent. It can also be propagated through seeds, however, seed set and seed germination is poor. Micropropagation can provide the opportunity to obtain large number of homogeneous plants and there are no reports of micropropagation of this species. So, we attempted for *in vitro* propagation of this species and describe here an efficient and rapid propagation method.

Nodal segments (1 cm long) of *Vitex trifolia* L. were obtained from 8-year-old tree growing in the Botanical garden, Karnatak University, Dharwad, India. Explants were thoroughly washed under running tap water and with detergent (*Laboline*, 0.1 %, v/v) for 10 min, then surface disinfested with 70 % alcohol for 5 min followed by immersion in 0.1 % (m/v) mercuric chloride for 5 min. After washing several times with distilled water, the cut edges of the explants were trimmed and cultured on Murashige and Skoog (MS, 1962) medium containing 3 % sucrose, 0.8 % agar (*Hi-media*, Mumbai, India), supplemented with different concentrations of cytokinins: benzylaminopurine (BAP), kinetin (KN), thidiazuron (TDZ), adenine (ADE) and 2-isopentenyladenine (2-iP) (0.25, 0.5, 1.0, 2.0, 5.0 and 10.0 μM). A set containing MS medium without growth regulators served as control. The medium pH was adjusted to 5.8 prior to the addition of agar. Medium was distributed into culture tubes (23 × 150 mm, *Borosil*, Mumbai, India) and plugged with non-absorbent cotton wrapped with one layer of cheesecloth. Culture media was autoclaved at 120 °C for 20 min and cultures were incubated at 25 ± 2 °C under 16-h photoperiod (cool, white fluorescent tubes, irradiance of 40 μmol m^{-2} s^{-1}). Explants were subcultured once in four weeks to fresh medium.

_____

Received 21 May 2004, *accepted* 20 June 2005.
Abbreviations: BAP - N^6-benzylaminopurine; KN - kinetin; TDZ - thidiazuron; ADE - adenine; 2-iP - 2-isopentenyladenine; NAA - 1-naphthaleneacetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog (1962) medium.
Acknowledgements: H.N. Murthy is thankful to Department of Biotechnology, New Delhi, India for research grants (Project No. BT/R&D/19/94 and No. BT/PR/3153/AGR/16/254/2002).
[1] Corresponding author; fax: (+91) 836 2747884, e-mail: nmurthy60@yahoo.co.in

Shoots attained 4 - 5 cm in height were transferred to full, half and quarter strength MS medium containing 2 % sucrose, and with or without 1-naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) at various concentrations (0.25, 0.5, 1.0 and 2.0 μM) to test their rooting efficiency. After 4 weeks in rooting medium, the rooted plantlets were transferred to plastic vessels containing sterilized soil and vermiculite (1:1) under controlled growth chamber conditions (25 ± 2 °C, 16-h photoperiod, 80 % relative humidity and irradiance of 50 μmol m^{-2} s^{-1}). The potted plants were irrigated with Hoagland solution once in week. After 2 weeks, the plantlets were kept under shade for 2 weeks and then placed outdoors under full sun.

Cultured explants were fixed in FAA (10 cm^3 of formalin : 85 cm^3 of 70 % ethyl alcohol : 5 cm^3 of glacial acetic acid) for 12-h at room temperature, dehydrated by ethanol-butyl alcohol series and embedded in paraffin wax. The material sectioned (thickness of 6 μm), stained with 0.05 % toluidine-blue solution (Fowke and Rennie 1996) and examined under compound microscope (*Nikon*, Tokyo, Japan).

A complete randomized design was used for all the treatments. There were 12 replicates for each treatment and the experiment was repeated twice. The cultures were observed periodically and morphological changes were recorded at weekly intervals. Results were subjected to analysis of variance (*ANOVA*) and mean values were separated according to Duncan's multiple range test at $P = 0.05$.

The explants cultured on MS medium showed their first response by initial enlargement of the existing axillary bud and later shoot sprouting was initiated after 2 - 3 weeks of culture. The percentage of response varied with the type of growth regulator used, and its concentration. Among the various cytokinins tested BAP alone resulted in maximum number of explants initiating shoots (Table 1). On MS medium supplemented with BAP, multiple shoots were observed directly without the intervention of callus. Of the various levels of BAP tested, 5.0 μM proved to be most effective, as on this medium the maximum of nine shoots were developed per explant (Fig. 1*C*). Histological observation revealed that direct development of shoots occurred from the nodal region without callus phase (Fig. 1*B*). On lowering the concentration of BAP from 1.0 μM to 0.5 μM, the number of shoots per culture was reduced. Similarly on higher concentrations of BAP (10.0 μM) the number as well as percent response was drastically reduced (Table 1). Reduction in the number of shoots generated from each node at BAP concentration higher than the optimal level was also reported for several medicinal plants (Kukreja *et al.* 1990, Sen and Sharma 1991, Vincent *et al.* 1992). TDZ (0.25 - 1.0 μM) supplemented to MS medium induced development of shoots from nodal explants and optimum of 3.91 shoots was developed at 1.0 μM TDZ (Fig. 1*A*). The cytokinin, 2-iP also induced multiple shoots, but not as effective as the BAP treatments. Some treatments caused a considerable callusing at the base of explants. KN and ADE containing MS medium also induced shoots from the explants but the number was not significant compared to BAP treatment. Of the five cytokinins (BAP, TDZ, KN, ADE and 2-iP) tested, BAP was most effective in inducing multiple shoot formation. The stimulating effect of BAP on multiple shoot formation has been reported earlier for several medicinal plant species including *Ocimum* spp. (Pattnaik and Chand 1996), *Piper* spp. (Bhat *et al.* 1995), *Vitex negundo* (Sahoo and Chand 1998), *Feronia limonia* (Hiregoudar *et al.* 2003), and *Sesbania drummondii* (Cheepala *et al.* 2004).

The shoot cultures were multiplied by repeatedly subculturing the original nodal explants on shoot

Table 1. Effect of cytokinins in different concentrations on shoot regeneration from nodal explants of *Vitex trifolia* L. Data were collected after 6 weeks of culture. No response was observed on MS basal medium. Mean values followed by the same letter are not significantly different according to Duncan's multiple range test at $P = 0.05$.

Growth regulators	[μM]	Response [explant^{-1}]	Number of shoots [explant^{-1}]
BAP	0.25	58.33	0.58 l
	0.50	66.66	1.24 h
	1.00	75.00	2.16 e
	2.00	83.33	5.33 b
	5.00	83.33	9.08 a
	10.00	66.66	3.91 c
KN	0.25	44.44	0.44 m
	0.50	55.55	0.60 l
	1.00	44.44	0.44 m
	2.00	22.22	0.38 mn
	5.00	22.22	0.38 mn
	10.00	16.66	0.33 n
TDZ	0.25	75.00	2.08 f
	0.50	75.00	2.74 d
	1.00	83.33	3.91 c
	2.00	58.33	0.74 k
	5.00	41.66	0.41 mn
	10.00	41.66	0.41 mn
ADE	0.25	50.00	0.60 l
	0.50	66.66	0.88 j
	1.00	66.66	1.00 I
	2.00	50.00	0.66 kl
	5.00	50.00	0.66 kl
	10.00	50.00	0.66 kl
2-ip	0.25	58.33	0.58 l
	0.50	75.00	1.33 g
	1.00	83.33	2.08 f
	2.00	75.00	1.00 I
	5.00	50.00	0.58 l
	10.00	41.66	0.41 mn

multiplication medium (MS supplemented with 5 µM BAP) after harvesting newly formed shoot. On average of 10 - 22 shoots were regenerated per explant up to three subcultures. A similar finding of subculturing the *in vitro* generated nodal explants to fresh shoot multiplication medium was reported in pistachio (Onay 2000), *Leptadenia reticulata* (Arya *et al*. 2003), *Sophora flavescens* (Zhao *et al*. 2004), *Calendula officinalis* (Çöçü *et al*. 2004) and *Sesbania rostrata* (Jain *et al*. 2004). After the three sub-culture the shoot multiplication rate was declined with the explants. A similar result was recorded in *Gardenia* (George *et al*. 1993) and *Crossandra* spp. (Girija *et al*. 1999). In many plants species (Debergh and Maene 1981) it was reported that micropropagation requires two media *i.e*. a propagation medium and a shoot elongation medium, which makes the micropropagation procedures cumbersome and uneconomical. Some researchers have added gibberellic acid (GA_3) to the shoot multiplication medium to achieve simultaneous shoot multiplication and elongation; however, this has drastically affected the shoot multiplication rate (Sahoo and Chand 1998). In the present studies shoot multiplication and subsequent elongation was achieved on the same medium.

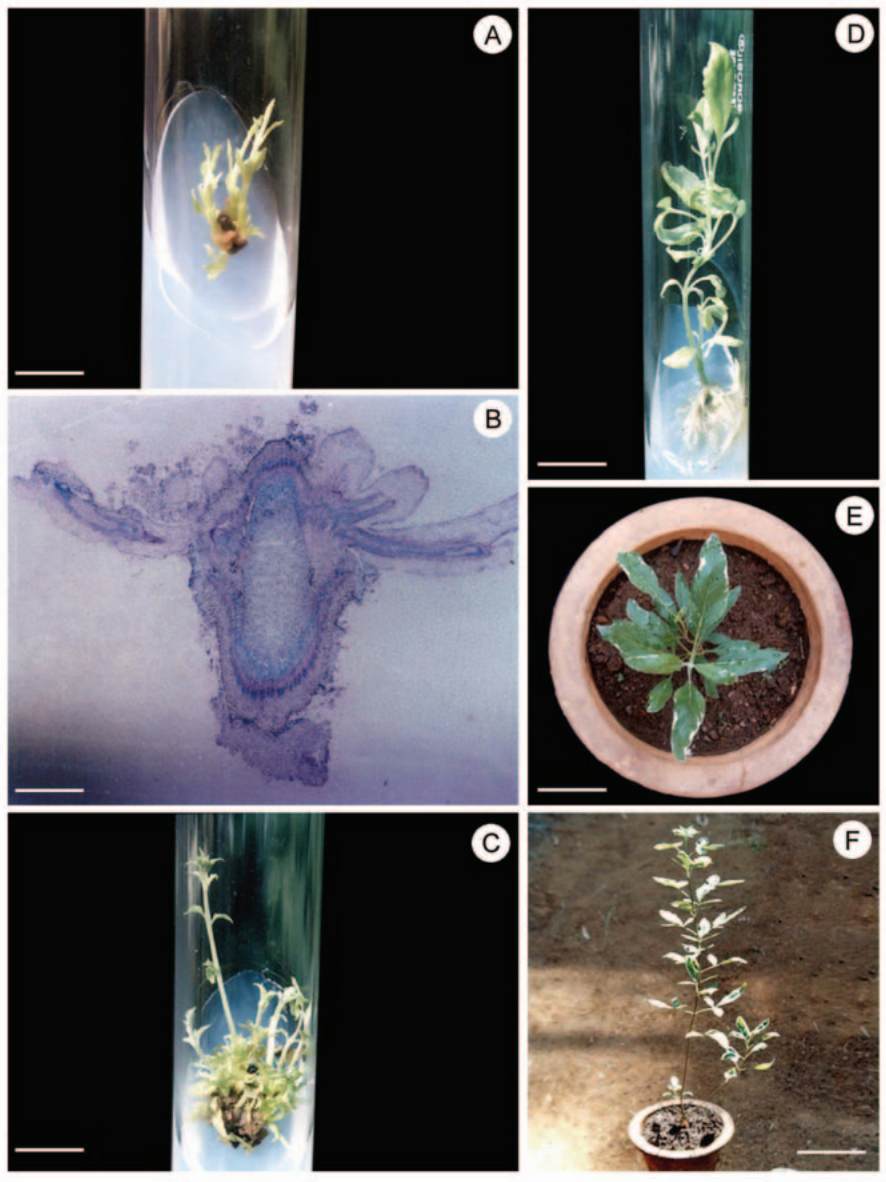

Fig. 1. *A* - Four weeks old culture showing multiple shoots on MS medium supplemented with 1.0 µM TDZ (*bar* = 0.8 cm); *B* - longitudinal section of nodal explant showing direct development of shoots (*bar* = 2 mm); *C* - 6-week-old culture showing multiple shoots on MS medium supplemented with 5.0 µM BAP (*bar* = 0.87 cm); *D* - a rooted shoot on half strength MS medium supplemented with 0.25 µM NAA (*bar* = 1.12 cm); *E* - *in vitro* developed plantlets transferred to pot (*bar* = 1.6 cm); *F* - regenerated plants, 8 weeks after transfer from culture tubes to soil (*bar* = 7 cm).

Shoots of 3 - 5 cm were transferred to full, half and quarter strength MS basal medium supplemented or not with NAA and IBA (0.25, 0.5, 1.0 and 2.0 μM) for the induction of roots. No roots were developed from regenerated shoots maintained on full strength MS basal medium and MS medium supplemented with NAA. Root formation was achieved within two weeks, from the bases of the shoots on half strength or quarter strength MS medium and the best rooting was achieved in these media fortified with 0.25 μM NAA (shoots induced 6.91 and 6.75 roots, respectively; Fig. 1*D*). Similarly, NAA has been used for *in vitro* rooting of shoots in a number of woody plant species (Barlass and Skene 1982, Rout *et al.* 1990, Naik *et al.* 2000).

The rooted plantlets were taken out gently from the test tubes and washed with sterile water to remove adhered agar and traces of the medium to avoid contamination. They were then transferred to plastic vessels containing sterile soil and vermiculite (1:1). Pots were irrigated with Hoagland solution once in a week and maintained in controlled environmental conditions in growth chambers for two weeks. Later pots were taken to greenhouse for a week followed by transfer of plants to out door. Survival rate of plants was 90 %. Plants transferred to the field have established themselves in the soil and were growing well. The regenerated plants did not show any immediately detectable phenotypic variation (Fig. 1*E*,*F*).

Use of axillary nodes for micropropagation is beneficial than other explant types. MS medium containing 5 μM BAP is best for multiple shoot induction and shoot proliferation of *Vitex trifolia* from the node explants of mature trees. Natural stand of *Vitex trifolia* are fast disappearing in India because of indiscriminate collection and over exploitation of natural resources for commercial purposes and the outlined procedure here offers a potential system for mass multiplication and conservation of *Vitex trifolia.*

References

Arya, V., Shekhawat, N.S., Singh, R.P.: Micropropagation of *Leptadenia reticulata* - a medicinal plant. - *In Vitro* cell. dev. Biol. Plant **39**: 180-185, 2003.

Barlass, M., Skene, K.G.M.: *In vitro* plantlet formation from *Citrus* species and hybrids. - Sci. Hort. **17**: 333-341, 1982.

Bhat, S.R., Chandel, K.P.S., Malik, S.R.: Plant regeneration from various explants of cultivated *Piper* species. - Plant Cell Rep. **14**: 395-402, 1995.

Chadha, Y.R.: The Wealth of India, Raw Materials. Vol. 10. - Publication and Information Directorate, CSIR, New Delhi 1976.

Cheepala, S.B., Sharma, N.C., Sahi, S.V.: Rapid *in vitro* regeneration of *Sesbania drummondii*. - Biol. Plant. **48**: 13-18, 2004.

Çöçü, S., Uranbey, S., İpek, A., Khawar, K.M., Sarihan, E.O., Kaya, M.D., Parmaksiz, İ., Özcan, S.: Adventitious shoot regeneration and micropropagation in *Calendula officinalis* L. - Biol. Plant. **48**: 449-451, 2004.

Debergh, P.C., Maene, L.J.: A scheme for commercial propagation of ornamental plants by tissue culture. - Sci. Hort. **14**: 335-345, 1981.

Fowke, L.C., Rennie, P.J.: Botanical microtechnique for plant culture. - In: Gamborg, O.L., Phillips, G.C. (ed.): Plant Cell, Tissue and Organ Culture. Pp. 217-228. Narosa Publishing House, New Delhi 1996

George, P.S., Ravishankar, G.A., Venkataraman, L.V.: Clonal multiplication of *Gardenia jaminoides* Ellis through axillary bud culture. - Plant Cell Rep. **13**: 59-62, 1993.

Girija,S., Ganapathi, A., Vengadesan, G.: Micropropagation of *Crossandra infundibuliformis* (L.) Nees. - Sci. Hort. **82**: 331-337, 1999.

Hiregoudar, L.V., Murthy, H.N., Hema, B.P., Hahn, E.J., Paek, K.Y.: Multiple shoot induction and plant regeneration of *Feronia limonia* (L.) Swingle. - Sci. Hort. **98**: 357-364, 2003.

Jain, N., Jha, A.K., Prakash, S., Nanda, K., Gupta, S.C.: Micropropagation of *Sesbania rostrata* from the cotyledonary node. - Biol. Plant. **48**: 289-292, 2004.

Kukreja, A.K., Mathur, A.K., Zaim, M.: Mass production of virus free patchouli plants [*Pogostemon cabin* (Blanco) Benth] by *in vitro* culture. - Trop. Agr. **67**: 101-104, 1990.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue culture. - Physiol. Plant. **15**: 437-497, 1962.

Naik, S.K., Pattnaik, S., Chand, P.K.: High frequency axillary shoot proliferation and plant regeneration from cotyledonary nodes of pomegranate (*Punica granatum* L.) - Sci. Hort. **85**: 261-270, 2000.

Onay, A.: Micropropagation of pistachio from mature trees. - Plant Cell Tissue Organ Cult. **60**: 159-162, 2000.

Pattnaik, S., Chand, P.K.: *In vitro* propagation of the medicinal herbs *Ocimum americanum* L. Syn. *O. canum* Sims. (hoary basil) and *Ocimum sanctum* L. (holy basil). - Plant Cell Rep. **15**: 846-850, 1996.

Rout, G.R., Debata, B.K., Das, P.: *In vitro* clonal multiplication of roses. - Proc. nat. Acad. Sci. India **60**: 311-318, 1990.

Sahoo, Y., Chand, P.K.: Micropropagation of *Vitex negundo* L., a woody aromatic medicinal shrub, through high frequency axillary shoot proliferation. - Plant Cell Rep. **18**: 301-307, 1998.

Sen, J., Sharma, A. K.: Micropropagation of *Withania somnifera* from germinating seeds and shoot tips. - Plant Cell Tissue Organ Cult. **26**: 71-73, 1991.

Vincent, K.A., Mathew, K.M., Hariharan, M.: Micro-propagation of *Kaempferia galanga* L. – a medicinal plant. - Plant Cell Tissue Organ Cult. **28**: 229-230, 1992.

Zhao, D.L., Guo, G. Q., Wang, X. Y., Zheng, G. C.: *In vitro* propagation of a medicinal plant species *Sophora flavescens*. - Biol. Plant. **47**: 117-120, 2003/4.