Journal of Plant Biology, March 2004, 47(1) : 48-51 **SHORT COMMUNICATION**

High Frequency In Vitro Regeneration of *Kigelia pinnata* L. Via Organogenesis

Jos T. Puthur and T. Dennis Thomas*
Postgraduate and Research Department of Botany, St. Thomas College, Pala, Arunapuram (P.O.), Kottayam (Dt.), PIN- 686 574, Kerala, India

An effective and reproducible protocol for the micropropagation of *Kigelia pinnata* L. through high frequency callus regeneration is described. Seeds were surface sterilized before culturing on Gamborgs basal medium (B5 medium). After two weeks the cotyledonary node along with a portion of the hypocotyl were carefully excised from well-developed embryos and subcultured on B5 medium supplemented with different concentrations of 2,4-D and BAP. The cultured cotyledonary node explants showed callus formation at the base of the lower cut end of the hypocotyl. This callus showed shoot initiation after two weeks of subculture on the regeneration medium supplemented with various concentrations of BAP alone or in combination with NAA. The highest number of shoot regeneration occurred on medium containing 5 µM BAP and 0.1 µM NAA. The optimum rooting of the regenerated shoots was observed on ½B5 medium supplemented with 4 µM IBA. Micropropagated plants were successfully established in soil in field condition with a survival frequency of 100%.

Keywords: acclimatization, bignoniaceae, *Kigelia pinnata*, micropropagation, rooting

Trees are essential component of forest ecosystem playing a significant role in maintaining the ecological balance. But trees are overexploited by man for various purposes such as food, fuel, timber and other minor products (Purohit et al., 1998). This overexploitation has led to their depletion at an alarming rate throughout the world, especially in the tropical countries. The rapid depletion of multipurpose tree species is posing a great threat in terms of erosion of genetic variability for a given species. In addition, the decrease of forest areas and plant biomass, may be responsible for climatic changes on different regions of the world (Kozai and Kubota, 2001).

In order to address the above mentioned serious problems, conservation of biodiversity by both *in situ* and *ex situ* means is necessary and large-scale reforestation is required annually. Moreover in future, demand for woody transplants for use in reforestation will increase because of their utilization in pulp, paper, timber and furniture industry as well as plantation, horticulture and desert rehabilitation. Propagation through conventional methods is difficult in the case of most tree species where there is poor seed viability, recalcitrant seeds and weak root establishment. In addition, selection and maintenance of elite genotypes using conventional breeding methods like obligate outcrossing are time consuming and difficult for tree species because of their very long breeding cycles (Morton, 1996). In vitro techniques are being increasingly applied for clonal propagation of tree species to supplement the conventional methods and for rapid multiplication and production of a large numbers of plants throughout the year.

Kigelia pinnata, belonging to the family Bignoniaceae, is a fast growing multipurpose tree used for ornamental and roadside planting,. Traditional healers in India have used various parts of this plant to treat wide range of skin ailments, from relatively mild complaints, such as fungal infections, boils and psoriasis, to more serious diseases like leprosy, syphilis and skin cancer. It has been also used in other medicinal applications such as treatment of dysentery, ringworm, tapeworm, malaria, diabetes, pneumonia and toothache. There have been reports of extraction of lapachol, an antimalarial compound from the root (Binutu et al., 1996), and other quinones from the wood whose antimalarial activity against drug resistant strains of *Plasmodium falciparum* was superior to that of chloroquine and quinine (Carvalho et al., 1988). Present study was carried out to standardize a technique for micropropagation of *K. pinnata* through high frequency callus regeneration.

*Corresponding author; fax +91-04822-216313
e-mail den_thuruthiyil@yahoo.com

Abbreviations: BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxy acetic acid; IBA, indole-3-butyric acid; NAA, 1-naphthalene acetic acid; B5 medium, Gamborg's basal medium

MATERIALS AND METHODS

Seeds of *K. pinnata* were obtained from Pratap nursery, Dehradun, India. Healthy seeds were cleaned and stored in a refrigerator at 5°C. Prior to use, they were washed under running tap water for 15 min and surface sterilized by soaking in ethanol for 2 min followed by immersion in 0.1% mercuric chloride and rinsing with sterile distilled water three times. The sterilized seeds were cultured on B5 (Gamborg's medium) basal medium (Gamborg et al., 1968). After 15 days, cotyledonary node containing two cotyledons and a portion of hypocotyl from the well developed embryos were cultured on B5 medium supplemented with various concentrations of 2,4-D and BAP for four weeks.

All the media were fortified with 20 g/L sucrose and gelled with 0.8% agar and the pH was adjusted to 5.8 after adding the growth regulators. The media were steam sterilized in an autoclave under 1.5 $kg \cdot cm^{-2}$ and 121°C for 15 min. All the cultures were grown at 25±2°C under 16 h photoperiod, supplied by two Philips TL 40W fluorescent tubes. At least twenty-four cultures were raised for each treatment, and all experiments were repeated three times. Analysis of variance and Duncans multiple range tests were used for comparison between treatments.

The callus portion below the shoots at the cotyledonary node was excised and cultured on B5 medium supplemented with various concentrations of BAP alone or in combination with NAA for six weeks to regenerate calli. Keeping the regenerated shoots on the same regeneration medium resulted in the browning and ultimate death of the shoots. Hence the regenerated shoots were excised and cultured on B5 medium supplemented with 7 µM BAP for further growth. For rooting, the multiplied shoots measuring about 3.5

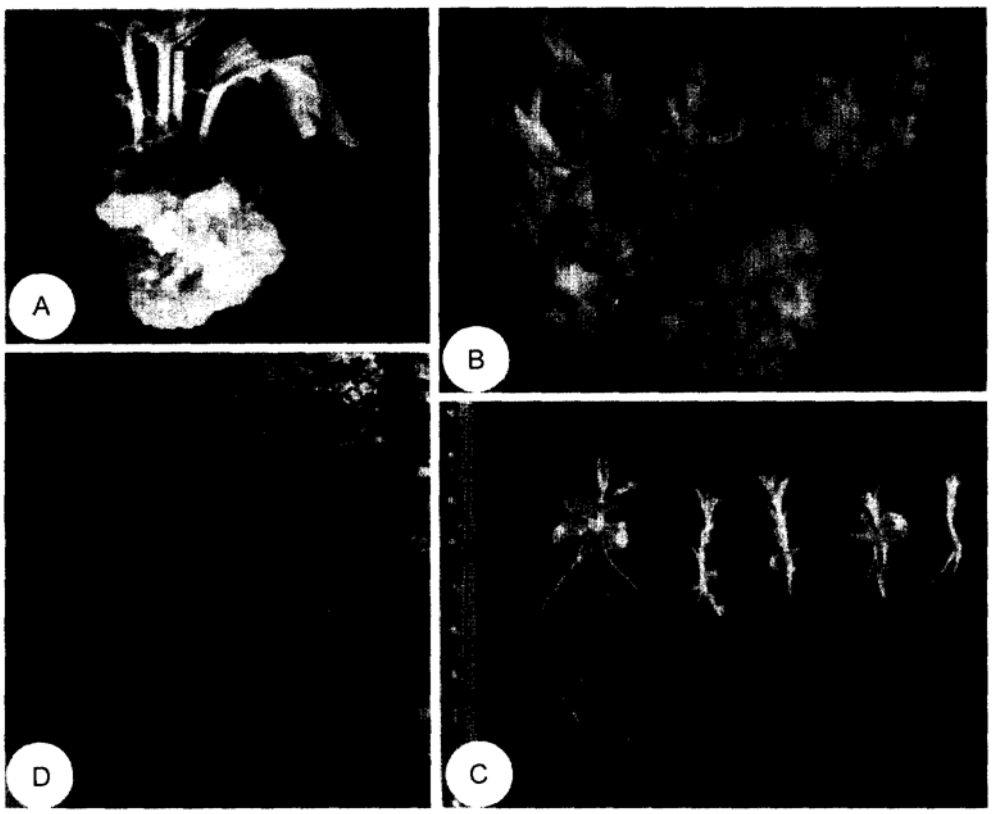

Figure 1. Different stages in the micropropagation of *K. pinneta* L. through callus regeneration. **A.** Photograph showing calli derived from the four-week-old cotyledonary node on B5 medium supplemented with BAP (10 µM) and 2,4-D (0.5 µM). A cotyledon and a number of shoots originated from the cotyledonary node were also seen. One of the cotyledons was removed for the better view of the shoots. **B.** Multiple shoot formation derived from callus of cotyledonary node on B5 medium supplementec with BAP (5 µM) and NAA (0.1 µM). Note the emergence of a large number of healthy shoots. The photographs were taken 4 weeks after callus culture. **C.** The rooting of the shoots on B5 medium supplemented with 4, 5, 3, 2, or 1 µM IBA (from left to right) four weeks after culture. Note the best rooting on B5 medium supplemented with 4 µM IBA. **D.** Hardened plants three months after transplantation to garden pots containing garden soil.

cm in height were transferred to ½B5 medium supplemented with IBA or NAA in the range of 1-5 µM.

To remove agar, the rooted shoots were thoroughly washed with water. Utmost care was taken not to damage the roots while removing agar. The shoots were transferred to plastic cups containing a mixture of autoclaved vermiculate and sand (2:1). The plantlets were transferred to garden pots one month after transplantation.

RESULTS AND DISCUSSION

Well developed embryos were emerged within 15 days from seed germination. The cotyledon and hypocotyl regions showed rapid growth, and within two weeks apical bud was initiated. The cotyledonary node taken from such well developed embryos, after four weeks of culture on B5 medium supplemented with various concentrations of BAP and 2,4-D, showed callus formation from the base of the node and induction of axillary shoot. Number of axillary shoots induced from the cotyledonary node and formation of callus from the base of the node were the highest on a medium containing 10 µM BAP and 0.5 µM 2,4-D (Fig. 1A). Cytokinins have been used for high frequency induction of bud from cotyledonary nodes in a number of plants (Bretague et al., 1994; Kodja et al., 1998), and 2,4-D has been used to induce callus formation (Frello et al., 2002).

Shoot initiation was achieved on callus after two weeks of culture on the regeneration medium supplemented with various concentrations of BAP alone or in combination with NAA. The highest number of shoot regeneration was observed on a medium supplemented with 5 µM BAP and 0.1 µM NAA (Fig. 1B, Table 1). The present study is in agreement with reports of auxin and cytokinin combinations supporting organogenesis in several species (Pereira et al., 2000; Koroch et al., 2002).

To achieve better shoot elongation and multiplication, the regenerated shoots measuring about 1 cm in length were subcultured on B5 medium supplemented with 7 µM BAP. On this medium the shoots multiplied 2.5 times every four weeks. Cytokinins, especially BAP, were effective to multiple shoot induction in some plants (Polisetty et al., 1997).

The multiplied shoots measuring about 3.5 cm in length were used for rooting. Since the full strength B5 medium caused callusing at the base of the shoots, ½B5 medium supplemented with IBA or NAA was used for rooting. There was no rooting on the basal medium. The optimum rooting was observed on ½B5 medium

Table 1. Effect of B5 medium supplemented with various concentrations of BAP and NAA on callus regeneration after 45 days in culture. The values represent the mean ± S. E. of three independent experiments. At least 24 cultures were raised for each experiment. Means within a column followed by the same alphabet are not significantly different by Duncan's multiple range test ($P>0.05$).

No:	Growth regulators (µM)	Cultures showed shooting (%)	Mean No. of shoots per explant	Length of shoot (cm)
1	BAP (0.1)	50a	2.6 ± 0.4a	1.8 ± 0.2b
2	BAP (0.5)	56b	6.2 ± 0.3d	1.3 ± 0.1b
3	BAP (1.0)	71d	12.4 ± 0.2e	0.9 ± 0.4a
4	BAP (5.0)	100g	16.1 ± 0.5f	0.5 ± 0.4a
5	BAP (10.0)	100g	18.2 ± 0.6g	0.7 ± 0.6a
6	BAP (0.1) + NAA (0.01)	53a	3.3 ± 0.5b	1.9 ± 0.5b
7	BAP (0.1) + NAA (0.1)	66c	3.5 ± 0.4b	1.8 ± 0.2b
8	BAP (0.1) + NAA (1.0)	58b	5.7 ± 0.9c	1.7 ± 0.1b
9	BAP (0.5) + NAA (0.01)	72e	4.6 ± 0.7b	1.9 ± 0.6b
10	BAP (0.5) + NAA (0.1)	78e	3.7 ± 0.2b	1.7 ± 0.7b
11	BAP (0.5) + NAA (1.0)	81f	5.9 ± 0.3c	1.6 ± 0.5b
12	BAP (1.0) + NAA (0.01)	100g	15.3 ± 1.2f	1.1 ± 0.4b
13	BAP (1.0) + NAA (0.1)	100g	12.2 ± 1.8e	1.0 ± 0.4b
14	BAP (1.0) + NAA (1.0)	100g	10.3 ± 1.3e	1.2 ± 0.6b
15	BAP (5.0) + NAA (0.01)	100g	15.3 ± 1.1f	1.2 ± 0.6b
16	BAP (5.0) + NAA (0.1)	100g	24.7 ± 1.3i	1.6 ± 0.3b
17	BAP (5.0) + NAA (1.0)	100g	16.2 ± 1.6f	0.8 ± 0.8a
18	BAP (10.0) + NAA (0.01)	100g	17.4 ± 1.0g	1.3 ± 0.2b
19	BAP (10.0) + NAA (0.1)	100g	20.3 ± 1.9h	0.7 ± 0.1a
20	BAP (10.0) + NAA (1.0)	100g	18.2 ± 2.0g	0.5 ± 0.2a

Table 2. Effect of half strength B5 medium supplemented with various concentrations of IBA and NAA on rooting of the shoots in *K. pinnata* after 45 days in culture. The values represent the mean ± S.E. of three independent experiments. At least 24 cultures were raised for each experiment. Means within a column followed by the same alphabet are not significantly different by Duncan's multiple range test ($P>0.05$).

Auxin treatment (μM)	Shoots rooted (%)	No. of roots per shoot	Mean root length (cm)	Time required for root initiation (days)
		IBA		
0.0	0	0	0	0
1	45b	2.0 ± 0.3c	0.7 ± 0.3a	9 ± 1.9c
2	60d	2.5 ± 0.4c	1.0 ± 0.8b	8 ± 2.1c
3	80e	2.9 ± 0.2c	1.7 ± 0.2b	7 ± 2.2b
4	100f	4.6 ± 0.2e	3.0 ± 0.5c	7 ± 1.8b
5	100f	3.0 ± 0.6d	2.5 ± 1.2d	5 ± 1.4a
		NAA		
0.0	0	0	0	0
1	33a	1.2 ± 0.3a	0.55 ± 0.2a	13 ± 2.8e
2	44b	1.6 ± 0.2a	0.89 ± 0.4a	11 ± 2.6d
3	58c	1.9 ± 0.6b	1.0 ± 0.1b	10 ± 2.8d
4	54c	1.4 ± 0.4a	0.9 ± 0.3a	11 ± 1.7d
5	55c	1.4 ± 0.2a	0.7 ± 0.4a	11 ± 1.9d

supplemented with 4 μM IBA (Fig. 1C). On this medium, shoots rooted with an average of 4.6 roots per shoot and an average root length of 3 cm. Comparatively IBA was better than NAA (Table 2). The roots were whitish in color with root hairs at the tip. Rooting induced by IBA and NAA has been reported in several plants including some tree species (Cid et al., 1999; Agarwal et al., 1997). The acclimatized plantlets with 3-4 roots showed obvious growth 7 days after they were transplanted in autoclaved vermiculate and sand (2:1) in plastic cups. The plantlets were watered once in every two days and were eventually transferred to garden pots with a survival frequency of 100% (Fig. 1D).

Received November 29, 2003; accepted January 9, 2004.

LITERATURE CITED

Agarwal DC, Banerjee AK, Kolala RR, Dhage AB, Kulkarni AV, Nalawade SM, Hazra S, Krishnamurthy KV (1997) In vitro induction of multiple shoots and plant regeneration in cotton (*Gossypium hirsutum* L.). Plant Cell Rep 16: 647-652

Binutu OA, Adesogan KE, Okogun JI (1996) Antibacterial and antifungal compounds from *Kigelia pinnata*. Planta Medica 62: 352-353

Bretague B, Chupeau MC, Chupeau Y, Fouilloux G (1994) Improved flax regeneration from hypocotyls using thidiazuron as a cytokinin source. Plant Cell Rep 14: 120-124

Carvalho LH, Rocha EMM, Raslan DS, Oliveira AB, Krettli AU (1988) In vitro activity of natural and synthetic naphthoquinones against erythrocytic stages of *Plasmodium falciparum*. Brazillian J Med Biol Res 21: 485-487

Cid LPB, Machado ACMG, Carvalheira SBRC, Brasileiro ACM (1999) Plant regeneration from seedling explants of *Eucalyptus grandis* X *E. urophylla*. Plant Cell Tiss Org Cult 56: 17-23

Frello S, Stummann BM, Serek M (2002) Shoot regeneration of *Campanula carpatica* Jacq. (Campanulaceae) via callus phase. Sci Hort 93: 85-90

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soyabean roots. Exp Cell Res 50: 151-158

Kodja H, Govinden-Soulange J, Gurib-Fakim A, Robene-Soustrade I, Humeau L, Figier J (1998) Micropropagation of *Psiadia arguta* through cotyledonary axillary bud culture. Plant Growth Reg 25: 75-80

Koroch A, Juliani HR, Kapteyn J, Simon JE (2002) In vitro regeneration of *Echinacea purpurea* from leaf explants. Plant Cell Tiss Org Cult 69: 79-83

Kozai TA, Kubota C (2001) Developing a phototrophic micropropagation system for woody plants. J Plant Res 114: 525-537

Morton JF (1996) The jackfruit (*Artocarpus heterophyllus* Lam.): Its culture, varieties and utilizations. Proc Fla State Hort Soc 78: 336-344

Pereira AM, Bertoni BW, Appezzato-da-Gloria B, Araujo ARB Januario AH, Lourenco MV, Franca SC (2000) Micropropagation of *Pothomorphe umbellate* via direct embryogenesis from leaf explants. Plant Cell Tiss Org Cult 60: 47-53

Polisetty R, Paul V, Deveshwar JJ, Khetarpal S, Suresh K, Chandra R (1997) Multiple shoot induction by benzyladenine and complete plant regeneration from seed explants of chickpea (*Cicer arietinum* L.). Plant Cell Rep 16: 565-571

Purohit SD, Tak K, Joshi P, Samar D (1998) Micropropagation of some woody tree species of Aravallis in Rajasthan. *In*: PS Srivastava, ed, Plant tissue culture and molecular biology applications and prospectus. Narosa Publishing House, New Delhi, pp 84-125