In vitro plantlet regeneration of *Pterocarpus marsupium* Roxb., an endangered leguminous tree

Pterocarpus marsupium Roxb. (Fabaceae), commonly known as Bijasal, is one of the most valuable multipurpose forest trees that yields excellent timber for the international trade market. The plant also has useful medicinal properties and is used as a powerful astringent for diarrhoea, dysentery, fever and toothache. It is also reported that an aqueous infusion of the wood is of use in diabetes and water stored in vessels made of the wood is reputed to have antidiabetic qualities[1].

The native natural stands of this tree are fast disappearing. The winged fruit is the only propagating material, but its germination is low (only 30%)[2]. Hard fruit coat, less germinability coupled with poor seed viability is responsible for its diminishing population size. Due to its significant multipurpose properties, *P. marsupium* has been overexploited, which in turn has led to its inclusion in the list of endangered plant species.

In view of its inherent qualities and restricted distribution, rapid *in vitro* clonal multiplication of this endangered tree species is needed. Micropropagation of tree species offers a rapid means of producing clonal planting stock for afforestation, woody biomass production and conservation of elite and rare germplasm[3,4].

Forest trees in general and Fabaceae in particular, have proved to be recalcitrant for mass propagation by tissue culture[5]. Attempts have already been made in the recent past to micropropagate leguminous tree species, namely *Dalbergia latifolia*[6], *Dalbergia sissoo*[7], *Sesbania sesban*[8] and these species responded positively. There are few preliminary reports on tissue culture studies of *P. marsupium*[9,10], but detailed information regarding regeneration steps has not been provided. Such information is necessary for developing complete micropropagation protocol. Das and Chatterjee[9] tried micropropagation both through seedlings as well as coppiced shoot explants, but did not get any response. Kalimuthu and Lakshmanan[10] used only seedling-derived nodal explants and reported a maximum induction of only two shoots per explant.

In the present communication, we report development of plantlets via induction of multiple shoots from seedling-derived cotyledonary node explant and their successful rooting and acclimatization.

Fruits of *P. marsupium* were obtained through the courtesy of Tropical Forest Research Institute (TFRI), Jabalpur. The stony fruit coats were removed manually with the help of a cutter and the seeds were kept in running tap water for 30 min, and then soaked for 24 h in distilled water. Seeds were then treated with liquid detergent (Teepol 5% v/v) for 5 min followed by thorough washing. They were then surface sterilized with 0.1% (w/v) aqueous $HgCl_2$ for 5 min, washed 4–5 times in sterile double-distilled water and then were implanted aseptically on half strength Murashige and Skoog (MS)[11] basal medium containing 3% sucrose and gelled with 0.8% agar (Qualigens, India).

When the seedling grew up to 5 cm, the cotyledons, nodal segments, cotyledonary nodes (CNs), and shoot tips derived from 18-day-old aseptic seedlings were excised and used to initiate culture. The MS medium was variously supplemented with cytokinins (BA, KN) or auxins (IAA,

Figure 1. Direct multiple shoot regeneration from cotyledonary node explant and complete plantlet establishment of *Pterocarpus marsupium*. ***a***, Induction and proliferation of multiple shoots from cotyledonary node explant on MS + BA (10 µM). ***b***, Shoot elongation on MS + BA (5 µM) + IAA (0.25 µM). ***c***, Multiple shoot formation on MS + BA (5 µM) after 6 weeks. ***d***, Growth and proliferation of shoots on the same medium after 8 weeks. ***e***, Normal rooted shoot developed on ½MS + IBA (0.5 µM) through a two-step culture procedure.

Table 1. Effect of cytokinin on shoot proliferation from cotyledonary nodes of *Pterocarpus marsupium* in MS medium

Growth regulator	Percentage explants showing shoot proliferation	Mean number of shoots produced/explant	Mean length of shoots (mm)
BA (1.0 μM)	40	$1.16 \pm 0.17_{cd}$	$21.2 \pm 0.03_{c}$
BA (2.5 μM)	60	$2.00 \pm 0.26_{c}$	$15.6 \pm 0.04_{d}$
BA (5.0 μM)	85	$7.83 \pm 0.30_{a}$	$54.3 \pm 0.04_{a}$
BA (10 μM)	70	$4.66 \pm 0.21_{b}$	$37.8 \pm 0.05_{b}$
Kn (1.0 μM)	30	$0.33 \pm 0.21_{d}$	$7.3 \pm 0.03_{f}$
Kn (2.5 μM)	50	$0.66 \pm 0.21_{d}$	$8.0 \pm 0.06_{d}$
Kn (5.0 μM)	55	$0.83 \pm 0.17_{d}$	$12.5 \pm 0.05_{e}$
Kn (10 μM)	40	$0.50 \pm 0.22_{d}$	$7.4 \pm 0.03_{b}$

Values represent mean ± SE of ten replicates per treatment.
Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level.

Table 2. Effect of IAA/NAA with optimal concentration of BA on shoot proliferation from cotyledonary nodes of *P. marsupium* in MS medium

Composition	Percentage explants showing shoot proliferation	Mean number of shoots produced/explant	Mean length of shoots (mm)
BA (5 μM) + IAA (0.25 μM)	70	$4.16 \pm 0.30_{a}$	$40.1 \pm 0.06_{a}$
BA (5 μM) + IAA (0.50 μM)	60	$1.33 \pm 0.21_{b}$	$16.7 \pm 0.03_{b}$
BA (5 μM) + IAA (1.00 μM)	50	$1.00 \pm 0.25_{bc}$	$10.6 \pm 0.3_{bc}$
BA (5 μM) + NAA (0.25 μM)	50	$0.50 \pm 0.22_{bc}$	$7.5 \pm 0.25_{cd}$
BA (5 μM) + NAA (0.50 μM)	30	$0.33 \pm 0.21_{bc}$	$5.6 \pm 0.18_{cd}$
BA (5 μM) + NAA (1.00 μM)	30	$0.20 \pm 0.16_{bc}$	$0.38 \pm 0.19_{d}$

Values represent mean ± SE of ten replicates per treatment.
Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level.

IBA, NAA), either singly or in combination. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 min. Borosil test tubes containing 15 ml of the medium were used, covered with cotton plugs wrapped in cheese cloth. All cultures were incubated at 25 ± 2°C in a photoperiod of 16 h for a day under fluorescent light (about 1200 lux) and with 55–60% relative humidity.

In vitro differentiated shoots measuring 3–4 cm were excised and subjected to *in vitro* rooting. For rooting, half and full strength MS medium augmented with various auxins (IAA, IBA, NAA) along with a phenolic acid were used.

All experiments were repeated thrice and ten replicates per treatment were taken. The data were analysed using one way analysis of variance (ANOVA) followed by Tukey's test with a confidence limit of 0.05.

Among various explants used, CN showed excellent response. The CN explants inoculated on MS medium responded differently to various cytokinins singly or in combination (Table 1). Among different concentrations of cytokinin tested, maximum shoot multiplication (7.83 ± 0.30) per explant was achieved on MS medium fortified with BA (5 μM) within 6 weeks (Figure 1 *c*). Multiple shoots obtained were divided into 2–3 clumps for further proliferation and to increase the number of shoots. These in turn proliferated into multiple shoots on the same concentration of BA (Figure 1 *d*). Regular subculturing was done every 3–4 weeks onto fresh medium. During initial subculturing, the mother explant was kept intact with proliferated shoots. Increasing the concentration of BA (10 μM) resulted in a decrease in the rate of shoot regeneration ability. Only 4.66 ± 0.21 shoots could be recorded after 6 weeks (Figure 1 *a*). Inhibitory effect of higher concentrations of BA on shoot formation has also been reported in *Albizia chinensis*[12].

When kinetin was used as the sole cytokinin (1.0–10 μM), only elongation of single shoot took place and no significant response was observed. Explants grown on BA-supplemented medium showed better growth and elongation, and were found to be more responsive to BA than kinetin. BA-induced shoot proliferation from CNs has also been reported in *Dalbergia sissoo*[13] and *A. chinensis*[12]. Superiority of BA for induced shoot multiplication in *Pterocarpus* species[14,15] has also been reported earlier.

Using BA (5 μM) as optimum for maximum shoot bud induction, different auxins (IAA and NAA) at 0.25–1.0 μM were added to the medium along with BA to observe the synergistic effect of auxin and cytokinin. Low level of IAA (0.25 μM) showed the effect on shoot bud induction, while higher levels (0.5–1.0 μM) were not found beneficial, as the callus was observed at the base of the explant. A maximum of 4.16 ± 0.75 shoots per explant were differentiated on MS medium supplemented with BA (5 μM) and IAA (0.25 μM) within 5–6 weeks (Figure 1 *b*). However, on BA (5 μM) + NAA (0.25–1.0 μM) supplemented medium, no significant results were observed (Table 2).

The addition of IAA and NAA with optimal concentration of BA significantly reduced the frequency of shoot formation compared to BA alone. This is in contrast with the result reported in *Wrightia tinctoria*[16] and *Acacia catechu*[17], where maximum number of shoot buds were initiated in the BA and NAA combination.

Healthy and sturdy shoots (4–5 cm long) were transferred to rooting medium

Figure 2. Established plantlet after 8 weeks under *ex vitro* conditions.

containing different concentrations of MS salts (¼, ½and full strength) and different auxins (IAA, IBA and NAA) applied singly at various concentrations. After 3–4 weeks of incubation, no rooting took place and callusing was observed at the base of the shoots.

The isolated microshoots were rooted to 40–50% success in 15–17 days by the two-step culture procedure and using a strategy of giving pulse treatment of an auxin (IBA; 200 µM) together with a phenolic acid for 5 days and subsequent transfer of such shoots to lower concentration of auxin IBA (0.5 µM) on ½MS medium. A single thick root induction was observed (Figure 1 *e*). The requirement of half strength culture medium for *in vitro* rooting has been reported in many woody species[18,19]. The two-step culture procedure for rooting was also reported in *Albizia*[12]. The incorporation of an auxin in the medium generally promotes rooting, while in the present study auxin alone in the MS medium was found to be ineffective for rooting. This is contrary to an earlier report, where IAA alone promoted rooting in *P. marsupium*[15]. However, addition of IBA with a phenolic acid for root initiation has also been demonstrated in Apple[20], *Mallotus philippensis*[21] and *Shorea robusta*[22].

Thus, eight to nine true-to-type plantlets of *P. marsupium* were raised *in vitro* from single cotyledonary node explant, transferred to perforated paper cups for gradual acclimatization and irrigated with nutrient solution every alternate day (Figure 2). Regenerated plantlets are in the hardening process.

Thus, our results indicate the direct regeneration of multiple shoots from CN explants of this endangered legume tree. Direct regeneration from the CN explants will ensure the cloning stocks[4] of this valuable tree. Reproducibility and plantlet regeneration will surely lead to the use of this system in afforestation programmes.

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M. ANIS*
MOHD KASHIF HUSAIN
ANWAR SHAHZAD

Plant Tissue Culture Laboratory,
Department of Botany,
Aligarh Muslim University,
Aligarh 202 002, India
**For correspondence.*
e-mail: anism1@rediffmail.com