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Micropropagation of Red Kino Tree (*Pterocarpus marsupium* Roxb.): A Medicinally Important Plant

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ABSTRACT

An efficient protocol is described for the rapid *in vitro* multiplication of an endangered highly valuable medicinal plant, *Pterocarpus marsupium* Roxb., through cotyledonary nodes of immature seeds (IS). High frequency of direct shoot regeneration was induced from cotyledonary nodes of IS on Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (BA). Among the various cytokinins tested BA, Kinetin (Kn), Zeatin (ZEA), BA proved to be most effective. The direct shoot regeneration capacity of the IS was influenced by the BA concentrations (0.44-8.87 μM), and the optimal response was observed at 4.44 μM BA, which induced maximum number of multiple shoots (12.9 ± 0.21) with highest shoot length (3.8 ± 0.03) in 100% of the cultures, within 4 weeks. Significant differences were recorded in terms of average number of shoots per explant (1.9-12.9) among the different concentrations of BA investigated. Concentrations of all cytokinins tested reached a level that can be considered above the optimum level, as marked by a reduced frequency of shoot regeneration. A proliferating shoot culture was established by repeatedly subculturing the IS explants on 4.44 μM BA. Rooting of regenerated shoots was achieved under *in vitro* conditions by a two-step procedure employing a pulse treatment with indole-3-butyric acid (IBA) and subsequent transfer to growth regulator free half-strength MS medium. The most effective first-step treatment was found to be 49.00 μM IBA for 24 h, which initiated rooting at a frequency of 68%. *In vitro* raised plantlets were transferred to pots containing sterilized soil and vermiculite mixture (1: 1), and then transferred to the greenhouse. Plantlets established in pots exhibited a 75% survival rate. This procedure is suitable for use in large-scale production of plants and may have potential application to other *Pterocarpus* species.

Keywords: cotyledonary node, immature seed, multiple shoots, Papilionaceae
Abbreviations: BA, 6-benzyladenine; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **Kn**, kinetin; **NAA**, α-naphthalene acetic acid; **ZEA**, zeatin

INTRODUCTION

Pterocarpus marsupium Roxb., popularly known as "red kino tree" and a member of the Papilionaceae family, is a widely used medicinal species useful in the treatment of diabetes, dysentery, stomachache, elephantiasis, leucoderma, cholera, urinary complaints and cough (Tiwari *et al.* 2004). The gum obtained from the stem is used as an astringent, in diarrhoea and for toothache and the leaves are useful for external applications of boils, sores and skin diseases (Mankani *et al.* 2005). Heart wood of *P. marsupium* has been used in Ayurvedic medicine for centuries for its anti-hyperglycemic activity. Earlier studies reported that the phenolic constituents of *P. marsupium* significantly lowered the blood glucose level in diabetic rats (Manikam *et al.* 1997). The flowers are used in fever and tree is valued for excellent timber that ranks next to teak (Chand and Singh 2004).

P. marsupium's natural resurgence is through seeds. However, the germination percentage is very poor (30%) (Husain *et al.* 2008) and propagation through stem cutting is also difficult. Poor propagation coupled with overexploitation for pharmaceutical use and timber, this species has become endangered hence, it is essential for the conservation of *P. marsupium* using *in vitro* techniques. The development of a rapid mass propagation system through tissue culture is not only a powerful tool for commercial propagation but also an important step that will open new field of research in *P. marsupium.* Alternative propagation methods would be beneficial in accelerating large-scale multiplication, improvement and conservation of the plant.

In vitro propagation of *P. marsupium* using mature seeds (Anuradha and Pullaiah 1999), *in vitro* raised nodal explants (Tiwari *et al.* 2004; Husain *et al.* 2008) and cotyledonary nodes of mature seeds (Chand and Singh 2004; Anis *et al.* 2005; Husain *et al.* 2007) have been reported. However, these published protocols involve several stages, which resulted in relatively low number of shoots per explant. The present investigation describes for the first time, an efficient and rapid *in vitro* propagation of endangered *P. marsupium* using immature seeds (IS).

MATERIALS AND METHODS

Green pods of *P. marsupium* Roxb were collected after 7-9 weeks of pollination from trees growing in the forest department campus at Warangal, A.P, India. The pods were washed thoroughly for 20 min under running tap water to remove dust particles and treated for about 10 min with laboline (10 ml/l) detergent (Qualigens Fine Chemicals, Mumbai, India), followed by Tween-20 for 4 min. This was followed by washing under tap water for removal of detergents and disinfectants. The collected pods were disinfected with an aqueous solution of 0.1% (w/v) freshly prepared aqueous mercuric chloride solution ($HgCl_2$) for 15 min and further rinsed with autoclaved distilled water five to six times and dried on sterile tissue paper.

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Original Research Paper

Multiple shoot induction

After removing the seed coat, the IS were excised under a laminar airflow cabinet and cultured on MS basal media (Murashige and Skoog 1962) containing 3% sucrose (Himedia, India) supplemented with plant growth regulators (PGRs), 6-benzyladenine (BA) (0.44, 2.22, 4.44, 6.66 and 8.87 μM), or Kinetin (Kn) (0.46, 2.32, 4.65, 6.97 and 9.29 μM) or zeatin (ZEA) (0.46, 2.28, 4.56, 6.84 and 9.12 μM). MS medium without PGRs served as controls for shoot initiation.

Rooting medium and greenhouse transfer

Well developed shoots of 1 cm size were rooted by a two-step culture method. In the first step, shoots were kept in half-strength MS liquid medium containing indole-3-butyric acid (IBA) at 4.90, 24.5, 49.00 or 98.00 μM for 24 or 48 h. In the second step, auxin-treated shoots were transferred onto PGR-free half-strength MS medium. The same procedure was also used for indole-3-acetic acid (IAA) and α-naphthalene acetic acid (NAA). *In vitro*-rooted plantlets (3-4 weeks old) were weaned and washed repeatedly in tap water to remove agar and medium constituents, potted in pots (20 cm in length, 15 cm in diameter, 2-3 cm deep; one plant per pot) containing sterilized soil and vermiculite mixture (1: 1). The potted plants were covered with polyethylene sheets to reduce the loss of moisture and acclimatized in a greenhouse [28°C during the day, 16-18°C at night, 65% relative humidity (RH)].

The pH of all media was adjusted to 5.8 with 1 M KOH prior to adding 0.8% agar (Himedia,-RM 301, Mumbai) and the media were autoclaved at a pressure of 15 lbs for 15 min at 121°C. All cultures were incubated at 25 ± 2°C with a 16-h photoperiod under white fluorescent light (40-60 μmol m^{-2} s^{-1}; IS 2416 L 7434877, two bulbs model, Phillips, India). All data were statistically analyzed using analysis of variance (ANOVA) and the means were compared using Duncan's multiple range test (DMRT). For *in vitro* shoot multiplication, 20 explants were used in each of two replicates for each treatment and the experiment was repeated twice.

RESULTS AND DISCUSSION

In the present investigation we have developed a reproducible protocol for direct shoot regeneration from cotyledonary nodes of immature seeds (IS) of *P. marsupium*. Using this protocol, within 28-30 days, 12-13 healthy plants were recovered. There are no reports available on *in vitro* propagation using IS explant in this plant species.

Multiple shoot induction

IS explant cultured on MS basal medium (without cytokinins) developed into a single plant- let (a shoot with root system) (**Fig. 1B**), but on cytokinin amended medium, multiple shoots were produced. Of the three cytokinins tested, BA was more effective than Kn or ZEA in producing multiple shoots and shoot growth. Multiple shoots emerged directly from cotyledonary nodes within one week of culture on BA containing media. The frequency of direct shoot induction and the number of shoots per explant increased with increasing concentration of BA (**Table 1**). Highest shoot regeneration frequency (100%) with 12.9 ± 0.21 shoots/explant and 3.8 ± 0.03 shoot length was observed on BA 4.44 μM (**Fig. 1C, 1D**). Similar results were also observed in bael (Raglili *et al.* 2007).

Chand and Singh (2004) and Anis *et al.* (2005) reported 9.5 shoots and 7.8 shoots/cotyledonary node of mature seed explant of *P. marsupium*, respectively. However, we have reported 12.9 shoots/cotyledonary node of IS explant. This difference could be due to progressive decline in regeneration capacity of mature seed; IS are meristematic and totipotent. In Southern Pines immature explant produced more shoots than mature explant (Greenwood *et al.* 1991).

Barmukh and Nikam (2008) reported that natural resurgence in *P. marsupium* is poor and that nursery germination is unpredictable; however, using wet, heat, physical and acid scarification it is possible to improve percentage of seed germination. Hence IS could be a choice explant for obtaining higher shoots/explant.

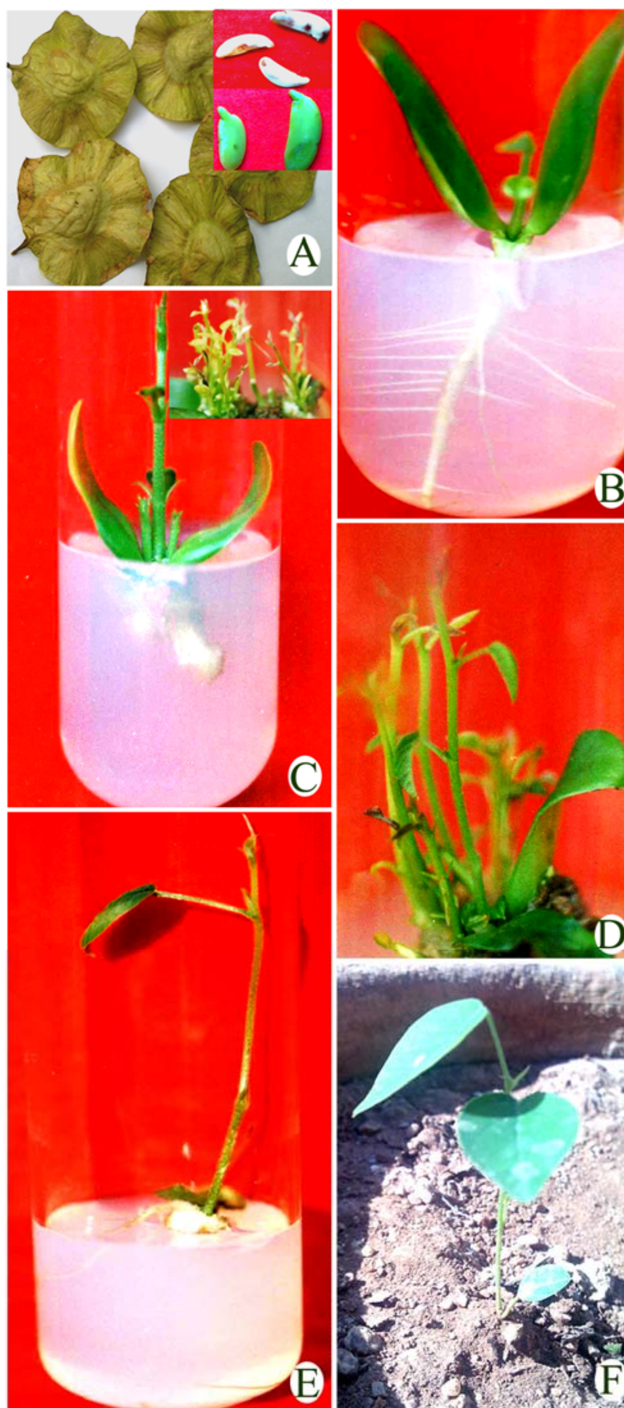

Fig. 1 *In vitro* multiplication of *Pterocarpus marsupium* from cotyledonary nodes of immature seeds and plant establishment. (**A**) Green pods of *Pterocarpus marsupium*; Inset: Immature seeds; (**B**) Induction of single shoot with complete root system on MS medium without growth regulators; (**C**) Multiple shoot induction on MS + BA (2.22 μM) (10 days); Inset: MS+BA (4.44 μM) (18 days); (D) Elongated shoots on MS + BA (4.44 μM) (28 days); (**E**) Rooted shoots on growth regulator free half strength MS medium through a two-step procedure; (**F**) Regenerated plantlet under field conditions 35 days after the rooting period.

Tiwari *et al.* (2004) reported development of 3.25 shoots/nodal explant and Hussain *et al*. (2008) reported the development of 4.8 shoots/nodal explant, respectively. Nodal explants have tiny preformed shoot buds which elongate on tissue culture medium; this is not the case in *de novo* regeneration of shoot buds as in IS or mature seed explants. The number of shoots produced per nodal explant

Table 1 Effect of plant growth regulators on direct shoot regeneration from cotyledonary nodes of immature seeds of *Pterocarpus marsupium* (28 days).

PGRs (µM)	Frequency of shoot regeneration (%)	Morphogenic response	No. of shoots/explant	Shoot length (cm)
0	50	S+R	0. 8 ± 0.20 e	1.6 ± 0.05 f
BA				
0.44	60	S	1.9 ± 0.11 b	2.3 ± 0.04 d
2.22	82	S	6.5 ± 0.30 n	3.0 ± 0.05 g
4.44	100	S	12.9 ± 0.21 f	3.8 ± 0.03 b
6.66	90	S	8.3 ± 0.23 i	2.5 ± 0.06 i
8.87	75	C+S	5.1 ± 0.19 d	1.0 ± 0.04 c
Kn				
0.46	50	S	1.0 ± 0.30 m	1.2 ± 0.08 l
2.32	58	S	2.3 ± 0.23 h	1.7 ± 0.03 a
4.65	70	S	6.3 ± 0.34 p	2.4 ± 0.06 i
6.97	60	C+S	4.0 ± 0.30 l	1.0 ± 0.05 e
9.29	52	C+S	1.5 ± 0.24 j	0.7 ± 0.04 c
Zeatin				
0.46	52	S	1.0 ± 0.10 a	1.8 ± 0.03 a
2.28	70	S	4.1 ± 0.22 g	2.2 ± 0.07 k
4.56	90	S	8.2 ± 0.14 c	2.7 ± 0.04 d
6.84	80	C+S	5.4 ± 0.28 k	1.7 ± 0.06 h
9.12	62	C+S	2.1 ± 0.32 o	0.8 ± 0.07 j

Data were collected after 28 days of incubation and are expressed as the mean of two replicates of 20 explants ± standard error; In each column, means followed by the same letter were not significantly different ($p \leq 0.05$) according to Duncan's multiple range test; S, shoot, R, root, C, callus

is less compared to IS or mature seeds. Thorpe *et al.* (2005) reported that micropropagation in woody species is that most successfully achieved with juvenile tissue and not from proven mature tissues.

Cytokinins have been used by all researchers who worked on the micropropagation of *P. marsupium.* Chand and Singh (2004) and Anis *et al.* (2005) used 5 µM BAP and 4.44 µM BAP + 0.26 µM NAA, respectively. We used 4.44 µM BAP. These comparisons show that BAP along with NAA is necessary for inducing 9.5 shoots in MS explant but only BAP is necessary for inducing 12.9 shoots in IS explant. Endogenous cytokinins in IS explant could explain the increase in the number of shoots.

Tiwari *et al.* (2004) used 13.31 µM BAP + 2.69 µM NAA for elongating shoots in nodal cultures of *P. marsupium*. Husain *et al.* (2008) used 20 µM adenine sulphate in addition to 4.0 µM BAP and 0.5 µM IAA. For elongation of tiny shoot buds in nodal explants, it appears that endogenous auxins are necessary (Nanda *et al.* 2004).

We have studied the effect of cytokinins viz. BA, Kn and ZEA on shoot regeneration from cotyledonary node of IS explants of *P. marsupium*. Shoot regeneration frequency was 100% in BA-amended medium while it was 70 and 90% for Kn and ZEA, respectively. BA at 4.44 µM induced 12.9 shoots/explant, while Kn or ZEA at 4.65 and 4.56 µM induced 6.3 and 8.2 shoots/explant, respectively. BA and Kn are synthetic cytokinins and ZEA is natural. It can be inferred from the above data that all three cytokinins induce shoots in IS explants of *P. marsupium* but BA induced more shoots than Kn and ZEA. The use of BA promoted regeneration through organogenesis while Kn and ZEA promoted organogenesis as well caulogenesis. Similar results were observed in *Eucalyptus* (Rodriguez and Vendrame 2003).

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 cotyledonary nodes has also been reported in *Dalbergia sissoo* (Pradhan *et al.* 1998) and *A. chinensis* (Sinha *et al.* 2000). Superiority of BA for induced shoot multiplication in *Pterocarpus* spp. has been reported (Patri *et al.* 1988; Anuradha and Pullaiah 1999; Anis *et al.* 2005). With an increase of BA concentration from 4.44 to 8.87 µM there was a decrease in the number of shoots (5.1+0.19); the tendency to form callus was also observed (**Table 1**). Moreover, the shoots that regenerated at higher concentrations of BA were smaller and senesced. An inhibitory effect of higher concentrations of BA on shoot induction and elongation has also been reported in bael (Raglili *et al.* 2007).

Shoot formation was 100% on 4.44 µM BA while on 4.65 µM Kn and 4.56 µM ZEA it was 70 and 90%, respectively. Mean shoot length was 3.8 cm in BA (4.44 µm), while in Kn (4.65 µM) and ZEA (4.56 µM) it was 2.4 and 2.7 cm, respectively. Shoots produced on Kn- and ZEA-supplemented media were small and tiny, respectively.

The frequency of shoot induction was low with fewer shoots per explant and a considerable amount of callus when IS explants were cultured on Kn or ZEA (**Table 1**). The shoots that developed on medium containing Kn or ZEA were stunted and subsequently failed to elongate. Development of stunted shoots on Kn-supplemented medium has been reported in tree species such as *Paulownia tomentosa* (Rout *et al.* 2001). Among the three cytokinins BA is the most effective PGR, for multiple shoot induction, indicating BA specificity of IS explant of *P. marsupium.* The superiority of BA over other cytokinins in direct shoot regeneration from seedling explants has been well documented in medicinal plants (Das *et al.* 1999).

Rooting of shoots

For a successful micropropagation protocol, establishment of *in vitro* rooting of shoots is crucial. IBA was preferably used for the development of adventitious roots *in vitro* for many tree species e.g. *Prunus mume* (Ning *et al.* 2007) and *Sterculia urens* (Hussain *et al.* 2008). When *in vitro* regenerated shoots failed to root on MS medium or MS medium with auxins (IAA, IBA and NAA), they were pulse treated with IBA and planted on PGR-free half-strength MS medium, and within 8 days they produced adventitious roots. The best rooting (68%) was obtained for 24 h treat-

Table 2 Root induction of *in vitro* excised shoots of *P. marsupium* under the influence of different concentration of IBA and time (24 h) of exposure (20 days).

Auxins (µM)	Root induction (%)	No. roots/shoot	Length of roots (cm)
0	0	0	0
IBA*			
4.9	0	0	0
24.5	14	0.8 ± 0.16 a	1.1 ± 0.12 b
49	68	2.1 ± 0.24 b	2.7 ± 0.08 a
98	20	1.0 ± 0.12 a	0.8 ± 0.10 b

*half-strength MS medium; Shoots were treated with IBA for 24 h, before transferring to PGR-free medium

Data were collected after 28 days of incubation and are expressed as the mean of two replicates of 20 explants ± standard error

In each column, means followed by the same letter were not significantly different ($p \leq 0.05$) according to Duncan's multiple range test

ment with 49.00 µM IBA (**Table 2**). All the shoots remained green and healthy at 24 h treatment with IBA at 49.00 µM. However, the shoots turned brown and callus formation was observed at the cut end of shoots when treated for 48 h (data were not shown). The number of roots per shoot was 2.1 ± 0.24 that attained an average length of 2.7 ± 0.08 cm (**Table 2, Fig. 1E**). Extensive callusing at the base of the shoots was noticed without any root formation when IAA or NAA was used. A two-step culture procedure for rooting was also reported in *Quercus semecarpifolia* (Tamta *et al.* 2008). *In vitro*-raised plants were successfully transferred to pots and hardened under greenhouse conditions at 65% relative humidity. Five weeks after transplantation, 75% of plantlets survived (**Fig. 1F**). All the tissue-cultured plantlets had normal leaf development and lacked detectable morphological variation and showed apparently uniform growth and morphology.

The present study reports for the first time, an efficient and simple method for high frequency direct shoot regeneration of *P. marsupium* from cotyledonary nodes of IS explant. About 4 weeks duration is required from culture initiation to plant regeneration. Using this protocol, about 35-40 plants can be obtained from a single cotyledonary node of IS explant within 3 months.

In vitro conservation of traditional medicinal plant germplasm is important to support chemical analysis and pharmacological and genetic transformation studies. With resurgence of public interest in plant based medicine and rapid expansion of pharmaceutical industries, *in vitro* micropropagation will be useful.

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