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ORIGINAL PAPER

Somatic embryogenesis and plant regeneration in *Pterocarpus marsupium* Roxb.

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Abstract Somatic embryogenesis (SE) has been achieved from hypocotyl-derived callus culture in *Pterocarpus marsupium*. Ninety percent of hypocotyl explants (excised from 12-day-old in vitro germinated axenic seedlings) produced callus on Murashige and Skoog medium supplemented with 5 μM 2,4-dichlorophenoxyacetic acid and 1 μM a 6-benzyladenine (BA). Induction of SE occurred after transfer of callus clumps (200 ± 20 mg fresh mass) to MS medium supplemented with BA at 2.0 μM, where a maximum of 23.0 ± 0.88 globular stage embryos per callus clump were observed after 4 weeks of culture. Subculturing of these embryos on MS medium supplemented with 0.5 μM BA, 0.1 μM α-naphthalene acetic acid and 10 μM abscisic acid significantly enhanced the maturation of somatic embryos to early cotyledonary stage, where 21.4 ± 0.32 embryos per callus clump were recorded after 4 weeks of culture. Of 30-well developed somatic embryos, 16.6 ± 0.33 germinated and subsequently converted into plantlets on half-strength MS medium supplemented with 1.0 μM BA. The morphologically normal plantlets with well-developed roots were first transferred to 1/4-liquid MS medium for 48 h and then to pots containing autoclaved soilrite and acclimatized in a culture room. Thereafter, they were transferred to a greenhouse, where 60% of them survived.

Keywords Hypocotyl · Leguminous tree · Malabar kino · Embryo germination · Seedlings · Embryogenic callus

Abbreviations

ABA	Abscisic acid
AC	Activated charcoal
BA	6-Benzyladenine
2,4-D	2,4-Dichlorophenoxyacetic acid
MS	Murashige and Skoog medium
NAA	α-Naphthalene acetic acid
PG	1,2,3-Trihydroxy benzene
PGRs	Plant growth regulators
IBA	Indole-3-butyric acid
2iP	2-Isopentenyladenine
SE	Somatic embryogenesis

Introduction

Pterocarpus marsupium Roxb. (Fabaceae) commonly known as Malabar Kino is one of the most economically important timber-yielding tree of India, characterized for its quick growth, quality of timber and disease resistance. The tree possesses gum-kino, which is a powerful astringent and used to cure various diseases. Traditionally in India, an aqueous infusion of the wood is used to treat diabetes and water stored in vessels made of the wood is

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reputed to have anti-diabetic properties (Anonymous 2003). Phenolic constituents (marsupsin and pterostilbene) isolated from the heartwood and aqueous extract of stem bark of *P. marsupium* have shown to possess antihyperglycemic activity (Manickam et al. 1997; Vats et al. 2002). A herbal product 'Vijayasar' extracted from the bark of this tree has shown positive results in the treatment of diabetes in several trials conducted by Indian Council of Medical Research (ICMR) with no side effects (Chaudhury 2004).

The conventional method of propagation of *P. marsupium* through seeds is not efficient because the germination percentage is low (only 30%) due to hard fruit coat and poor viability (Kalimuthu and Lakshmanan 1995). In consequence, the regeneration rate of leguminous trees in natural habitat is low (Husain et al. 2007). The exploitation of this medicinally as well as economically important tree from the natural habitat and inadequate efforts for its cultivation resulted in marked decline in the population of the species and has, therefore, been included on the list of depleted plant species (Chaudhuri and Sarkar 2002).

Although, some investigators have successfully achieved in vitro plant regeneration in *P. marsupium* using cotyledonary or nodal explants (Chand and Singh 2004; Anis et al. 2005; Husain 2007; Husain et al. 2007, 2008), SE has not been reported up to date. Regeneration of plants through SE, if optimized, is an efficient method of rapid propagation of selected genotypes and chemotypes.

In this study, we report for the first time a plant regeneration protocol through SE from hypocotyl-derived callus in *P. marsupium*.

Materials and methods

Explant source and disinfection

The mature winged fruits of *P. marsupium* were obtained from the Tropical Forest Research Institute, Jabalpur, India. The healthy seeds were excised from the fruits and washed thoroughly in running tap water for 30 min followed by rinsing with 5% (v/v) Teepol (a liquid detergent, Glaxo, India) for 10 min. The treated seeds were agitated in distilled water (10 seeds/100 ml) for 24 h to remove the chemical inhibitors of germination. The leachates were replaced with sterile distilled water. Seeds were surface disinfested with 70% (v/v) ethanol for 30 s, followed by 0.1% (w/v) $HgCl_2$ solution for 6 min and finally rinsed six times with sterile distilled water. An average of 3–5 seeds were germinated aseptically in a culture flask (100 ml, Borosil, India) containing 35 ml of PGR-free half-strength Murashige and Skoog (1962) medium with 3% (w/v) sucrose (Hi-media, India) and 0.7% (w/v) agar (bacteriological grade, Hi-media, India). The culture flasks were closed with non-absorbent cotton plugs and incubated in culture room conditions specified below. Aseptically germinated seedlings were grown until they attained a length of 2–4 cm. Hypocotyl segments (0.5 cm) excised from 12-day-old axenic seedlings were used as explants.

Media and conditions for explant culture

Murashige and Skoog medium containing 3% (w/v) sucrose and 0.7% (w/v) agar was used in all the experiments unless otherwise specified. Plant growth regulators at different concentrations were incorporated into MS medium specified below. The pH of the medium was adjusted to 5.8 by 1 N NaOH or 1 N HCl before autoclaving at 1.06 kg cm^{-2} (121°C) for 20 min. The medium was dispensed into 25 × 150-mm test tubes (Borosil, India) and plugged with non-absorbent cotton wrapped in muslin cloth. All the culture were incubated under 16 h photoperiod at 50 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by cool white fluorescent tubes (40 W, Philips, India) at 25 ± 2°C in a culture room with 55 ± 5% of relative humidity (RH).

Induction of callus and somatic embryogenesis

The hypocotyl segments excised from 12-day-old axenic seedlings were cultured on MS medium supplemented with various concentrations (1, 2, 5 and 10 μM) of 2,4-D either alone or in combination with 1, 2 and 5 μM BA to induce callus. Explants forming callus were counted 4 weeks after culture initiation.

For the induction of SE, the calli (200 ± 20 mg fresh mass) were transferred to MS medium supplemented with different concentrations of BA (0.5, 1.0, 2.0 and 5 μM) and 2iP singly. The induction frequency (%) of somatic embryo formation was recorded after 4 weeks of culture.

Maturation of somatic embryos

Embryogenic calli with globular embryos were subcultured on embryo maturation medium consisting of MS salts without PGRs (MSO, control) or MS supplemented with 0.5 μM BA, 0.1 μM NAA, 5, 10 and 15 μM ABA and AC at 1 and 2 g l^{-1} either alone or in combinations (Table 2). The average number of early cotyledonary-stage embryos was counted using a stereozoom microscope (Motic-SMZ 143, Japan) attached to a computer.

Germination of somatic embryos and conversion into plantlets

At least 30 cotyledonary-stage somatic embryos were individually transferred to full and half-strength MS (1/2 MS)

without PGRs (MSO, control) or with BA (0.5, 1.0 and 2.0 μM), NAA (0.1 μM) and AC (2 g l^{-1}) either alone or in combinations (Table 3). The frequency of germination of somatic embryos and conversion into plantlets was recorded after 4 weeks of culture on the germination medium.

Acclimatization of plantlets

Plantlets with well-developed shoots and roots were transferred to test tubes containing 1/4 strength liquid MS medium for 48 h, followed by their transfer to thermocol cups containing autoclaved soilrite mixture (Keltech Energies Pvt. Ltd, Karnataka, India) for acclimatization and finally to the clay pots.

Statistical analysis

The experiments were conducted according to a complete randomized design taking ten explants per treatment with three repetitions (=three replicates, 30 explants per treatment). The percentage data obtained for various parameters (callus induction, embryo induction, maturation and germination) was converted to arc sine transformations prior to statistical analysis employing ANOVA. Tukey's test ($P = 0.05$) of significance was applied to separate treatment means. The statistical analysis was done using SPSS version 12 (SPSS Inc., Chicago IL, USA). Original percentage data (untransformed) are then presented in tables and figure.

Results

Induction of callus and somatic embryogenesis

The hypocotyl segments showed no response on PGR-free MS medium even after 4 weeks of culture. However, with the addition of PGRs (2,4-D and BA), callus was induced initially from the cut ends of hypocotyl explants, which subsequently spread over the entire surface within 3 weeks of culture. The frequency of callus induction ranged from 41 to 90%, depending on the concentration and combination of PGRs (Fig. 1). The best among tested concentrations of 2,4-D, 5 μM was used in combination of BA. A maximum response (90%) for callus formation was observed on MS medium supplemented with 5 μM 2,4-D and 1 μM BA (Fig. 2a). On medium with a higher concentration of 2,4-D, the frequency of callus formation was reduced to 58%. The combination of 2,4-D (5 μM) with BA above 1 μM reduced the frequency of callus formation progressively. The induced calli were further proliferated on the medium containing 2,4-D (5 μM) and BA (1 μM).

No organogenesis or SE occurred in the induced callus. The calli from 2,4-D and BA combination became

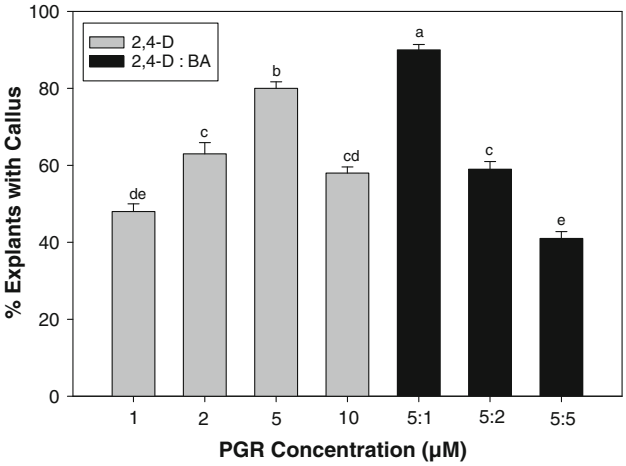

Fig. 1 Effect of PGRs on callus induction from hypocotyl explants of *P. marsupium* on MS medium, after 4 weeks of cultures (*bars* means ± SE). *Bars* denoted by the *same letter* are not significantly different ($P = 0.05$) by Tukey's test

embryogenic only after transfer to a medium supplemented with BA (0.5–5 μM) and 2iP (0.5–5 μM) and were characterized by yellowish to green in color, fast growth and friable to wet in consistency. Globular and heart-shaped somatic embryos appeared on the surface of calli within 2 weeks. The response for somatic embryo induction frequency varied on different concentrations of PGRs (Table 1). The highest frequency of somatic embryo induction was 62% on MS medium supplemented with 2 μM BA after 4 weeks. The induction frequency of somatic embryo formation was reduced to 26% on 5 μM BA. The frequency of somatic embryo formation from hypocotyl-derived callus on medium with 2iP was in the range of 9–52%. A maximum response was observed at 1 μM 2iP, while higher concentration reduced the induction. A maximum number of somatic embryos per callus clump (200 ± 20 mg) was recorded on MS medium supplemented with BA at 2 μM, after 4 weeks of culture (Fig. 2b).

Maturation of somatic embryos

The frequency of maturation of somatic embryos varied from 11 to 51% depending on the type of PGR or additives used (Table 2). A medium supplemented with ABA (10 μM), BA (0.5 μM) and NAA (0.1 μM) resulted in enhanced production of cotyledonary-stage embryo, on which 21 somatic embryos per culture was observed after 4 weeks. Immature somatic embryos enlarged and passed through the typical developmental stages, i.e. globular, heart, and torpedo stage (Fig. 2c, d, 3a, b) before developing to cotyledonary stage (Fig. 3c). Most of the mature somatic embryos had two cotyledons, but some of them had only one cotyledon or cotyledons differing in size and

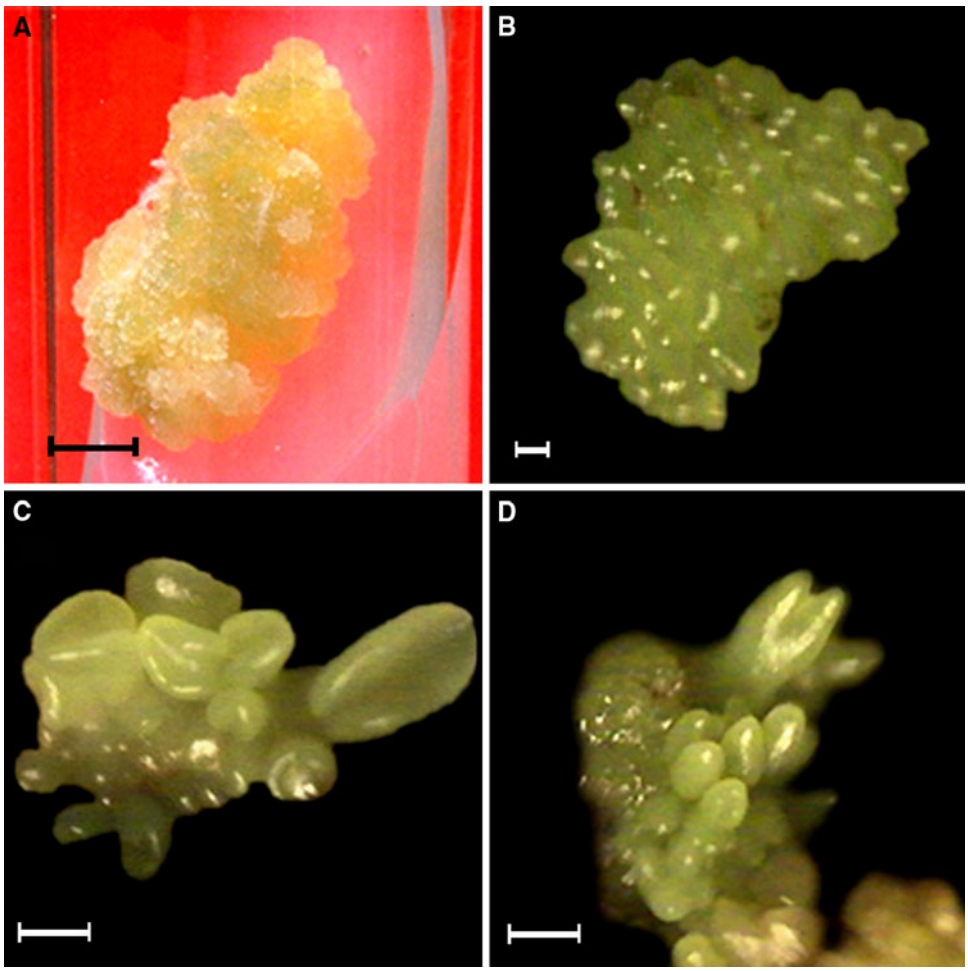

Fig. 2 Induction of indirect somatic embryogenesis in *Pterocarpus marsupium*. **a** *Light yellow* callus induced from hypocotyl explant on MS + 2,4-D (5 μM) + BA (1 μM) (*bar* 5 mm), **b** cluster of globular somatic embryos developed over the surface of callus on MS + BA (2 μM). (*bar* 1 mm). **c**, **d** Somatic embryos at the heart-shaped stage grown on MS + BA (0.5 μM) + NAA (0.1 μM) + ABA (10 μM) (*bar* 1 mm) (color figure online)

Table 1 Effect of PGRs on induction of somatic embryo from hypocotyl-callus (induced on BA + 2,4-D) of *P. marsupium* on MS medium, after 4 weeks of culture

Plant growth regulators (μM)		Induction frequencies of somatic embryos (%)	Average no. of somatic embryos/callus clump
BA	2iP		
0.5		18.0 ± 1.73^{de}	4.6 ± 0.71^{f}
1.0		20.0 ± 1.75^{d}	8.2 ± 0.57^{e}
2.0		62.0 ± 1.45^{a}	23.0 ± 0.88^{a}
5.0		26.0 ± 0.88^{c}	14.4 ± 0.14^{c}
	0.5	13.3 ± 0.66^{e}	9.1 ± 0.17^{e}
	1.0	52.3 ± 1.45^{b}	17.2 ± 0.19^{b}
	2.0	32.0 ± 0.57^{c}	12.1 ± 0.26^{d}
	5.0	9.0 ± 1.00^{f}	3.3 ± 0.23^{g}

Values represent means ± SE. Each replicate consisted of 200 ± 20 mg fresh mass of callus tissue. Means followed by the same letter within columns are not significantly different ($P = 0.05$) by Tukey's test

shape. ABA and AC alone also induced the maturation of somatic embryos, albeit not as many as the BA, NAA and ABA combination.

Germination of somatic embryos and conversion into plantlets

Somatic embryo started germinating within 2 weeks of culture on germination medium. The frequency of germination was 25% on MS0 and 41% on 1/2 MS0 medium. BA at 1.0 μM promoted the highest germination frequency (56%) and also the maximum average number (16.6) of germinated embryos that later converted into plantlets (Table 3).

Somatic embryos, which developed in groups, were difficult to separate as they were fused to each other. Such embryos showed recurrent embryogenesis, frequently from the plumular part of the embryo. Around 30% of somatic

Table 2 Maturation of somatic embryos on MS medium supplemented with different PGRs/additive, after 4 weeks of culture in *P. marsupium*

Treatments	Maturation (%)	Mean no. of somatic embryos[A]
MS (control)	40 ± 1.15[b]	12.9 ± 0.21[c]
BA (0.5 μM) + NAA (0.1 μM)	42 ± 1.19[b]	16.8 ± 0.37[b]
BA (0.5 μM) + NAA (0.2 μM)	35 ± 1.43[bc]	8.4 ± 0.29[d]
ABA (5 μM)	29 ± 1.45[cd]	6.2 ± 0.27[e]
ABA (10 μM)	43 ± 2.18[ab]	17.5 ± 0.37[b]
ABA (15 μM)	22 ± 1.35[d]	3.7 ± 0.24[f]
BA (0.5 μM) + NAA (0.1 μM) + ABA(10 μM)	51 ± 1.76[a]	21.4 ± 0.32[a]
AC (1 g l^{-1})	12 ± 1.21[e]	1.9 ± 0.11[g]
AC (2 g l^{-1})	13 ± 1.52[e]	2.6 ± 0.20[g]
AC (4 g l^{-1})	11 ± 1.11[e]	1.5 ± 0.25[g]

Values represent means ± SE. Means followed by the same letter within columns are not significantly different ($P = 0.05$) by Tukey's test

[A] Number of somatic embryos at early cotyledonary stage

embryos with recurrent embryogenesis did not germinate unless their plumular part (shoot apex) was excised and elongated on 1/2 MS medium supplemented with BA (2 μM) and NAA (0.1 μM) for 3 weeks. The elongated shoots rooted on 1/2 MS medium with PG (0.2 μM) and IBA (0.2 μM) and with 2% sucrose (Fig. 3d).

The plantlets were first transferred from test tubes to 1/4 MS liquid medium for 48 h followed by their acclimatization in soilrite mixture (Fig. 4) and finally to clay pots containing garden soil. Out of 90 transferred plants 54, i.e. nearly 60% survived under greenhouse conditions.

Discussion

The progress of SE in woody taxa of Fabaceae has not been achieved with the same pace as in the crop plants. Generally, there are two well-defined routes for SE in plants; somatic embryos can either differentiate directly from the explants without any intervening callus phase or indirectly after callus phase (Williams and Maheshwaran 1986). Moreover, SE can be induced from a wide range of tissues;

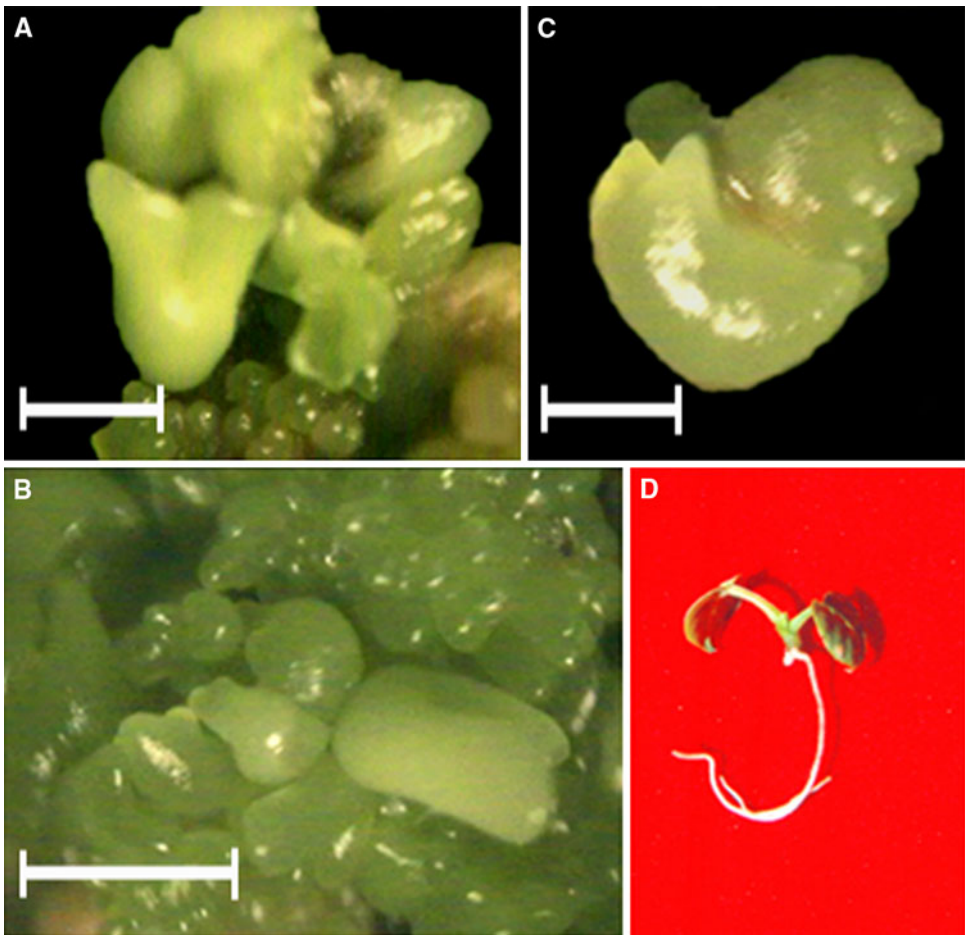

Fig. 3 Different stages of somatic embryogenesis and plantlet regeneration in *Pterocarpus marsupium*. **a**, **b** Differentiation of heart- and torpedo-shaped embryos on MS + BA (0.5 μM) + NAA (0.1 μM) + ABA (10 μM) (*bar* 1 mm). **c** Somatic embryo at the cotyledonary stage (*bar* 1 mm). **d** The elongated plumular part (shoot apex) of a somatic embryo rooted on 1/2 MS + IBA (0.2 μM) + PG (0.2 μM): conversion into a plantlet

Table 3 Germination and plantlet conversion of somatic embryos on MS medium (sucrose 2%) supplemented with different PGRs/additive, after 4 weeks of culture in *P. marsupium*

Treatments	Germination frequency (%)	Mean no. of somatic embryos germinated (plantlet conversion)
MSO	25 ± 1.73^{de}	7.4 ± 0.23^{e}
1/2 MSO	41 ± 2.00^{b}	12.1 ± 0.44^{c}
1/2 MS + BA (0.5 μM)	30 ± 1.02^{cd}	9.7 ± 0.28^{d}
1/2 MS + BA (1.0 μM)	56 ± 2.60^{a}	16.6 ± 0.33^{a}
1/2 MS + BA (2.0 μM)	38 ± 1.45^{bc}	12.2 ± 0.68^{c}
1/2 MS + BA (1.0 μM) +NAA (0.1 μM)	22 ± 1.53^{f}	6.8 ± 0.21^{e}
1/2 MS + BA (1.0 μM) + AC (2 g l^{-1})	41 ± 1.45^{b}	13.8 ± 0.37^{b}

Each treatment consisted of 30 somatic embryos, and experiment was repeated thrice. Values represent means ± SE. Means followed by the same letter within columns are not significantly different ($P = 0.05$) by Tukey's test

Fig. 4 Acclimatized plantlets of *P. marsupium*

however, immature/juvenile plant parts are generally reported to be more responsive. Hypocotyl segments, excised from axenic seedlings, have been used effectively by many workers to achieve indirect SE in several woody and herbaceous plant species (Sunnichan et al. 1998; Ashok Kumar et al. 2002; Junaid et al. 2006).

In *P. marsupium*, callus was induced from hypocotyl explants by the application of 2,4-D and BA either alone or in combination. The 2,4-D, an auxin analog used as herbicide has been shown to play signaling role in induction of SE in many plant systems (Nomura and Komamine 1995). In tree species like *Dalbergia sissoo* (Singh and Chand 2003) and *Buchanania lanzan* (Sharma et al. 2005), 2,4-D in combination with cytokinin (BA/KIN) has induced embryogenic callus that later differentiated into somatic embryos. In the present study, the callus was produced either in the presence of auxin (2,4-D) or auxin and cytokinin (2,4-D:BA) combinations, but the induced callus remained nonembryogenic until it was subcultured to a medium with a cytokinin (BA or 2iP), where the callus became embryogenic and produced somatic embryos. The continuous presence of 2,4-D was detrimental to SE. Removal of auxin from the culture is considered to be essential for the inactivation of several genes or for the synthesis of new gene-products necessary for embryo development (Zimmerman 1993).

On maturation medium, majority of somatic embryos developed to the cotyledonary stage; however, a few had one to several cotyledons. This type of abnormality has been well documented for several other tree species including *Sesbania sesban* (Shahana and Gupta 2002) and *D. sissoo* (Singh and Chand 2003).

In some cases maturation of somatic embryos was promoted by ABA, a growth inhibitor/retardant, which creates stress conditions that are conducive to development and maturation of somatic embryos of several plant species (Husain 2007). In *Pterocarpus*, a combination of ABA at 10 μM with BA and NAA resulted in enhanced production of cotyledonary-stage embryo as compared with ABA alone. It seems that ABA acted synergistically with auxin and cytokinin and promoted a higher number of somatic embryos maturation. Similar type of response has been reported by Sahrawat and Chand (2001) in *Psoralea corylifolia*.

In the present study, the highest germination rate of somatic embryos occurred in the presence of BA (1.0 μM) only. It appears that cytokinins such as BA and kinetin have certain regulatory functions during germination and conversion. This agrees with earlier reports that this group of PGRs has a positive effect on embryo germination in woody plant taxa including *Acer palmatum* (Vlasinova and Havel 1999) and *Paulownia elonagta* (Ipecki and Gozukirmizi 2003).

In conclusion, the present investigation clearly indicates that seedling explants (hypocotyls) of *Pterocarpus marsupium* are a suitable source of tissue for somatic embryo

induction and plantlet regeneration. Attempts are continuing to induce SE from the explants taken from adult trees.

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