Physiol Mol Biol Plants (March–April 2019) 25(2):569–579
https://doi.org/10.1007/s12298-018-0602-8

RESEARCH ARTICLE

# Assessment of genetic stability in somatic embryo derived plantlets of *Pterocarpus marsupium* Roxb. using inter-simple sequence repeat analysis

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Received: 21 July 2017 / Revised: 10 July 2018 / Accepted: 6 September 2018 / Published online: 16 November 2018


**Abstract** An efficient plantlet regeneration protocol using immature zygotic embryos (IZEs) via somatic embryogenesis has been developed in *Pterocarpus marsupium* Roxb. The regenerated plantlets were evaluated for their genetic stability. IZEs were incubated on Murashige and Skoog (MS) media augmented with 1.07–16.11 μM naphthalene acetic acid (NAA) or 0.90–13.97 μM 2,4-dichlorophenoxyacetic acid. The optimum callus induction (96.6%) was observed on MS medium augmented with 5.37 μM NAA. Induction of somatic embryos (SEs) was observed after subculture of calli on medium with decreased concentrations of NAA (0.54–5.37 μM), either alone or 2.69 μM NAA in combination with 2.22–8.90 μM benzyladenine (BA) or 2.32–9.30 μM Kinetin. Maximum number (33.4 ± 0.85) of SEs occurred on MS medium augmented with 2.69 μM NAA + 4.40 μM BA + 3% sucrose. Highest percentage (67.3 ± 0.37) of SEs matured and developed into cotyledonary stage by subsequent subculture on the same medium. SE formation and maturation decreased when sucrose concentrations were higher than 3%. Seventy percent of mature somatic embryos developed into plantlets on half strength MS medium augmented with 5.80 μM gibberellic acid. The various stages of development during somatic embryogenesis include globular, heart, torpedo and mature stages as revealed by the stereomicroscopic and histological studies of explants. Plantlets derived from SEs were successfully acclimatized in the greenhouse with a survival rate of 78%. Among the survived plantlets, 9 plantlets were randomly selected for inter-simple sequence repeat (ISSR) analysis. Of the 13 primers used, 8 produced reproducible and monomorphic bands. ISSR analysis revealed a homogenous amplification profile for all regenerated plantlets analyzed validating the genetic stability of somatic embryo derived plantlets.

**Keywords** Indian Kino tree · Immature zygotic embryos · Somatic embryogenesis · Plantlet conversion · Genetic stability

**Abbreviations**

| | |
|---|---|
| MS | Murashige and Skoog |
| PGR | Plant growth regulators |
| BA | 6-Benzyladenine |
| $GA_3$ | Gibberellic acid |
| Kn | Kinetin |
| NAA | Naphthaleneacetic acid |
| IZE | Immature zygotic embryo |
| SE | Somatic embryo |
| ISSR | Inter-simple sequence repeats |

## Introduction

The development of an efficient plant regeneration system via somatic embryogenesis is one of the main requirement for the potential applications of propagation, genetic

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s12298-018-0602-8) contains supplementary material, which is available to authorized users.

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manipulation and germplasm preservation of trees species under in vitro conditions (Anjaneyulu and Giri 2008). *Pterocarpus marsupium* Roxb. (Fabaceae) is one of the most valuable multipurpose tree, commonly known as Indian Kino tree or Malabar Kino. In India, it is found in hilly regions throughout the Deccan Peninsula, Gujarat, Madhya Pradesh, Uttar Pradesh, Bihar, Odisha, Telangana & Andhra Pradesh and in other countries like Nepal and Srilanka. *P. marsupium* is an economically important timber-yielding tree valued for construction and ornamental wood works. As per ayurveda, *P. marsupium* is one of the most versatile plant with a wide spectrum of biological activities and all parts possess medicinal value (Rizvi and Mishra 2013). Traditionally, the heartwood is the potential source of drugs (major bioactive constituents are polyphenols) for treatment of diabetes, diarrhoea, asthma, bronchitis, skin disease, leprosy, elephantiasis and jaundice (Kirtikar and Basu 2012). The modern pharmacology research has indicated that *P. marsupium* possess a wide variety of activities, including anti-hyperglycemic (Manickam et al. 1997), anti-proliferative (Chakraborty et al. 2010), dipeptidyl peptidase-4 inhibition (Kosaraju et al. 2014). Our recent studies on this plant species have reported antioxidant, analgesic (Radhika et al. 2010), anti-proliferative, anti-mitotic and anti-telomerase activities (Radhika et al. 2013a, b).

Indiscriminate felling of *P. marsupium* from the natural habitat by commercial operators and little efforts made for its large scale cultivation by R&D centers and universities, has resulted its inclusion in the list of vulnerable plant species (VU A1cd v2.3) (World Conservation Monitoring Centre 1998, IUCN Red List of Threatened Species. Version 2006). Conventional propagation of this tree is quite difficult, because of its hard seed coat, poor seed viability and low rate of germination (Anis et al. 2005). Propagation through seed is unreliable as seeds are often damaged by pod borers (Fig. 1) and secondary infections are also caused by bacteria and fungi (Radhika et al. 2013a, b). Thus, an efficient in vitro regeneration system may play a vital role in rapid propagation and conservation of this medicinally important tree.

Plant regeneration efficiency depends on the availability of totipotent cells in the explant. However, legume tree species are in general highly recalcitrant to somatic embryogenesis (Rutledge et al. 2013). So, an explant source with meristematic cells such as immature zygotic embryos (IZEs) may be an ideal explant for plantlet regeneration (Yang et al. 2012). Several regeneration attempts have been made in *P. marsupium* (Chand and Singh 2004; Tiwari et al. 2004; Anis et al. 2005; Husain et al. 2008); only a single study reported plantlet regenerated from hypocotyl derived callus via somatic embryogenesis, wherein 51% somatic embryos (SEs) matured and 56% of the SEs were converted into plantlets (Husain et al. 2010).

In the present investigation, an efficient plantlet regeneration protocol was developed from IZEs explant callus via somatic embryogenesis and the genetic stability of the regenerated plantlets was assessed using ISSR markers in *P. marsupium.*

## Materials and methods

### Plant materials and explant preparation

Immature zygotic embryos (IZEs) of *P. marsupium* were used as the source material for callus induction. Immature green pods were collected from the 27 years old tree growing (height-16.4 m; cbh-106 cm) in the Kakatiya University campus at Warangal, Telangana, India. For somatic embryogenesis, green pods were collected at about 7 day's interval from Dec 22nd to Jan 25th. They were washed thoroughly under running tap water for 15 min and then washed with 2% (w/v) detergent solution (Teepol) for 10 min followed by Tween20 for 4 min. Further sterilization was done with freshly prepared 0.1% (w/v) aqueous mercuric chloride solution ($HgCl_2$) for 6 min. with constant shaking. The green pods were then rinsed 4–5 times with sterile distilled water and dried on sterile tissue paper. All the explants were collected from the same donor plant in the present investigation.

### Experimental conditions

Murashige and Skoog medium (MS) (1962) containing 3% sucrose w/v (HiMedia; Mumbai, India), 0.8% w/v agar (HiMedia; Mumbai, India) and plant growth regulators at different concentrations was used in all experiments. The pH of the medium was adjusted to 5.8 with 1 N NaOH before autoclaving at 121 °C for 15 min. The medium was dispensed into culture tubes and plugged with non-absorbent cotton plug. All the cultures were maintained at 25 ± 2 °C with 16 h photoperiod under 10–15 µmol $m^{-2}$ $s^{-1}$ light intensity provided by cool white fluorescent tube lights (Philips Electronics India Ltd.) and 55–60% relative humidity.

### Induction of callus

For callus induction, the green pods collected on different dates (Dec, 22nd, 29th, Jan, 4th, 11th, 18th, 25th) were opened aseptically and IZEs were inoculated on MS medium supplemented with different concentrations of α-naphthalene acetic acid (NAA; 1.07, 2.69, 5.37, 10.74, 16.11 µM) or 2,4-dichlorophenoxyacetic acid (2,4-D; 0.90,

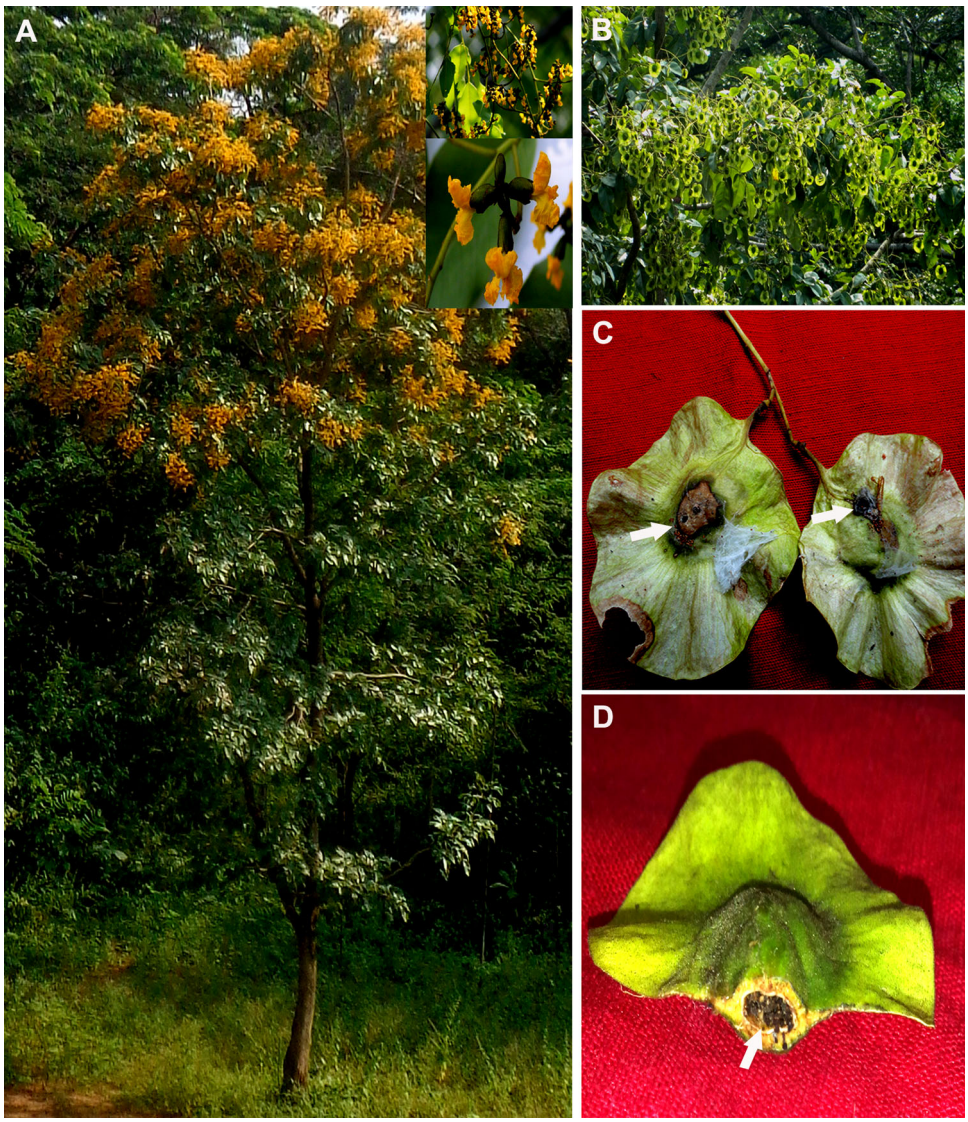


**Fig. 1** **a**, **b** *Pterocarpus marsupium* tree growing in the Kakatiya University research field, Warangal, Telangana, India on which flowers and green pods were produced, **c**, **d** borer infected seeds (arrows)

2.26, 4.52, 9.05 13.97 μM). Explants forming calli were recorded after 4 weeks of culture. Callus was sub-cultured at 3 weeks intervals.

### Induction of somatic embryos

For somatic embryos (SEs) induction, the calli were transferred to MS medium supplemented with reduced concentrations of NAA (0.54, 1.07, 2.69, 5.37 μM) either alone or 2.69 μM NAA in combination with benzyladenine (BA; 2.22, 4.40, 8.90 μM) or Kinetin (Kn; 2.32, 4.60, 9.30 μM). Data on number of cultures showing SEs and number of SEs per culture were scored using a stereomicroscope (Nikon, Japan) after 6 weeks of transfer.

### Maturation of somatic embryos

Somatic embryos obtained on induction medium were sub-cultured on MS medium supplemented with NAA (2.65 μM) and BA (4.40 μM) for maturation. Further investigations were carried out to determine the effect of sucrose on embryo maturation. Various concentrations of sucrose (1%, 2%, 3%, 4%) was added to MS medium supplemented with 2.65 μM NAA and 4.40 μM BA. The number of early cotyledonary stage embryos were counted using a stereomicroscope after 4 weeks of culture.

### Somatic embryos conversion to plantlets

Cotyledonary embryos were individually transferred to MS basal medium, ¼ MS basal medium and ½ MS basal medium supplemented with three different concentrations

of $GA_3$ (2.90, 5.80, 11.60 μM). Germination and percentage of conversion of SEs into plantlets was scored after 4 weeks of culture.

### Microscopic observations

The cultured materials were examined under a stereomicroscope (Nikon, Japan) and photographed using camera (Nikon, Japan). For histological studies, explants were sampled at different time intervals. Five explants were sampled for each period. The materials sampled for histological studies were fixed in formaldehyde/glacial acetic acid/ethanol (FAA, 5:5:90, v/v/v) for 1 day, dehydrated through a graded tertiary butyl alcohol (TBA) series, each for 1 day and embedded in a saturated paraffin wax. Embedded materials were sectioned at 5 μm thickness on a rotary microtome and mounted on glass slides. Paraffin wax was removed by xylene prior to rehydration of the tissues in a graded ethanol series and the tissue was stained with 1.0% (w/v) safranin. Tissues were washed briefly in water to remove excess stain and then dehydrated in a graded ethanol series. The slides were examined under a light microscope (Nikon, Japan) and photographed using camera (Nikon, Japan).

### Transplantation and acclimatization of plantlets

Plantlets were washed thoroughly with water before transplanting into paper cups containing mixture of sterilized sand and garden soil (1:1) and irrigated with 1/10 MS liquid medium and they were subsequently transferred to clay pots containing garden soil, sand, compost (2:1:1) and established in green house (temperature 25 ± 2 °C; relative humidity 85 ± 5%; light-natural light diffuse) for 9 months.

### Genetic stability of plantlets using ISSR marker

Genetic stability of in vitro regenerated plantlets and mother plant was tested using PCR-based ISSR analysis. For this purpose, total genomic DNA was isolated from the leaves of mother plant and randomly chosen in vitro regenerated plantlets using the modified CTAB method (Doyle and Doyle 1990). DNA quality was assessed by gel electrophoresis (Owl Scientific, Germany).

After preliminary screening of ISSR primers (Bioserve, Hyderabad, India), eight primers were selected for analyzing genetic stability of in vitro regenerated plantlets. DNA amplification was carried out using DNA Thermal Cycler (Bio-Rad, USA). PCR was carried out in a 25 μL reaction volume containing 2.0 μL of 1.25 mM each of dNTP's, 1 μL of the primer, 1 × PCR buffer (Genei, India), 0.5 U of Taq DNA polymerase (Genei, India) and 2 μL (30 ng) of genomic DNA. DNA amplification was initiated by denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, 1 min annealing at 37 °C and 2 min extension at 72 °C, followed by one final extension at 72 °C for 10 min. PCR products were resolved by electrophoresis on 1.2% (w/v) agarose (Sigma, USA) gel in Tris–Borate EDTA (TBE) buffer, stained with ethidium bromide and image was taken under Gel Documentation system (Bio-Rad, USA).

### Statistical analysis

In this study, each experiment was repeated four times and each replicate contained 12 cultures. All the data were subjected to one way ANOVA followed by the statistical significance test. The significant difference among the mean ± standard error was carried out using Duncan's Multiple range test (DMRT) at significance level of $P < 0.05$.

## Results

### Induction of callus

IZEs showed no response on MS basal medium even after 4 weeks of culture. The percentage of callus induction and type of callus varied depending on date of collection of explant and concentrations of NAA and 2,4-D used. IZEs were incubated on MS medium augmented with NAA (1.07–16.11 μM) or 2,4-D (0.90–13.97 μM). The optimum callus induction (96.6%) was observed on MS medium augmented with 5.37 μM NAA from third collection date of IZEs (Jan 4th) (Table 1; Fig. 2a). For further studies, this callus was used. When the IZEs were cultured on 2,4-D containing medium, it resulted in poor callusing and somatic embryos or adventitive organs were not observed even after 9 weeks of sub-culture.

### Induction of somatic embryo

When 4 weeks old callus was transferred to a medium containing reduced concentration of NAA (0.54–5.37 μM) alone or NAA (2.69 μM) in combination with BA (2.22–8.90 μM) or Kn (2.32–9.30 μM), embryogenic callus was developed. After 1 week of sub-culture, globular embryo like structures developed on the surface of callus and as the time progressed, the globular (Fig. 2b–d) and heart-shaped (Fig. 2e) somatic embryos developed into torpedo-shaped embroys (Fig. 2f, g). Of different concentrations tested, 2.69 μM NAA and 4.40 μM BA were found most effective, and an optimum number (33.4 ± 0.85) of embryos with highest (79.1%) percentage of embryo

**Table 1** Effect of various concentrations of NAA and 2,4-D on callus induction from IZE explants of *P. marsupium* after 4 weeks of culture

| NAA (μM) | 2,4-D (μM) | Explants forming callus (%) |
|---|---|---|
| 0 | | 0 |
| 1.07 | | 21.6 ± 0.82[b] |
| 2.69 | | 35.0 ± 1.08[c] |
| 5.37 | | 96.6 ± 0.82[e] |
| 10.74 | | 73.3 ± 0.70[e] |
| 16.11 | | 56.6 ± 0.49[d] |
| | 0.90 | 13.3 ± 1.21[a] |
| | 2.26 | 23.3 ± 0.49[b] |
| | 4.52 | 40.0 ± 0.99[c] |
| | 9.05 | 51.6 ± 1.46[d] |
| | 13.97 | 35.0 ± 0.82[c] |

The values present the mean ± SE of four independent experiments. At least 12 cultures were raised for each experiment

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

induction per callus was obtained (Table 2). Medium supplemented with 2.69 μM NAA and 4.60 μM Kn induced less number (19.0 ± 0.51) of embryos in 58.3% cultures. Only 35% of cultures showed SE induction on MS medium supplemented with 2.69 μM NAA alone with an average of 14.9 embryos per culture.

### Maturation of somatic embryos

On continuous exposure of somatic embryos to 2.69 μM NAA and 4.40 μM BA medium with 3% sucrose, torpedo shaped embryos developed to maturity (cotyledonary stage) (Fig. 2h, i). The effect of sucrose concentrations on embryo maturation was also evaluated. The percentage of embryo maturation varied from 19.5 to 67.3 depending on the concentration of sucrose. Maximum percent of embryo maturation (67.3) was noticed on MS medium supplemented with 3% sucrose and 2.69 μM NAA and 4.40 μM BA (Table 3). At 4% sucrose concentration, 37.16% of maturation & 5.8 somatic embryos with cotyledons were produced. Least number of somatic embryos with cotyledons (1.8) and lowest percentage of maturation (19.50) were observed in 1% sucrose concentration. Occasionally abnormal somatic embryos with multiple cotyledons were also observed in this study (Fig. 2j).

### Somatic embryo conversion into plantlets

The SEs converted into plantlets when cultured on MS basal medium, ¼ MS basal medium and ½ MS basal medium, with or without plant growth regulators, within 4 weeks of culture (Fig. 2k–m). The presence of shoot and root pole were the distinguishing feature of the germinating the embryo (Fig. 2n). The conversion percentage varied from 22 to 70%, depending on the medium composition and growth regulator concentrations used. The best result was obtained on ½ MS basal medium augmented with 5.80 μM $GA_3$ with highest (70.0 ± 1.72) percentage of SEs into plantlets (Table 4). The same basal medium, when supplemented with higher concentration (11.6 μM) of $GA_3$, showed reduced percentage of conversion (46.6 ± 1.59). The number of plantlets produced through this method and green house survival is approximately 78% (Fig. 2o, p). All the regenerated plantlets showed normal leaf development and were morphologically similar to the mother plant.

### Microscopic observations

Histological examination showed that somatic embryos originated from surface of the callus or internal cells of callus and their development passed through the globular-shaped embryo (Fig. 3a, b) heart-shaped embryo (Fig. 3c) and torpedo-shaped embryo (Fig. 3d) with shoot and root poles and embryo with folded cotyledons (Fig. 3e) and subsequently developed into whole plantlet.

### Genetic stability: ISSR analysis

In the present study, DNA of 9 randomly selected in vitro regenerated plantlets and mother plant was subjected to ISSR analysis to assess the genetic stability. A total of 13 ISSR primers were used for primary screening, but only 8 primers generated clear, distinct and scorable bands with an average of 4.75 bands per primer and a total of 38 bands. Each primer generated monomorphic banding patterns with size ranging from 300 to 2000 bp. The number of scorable bands from each ISSR primer varied from 2 (ISSR 7) to 7 (ISSR 4). The number of scorable bands for each ISSR primer has been presented in Table 5. ISSR profiles obtained with primer 8 and 6 are shown in Fig. 4a, b, respectively. This analysis clearly indicated that there was no DNA polymorphism and all the regenerated plantlets via somatic embryogenesis are genetically identical to mother plant.

## Discussion

In this study, efficient plant regeneration system via somatic embryogenesis from immature zygotic embryo (IZE) derived callus explants was achieved in *P. marsupium* and analyzed for genetic stability of regenerated plantlets using ISSR markers. To date, there is no report

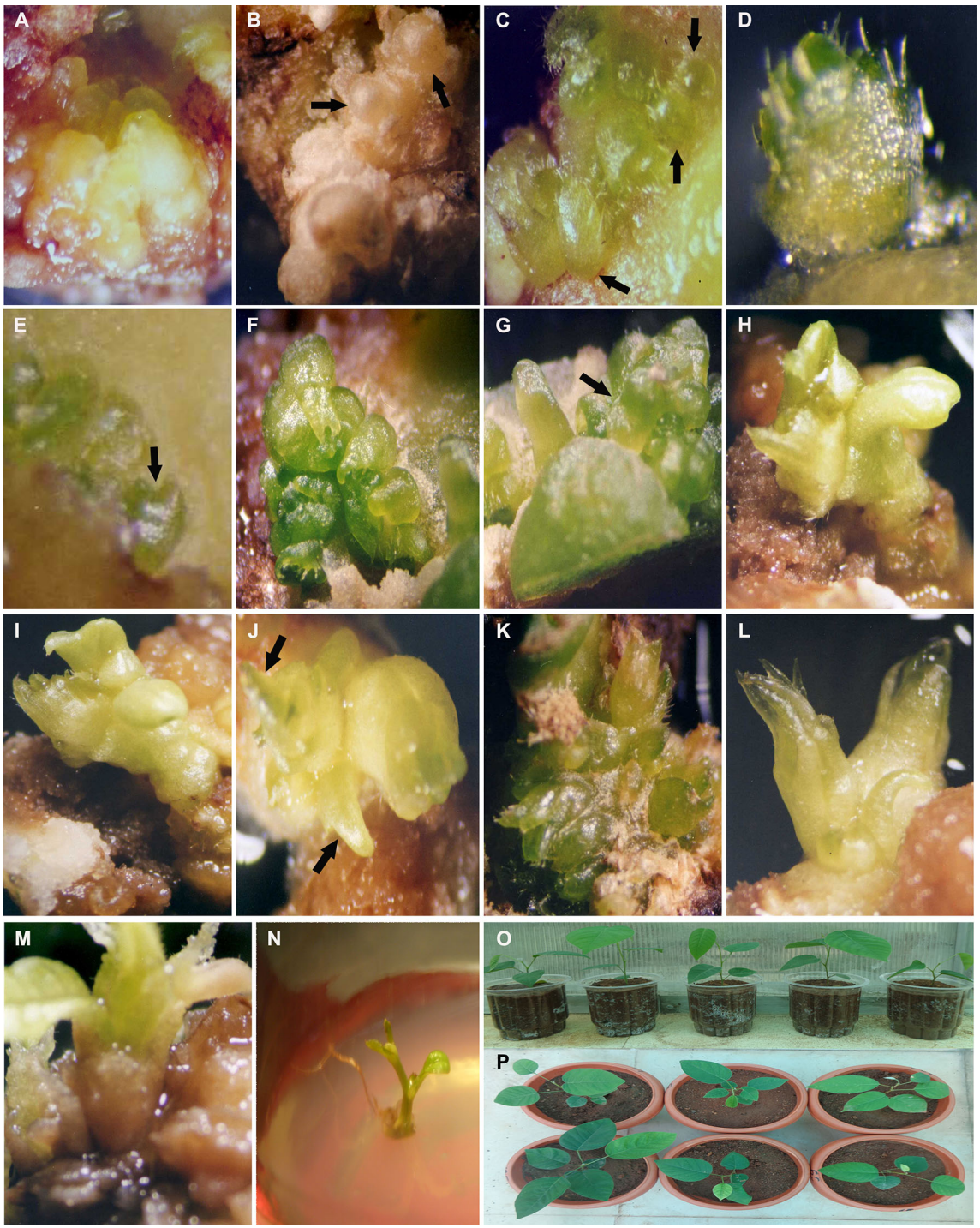


**Fig. 2** Plant regeneration from immature zygotic embryo derived calluses of *P. marsupium* via somatic embryogenesis. **a** Proliferation of embryogenic callus on MS medium containing 5.37 µM NAA, **b** clear view of somatic embryo development from the callus surface on MS medium containing 2.69 µM NAA and 4.40 µM BA (arrows), **c**, **d** globular-shaped somatic embryos (arrows), **e** heart-shaped somatic embryo with notch (arrow), **f** intermediate stage between heart and torpedo shaped, **g** maturation of somatic embryos, globular, short (arrow) and elongated torpedo shaped, **h** cotyledonary stage somatic embryo with two cotyledons (arrows), **i** abnormal somatic embryo with multiple cotyledons (arrows), **j–l** conversion of somatic embryos into plantlets upon culture on ½ MS medium supplemented with 5.80 µM $GA_3$, **m** Four weeks old embryo germinated plantlet in culture media, **n**, **o** somatic embryos derived plantlets growing in paper cups (4 months old)/clay pots (9 months old) under greenhouse conditions

**Table 2** Effect of different plant growth regulators (μM) upon the somatic embryogenesis from the IZE callus of *P. marsupium* on MS basal medium containing 3% sucrose and 0.8% agar

| NAA | BA | Kn | Embryo induction (%) | No. of embryos/explant |
|---|---|---|---|---|
| 0 | | | 0 | 0 |
| 0.54 | | | 10.4 | 3.8 ± 0.32[a] |
| 1.07 | | | 16.6 | 5.6 ± 0.25[a] |
| 2.69 | | | 35.4 | 14.9 ± 0.40[b] |
| 5.37 | | | 20.8 | 7.1 ± 0.36[b] |
| 2.69 | 2.22 | | 43.7 | 17.2 ± 0.37[c] |
| 2.69 | 4.40 | | 79.1 | 33.4 ± 0.85[e] |
| 2.69 | 8.90 | | 66.6 | 22.1 ± 0.57[d] |
| 2.69 | | 2.32 | 37.5 | 11.6 ± 0.66[b] |
| 2.69 | | 4.6 | 58.3 | 19.0 ± 0.51[c] |
| 2.69 | | 9.3 | 33.3 | 16.2 ± 0.63[c] |

The values present the mean ± SE of four independent experiments. At least 12 cultures were raised for each experiment

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

**Table 3** Effect of various concentrations of sucrose on maturation of somatic embryos in *P. marsupium* callus cultures (MS + 2.69 μM NAA + 4.40 μM BA)

| Sucrose (%) | Globular and heart | Torpedo | Cotyledonary | Maturation (%) |
|---|---|---|---|---|
| 1 | 9.3 ± 0.39[a] | 5.2 ± 0.51[a] | 1.8 ± 0.17[a] | 19.5 ± 1.20[a] |
| 2 | 18.2 ± 0.12[b] | 12.5 ± 0.18[b] | 8.2 ± 0.23[c] | 45.0 ± 1.02[c] |
| 3 | 33.6 ± 0.20[c] | 26.9 ± 0.12[c] | 22.6 ± 0.22[d] | 67.3 ± 0.37[c] |
| 4 | 15.8 ± 0.41[a] | 10.1 ± 0.43[b] | 5.8 ± 0.12[b] | 37.1 ± 0.97[b] |

The values present the mean ± SE of four independent experiments. At least 12 cultures were raised for each experiment

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

**Table 4** Germination and plantlet conversion frequency of somatic embryos on different strengths of MS medium supplemented with different concentrations of $GA_3$ in *P. marsupium* after 4 week of culture

| PGRs (μM) | Number of embryos cultured | Mean number of embryos germinated | Conversion (%) |
|---|---|---|---|
| MSO | 40 | 9.3 | 23.3 ± 0.82[a] |
| ½ MS | 40 | 16.6 | 41.6 ± 1.38[c] |
| ¼ MS | 40 | 12.0 | 30.0 ± 1.18[b] |
| ½ MS + $GA_3$ (2.90) | 40 | 20.0 | 50.0 ± 1.97[d] |
| ½ MS + $GA_3$ (5.80) | 40 | 28.0 | 70.0 ± 1.72[d] |
| ½ MS + $GA_3$ (11.60) | 40 | 18.6 | 46.6 ± 1.59[c] |

*PGRs* plant growth regulators

The values present the mean ± SE of four independent experiments. At least 12 cultures were raised for each experiment

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

describing in detail the study of somatic embryogenesis in this species using IZE explants. The immature zygotic embryo explant is ideal for culture initiation and multiplication under in vitro conditions (Chaturani et al. 2006). In recent years, there are several reports on the use of IZE explants in somatic embryogenesis of tree species (Lara-Chavez et al. 2011; Yang et al. 2012).

Plant growth regulators are an important factor in inducing unorganized callus or polarized growth which leads to somatic embryogenesis (Arnold et al. 2002). Plant growth regulator concentration, combination and explant

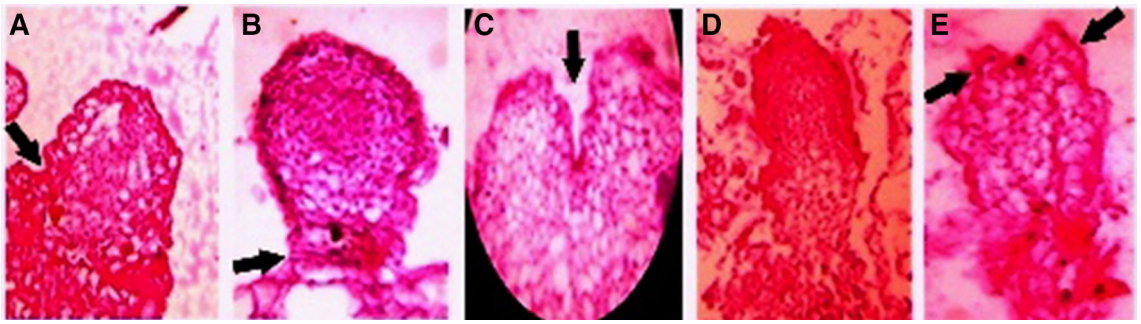


**Fig. 3** Histological observations of somatic embryogenesis in callus of *P. marsupium*. **a** Somatic embryo at early globular-shape, **b** globular-shaped embryo with suspensor (arrow), **c** heart-shaped somatic embryo with a notch (arrow), **d** torpedo-shaped somatic embryo, **e** cotyledonary stage somatic embryo with two cotyledons (arrows)

**Table 5** ISSR primers used for assessment of genetic stability somatic embryo derived plantlets and mother plant of *P. marsupium*

| S. no. | Primer sequence (5′–3′) | Total no. of amplified products |
|---|---|---|
| 1 | AGAGAGAGAGAGAGAGC | 5 |
| 2 | GAGAGAGAGAGAGAGAT | 6 |
| 3 | TCTCTCTCTCTCTCTCC | 4 |
| 4 | TCTCTCTCTCTCTCTCG | 7 |
| 5 | TCTCTCTCTCTCTCTCRG | 3 |
| 6 | GATAGATAGATAGATA | 5 |
| 7 | GCCGAGAGAGAGAGA | 2 |
| 8 | ACTGCTAGAGAGAGAGAG | 6 |

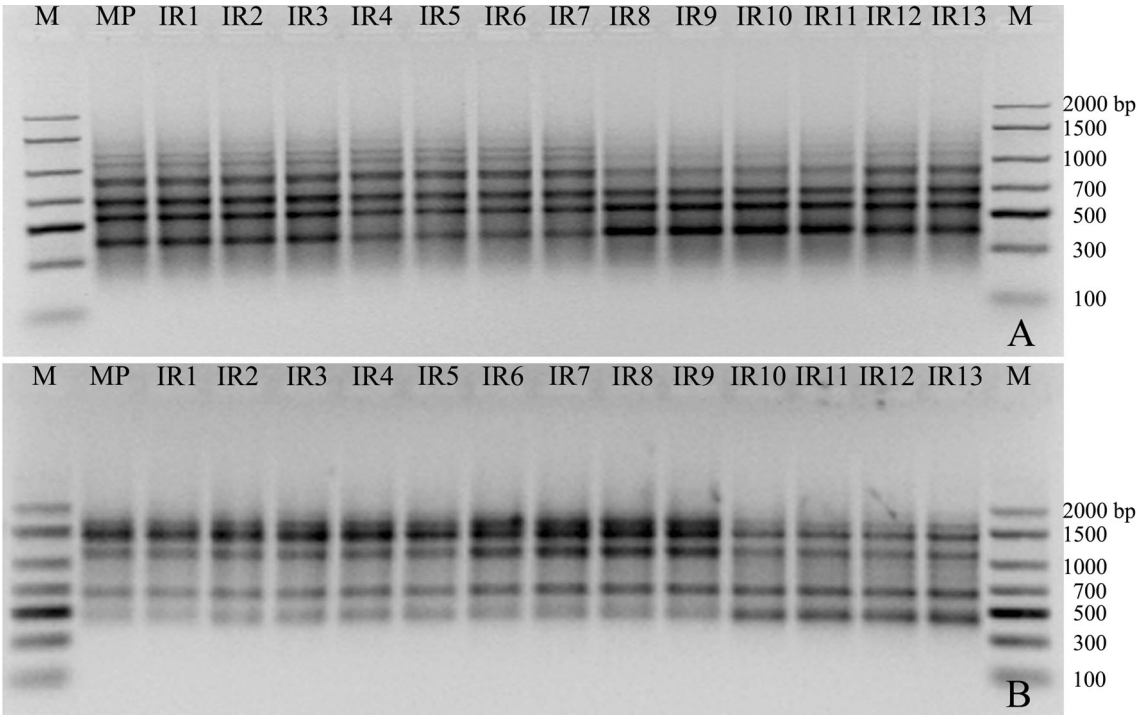


**Fig. 4** Assessment of genetic stability of in vitro regenerated plants of *P. marsupium* with their mother plant. **a**, **b** ISSR amplification profile with primers 8 and 6. lanes, *M* marker, *MP* mother plant, *IR1–IR12* in vitro regenerated plantlets, *IR13* acclimatized plantlet

type influenced callus induction and somatic embryogenesis. In recent years, 2,4-D has been used for the more potent and effective callus induction in many tree species (Husain et al. 2010; Naz et al. 2016). However, in the present investigation, MS medium supplemented with NAA responded better than 2,4-D for callus induction and

2,4-D was not a prerequisite for embryo induction. Similar observations were also noticed in *Simarouba glauca* (Das 2011).

When calli obtained from NAA containing media were subsequently transferred on to the NAA alone (0.54–5.37 μM) or reduced concentration of NAA (2.69 μM) in combination with BA (2.22–8.90 μM) or Kn (2.32–9.30 μM), SEs were induced. Medium augmented with 2,4-D resulted in less callusing. The combination of NAA and BA has proven to be more potent and effective for SE induction in many tree species (Thengane et al. 2006; Yang et al. 2012). Cytokinin type and concentration had significant effect on the induction of embryos and subsequent maturation. In the present study, NAA and BA combination was more effective than NAA alone or NAA and Kn, as only a few embryos were observed on MS medium augmented with NAA alone or NAA and Kn. The highest number of matured embryos were recovered in NAA (2.69 μM) and BA (4.40 μM) containing media. It is well documented that combination of auxin and cytokinin in the culture media regulate various aspects of plant cell dedifferentiation and differentiation (Woodward and Bartel 2005; Sakakibara 2006). For callus induction and proliferation, usually auxins are used and for redifferentiation of callus into organized cell, both auxin and cytokinins are used (Wang et al. 2008).

To study the effect of sucrose on SE maturation, the embryos were subcultured on MS medium supplemented with NAA (2.69 μM) and BA (4.40 μM) and varying concentrations (1–4%) of sucrose. Highest percentage of SE maturation was observed at 3% sucrose. There was a reduction in the maturation of SEs above 3% sucrose. Similar observations were noticed by Kim et al. (2007) in *Magnolia obovata* Thunberg tree, where 3% of sucrose was optimum for embryo maturation. This work indicated that NAA and BA was not only used for SE induction but also used for embryo maturation.

In the present study, globular embryos developed into heart, torpedo and majority to cotyledonary stages. Great variability was found in their morphology. Occasionally, a few abnormal SEs with several cotyledons were observed in the present study. Similar type of abnormality has been reported in other tree species, *Dalbergia sissoo* (Singh and Chand 2003), *Fraxinus mandshurica* (Yang et al. 2013).

When the propagation is based on somatic embryogenesis, it is important to choose the explant with high regeneration capacity (Prakash and Gurumurthi 2010). For the induction of somatic embryogenesis in several tree species, the collection date, age and developmental stage of the explant is critical (Mauri and Manzanera 2004). In the present study, collection date had a great effect on callus induction and somatic embryogenesis. IZEs that were collected from the beginning of Jan (4th) yielded a highest frequency of embryo induction with maximum number of SEs than other collection dates (supplementary data). These results were consistent with reports in other tree species like *Manchurian ash* (Kong et al. 2012), *Cyathea delgadii* (Mikuła et al. 2015), where collection date and age of the explant play major role in the induction of somatic embryogenesis.

Conversion of SEs into plantlets is a major problem in tree species (Corredoira et al. 2015). Maximum number of embryo germination (root and shoot formation) was observed when cotyledonary embryos were transferred to ½ MS medium containing different concentrations of $GA_3$. Conversion percentage of SEs was maximum (70%) on ½ MS medium containing $GA_3$ (5.80 μM). The embryo germination was enhanced and root initiation and conversion of embryos to complete plantlets was increased by the addition of $GA_3$. The requirement of $GA_3$ to trigger germination of SE has also been reported in *Magnolia obovata* Thunberg (Kim et al. 2007), *Albizia lebbeck* (Saeed and Shahzad 2015). In the present study, germination with enhanced root formation observed may be due to the endogenous level of plant growth regulators.

Heterogeneity in tissue cultured plantlets can seriously limit the purpose of in vitro regeneration system. Therefore, it is advisable to test the genetic stability of in vitro regenerated plantlets. Molecular markers are more reliable than phenotypic observations for identification of genetic uniformity. In the present investigation, genetic stability of in vitro regenerated plantlets was assessed by PCR based ISSR markers. The utility of this marker, which amplifies diverse regions of the genome, allows better chances for the detection of genetic changes in the regenerated plantlets (Martin et al. 2004). Molecular markers, mainly ISSR, have been widely used for identifying the genetic stability among in vitro propagated plantlets in majority of tree species (Siragusa et al. 2007; Rai et al. 2012). However, our results clearly confirmed that all the in vitro raised plantlets showed genetic stability. It is apparent that SEs derived from *P. marsupium* plants are true to type. The markers are useful for identifying developing clonal lines and could be useful for long-term quality control of regenerated plantlets of *P. marsupium.*

## Conclusion

*Pterocarpus marsupium* has a long juvenile phase of 20 years for attaining maturity. Poor seed germination, attack by fungi, sap suckers and wood borer, incidence of seeds/seedlings damage due to forest fires, low rate of propagation through stem cuttings has resulted in inclusion in the list of vulnerable plant species of IUCN red data book. In this paper, we developed a protocol for

regeneration of plantlets from callus of IZEs and 78% of *P. marsupium* plantlets survived in green house conditions. The markers were useful for developing clonal lines and could be useful for long-term quality control of regenerated plantlets of *P. marsupium.* Therefore, the above said protocol is useful for conservation of the vulnerable medicinal plant, *P. marsupium*.

**Acknowledgements** T. Radhika is thankful to Jawaharlal Nehru Memorial Fund, New Delhi, India for financial support (Ref: SU-A/180/2011-12/404). The authors gratefully acknowledge Prof. A. Sadanandam, Department of Biotechnology, Kakatiya University, Warangal, Telangana, India for providing ISSR primers.

**Author contributions** The authors have made the following declarations regarding their contributions. CT, RSN: Conceived and designed the experiments. RT: Performed the experiments. RT, PM: Analyzed the data. CT, RSN: Contributed reagents/materials. RT: Wrote the paper. CT, RSN, PM: Reviewed the manuscript. All the authors have read and approved the final manuscript.

**Compliance with ethical standards**

**Conflict of interest** None of the authors of the submitted manuscript has declared any conflict of interest.

**Practical application** The protocol is useful for mass multiplication, genetic engineering, synthetic seed preparation and cryopreservation of *P. marsupium* plants.

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