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Indirect somatic embryogenesis and genetic homogeneity assessment in *Plectranthus bourneae* Gamble- an endemic plant to Western Ghats of Tamil Nadu, India

R. Thaniarasu, T. Senthil Kumar*and M.V. Rao

Department of Botany Bharathidasan University, Tiruchirappalli- 620024, Tamil Nadu, India.
**E-mail: senthilbdc@bdu.ac.in*

Abstract

An effective somatic embryogenesis protocol was established for *Plectranthus bourneae,* an endemic plant to Western Ghats of Tamil Nadu, India. High frequency (77.28 %) of embryogenic callus was achieved from leaf explants on Murashige and Skoog (MS) medium supplemented with 0.5 mg/L 2,4- dichlorophenoxyacetic acid (2,4-D). The embryogenic callus was subcultured in a combination of 0.5 mg/L 2,4-D and 50 mg/L glutamine (GLU) which produced somatic embryos (84.22 %). Different stages of embryos (globular, heart, torpedo and cotyledonary stage) were observed. Maturation and the frequency of somatic embryo germination increased when transferred onto half-strength MS medium containing 0.5 mg/L gibberellic acid (GA_3) and 0.5 mg/L 6-benzyladenine (BA). Plantlets were acclimatized successfully with 90 % survival. Histological studies revealed the development of primary and secondary embryos. The genetic fidelity of mother plants and *in vitro* raised plants was established by inter-simple sequence repeat (ISSR) markers. The established protocol assists the rapid production of true-to-type plants by somatic embryogenesis and hence could provide a valuable target material for genetic transformation experiments.

Key words: *Plectranthus bourneae*, embryogenic callus, somatic embryogenesis, leaf explant, glutamine, ISSR

Introduction

Plants are the benefactors of various constituents and effective medicines. Swift intensification of the human population, the devastation of natural habitats, over-exploitation of natural resources and the establishment of exotic species have menaced a large number of species with extinction (Lampert, 2019). Endangered and endemic species are significant factors of forest ecosystems. Biotechnological studies overtly and obliquely support biodiversity conservation. The family Lamiaceae includes commercially significant genera, such as *Plectranthus*, *Ocimum, Mentha* and *Salvia.* which are recognized with a valuable assortment of ethnobotanical assistances. Several studies on the family Lamiaceae have been conducted to investigate secondary metabolites, such as essential oils (Uritu *et al*., 2018), terpenoids, flavonoids (Grayer *et al.,* 2010) and antioxidants, and compounds having pharmacological activity.

Plectranthus is one of the medicinally significant genera, recognized for its alimentary properties and horticultural importance for their aromatic nature and essential oil producing competence (Arumugam *et al.,* 2016). Various *Plectranthus* species were scrutinized for their antimicrobial properties (Crevelin *et al.,* 2015), antioxidant activity (Muhamad and Mat Ali, 2019), anti-inflammatory activity (Oguntibeju, 2018), schistosomicidal activity (Caixeta *et al.,* 2011) and antiprotozoal activity (Mothana *et al.,* 2014). Several *Plectranthus* species are used as ornamental plants which include *P. tenuiflorus, P. ciliates* and *P. madagascariensis* (Abdel Khalik, 2016; Salachan *et al.,* 2015 and Kubinova *et al.,* 2014).

Plectranthus bourneae Gamble is an endemic plant species with minimal distribution in the Pambar Shola of Kodaikanal (Palni hills), Western Ghats of Tamil Nadu, India (Matthew,1993). Poor seed germination and slow growth rate are threats to this species, therefore an immediate conservation strategy *viz.,* tissue culture technique, must be developed for restitution.

Biotechnological applications such as micropropagation and somatic embryogenesis afford several conceivable benefits for propagation of rare and valuable plants (Baskaran and Jayabalan 2009; Egertsdotter *et al.,* 2019). One of the intense significant features of *in vitro* techniques for germplasm conservation is the attention to the genetic stability of conserved plants (Baskaran *et al.,* 2018; Acuña *et al.,* 2019). The genetic stability of tissue cultured plantlets is crucial to establish the true-to-type nature of the plantlets. Polymerase chain reaction (PCR) based markers (Random Amplified Polymorphic DNA [RAPD] and Inter-simple Sequence Repeat [ISSR]) are extremely valuable in resolving the genetic stability of *in vitro* regenerated plantlets in several plant species (Vinoth and Ravindhran, 2015; Soares *et al.,* 2016). Of the molecular markers, ISSR have been applicable for repetitive assessment of genetic stability of plantlets (Saha *et al.,* 2016). ISSRs have effectively proved the genetic integrity of *Canna indica* plantlets derived from somatic embryos (Mishra *et al.,* 2015), *Vanilla planifolia* Jacks. Plantlets derived from indirect organogenesis (Ramirez-Mosqueda and Iglesias-Andreu, 2015) and micropropagated plantlets of *Nothapodytes nimmoniana* (Prakash *et al.,* 2016).

The commencement of a somatic embryo propagation method will provide an expeditious platform for clonal production, genetic transformation and germplasm conservation (Lu *et al.,*

2017; Bhavana *et al.,* 2018). Currently, few reports are available on somatic embryogenesis in *Plectranthus*. However, the present study was designed to establish a simple and effective protocol for the somatic embryogenesis of *P. bourneae* and verify the clonal fidelity of *in vitro* plants by using ISSR markers.

Materials and methods

Plant material and culture initiation: Young leaves of *P. bourneae* were used as initial explant. The plants were collected and transplanted from wild habitat Pambar Shola (10°5′-10°25′ N, 77°50′ E at an altitude of 2020 m) in the Western Ghats of Tamil Nadu, India, to a greenhouse at Department of Botany, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. The plants were maintained in earthen clay pots (17×15×17 inches) in the green house. For sterilization, the explants (young leaves 4 cm) were washed under running tap water for 15 to 20 min, followed by 70 % (v/v) ethanol for 15 s and 0.1 % mercuric chloride solution for 2 min and rinsed with sterile distilled water thrice after each treatment.

After excision, the sterilized leaf explants (about 2 cm^2) were inoculated by the leaf adaxial side facing upwards on the basal Murashige and Skoog (MS) (Murashige and Skoog 1962) medium fortified with 30 g/L sucrose (Himedia, India) and solidified with 0.8 % (w/v) agar (Himedia, India). The pH of the medium was adjusted to 5.7 ± 0.2 by adding 0.1 N NaOH or 0.1 N HCl after adding the different concentrations of plant growth regulators into the medium. The medium was apportioned into test tubes and autoclaved at 15 psi and 121 °C for 20 min and all the cultures were maintained in culture room at 26 ± 2 °C, under 16 h photoperiod at approximately 60 μmol^{-2} s^{-1} light intensity provided by cool-white fluorescent lamps and with 55 to 60 % relative humidity. All the cultures were subcultured on the same medium every two weeks.

Callus induction and proliferation: The callus induction medium, MS supplemented with different auxins (2,4-D, α-Naphthalene acetic acid (NAA), Picloram (PIC) -0.1 to 2.0 mg/L) (Himedia, India). The cultures were kept dark for the first seven days and then incubated under 14-h regular photoperiod of cool fluorescent light for four weeks to induce callus formation. The callus induction frequency from each treatment was recorded after four weeks.

Somatic embryo induction and maturation: The embryogenic callus was subcultured on MS medium supplemented with 2,4-D (0.5 mg/L) and GLU (25-100 mg/L) for somatic embryo induction and maturation. The frequency of somatic embryo development and number of somatic embryos was recorded after eight weeks of culture.

Somatic embryo germination: Torpedo stage embryos were detached from the embryogenic callus mass and cultured on half-strength MS medium (macro nutrients and boric acid) supplemented with different concentration (0.1-2.0 mg/L) of GA_3 and cytokinin BA (0.5 mg/L) for germination. The frequency of somatic embryo development and number of plantlets was recorded after six weeks of culture.

Hardening and acclimatization: Well germinated plantlets were removed gently and thoroughly washed with sterile water and transferred to plastic pots (6.5 cm diameter) containing red soil, sand and coir pith (1:1:1). Each plantlet was covered with polythene bag in order to maintain controlled growth conditions of 26 ± 2 °C, 16 h photoperiod, 80–85 % relative humidity and 60 $\mu mol\ m^{-2}\ s^{-1}$ light intensity. After three weeks, the polythene covers were removed gently and the plants were gradually exposed to greenhouse condition. The plants were frequently irrigated with sterile water, every three days for four weeks. Well-developed plants were finally transferred to the field.

Histological investigations: Callus with somatic embryos were employed for histological investigation to observe various stages of somatic embryo formation. The cultures were fixed in formaldehyde: alcohol (1:2) solution, then dehydrated in ethanol-xylol series, sectioned at 10 µm thickness and stained with safranin, and finally fixed in Distyrene Plasticizer Xylene (DPX) mount. Permanent slides were observed with a light microscope equipped with camera (GTR – 22A, Getner Instrument Industries, Haryana, India).

Genetic fidelity analysis using ISSR markers: Genomic DNA was isolated from the leaves of the somatic embryo derived plants (9-wk-old *in vitro*) and mother (*ex vitro*) plants (8-wk old) using Cetyl trimethyl ammonium bromide (CTAB) technique (Doyle and Doyle,1990). *In vitro* raised and mother plants were analyzed by using ISSR (Eurofins Genomics, Bangalore, India) primers. ISSR reaction incorporating in a volume of 10 µL containing 1 µL genomic DNA (100 ng), 5 µL 2 × master mix (Takara, United States of America), 0.8 µL of 10 pmol ISSR primers and 3.2 µL double distilled water. PCR amplifications included an initial denaturation step at 98 °C for 2 min, 30 cycles of denaturation at 55 °C for 90 sec, annealing at 34-57 °C for 90 sec min, and extension at 72 °C for 60 secs, followed by a final extension at 72 °C for 5 min. Amplifications were employed on a thermo cycler (PCR master cycler personal, Eppendorf, Hamburg, Germany) and the PCR products were electrophoresed in 0.8 % [w/v] agarose gel in 1× TAE buffer. PCR amplicons were photographed using a gel documentation system (Lark innovative Fine Tek knowledge, Tamil Nadu, India). The sizes of the amplicons were assessed by assorting with a 1 kb DNA ladder (Thermo scientific, USA).

Statistical analysis: The data on the influence of PGRs on various stages of callus induction and proliferation were expressed as mean± standard error. Each set of experiment consisted of seven replicates and each experiment was repeated thrice. The mean values of the repeated experiments were compared by DMRT at 5 % level of significance, using statistical software SPSS Ver.17.0.

Result and discussion

Callus induction and proliferation: Callus induction was observed from cut margins of leaf explants of *P. bourneae* after two weeks of incubation cultured on MS medium supplemented with auxins *viz.* 2,4-D, NAA and PIC (0.1-2.0 mg/L), under the partial incubation in dark (Fig. 1a). The occurrence of callus induction varied between genotypes and supplement of culture media with reference to the percentage of calli developed, morphology, quantity and type of embryogenic callus (Koufan *et al.,* 2020). Leaf explants enlarged and curled during the initial period, forming yellow, compact callus from the cut end. Of the various PGRs tested, MS medium supplemented with 2,4-D gave maximum response of embryogenic callus proliferation (Table 1). Globular somatic embryos were developed on the surface of the calli (0.5 mg/L 2,4-D) within four weeks (Fig. 1b

and f). Callus induced on 2,4-D at lower concentration produced greenish white nodular compact (GWNC) (Table 1). Among the various concentrations tested, the maximum frequency of 77.28 % embryogenic callus was obtained on MS medium supplemented with 0.5 mg/L of 2,4-D. 2,4-D play a significant role in the initial developmental stage of embryogenic callus induction.

Table 1. Effect of different concentrations of auxins on callus induction from leaf explants of *P. bourneae*

Auxins (mg/L)		Callus (%)	Callus morphogenesis
2,4-D	Control	00.0±0.0	-
	0.1	63.42 ± 0.64^{c}	WEF
	0.5	77.28 ± 0.64^{a}	GWNC
	1.0	65.42 ± 0.98^{c}	GWNC
	1.5	60.42 ± 1.23^{d}	YWEF
	2.0	58.42 ± 0.86^{d}	YWEF
NAA	0.1	52.14 ± 1.18^{ef}	GC
	0.5	68.00 ± 0.87^{b}	GC
	1.0	53.14 ± 0.70^{ef}	GC
	1.5	49.00 ± 1.51^{ij}	YWC
	2.0	44.28 ± 0.68^{lm}	YWC
PIC	0.1	42.71 ± 0.52^{m}	WEF
	0.5	48.42 ± 0.99^{ij}	WEF
	1.0	64.57 ± 1.10^{c}	WEF
	1.5	54.00 ± 1.00^{e}	WN
	2.0	50.71 ± 1.45^{gh}	WN

Values are mean ± S.E. from 7 replicates per treatment and all the experiments were repeated thrice. Means followed by the same letters in each column are not significantly different (P = 0.05) using Duncan's multiple range test. WEF – White embryogenic friable callus, GWNC – Greenish white nodular callus, YWEF – Yellowish white embryogenic friable callus, GC – Green compact callus, YWC – Yellowish white compact callus, WEF – White embryogenic friable callus, WN – White nodular callus.

The 2,4-D has been effectively utilized for embryogenic callus induction in other plant species (Pantha *et al*., 2016; Satish *et al.,* 2015; Silveira *et al.,* 2013). Our study exhibited that a lower concentration of 2,4-D (0.5 mg/L) produced a higher percentage of embryogenic callus. Lower concentration of plant growth regulators or deficient plant growth regulators in medium support the unicellular origin of somatic embryos and the possibility of reducing the somaclonal variation (Tomiczak *et al*., 2019). Several investigations have displayed the vital role of the exogenous treatment of auxins, mainly 2,4-D is deliberated to be one of the major assisting factors for somatic embryogenesis (Hazubska-Przybył *et al*., 2020). The function of 2,4-D in cell division and embryogenic capability was reported by Raghavan (2004). The greenish white nodular compact embryogenic callus exhibits more number of globular embryos. Increase in concentration of 2,4-D showed yellowish white friable embryogenic callus. Generally, the callus induced on NAA containing medium was smooth and somatic embryos were not developed, rather than, all explants produced adventitious shoots. NAA at all concentrations produced only green compact (GC) organogenic calli (Table 1). The medium containing higher concentrations of NAA developed glossy, slow-growing and non-embryogenic callus cultures which eventually turned brown or black. Similar negative effects of NAA on organogenic callus of *P. bourneae* at higher concentrations have already been reported by Thaniarasu *et al.* (2016). The auxin PIC also induced embryogenic callus like white nodular callus but it was substantially low as compared to 2,4-D (Table 1). Similar result was obtained from *Podophyllum hexandrum* (Rajesh *et al.,* 2014). In contrast, influence of PIC displayed a significant effect over 2,4-D on the development of somatic embryos in *Agapanthus praecox* (Yaacob *et al.,* 2012).

Somatic embryo induction and maturation: The supplement of an organic nitrogen form has a positive effect on somatic embryogenesis (Kim and Moon, 2014; Truong *et al.,* 2013; Sezgin and Dumanoğlu, 2013). For somatic embryo induction MS medium containing 2,4-D (0.5 mg/L) was supplemented with glutamine at different concentrations (25-200 mg/L). After four weeks of culture, embryogenic calli with conspicuous globular somatic embryos were developed.

Embryogenic calli with globular somatic embryos were sub-cultured for another four weeks on the same medium for further growth. Consequently, a total of eight weeks was required for induction of somatic embryo from embryogenic calli. Of the different concentrations of glutamine examined, somatic embryos developed only on MS medium supplemented with 0.5 mg/L 2,4-D + GLU 50 mg/L. The glutamine supplement (50 mg/L) in media employed significant somatic embryo establishment in *P. bourneae* (Table 2). Various developmental stages of somatic embryos (globular, heart, torpedo and cotyledonary) were recorded after eight weeks of culture (Fig. 1b, c, d, f, g, h, and i). Our result showed that the glutamine was suitable for somatic embryo induction and maturation. Similarly, several reports confirmed formation of somatic embryo by glutamine (Bajpai *et al.,* 2016; Morais-Lino *et al.,* 2016; Baskaran and Van-Staden, 2014). Medium comprising 200 mg/L glutamine confirmed a substantial decrease in the enhancement of somatic embryos when compared with control (Table 2). Therefore, the results of present investigation agreed with the effective concentration of glutamine to develop somatic embryogenesis of *P. bourneae*.

Table 2. Effect of different concentrations of glutamine with 2,4-D (0.5 mg/L) on induction of somatic embryos after 8 weeks of culture

2,4-D (0.5 mg/L) + Glutamine (mg/L)	% of embryogenic callus formation	Mean number of somatic embryos
0.0	32.56 ± 2.13^{de}	7.71 ± 0.74^{cd}
25	75.16 ± 1.34^{b}	23.71 ± 0.99^{b}
50	84.22 ± 1.21^{a}	32.57 ± 0.75^{a}
100	52.26 ± 2.24^{c}	15.14 ± 0.73^{c}
150	41.34± 1.43^{cd}	9.56± 0.44^{d}
200	24.71± 2.10^{e}	4.53± 0.32^{e}

Values are mean ± S.E. from 7 replicates per treatment and all the experiments were repeated thrice. Means followed by the same letters in a column are not significantly different (P = 0.05) using Duncan's multiple range test.

Germination of somatic embryos: Somatic embryos are incapable to germinate on the same embryogenic callus induction medium. To regenerate into a whole plant, torpedo stage somatic embryos were transferred to half- MS basal medium containing different concentrations of GA_3 (25 – 200 mg/L) with BA (0.5 mg/L). Cotyledonary embryos showed plantlet conversion on transfer to the germination medium. Addition of GA_3 in the germination medium provided a significant result on the rate of somatic embryo conversion into plantlet. Maximum of 19.57 average number of cotyledon embryos were obtained at 1.0 mg/L GA_3 + 0.5 mg/L BA.

The cotyledon development initiated within 2-3 weeks of transfer (Fig. 1e). Consequently, the embryos develop a shoot and root

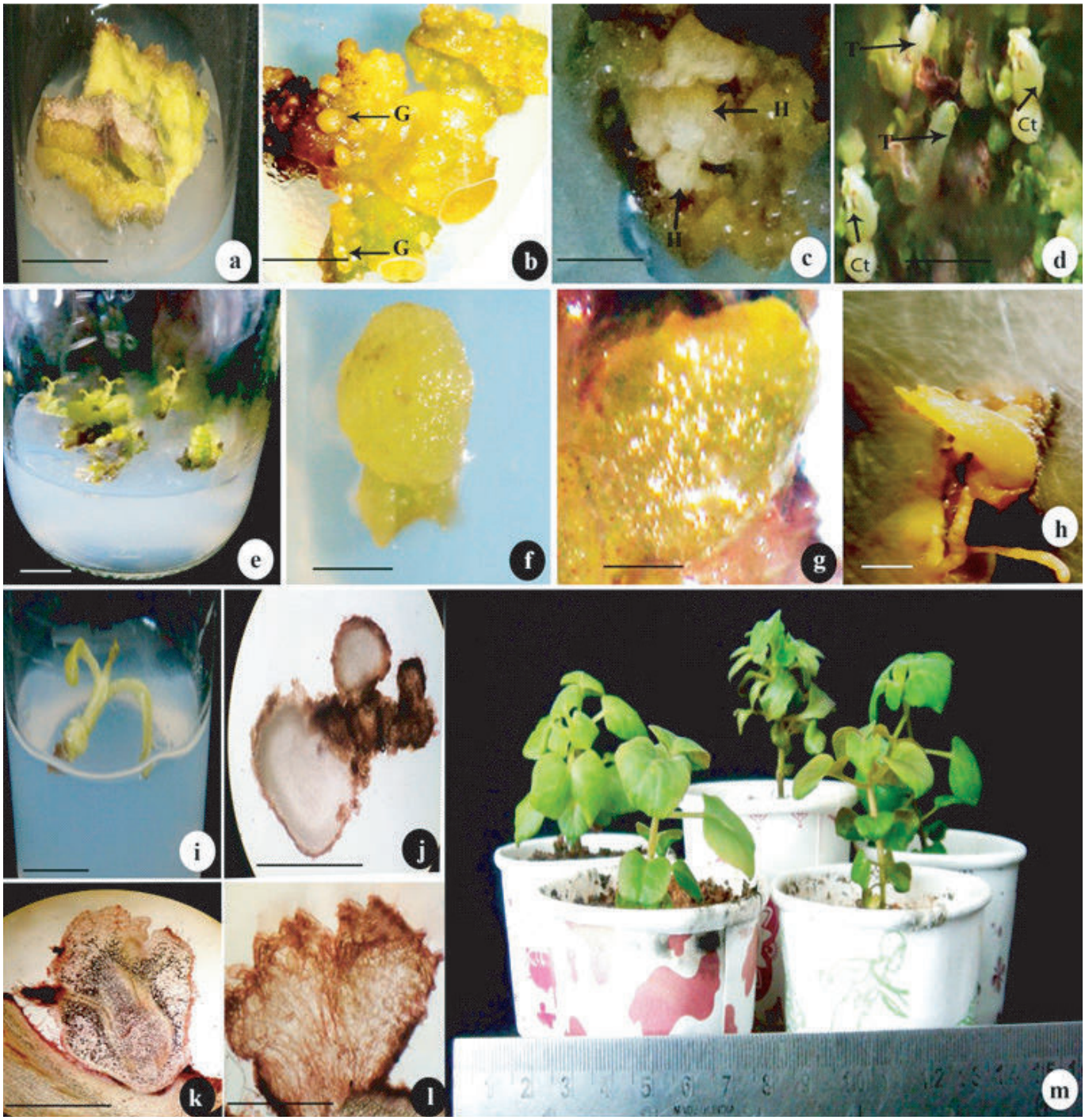

Fig. 1. Embryogenic callus formation and developmental stages of somatic embryos of *P. bourneae*. a) Leaf explant, b) Proliferation of embryogenic callus with globular embryo on MS medium containing 2,4-D 0.5 mg/L + GLU 50 mg/L, c) Heart shape embryo on MS medium containing 2,4-D 0.5 mg/L + GLU 50 mg/L, d) Torpedo shape embryo on MS medium containing 2,4-D 0.5 mg/L + GLU 50 mg/L, e) Cotyledonary stage embryos on ½ MS medium containing GA_3 0.5 mg/L + BA 0.5 mg/L, f) Germination of somatic embryos on ½ MS medium supplemented with GA_3 0.5 mg/L + BA 0.5 mg/L, f) Enlarged view of globular embryo, g) Enlarged view of heart stage embryo, h) Enlarged view of torpedo stage embryo with juvenile root, i) Cotyledonary stage embryo, j) Microscopic view of globular and early heart shape embryos, k) Microscopic view of heart shape embryo, l) Microscopic view of torpedo shape embryo. m) Hardened plantlets. The *bar* represents 1.0 cm in a-d, 2.0 cm in e, 0.5 mm in f-h**,** 1.0 mm in i,25 µm in j-l.

axis, and established a complete plantlet within 4-6 weeks of culture (Fig. 1h and i). The essential stimulatory effect of GA_3 for somatic embryo germination was confirmed in *Papaver nudicaule* (Yang *et al.,* 2010). The addition of BA (0.5 mg/L) with different concentrations of GA_3 considerably increased the germination of somatic embryos. Similarly, the accumulation of BA with GA_3 for higher somatic embryo formation was established in Okra (Daniel *et al.,* 2018).

The maximum frequency (61.56 %) of germination was observed on half-strength MS medium containing 0.5 mg/L GA_3 and 0.5 mg/L BA. Similar effect of BA in combination with other plant growth regulators increased somatic embryo development and plantlet conversion have also been reported in *Coriandrum sativum* (Ali *et al.,* 2016) and *Schisandra chinensis* (Chen *et al.,* 2010). The highest germination rate of 14.85 was observed at 1.0 mg/L GA_3 + 0.5 mg/L BA (Table 3). The germination decreased with increasing concentration of GA_3. Combined effect of

cytokinin and gibberellin in promoting the conversion of embryos to plantlets has been reported in *Cnidium officinale, Wedelia calendulacea* and *Capsicum baccatum* (Lee *et al.,* 2009; Sharmim *et al.,* 2014; Venkataiah *et al.,* 2016). GA_3 also has been used in the elongation of regenerated shoots (Thaniarasu *et al.,* 2015).

Table 3. Effects of half-strength MS medium containing different concentrations of GA_3 with BA (0.5 mg/L) on germination and plantlet conversion of somatic embryos after 6 weeks of culture

Plant growth regulators	Germination frequency (%)	Mean number of cotyledonary stage	Mean number of embryo germination
GA_3 (mg/L)			
0.0	12.61 ± 0.45^{e}	3.23 ± 0.22^{d}	1.64 ± 0.54^{d}
0.1	44.32 ± 0.74^{c}	11.71 ± 0.47^{c}	5.14 ± 0.50^{c}
0.5	61.56 ± 0.22^{a}	19.57 ± 0.71^{a}	14.85 ± 0.40^{a}
1.0	48.56 ± 0.71^{b}	15.28 ± 0.68^{b}	9.14 ± 0.63^{b}
2.0	34.91 ± 0.54^{d}	10.14 ± 0.82^{cd}	6.71 ± 0.64^{bc}

Values are mean ± S.E. from 7 replicates per treatment and all the experiments were repeated thrice. Means followed by the same letters in each column are not significantly different (P = 0.05) using Duncan's multiple range test.

Hardening and acclimatization: The plantlets were successfully acclimatized in a greenhouse under *ex vitro* conditions (Fig. 1m) with a survival frequency of 90 %. Combination of red soil, sand and coir pith was realized appropriate for hardening and acclimatization. There was no distinct phenotypic dissimilarity amongst the original greenhouse-grown plants and those regenerated from somatic embryos in this study, exhibiting that the regenerated plantlets were true-to-type.

Histological investigations: Anatomical sections prepared from the embryogenic cultures of *P. bourneae* comprising somatic embryos at different developmental stages showed the initiation of somatic embryos. Similarly, histological and ultrastructural studies confirmed different developmental stages of somatic embryo for clonal production in other plant species (Baskaran *et al.,* 2016; Baskaran and Staden, 2017). The histological sections displayed groups of meristematic cells developed at the marginal region of embryogenic callus. These meristematic cells were delicate, compact and densely cytoplasmic with distinct nuclei. Moreover, the differentiation of these structures focused to the development of globular, oval shaped embryos on the surface of the callus (Fig. 1j). Certain callus tissues exhibited bilaterally symmetrical heart-shape embryos (Fig. 1k). Through this stage, the improvement of precise bipolar embryos with systematic shoot and root portion was observed (Fig. 1k). The embryo was attached to the embryogenic callus with a conspicuous multicellular stalk, the suspensor. Consequently, embryos developed as exempted structures deprived of vascular connections among them. Similar findings are reported in several plant species *Lilium pumilum, Justicia gendarussa* and *Cleome rosea*(Zhang *et al.,* 2016; Bhagya *et al.,* 2012; Simoes *et al.,* 2010).

Genetic fidelity analysis using ISSR markers: DNA was isolated from both somatic embryos derived from *in vitro* plants and mother plant to authenticate the genetic stability. Ten primers delivered specific and reproducible bands out of 15 ISSR primers employed in the initial assessment. The optimum annealing temperature for ISSR markers is diverse from 32 to 34 ^{o}C (Table 4). The ten ISSR primers provided 55 conspicuous and scorable bands in size range of 200 bp (ISSR - 07) to 2,800 bp (ISSR - 08). All banding reports from somatic embryo-derived plantlets were monomorphic and correspond to the mother plant's (Fig. 2). Similar findings of genetic assessment have been described by Konar *et al.* (2018) in *Psidium guajava,* Heikrujam *et al.* (2014) in *Calliandra tweedii* (Benth.) and Singh *et al.* (2016) in *Sapindus mukorossi*.

Table 4. List of 10 primers, their sequences, number and size of the amplified fragments generated by ISSR primers

Primer name	Sequence 5' – 3'	Annealing temperature (^{o}C)	Number of scorable band per primer	Size range of amplified product (bp)
ISSR 1	$(GA)_8A$	32	07	2000 – 300
ISSR 2	$(AC)_8C$	32	04	2500 – 400
ISSR 3	$(GA)_8CC$	34	06	2000 – 400
ISSR 4	$(CTC)_6$	32	03	1800 – 400
ISSR 5	$(AC)_8AT$	32	05	1500 – 1000
ISSR 6	$(AC)_8AG$	32	06	2800 – 500
ISSR 7	$(AC)_8TG$	34	10	2000 – 200
ISSR 8	$(AG)_8CC$	34	05	1400 – 500
ISSR 9	$(AC)_8CC$	32	04	1800 – 500
ISSR 10	$(ACTG)_4$	32	05	2000 – 400

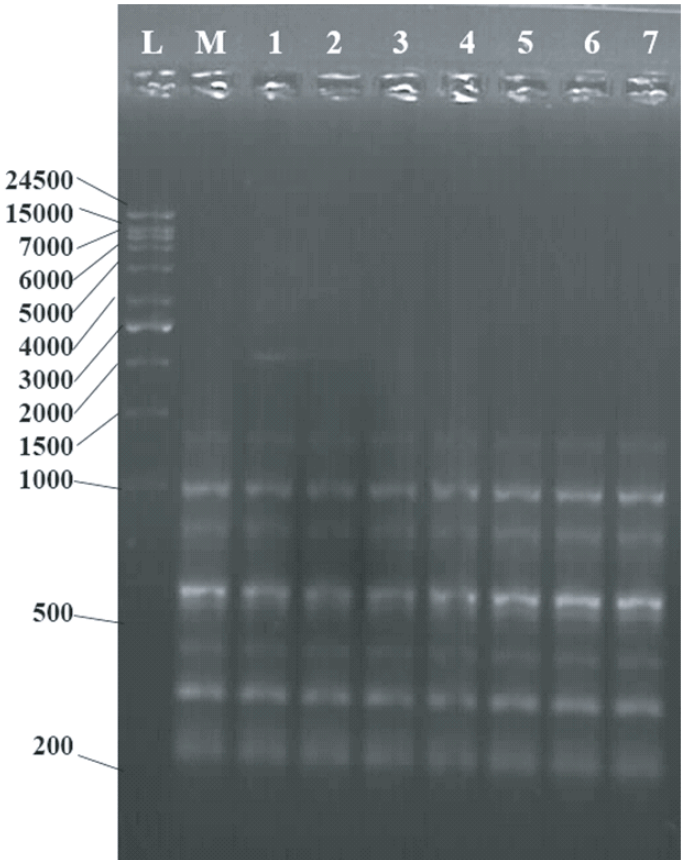

Fig. 2. Polymerase chain reaction (PCR) amplification products obtained with ISSR (inter-simple sequence repeat) primer (ISSR – 7). Lane L1 represents 1 kb ladder, Lane M represents mother plant, Lane 1 to 7 represent *in vitro* raised clones of *P. bourneae*.

The present study observed that 12 weeks required to develop a complete plantlet of *P. bourneae via* somatic embryogenesis system. The regenerated plantlets were healthy and ISSR evaluation displayed that they are genetically stable and equivalent

to their parental counterpart. The present technique was established for the rapid regeneration and mass multiplication of *P. bourneae* clonal production through somatic embryogenesis.

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