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RESEARCH ARTICLE

Mass propagation of *Plectranthus bourneae* Gamble through indirect organogenesis from leaf and internode explants

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Abstract The present study describes the plant propagation via indirect organogenesis from in vitro derived leaf and internode explants of *Plectranthus bourneae*, an endemic plant to south India. Leaf and internodal explants successfully callused on Murashige and Skoog medium (MS) supplemented with different concentrations of auxins [2,4-D (2, 4-dichlorophenoxyacetic acid), NAA (α-naphthalene acetic acid), IAA (indole-3 acetic acid), IBA (indole-3-butyric acid) and PIC (Picloram); 0.1–2.0 mg/l] in combination with BA (6-benzyladenine) (0.5 mg/l). Maximum callus induction (98 %) was achieved from leaf explant followed by internodal explant (89 %) at 1.0 mg/l NAA, 0.5 mg/l BA. Leaf derived callus showed better shoot regeneration (29.71 shoots) on MS medium containing 1.0 mg/l KN (kinetin), 0.7 mg/l NAA, and 50 mg/l CH (casein hydrolysate) followed by internodal callus (19.71). A maximum of 19.14 roots/shoot was observed at 1.0 mg/l IBA. The rooted plantlets were successfully hardened and transferred to greenhouse condition with 80 % survival. This system could be utilized for large-scale multiplication of *P. bourneae* by tissue culture.

Keywords Callus induction · Organogenesis · *Plectranthus bourneae* · Endemic plant · Growth regulators

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Abbreviations

MS	Murashige and Skoog medium
BA	6-Benzyl adenine
IAA	Indole-3 acetic acid
IBA	Indole-3-butyric acid
KN	Kinetin
NAA	α-Naphthalene acetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
PIC	Picloram
AA	Ascorbic acid
CH	Casein hydrolysate
AC	Activated charcoal
AdS	Adenine sulphate

Introduction

The implements of contemporary biotechnology are being progressively applied for plant diversity depiction, indisputably they have a major role in assisting plant conservation programmes. Consequently, special solicitude should be remunerate to protect and preserve the endemic flora that is normally found in limited areas (Fay 1992; Sarasan et al. 2006). Conservation of endemic species by plant tissue culture has attracted the attention of many researchers (Mallon et al. 2010; Piovan et al. 2010). Lamiaceae herbs namely basil, oregano, sage, marjoram and rosemary are popular aromatic herbs. The food industry is becoming increasingly interested in aromatic herbs, mainly of the Lamiaceae family due to growing consumer demands for healthy natural foods. In addition to the food industry, Lamiaceae herbs are also of high demand in dyeing, fragrances, cosmetics, beverages, confectionary and Pharmaceutical industries (Zaidi and Dahiya 2015). The genus *Plectranthus*, comprising of 300 species, belongs to around 40 % of the Lamiaceae genera, possess

aromatic properties (Lawrence 1992). Several *Plectranthus* species are used to treat skin infections, peptic ulcers, constipation, flatulence and stomach ache (Kokwaro 1993; Lukhoba et al. 2006). *Plectranthus* leaves contain essential oils, flavonoids, cinaminics and terpene derivatives, all of which have antineoplastic properties (Brandao et al. 2013).

Plectranthus bourneae Gamble is an endemic plant species, has very restricted distribution only in the Pambar Shola of Kodaikanal (Plalni hills), Western Ghats of Tamil Nadu, India (Matthew, 1993). It is a well branched, compact shrub, branchlets subsucculent. Our previous study on antibacterial effect of this plant extract showed prominent results on different human pathogenic bacteria (Thaniarasu et al. 2015). Matthew (1999) has reported this species as to the plant become threatened and highly vulnerable in position due to habitat destruction at Palni hills. Peoples settled from overseas since the mid-19th century, introduced many plants from the temperate countries, by which the percentage of alien plants now is 20 % of the total and these have also become a standing threat to the native species, by altering and disturbing the very visage of the hills and their primary vegetation (Matthew 1999).

Low seed germination and slow growth rate impends threat to this species hence required an alternative conservation strategies viz. tissue culture technique to ensure its restoration. Tissue culture methods offer highly efficient tools for germplasm conservation and mass multiplication of many threatened plant species (Salehi et al. 2014; Ozel et al. 2015). Sreedevi et al. (2013) and Thangavel et al. (2014a) have previously reported successful organogenesis from *Plectranthus barbatus* using leaf explants. In the present study, an exertion has been made to develop an efficient in vitro organogenesis method of multiple shoot formation through callus culture from leaf and internode explants of *Plectranthus bourneae*.

Materials and methods

Plant material and surface sterilization

The plants of *P. bourneae* collected from Pambar Shola (10° 5′-10°25′ N, 77°50′ E at an altitude of 2020 m) in the Western Ghats of Tamil Nadu, India, being maintained in the glass house of Bharathidasan University, Tiruchirappalli, were used as the explant source. Leaf and internode explants (4 cm length) were washed under running tap water for 20 to 30 min. The internode explants were sterilized with 70 % ethanol for 30 s followed by 0.1 % bavistin treatment for 5 min and finally 0.1 % mercuric chloride treatment for 2 min. Surface sterilization of the leaf explants were similar to the internodes, but the ethanolic treatment was reduced to 10 s, since over exposure of ethanol to the leaf tissue caused early browning of explants. The explants were rinsed with sterile distilled water thrice after each treatment. These sterilized explants were inoculated on culture medium. The sterilized leaves (about 2 cm^2) and internodes (2 cm) after excision were inoculated (leaves - adaxial side facing upwards) on the callus induction medium. This whole process was carried out under the laminar air flow chamber.

Culture conditions

The basal MS (Murashige and Skoog 1962) medium fortified with 30 g/l sucrose (Himedia, India) and gelled with 0.8 % (*w*/*v*) agar (Himedia, India), and the pH of the medium was adjusted to 5.7 ± 0.2 using 0.1 N NaOH or 0.1 N HCl after addition of the plant growth regulators. The medium was autoclaved at 121 °C and pressure for 30 min and maintained. All the cultures were maintained in culture room at 26 ± 2 °C, under 16/8 h light regime provided by cool white fluorescent light (60 μmol^{-2} s^{-1} light intensity) and with 55 to 60 % relative humidity.

Callus induction and regeneration of shoots

Sterilized, leaf and internode explants were cultured on MS medium supplemented with auxins (NAA, IAA, IBA, 2, 4-D, PIC - 0.1 mg/l to 2.0 mg/l) (Himedia, India) and cytokinin BA (0.5 mg/l) for callus induction. The cultures were initially kept in dark for 7 days and then transferred to light for 4 weeks. The fully matured green compact calluses (1.0 mg/l NAA with 0.5 mg/l BA) obtained from both the explants were transferred to regeneration medium (BA, KN, and TDZ 0.1, 0.5, 1.0, 1.5 and 2.0 mg/l) (Himedia, India). The number of regenerated shoots from each explant callus was recorded after 4 weeks.

Effect of auxins on shoot regeneration

The fully matured green compact calluses were further cultured on KN (1.0 mg/l) supplemented MS medium with different auxins (NAA, IBA, and IAA) at 0.1, 0.5, 0.7, 1.0, 1.5, and 2.0 mg/l for shoot multiplication. The multiplied plantlets continued to grow on the same medium for shoot elongation. The data on multiple shoot formation, shoot number and shoot lengths were recorded after 4 weeks of culture.

Influence of additives on shoot multiplication

The effect of various additives on improved shoot multiplication was studied after determining the optimum cytokinin combination for the shoot bud induction and multiplication from callus. The leaf and internode derived fully matured green compact callus were further cultured on MS medium containing KN (1.0 mg/l), NAA (0.7 mg/l) along with different concentrations of

(25, 50, 75, 100 mg/l) AA, CH, AC, and AdS (Himedia, India). After 6 weeks of culture, the percentage of shoot formation, shoot number and the length of newly formed shoots were recorded.

Root induction and acclimatization

In vitro raised, elongated shoots (~3-4 cm) obtained from MS medium supplemented with KN (0.1 - 2.0 mg/l) + NAA (0.1 - 2.0 mg/l) + CH (25 - 100 mg/l) were transferred to root induction on half strength MS medium supplemented with auxins IBA, IAA and NAA (0.1, 0.5, 1.0, 1.5 and 2.0 mg/l). Rooting percentage, number of roots and root length were recorded after 3 weeks of culture.

The well rooted healthy plantlets were gently picked up from culture vessels without any damage to the roots and washed carefully with distilled water and subsequently transferred to paper cups (6.5 cm diameter) containing red soil, sand and coconut coir (1:1:1). Potted plantlets were covered with transparent polythene bags and maintained under controlled growth conditions of 26 ± 2 °C, 16 h photoperiod, 80–85 % relative humidity and 60 μmol^{-2} s^{-1} light intensity. The plantlets were frequently irrigated with sterile water every three days for four weeks. Well established plantlets were finally transplanted to the field.

Statistical analysis

All the treatments were consisted a minimum of 7 test tubes with single explant and each experiment was repeated three times. The data on frequency of explants responding callus, average number and length of shoots and roots were recorded regularly at weekly intervals. The mean values of the repeated experiments were compared by DMRT test at 5 % level of significance, using statistical software SPSS Ver.17.0.

Results and discussion

Callus induction was observed from cut margins of leaf and internode explants of *P. bourneae* after 2 weeks of incubation, cultured on MS medium supplemented with auxins viz. 2, 4-D, IAA, IBA, NAA and PIC (0.1–2.0 mg/l) along with BA (0.5 mg/l), under a partial incubation in dark (Table 1). Initial responses like leaf curl followed by swelling of explants were observed from the 6th day of culture period (Fig. 1a and d). Of the various treatments, NAA along with BA gave the best callus initiation and proliferation followed by 2, 4-D, IAA, IBA and PIC (Table 1, Fig. 1b and e). Depending upon the concentration and combination of plant growth regulators used, a wide range of variation in frequency of callus formation and nature of callus was observed. Fresh weight and dry weight of callus biomass for leaf and internode explants of *P. bourneae* are shown in Table 1. The highest fresh weight was achieved on 0.5 mg/l BA and 1.0 mg/l NAA in both of the explant.

2, 4-D at lower and higher concentrations produced light yellow to brown friable or nodular callus from both internode and leaf explants, which were failed to respond further (Table 1). In contrast 2, 4-D was reported to develop embryos with irregular morphology (Passinho-Soares et al. 2013). Medium containing 1.0 mg/l IAA and 0.5 mg/l BA produced green organogenic callus was obtained from leaf explants, whereas IBA (1.0 mg/l) with BA (0.5 mg/l) produced dark brown callus with rooting. Similarly Soh et al. (1998) suggest that the modified callus did not form adventitious roots on medium with auxins but only with cytokinins, therefore it is suggested that cytokinin have stimulate effect on root formation from callus. The highest frequencies of 98 % and 89 % of well developed, dark green organogenic calluses were induced from leaf and internode explants respectively on MS medium supplemented with 1.0 mg/l NAA and 0.5 mg/l BA (Fig. 1b and e). Lower concentrations of NAA (0.1–1.0 mg/l) along with BA (0.5 mg/l) formed profuse amount of callus in contrast to higher concentrations (Table 1).

Among the different concentrations of auxin tested, NAA with BA proved to be the best for high frequency of greenish compact callus induction. Earlier reports by Erisen et al. (2010); Bakar et al. (2014); Thangavel et al. (2014b) confirmed the same. All the callus cultures were sub-cultured every 15 days on to a fresh medium containing the same Plant growth regulators (PGR) composition. Most of the calli induced and developed as yellow in colour become green compact organogenic callus in 4 weeks. Only green compact callus was examined for further study.

In comparison, leaf explants produced significantly more frequency of callus (32.0–98.0 %) than the internode explant (32.0–89.0 %). It has been revealed from the present study that explant nature is a significant feature and responsible for the rate of achievement in callus induction.

Effect of cytokinins on shoot regeneration

The well-established callus transferred to the shoot regeneration medium induced multiple shoots within two weeks of culture. The leaf derived callus produced a highest number of 10.4 shoots than the internode derived callus (6.2 shoots) on KN (1.0 mg/l) (Table 2, Fig. 1c). Above this optimum concentration (1.0 mg/l)

Table 1 Callus induction from leaf and internode explants of *P. bourneae* cultured on MS medium supplemented with different concentrations of auxins with BA, after 4 weeks of culture

PGR's (mg/l)	Percentage of response		Callus weight mg/explant			
	Leaf	Internode	Leaf callus fresh weight (mg)	Leaf callus dry weight	Internode callus fresh weight	Internode callus dry weight
Control	00.0 ± 0.0	00.0 ± 0.0	00.0 ± 0.0	00.0 ± 0.0	00.0 ± 0.0	00.0 ± 0.0
2,4-D+ BA (0.5)						
0.1	49.0 ± 1.0^{gh}	35.0 ± 1.6^{ghi}	328.5 ± 0.90^{j}	81.0 ± 0.53^{i}	308.9 ± 0.38^{k}	74.4 ± 0.65^{m}
0.5	79.0 ± 1.0^{b}	39.0 ± 1.0^{fg}	338.4 ± 2.45^{i}	86.7 ± 0.59^{h}	301.1 ± 0.76^{l}	69.3 ± 0.65^{n}
1.0	55.0 ± 1.0^{ef}	46.0 ± 1.6^{de}	504.7 ± 1.33^{e}	105.9 ± 2.15^{d}	323.8 ± 0.55^{i}	77.6 ± 0.58^{l}
1.5	43.0 ± 1.5^{jk}	44.0 ± 1.6^{de}	406.1 ± 1.59^{f}	99.0 ± 2.09^{f}	230.2 ± 0.41^{p}	52.4 ± 0.71^{p}
2.0	42.0 ± 1.3^{k}	33.0 ± 1.5^{hi}	400.4 ± 1.64^{g}	94.5 ± 0.96^{g}	228.4 ± 0.49^{p}	47.5 ± 0.52^{q}
NAA+ BA (0.5)						
0.1	63.0 ± 1.5^{c}	55.0 ± 1.6^{c}	405.9 ± 1.20^{f}	85.2 ± 1.05^{h}	553.2 ± 0.51^{ef}	128.8 ± 0.35^{e}
0.5	78.0 ± 1.3^{b}	75.0 ± 1.6^{b}	508.5 ± 1.26^{d}	102.9 ± 1.50^{e}	567.5 ± 0.52^{d}	130.9 ± 0.27^{d}
1.0	98.0 ± 1.3^{a}	89.0 ± 1.0^{a}	1698.6 ± 1.90^{a}	255.6 ± 1.46^{a}	885.3 ± 0.42^{a}	211.6 ± 0.61^{a}
1.5	58.0 ± 1.3^{de}	53.0 ± 1.5^{c}	815.7 ± 0.68^{b}	173.3 ± 0.77^{b}	874.8 ± 0.26^{b}	202.9 ± 1.01^{b}
2.0	48.0 ± 1.3^{hi}	38.0 ± 1.3^{gh}	803.6 ± 2.63^{c}	169.9 ± 0.78^{c}	783.6 ± 1.03^{c}	184.0 ± 0.73^{c}
IAA + BA (0.5)						
0.1	32.0 ± 1.3^{l}	37.0 ± 1.5^{ghi}	235.3 ± 0.44^{o}	54.6 ± 0.90^{n}	346.8 ± 0.38^{g}	86.2 ± 0.35^{k}
0.5	43.0 ± 1.5^{jk}	48.0 ± 1.3^{d}	275.8 ± 0.66^{m}	52.3 ± 0.77^{o}	343.7 ± 0.55^{h}	90.9 ± 0.23^{i}
1.0	47.0 ± 1.5^{hij}	55.0 ± 2.2^{c}	280.9 ± 1.40^{l}	64.1 ± 0.56^{l}	356.0 ± 0.63^{e}	103.7 ± 0.98^{f}
1.5	44.0 ± 1.6^{ijk}	46.0 ± 1.6^{de}	276.0 ± 0.68^{m}	61.1 ± 037no	341.9 ± 0.40^{h}	98.6 ± 0.47^{g}
2.0	34.0 ± 1.6^{l}	37.0 ± 1.5^{ghi}	268.1 ± 0.64^{n}	59.1 ± 0.54^{n}	308.2 ± 1.08^{k}	95.7 ± 0.30^{h}
IBA + BA (0.5)						
0.1	47.0 ± 1.5^{hij}	35.0 ± 1.6^{ghi}	145.2 ± 0.90^{q}	34.9 ± 0.62^{q}	143.0 ± 0.53^{q}	38.8 ± 0.38^{s}
0.5	42.0 ± 1.3^{k}	37.0 ± 1.6^{ghi}	187.7 ± 0.97^{p}	40.6 ± 0.45^{p}	154.3 ± 0.55^{p}	43.0 ± 0.53^{r}
1.0	32.0 ± 1.3^{l}	43.0 ± 1.5^{f}	130.1 ± 0.80^{r}	31.1 ± 0.52^{r}	160.7 ± 0.68^{o}	46.4 ± 0.71^{q}
1.5	-	34.0 ± 1.6^{ghi}	-	-	142.4 ± 1.21^{q}	37.0 ± 0.68^{t}
2.0	-	32.0 ± 1.3^{i}	-	-	127.4 ± 0.49^{r}	36.3 ± 0.76^{t}
PIC + BA (0.5)						
0.1	43.0 ± 1.5^{jk}	33.0 ± 1.5^{hi}	327.3 ± 0.42^{j}	85.0 ± 0.57^{h}	293.7 ± 0.57^{m}	73.1 ± 0.37^{m}
0.5	61.0 ± 1.0^{cd}	44.0 ± 1.6^{de}	331.3 ± 1.07^{jk}	79.9 ± 0.99^{i}	308.2 ± 1.00^{k}	66.8 ± 0.53^{o}
1.0	53.0 ± .15^{fg}	38.0 ± 1.3^{gh}	353.2 ± 0.98^{h}	73.4 ± 0.22^{j}	319.9 ± 0.43^{j}	88.8 ± 0.51^{j}
1.5	45.0 ± 1.6^{hij}	35.0 ± 1.6^{ghi}	311.6 ± 2.02^{jk}	69.6 ± 0.52^{k}	286.0 ± 0.33^{n}	86.8 ± 0.53^{k}
2.0	42.0 ± 1.3^{k}	33.0 ± 1.5^{hi}	275.0 ± 0.67^{m}	63.5 ± 0.76^{mn}	282.8 ± 0.48^{o}	70.6 ± .40^{n}

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

of KN decrease in shoot development was observed similar to *Plectranthus rotundifolius* (Asha et al. 2013). KN has been most frequently used for the in vitro shoot regeneration and multiplication of several plants (Akbar et al. 2003; Libin et al. 2012). MS medium supplemented with BA exhibited noticeable effect on shoot development in both explants but was less effective in shoot multiplication than KN. Similar effect of BA was previously recorded on *Mentha piperita* (Sunandakumari et al. 2004). TDZ responded similar to BA on shoot induction, while shoot development was comparatively low (Table 2).

Effect of auxins on shoot regeneration

The occurrence of cytokinin along with auxin is required for indirect adventitious shoot multiplication. Accordingly, the effect of auxins NAA, IAA, and IBA were tested along with the established best KN concentration (1.0 mg/l) for shoot multiplication. KN at

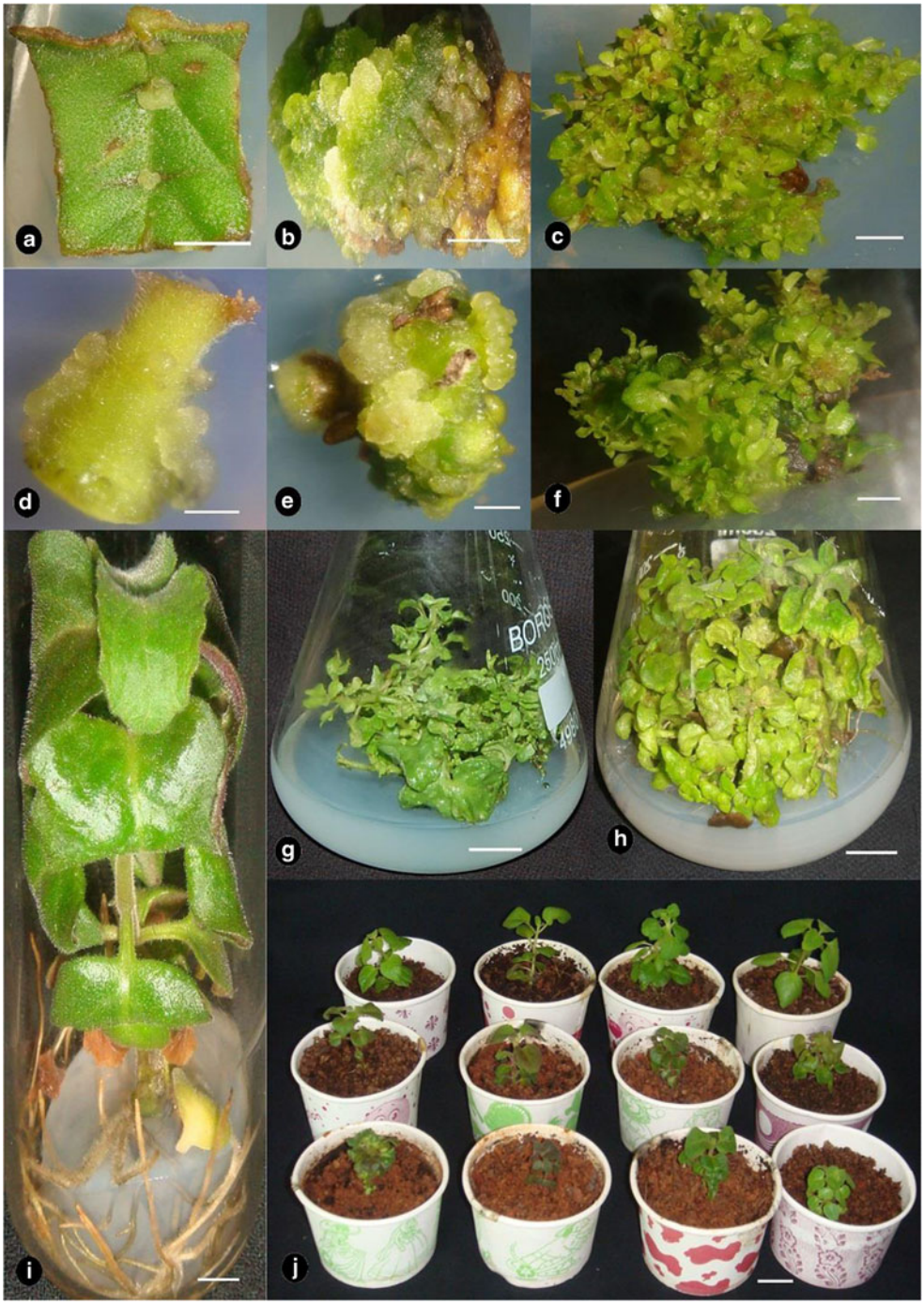

Fig. 1 Callus induction and plant regeneration from leaf and internode explants of *P. bourneae*. **a** leaf explant showing callus initiation, after 2 weeks of inoculation, **b** Callus proliferation on MS + NAA (1.0 mg/l) + BA (0.5 mg/l), after 4 weeks, **c** Shoot proliferation on MS + KN (1.0 mg/l), after 4 weeks of culture, **d** Internode explant showing callus initiation, after 2 weeks of inoculation, **e** Callus proliferation on MS + NAA (1.0 mg/l) + BA (0.5 mg/l), after 4 weeks, **f** Shoot proliferation on MS + KN (1.0 mg/l), after 4 weeks of culture, **g** & **h** Shoot multiplication and elongation on MS + KN (1.0 mg/l) + NAA (0.7 mg/l) + CH (50 mg/l) from internode and leaf derived callus, after 6 weeks of culture, **i** Rooting on MS + IBA (1.0 mg/l), after 3 weeks, **j** Hardening of in vitro raised plantlets, after 1 month. The *bar* represents 1.0 cm in **a**-**f**, 1.0 cm in **g**, 1.5 cm in **h**, 0.4 cm in **i** and 1.0 cm in **j**

1.0 mg/l in combination with 0.7 mg/l NAA responded highest shoot regeneration (89 %) with highest number of shoots (25.57) and shoot length (3.87) on leaf derived callus (Table 3). Similar results have also been observed in leaf explants of in *Peperomia obtusifolia* (Naggar and Osman 2014) and *Ziziphora tenuior* (Dakah et al. 2014). Likewise, internodal explants cultured on MS medium supplemented with KN (1.0 mg/l) and NAA (0.7 mg/l) showed higher regeneration frequency (85 %) and highest number (16.42) of shoots and shoot length (2.94). This result agreement with the report by Tsegaw and Feyissa (2014) who obtained the highest number of shoot multiplication on combination of higher concentration of KN and lower concentration of NAA in *Plectranthus edulis*. IAA was less effective in shoot production and the quality of shoots were also poor (Table 3). IBA combination on MS + KN 1.0 mg/l formed less number of shoots. The interaction between various growth regulators and the two explants revealed that, the 1.0 mg/l KN with 0.7 mg/l NAA was the best

Table 2 Shoot regeneration from callus of *P. bourneae* on MS medium supplemented with different concentrations of cytokinins, after 4 weeks of culture

Plant growth regulators (mg/l)				Regeneration frequency (%)		Mean number of shoots per callus		Shoot length (cm)	
control	BA	KN	TDZ	Leaf	Internode	Leaf	Internode	Leaf	Internode
0.0				00.00	00.00	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
	0.1			57.14^{bcd}	48.97^{cde}	3.85 ± 0.14^{f}	3.14 ± 0.26^{g}	1.30 ± 0.21^{h}	0.97 ± 0.35^{de}
	0.5			69.38^{ab}	63.26^{b}	7.85 ± 0.14^{b}	4.42 ± 0.20^{de}	2.12 ± 0.35^{c}	1.38 ± 0.40^{b}
	1.0			57.14^{bcd}	55.10^{bcd}	4.71 ± 0.18^{e}	4.57 ± 0.20^{cd}	1.68 ± 0.26^{e}	1.08 ± 0.93^{cd}
	1.5			53.06^{bcd}	44.89^{def}	3.71 ± 0.18^{f}	3.42 ± 0.36^{fg}	1.52 ± 0.18^{fg}	0.87 ± 0.42^{ef}
	2.0			48.97^{cd}	40.81^{ef}	3.14 ± 0.14^{g}	3.28 ± 0.14^{fg}	1.45 ± 0.36^{g}	0.77 ± 0.42^{fg}
		0.1		57.14^{bcd}	48.97^{cde}	5.28 ± 0.18^{d}	4.28 ± 0.18^{de}	1.50 ± 0.21^{fg}	1.11 ± 0.40^{cd}
		0.5		61.22^{bcd}	61.22^{b}	6.57 ± 0.20^{c}	5.57 ± 0.36^{b}	2.05 ± 0.29^{c}	0.90 ± 0.40^{ef}
		1.0		83.67^{a}	75.51^{a}	10.42 ± 0.29^{a}	6.42 ± 0.42^{a}	2.84 ± 0.42^{a}	1.70 ± 0.61^{a}
		1.5		67.34^{b}	57.14^{bc}	7.42 ± 0.20^{b}	4.00 ± 0.21^{ef}	2.35 ± 0.42^{b}	1.28 ± 0.12^{bc}
		2.0		65.30^{bc}	55.10^{bcd}	5.42 ± 0.20^{d}	3.14 ± 0.14^{g}	1.85 ± 0.36^{d}	0.71 ± 0.10^{gh}
			0.1	46.93^{d}	36.73^{f}	3.42 ± 0.20^{f}	2.14 ± 0.26^{h}	1.04 ± 0.29^{i}	0.42 ± 0.40^{i}
			0.5	57.14^{bcd}	48.97^{cde}	4.71 ± 0.18^{e}	3.28 ± 0.18^{fg}	1.25 ± 0.29^{h}	0.51 ± 0.55^{hi}
			1.0	63.26^{bcd}	61.22^{b}	7.57 ± 0.20^{b}	5.28 ± 0.18^{bc}	1.58 ± 0.55^{f}	1.31 ± 0.79^{bc}
			1.5	57.14^{bcd}	53.06^{bcd}	5.71 ± 0.18^{d}	4.28 ± 0.28^{de}	1.34 ± 0.29^{h}	1.10 ± 0.11^{cd}
			2.0	55.10^{bcd}	48.97^{cde}	3.85 ± 0.14^{f}	3.71 ± 0.18^{fg}	1.08 ± 0.40^{i}	0.81 ± 0.96^{fg}

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

Table 3 Shoot regeneration from callus of *P. bourneae* on MS medium supplemented with KN (1.0 mg/l) in combination with auxins, after 4 weeks of culture

Plant growth regulators (mg/l)				Regeneration frequency (%)		Mean number of shoots per callus		Shoot length (cm)	
control	NAA	IAA	IBA	Leaf	Internode	Leaf	Internode	Leaf	Internode
0.0				38.00^{f}	32.00^{h}	5.60 ± 0.30	3.28 ± 0.43	1.50 ± 0.43^{h}	1.36 ± 0.20^{gh}
	0.1			55.10^{de}	51.02^{fg}	12.42 ± 0.48^{def}	8.28 ± 0.96^{ef}	3.22 ± 0.11^{bcd}	1.98 ± 0.22^{def}
	0.5			67.34^{bcd}	53.06^{ef}	13.57 ± 0.75^{def}	12.42 ± 0.84^{bc}	3.14 ± 0.20^{cde}	2.42 ± 0.09^{bc}
	0.7			89.79^{a}	85.71^{a}	25.57 ± 1.04^{a}	16.42 ± 1.28^{a}	3.87 ± 0.15^{a}	2.94 ± 0.09^{a}
	1.0			69.38^{bcd}	75.51^{bc}	22.00 ± 0.61^{ab}	11.71 ± 0.77^{cd}	3.45 ± 0.19^{ab}	2.40 ± 0.08^{bc}
	1.5			65.30^{cd}	65.30^{cd}	18.42 ± 1.34^{bc}	9.28 ± 1.18^{def}	3.02 ± 0.16^{cde}	2.11 ± 0.15^{cd}
	2.0			59.18^{cde}	63.26^{de}	15.85 ± 0.88^{cde}	8.28 ± 0.80^{ef}	2.85 ± 0.11^{de}	1.81 ± 0.10^{fg}
		0.1		48.97^{e}	46.93^{g}	9.57 ± 0.42^{f}	6.85 ± 0.55^{f}	3.14 ± 0.19^{bcd}	1.87 ± 0.21^{ef}
		0.5		71.42^{bc}	61.22^{de}	11.28 ± 0.77^{def}	11.28 ± 1.08^{cde}	3.07 ± 0.10^{bcd}	2.40 ± 0.07^{bc}
		0.7		63.26^{cd}	63.26^{de}	13.57 ± 0.61^{def}	11.14 ± 0.88^{cde}	2.95 ± 0.13^{de}	2.12 ± 0.04^{cd}
		1.0		59.18^{cde}	77.55^{ab}	15.42 ± 2.36^{cde}	8.85 ± 0.93^{def}	2.70 ± 0.12^{ef}	1.92 ± 0.10^{def}
		1.5		55.10^{de}	65.30^{cd}	13.28 ± 2.11^{def}	7.85 ± 0.88^{f}	2.34 ± 0.11^{fg}	1.77 ± 0.14^{fg}
		2.0		55.10^{de}	57.14^{ef}	10.00 ± 1.36^{f}	7.00 ± 0.61^{f}	2.15 ± 0.12^{g}	1.57 ± 0.10^{g}
			0.1	63.26^{cd}	51.55^{fg}	11.71 ± 0.71^{f}	9.85 ± 0.88^{cde}	2.87 ± 0.17^{de}	2.31 ± 0.07^{cd}
			0.5	79.59^{ab}	75.51^{ab}	15.71 ± 2.63^{cde}	15.00 ± 1.17^{ab}	3.25 ± 0.23^{bc}	2.71 ± 0.13^{ab}
			0.7	67.34^{bcd}	67.34^{cd}	13.57 ± 1.98^{def}	11.57 ± 0.84^{cd}	2.94 ± 0.18^{de}	2.31 ± 0.07^{cd}
			1.0	63.26^{cd}	61.22^{de}	13.57 ± 0.84^{def}	11.71 ± 1.06^{cd}	3.11 ± 0.20^{bcd}	2.14 ± 0.08^{cde}
			1.5	67.34^{bcd}	55.10^{fg}	12.57 ± 1.25^{def}	9.00 ± 0.57^{de}	2.84 ± 0.14^{de}	1.82 ± 0.10^{ef}
			2.0	59.18^{cde}	51.02^{fg}	10.28 ± 0.64^{f}	8.42 ± 0.86^{ef}	2.62 ± 0.12^{ef}	1.71 ± 0.07^{fg}

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

Table 4 Influence of Adenine sulphate, Casein hydrolysate, Citric acid and Yeast extract along with KN (1.0 mg/l) + NAA (0.7 mg/l) on shoot regeneration from callus, after 6 weeks of culture

Additives (mg/l)					Percentage of response		Mean number of shoots		Shoot length (cm)	
control	AC	AA	AdS	CH	Leaf	Internode	Leaf	Internode	Leaf	Internode
00					40.10^{h}	33.46^{g}	6.32 ± 0.57^{j}	3.40 ± 0.62^{hi}	2.00 ± 0.10^{j}	1.30 ± 0.13^{ef}
	25				51.02^{g}	40.81^{f}	9.14 ± 0.67^{ef}	5.00 ± 0.57^{h}	2.60 ± 0.61^{gh}	1.92 ± 0.05^{e}
	50				63.26^{de}	53.06^{de}	10.57 ± 0.84^{hi}	12.14 ± 0.50^{c}	2.88 ± 0.10^{e}	2.25 ± 0.06^{d}
	75				65.30^{cd}	59.18^{de}	12.57 ± 0.64^{hi}	8.28 ± 0.64^{fg}	2.31 ± 0.82^{ij}	1.91 ± 0.09^{e}
	100				53.06^{fg}	53.06^{ef}	10.28 ± 0.68^{i}	7.28 ± 0.77^{g}	2.08 ± 0.76^{j}	1.90 ± 0.07^{e}
		25			51.02^{g}	46.10^{ef}	11.14 ± 0.55^{hi}	8.85 ± 0.55^{fg}	2.40 ± 0.12^{hi}	1.90 ± 0.04^{e}
		50			61.22^{ef}	57.14^{de}	13.85 ± 1.18^{ef}	10.85 ± 0.50^{cd}	2.34 ± 0.61^{ij}	2.88 ± 0.07^{b}
		75			65.30^{cd}	67.34^{bc}	14.85 ± 0.45^{ef}	15.42 ± 0.89^{b}	3.21 ± 0.55^{e}	2.54 ± 0.13^{c}
		100			57.14^{ef}	55.10^{de}	13.71 ± 0.47^{fg}	7.85 ± 0.79^{f}	2.62 ± 0.13^{fg}	2.21 ± 0.09^{de}
			25		63.26^{de}	51.02^{ef}	16.42 ± 0.99^{de}	11.28 ± 0.56^{cd}	2.57 ± 0.74^{gh}	1.94 ± 0.12^{e}
			50		71.42^{cd}	59.18^{de}	13.85 ± 0.63^{ef}	14.85 ± 0.76^{b}	3.65 ± 0.81^{d}	3.74 ± 0.14^{a}
			75		75.51^{bc}	65.30^{cd}	21.00 ± 0.69^{c}	9.14 ± 0.59^{ef}	3.35 ± 0.92^{e}	2.68 ± 0.14^{bc}
			100		63.26^{de}	53.06^{de}	10.28 ± 0.52^{i}	4.85 ± 0.55^{h}	2.75 ± 0.11^{fg}	1.92 ± 0.06^{e}
				25	65.30^{cd}	55.10^{de}	18.42 ± 1.92^{d}	11.14 ± 0.45^{cd}	3.25 ± 0.81^{e}	2.71 ± 0.14^{bc}
				50	91.83^{a}	81.63^{a}	29.71 ± 0.52^{a}	19.71 ± 0.68^{a}	4.95 ± 0.71^{a}	3.75 ± 0.08^{a}
				75	79.59^{b}	69.38^{ab}	23.71 ± 1.04^{b}	12.57 ± 0.52^{c}	4.70 ± 0.69^{b}	2.90 ± 0.09^{b}
				100	71.42^{cd}	63.26^{cd}	17.85 ± 0.40^{d}	9.57 ± 0.48^{de}	4.22 ± 0.11^{c}	2.68 ± 0.06^{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

combination for shoot multiplication and elongation in *P. bourneae*. The consistency of inducing shoot elongation by NAA was previously reported in other plants such as, *Fuchsia magellanica* (Parveen and

Table 5 *In vitro* rooting of callus derived shoots of *P. bourneae* on MS medium supplemented with different concentrations of auxins, after 3 weeks of culture period

Growth regulators (mg/l)				Percentage of response	Mean number of roots	Mean root length (cm)
control	IAA	IBA	NAA			
0.0				18.04^{f}	1.82 ± 0.10^{k}	1.00 ± 0.22^{i}
	0.1			51.02^{de}	5.42 ± 0.36^{i}	4.05 ± 0.25^{g}
	0.5			55.10^{de}	7.57 ± 0.36^{h}	6.77 ± 0.47^{d}
	1.0			63.26^{cde}	8.71 ± 0.18^{f}	9.02 ± 0.30^{b}
	1.5			53.06^{de}	6.00 ± 0.72^{fg}	6.31 ± 0.26^{de}
	2.0			48.97^{e}	4.28 ± 0.35^{j}	4.74 ± 0.35^{gh}
		0.1		59.18^{cde}	8.00 ± 0.30^{gh}	6.38 ± 0.19^{de}
		0.5		67.34^{bcd}	15.71 ± 0.35^{b}	6.58 ± 0.19^{de}
		1.0		97.75^{a}	19.14 ± 0.34^{a}	10.85 ± 0.29^{a}
		1.5		65.30^{cde}	14.85 ± 0.26^{b}	7.77 ± 0.15^{c}
		2.0		59.18^{cde}	10.28 ± 0.35^{e}	6.97 ± 0.12^{d}
			0.1	53.06^{de}	5.42 ± 0.20^{i}	5.80 ± 0.16^{ef}
			0.5	59.18^{cde}	8.14 ± 0.34^{gh}	5.91 ± 0.06^{ef}
			1.0	75.51^{abc}	11.57 ± 0.36^{d}	6.31 ± 0.11^{de}
			1.5	81.63^{ab}	13.14 ± 0.34^{c}	5.85 ± 0.22^{ef}
			2.0	67.34^{bcd}	9.71 ± 0.28^{ef}	5.35 ± 0.45^{fg}

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

Rasheed 2013) and *Cajanus cajan* (Lakshmi Sita and Venkatachalam 2008). The proficiency to regenerate shoots from callus has various benefits. Numerous shoots can be formed from an explant through callus induction and shoot development.

Influence of additives on shoot regeneration

Shoot multiplication and development of *P. bourneae* was highly influenced by additives. Four different additives AA, AC, AdS and CH were tested further on callus derived shoots to enhance the shoot multiplication frequency. The maximum number of 29.71 and 19.71 shoots were obtained from leaf and internode derived callus, at 50 mg/l of CH supplemented medium (Table 4, Fig. 1g and h). CH is an intricate assortment of amino acids and ammonium salts, which together with the combination of KN (1.0 mg/l) and NAA (0.7 mg/l) supported to increase the shoot multiplication rate in *P. bourneae*. Similar effect of CH have also been reported in *Stevia rebaudiana* (Sridhar and Aswath 2014), *Digitalis lanata* (Fatima et al. 2009). The shoots cultured on medium containing AC, failed to develop more number of multiple shoots, because AC is known to absorb high concentrations of growth regulators and may reduce shoot regenerative response (Constantin et al. 1977; Weatherhead et al. 1978). The AA also failed to enhance the multiple shoots, a maximum number of 15.42 and 14.85 shoots were produced from the shoot explant derived from internode and leaf callus respectively. Even though, AdS did not enhance the multiple shoots, at 75 mg/l it was marginally contributed to shoot multiplication (21.00) from leaf callus derived shoots.

The higher frequency of callus formation and shoot bud regeneration ability of leaf over internode explant is related to the densely packed meristamatic cells possessing higher proliferation ability. Similar result of high callus formation and shoot bud regeneration was recorded on the leaf explant in *P. barbatus* (Thangavel et al. 2014b). Subculture of leaf and internode derived callus on 1.0 mg/l KN, 0.7 mg/l, NAA and 50 mg/l CH yielded a higher number of adventitious shoots which increased gradually from subculture to subculture. On this medium, from the initial stage of 4–6 regenerated shoot buds (2 weeks), a cluster of 25–30 shoots were produced from leaf and 15–20 shoots were produced from internode derived callus within 6 weeks.

Overall, leaf derived callus showed more regeneration ability than the internode. This may be attributed to the variations of endogenous growth regulator levels in the explant or the difference in tissue sensitivities to these plant growth regulators (Lisowska and Wysokinska 2000).

Rooting of regenerated shoots

In vitro root induction of shoots occurred on half MS medium containing IBA, NAA and IAA, within a week period followed by efficient root system formation in 15 days. The maximum rooting response (97.75 %) and number of roots per shoot (19.14) was recorded on ½ MS medium containing 1.0 mg/l IBA (Table 5, Fig. 1i). The superiority of IBA over other auxins on in vitro rooting has been reported in *Plectranthus ambonicus* (Rhaman et al. 2015) and *Glinus lotoides* (Teshome and Feyissa 2015). The number of roots per shoot was increased with increasing concentration of IBA from 0.1 to 1.0 mg/l, whereas subsequent concentrations decreased the frequency of rooting. NAA at 1.5 mg/l presented second highest rooting response (81.63 %) as good as IBA. IAA at all concentrations, showed lower response of root formation, whereas highest length of roots (9.02 cm) formed only in IAA at 1.0 mg/l. Of the three auxins used, IBA was the most effective for rooting in *P. bourneae*. The probable reason could be that, IBA is more insistent than IAA or NAA to chemical degradation in tissue culture media, both during auto-claving and at room temperature (Cuenca et al. 1999).

Acclimatization of regenerated plants

Well rooted plantlets (70 plantlets) were successfully hardened and acclimatized with a maximum survival of 90 % (63 plantlets) in vitro. After a month of hardening plantlets were transferred to greenhouse condition, where 56 (80 %) of the rooted plantlets survived and showed no morphological variations (Fig. 1j).

Conclusion

This is the first report describing a protocol for indirect organogenesis of *P. bourneae*. The present study demonstrates a reliable method for shoot regeneration through leaf and internode derived callus cultures. Prominent callus response from leaf explant was observed under the influence of BA and NAA combinations. A combination of 1.0 mg/l KN, 0.7 mg/l, NAA and 50 mg/l CH was effective for multiple shoot production from leaf and internode derived callus. Best rooting response was achieved on half-strength MS medium supplemented with 1.0 mg/l IBA and the plantlets were successfully hardened and established in the field. The organogenesis and plant regeneration system developed in this study could be utilized in future for the in vitro production, transformation and secondary metabolites production studies for this genus.

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