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In vitro Propagation of *Plectranthus bourneae* Gamble- An Endemic Red Listed Plant

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Key words: *Plectranthus bourneae*, Endemic plant, Lamiaceae, *in vitro* propagation, axillary bud.

Abstract

An efficient protocol of *in vitro* propagation of *Plectranthus bourneae* Gamble (Lamiaceae), a valuable medicinal important and endemic Red listed plant of Western Ghats, (Tamil Nadu, India) was standardized by improved shoot multiplication from axillary bud explant. An *in vitro* propagation system has been reconnoitered on MS with the effective concentration BA (0.7 mg/l) followed by a combination of BA (0.7 mg/l) and TDZ (1.0 mg/l) which promoted high number of shoots. The multiple shoot rate was enhanced further by adding AdS (50 mg/l). Beneficial shoot length was achieved when cultured on MS containing GA_3 (0.5 mg/l). Rooting was increased on MS augmented with IBA (1.5 mg/l). Micropropagated plants were acclimatized and the survival rate was 80%. Acclimatized *P. bourneae* plants can be used as substitute alternative to natural populations. Using this protocol the propagated plants can be used for conservation strategies.

Introduction

The genus *Plectranthus* (Lamiaceae) comprises of about 300 species distributed through the tropical and warm regions of the Old World including Africa, India and Australia (Retief 2000). *Plectranthus* species are mostly soft, low growing, semi-succulent to succulent herbs or shrubs. These species are used for medicinal purposes against vomiting and nausea, to relive toothache, headache, sores, and burns, or as antiseptic, respiratory diseases (Rivera and Obon 1992a, b, Vera et al.

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1993, Bown 1995 and Lukhoba et al. 2006). The leaf extract of *P. tenuiflorus* is used to treat ear infections in Saudi Arabia (Abulfaith et al. 1987). *P. cylindraceus* has a pleasant aroma similar to commercial product Vicks (Marwah et al. 2007) and its leaves used as a body disinfectant and deodorant (Miller et al. 1998).

Plectranthus bourneae Gamble is an endemic plant species, has very restricted distribution only in the Western Ghats of Tamil Nadu, India. It is located in Pambar Shola, Kodaikanal, Tamil Nadu (Matthew 1993 and 1998). The plant is a well branched compact shrub (Fig. 1a). This species is in highly vulnerable status due to habitat destruction (Matthew 1999) and is included in the Red List of India (Nayar et al. 1990). Hence, a need for the development of alternative mass propagation system for this valuable species. Conventional enhancement methods and *in vitro* practices can be employed in further improvement of valuable *Plectranthus*. The conventional method of propagating this species is through seeds, but seed viability is very poor and low germinating limits its multiplication.

Plant tissue culture (PTC) offers a viable alternative method of propagation. It is used widely in the conservation of rare, endangered and medicinal plants and also commercial propagation (Rout et al. 2000). PTC constitutes a way to maintain available competent explants and free from contamination for use *in vitro* propagation and genetic transformation, besides being highly convenient for conservation of *in vitro* germplasm and cryopreservation. Very few reports are available on *in vitro* propagation of genus *Plectranthus* species such as *P. ventiveroides* (Sivasubramanian et al. 2002), *P. barbatus* (Thangavel et al. 2011). The aim of this study was to establish an efficient and reproducible *in vitro* propagation method from axillary bud of *P. bourneae*.

Materials and Methods

The plants of *P. bourneae* were collected from Pambar Shola of Kodaikanal hills (10º 5′-10º 25′ N, 77º 50′ E at an altitude of 2020 m) in the Western Ghats of Tamil Nadu, India and maintained in earthen pots in the glass house of Bharathidasan University, Tiruchirappalli under controlled condition (26 ± 2°C and RH 70%). Axillary bud explants (1 cm) were washed under running tap water for 30 min and decontaminated with 70% ethanol for 30s followed by 0.1% bavistin for 5 min and 3% NaOCl for 2 min. The explants were washed thoroughly with sterile distilled water minimum thrice. These sterilized explants were inoculated on culture medium.

MS was 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 with 0.1 N NaOH or 0.1 N HCl after addition of the growth regulators. The

medium was autoclaved at 121°C, 15 lb pressure for 15 min. All the cultures were maintained in aseptic growth room conditions [26 ± 2°C, under 16/8 hrs light regime provided by cool white fluorescent light (60 μmol^{-2} sec^{-1} light intensity) and with 55 to 60% relative humidity].

Axillary bud explants were inoculated on MS medium containing different concentrations (0.1 mg/l to 2.0 mg/l) of cytokinins BA, Kn, TDZ and 2-iP alone and in combination for shoot initiation and multiplication. Data on frequency of shoot induction, multiple shoot induction, number and length of shoots were recorded after six weeks of culture.

Influence of various additives was studied after determining the optimum cytokinin combination level for the shoot bud induction to enhance the range of multiple shoots. Explants were cultured on MS supplemented with BA (0.7 mg/l) and TDZ (1.0 mg/l) along with different concentrations (25, 50, 100 mg/l) of adenine sulphate (AdS), glutamine, citric acid (CA) and yeast extract (YE) were used to study for the efficiency of shoot multiplication.

The microshoots (~1 cm) obtained from the above experiments were transferred to shoot elongation medium (MS) containing GA_3 (0.1, 0.5, 1.0, 1.5 and 2.0 mg/l). MS devoid of plant growth regulators (PGRs) was used as control.

For rooting, *in vitro* raised shoots (5 - 8 cm) were harvested after six weeks and then inoculated on half strength MS supplemented with different levels (0.5, 1.0, 1.5 and 2.0 mg/l) of IBA, IAA and NAA. Rooting was observed from 10 to 20 days. MS devoid of PGRs was used as control. Rooting percentage, number, and length were recorded after four weeks of culture. The rooted plantlets were washed and subsequently transferred to red soil: sand: coconut coir (1: 1: 1) mixture. Potted plants were covered with transparent polythene membrane under controlled growth chamber conditions of 26 ± 2ºC, 16 hrs photoperiod, 80-85% relative humidity and 60 $\mu mol^{-2}s^{-1}$ light intensity. They were frequently irrigated with sterile water every three days for four weeks. Plantlets were transplanted to the field.

All the experiments were conducted with a minimum of ten replicates per treatment and each experiment was repeated three times. DMRT was performed using SPSS version 16.0, mean and standard error (SE) followed by different letters were assigned to denote significance with $p < 0.05$.

Result and Discussion

Axillary bud explants were used for shoot initiation and multiplication (Fig. 1b). Shoot initiation was achieved in MS containing various concentrations of BA, Kn, 2-iP and TDZ (Fig. 1c). No regeneration was observed in control treatment.

Among four cytokinins, BA (0.7 mg/l) produced 3.53 shoots/explant (Table 1). The BA higher than 0.7 mg/l reduced shoot number and frequency. For bud break Kn was found best and BA responded better than Kn in terms of shoot

Fig. 1. *In vitro* propagation of *P. bourneae* (a) Habitat of *P. bourneae*, (b) Axillary bud explant. (c) Initiation of shoots from axillary bud explant. (d) Multiple shoots on MS with BA 0.7 mg/l + TDZ 1.0 mg/l+ AdS 50 mg/l. (e) Shoot elongation. (f) Root formation on half strength of MS with IBA 1.5 mg/l. (g) Hardened *in vitro* plant.

Table 1. **Shoot bud initiation from axillary bud explants of *P. bourneae* on MS supplemented with cytokinins, after 45 days.**

Cytokinin (mg/l)	Shoots/explant	Shoot length (cm)	Frequency of response (%)
BA			
0.1	1.87 ± 0.12^{de}	0.70 ± 0.09^{j}	42.85 ± 2.63^{hi}
0.5	2.87 ± 0.47^{b}	1.27 ± 0.15^{de}	59.18 ± 3.72^{de}
0.7	3.53 ± 0.46^{a}	3.08 ± 0.37^{a}	87.75 ± 2.85^{a}
1.0	2.37 ± 0.18^{bcd}	1.50 ± 0.25^{cd}	73.46 ± 3.72^{bc}
1.5	1.75 ± 0.16^{ef}	1.58 ± 0.20^{bc}	67.34 ± 2.08^{bc}
2.0	1.37 ± 0.18^{g}	1.55 ± 0.09^{bc}	51.02 ± 2.88f^{gh}
KN			
0.1	1.87 ± 0.12^{de}	2.07 ± 0.13^{b}	57.14 ± 3.11^{de}
0.5	1.87 ± 0.12^{de}	1.72 ± 0.17^{bc}	65.30 ± 2.24^{cde}
0.7	2.00 ± 0.00^{cd}	1.88 ± 0.17^{bc}	61.22 ± 2.08^{de}
1.0	2.50 ± 0.26^{bc}	1.84 ± 0.14^{bc}	77.55 ± 2.24^{ab}
1.5	2.00 ± 0.00^{cde}	1.77 ± 0.17^{bcd}	63.26 ± 2.88^{cde}
2.0	2.00 ± 0.00^{cde}	1.61 ± 0.09^{bcd}	48.97 ± 2.88^{gh}
2iP			
0.1	1.75 ± 0.16^{ef}	0.97 ± 0.16^{hi}	22.44 ± 3.26^{k}
0.5	1.37 ± 0.18^{g}	0.95 ± 0.07^{hi}	32.65 ± 4.08^{ij}
0.7	1.62 ± 0.18^{fg}	1.20 ± 0.08^{ef}	32.65 ± 2.63^{ij}
1.0	2.00 ± 0.00^{cd}	0.95 ± 0.07^{hi}	34.69 ± 2.88^{ij}
1.5	1.62 ± 0.18^{fg}	1.07 ± 0.11^{gh}	36.73 ± 2.88^{i}
2.0	1.75 ± 0.25^{ef}	1.04 ± 0.15^{gh}	24.48 ± 2.63^{jk}
TDZ			
0.1	1.62 ± 0.18f^{g}	1.24 ± 0.14^{de}	55.10 ± 2.04^{efg}
0.5	2.25 ± 0.16^{de}	0.94 ± 0.16^{ij}	59.18 ± 3.72^{de}
0.7	1.87 ± 0.12^{de}	1.07 ± 0.12^{fg}	65.30 ± 2.88^{cde}
1.0	2.62 ± 0.26^{bc}	1.28 ± 0.08^{de}	69.38 ± 2.85^{bc}
1.5	2.37 ± 0.26^{bcd}	1.05 ± 0.17^{gh}	65.30 ± 2.88^{cde}
2.0	2.12 ± 0.12^{cde}	1.42 ± 0.04^{cd}	48.97 ± 2.88^{g}

initiation and multiplication. Comparing the number of shoots using optimal concentration of Kn (1.0 mg/l) with that using BA (Table 1), it was clear that fewer shoots were produced by using Kn. BA was the most effective to overcome apical dominance, and promote multiple shoot formation (Fatima et al. 2012). TDZ induced the formation of shoots at all concentration. Moreover, the proportion of stunted shoots increased with increased concentration. When 2-iP

was used alone, a slight basal callusing was observed with a low regeneration frequency. Similar result was also reported by Khattab (2011) and Rafique Ahmed et al. (2013). In the present study, optimal concentration of 0.7 mg/l BA was selected further and used in combination with Kn, 2-iP and TDZ to enhance shoot multiplication rate. Similarly many workers reported stimulatory effect of BA on multiple shoot induction in other medicinally important plants species such as *Salacia reticulata* (Dhanasri et al. 2013) and *Decalepis hamiltonii* (Sharma et al. 2014).

Values represent means ±SE. Values followed by the same letter are not significantly different at $p < 0.05$ according to DMRT. In this study, 2-iP produced lower shoot regeneration than other combinations. High shoot formation (12.14 ± 0.55) was observed in the concentration of BA (0.7 mg/l) and TDZ (1.0 mg/l) and it can be attributed to the synergistic effect of the combination (Table 2). The combination of BA and TDZ showed an increase in shoot numbers and decreased at higher concentration. It is widely documented by many researchers that TDZ is equal in effective than BA (Tawfik and Mohamed 2005, Nagar et al. 2015). The result produced on BA and TDZ combination was vastly greater than any other cytokinin single or combination. BA combined with Kn showed appropriate result of shoot multiplication, however, it was not as efficient as TDZ (Table 2). Frequently, cytokinins have a major role on plant development, such as the regulation of shoot development and multiplication and the improvement of cell division and expansion (Mok and Mok 2001).

Effect of additives like adenine sulphate, glutamine, citric acid, yeast extract in different concentrations of 25 to 100 mg/l were tested in combination with BA (0.7 mg/l) and TDZ (1.0 mg/l) to enhance *in vitro* shoot multiplication after determining optimal cytokinins level for shoot proliferation, multiplication and quality of shoots. The medium supplemented with AdS showed significant increase in the rate of shoot multiplication. Benefits are often only observed when adenine is administered together with a cytokinin (George et al. 2007). In our study, we found 50 mg/l of AdS was highly effective for enhancing shoot multiplication rate (19.57 ± 0.64) (Table 3, Fig. 1d). AdS can augment cell growth and shoot multiplication feasibly by performing as organic nitrogen source and or as precursor for natural cytokinin production. The stimulative role of AdS in shoot multiplication has been reported by many workers (Husain et al. 2008, Siwach et al. 2011). Nitrogen is the vital factor for appropriate plant growth and development.

The efficacy of organic nitrogen source like glutamine exhibit moderate number of shoots (15.14 ± 0.70) due to its stimulative effect, glutamine directly

involved in the assimilation of ammonia. The nitrates and ammonium salts have been widely used for *in vitro* studies as nitrogen source, many reports suggest the use of adenines and amino acids can adjust the nitrogen utilization of *in vitro* growth regulation in many plant species (Shrivastava et al. 2008, Jayakumar et al. 2013). CA controls browning of culture medium and also plays a significant role in the multiplication of shoots in many plants. Accordingly, their ability on shoot multiplication was examined. CA in combination with BA (0.7 mg/l) + TDZ (1.0 mg/l) enhance shoot multiplication frequency and number of shoot production per explant was lower when compared to that of AdS and glutamine (Table 3). YE did not significantly improve the shoot development frequency, shoot number and shoot length. At higher concentration of YE browning of tissues resulted with the suppression of shoot proliferation (Sridhar et al. 2014). The shoots (1 - 2 cm) were excised from the axillary bud shoot clusters raised from BA (0.7 mg/l), TDZ (1.0 mg/l) and AdS (50 mg/l) combination, and were elongated on MS media with various concentrations of GA_3 (0.1 - 2.0 mg/l).

Among various combinations used GA_3 0.5 mg/l produced maximum shoot length (5.87 ± 0.31 cm**)** and number of leaves per shoot (11.70 ± 0.15). When the GA_3 concentration was increased, the shoot length also increased up to the optimum level (1.5 mg/l) afterwards it decreased with further increased. However, shoot length and frequency considerably decreased in higher concentration of GA_3 (Table 4, Fig. 1e). Similar results also were reported in *Graptophyllum pictum* (Justin and Wilson 2010) and *Andrographis echoides* (Hemalatha and Vadivel 2010). Following this experiment elongated shoots were transferred to root induction medium.

Elongated shoots (4 - 6 cm) were excised and placed on half and full strength MS supplemented with various concentrations of IBA, IAA and NAA for root induction. Full strength MS containing auxins showed very poor response in rooting, but half strength MS supplemented with auxins were achieved well developed roots within 20 days. All the micro-cuttings show rooting response against all the three auxins tested. Half strength MS fortified with different concentrations of IBA (1.5 mg/l) had radical effect and their supremacy was absolute for all the other treatments, followed by different concentrations of NAA and IAA, respectively. The current results were further confirmed by the previous findings of *Trichosanthes dioica* (Awal et al. 2005) and Ginger (Rajani and Patil 2009), who suggested IBA as the best auxin for root induction and development in various plant species. Among the different auxins, IBA was found to be most effective for root induction where NAA gave

moderate number of roots. The highest number of roots per shoot (12.85 ± 0.82), root length per shoot (5.11 ± 0.28) and high frequency was obtained in IBA (91.83 ± 4.24). Further increase in auxin concentration showed a decrease in the frequency, number of roots and root length (Table 5, Fig. 1f).

Table 2. Shoot multiplication from shoot buds initiated of axillary bud explants of *P.bourneae* on MS medium supplemented with BA (0.7 mg/l) and other cytokinins, after 45 days.

Growth regulators (mg/l)	Shoots/ explant	Shoot length (cm)	Frequency of response (%)
Kn			
0.5	4.14 ± 0.26^{cd}	2.92 ± 0.31^{ab}	55.10 ± 2.04^{ef}
1.0	6.42 ± 0.36^{b}	3.22 ± 0.24^{a}	71.42 ± 3.11^{bc}
1.5	4.14 ± 0.40^{cd}	2.34 ± 0.13^{cd}	53.06 ± 2.63^{ef}
2.0	3.71 ± 0.28^{d}	2.55 ± 0.21^{bc}	48.97 ± 2.88^{f}
TDZ			
0.5	7.85 ± 1.14^{b}	1.74 ± 0.12^{de}	63.26 ± 2.88^{cd}
1.0	12.14 ± 0.55^{a}	1.90 ± 0.15^{de}	85.71 ± 3.11^{a}
1.5	6.71 ± 1.45^{b}	1.60 ± 0.11^{e}	73.46 ± 3.72^{b}
2.0	6.00 ± 0.61^{b}	1.54 ± 0.05^{e}	59.18 ± 3.72^{de}
2iP			
0.5	2.85 ± 0.26^{d}	2.35 ± 0.30^{cd}	40.81 ± 3.73^{h}
1.0	2.71 ± 0.28^{d}	2.12 ± 0.20^{cd}	38.77 ± 2.08^{h}
1.5	3.28 ± 0.28^{d}	1.64 ± 0.23^{e}	46.93 ± 2.08^{gh}
2.0	3.00 ± 0.30^{d}	1.61 ± 0.23^{e}	42.85 ± 2.40^{gh}

Values represent means ± SE. Values followed by the same letter are not significantly different at $p<0.05$ according to DMRT.

Plantlets were removed from the rooting medium, washed properly under running tap water to remove any adherent gel and transferred to paper cups (6.5 cm diameter) containing sterilized soil (Fig. 1g). Paper cups were covered with polythene bag and kept for 15 days. Small holes were made on the polythene bag to reduce the relative humidity. Slowly the width of the holes was increased until the relative humidity inside the polythene bag and outside the chamber comes to equal. The plants were maintained under controlled temperature (25 ± 2°C) with 90% humidity. Finally polythene bags were removed and the pots were kept in

Table 3. Shoot multiplication from shoot buds initiated from axillary bud explants of *P. bourneae* on MS supplemented with BA (0.7 mg/l), TDZ (1.0 mg/l) and additives, after 45 days.

Additives (mg/l)	Shoot length (cm)	Shoots/ explants	Frequency of response (%)
Adenine sulphate			
25	1.10 ± 0.24^{c}	10.42 ± 0.42^{d}	66.00 ± 1.63^{bc}
50	1.94 ± 0.31^{a}	19.57 ± 0.64^{a}	92.00 ± 1.33^{a}
100	1.11 ± 0.20^{c}	9.85 ± 0.50d^{e}	64.00 ± 1.63^{c}
Glutamine			
25	1.00 ± 0.13^{c}	8.71 ± 0.35^{ef}	43.00 ± 1.52^{ef}
50	1.90 ± 0.04^{a}	15.14 ± 0.70^{b}	70.00 ± 1.49^{b}
100	1.45 ± 0.40^{b}	13.42 ± 0.68^{c}	58.00 ± 1.33^{d}
Citric acid			
25	1.15 ± 0.15^{c}	9.57 ± 0.48d^{e}	32.00 ± 1.33^{g}
50	1.12 ± 0.26^{c}	7.57 ± 0.20^{fg}	43.00 ± 1.52^{ef}
100	0.67 ± 0.20^{d}	7.28 ± 0.52^{gh}	42.00 ± 1.33^{f}
Yeast extract			
25	1.58 ± 0.23^{b}	7.14 ± 0.26^{gh}	33.00 ± 1.52^{g}
50	1.45 ± 0.19^{b}	6.00 ± 0.30^{hi}	47.00 ± 1.52^{e}
100	1.14 ± 0.26^{b}	5.57 ± 0.29^{j}	34.00 ± 1.63^{g}

Values represent means ± S.E. Values followed by the same letter are not significantly different at $p \leq 0.05$ according to DMRT.

Table 4. Effect of different concentrations of GA_3 on *in vitro* shoot elongation.

Growth regulator GA_3 (mg/l)	No. of nodes/ explant	Shoot length (cm)	No. of leaves/ explant
0.1	3.60 ± 0.16^{c}	2.40 ± 0.16^{d}	7.70 ± 0.48^{c}
0.5	7.60 ± 0.16^{a}	5.87 ± 0.31^{a}	11.70 ± 0.15^{a}
1.0	5.60 ± 0.16^{b}	4.67 ± 0.27^{b}	9.40 ± 0.30^{b}
1.5	3.70 ± 0.15^{c}	3.92 ± 0.19^{c}	7.60 ± 0.16^{c}
2.0	2.50 ± 0.16^{d}	2.27 ± 0.15^{d}	5.80 ± 0.13^{d}

Values represent means ± SE. Values followed by the same letter are not significantly different at $p < 0.05$ according to DMRT.

diffused light for 15 days and finally plants were shifted to pots for acclimatization and they were exposed to the sunlight. The percentage of plant survival was calculated after two months. The *in vitro* rooted plantlets were

successfully acclimatized with around 80% survival rate. The regenerated plants did not show any noticeable morphological variations when compared with mother plant. Frequent observations were made to inspect drying of plant parts and they were slowly introduced to a glass house environment.

Table 5. Rooting of shoots obtained from axillary bud explants of *P.bourneae* on half strength MS medium supplemented with auxins, after 20 days. [[

Auxins (mg/l)	Frequency of rooting (%)	No. of roots/ explant	Root length (cm)
IAA			
0.5	46.93 ± 2.63^{c}	3.57 ± 0.20^{cde}	3.48 ± 0.11^{cd}
1.0	51.02 ± 2.88^{c}	3.42 ± 0.20^{cde}	3.52 ± 0.12^{cd}
1.5	51.02 ± 2.88^{c}	2.85 ± 0.26^{de}	3.30 ± 0.04^{cde}
2.0	46.93 ± 2.63^{c}	2.42 ± 0.20^{e}	2.87 ± 0.17^{e}
IBA			
0.5	71.42 ± 2.24^{b}	4.14 ± 0.26^{c}	4.14 ± 0.14^{b}
1.0	63.26 ± 2.88^{b}	6.57 ± 0.29^{b}	3.68 ± 0.10^{bc}
1.5	91.83 ± 4.24^{a}	12.85 ± 0.82^{a}	5.11 ± 0.28^{a}
2.0	65.30 ± 2.88^{b}	5.57 ± 0.52^{bh}	3.47 ± 0.11^{cd}
NAA			
0.5	44.89 ± 2.04^{c}	3.57 ± 0.20^{cde}	3.51 ± 0.22^{cd}
1.0	46.93 ± 2.63^{c}	4.00 ± 3.77^{cd}	3.54 ± 0.25^{cd}
1.5	42.85 ± 3.11^{cd}	3.28 ± 0.28^{cde}	3.51 ± 0.10^{cd}
2.0	34.76 ± 1.82^{d}	2.57 ± 0.20^{e}	3.02 ± 0.18^{e}

Values represent means ± SE. Values followed by the same letter are not significantly different at $p < 0.05$ according to DMRT.

The present study demonstrates that axillary bud explants of *Plectranthus bourneae* are a good starting material for *in vitro* propagation. Combination of BA, TDZ and AdS was more effective in shoot multiplication and GA_3 enhanced shoot elongation. Best rooting was achieved with IBA. IBA can be considered for root production in *P. bourneae*. The present micropropagation system can be recommended for the mass propagation of *P. bourneae* plants for conservation strategies. The acclimatized *in vitro*-derived plants can be used as an alternative to natural populations of *P. bourneae*.

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