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MICROPROPAGATION, ANTIOXIDANT AND ANTIMICROBIAL EFFECT OF *PLECTRANTHUS BOURNEAE* GAMBLE: AN ENDANGERED MEDICINAL PLANT

Rajaram K, Sriramji P, Sureshkumar P*
Department of Biotechnology, Bharathidasan Institute of Technology, Anna University, Tiruchirappalli-620 024, Tamil Nadu, India.
***Corresponding author: Email Id: drsureshbiotech2003@gmail.com; biorajaram@gmail.com**

ABSTRACT

An efficient *in vitro* regeneration protocol was developed for an endangered medicinal plant *Plectranthus bourneae* Gamble by inducing multiple shoots from the nodal and shoots tip explants. The shoot tip and nodal segments were cultured on MS medium supplemented with various concentrations of BAP, KN and ADS. Maximum shoot proliferation was achieved at 0.1 mg/l BAP in the nodal explants with higher regeneration efficiency (8.67±0.28 shoots/explant) when compared to shoot tip explants (4.67±0.15 shoots/explant). Elongation of the regenerated shoots was achieved at 0.5 mg/l GA_3 (5-6 cm). Effective rooting of the elongated plantlets was induced by 0.5 mg/l of IBA. The plantlets were hardened in the plant growth chamber and the maximum survival rate was 83.2%. This was the first report on the micropropagation of *Plectranthus bourneae*. The *Plectranthus bourneae* was collected and extracted with different solvents (Chloroform, Ethyl acetate and Methanol). The maximum antioxidant activity was observed in methanol extract and also the highest antibacterial activity obtained in methanol extract against all clinical strains (*K. pneumoniae, P. aeruginosa, E. coli, S. aereus and P.vulgaris*) than other extracts (Ethyl acetate and Chloroform).

***Key words**:* Adenine sulphate, Micropropagation, Nodal segment, *Plectranthus bourneae*, Shoot tip

INTRODUCTION

Medicinal plants are the best natural resource for the treatment of many hazardous diseases since time immemorial (Constable, 1990). Almost 80% of the world's total population uses traditional medicines which involve the use of plant extracts (Vieira and Skorupa, 1993). While extinction is a natural process, human impacts have increased the rate of extinction by several thousand times, the natural rate. It is estimated that there are more than 2, 70,000 plant species in existence and about 34, 000, i.e. 1 in 8 of these are endangered (Groombridge and Jenkins, 2000). In this aspect, plant tissue culture holds a key role in the conservation of germplasm through *in vitro* propagation and *in vitro* seed germination has been extensively used for multiplication of a large number of endangered plant species. *Plectranthus bourneae* an endangered medicinal plant belonging to the family *Lamiaceae* comprises of more than 3,000 species including several with commercial importance (Catherine *et al.,* 2006). It was first collected by Gamble in 1883 from Coonor, Nilgiris hills, India, but not known to have been collected thereafter from the hills. The status of this plant is indeterminate and it is not collected out in recent decades in Palani and Nilgiris hills. The main causes of its depletion may be over exploitation for medicinal purpose by local ethnic people and but forest cleaning for plantation crops largely accounts for its depletion (Nayar and Sastry, 1990).

Several species of *Plectranthus* are grown as ornamental plants, leaf vegetables and root vegetables for their edible tubers and as medicine, which is used in the treatment of diseases like Rheumatoid Arthritis (Jia-Ming et al. 2007), nausea (Dash and Kashyap, 1987) and used as an antioxidant (Salman *et al.,* 1996) and antimicrobial agents (Anita, 2010). This potent medicinal plant (*Plectranthus bourneae)* wildly used as ancient folklore for curing cough and fever. However, till now no scientific basis is available on *Plectranthus bourneae* about its medicinal use and *in vitro* propagation. There are several reports available on the *in vitro* propagation of *Plectranthus* species (Faure *et al.,* 1998; Irina *et al.,* 2004; Charleson *et al.,* 2006; Sadia *et al.,* 2009; Kumaraswamy *et al.,* 2010). Hence, an attempt has been made to scientifically document the medicinal properties (antioxidant and antimicrobial activity) and micropropagation of this endangered plant.

MATERIALS AND METHODS

Plant collection: *P.bourneae* was recorded as endangered medicinal plants in the red data book of Indian plants (Nayar and Sastry, 1990). The plant was collected and permitted by the Vattakanal Conservation Trust, Kodaikanal for micropropagation and it was established in the green house at Anna University, Tiruchirappalli, Tamil Nadu, India.

Micropropagation:

Establishment of aseptic culture: The young shoot tips and nodal segments of size (0.5−1.0 cm) were placed in a beaker and washed in running tap water for 5−10 min to remove soil and other fine particles and washed thoroughly with two drops of Tween 20 (Himedia, Mumbai, India) for 15 min and then treated with antifungal agent Bavastin (0.1%; w/v) for 10 min. The treated explants were washed thoroughly under running tap water and rinsed with sterile distilled water.

Surface Sterilization: The explants were immersed in aqueous Hg_2Cl_2 (0.1%; w/v) for 3 min. After this treatment, the explants were thoroughly rinsed with distilled water. Further the explants were trimmed into pieces of about 1 cm and then inoculated into the culture media.

Media and culture conditions: The basal medium used in this investigation consists of MS (Murashige and Skoog 1962) supplemented with 30 g/l sucrose (Himedia, Mumbai, India) and 0.8% agar (Himedia, Mumbai, India) was used as the gelling agent on respective media. The pH of the media were adjusted between 5.7−5.8 using 0.1N NaOH or 0.1N HCl before solidification. The media were autoclaved at 121°C for 15 min for sterilization. The medium was distributed to the test tubes and plugged with cotton wrapped in gauss cloth. The tubes were autoclaved and used. All the culture vials were incubated in culture room at 25 ± 2ºC under 16/8 hr (light/dark) cycle with a light intensity of 50 $\mu mol^{-2}\ s^{-1}$ supplied by cool white fluorescent lamps and with 60–65% relative humidity.

Shoot induction and multiplication: Nodal segments and shoot tips were placed on MS medium with various plant growth regulators (BAP, KN and ADS; Himedia, Mumbai, India) at different concentrations (0.01, 0.05, 0.1 and 1.0 mg/l) as listed in Table 1. Sub culturing onto the same medium was performed every 2 weeks.

Shoot elongation: The young shoots were elongated in MS medium supplemented with GA_3 (0.1, 0.5, 1.0 mg/l; Himedia, Mumbai, India). The frequency of explants producing shoots, number of shoots per explants and shoot length were recorded (Table 2) and all the experiments were repeated thrice.

Rooting: Healthy and well-elongated shoots (5–6 cm) were excised from culture and transferred to rooting media composed of MS medium with different concentrations of auxins (IAA, IBA and NAA; Himedia, Mumbai, India) as illustrated in Table 3. Data on percentage of rooting, number of roots and mean root length per shoot were recorded after 4 weeks of transfer to rooting media and all the experiments were repeated thrice.

Acclimatization: Plantlets with well-developed shoots and roots were removed from the culture medium, washed gently under running tap water and transferred to plastic cups containing sterile sand, garden soil and vermiculite (1:1:1) mixture under diffused light (16:8 h photoperiod). Potted plantlets were covered with transparent polythene bags to ensure high humidity conditions and it is maintained in a plant growth chamber (Colton, India) and watered every alternate day for 2 weeks. Thereafter, the bags were removed in order to harden the plants to field conditions. After 4 weeks, the acclimatized plants were transferred to pots containing normal garden soil and maintained in a greenhouse.

Statistical analysis: Each treatment had three replicates containing 50 explants and all the experiments were repeated thrice. The data on various parameters were subjected to one-way Analysis of Variance (ANOVA) using SPSS version 17.0 (SPSS Inc., Chicago, USA). The significance of differences among means was carried out using Duncan's multiple range test at P = 0.05 (Gomez and Gomez 1976).

Preparation of extracts and collection of microorganisms: The plants were thoroughly washed and each parts of the plant were cut into pieces, dried in shade condition and pulverized together. Solvent extractions based on polarity (chloroform, ethyl acetate and methanol; Himedia, Mumbai, India) for 24 h in a soxhlet extractor. Thereafter the solvent was removed under vacuum. The crude extracts were kept in a sample bottles and stored at −4°C. Five microorganisms that are *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa* and *Proteus vulgaris* were obtained from the Department of Microbiology, Bharathidasan University.

Determination of antimicrobial activity: The antibacterial potency of extract was determined by using the filter paper disc diffusion method (Anita, 2010). Positive controls included streptomycin and a solvent control was included with the assay since DMSO (Himedia, Mumbai, India) has antimicrobial activity. The lawn of test bacterium that is *S. aureus, E. coli, K. pneumoniae, P. aeruginosa* and *P. vulgaris* were prepared using glass spreader. The filter paper disc (3 mm) which were presoaked in respective extract (100µg) for 20 min (excess solvent air dried) were kept onto the bacterial lawn. The plates were left on bench for one hr before incubation at 37°C for 24 h to allow prediffusion of extract (Esimone *et al.,* 1998). The zone of inhibition (in mm) was measured using zone measuring scale (Table 4) and compared with control set (disc with solvent) (EUCAST, 2000).

Determination of radical scavenging activity using DPPH method: DPPH assay is the simplest method to measure the ability of antioxidants to intercept free radicals. Antioxidants react with DPPH, which is a stable free radical, and then scavenge this radical by converting it to α, α-diphenyl-β-picryl hydrazine (Himedia, Mumbai, India) due to their H-donating ability. The degree of discoloration indicates the scavenging potential of the antioxidant compounds. Experiments were performed according to Gaulejac *et al.,* (1998). Different concentrations of test compounds mixed with methanol solution of DPPH ranging from 50, 100, 200, 500, 1000 µg/ml were tested. Changes in the absorbance of the samples were measured at 517 nm. Inhibition of DPPH radical was calculated as follows:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 and A_1 are the absorbance values of the control and of the sample at 30 min respectively. Radical scavenging activity was expressed in terms of IC_{50} (concentration in mmol/l required for a 50% decrease in absorbance of DPPH radical). Vitamin C served as reference compounds (Table 5).

RESULTS AND DISCUSSION

Micropropagation of *P.bourneae*:

Initiation of shoot bud and multiple shoot induction: *In vitro* cell and tissue culture methodology plays a key role in germplasm conservation to enhance the survival of endangered plant species, rapid mass propagation and for genetic manipulation studies. The combinations of *in vitro* propagation techniques and cryopreservation help in the conservation of biodiversity of medicinal plants (Fay, 1992). In the present study, *in vitro* propagation of *P. bourneae* was attempted using shoot tip and nodal explants. Initially, when cultured in MS medium without PGR both the explants failed to induce shoots even after 5 weeks of incubation, therefore necessitating the need for cytokinins for inducing shoots. However, its effective type and optimal concentration varies with the system (Park *et al.*, 2008). A varied response was observed with different concentrations of BAP, KN and ADS (Table 1). Among the various PGRs tested, MS supplemented with BAP (0.1 mg/l) was found to be the optimum for maximum shoot induction in nodal (8.67±0.28 shoots/explant) and in shoot tip explants (4.67±0.15 shoots/explants) respectively (Table 1) (Fig. 1b, 1d). However further increase in the concentration of BAP (0.1 mg/l) have no significant increase in the shoot proliferation (Kiran *et al.*, 2004).

In the present study higher multiplication of shoots was achieved from nodal explants. Our results were in accordance with the earlier reports in the micropropagtion of medicinal plants such as *Rauwolfia serpentia* (Roy *et al.*, 1995), *Emblica officinalis* (Rahman *et al.*, 1999), *Enicostemma hyssopifolium* (Seetharam *et al.*, 2002), *Mentha piperita* (Kiran *et al.*, 2004) and *Andrographis lineata* (Sudarshana *et al.*, 2011).

Elongation of shoots: MS augmented with GA_3 at 0.5mg/l concentration showed maximum elongation of shoots (5.17 cm) in both the explants cultured (Table 2; Fig. 1e). Similar results were observed in *Mentha piperita* by Kiran *et al.*, (2004).

Rooting: Rooting of *in vitro* raised shoots were carried out by transferring the healthy, well elongated shoots to rooting media composed of MS with different concentrations of auxins. IBA at 0.5 mg/l produced maximum number of roots per shoot (5.33) without any basal callus (Table 3; Fig. 1f). Sadia *et al.*, (2009) have also observed similar results in *Mentha piperita*.

Hardening and acclimatization: The rooted plantlets were successfully hardened inside the plant growth chamber in a selected planting substrate with the mixture of sand, garden soil, vermiculite (1:1:1; Fig. 2). The maximum survival rate was 83.2%. The plantlets were subsequently transferred to pots containing garden soil followed by shifting to green house.

Qualitative analysis of phytochemicals, antimicrobial and antioxidant activity of *P.bourneae*: Qualitative analysis of phytochemicals was carried out by Brinda *et al.*, (1981) method in the *P. bourneae*. Maximum extraction of phytochemicals was found to be present in methanol extract when compared with chloroform and ethyl acetate of *P. bourneae*. The phytochemical screening of methanol extract showed that the whole plant was rich in alkaloids, tannin, flavonoids, phlobatanins, glycosides, terepenoids and saponins. This analysis shows the absence of steroids and other acidic compounds which is in accordance with the earlier report on *Plectranthus amboinicus* by Manjamalai *et al.*, (2010).

The antibacterial activity of *P.bourneae* was tested in five microorganisms using ethyl acetate, chloroform and methanol extracts (Table 4). The result indicates that the highest antibacterial activity was shown in methanol extract when compared to ethyl acetate and chloroform extract (Anita, 2010). The free radical scavenging activity of methanol, ethyl acetate and chloroform extracts of *P. bourneae* were measured by the DPPH method (Table 5). Methanol extract exhibited maximum antioxidant activity (IC_{50}:46.67µg/ml) than other extracts (Standard: Ascorbic acid: IC_{50}:30.4µg/ml).

These results indicate that the essential oils of this endangered species could serve as a safe antimicrobial and antioxidant agents in preventing deterioration of foodstuffs, beverages and pharmaceuticals. Also, consumption of food produced with natural essential oils or aromatic plant extracts (functional foods) is expected to prevent the risk of free radical dependent diseases and also as important functional food in the prevention and treatment of various human diseases (Biljana, 2010; Rajaram and Suresh, 2011). Hence this medicinally important endangered species require conservation measures and plant tissue culture holds good for the micropropagation of this plant and an attempt was made to propagate this plant (Rajaram *et al.*, 2012).

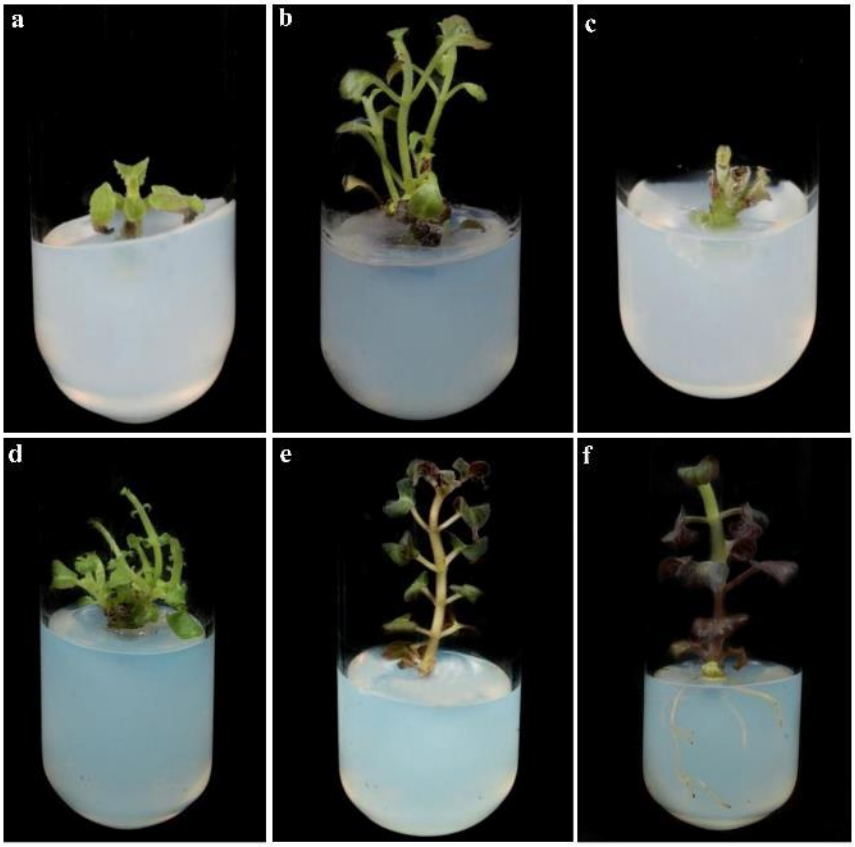

Fig. 1 *In vitro* regeneration of *Plectranthus bourneae* from shoot tip and nodal explants. a. Shoot tip explant (MS+0.1 mg/l BAP); b. Shoot proliferation in shoot tip explants; c. Nodal explant (MS+0.1 mg/l BAP); d. Shoot proliferation in nodal explant; e, Shoot elongation (MS+0.5 mg/l GA_3); f. Root induction (MS + 0.5 mg/l IBA)

Fig.2.An acclimatized plantlet in plastic cup containing sand, garden soil, vermiculite (1:1:1)

Table.1.Effect of different concentrations of plant growth regulators on multiple shoot regeneration from nodal segments of *Plectranthus bourneae* in MS medium

Plant Growth Regulators (mg/l)	Source of explants					
BAP	Shoot tip			Nodal segments		
	Regeneration (%)	Mean no. of shoots	Mean length of shoots/explants (cm)	Regeneration (%)	Mean no. of shoots	Mean length of shoots/explants (cm)
0.01	53.33±0.33bc	2.67±0.32bc	0.73±0.15bcd	73.33±0.32bc	4.33±0.28cde	1.33±0.38cd
0.05	56.67±0.43b	2.87±0.38b	0.87±0.15abcd	76.67±0.43abcd	4.67±0.18c	1.40±0.36c
0.1	66.67±0.47a	4.67±0.15a	1.57±0.49a	86.67±0.45a	8.67±0.28a	2.70±0.20a
1.0	43.33±0.37cd	2.33±0.48c	0.81±0.17b	66.67±0.15cde	5.33±0.38abc	1.53±0.27bc
KN						
0.01	46.67±0.32c	1.67±0.38de	0.72±0.20bcde	71.34±0.12c	4.37±0.38cd	1.13±0.32d
0.05	50.00±0.42bcd	2.38±0.28bcd	0.80±0.15bc	75.33±0.15b	4.77±0.28bcd	1.43±0.45bcd
0.1	63.33±0.31ab	3.33±0.15ab	0.93±0.17ab	81.33±0.42ab	5.67±0.38ab	1.83±0.49ab
1.0	41.33±0.37d	2.00±0.12d	0.69±0.35cd	68.33±0.23cd	5.00±0.14b	1.57±0.30b
ADS						
0.01	36.67±0.29def	1.33±0.10e	0.63±0.34e	63.33±0.48de	4.00±0.13d	1.10±0.10def
0.05	44.43±0.34cd	1.47±0.2def	0.70±0.24c	72.23±0.34bcd	3.97±0.38de	1.31±0.30cde
0.1	61.33±0.39abc	3.11±0.38abc	0.90±0.17abc	74.47±0.28abc	4.87±0.28bc	1.67±0.21abc
1..0	39.33±0.37de	2.23±0.48cd	0.67±0.15cde	64.43±0.38d	3.11±0.12def	1.23±0.11de

Values within each column were represented as means ± standard error. Means sharing the same letter are not significantly different (P = 0.05) using Duncan's multiple range test.

Table.2.Effect of GA_3 on *in vitro* shoot elongation of *P.bourneae*

PGR (mg/l)	Regeneration (%)	Mean length of shoot/explant (cm)
GA3		
0.1	76.67±0.58b	3.77±0.38b
0.5	83.33±0.58a	5.17±0.26a
1.0	66.67±1.15c	2.80±0.36c

***PGR- Plant Growth Regulator; Values within each column were represented as means ± standard error. Means sharing the same letter are not significantly different (P = 0.05) using Duncan's multiple range test.**

Table.3.*In vitro* rooting of regenerated shoots of *P.bourneae* using different auxins.

PGR (mg/l)			Regeneration (%)	Mean no. of roots	Mean length of roots/explants(cm)
IBA	IAA	NAA			
0.1	-	-	76.67±0.18b	3.33±0.28b	0.90±0.20ab
0.5	-	-	86.67±0.38a	5.33±0.38a	1.40±0.36a
1.0	-	-	66.67±0.15cd	4.33±0.48ab	0.57±0.42cde
-	0.1	-	73.33±0.45bc	2.33±0.28cd	0.73±0.25c
-	0.5	-	80.00±0.44ab	3.67±0.18abc	0.87±0.15abc
-	1.0	-	70.00±0.34c	3.00±0.24bc	0.63±0.43cd
-	-	0.1	63.33±0.48cde	2.37±0.35c	0.85±0.15b
-	-	0.5	77.78±0.34abc	2.67±0.34bcd	0.80±0.10bcd
-	-	1.0	71.33±0.35bcd	2.00±0.34cde	0.83±0.20bc

****PGR- Plant Growth Regulator; Values within each column were represented as means ± standard error. Means sharing the same letter are not significantly different (P = 0.05) using Duncan's multiple range test***

Table.4.Antimicrobial sensitivity testing of methanol, ethyl acetate and chloroform extracts of *P.bourneae*

Microorganism	Streptomycin (standard) (mm)	Ethyl acetate Extract (mm)	Methanol extract(mm)	Chloroform (mm)
K.pneumoniae	23	10	14	10
P.aeruginosa	18	10	12	9
E.coli	16	9	11	8
S.aereus	20	10	13	6
P.vulgaris	21	9	12	8

Antimicrobial Control – Streptomycin (10 µg), Solvent control (DMSO). Experiment is repeated thrice.

Table.5.Antioxidant activity of methanol, ethyl acetate and chloroform extracts.

Extracts	Antioxidant activity (DPPH Assay) IC_{50}(µg/ml)
Methanol	47.67±1.52
Ethyl acetate	202.5±1.61
Chloroform	993.2±1.25
Ascorbic acid (Positive Control)	30.4±0.00

Values within the column were represented as mean ± standard error. Experiment is repeated thrice.

CONCLUSION

The present study demonstrates the presence of vital phytochemicals, antimicrobial activity and antioxidant activity of *P. bourneae*. As this plant species is endangered, we have evolved micropropagation strategy to mass multiply this rare species. The development of this *in vitro* protocol plays an important role in the conservation of this rare medicinal plant and it can be used as a basic tool for the commercial cultivation of this plant.

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