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Plant regeneration from leaf-derived callus in *Plectranthus barbatus* Andr. [Syn.: *Coleus forskohlii* (Wild.) Briq.]

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A protocol was developed for *Plectranthus barbatus* for high frequency shoot organogenesis from leaf derived callus of aseptically grown plantlets derived from mature plant explants of high yielding clones (yield of forskolin 1.5 to 1.9%) on Gamborg medium (B_5) medium supplemented with 2 mg/l 2,4- dichloro phenoxy acetic acid (2,4-D). Shoots were regenerated from the callus on MS medium supplemented with 6-benzyl amino purine (BAP) (2 mg/l) + naphthalene acetic acid (NAA) (1 mg/l). The highest rate of shoot multiplication was achieved at the 6th subculture and more than 2000 shoots were produced from callus clump. Roots were induced from shoots of *in vitro* grown plantlets on basal half strength MS medium and high rooting frequencies were obtained. Regenerated plants were easily acclimatized in greenhouse conditions and later transferred to soil with 100% survival. The procedure here allows the micropropagation of *P. barbatus* in five months of culture and proliferated cell masses which could be used for studies of organic compounds of pharmaceutical interest.

Key words: Callus culture, medicinal plant, root induction, shoot organogenesis.

INTRODUCTION

Plectranthus barbatus Andr. [Syn.: *Coleus forskohlii* (Willd.) Briq.] is a perennial herb of the family Lamiaceae. The plant originated from the Indian subcontinent, but has been distributed to Egypt, Arabia, Ethiopia, East Africa and Brazil (Valdes et al., 1987). *P. barbatus* produces an ancient root-derived drug traditionally used in the treatment of heart diseases, abdominal colic, respiratory disorder, insomnia, convulsions, asthma, bronchitis, intestinal disorders, burning sensation, constipation, epilepsy and angina (Ammon and Műller, 1985). Recently, an array of plant products are added to the list which were identified as nutraceuticals and received global attention such as Guggulsterones, Bacosides, Hydroxy citric acid, etc. One such new compound recently added is Forskolin obtained from *P. barbatus.* An Indian based American company by the name Sabinsa got best product award for introducing Forslean ® standardized extract of *P. barbatus* containing the compound forskolin. The novel feature of forskolin has opened up vistas for bio medical research.

The fasciculated tubers of *P. barbatus* on drying yield a unique labdane diterpenoid called forskolin (Dubey et al.,

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Abbreviations: AC, Activated charcoal; **BAP,** 6-benzyl amino purine; **B_5,** Gamborg medium; **IAA,** indole acetic acid; **IBA,** indole butyric acid; **Kn,** Kinetin; **MS,** Murashige and Skoog medium; **NAA,** naphthalene acetic acid; **PVP,** poly vinyl pyrrolidine; **2,4-D,** 2,4-dichloro phenoxy acetic acid; **2,4,5-T,** 2,4,5-trichloro phenoxy acetic acid; **2,4,5, TP,** 2,4,5-trichloro phenoxy propionic acid.

1981). Ayurvedic practitioners in India have used this plant to treat illness and various diseases including cardiac troubles since ancient times. The plant is valued, as forskolin drug is used for the treatment of glaucoma, congestive cardiomyopathy and asthma (De souza et al., 1986; Valdes et al., 1987; Hussain et al., 1992). Using the adenylate cyclase stimulant activity, it is also valued for antiallergic activity and suppressing hair graying (Keikichi et al., 1988). The leaves are used as an expectorant and diuretic. Although diterperoids are found in almost all parts of the plant, the roots are the main source (Shah et al., 1980), the major diterperoid being forskolin (coleonol). The secondary metabolites are stored mainly in the cytoplasmic vesicles of cork cells in both fibrous and tuberous root (Abraham et al., 1988). Forskolin possesses positive inotropic and blood pressure-lowering activities through intra venous administration in a central nervous system (CNS) depressant, bronchodilator (Lichey et al., 1984) serves nerve regeneration and lowers intra ocular pressure (Caprioli and Sears, 1983). Owing to its unique pharmacological properties and its potential, the plant has attracted worldwide attention in recent years. It is only source of this compound detected so far. Indiscriminate collection of this plant from their natural habitat is leading to depletion of the resources (Vishwakarma et al*.,* 1988). Studies on regeneration of plants *in vitro* are few and scanty (Reddy et al., 2001; Balasubramanya et al., 2012). Hence, a procedure for rapid *in vitro* propagation of this species has been described in this paper. The scope of this study includes conditions of explanting for callus induction, effects of hormones on shoot multiplication and plantlet regeneration.

MATERIALS AND METHODS

From nature, healthy mother plants fresh, young and juvenile shoots were collected and cut into 5 to 6 mm long nodal segments with a single axillary bud and shoot tips. These explants were surface sterilized in a 0.05% $HgCl_2$ (7 min) followed by 0.01% $HgCl_2$ (4 min) and aseptically cultured on to the Murashige and Skoog (MS) medium supplemented with 0.5 mg/l BAP for 45 days. After bud break, the explants were grown for another 45 days for multiple shoot production on MS medium supplemented with BAP (0.5 mg/l). These shoots were excised and transferred to ½ MS medium supplemented with 0.5 mg/l indole acetic acid (IAA) for rhizogenesis. The cultures were maintained uniformly with 0.8% agar, pH 5.6, 16 + 8 h light/dark conditions at 26 ± 2°C. From these *in vitro* raised plantlets, different explants such as root, node, leaf, petiole and internode were excised and cultured on MS and B_5 media fortified with 2 mg/l 2,4-dichlorophenoxyacetic acid. The pH was adjusted to 5.6 before autoclaving at 121°C for 15 min. The frequency of callus formation and nature of response was determined eight weeks after culture initiation. The calli derived from the different explants were transferred to MS medium supplemented with 1.5 mg/l BAP, 3% sucrose and 0.8% agar (bacteriological grade) for shoot production. After screening of suitable medium for callus induction, the explants were studied with different growth regulators using 2,4-D, 2,4,5-T, 2,4,5-TP and IAA (0.5 to 3.0 mg/l) individually. Cultures were maintained under light or dark conditions at 26 ± 2°C. The light intensity was 30 m mol m^2 S^1. Leaf explants, considering the faster response, were selected and cultured on media to assess initiation and nature of response.

Shoot induction and multiplication

To induce and proliferate shoots, the calli were transferred to shoot induction medium containing MS (Murashige and Skoog, 1962) salts, vitamins, 0.5 to 2.0 mg/l BAP or Kn and in different combinations of auxins (NAA, IAA and IBA) and cytokinin (BAP), 3% sucrose and 0.8% agar with pH. 5.6. Six weeks old leaf derived calli were kept under the same light/darkness condition for shoot initiation on shoot induction medium. The number of shoots per treatment was recorded at 6 and 10 weeks after transferring the calli to shoot induction medium.

Rooting

For the induction of roots, single shoots with three to four nodes were excised from callus cultures and transferred to rooting medium. In order to induce roots, MS medium ½ strength with or without AC was supplemented with or without hormones; 0.5 to 0.2 mg/l NAA, IAA and IBA, 3% sucrose, 0.8% Agar and pH 5.6. Both *in vitro* and *ex vitro* rooting methods were adopted. To induce rooting under *ex vitro* conditions, shoots measuring 6 to 7 cm with five to six nodes were excised from the cultures and pulsed with auxins (NAA, IAA and IBA) at concentrations of 0.25 to 0.5 mg/l. The auxin pulsed shoots were then transferred to net pots containing sterile soil rite. ½ MS salt solution without sucrose and vitamins was given to moisten the soilrite. These were placed in culture room for 15 days and then transferred to greenhouse. Data were recorded after four weeks of culture. Frequencies of rooting and average number of roots per shoot were determined.

In vitro rooted plantlets were transferred to a moistened and autoclaved soilrite placed in protrays containing netpots. These protrays were placed in polyhouse with mist facility. The side covers of polyhouse were rolled periodically to allow air exchange. High humidity (90%) was maintained with optimum temperature (26 + 2°C). Plantlets were slowly acclimatized by increasing the exposure period from 1 to 7 h. The percentage of survival was recorded after two weeks. All experiments were repeated at least three times and statistically analyzed.

Statistical analysis

Means of callus induction frequency, number of induced shoots, frequency of rooting and number of induced roots were analyzed by using ANOVA.

RESULTS AND DISCUSSION

Callus initiation

Various explants such as petiole, leaf, node, internode and root from aseptically grown plantlets of *P. barbatus* were used as source of material and implanted on MS and B_5 media fortified with 2 mg/l 2,4,-D. Good callus proliferation was observed on B_5 medium. Calli were obtained only in the presence of 2,4-D (2 mg/l) using either MS and B_5 medium (Gamborg et al., 1968). Thus,

Table 1. Influence of medium composition on the nature of morphogenic response in callus cultures in *P. barbatus* (syn.:*Coleus forskohlii*).

Medium + 2,4-D (2 mg/l)	Explant	Percentage of response	Nature of callus response
MS	Leaf	86.0	Greenish white nodular
	Node	70.0	Pale greenish brown friable
	Internode	68.0	White compact
	Petiole	56.0	Black compact
	Root	34.0	Brown nodular
B_5	Leaf	98.0	Greenish white nodular
	Node	94.0	Pale greenish friable
	Internode	88.0	White compact
	Petiole	72.0	Black compact
	Root	36.0	Brown nodular
F-Value		59.37	
SEM±		5.35	
Critical difference at 5%level		10.49	

**Significant at 1%level. Data represents average of three experiments with 20 replications.

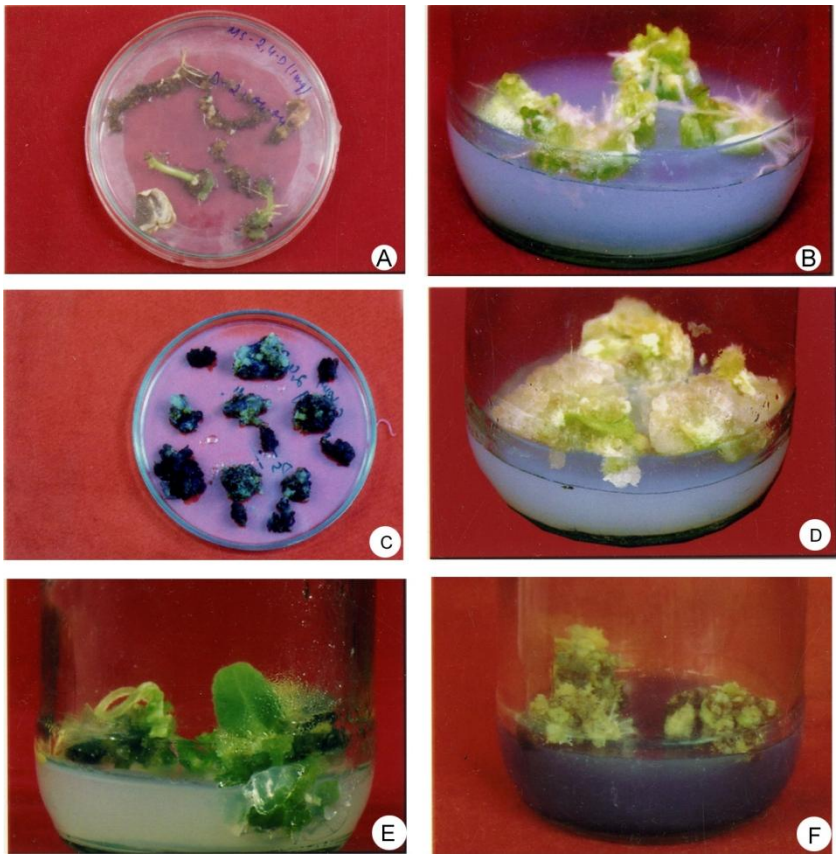

Figure 1. Regeneration of different type of calli from different explants in *P. barbatus*. **A,** Callus from roots; **B,** callus from leaf explants with rhizogenesis; **C,** black callus from internode; **E,** green callus from leaf; **F,** brown from node.

B_5 medium was used for callus induction which responded best for all explants (Table 1; Figures 1A to F), but the most interesting observation in this plant was the media dependent responses. Better multiplication

Table 2. Morphogenic response of calli derived from different resource of explants on MS medium supplemented with 1.5 mg/l BAP in *P. barbatus* (syn.: *Coleus forskohlii*).

Nature of explant	Explants that responded (%)	Nature of response
Internode	76.65	Multiple shoot buds
Leaf	91.65	Multiple shoot buds
Root	25.00	Callus
Petiole	43.35	Multiple shoot buds
Node	78.35	Multiple shoot buds
F-Value	267.86**	
SEM±	2.10	
Critical difference at 5% level	4.68	

**Significant at 1%level. Data represents average of 3 experiments with 20 replications.

rate and healthy shoot buds were developed on MS medium. Thus, organogenesis was done in two steps. First step was the usage of B_5 medium for callus induction and the second was the usage of MS medium for multiplication stage. Among the various explants tested, leaf explants favoured good callogenic response (98.0%) and further experiments were carried out with calli derived from leaf explants only. The leaf explant showed greenish, white nodular callus, internode with white, compact callus, node with pale greenish, brown friable callus, petiole with black, compact callus and root with brown nodular callus (Table 2).

Callus induction and proliferation systems are known to be very useful for the study of bio-synthesis of natural products. In *P. barbatus,* this approach of synthesis of forskolin from callus has been used (Sen et al., 1992; Mukherjee et al., 1996; Reddy et al., 2001). Hence the optimization of cellular proliferation is the first essential step to establish cultures from plant tissues for the production of natural products. Our results show that callus growth of *P. barbatus* was markedly affected by the growth regulators and morphogenic response of calli significantly varied to a greater extent according to explants type. High callus proliferation was observed when explants were cultured in darkness. The endogenous balance between Auxin/cytokinin may become altered and consequently decrease the callus growth. Leaf callus is nodular, white and green in colour and considered as most suitable for establishing callus cultures. Leaf explant not only formed a good source for callus induction but also exhibited maximum percent frequency of shoot bud differentiation. Earlier reports also confirmed the advantage of leaf being the source of the dedifferentiation and callus formation in other medicinal plants such as *Tylophora indica* (Rao et al., 1970), *Cimcifuga racemosa* (Lata et al., 2002) and for *P. barbatus* (Reddy et al., 2001).

Leaf segments when implanted on B_5 medium supplemented with various growth regulators viz., 2,4-D, 2,4-5T, 2.4-5TP, IAA at varied concentrations (0.5 to 3mg/l) (Table 3) exhibited differential response both in terms of morphology and biomass. The best response was on 2 mg/l 2,4-D B_5 medium. At lower concentrations, 2,4-D induced two types of callus, viz., yellow, friable and greenish white and nodular. Only greenish white and nodular callus was able to regenerate adventitious shoots after being transferred to shooting medium (MS medium with 1.5 mg/l BAP). IAA induced green compact callus at lower concentration (0.5 mg/l) which resulted in root formation from the midrib. At higher concentration (3 mg/l), 2,4-D was found to be toxic to explants which turned brown after two weeks of incubation. Later, it turned to black at the base after four weeks of culture. The most effective nature of 2,4-D to induce callus was observed in *Citrus* (Ling and Iwamasa, 1997) and Pommello (Goh et al., 1995).

Shoot induction

Adventitious shoots were initiated either directly on the explant or indirectly in the callus that is produced from the leaf explant. Explants taken from *in vitro* shoot cultures were found to be superior plant material for shoot regeneration as they are maintained in sterile environment.

Green, compact, nodular leaf callus that developed on B_5 medium supplemented with 2 mg/l 2,4-D underwent organogenesis after two subcultures and transferred onto MS medium fortified with various concentrations of BAP and kinetin alone (0.5 to 2.0 mg/l) (Table 4). The best shoot bud induction response over the explant surface area was obtained on the medium containing 2 mg/l BAP with higher percent (53.33%) and number of multiple shoot buds (11 to 12). In contrast, the frequency of shoot bud differentiation (36.67%) and eight to nine number of shoots were lesser in 2 mg/l Kn concentration. The effect of NAA, IAA and IBA in combination with BAP was also studied for shoot regeneration from the callus. It was found that MS medium with BAP (2 mg/l) + NAA (1 mg/l) concentration was the best suitable medium (86.67%) in emerging shoot buds (44 in numbers) from leaf callus.

Table 3. Effect of different growth regulators on callus induction of the leaf explants in *Plectranthus barbatus* (syn.:*Coleus forskohlii*).

Hormone	Treatment (mg/l)	Percentage of explants that responded	Nature of response
2,4-D	0.50	3.3	+
	1.00	53.33	++
	1.50	76.7	++
	2.00	100.0	+++
	2.50	96.7	+
	3.00	96.7	+
2,4,5-D	0.50	0.0	+
	1.00	0.0	++
	1.50	16.7	++
	2.00	50.0	+++
	2.50	50.0	+
	3.00	56.7	+
2,4,5-TP	0.50	0.0	+
	1.00	10.0	++
	1.50	36.7	++
	2.00	60.0	+++
	2.50	70.0	+
	3.00	73.3	+
IAA	0.50	23.3	+
	1.00	33.3	+
	1.50	43.3	++
	2.00	56.7	++
	2.50	63.3	++
	3.00	63.3	++
F-Value		37.7**	
SEM±		13.73	
Critical difference at 5% level		26.92	

**Significant at 1% level. Data represents average of 3 experiments with 10 replications. +, Little amount; ++, medium; +++, profuse proliferation.

This response was better than the ones described by Reddy et al. (2001) and Balasubramanya et al. (2012). The morphogenic response of node callus (78.35%) was also observed and it was found lesser than leaf and better than for other explants (Table 5; Figures 2A to C). The number of shoots regenerated per callus increased drastically from 1st to 6th subculture (50 to 200). This is because of the passage of some intermodal components from pre-existing axillary buds that are essential to evoke caulogenesis (Martin, 2002).

Rooting of shoots

The basal half strength MS medium is most suitable for healthy and high frequency root production (86.67%) (Table 6). IAA (0.5 mg/l) resulted in 76.65% than NAA (0.5 mg/l) with 51.65% and IBA (0.5 mg/l) with 61.65%. Reddy et al. (2001) in their study in *P. barbatus* also confirmed the advantage of using basal medium for rooting. However Sharma et al. (1991) and Bhattacharya and Bhattacharya (2001) reported better rooting response in *P. barbatus* on media containing IAA. Success of auxin free medium for efficient root induction was also reported in *Clerodendrum colebrookianun* (Monier and Ochatt, 1995). The incidence of root formation in auxin free medium may be due to the presence of higher quality of endogenous auxin in *in vitro* raised shootlets (Minocha, 1987). After a week, 90% of the shoots developed two to three roots per plantlet. Regenerated plants were transferred to non-aseptic conditions for acclimatization and then to conditions of progressively lower humidity levels. Completely adapted plants were transferred to field with 100% survival rate.

Table 4. Effect of cytokinins on shoot regeneration of callus cultures in *P. barbatus* (syn.: *Coleus forskohlii*).

Treatment (mg/l)	Percentage of regeneration of shoot	Average number of shoots/callus	Average length of shoot (cm)
BAP			
0.5	6.67	1.33	1.00
1.0	16.67	1.67	1.67
1.5	23.33	5.67	2.00
2.0	53.33	11.33	3.33
Kn			
0.5	3.33	1.00	1.00
1.0	6.67	1.33	1.33
1.5	16.67	2.67	1.67
2.0	36.67	8.67	3.33
F-Value	26.41**	111.53**	12.69**
SEM ±	4.71	0.53	0.37
Critical difference at 5% level	9.98	1.12	0.78

**Significant at 1% level. Data represents average of 3 experiments with 10 replications.

Figure 2. Induction of shoot buds from leaf callus of *P. barbatus.* **A and B**, Development of multiple shoots from leaf derived callus culture. **C,** adventitious shoot bud regeneration from node explants.

Table 5. Effect of cytokinins and auxins on shoots regeneration of callus cultures in *Plectranthus barbatus* (syn.: *Coleus forskohlii*).

Treatment (mg/l)	Percentage of regeneration of shoots per callus	Average number of shoots per callus	Average number of root	Average length of shoot
BAP (0.5)+NAA (0.5)	66.67	22.00	6.00	2.33
BAP (0.1)+NAA (0.5)	23.33	12.67	2.33	1.67
BAP (2.0)+NAA (1.0)	86.67	44.00	7.33	3.67
BAP (0.5)+IAA (0.5)	63.33	17.33	5.67	1.00
BAP (0.1)+IAA (0.5)	30.00	13.33	3.33	2.33
BAP (2.0)+IAA (0.1)	73.33	24.67	12.00	2.67
BAP (0.5)+IBA (0.5)	46.67	19.00	4.33	1.67
BAP (0.1)+IBA (0.5)	26.67	12.00	4.67	1.00
BAP (2.0)+IBA (1.0)	63.33	21.67	8.67	2.67
F-Value	12.94**	177.18**	32.26	10.80
SEM ±	8.91	1.76	0.74	0.39
Critical difference at 5% level	18.72	3.70	1.55	0.82

**Significant at 1% Level. Data represents average of 3 experiments with 10 replications.

Table 6. Effect of different hormone treatments (with activated charcoal) for in vitro rooting in *P. barbatus* (syn.: *Coleus forskohlii*).

Media + hormone + 0.05% A.C.	Percentage of explants showing rooting	Percentage of explants showing callus	Average number of roots/explant	Average root length (cm)
1/4MS +0.5	73.35	26.65	15.33	10.67
NAA	76.65	23.35	12.33	10.33
1/2MS + 0.5	86.65	13.35	17.67	12.00
NAA	88.33	26.67	16.33	11.33
¼ MS+0.5 1AA	68.35	31.65	11.67	9.67
½ MS+0.5IAA	76.65	23.35	10.33	8.67
¼ MS+0.5 IBA	93.35	6.65	17.33	11.33
½ MS+0.5 IBA				
½ MS control	51.67	20.00	9.33	8.67
¼ MS control				
F-Value	33.60 **	29.58**	31.83**	15.66**
SEM ±	2.58	2.22	0.78	0.44
Critical difference at 5% level	5.62	4.84	1.71	0.96

**Significant at 1% level. Data represents average of 3 experiments with 20 replications.

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