In vitro direct shoot regeneration from proximal, middle and distal segment of *Coleus forskohlii* leaf explants

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ABSTRACT

Coleus forskohlii is an endangered multipurpose medicinal plant that has widespread applications. In spite of this, there have been few attempts to propagate its cultivation in India. The present communication presents an *in vitro* rapid regeneration method using leaf explants of *Coleus forskohlii* through direct organogenesis. Leaf explants that were excised into three different segments i.e. proximal (P), middle (M) and distal (D) were cultured on Murashige and Skoog (MS) basal medium supplemented with cytokinins. MS Media containing 5.0 mg L⁻¹ BAP (6-Benzylaminopurine) promoted regeneration of multiple shoots through direct organogenesis from the leaf, which were further elongated on MS media augmented with 0.1 mg L⁻¹ BAP and 0.1 mg L⁻¹ IAA (Indole-3-acetic acid), cytokinin and auxin combination. Regenerated and elongated shoots, when transferred to ½ MS with 1.5 % sucrose resulted in profuse rooting plants that were transferred to soil after acclimatization and maintained in a green house. The current protocol offers a direct, mass propagation method bypassing the callus phase of *C. forskohlii* and is suitable for conservation, large-scale commercial cultivation, and genetic transformation with agronomically desirable traits. [Physiol. Mol. Biol. Plants 2010; 16(2) : 195-200] E-mail : gauravkris@gmail.com

Keywords : 6-benzylaminopurine, *Coleus forskohlii*, cytokinin, tissue culture, multiple shoots.

Abbreviations: BAP: 6-benzylaminopurine, IAA: indole-3-acetic acid, KIN: kinetin, NAA: α -naphthalene acetic acid, TDZ: thidiazuron

INTRODUCTION

Coleus forskohlii (Family: Lamiaceae) is a herbaceous plant that usually grows at a height of 600 - 1500 ft and is spread over the subtropical warm temperate climatic zones of India, Nepal, Myanmar, Srilanka, Thailand and Africa. In India, it is widely distributed in dry hills of Western Uttar Pradesh, Gujarat, parts of Orissa, and Western Ghats. It is highly valuable as a medicinal plant due to a biologically active compound labdane diterpene present in the root tubers called 'forskolin' (Bhat *et al.*, 1977). In animal systems, 'forskolin' has been shown to directly stimulate the synthesis of adenylate cyclase (Seamon & Daly 1981), and also exhibits an anti-

inflammatory property (Rupp *et al.*, 1986). Another product of the plant namely 'ditermene coleonol' was found to reduce the blood pressure in experimental rats and cats (Dubey *et al.*, 1981) that makes it an attractive target for treatment of hypertension and related diseases.

In spite of the tremendous promise it holds in medicinal formulations, very few attempts have been made for its replenishment or cultivation and it is currently listed as one of the plant species vulnerable to extinction in India. Traditionally, the propagation of *C. forskohlii* is carried by vegetative propagation, however, this process is time consuming and results in limited number of propagules. *In vitro* plant tissue culture technique has been previously shown to play an important role in rapid clonal multiplication and reforestation (Biondi and Thorpe, 1982; Farnum *et al.*, 1983; Reddy *et al.*, 2001), although there are few reports on *in vitro* multiplication of *C. forskohlii* such as

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regeneration of shoots from the axillary buds (Sharma *et al.*, 1991), stem tips (Rajasri and Sabita, 2001), and leaf-derived calli (Reddy *et al.*, 2001). Despite effective and surrogate method of large-scale production of plantlets, direct shoot regeneration pathway has not been yet reported for *C. forskohlii*. Direct regeneration of shoots without intermediate callus stage is a faster way of multiplying clones precluding somaclonal variation. Therefore, the present investigation is aimed at optimizing a protocol for faster and large-scale direct shoot regeneration of *C. forskohlii*.

MATERIALS AND METHODS

C. forskohlii collected from the fields of Adilabad district, Andhra Pradesh, India was established in the greenhouse of JK Agri-Genetics Ltd., Hyderabad. Young leaves were used as explants with cut pieces of (2"x 2") from the proximal (P), distal (D) and middle (M) segments (Fig. 1), which were surface sterilized with 0.1 % HgCl₂ for 4 min, followed by 5 washes in sterile

double distilled water. The leaf segments were slit through the midrib and inoculated on the medium.

Media and culture conditions

MS medium supplemented with BAP (5 concentration), KIN and TDZ (3 concentration) alone, and with BAP + NAA were used for direct shoot regeneration. The regenerated shoots from 0.5, 1.0, 2.5, and 5.0 mg L⁻¹ were placed for elongation on MS medium comprising BAP, KIN + NAA and KIN + IAA. However, among these combinations, BAP (@0.1 mg L⁻¹), KIN (@0.5 mg L⁻¹), NAA (@0.5 mg L⁻¹), and IAA (@0.1 mg L⁻¹) were utilized. Finally MS basal, ½ MS, and ¼ MS media, without plant growth regulators were used for inducing roots from the regenerated shoots. All the media were prepared in culture bottles by autoclaving at 121 °C under 15 lb pressure, and the cultures were incubated at 25±2 °C and photoperiod (16L:8D). Rooted plants were shifted to the greenhouse after hardening in autoclaved soil.

Table 1. Effect of plant growth regulators on shoot regeneration from leaf explants

Plant growth regulator (mg L ⁻¹)				No. of explants inoculated	Shoot regeneration (%)	Type of Response
BAP	TDZ	KIN	NAA			
0.1	-	-	-	45.0	0.0	C
0.5	-	-	-	51.0	13.0	S
1.0	-	-	-	42.0	35.8	S
2.5	-	-	-	42.0	42.3	S
5.0	-	-	-	45.0	76.4	S
-	0.1	-	-	41.0	10.1	S+C
-	0.5	-	-	42.0	5.2	S+C
-	1.0	-	-	51.0	0.0	C
-	-	0.1	-	50.0	0.0	C
-	-	0.5	-	44.0	0.0	C
-	-	1.0	-	43.0	15.1	S+C
0.1	-	-	0.5	41.0	10.6	S+C
0.5	-	-	0.5	44.0	10.0	S+C
1.0	-	-	0.5	51.0	25.2	S+C
2.5	-	-	0.5	49.0	23.7	S+C
5.0	-	-	0.5	42.0	37.6	S+C

S - Shoot regeneration; C - Only callus formation, S + C - Callus mediated shoot regeneration

RESULTS AND DISCUSSION

Plant micropropagation *via* direct shoot regeneration allows large-scale multiplication of plantlets *in vitro* by preventing clonal variation as opposed to regenerated from calli alone, which often leads to somaclonal variation (Reddy *et al.*, 2001). In this study, leaf segments excised from different regions (Fig. 1) were tested for their response to plant growth regulators at different concentrations. After incubation over a period of 28 days, an optimal regeneration of shoots (76.4 %) was achieved on MS medium fortified with BAP (@ 5.0 mg L⁻¹), in comparison with other plant growth regulators (Table 1). Generally the usage of BAP is considered as most suitable for promoting large-scale multiplication and micropropagation of various plant species (Shrivastava and Banerjee, 2008). In addition, BAP alone was shown to promote multiple shoots formation from the nodal segments and shoot tips in several species of *Coleus*, including *C. forskohlii* (Sen and Sharma, 1991; Ponsamuel *et al.*, 1994). In the current study, MS medium fortified with BAP (@ 2.5 mg L⁻¹) resulted in reduced regeneration efficiency by 55 %, followed by poor response at further lower concentrations. Moreover, the explants of *C. forskohlii* grown on MS medium fortified with BAP (@ 0.1 mg L⁻¹) showed only callus formation but not of shoots. Alternatively, the leaf explants at the same concentration of BAP combined with NAA responded with 10.0 - 37.6 % shoot regeneration and concurrent callus proliferation. Similar combination of plant growth regulators were previously found to be responsive to callus and plantlet initiation in *C. blumei* (Ibrahim *et al.*, 1992).

Some explants regenerating shoots showed small calli formation at various concentration of BAP, including 5.0 mg L⁻¹, which could be due to the variation in differentiation process of leaf age. These calli occasionally regenerated few small shoot buds, however, due to the dominating fast growth of shoot regenerating directly from explants, they could not complete the growth cycle. Interestingly, the leaf explants showed

Fig. 1. Diagrammatic representation of proximal (■), middle (□) and distal (▒) segments used from leaf explant tissue.

10.1 and 5.2 % regeneration efficiency respectively with TDZ (@ 0.1 and 0.5 mg L⁻¹); and 15.1 % with KIN (@ 1.0 mg L⁻¹). The shoot formation induced by TDZ showed hyperhydricity that were in line with earlier reports that reported the explant's inability to degrade TDZ, leading to its further accumulation (Srinivasan *et al.*, 2006, Mok and Mok, 2001). Briggs *et al.*, (1988) had earlier observed improved shoot quality with TDZ in combination with BAP.

On the other hand TDZ (@1.0 mg L⁻¹) as well as KIN (@0.1 and 0.5 mg L⁻¹) mimicked ineffective response, although callus proliferation was observed. Sharma *et al.* (1991) had previously reported increase in callusing frequency from leaf explants on media enriched with KIN. Conversely, incubation of shoot tips and nodal segments in the media containing KIN achieved low morphogenetic response (Rajasri and Sabita, 2001), while Reddy *et al.* (2001) reported dose-dependent increase in callus growth by BAP, KIN, and TDZ. However, among the cytokinins, it was been observed that except BAP, all others achieved shoots with callus-mediation. Therefore, further experiments were continued with BAP only.

Comparison of shoot regeneration response from different leaf segments at 5.0 mg L⁻¹ BAP showed that the distal end was comparatively higher with 45 shoots than other leaf segments (Table 2). However, BAP (@ <0.5 mg L⁻¹) affected shoot regeneration and resulted in formation of five shoots from the distal end of the leaf explants, while shoot regeneration from the leaf explants of middle and distal ends of BAP (@ 1.0 and 2.5 mg L⁻¹) was realized with a similar response. Furthermore, it was observed that the proximal segments showed shoot regeneration from all the sides compared to other segments, which may be due to large

Table 2. Effect of BAP concentrations on shoot bud regeneration from different segments of leaf explants in *C. forskohlii*

Plant growth regulator (mg L ⁻¹)	Proximal (P) (Mean ± SE)	Middle (M) (Mean ± SE)	Distal (D) (Mean ± SE)
0.5 BAP	5.0 ± 0.1 ^b	2.5 ± 0.05 ^c	5.0 ± 0.1 ^b
1.0 BAP	12.5 ± 0.7 ^{ab}	22.0 ± 1.0 ^{ab}	21.0 ± 1.2 ^{ab}
2.5 BAP	5.0 ± 0.2 ^b	12.5 ± 0.6 ^{abc}	13.0 ± 0.8 ^b
5.0 BAP	21.0 ± 1.2 ^a	23.0 ± 1.3 ^a	45.0 ± 2.0 ^a

Means in the same column followed by same letter are not significantly different (DMRT >0.05)

Fig. 2. Elongation of regenerated shoots from the proximal (■), middle (□) and distal (▒) segments of leaf explants derived by different combinations of cytokinins and auxins in *C. forskohlii*.

regenerating meristematic tissues at the proximal segment of the leaf.

Subculturing of regenerated shoots on MS medium supplemented with BAP or KIN in combination with NAA or IAA resulted in shoot elongation and was effective on BAP (@ 5.0 mg L⁻¹) (Fig. 2 and 3). The proximal and middle segments of the leaf showed varied elongation (20 - 80 %), which was comparatively higher from the distal end segment. In addition, the elongation frequency was higher on MS medium supplemented with 0.1 mg L⁻¹ BAP and IAA. Using nodal, internodal, and leaf explants of *C. blumei* Zagajski *et al.* (1997) had demonstrated that BAP and IAA combinations were effective in shoot induction. However, the shoot elongation was reported to be minimum with KIN (@ 0.5 mg L⁻¹) and IAA (@ 0.1 mg L⁻¹) (Rajasri and Sabita, 2001). Similar combinations in MS medium were suitable for shoot induction and

multiplication from nodal segments and shoot tips of *C. forskohlii* (Sharma *et al.*, 1991); whereas the medium enriched with BAP (@ 0.1 mg L⁻¹) and NAA (@ 0.5 mg L⁻¹) showed relatively higher elongation compared to KIN (@ 0.1 mg L⁻¹) and NAA (@ 0.5 mg L⁻¹). These observations are consistent with earlier studies where addition of NAA promoting the proliferation and elongation of shoots in *Petasites hybridus* (Wldi *et al.*, 1998), *Eucalyptus grandis* (Luis *et al.*, 1999) and *Hybanthus enneaspermus* (Prakash *et al.*, 1999) was shown.

In the present investigation, the shoots grew to a length of 2-3 cm over an incubation period of 15 days. They were separated and inoculated on ½ MS medium for profuse rooting, which was evident in 10 - 15 days. All the shoots were rooted and established in the greenhouse after hardening (Fig. 3). The rooting frequency was around 90% on ½ MS as against ¼ MS

Table 3. Effect of MS medium concentrations on root induction from *in vitro* raised shoots in *C. forskohlii*

MS medium concentration	Shoot root ⁻¹ (no.)	Roots length (cm)	Rooting (%)
MS Basal	9.0 ± 0.5 ^a	4.0 ± 0.1 ^a	70
½ MS	23.0 ± 1.2 ^a	7.0 ± 4.2 ^a	90
¼ MS	12.0 ± 0.3 ^a	3.0 ± 0.5 ^a	50

Means in the same column followed by same letter are not significantly different (DMRT >0.05)

(Table 3). It has been reported that IAA (@ 1.0 mg L⁻¹) with MS medium is required for the induction of roots in *C. forskohlii* (Sharma *et al.*, 1991). However, the success of an auxin-free basal medium in the present study for efficient root induction (Monier and Ochatt, 1995; Mao *et al.*, 1995) validated our own observations. This discrepancy can be attributed in part to the availability of higher quantity of endogenous auxins present in *in vitro* regenerated shoot lets (Minocha, 1987). Although 70 % rooting was observed on MS media it yielded a small number of calli. The number and length of roots developed on ½ MS media was also found to be more as compared to MS and ¼ MS media.

Fig. 3. Direct regeneration of shoots from leaf explants of *Coleus forskohlii*: A. Shoot buds regenerated on MS medium with 5.0 mg L⁻¹ BAP after 28 days; B. Shoot bud elongation on MS medium with 0.1 mg L⁻¹ BAP and 0.1 mg L⁻¹ IAA; C. Rooting of regenerated shoot on ½ MS media; D. Plants transferred in green house.

In conclusion, the present approach offers an efficient protocol for the direct and large-scale mass propagation of *C. forskohlii* by circumventing the callus phase that would find application in conservation, large-scale commercial cultivation, and genetic transformation efforts.

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