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In Vitro Regeneration and Conservation of Three *Coleus* Species

P. E. Rajasekharan* • **S. Ganeshan** • **Sunitha Bhaskaran**

In Vitro Conservation and Cryopreservation Laboratory, Division of Plant Genetic Resources
Indian Institute of Horticultural Research, Hessaraghatta Lake P.O, Bangalore 560 089, India
Corresponding author: * **rajasekharan.pe@gmail.com**

ABSTRACT

Three species of the genus *Coleus* (Lamiaceae), *Coleus forskohlii* Briq. (syn. *C. barbatus* Benth.), *Coleus parviflorus* Benth., and *Coleus zeylanicus* Benth. L.H. Crammer were cultured *in vitro* on half-strength Murashige and Skoog (MS) medium supplemented with 8.87 µM BAP in combination with 0.54 µM NAA. Direct regeneration was encouraged to avoid a callus phase and to maintain true-to-type cultures. Concomitant rooting in multiplication media enabled successful establishment *ex vitro* with 85% survival. Similar rooted cultures were conserved *in vitro* at 10°C for a minimum period of 6 months, without any subculture and at standard culture conditions in MS or ½MS basal medium devoid of plant growth regulators, growth retardants or osmoticum to avoid somoclonal variation.

Keywords: *Coleus forskohlii*, *Coleus parviflorus*, *Coleus zeylanicus*

INTRODUCTION

The genus *Coleus*, member of the Lamiaceae, formerly Labiatae, or mint family, contains more than 300 species. Most of these are annual or perennial herbs native to diverse locations such as Africa, Australia, East Indies, Malay Archipelago and the Philippines. Though most *Coleus* species are grown as foliar ornamentals, this genus also contains several medicinal (*C. forskholii*) and edible species *C. parviflorus* (Nagpal *et al*. 2008). Three *Coleus* species were selected for *in vitro* conservation studies.

1) *Coleus forskohlii* Briq. (syn. *C. barbatus* Benth) is an aromatic herb found in sub-tropical Himalayas, Bihar and Gujarat. It is being cultivated to a limited extent for root tubers in the states of Gujarat, Maharashtra and Karnataka in India. *C. forskohlii* is chiefly propagated through seeds and stem cuttings during June-July (Anonymous 1950). It is an ancient root drug recorded in Ayurvedic *materia medica* (Raj Nighantu Samhita Dhanvantariya Nighantu 1896), under the Sanskrit names 'Makandi' and 'Mayini', claimed to improve appetite, facilitate digestion, increase vitality, useful in anemia, inflammation, flatulence, dropsy, fevers infestation of worms, spleenomegaly, dysentery, chronic abdominal problems, colitis and piles. The root accelerates cellular metabolism, is used as a cardio-vascular tonic, lowers blood pressure and relieves uterine cramps. Leaves are indicated for stomach and respiratory problems. Roots are a rich and exclusive source of forskohlin (diterpenoid) used in preparation of drugs against cardiomyopathy, glaucoma (Anonymous 1950). Commercial exploitation of the tuberous roots from wild has increased in response to the growing demand for forskohlin, thereby listing this indigenous species as endangered (Vishwakarma *et al*. 1988).

2) *Coleus parviflorus* Benth is a small prostrate herb

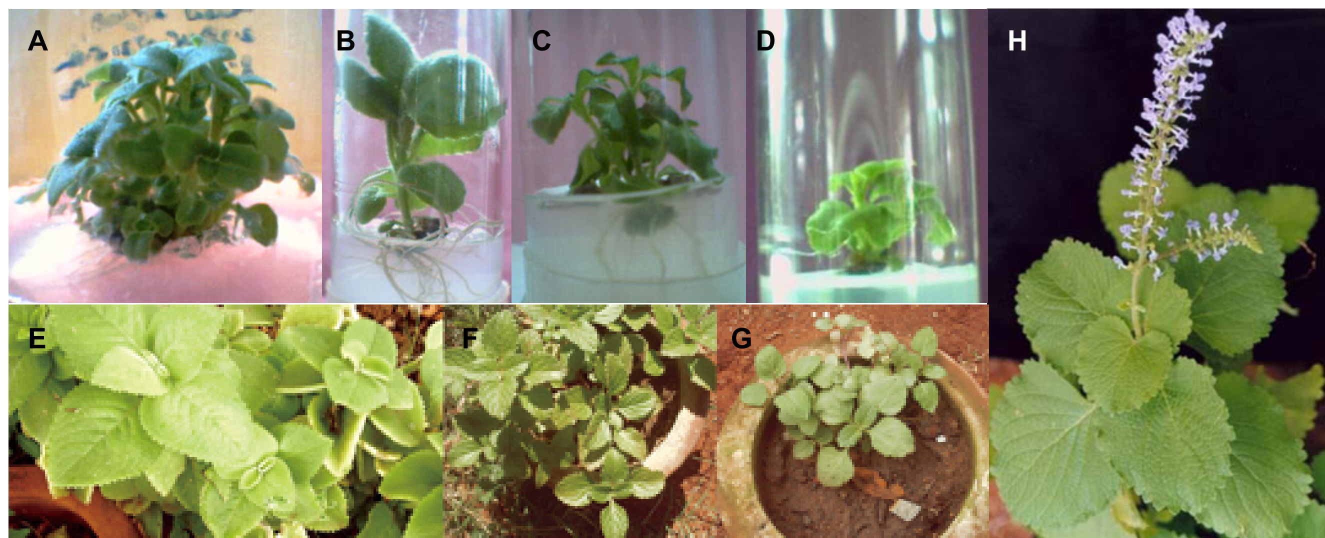

Fig. 1 ***Coleus* spp. *in vitro*.** (**A**) *C. aromaticus* multiplication *in vitro*; (**B**) *C. aromaticus* single plantlet with roots; (**C**) *In vitro* rooted plant of *C. aromaticus*; (**D**) *C. zeylanicus in vitro*; (**E**) *C. aromaticus* established *ex vitro*; (**F**) *C. parviflorous* established *ex situ*; (**G**) *C. parviflorous* established *ex situ*; (**H**) *C. zeylanicus* established *ex vitro*.

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bearing dark brown tuberous roots known as 'Inala' or 'country potato'. This species is grown in the Deccan peninsula, Quilon and southern districts of India, mainly Kerala, Sri Lanka, Java, Indo-China and parts of tropical Africa for edible tubers. It is cultivated to a large extent in Sri Lanka and Malabar region as a monsoon crop. It is considered to be an underutilized tuber crop (Anonymous 1950).

3) *Coleus zeylanicus* (Benth.) L.H. Crammer is a profusely branched, semi-succulent, strongly aromatic, soft-tomentose herb with fibrous roots and pale blue flowers. It is a native of Sri Lanka (but presently not available in the wild) (Sivarajan and Balachandran 1998) and naturalized in India. Medicinally this species is used in bowel complaints, as a coolant, carminative and tonic. It cures dyspepsia, indigestion, dysentery, vomiting, fever, dermatitis ulcers, bleeding disorders and quenches thirst.

Two accessions of *C. forskohlii* (Indian Institute of Horticultural Research, Bangalore-89 and Gandhi Krishi Vigyan Kendra, University of Agricultural Science, Bangalore-65), one variety of *C. parviflorus* (var. 'Shridhar') released by the Central Tuber Crops Research Institute, Thiruvananthapuram (Kerala state, India) and one accession of *C. zeylanicus* sourced from the Tropical Botanical Garden and Research Institute, Thiruvananthapuram (Kerala State, India) and successfully established in the Field Gene Bank at IIHR, Hessaraghatta, Bangalore, prior to accessing explants for *in vitro* culture. *In vitro* conservation techniques are very useful for vegetatively propagated crops, particularly where propagation by conventional means is not reliable due to total lack of seed production, or production of non-viable seeds. For clonally propagated plants, *in vitro* conservation is recognized as an alternative to conventional field maintenance, in order to safeguard against pests and natural disasters (Dodds 1991). While micropropagation of *Coleus forskohlii* (Sen and Sharma 1991; Sharma *et al.* 1991; Suryananarayana and Jagadish 1998) is well established, protocols for *in vitro* conservation still remains to be optimized. Research is in progress on phytochemistry of forskohlin (Tripathi *et al.* 1995; Yanagihara *et al.* 1995; Suryanarayana and Jagdish 1999) which could enable sustainable extraction and use of the active ingredient from elite chemotypes, when protocols are intercalated with *ex situ* conservation programs. Bhattacharya and Bhattacharya (2001) developed a protocol that led to the development of complete *C. forskohlii* plantlets within 35 ± 40 d by culturing stem tip explants in MS medium containing 0.57 mM indole-3-acetic acid and 0.46 mM kinetin through direct multiplication at a rate of 12.5 shoot explants. A detailed pharmacognostical study of the roots of *C. forskohlii* procured from different geographical zones of India was carried out by Srivastava (2002). The growth and rosmarinic acid production by *C. forskohlii* hairy root cultures in various liquid media were examined by Li *et al.* (2005).

In the present investigation, a protocol for *in vitro* conservation was optimized, and which could be adopted by *in vitro* active gene banks.

MATERIALS AND METHODS

Plant material

Juvenile shoot tips and nodal segments of *C. forskholii*, *C. zeylanicus* and *C. parviflorus* formed the choice explants for *in vitro* establishment of cultures. Explants were cut into 1 cm pieces and washed thoroughly with water for 15 min and with 4% Cleansol (detergent) for 15 min. Subsequently, the explants were pretreated with 70% ethanol for 90 sec, prior to sterilization with 0.01% $HgCl_2$ for 5 min. This was followed by three to five rinses with sterile distilled water in the laminar airflow cabinet. The sterilized explants were further trimmed to 0.5-cm pieces prior to inoculation.

Culture media, initiation and multiplication

Explants were cultured under aseptic conditions on Murashige and Skoog (MS) medium (1962) devoid of plant growth regulators supplemented with 3% sucrose and 0.7% agar for both MS and ½MS basal media. The pH of the medium was adjusted to 5.8 before autoclaving at 121 psi for 20 min. Multiple shoots were obtained on ½MS medium supplemented with various concentrations of BAP (benzyl amino purine) and NAA (α-naphthalene acetic acid) (i.e. ½MS+ 8.87 µM BAP + 0.54 µM NAA; ½MS + 4.44 µM BAP + 0.54 µM NAA). The cultures were incubated at 25 ± 2°C with a 16-h photoperiod. A minimum of 100 cultures in test tubes were placed at standard culture conditions (SCC; 25 ± 2°C; 31.55 $\mu mol^{-2} s^{-1}$) and 10°C with low light intensity (2.97 $\mu mol^{-2} s^{-1}$). *In vitro* cultures were considered established with a good shoot root ratio (2: 1) after 4 weeks, after which conservation treatments were imposed.

Rooting and hardening

Rooting occurred concomitantly with shoot growth; hence no separate medium was required. Rooted cultures were hardened in polythene bags containing 'Soilrite' (vermiculite potting mixture) and maintained in a glasshouse with normal light and temperature.

Data analysis

The results recorded by observing growth parameters (**Table 1**) with respect to species, media, and storage period under SCC and after storage at 10°C were analyzed using Completely Randomized Design and ANOVA without transformation.

Table 1 Media, growth parameters and culture conditions for the 3 *Coleus* species.

Species	Medium	Explant	Culture conditions	Observations recorded
Coleus forskholii (IIHR)	MS Basal	Nodes	SCC	Plant height, number of shoots, number of roots, number of nodes and internodal length (all in cm)
	HMS Basal	Nodes	SCC	
	HMS+8.87µM BAP+0.54µM NAA	Nodes	SCC	
	MS Basal	Nodes	10ºC	
Coleus forskholii (GKVK)	MS Basal	Nodes	SCC	
	HMS Basal	Nodes	SCC	
	HMS+8.87µM BAP+0.54µM NAA	Nodes	SCC	
	MS Basal	Nodes	10ºC	
Coleus zeylanicus	MS Basal	Nodes	SCC	
	HMS Basal	Nodes	SCC	
	HMS+8.87µM BAP+0.54µM NAA	Nodes	SCC	
	MS Basal	Nodes	10ºC	
Coleus parviflorus	MS Basal	Nodes	SCC	
	HMS Basal	Nodes	SCC	
	HMS+8.87µM BAP+0.54µM NAA	Nodes	SCC	
	MS Basal	Nodes	10ºC	

Table 2 Mean values of the 3 species, with regard to growth parameters under SCC.

Species	No. of shoots	No. of roots	Shoot length	Internode length	No. of nodes
C. forskohlii (IIHR)	3.833 a	15.500 a	6.0833 a	0.803 b	4.350 c
C. forskohlii (GKVK)	3.200 a	15.200 a	3.1933 a	0.336 a	3.466 a
C. zeylanicus	3.133 a	13.433 a	4.153 b	1.163 c	3.766 ab
C. parviflorus	3.700 a	13.706 a	3.916 ab	0.823 b	4.200 bc
Significance at 5%	NS	NS	**	**	**
SeM	0.3246	0.6817	0.2979	4.404E-02	0.1980
CD 5%	NS	NS	0.8375	0.1223	0.5567

In each column, mean values super scribed by the same letters are not significantly different at $P = 0.05$.

Table 3 Mean values, of storage period, with regard to growth parameters under SCC.

Storage period (in months)	No. of shoots	No. of roots	Shoot length	Internode length	No. of nodes
1 month	2.733 a	11.400 a	2.9756 a	0.7183 a	3.075 a
3 months	4.200 b	17.520 b	5.6983 b	0.8450 b	4.816 b
Significance at 5%	**	**	**	**	**
SeM	0.2295	0.4820	0.2106	3.114E-02	0.1400
CD 5%	0.6453	1.3550	0.5922	8.755E-02	0.3936

In each column, mean values super scribed by the same letters are not significantly different at $P = 0.05$.

Table 4 Mean values, of the different medium, with regard to growth parameters under SCC.

Media	No. of shoots	No. of roots	Shoot length	Internode length	No. of nodes
MS Basal	2.450 a	14.025 a	4.675 b	0.935 b	3.622 a
½MS Basal	3.025 a	15.175 a	4.800 b	0.962 b	3.800 a
HMS+8.87µM BAP +0.54µM NAA	4.925 b	14.180 a	3.535 a	0.447 a	4.375 b
Significance at 5%	**	NS	**	**	**
SeM	0.2811	0.5903	0.2580	3.814E-02	0.1715
CD 5%	0.7903	NS	0.7253	0.1072	0.4821

In each column, mean values super scribed by the same letters are not significantly different at $P = 0.05$.

Table 5 Mean values of the species with regard to growth parameters during storage at 10ºC.

Species Name	No. of shoots	No. of roots	Shoot length	Internode length	No. of nodes
C.forskohlii (IIHR)	4.6 c	17.1 c	4.49 a	1.29 b	3.4 a
C.forskohlii (GKVK)	1.2 a	7.9 a	6.8 b	1.49 b	4.0 b
C.zeylanicus	1.9 ab	6.4 a	4.59 a	0.75 a	4.2 b
C.parviflorus	2.0 b	13.4 b	6.27 b	1.8 c	4.4 b
Significance at 5%	**	**	**	**	**
SeM	0.2524	0.6745	0.4762	0.0819	0.2061
CD 5%	0.7275	1.9436	1.3722	0.2360	0.5940

In each column, mean values super scribed by the same letters are not significantly different at $P = 0.05$.

Table 6 Mean values of storage period with regard to growth parameters during storage at 10ºC.

Storage period (in months)	No. of shoots	No. of roots	Shoot length	Internode length	No. of nodes
1 month	2.3a	8.7 a	2.715 a	0.63 a	3.75 a
6 months	2.55 a	13.7 b	8.36 b	2.033 b	4.25 b
Significance at 5%	NS	**	**	**	**
SeM	0.1785	0.4769	0.3367	5.793E-02	0.1457
CD 5%	0.5144	1.3743	0.9703	0.1669	0.4200

In each column, mean values super scribed by the same letters are not significantly different at $P = 0.05$.

RESULTS

Aseptic cultures were established from nodal explants up to 98% by using 0.01% $HgCl_2$ for 5 min. Nodal segments *C. forskohlii*, *C. zeylanicus* and *C. parviflorus* cultured on MS basal, ½MS basal, ½MS + 8.84 µM BAP + 0.54 µM NAA, ½MS + 4.44 µM BAP + 0.54 µM NAA with sucrose at 3% (30 g/l) produced 2 shoot buds after 8 to 10 days of culture. Within 90 days *C. forskohlii* (IIHR) achieved a height of 3.53 to 4.80 ± 0.29, *C. zeylanicus* 4.15 ± 0.29, *C. parviflorus* 3.91 ± 0.29 and *C. forskohlii* (GKVK) 3.19 ± 0.29 in MS and ½MS media. Representative images of *in vitro* cultures of these *Coleus* spp. are shown in **Fig. 1**.

Storage under SCC

The three *Coleus* species did not differ significantly ($P = 0.05$) with respect to number of shoots and roots under SCC. But significant differences were observed for shoot and internodal length, and number of nodes (**Table 2**). After 3 months of storage, there was a significant increase in all parameters studied (**Table 3**). With an increase in storage period there was an increase in the number of roots observed, which after 3 months reached a value of 17.52 ± 0.48. When three media combinations used in this study were compared, significant differences were observed for all parameters except for the number of roots, which remained uniform irrespective of the media used. Addition of growth regulators in ½MS did not yield an increase in the number of roots (**Table 4**), but reduced shoot length significantly, although maximum number of shoots were produced (4.925 ± 0.28).

Storage under reduced temperature and light conditions (10°C)

Among species, under reduced temperature and light conditions (10°C and 2.97 $\mu m^{-2}s^{-1}$ light intensity) production of multiple shoots, roots, shoot and internodal length and nodes all differed significantly (**Table 5**). After 6 months of storage the number of shoots produced was not significantly altered (2.55 ± 0.17). But, there was a significant increase ($P = 0.05$) in the number of roots, shoots and internodal length and number of nodes after 6 months of storage (**Table 6**).

The number of multiple shoots formed was not signifi-

cant even after 6 months of storage. However, other parameters showed significant differences over 6 months of storage. Six months of storage under reduced temperature and light storage under SCC for a period of 3 months produced similar results. There was a maximum increase in the number of roots in *C. forskohlii* and *C. parviflorus* during storage due to the occurrence of tubers in these two species. Shoot length increased at 10°C due to increased internodal length, however, storage at 10°C and low light could delay subculture further by a further 3 months. These species showed significant differences in growth parameters at 10°C and low light with respect to number of shoots and roots.

All rooted *in vitro* cultures were easily established *ex vitro* in 'Soilrite' and later transplanted in the field from glass house with less than 5% mortality in all species. 100% survival was observed in the case of *C. zeylanicus*.

DISCUSSION

All three *Coleus* species in this study could be maintained at SCC for a period of 3 months and at 10°C with low light intensity for a period of 6 months without subculture. However, after 3 months at 10°C, the cultures had to be brought to SCC for 2 days and subsequently relocated to 10°C in order to maintain them at low temperature. Etiolation of older leaves was common in all 3 species; however, the terminal ends remained green. At 10°C storage, the cultures were maintained in full-strength MS basal medium in order to prevent somaclonal variation during storage which was more likely to occur when plant growth regulators, growth retardants and osmoticum, etc. are used (Ashmore 1997).

There is a reduction in shoot length during storage, obtained in MS basal media at 10°C after 6 months, which averages 1.39 cm growth per month compared to that obtained after 3 months storage under SCC, averaging 1.79 cm. An increase in internodal length was also observed, a feature commonly noticed when such storage treatments were imposed with reduced light intensity. Maintenance of cultures at 10°C proved effective in reducing subculture frequency and increasing storage period. *In vitro* slow growth storage and cryopreservation are the only ways of keeping material. Slow growth culture is a medium term method using for *in vitro* culture of organs at low temperatures (Keller *et al*. 2006).

Ex situ conservation of plant species in field gene banks are prone to diseases or damage through natural disasters and stress factors (Ashmore 1997). In living plant collections, vegetatively propagated species are outstanding material with respect to vulnerability and amount of labour. Therefore, *in vitro* storage techniques offer greater security for germplasm. *Ex situ* techniques can be used to complement *in situ* methods and, in some instances, may be the only option for some species (Rao and Hodgkin 2002). Nodal segments of *Coleus* species were observed to be the most appropriate explant source for initiation of cultures, since they withstood sterilization process better than apical shoots, exhibiting maximum survival frequency. A similar result was reported for *C. forskohlii* by Sharma *et al.* (1991) and for *C. blumei* (Zagrajski *et al.* 1997). Besides this, in the present study, bud break was more commonly observed with nodal explants rather than shoot tips, as the latter turned brown due to excess polyphenol exudation and failed to establish *in vitro*.

In vitro conservation aims to reduce the growth rate to maintain *in vitro* culture cultures in a slow growth phase, in order to avoid frequent subculture. Conservation at low temperature is known to influence the growth rate by lowering the overall cell metabolic activity (Grout 1995). This may be due to a decrease in overall *in vitro* photosynthetic activity (Desjardins 1995; Zimmerman 1995) with a reduction in leaf size; total leaf area was reduced from 1.30 cm^2 (SCC) to 0.3 cm^2 during storage at 10°C. Increased survival post low temperature storage can also be attributed to concomitant rooting observed among the 3 *Coleus* species studied. Rooting *in vitro* regulates absorption of water and nutrients from the culture medium, which is a limiting factor in shoot cultures (Hazarika 2003). Many more problems are encountered with respect to plant germplasm, which cannot be stored as seeds, because either it does not form seeds at all or their seeds are not storable (recalcitrant) or do not represent genetic identity with the parental material because of heterozygousity like in many clonal crops.

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