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Influence of explants type and phytohormones on *In vitro* callogenesis and plantlet regeneration of patharchur (*Coleus barbatus* L.), an endangered ethnomedicinal plant

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Abstract
The present study was conceptualized to study the effect of explants and plant growth regulators on organogenesis to establish an efficient regeneration protocol for *Coleus barbatus*, an endangered ethnomedicinal Plant. In course of this, sterilized explants were cultured onto MS medium augmented with different concentrations and combinations of auxin (IAA) and cytokinin (BAP). The best callogenic response was observed on media M_4 followed by M_9 and M_5 in terms of Days of callogenesis, degree of response, colour and texture, considering all the three explants. Regenerated shoots from embryogenic calli were further multiplied on fresh media. Highest per cent shoot regeneration response was obtained on medium M_{12} (84.7%) and M_7 (84.7%) followed by media M_6 (81.4%), whereas in terms of number of shoots per culture, shoot apex (0.0-3.7) and internodal stem (0.0-3.7) exhibited as better explant then young leaf (0.0-1.7). The long and healthy shoots were harvested and subcultured on rooting media for root induction. Maximum per cent response of root proliferation were obtained on medium M_{13} (85.2%) followed by media M_9 (84.4%) and M_8 (84.1%), whereas for highest frequency of root per elongated shoot, shoot apex (0.0-10.3) exhibited as better explant then internodal stem (0.0-9.7) and leaf explants (0.0-7.3). Hence, the *In vitro* regenerated plantlets using this established micropropagation protocol can be used for large-scale commercial cultivation, *in situ* conservation, and genetic characterization of this endangered medicinal plant.

Keywords: *Coleus barbatus*, ethnomedicinal, sterilants, callogenic response, organogenesis

Introduction
Medicinal plants are the finest and fascinating gifts of nature and are extensively used as herbal medicine over the globe since ancient times. As per estimates, over 80% of the world's population still relies on plant-derived medicine (traditional healers) for their basic health-care needs. In fact, Herbal remedies provide safe and effective drugs and have less or no side effects then synthetic modern medicine (Praveena *et al*, 2012; Thirupathi *et al*. 2013) [22, 36]. *Coleus barbatus* Benth (Syn. *Coleus forskohlii* Briq.) is an important ethnomedicinal perennial herb, belongs to the mint family (Lamiaceae). This is a pungent aromatic herb, considered to be native to Indian sub-continent (Singh *et al.*, 2011) [34] and widely distributed across arid, tropical, subtropical and temperate regions of India, Sri Lanka, Nepal, Pakistan, East Africa, Thailand, Egypt, Arabia, and Brazil at an altitude of about 600-2400 mts. In India, the crop is commercially grown in hilly regions of Himalayas, Deccan peninsula, Rajasthan, Tamil Nadu, Karnataka, Maharashtra and Bihar (Yashaswini and Vasundhara, 2011; Khan *et al*, 2012) [35, 11]. It is a multipurpose medicinal plant and is commonly known as "*Green gold*" in Indian ayurvedic and siddha medicine (Maheswari *et al*, 2011; Khan *et al*, 2012) [15, 11]. The plant has known by numerous vernacular names viz., Coleus in English, Patharchur in Hindi, Pashanbhedi in Sanskrit, Makandiberu in Kannada, Maimnul in Marathi and Garmalu in Gujarati (Kotia *et al.*, 2014) [14]. In recent years coleus has gained ethnomedical importance as the only known natural source of biologically active compound called labdane diterpenoid, coleonol [Forskolin (7 β - acetoxy - 8, 13-epoxy-1α)] in the tuberous root (Bhat *et al.*, 1977; Rupp *et al*., 1986; Murugesan *et al*, 2012) [3, 24, 19]. The root extracts of the coleus were found to be rich in Forskolin (0.07 to 0.59%) (Maheswari *et al*, 2011) [15] and have many pharmacological activities such as antiinflammatory, antiglaucoma, antithrombotic, antiplatelet aggregation, antidepressant, and antidiuretic (Krishna *et al*, 2010; Sharma and Vasundhara, 2011; Mathur *et al*, 2011; Khan *et al*, 2012; Kotia *et al.*, 2014) [13, 30, 17, 11, 14].

Because of its unique ethnomedicinal properties, it has a great demand as a potential raw material in the pharmaceutical and food industries. This demand led to rapid depletion of wild sources, resulting in its listing as a plant under endangered species category (Maheswari *et al*, 2011; Murugesan *et al*, 2012; Kotia *et al.,* 2014) [15, 19, 14]. This situation encouraged the cultivation of *C. barbatus* on vast area, which is generally practiced by seeds and vegetative propagation method and is labour intensive, time consuming and results in lower rate of multiplication (Prajapati *et al*., 2003) [23]. Thus, *In vitro* regeneration can possibly eliminate these problems and might be considered as a handy tool for conservation of this important medicinal plant (Velmurugan *et al*, 2010; Kanungo *et al*., 2012) [38, 12]. In view of the above background the present study was conceptualized to study *in vitro* regeneration capabilities of *Coleus barbatus* for developing efficient protocol for large-scale commercial cultivation, *in situ* conservation, and genetic characterization of this endangered medicinal plant.

Materials and Methods

Collection and surface sterilization of explants

Shoot branches of about 15 cm lengths were collected from 4-6 months old young, healthy and disease free plants of *C. barbatus* maintained in the Polyhouse of Hi-Tech Horticulture, medicinal plant garden of Dr. Rajendra Prasad Central Agricultural University**,** Pusa, Bihar (Figure: 01 a, b). Plantlets collected from the field were washed thoroughly under running tap water (15-20 minutes) to remove the superficial dust particles. Shoot apex (1-2 cm), internodal (1.5-2.0 cm) segments with a single axillary and leaf lamina with mid-vein (2 cm) were used as excised explants in the present study (Figure: 01 e, f and g). Different concentration and combination of sterilants and detergents have been used to standardize successful sterilization (Table: 01). Explants were dressed, washed and treated with 0.1% Tween 20 (v/v) for 5-30 min, 1% Bavistin (w/v) (systemic fungicide) for 15-60 min and then washed with autoclaved distilled water (3-4 times) to steer noxious chemicals (Krishna *et al*, 2010; Pattar and Jayaraj, 2012; Shekhawat *et al.,* 2015) [13, 20, 31]. These pretreated explants were then disinfected with 0.1% $HgCl_2$ (w/v) for 2-5 min under aseptic conditions inside the laminar flow cabinet and again rinsed with sterile distilled water (3-4 times) (Maheswari *et al*, 2011; Sen *et al.,* 2014; Shekhawat *et al.,* 2015) [15, 26, 31]. Finally, these explants were immersed in 0.5% streptomycin (w/v) for 2-5 min followed by washing with sterile distilled water (3-4 times) (Bhattacharyya and Bhattacharya, 2001) [4] and were used in further *in vitro* studies after trimming its edge (Table: 01, Figure: 01 c, d).

Nutrient media and culture conditions

During present study, The Murashige and Skoog (1962) [18] basal medium (MS) supplemented with different concentrations and combinations of plant growth regulators viz., auxin (IAA: Indole-3-acetic acid) and cytokinin (BAP: 6-Benzylaminopurine) were used for establishment of culture (Table: 02). The $_{P}H$ of the media prepared in culture tubes were adjusted to 5.8 ± 0.02 before autoclaving at 121 ^{0}C under 15 lbs for 15-20 min. The surface sterilized explants were inoculated on different media and cultures were maintained at temperature (28 ± 2 ^{0}C), relative humidity (60 to 80%) under photoperiod (16L: 8D) provided by white fluorescent tubes (2500-3000 Lux) in the incubation chamber (Krishna *et al*, 2010; Shekhawat *et al.,* 2015) [13, 31].

Callus induction and organogenesis

The experiment was conducted to determine the effects of MS basal media, alone or supplemented with different plant growth regulators on different tissue culture responses. *In vitro* cultured explants of *Coleus barbatus* showed various responses viz., swelling of explants, callus induction (callogenesis), multiple shoot differentiation (caulogenesis) and root regeneration (rhizogenesis) which is largely depends upon nutrients fortified in the media (Cheruvathura *et al*, 2012) [5]. After callus initiation, frequency, colour and texture of callus were periodically assessed for each explant. The embryogenic calli showing multiple shoot differentiation were subcultured after 4-5 week on fresh medium (M_2, M_5, M_6, M_7, M_{10}, M_{11}, M_{12}, M_{16} and M_{17}) and observed for its relative frequency (Krishna *et al*, 2010; Velmurugan *et al*, 2010; Praveena *et al*, 2012) [13, 38, 22]. Finally, the differentiated shoots were transferred in the selected rooting medium (M_3, M_8 M_9, M_{13}, M_{14}, M_{15}, M_{18}, M_{19}, M_{20}, and M_{21}) and frequency of rooting was observed (Velmurugan *et al*, 2010; Singh *et al*, 2010; Praveena *et al*, 2012) [38, 33, 22]. MS basal media (full strength), without any plant growth regulators serve as control in callus induction, shoot and root regeneration studies (Table: 02) (Krishna *et al*, 2010; Dube *et al*, 2011; Praveena *et al*, 2012) [13, 7, 22].

Acclimatization and field transfer of regenerated plantlets

The well rooted plantlets were washed carefully with sterile distilled water to remove adhered medium from the roots. Plantlets were then transferred to small pots containing mixture of autoclaved soil, sand and vermicompost in 1:1:1 ratio and kept in acclimatization chamber for 10-15 days. After initial acclimatization, potted plantlets were shifted to the green house for 25-30 days (Bhattacharya and Bhattacharyya, 2001; Velmurugan *et al*, 2010) [4, 38]. During this practice the relative humidity (90-30%) was reduced gradually and periodical monitoring of pots were done for successful restoration. Afterwards, hardened plants were transplanted to the field conditions for further growth and development (Pattar and Jayaraj, 2012; Thirupathi *et al*, 2013; Shekhawat *et al.,* 2015) [20, 36, 31]. Care should be taken while transfer of plantlets to pots and soil conditions regarding minimum disturbance to root system.

Observations, data collection and statistical analysis

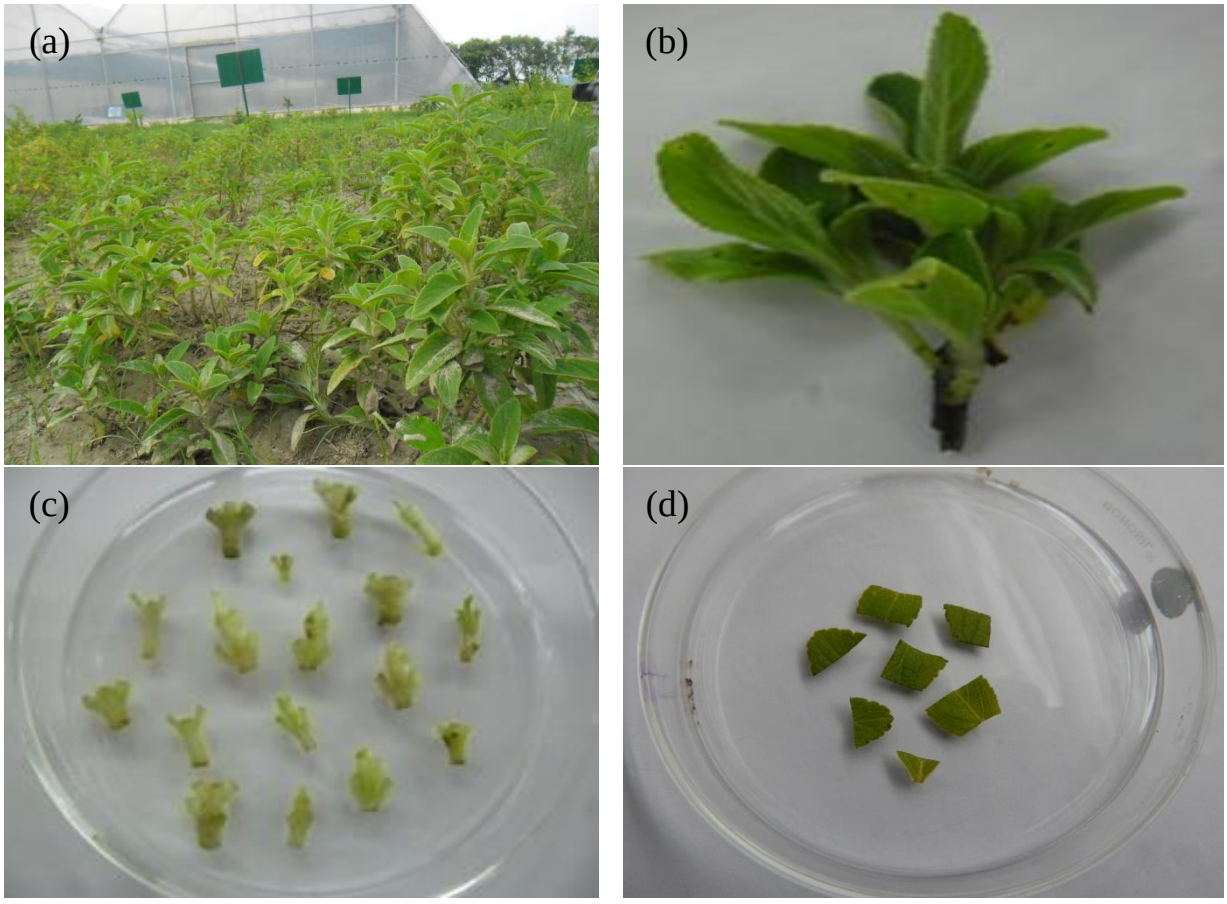
The culture was periodically observed for different tissue culture responses such as per cent survival, callus intensity and morphology, number and frequency of regenerated shoots and roots, etc. for each explant. Experiments were conducted in a completely randomized design (CRD) for studying the effects of single factor and were repeated thrice with three replicates per treatments (Thirupathi *et al*, 2013; Shekhawat *et al.,* 2015) [36, 31]. OPStat Excel version software package was used for statistical analysis like coefficient variation, mean, standard deviation (Sheoran *et al*. 1998) [32] and Excel software package of Microsoft was used for graphical representation.

Results and Discussion

Effect of surface sterilants on culture establishment

The cultures in which explants could not be established were discarded largely because of the infection of fungus and bacteria and rarely due to the necrosis of the explants resulting into their non-responsiveness and ultimately death. For solving these problem nine different explant pre-treatment

steps has been used in the way to establish an efficient protocol. The frequency of explants survival and response of explants for callus initiation varied with concentration of Tween-20, bavistin, and $HgCl_2$ respectively at varying sterilization time (Garla *et al*, 2011; Sen *et al*, 2014) [9, 26]. The data obtained from the surface sterilization of different explants were represented as graph: 01 and it reveals that the per cent survival of shoot apex (93.67%), internodal (85.00%) and leaf lamina (75.33%) were highest with treatment T_8 (0.1% Tween-20 for 5 min., 1% bavistin for 1 hour, 0.1% $HgCl_2$ for 2-3 min. and 0.5% streptomycin for 5 min., respectively) and was found significantly higher than all other treatments. The least survival rate of shoot apex (34.67%) was recorded with treatment T_2 (0.1% Tween-20 for 10 min., 1% bavistin for 15 min., and both 0.1% $HgCl_2$ and 0.5% streptomycin for 2-3 min., respectively). Whereas, Internodal (33.67%) and leaf lamina (16.67%) showed the least survival rate with treatment T_1 (0.1% Tween-20 for 5 min. 1% Bavistin for 1 hour, and 0.1% $HgCl_2$ for 2-3 min.). It was also observed that per cent survival of the explants were gradually increasing when the treatment time of sterilants were increased i.e., 0.1% Tween 20 for 5-30 min, 1% bavistin for 15-60 min., 0.1% $HgCl_2$ for 2-5 min. in combination with 0.5% streptomycin for 2-5 minutes. Therefore, treatment of T_8 was considered best for surface sterilization of explants and used in further *in vitro* studies (Table: 01, Graph: 01). Hence based on the above results, it was observed that surface sterilants play a significant role in reducing fungal or bacterial contamination and increases survival rate of the explants. These observations emphasized by previous reports like Bhattacharya and Bhattacharya (2001) [4] sterilized the shoot apex and internodal segments with 5% Teepol, 0.1 % bavistin, 0.02% streptocycline in combination with 0.01% $HgCl_2$ and observed a significant reduction in contamination. Shoot tips and nodal segments sterilized with 70 % alcohol for 30 min. and 0.1% $HgCl_2$ for 8 minutes reported better for culture establishment in coleus spp. (Kumar *et al*., 2006). Similarly, Leaf explants of coleus sterilized with 0.1% $HgCl_2$ for 4 minutes (Krishna *et al*, 2010) [13] and shoot tip for 5 min. gave the highest per cent survival (78%) with least contamination (20%) (Velmurugan *et al*., 2010) [38]. Surface sterilization of stem and leaf explants with 1% Laboline for 30 min. and 0.1% $HgCl_2$ for 2 min observed better for culture establishment in *H. indicum* (Bagadekar and Jayaraj, 2011) [2]. Explants pre-treated with 0.1% each of bavistin and streptomycin for 15-17 min. followed by 0.1% $HgCl_2$ for 5-7 min and 70% ethanol for 1 min was found to be reasonable in reducing contamination (Maheswari *et al*, 2011; Panwar *et al.*, 2012) [15, 21]. Much similar result was observed with 0.1% $HgCl_2$ for 1-2 min. and 50% alcohol for 2-3 minutes (Praveena *et al.*, 2012) [22]. Likewise, Explants treated with 0.5% bavistin for 10-12 min and 0.1% $HgCl_2$ for 2-3 min showed the highest per cent survival of internodal (94.60%) explants followed by leaf (76.07%) and root (40.20%), respectively in *A. aspera* (Sen *et al*, 2014) [26]. Surface sterilization of nodal shoots treated with 0.1% bavistin and 0.1% $HgCl_2$ for 5 min. each provides sufficient sterile condition for culture establishment in medicinal plants, *E. gerardiana* (Garla *et al*, 2011) [9] and *P. foetida* (Shekhawat *et al.*, 2015) [31].



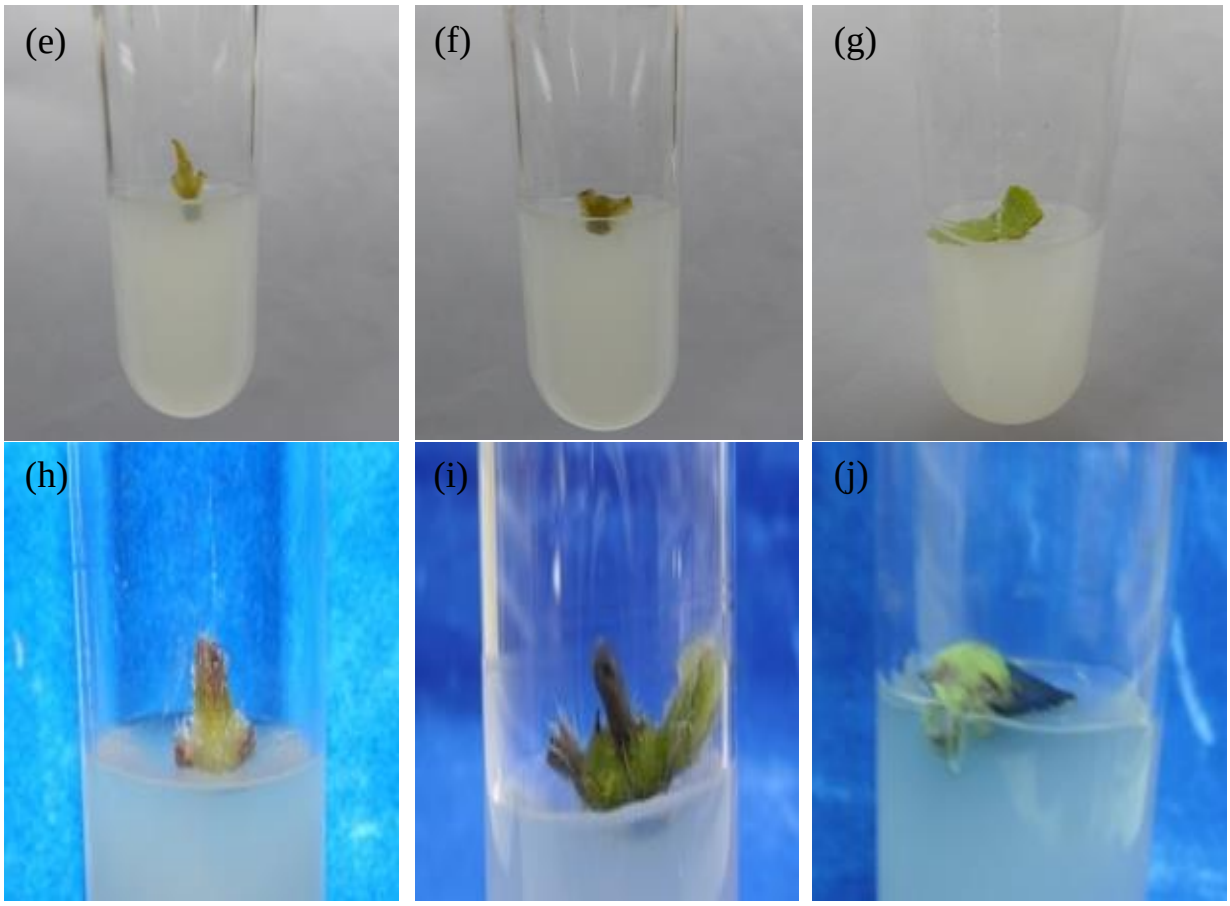
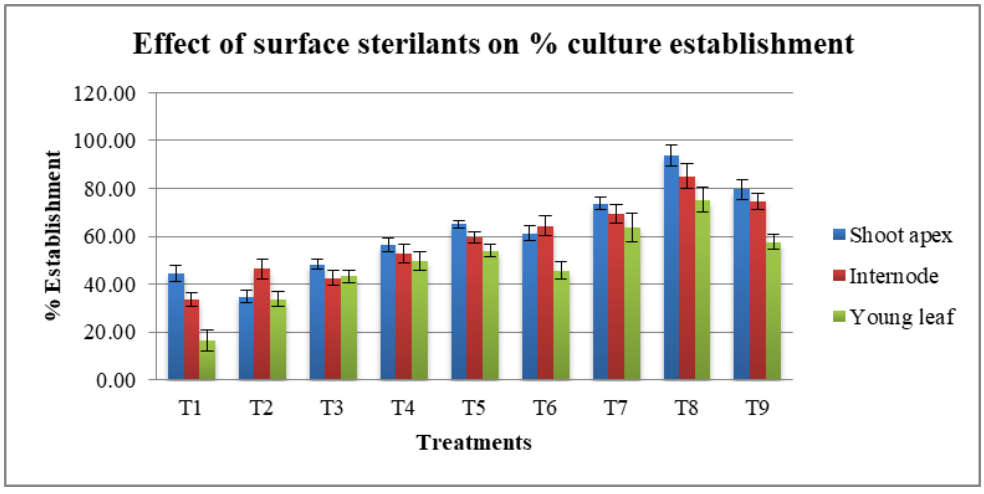


Fig 1: Plants and axillary branch of *coleus barbatus* collected from Hi-Tech Horticulture, RPCAU, Pusa (a, b), excised explants used for culture establishment (c, d, e, f and g) and swelled and non-swelled regions of explants for callus initiation (h, i and j).

Table 1: Different concentration and combination of sterilants used for establishment of aseptic culture at basal medium

Treatment	Different concentration and combination of sterilants					Edge cut
	Savlon (1 ml)	0.1% Tween-20 (v/v)	1% Bavistin (w/v)	0.1 % $HgCl_2$ (w/v)	0.5% Streptomycin (w/v)	
T_1	-	5 min	1 hrs	2-3 min	-	-
T_2	2-3 min	10 min	15 min	2-3 min	2-3 min	-
T_3	2-3 min	15 min	1 hrs	-	2-3 min	-
T_4	2-3 min	30 min	15 min	2-3 min	-	-
T_5	2-3 min	30 min (Altogether)		5 min	2-3 min	-
T_6	2-3 min	5 min	1 hrs	2-3 min	5 min	-
T_7	2-3 min	30 min (Altogether)		5 min	5 min	-
T_8	2-3 min	5 min	1 hrs	2-3 min	5 min	Yes
T_9	2-3 min	30 min (Altogether)		2-3 min	5 min	Yes



Graph 1: Effects of sterilants on surface sterilization of explants of *Coleus barbatus*

Table 2: Compositions of different nutrient media used for present study.

Media Code	Media composition	Plant growth regulators (conc. in mgL^{-1})	
		IAA	BAP
M_1	MS basal (Full strength)	-	-
M_2	MS + IAA + BAP	-	0.5
M_3		0.5	-
M_4		0.5	0.5
M_5		0.5	1
M_6		0.5	3
M_7		0.5	5
M_8		1	-
M_9		1	0.5
M_{10}		1	1
M_{11}		1	3
M_{12}		1	5
M_{13}		3	-
M_{14}		3	0.5
M_{15}		3	1
M_{16}		3	3
M_{17}		3	5
M_{18}		5	-
M_{19}		5	0.5
M_{20}		5	1
M_{21}		5	3
M_{22}		5	5

Effect of nutrient media and type of explant on callogenic response

Callus formation is an undesired response of cultured explants when the objective is regeneration and micro propagation of plant. Callus is an amorphous, coherent and unorganized tissue formed by the differentiated plant cells. Callus formation is considered to be an essential and significant feature for indirect organogenesis in tissue culture studies (Mahmood *et al*, 2012) [16]. In the present investigation, callus initiation was observed from cut surface of swelled and non-swelled regions of the explant within 6-18 days (Figure: 01 h, i and j). The explants (internode and leaf lamina) cultured on MS basal medium failed to generate callus whereas apical shoot gives callogenic response (Table: 03). It has been reported that degree of response, color and texture of callus depend on origin and type of explant as well as on the hormonal concentrations in the media (Cheruvathura *et al*, 2012) [5]. Explant from apical shoot and internode were found higher potential whereas explant from leaf and roots were less efficient for callogenic response (Sharma and Nautiyal, 2009; Mahmood *et al*, 2012) [29, 16]. Similar trend was observed for most of the callogenic response at different media combinations in the present study. For both days of callus initiation and degree of callogenesis, medium M_4 (MS + 0.5 mgL^{-1} IAA + 0.5 mgL^{-1}BAP) gave the best result followed by media M_9 (MS + 1.0 mgL^{-1} IAA + 0.5 mgL^{-1} BAP) and M_5 (MS + 0.5 mgL^{-1} IAA + 1.0 mgL^{-1}BAP), respectively whereas least response was found in medium M_6 (MS + 0.5 mgL^{-1}IAA + 3.0 mgL^{-1}BAP) considering all the three explants (Table: 03).

Among the selected explants, the shoot apex and internodal stem formed more callus compared to young leaf because these explants have higher proportions of meristematic tissues (Figure: 02). Krishna *et al*, (2010) [13] observed good callogenesis from leaf explants in *C. forskohlii* in MS medium fortified with 0.1 mgL^{-1} BAP and 5.0 mgL^{-1} BAP and for this response shoot tip was better than internodal stem as observed in the present study. Our results showed that media supplemented with lower concentration of auxin (0.5-1.5 mgL^{-1} IAA) and cytokinin (1.0-2.0 mgL^{-1} BAP) supports better callus formation in most of the explants whereas media fortified with higher concentration of cytokinin resulted in less callus formation (Table: 03, Figure: 02). This is in accordance with many previous reports like *C. borivillianum* (Haque *et al.*, 2009) [10], *J. curcas* (Varshney and Johnson, 2010), *P. nudicaule* (Yang *et al*., 2010), *E. gerardiana* (Garla *et al*, 2011) [9], *A. aspera* (Sen *et al*, 2014) [26] and *P. foetida* (Shekhawat *et al.*, 2015) [31]. Almost similar conclusion was drawn by Praveena *et al.* (2012) [22] in *C. forskohlii* and observed that lower concentration of 2,4-D (1-3.0 mgL^{-1}) along with BAP (0.5-2.0 mgL^{-1}) and IBA (0.5-1.0 mgL^{-1}) were potent for profuse callusing in tissue explants.

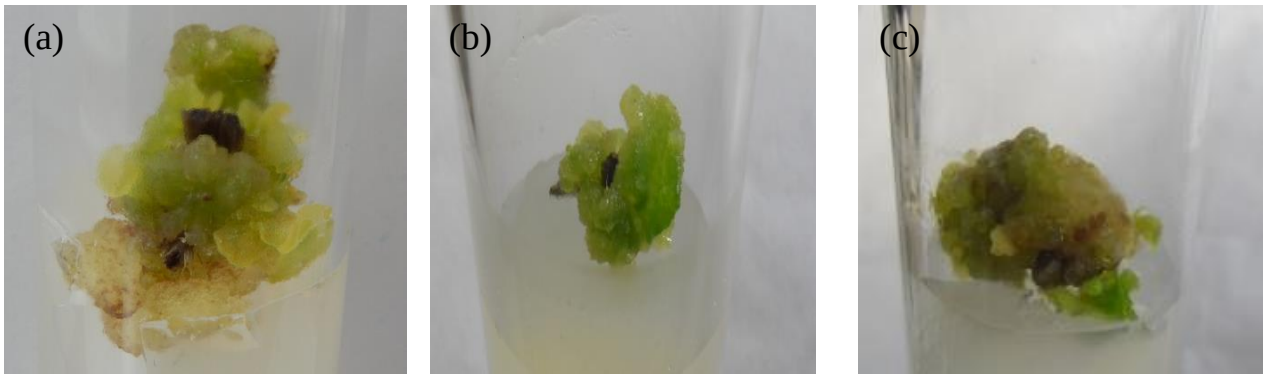
The present investigation also revealed that, *C. barbatus* formed greenish, brownish, creamish to pale yellow coloured, friable to compact and embryogenic calli in cultured explants. The MS medium supplemented with high concentrations of IAA and BAP (above 3.0 mgL^{-1}) produced more compact callus and are not embryogenic whereas friable and embryogenic calli were observed in lower IAA and BAP fortified media (Table: 03, Figure: 02). Earlier studies also reported that, high concentration of phytohormones like IAA, 2,4-D, NAA and IBA (3.0-5.0 mgL^{-1}) produces less callus friability and more compactness in *E. gerardiana* (Garla *et al*, 2011) [9], *H. indicum* (Bagadekar and Jayaraj, 2011) [2], *P. ovate* (Mahmood *et al*, 2012) [16], *E. alba* (Sharma *et al*, 2013), *A. aspera* (Sen *et al*, 2014) [26] and *P. foetida* (Shekhawat *et al.*, 2015) [31].

Table 3: Effects of different plant growth regulators on callus proliferation and morphology of various explants of *Coleus barbatus*.

Media	Explants	Callogenic response			
		Callus initiation (Days)	Degree of response	Colour	Texture
M_1	Shoot apex	11.3 ± 0.8	+++	Light green	Friable and embryogenic
	Internode	0.0	No callusing	No callusing	No callusing
	Young leaf	0.0	No callusing	No callusing	No callusing
M_2	Shoot apex	9.3 ± 0.8	+++	Creamish	Friable
	Internode	10.3 ± 0.6	+++	Light green	Compact
	Young leaf	13.6 ± 1.4	++	Light green	Compact
M_3	Shoot apex	8.6 ± 0.8	++++	Light Green	Friable and embryogenic
	Internode	9.6 ± 0.8	+++	Brownish green	Compact
	Young leaf	14.3 ± 1.4	++	Brownish green	Compact
M_4	Shoot apex	7.3 ± 0.8	++++	Light Green	Friable and embryogenic
	Internode	7.6 ± 0.6	++++	Light Green	Friable and embryogenic
	Young leaf	12.6 ± 0.8	++	Brownish green	Compact
M_5	Shoot apex	9.3 ± 0.8	+++	Light green	Friable and embryogenic
	Internode	8.3 ± 0.6	++++	Light green	Friable and embryogenic
	Young leaf	11.3 ± 0.8	+++	Pale yellow	Friable

M_6	Shoot apex	11.6 ± 1.2	+++	Brownish green	Compact
	Internode	11.3 ± 0.8	+++	Light Green	Friable
	Young leaf	15.3 ± 0.8	+	Brownish green	Compact
M_7	Shoot apex	13.6 ± 1.4	++	Brownish green	Compact
	Internode	14.6 ± 0.8	++	Creamish	Friable
	Young leaf	0.0	No callusing	No callusing	No callusing
M_8	Shoot apex	9.3 ± 0.8	+++	Light green	Friable
	Internode	10.3 ± 0.6	+++	Brownish green	Compact
	Young leaf	12.3 ± 0.8	++	Brownish green	Compact
M_9	Shoot apex	8.6 ± 1.2	++++	Light Green	Friable and embryogenic
	Internode	8.3 ± 0.8	++++	Light Green	Friable and embryogenic
	Young leaf	12.6 ± 1.2	++	Brownish green	Friable
M_{10}	Shoot apex	9.3 ± 0.8	+++	Light green	Friable and embryogenic
	Internode	11.6 ± 0.8	+++	Light green	Friable
	Young leaf	16.3 ± 1.2	+	Brownish green	Compact
M_{11}	Shoot apex	9.6 ± 0.6	+++	Light green	Friable and embryogenic
	Internode	11.3 ± 0.8	+++	Brownish green	Compact
	Young leaf	0.0	No callusing	No callusing	No callusing
M_{12}	Shoot apex	12.3 ± 1.2	++	Creamish	Friable
	Internode	0.0	No callusing	No callusing	No callusing
	Young leaf	0.0	No callusing	No callusing	No callusing
M_{13}	Shoot apex	8.6 ± 1.2	++++	Light green	Friable and embryogenic
	Internode	10.6 ± 0.8	++	Creamish	Friable
	Young leaf	13.3 ± 0.8	++	Brownish green	Compact
M_{14}	Shoot apex	8.3 ± 1.2	++++	Light green	Friable and embryogenic
	Internode	12.3 ± 0.8	++	Brownish green	Compact
	Young leaf	14.6 ± 0.8	++	Brownish green	Compact
M_{15}	Shoot apex	8.6 ± 0.8	++++	Light green	Friable
	Internode	12.3 ± 0.8	++	Creamish	Friable
	Young leaf	15.3 ± 1.2	+	Brownish green	Compact
M_{16}	Shoot apex	10.6 ± 0.8	+++	Light green	Compact
	Internode	0.0	No callusing	No callusing	No callusing
	Young leaf	0.0	No callusing	No callusing	No callusing
M_{17}	Shoot apex	11.6 ± 1.4	+++	Light green	Compact
	Internode	0.0	No callusing	No callusing	No callusing
	Young leaf	0.0	No callusing	No callusing	No callusing
M_{18}	Shoot apex	10.6 ± 1.4	+++	Light green	Friable
	Internode	8.6 ± 0.8	++++	Light green	Compact
	Young leaf	15.3 ± 0.8	+	Brownish green	Compact
M_{19}	Shoot apex	11.3 ± 1.4	+++	Pale yellow	Friable
	Internode	7.6 ± 0.8	++++	Light green	Compact
	Young leaf	16.6 ± 0.8	+	Brownish green	Compact
M_{20}	Shoot apex	9.3 ± 0.3	+++	Light green	Compact
	Internode	12.3 ± 0.6	++	Creamish	Friable
	Young leaf	0.0	No callusing	No callusing	No callusing
M_{21}	Shoot apex	9.3 ± 0.8	+++	Light green	Friable
	Internode	0.0	No callusing	No callusing	No callusing
	Young leaf	0.0	No callusing	No callusing	No callusing
M_{22}	Shoot apex	11.6 ± 0.8	+++	Light green	Compact
	Internode	0.0	No callusing	No callusing	No callusing
	Young leaf	0.0	No callusing	No callusing	No callusing

Note: 0.0: No response; Poor: +, intermediate: ++, good: +++ and excellent: ++++



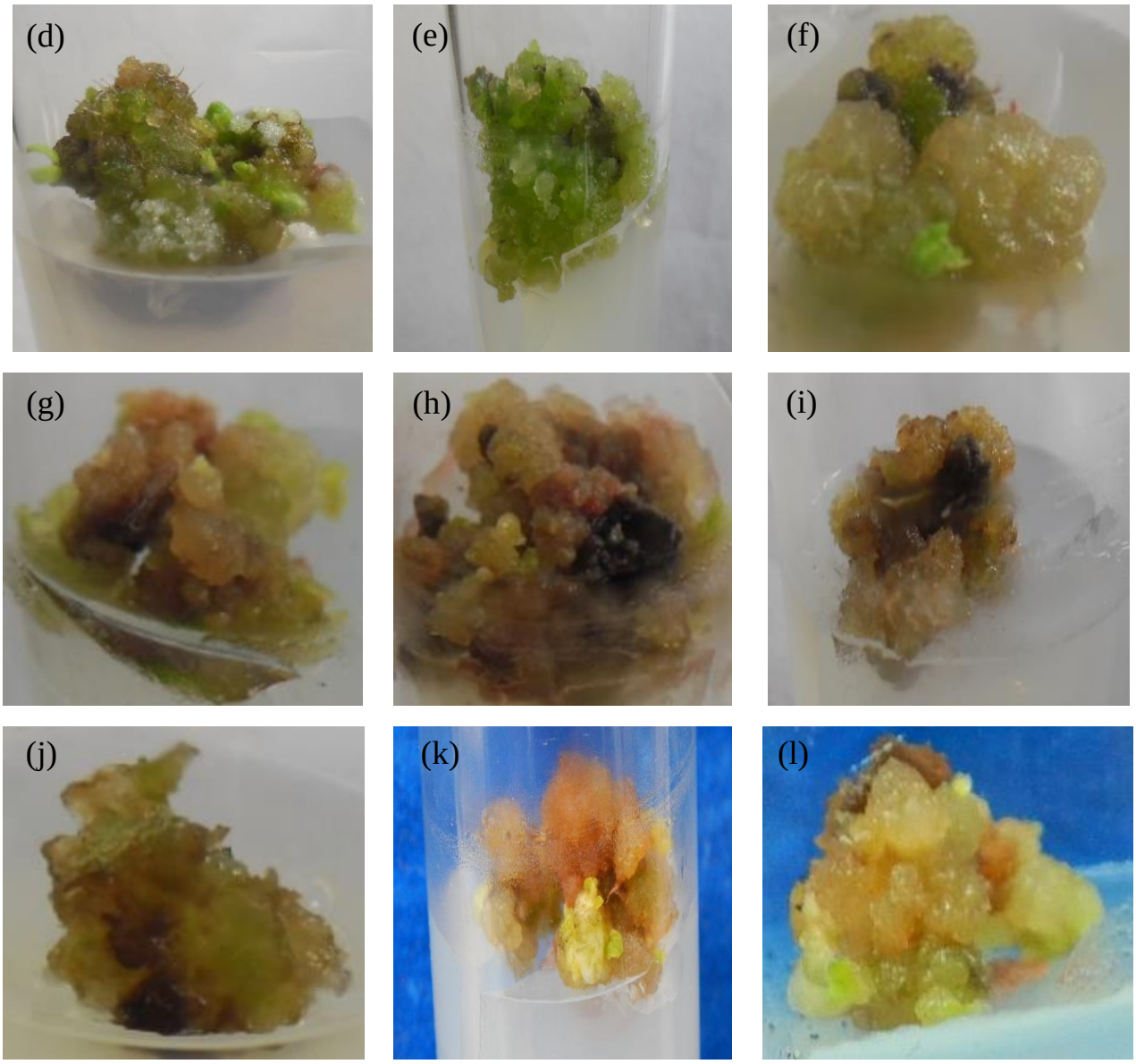


Fig 2: Cultured explants showing different callogenic responses in *Coleus barbatus i.e,* greenish (a, b, c, d, and e), brownish (f, j), creamish (g, h, and i) to pale yellow coloured callus (k, l); friable (a, b, c, d, e, g, h, i, j, k and l) & compact callus (f) and embryogenic in nature (a, c, d, e and l).

Callus mediated shoot regeneration

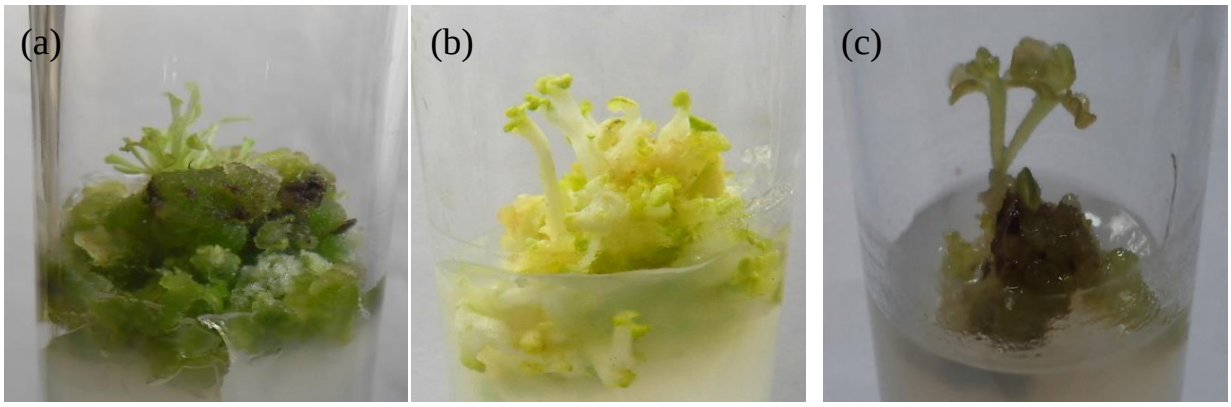
Shoot differentiation is the most frequent and essential process to facilitate plant regeneration. Rate of shoot multiplication ultimately decides the rate of propagules multiplication during the micropropagation of plants. In the present investigation, multiple shoots were differentiated from all the three explants through friable and embryogenic callus. Proliferated shoots were multiplied by repeated subculturing on fresh MS media fortified with a combination of IAA and BAP. The best per cent response of multiple shoot differentiation were obtained on medium M_7 (MS+0.5mgL^{-1}IAA+5.0mgL^{-1}BAP) and M_{12} (MS+1.0mgL^{-1}IAA+5.0mgL^{-1}BAP) followed by media M_6 (MS+0.5mgL^{-1}IAA+3.0mgL^{-1}BAP) and M_5 (MS+0.5mgL^{-1}IAA+1.0mgL^{-1}BAP), with minimum or no response on M_1 (MS basal) considering all the three explants. Likewise, among the selected explants, shoot apex (19.3-94.7%) and internodal stem (15.7-94.3%) explants exhibited superior multiple shoot differentiation response compared to leaf explants (0.0-72.7%) (Table: 04). Considering the number of shoot regenerated per cultured explant, the highest number of shoot per culture was observed on medium M_{12} (MS+1.0mgL^{-1}IAA+5.0mgL^{-1}BAP) followed by media M_7 (MS+0.5mgL^{-1}IAA+5.0mgL^{-1}BAP) and M_5 (MS+0.5mgL^{-1}IAA+1.0mgL^{-1}BAP), with minimum or no response on medium M_1, M_3, M_4, M_9, M_{14}, M_{18}, M_{20}, M_{21} and M_{22}, considering all the three explants. Similarly, among the selected explants, shoot apex (0.0-3.7) and internodal stem (0.0-3.7) explants exhibited highest number of shoots compared to leaf explants (0.0-1.7) which was statistically significant at 5% level (Table: 04, and Figure: 03 c, d and e). Similar result was observed in many previous studies. Sharma *et al*. (1991) [28] reported in *C. forskohlii* that nodal stem explants has higher morphogenic potential for multiple shoot differentiation in compared to shoot apex. Bhattacharya and Bhattacharyya (2001) [4] reported a maximum of 12.5 shoots per explant in *C. forskohlii* using shoot tip and nodal explant on medium (MS+0.46mgL^{-1}KIN +0.57mgL^{-1}IAA). Media supplemented with IBA (4.0mgL^{-1}) produces on an average 3.4 shoot from leaf explant in *R. Nasutus* (Cheruvathur *et al*, 2012) [5], BAP (4.0 mg L^{-1}) and KIN (0.5 mg L^{-1}) produces highest number of shootlets (4.83±0.17) in *A. aspera* (Sen *et al*, 2014) [26] and on media BAP (0.5 mg L^{-1}) and KIN (0.5 mg L^{-1}), 6.13-16.45 shoots per explant were regenerated using nodal explants in *P. foetida* (Shekhawat *et al.*, 2015) [31]. Sen.

et al (1992) obtained differentiation of multiple shoots in medium with slightly higher concentration of BAP (2.0 mg L^{-1}) in *C. forskohlii*. Gaurav *et al*. (2010) also observed that medium enriched with BAP (1.0 and 2.5 mg L^{-1}) showed relatively higher shoot regeneration in *C. forskohlii* using young leaf explant. Using shoot tips of *C. blumei*, Vasile *et al.* (2011) demonstrated that MS medium with BAP (1.0 and 1.5 mg L^{-1}) were effective in shoot multiplication. The beneficial effect of BAP further became evident when large number of multiple shoots gradually formed a compact colony comprising of shoots at different developmental stages (Dube *et al*., 2011) [7]. Previous reports also supports our finding that, media supplemented with lower concentration of phytohormones like IAA, 2,4-D, NAA and IBA (3.0-5.0 mgL^{-1}) and slightly higher concentration of BAP (0.5-5.0 mgL^{-1}) showed relatively higher shoot regeneration potential in *C. forskohlii* (Rajasekharan *et al*., 2005; Ashwinkumar, 2006; Velmurugan *et al*. 2010; *Praveena et al*. 2012) [38, 22], *E. gerardiana* (Garla *et al*, 2011) [9], *E. alba* (Sharma *et al*, 2013) [27], *A. aspera* (Sen *et al*, 2014) [26] and *P. foetida* (Shekhawat *et al*., 2015) [31].

Table 4: Effects of different plant growth regulators on shoot regeneration and rooting of various explants of *Coleus barbatus*.

Media	Shoot regeneration						Rooting					
	% response			No. of shoot/culture			% response			No. of root/culture		
	SA	N	L	SA	N	L	SA	N	L	SA	N	L
M1	24.3±3.5	16.7±2.4	0.0	0.7±0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
M2	81.7±2.0	77.7±2.2	62.7±2.3	1.0±0.6	0.7±0.3	0.0	28.3±2.0	22.7±1.8	19.3±3.0	2.3±0.3	2.3±0.3	1.3±0.3
M3	45.3±2.6	39.0±2.1	35.0±2.9	0.0	0.0	0.0	87.3±2.3	78.0±3.1	62.7±2.3	8.3±0.3	7.7±0.3	5.7±0.3
M4	31.0±2.1	24.3±2.3	21.7±3.3	0.0	0.0	0.0	29.3±3.0	0.0	0.0	2.3±0.3	0.0	0.0
M5	87.3±2.3	84.3±3.5	72.7±2.3	2.7±0.3	2.3±0.3	1.7±0.3	40.7±2.4	37.7±1.5	0.0	3.7±0.3	3.3±0.3	0.0
M6	90.7±3.5	92.7±2.3	65.3±3.7	2.7±0.3	2.3±0.7	1.3±0.3	36.0±2.3	32.0±3.1	25.7±2.3	3.3±0.3	2.7±0.3	2.3±0.3
M7	94.7±2.4	91.3±3.5	68.3±2.0	3.3±0.3	3,0±0.6	1.7±0.3	41.3±2.4	44.3±2.3	28.3±3.8	3.7±0.3	4.3±0.3	2.7±0.3
M8	56.0±3.5	50.7±3.0	40.7±3.0	1.0±0.0	0.3±0.3	0.7±0.3	92.7±1.8	91.3±3.5	68.3±2.0	10.3±0.9	9.7±0.3	6.3±0.3
M9	40.7±3.0	31.3±2.4	21.0±2.1	0.0	0.0	0.0	90.0±2.9	94.3±2.3	69.0±3.8	8.7±0.3	9.3±0.3	6.7±0.3
M10	63.7±3.0	67.3±3.9	39.3±3.0	1.0±0.0	1.3±0.3	0.0	40.3±3.9	35.0±2.9	31.7±3.8	3.3±0.3	3.7±0.3	2.3±0.3
M11	83.3±2.9	79.3±3.0	67.3±3.9	2.7±0.3	2.3±0.3	1.3±0.3	43.3±2.9	39.3±3.0	0.0	3.7±0.3	2.7±0.3	0.0
M12	90.7±3.0	94.3±2.3	69.0±3.8	3.7±0.3	3.7±0.3	1.7±0.3	40.7±3.0	34.3±3.5	25.0±2.9	3.3±0.3	3.0±0.6	2.3±0.3
M13	53.3±2.4	46.7±2.4	38.3±2.0	1.0±0.0	0.3±0.3	0.0	90.7±3.0	86.7±2.4	78.3±2.0	9.0±0.6	8.3±0.3	7.3±0.3
M14	43.0±3.6	44.3±3.5	38.0±3.6	0.0	0.3±0.3	0.0	83.3±2.9	81.0±2.1	72.7±1.5	7.7±0.3	8.0±0.6	6.7±0.3
M15	50.7±3.0	45.7±2.3	41.7±2.4	0.7±0.3	0.3±0.3	0.0	79.7±3.9	74.3±3.5	61.7±2.4	7.3±0.7	7.0±0.6	5.7±0.3
M16	65.7±3.5	60.7±3.0	50.7±1.8	1.0±0.0	1.0±0.0	0.7±0.3	35.7±2.3	37.3±3.9	0.0	3.3±0.3	3.3±0.3	0.0
M17	78.0±3.1	74.3±3.5	63.0±3.6	2.0±0.0	2.3±0.3	1.0±0.0	44.7±3.7	41.0±2.1	29.7±2.7	4.3±0.3	3.7±0.3	2.3±0.3
M18	35.7±2.3	38.7±2.0	30.7±2.3	0.0	0.0	0.0	78.0±3.1	75.7±3.5	64.0±2.1	7.7±0.3	7.3±0.3	6.3±0.3
M19	44.0±3.1	49.3±3.0	32.7±3.9	0.3±0.3	0.3±0.3	0.0	84.0±3.1	74.3±2.3	67.7±1.5	8.3±0.3	6.7±0.3	5.7±0.3
M20	35.7±2.3	34.7±4.2	25.7±2.3	0.0	0.0	0.0	90.7±3.0	84.7±3.3	70.0±2.9	9.3±0.7	8.7±0.3	6.3±0.3
M21	25.0±2.9	29.3±3.0	16.7±0.9	0.0	0.0	0.0	85.3±2.9	82.7±2.3	68.3±1.7	8.0±0.6	7.7±0.3	6.7±0.3
M22	19.3±3.0	15.7±2.3	16.3±1.2	0.0	0.0	0.0	59.3±3.0	47.3±3.9	40.7±3.0	5.3±0.3	3.7±0.3	2.3±0.3
Range	19.3-94.7	15.7-94.3	0.0-72.7	0.0-3.7	0.0-3.7	0.0-1.7	0.0-92.7	0.0-94.3	0-78.3	0.0-10.3	0.0-9.7	0.0-7.3
Average	56.35	54.02	42.42	1.08	0.94	0.45	59.15	54.27	41.02	5.61	5.14	3.59
CD at 5%	8.273	8.255	7.89	0.674	0.862	0.538	8.086	7.895	6.564	1.236	1.036	0.838
Mean±SE	2.893	2.887	2.759	0.236	0.302	0.188	2.828	2.761	2.295	0.432	0.362	0.293
CV (%)	8.892	9.257	11.468	37.95	55.593	71.647	8.279	8.81	9.692	13.356	12.22	14.113

Note: SA: Shoot apex, N: internodal stem, and L: young leaf



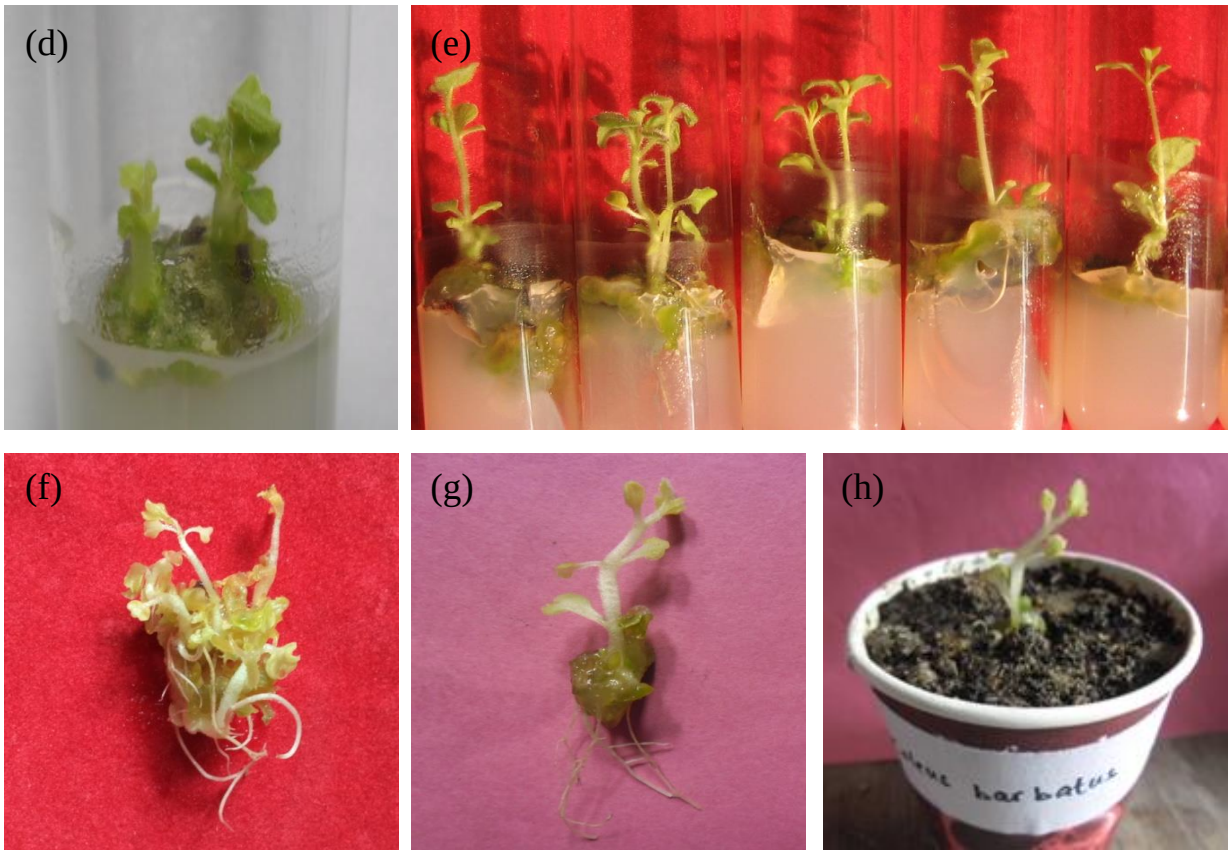


Fig 3: Different developmental stages of *in vitro* regenerated *Coleus barbatus:* Callus mediated multiple shoot regeneration from shoot apex on media M_7 and internodal stem on media M_{12} (a and b); subcultured on M_5 and M_{12} (c and d); shoot elongation on M_{12} (e); Rooting of regenerated shoot on M_8 and M_{13} (f and g), and Potted plantlet for hardening (h).

In vitro rooting and hardening of plantlets

Rhizogenesis or regeneration of proper rooting system from the base of regenerated shoots is the prerequisite to facilitate successful establishment in the soil. Exogenously supplied auxins in the medium played a major role in root proliferation by promoting cell division activities (Salman, 1988). In the present investigation, no root initiation response was observed on MS basal medium. So for successful establishment of roots, proliferated shoots were subcultured on rooting media which showed rooting response within two weeks of inoculation. The best per cent response of root proliferation were obtained on medium M_{13} (MS+3.0mgL^{-1}IAA) followed by media M_9 (MS+1.0mgL^{-1}IAA +0.5mgL^{-1} BAP) and M_8 (MS+1.0mgL^{-1}IAA), with minimum or no response on medium M_1 and M_4 considering all the three explants. Likewise, among the selected explants, internodal stem (0.0-94.3%) and shoot apex (0.0-92.7%) explants exhibited better root initiation response compared to leaf explants (0-78.3%) (Table: 04). Considering the number of root per culture, the highest frequency of root initiation per elongated shoot were observed on medium M_8 (MS+1.0mgL^{-1}IAA) followed by media M_{13} (MS+3.0mgL^{-1}IAA) and M_9 (MS+1.0mgL^{-1}IAA+0.5mgL^{-1}BAP), with minimum or no response on medium M_1 and M_4, considering all the three explants. Similarly, among the selected explants, shoot apex (0.0-10.3) and internodal stem (0.0-9.7) explants exhibited highest frequency of root compared to leaf explants (0.0-7.3) which was statistically significant at 5% level (Table: 04, and Figure: 03 f and g). Similar observation was made in *C. tamala* (Sharma and Nautiyal, 2009) [29], *C. forskohlii* (Velmurugan *et al*, 2010; Dube *et al*, 2011; Praveena *et al*, 2012) [38, 7, 22] and *S. rebaudiana* (Thiyagarajan and Venkatachalam, 2012) [37], *A. aspera* (Sen *et al*, 2014) [26] and *P. foetida* (Shekhawat *et al.*, 2015) [31] on MS medium fortified with IBA (0.2- 3.0 mgL^{-1}) and IAA (0.5- 3.0 mgL^{-1}). Effectiveness of IAA over other auxins (NAA and IBA) in terms of better root initiation response, higher frequency and length of roots were observed in previous reports in *C. cajon* (Dayal *et al*., 2003) [6], *M. konini* (Rout, 2005) [25], *Q. resiliencies* (Fleck *et al*., 2009) [8], and *R. nasutus* (Cheruvathur *et al*, 2012) [5]. It is exciting to note that the rooting frequency was decreased when the BAP concentration was increased from 0.5 to 3.0mgL^{-1} while keeping IAA concentration constant in rooting media and was inhibited at higher concentration (5.0mgL^{-1}). Almost similar trend was also observed in *D. hamiltonii* (Anitha and Pullaiah, 2002) [1], *C. forskohlii* (Dube *et al*, 2011; Praveena *et al*, 2012) [7, 22], and *S. rebaudiana* (Thiyagarajan and Venkatachalam, 2012) [37]. Well rooted plantlets with fully expanded leaves were transferred to pots for acclimatization and subsequently they were transplanted to the field condition after showing 70-85% survival.

Conclusion and future prospects

Coleus barbatus is only known source of labdane diterpenoid (coleonol), biologically active compound which is very useful for developing many life saving drugs. But due to its continuous depletion, it is enlisted as an endangered species category. Regeneration of healthy plantlets from promising shoot apex explants using media (MS+1.0mgL^{-1}IAA+5.0mgL^{-1} BAP) could open up new vistas for large-scale commercial cultivation, *in situ* conservation, and genetic characterization of this endangered medicinal plant. In essence, the present study could offer an efficient and commercially feasible approach for *in vitro* regeneration of this important medicinal plant.

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Author Contributions
AKC and KKR have contributed equally to this work. AKC, KG and KKR conceptualized the manuscript. Experiments designed by MK and HK and conducted by AKC. AKC and KG wrote the manuscript. KKR, MK and HK assisted and edited the manuscript. AKC and KG has statistically analyzed the data. AKC, KG and KKR contributed in critically revising the draft and updating the manuscript for publication.

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