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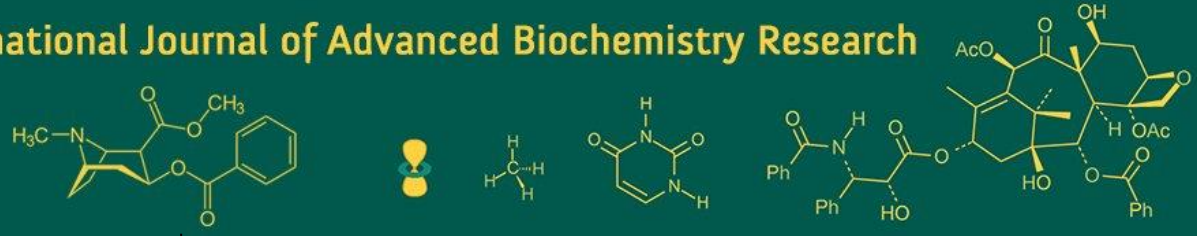

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Standardization protocol of medicinal coleus (*Coleus forskohlii)* for rapid regeneration under *in vitro* conditions and its effective use in bio-pharming

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Abstract
Coleus forskohlii is a medicinal plant that can be used as a plant system to produce recombinant proteins due to its short duration of 6 to 7 months and ability to grow in moderate climates with light, medium, and drained soil. Callus induction and proliferation systems are useful for genetic improvement of this endangered multipurpose medicinal plant systems are a promising direction of protein production technology, providing a wide variety of bacterial to complex multi subunit glycosylated human proteins. Plant systems provide the stable inherited expression of a foreign gene by transforming the nuclear DNA and fast, highly effective production of the desired protein by extra chromosomal amplicons. They also provide an cost-effective and safe platform for vaccine development due to ease in scalability and a low risk of contamination with endotoxins or human pathogens. Coleus is suitable for transformation due to its abundant protein accumulation in leaves and easy propagation using cuttings. The standardization of regeneration protocol of *Coleus forskohlii* using leaf explants was conducted, using various concentrations of growth hormones such as auxin (IAA) and cytokinin (BAP). The results showed that the mean number of shoots per callus differed significantly between treatments, with the highest shoot formation being obtained in the combination of hormones containing 9mg L^{-1} of BAP and 0.4 mg L^{-1} of IAA. The callus texture obtained during this experiment was compact and fragile and the color of the callus was pale green to dark green. Significantly highest per-cent of callus, mean number of shoots per callus and the rooting was obtained in the combination containing 9mg L^{-1} of BAP and 0.4 mg L^{-1} of IAA with 94 percent, 11.16, and 6.34 respectively. Rooting and hardening were achieved with different concentrations of BAP and IAA after sub-culturing at the 60th day.

Keywords: *Coleus forskohlii*, rapid regeneration, bio-pharming

Introduction
Coleus forskohlii is a medicinal plant which is a perennial that grows up to 45-60 cm tall and aromatic in nature. Leaves are usually pubescent, narrowed into petioles which are 7.5 to 12.5 cm in length and 3 to 5cm in width. Nodes are often hairy and have four angled stems that are branched. Inflorescence is a raceme, which is 15-30 cm in length and the flowers are stout, 2 to 2.5 cm in size, usually perfect and calyx hairy inside. Calyx is broadly ovate. The blue or lilac corolla is bilabiate. Lower lobes are concave and elongated so they can enclose the essential organs. The stigma is two lobed and ovary is four parted and the flower is cross-pollinated by wind or insects (Prajapati *et al.*, 2003) [6]. *Coleus forskohlii* can be used as a plant system to produce recombinant proteins because of its short duration of 6 to 7 months and it can be grown in moderate climate with light, medium and drained soil and produce enough biomass of about 400 to 600 kg dry wt /acre (Hegde *et al.*, 2009) [3]. Callus induction and proliferation systems are known to be very useful for the genetic improvement of *Coleus forskohlii* which is an endangered multipurpose medicinal plant that has widespread applications. Mersinger *et al.* (1988) [5] successfully obtained dark green to yellow green callus from *C. forskohlii* when placed on Gamborg's B5 medium supplemented with 0.5 mg L^{-1} 2, 4-D, and 0.2 mg L^{-1} kinetin. Callus cultures were also initiated on MS media containing NAA (2 mg/l) and BAP (1 mg/I) using 6- to 8-mm-long hypocotyl segments from 30-day-old aseptically grown seedlings (Sen *et al.*, 1991) [11].

The best medium for callus induction in *C. forskohlii* was reported by Tefera (1998) [13], which contained MS medium supplemented with 1.0 mg L^{-1} indole-3-acetic acid (IAA) and 1.5 mg L^{-1} benzyl adenine purine (BAP) for the formation of a green compact callus.

The highest rate of shoot multiplication, i.e., more than 150 shoots per callus, was produced in the MS medium supplemented with 4.6 mm kinetin and 0.54 mm NAA from the leaf-derived callus of *C. forskohlii* (Reddy *et al*., 2001) [8].

Recombinant proteins are primarily produced from bacteria cells, insect and cultures of mammalian cells. In recent years, the development of deconstructed virus-based vectors has allowed plants to become a viable platform for recombinant protein production, with advantages in versatility, speed, cost, scalability, and safety over the current production paradigms (Qiang and Huafang 2014) [16].

Biopharming is the use of plants for the production of heterologous proteins using genetic engineering techniques. It is widely used for scientific and medical purposes, including on an industrial scale. Plant systems are a promising direction of development of protein production technology (Fischer *et al.,* 2015 and Loos *et al.,* 2014) [1, 2].

A wide variety of bacterial to complex multi subunit glycosylated human proteins can be produced in plants (Vyacheslavova *et al.,* 2012) [14]. Plant systems provide the stable inherited expression of a foreign gene by transforming the nuclear DNA and fast highly effective production of the desired protein by extra chromosomal amplicons (Kawaka *et al*., 2017) [4].

Plants also provide a cost-effective and safe platform for vaccine development due to ease in scalability and a low risk of contamination with endotoxins or human pathogens. Hence this production system we can expect to convince the sceptic and can become approved for the use of emergency response therapeutics and vaccines in humans (Waheed *et al.,* 2016) [15].

Coleus is suitable for transformation as there is an abundant protein accumulation in leaves, further it can be easily propagated using cuttings and hence easy to mass propagate with the additional advantage of horizontal gene transfer since they do not produce any pollen.

Materials and Methods

The study pertaining to the research on "Standardization protocol of medicinal coleus (*Coleus forskohlii)* for rapid regeneration under *in vitro* conditions and its effective use in bio-pharming"was carried out in the Department of Plant Biotechnology, University of Agricultural Sciences, GKVK, Bengaluru. This research was carried to find out the optimal concentration of growth regulators for producing callus and multiple shoots from leaf explant of *C. forskohlii*. The following six treatments were formulated using MS as a basal media with varying concentrations of 6-benzylaminopurine (BAP) and Indole-3-acetic acid (IAA). Each treatment had three replications. The treatments are as follows.

T_1: MS basal medium with 4 mg L^{-1} BAP+0.4mgL^{-1} IAA
T_2: MS basal medium with 5 mg L^{-1} BAP + 0.4 mg L^{-1} IAA
T_3: MS basal medium with 6 mg L^{-1} BAP + 0.4 mg L^{-1} IAA
T_4: MS basal medium with 7 mg L^{-1} BAP + 0.4 mg L^{-1} IAA
T_5: MS basal medium with 8 mg L^{-1} BAP + 0.4 mg L^{-1} IAA
T_6: MS basal medium with 9 mg L^{-1} BAP + 0.4 mg L^{-1} IAA

Observation: The various parameters recorded for observations were

1. No. of explants responding
2. Average number of days taken for callus initiation
3. Callus intensity, color and texture.
4. Average number of days taken for shoot initiation
5. Average number of days taken for root initiation
6. Average number of shoots per explant
7. Average number of roots per explant

Preparation of explants

1. The fresh young leaves of *C. forskohlii* were collected from the green house and kept under running tap water overnight to remove the surface contaminants.
2. The leaves were then treated with 1 percent bavistin for 2 hr, rinsed with sterile water several times and transferred into sterile laminar airflow chamber.
3. The leaves were treated with 0.1 percent $HgCl_2$ thrice for 10 sec. inside the laminar airflow chamber.
4. The treated leaves were thoroughly washed 3-4 times with sterile distilled water.
5. The treated leaves were blot dried and were cut into pieces of 1 cm^2 - 1.25 cm^2 size by using scalpel.
6. The explants were then placed on tissue culture bottles containing MS media with different concentrations of growth hormones.
7. The bottles were then placed in the culture room by maintaining temperature at 25 °C and 16 h photoperiod with 65 percent of relative humidity.

Acclimatization

1) Washing of rooted plants

After the proper development of roots, the plants were taken out carefully from the culture bottles. Then the plants were placed under gentle flow of tap water for 2 h to remove adhering agar completely. Plants were then treated with a fungicide bavistin 0.4 percent for half an hour to prevent fungal contamination and then were transferred to varying potting mixture.

2) Planting in potting mixture

Vermicompost and sand were mixed in a proportion of 1:1 and autoclaved. This was done for adequate drainage of surplus water in tiny plastic cups with holes at the bottom. The plants were removed from the MS medium and moved to the plastic cups with correct root orientation. The pot mixture was replenished with half a MS medium (diluted 100 times) until it was moist. Plants were then covered with poly bags having holes in them for air circulation to maintain high humidity. All plastic cups were kept at about 20-22 °C in the green house, which was maintained by cooling pads and fans. Plants were irrigated according to their requirements.

Results and Discussions

Standardization of regeneration protocol of *Coleus forskohlii* using leaf explants

The standardization of regeneration protocol of *Coleus forskohlii* using leaf explants was conducted. The various concentration of growth hormones such as auxin (IAA) and cytokinin (BAP) was used and effects of these hormones were studied on callus initiation, shoot regeneration, and root regeneration. The results pertaining to this is given below.

Induction of callus

In vitro callus culture of *Coleus forskohlii* was established, which was prepared from leaf explants using MS media containing 0.4 mg $L^{-1}$of IAA with different concentrations of BAP (4 mg L^{-1} to 9 mg L^{-1}). The callus texture obtained during this experiment was compact and fragile and the color of the callus was pale green to dark green. Significantly highest percent of callus was obtained in the combination containing 9 mg L^{-1} of BAP and 0.4 mg $L^{-1}$of IAA with 94 percent response (Table 1, Fig. 1 and Plate 1).

Tefera (1998) [13], reported the formation of green compact callus using leaf as explants on the MS medium supplemented with 1.0 mg L^{-1} IAA and 1.5 mg L^{-1} BAP. Sanjay Rao (2013) [10], found that 6 mg L^{-1} BAP and 0.4 mg L^{-1} IAA was good for callus induction.

Shoot initiation

The mean number of shoots per callus differed significantly between the treatments. It was significantly highest in T_6 (11.16) (9 mg L^{-1} of BAP and 0.4 mg $L^{-1}$of IAA). In T_3 (6 mg L^{-1} of BAP and 0.4 mg $L^{-1}$of IAA) it was 9.00 which was significantly higher than that in T_4 (7.11) (7 mg L^{-1} of BAP and 0.4 mg $L^{-1}$of IAA) T_2 (4.53) (5 mg L^{-1} of BAP and 0.4 mg $L^{-1}$of IAA) T_5 (3.22) (8 mg L^{-1} of BAP and 0.4 mg $L^{-1}$of IAA) and T_1 (3.00) (4 mg L^{-1} of BAP and 0.4 mg $L^{-1}$of IAA). The percentage of shoot formation from callus ranged from 51.0 to 71.33 percent. Significantly high shoot formation of 71.33 percent was obtained for the combination of hormones containing 9mg L^{-1} of BAP and 0.4 mg $L^{-1}$of IAA (Table 2, Fig. 2, Fig. 3 and Plate 1).

An effective multiplication of shoots from the callus was observed in MS medium supplemented with 2 mg L^{-1}BAP and 0.4 mg L^{-1} NAA (Sandesh, 2007) [9]. Sreedevi and Pullaiah (2013) [12], cultured the nodal explants of *C. forskohlii* on MS medium supplemented with BAP (0.25 mg L^{-1}) and Kin (0.25 mg L^{-1}) resulting in average of 41 multiple shoots.

Table 1: Effect of different concentrations of BAP and IAA on callus formation from the leaf explants of *C. forskohlii*

Treatments (BAPmgL^{-1}+IAAmgL^{-1})	% of Callus response	Callus texture	Callus intensity	Callus color
T_1 (4+0.4)	61.073	Fragile	††	yellow
T_2 (5+0.4)	67.220	Fragile	††	Green
T_3 (6+0.4)	71.020	Compact	†††	Green
T_4 (7+0.4)	58.253	Compact	†	Pale green
T_5 (8+0.4)	53.100	Fragile	††	Pale green
T_6 (9+0.4)	94.000	Compact	†††	Green
C.V.	1.378	-	-	-
C.D.	1.672			
SEM	0.537			

Legends: + = Low ++ = Medium +++ = Good

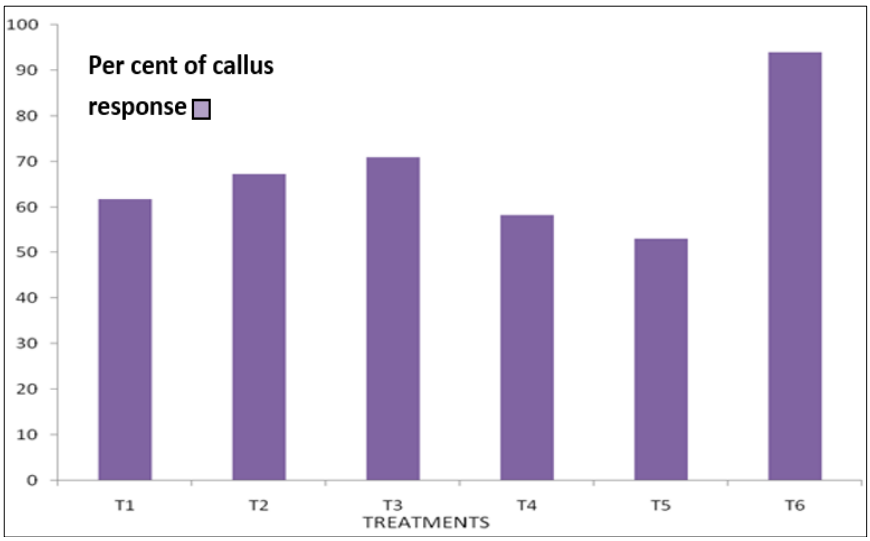

Fig 1: Effect of different concentrations of BAP and IAA on callus formation from the leaf explants of *C. forskohlii*

Table 2: Effect of different concentrations of growth regulators on number of days taken for multiple shoot production and root initiation from leaf explants of *C. forskohlii*

Treatments (BAP mgL^{-1}+ IAA mgL^{-1})	Mean no. of shoots per callus	No. of days taken for shooting	Mean no. of roots per shoot	No. of days taken for root initiation
T_1 (4+0.4)	3	35	3.21	82
T_2 (5+0.4)	4.53	33	5.44	81
T_3 (6+0.4)	9.00	30	4.65	82
T_4 (7+0.4)	7.11	35	5.22	83
T_5 (8+0.4)	3.22	30	3.8	85
T_6 (9+0.4)	11.17	33	6.34	80
C.D	1.78	-	0.16	-
CV	15.58	-	1.87	-
SEM	0.570	-	0.05	-

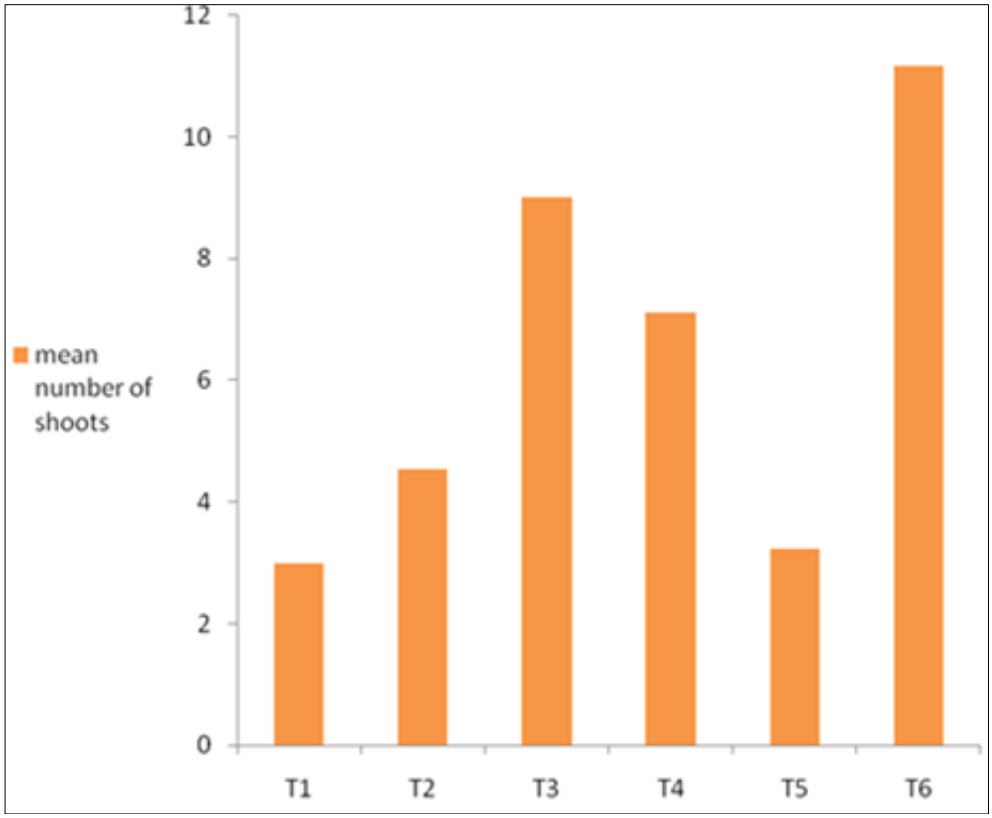

Fig 2: Effect of different concentrations of BAP and IAA on mean number of shoots per callus from the leaf explants of *C. forskohlii*

Table 3: Effect of different concentration of growth regulators on percent of callus regenerated into shoots from leaf explant of *C. forskohlii*

Treatments (BAP mg L^{-1} + IAA mg L^{-1})	Percent of callus regenerated into shoots at 60th day
T_1 (4+0.4)	51.00
T_2 (5+0.4)	53.00
T_3 (6+0.4)	58.33
T_4 (7+0.4)	65.00
T_5 (8+0.4)	67.67
T_6 (9+0.4)	71.33
C.D	8.41
C.V	7.65
SEM	2.69

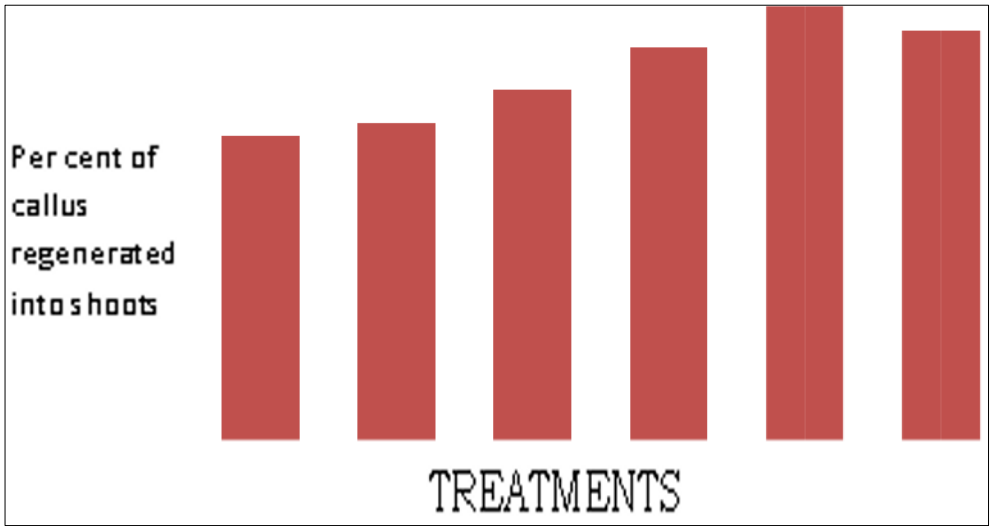

Fig 3: Effect of different concentrations of BAP and IAA on percent of callus regenerated into shoots at 60th day

Rooting and Hardening

Rooting was obtained with the MS media containing different concentrations of BAP ranging from 4 mg L^{-1} to 9 mg L^{-1} and IAA 0.4 mg L^{-1} after sub culturing at the 60th day. Rooting was significantly highest in T_6 (6.34) (9 mg L^{-1} of BAP and 0.4 mg L^{-1} of IAA). In T_2 (5mg L^{-1} of BAP and

0.4 mg $L^{-1}$of IAA) it was 5.44 which was significantly higher than that in T_4 (5.22) (7 mg L^{-1} of BAP and 0.4 mg $L^{-1}$of IAA) T_3 (4.65) (6 mg L^{-1} of BAP and 0.4 mg L^{-1} of IAA) T_5 (3.8) (8 mg L^{-1} of BAP and 0.4 mg $L^{-1}$of IAA) and T_1 (3.33) (4 mg L^{-1} of BAP and 0.4 mg $L^{-1}$of IAA). The rooted plants were transferred into the pot mixture containing peat and sand in 1:1 ratio. The plants were covered with polythene bags containing holes for maintaining the relative humidity (Table 2 and Plate 2).

Tefera (1998) [13], reported that although IAA at 1mg L^{-1} was effective in inducing roots, normal rooting was also observed in hormone free medium. Reddy *et al.* (2001) [8], cultured *in vitro* differentiated shoot lets of *C. forskohlii* measuring 3.5-4 cm in length, derived from the callus on MS medium supplemented with different concentration of IAA for rooting.

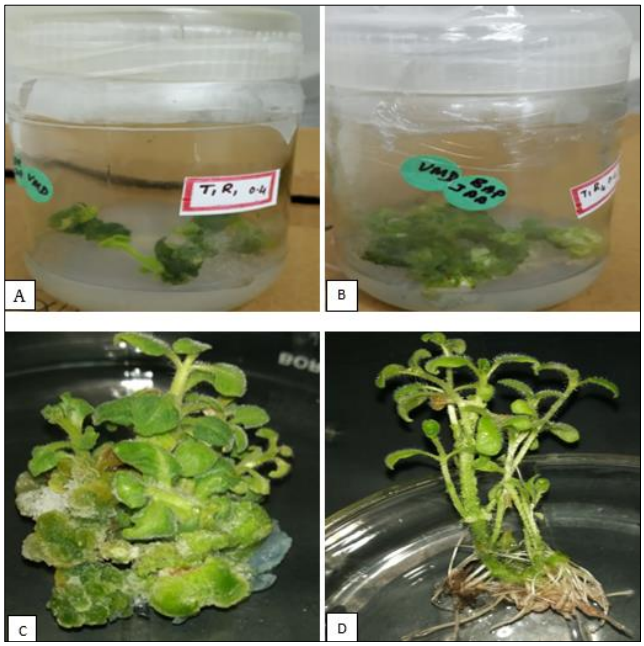

Plate 1: Different stages of regeneration of *C. forskohlii*, A) Explant response, B) Callus formation, C) Shooting, D) Rooting

Plate 2: Hardening of the regenerated *C. forskohlii* plants, A) Hardened tissue culture plantlet, B) Hardened tissue culture plant in green house

Conclusion

The standardization of regeneration protocol of *Coleus forskohlii* using leaf explants was conducted. The various concentration of growth hormones such as auxin (IAA) and cytokinin (BAP) was used and effects of these hormones were studied on callus initiation, shoot regeneration, and root regeneration. The callus texture obtained during this experiment was compact and fragile and the color of the callus was pale green to dark green. Significantly highest per-cent of callus, mean number of shoots per callus and the rooting was obtained in the combination containing 9 mg L^{-1} of BAP and 0.4 mg L^{-1} of IAA with 94 percent, 11.16, and 6.34 respectively.

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