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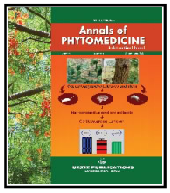

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In vitro regeneration of *Coleus aromaticus* (Benth.) using leaf disc and nodal segment explants

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Abstract

From ancient times, plants are major source of multiple pharmaceutical products. These products serve as antioxidants, antimicrobial and therapeutic agents. *Coleus aromaticus* (Benth.) is one of those plant which posses these activities and utilized in wide range of applications from treatment of various human health problems to the culinary use. The huge utilization of this plant makes it vulnerable to extinction and needs to apply new technologies for conservation of this plant. Plant tissue culture is one of those techniques which can be utilized to fulfill the demands of this plant at industrial level. In this context, investigation was carried out to regenerate *C. aromaticus* from leaf and nodal explants using indirect and direct regeneration system, respectively. Murashige and Skoog (MS) medium with 3 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) exhibited maximum callus induction from leaf disc explants. Callus transferred to medium containing 6-benzylaminopurine (BAP) showed induction of globular structures on the surface but failed to initiate shoots. MS medium with BAP and kinetin were used for direct shoot induction from nodal explants and medium containing BAP induced higher shoots per explants. Root induction was achieved using indole-3-butyric acid (IBA) in the medium and rooted plants were successfully transferred to the soil. Present study developed a potential protocol for callus induction as well as shoot induction from leaf disc and nodal segment, respectively.

1. Introduction

India has rich diversity of medicinal plants and these plants are important part of various medicines. From ancient times, these formulations were used to cure multiple human health problems. Multiple communities from rural and tribal areas utilize medicinal plants as ethnomedicine for human healthcare (Thakur *et al.*, 2016). Various novel medicines are derived from plant and herbal sources are considered safe for human consumption (Shamna and Poyil, 2021). In India, several medicinal plants are consumed everyday in culinary purpose. This increasing use of medicinal plants increased the demands of such plants in global market and bulks of medicinal plants were overexploited from wild resources. This overexploitation disturbs the natural habitat and most of the plants are at the level of extinction. There is an urgent need to apply new technologies for conservation of this plant. Plant tissue is one of those techniques which can be utilized to fulfill the demands of this plant at industrial level.

Coleus aromaticus (Benth) syn. *C. ambionicus* (Lour) Spreng or *Plectranthus ambonicus* (Lour) is a native shrub of India and Mediterranean. It is often found in tropical Africa, Asia and Australia (Lukhoba *et al.*, 2006). It is a perennial shrub with highly aromatic leaves containing a strong flavor of mixed herbs and makes it an excellent addition in stuffing for meat and poultry (Khare *et al.*, 2011). It is rich in various nutrients like proteins, vitamins, minerals and fibers (Gupta *et al.*, 2005). *C. aromaticus* is reported to contain antimicrobial, antiepileptic and antioxidant properties. Leaf extract of this shrub is helpful to treat digestive problems like constipation (Khare *et al.*, 2011). Some reports suggested its use in Indian folklore medicine as an antioxidant, nephroprotectant (Palani *et al.*, 2010), antimicrobial (Devi and Yogyarti, 2006), anti-leishmania (Tempone *et al.*, 2008), antitumor, antiepileptic activity (Gurgel *et al.*, 2009) and in the treatment for respiratory track, cold and epilepsy (Khare, 2007). The industrially important products of *C. aromaticus* are essential oil (terpenes) and phytochemicals such as flavones, salvigenin and luteolin (Khare *et al.*, 2011). These products have a huge impact in pharmaceutical and other industries.

For improvement of *Coleus* using biotechnological tools and production of either planting material or secondary metabolite in large scale. It is important to have an efficient regeneration protocol as well as callus induction system. Plant tissue culture is a technique also used for production of important phytochemicals in large scale irrespective of external environment (Sharma *et al.*, 2021).There are some reports for employment of direct and indirect regeneration system for *Coleus* regeneration system (Anbazhagan *et al.*, 2005; Rajasekharan *et al.*, 2005; Srinivasan *et al.*, 2006; Govindaraju and Arulselvi, 2018). Hence, keeping the demand of this shrub on industrial level, it is time to find out more efficient regeneration protocol for *C. aromaticus*. Present study has investigated the potential of leaf disc explants and nodal segment for regeneration of *Coleus* using indirect and direct regeneration system, respectively.

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2. Materials and Methods

2.1 Plant materials and explants sterilization

C. aromaticus plants were procured from Herbal and Medicinal Garden, Department of Plant Physiology, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur, Madhya Pradesh, India. Leaf disc and nodal segments were used in two different regeneration systems.The explants were disinfected by immersing inthe solution containing 8-10 drops of Tween-20 for 15 min, followed by 4-5 rinses with sterile distilled water. Further explants were treated with 70% v/v ethyl alcohol for 2 min, followed by $HgCl_2$ (0.3% w/v) for 3 min. After sterilization, leaves were trimmed into pieces of about 1 to 2 cm and then inoculated onto callus induction medium whereas individual nodal segment was inoculated on shoot induction medium for direct regeneration of multiple shoots.

2.2 Callus induction

For callus induction, MS medium (Murashige and Skoog, 1962) fortified with different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) was used. On an average, 15 leaf discs were inoculated in each plate, sealed with parafilm M® and incubated at 25 ± 2°C under 16/8 h photo period conditions. Explants were sub-cultured on similar medium after 21 days of incubation and observations were recorded after 45 days of initial inoculation.

2.3 Shoot induction from callus cultures

Well-developed calli were transferred to MS medium with different concentration of 6-benzylaminopurine (BAP). MS medium without any growth regulator was used as control. Cultures were sub-cultured after 21 days of incubation and observations were taken after 45 days of incubation.

2.4 Direct regeneration using nodal segments

For direct regeneration of shoots, individual nodal segments were inoculated on shoot MS medium fortified with different concentrations of BAP (1-5 mg/l) and kinetin (1-5 mg/l) and MS medium without any growth regulators used as control. After 21 days of incubation, explants were sub-cultured on similar medium. Observations were noted after 45 days of initial inoculation.

2.5 Root induction from regenerated shoots

After shoot induction, individual shoots were separated from the clumps and transferred in test tubes containing MS medium fortified with various concentrations of indole-3-acetic acid (IAA; 0.25 and 0.5 mg/l) and indole-3-butyric acid (IBA; 0.25 and 0.5 mg/l). MS medium without any growth regulator was also tested for root induction. Root induction per cent, root numbers per shoot and root length were recorded after 15 days of incubation

2.6 Hardening of plantlets

For adaptation in natural environment, plantlets were washed with tap water to remove adhered medium without damaging the roots. Then plantlets were transplanted in plastic pots filled with sterile mixture of sand, soil and compost (1:1:1) and covered with a transparent plastic bag for 10-12 days. The cover was gradually removed after twelve days, initially for 3 h, followed by 6 h and 12 h at three days intervals. Subsequently, the period of keeping the plantlets without any cover was gradually increased and after 21 days, they were brought outside under shade. Within next 10 days, these plants were ready to transfer in the field.

2.7 Statistical analysis of data

Each experiment was repeated three times and mean values and standard deviation were calculated. All data obtained were subjected to the single factor analysis of variance (ANOVA) using Microsoft excel. The critical difference (CD) values were calculated at p=0.05 level to find out the significant difference between the means of different treatments. The significantly different mean values are indicated by different letters.

3. Results

The present study has investigated potential of two different explants in two regeneration system.

3.1 Callus induction using leaf explants

While initiating callus induction from leaf disc explants, swelling of tissues was observed after 7 days of incubation. Callus induction was initiated from surrounding of the leaf disc. Green and compact callus were obtained at initial phase while calli were found loosed (Figure 1a) and whitish green after 45 days of incubation (Figure 1b). Among five different concentrations used, MS medium containing 3 mg/l 2,4-D has given highest level of callus induction (Figure 2A). Auxins alone or together with cytokinin were extensively studied plant growth regulators for induction of callus and subsequent organ regeneration in *Coleus*.

3.2 Organogenesis in callus cultures

After incubation of 20 days, globular structures appeared on callus cultures. These globular structures were also confirmed using histological analysis (Figure 1C). The callus cultures were transferred on to MS basal medium without growth regulators exhibited maximum morphogenic response. Morphogenic response was reduced as concentrations of BAP increased (Figure 2B). When these globular structures were separated from callus cultures, rhizogenesis was induced (Figure 1D). These cultures were failed to develop shoots after incubation of 30 days.

3.3 Direct regeneration using nodal segments

Nodal segments showed initial response of swelling after 10 days of incubation. Multiple shoots were observed after 21 days of inoculation (Figure 1E) and continued to multiply till observation taken in 45 days after inoculation (Figure 1F and G). The highest regeneration response (42.22 %) with 3.11 shoots per explants were noted on MS medium fortified with 1 mg/l BAP, followed by 33.33% regeneration response with 2.31 shoots per explants on MS medium fortified with 2 mg/l BAP (Table 1). The regeneration response and number of shoots per explants were decreased with the increase in concentration of BAP. The nodal segments cultured on MS medium fortified with BAP performed better then MS medium fortified with kinetin (Table 1).

3.4 Root induction in regenerated shoots and hardening of plantlets

All shoots showed root induction in all the tested treatments. Root induction initiated after 11 days of incubation and full length of

roots were obtained after 21 days of incubation. Maximum numbers of roots per shoot (5.78) were obtained on MS medium fortified with 0.25 mg/l IBA with average length of 5.35 cm, followed by 4.47 roots per shoot with 3.80 cm on MS medium supplemented with 0.25 mg/l IAA (Table 2). When transplanted in mixture of soil, sand and compost, more than 85% plantlets (data not shown) survived and resumed normal growth after one month of transplantation (Figure 1I).

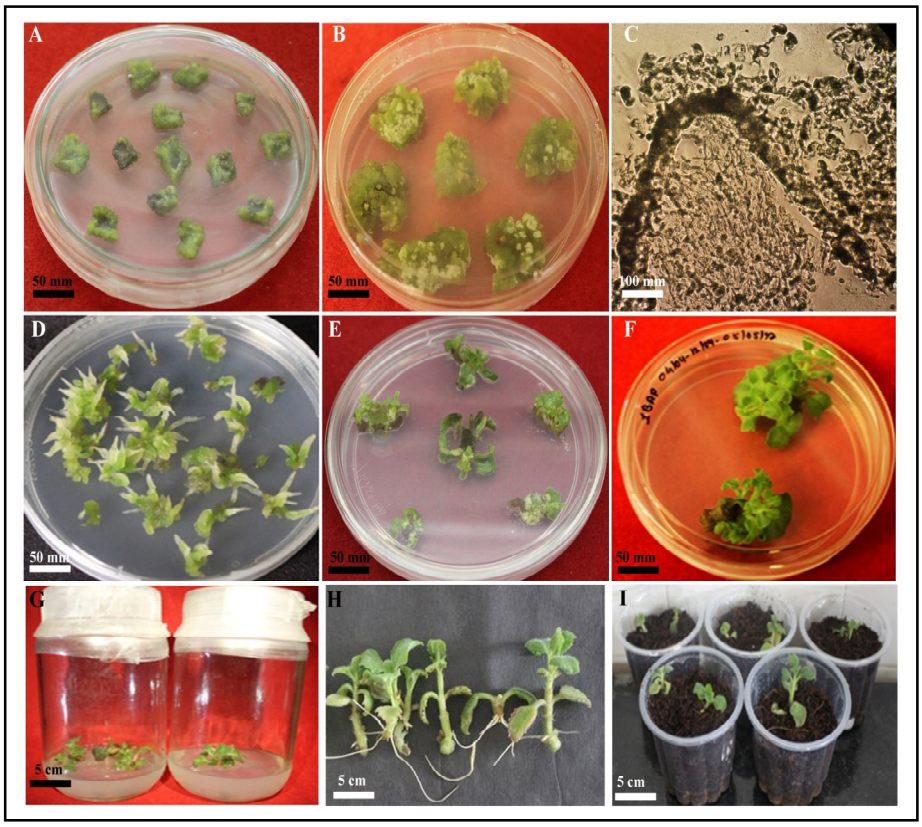
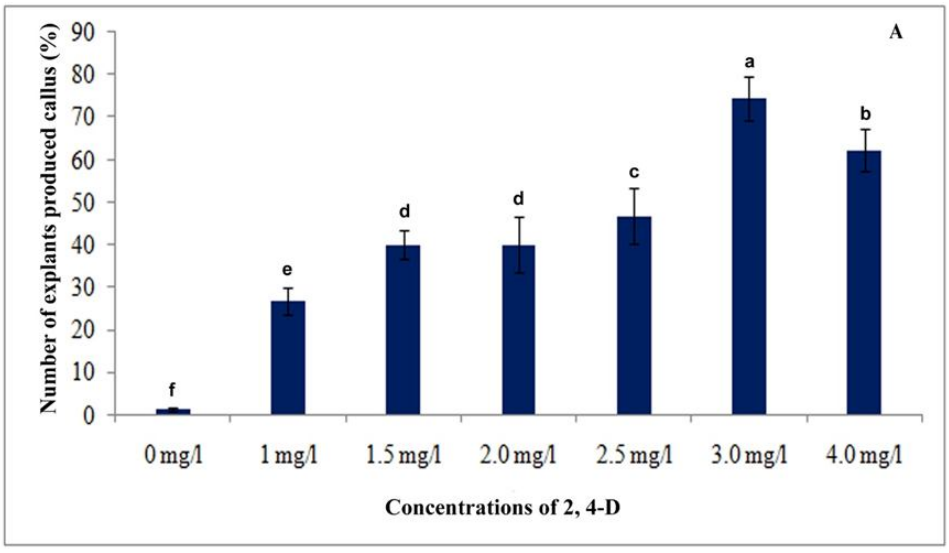


Figure 1: Callus induction and direct regeneration of *C. aromaticus*. (A) Callus cultures after 21 days of incubation. (B) Callus cultures after 45 days of incubation. (C) Histological analysis of morphogenic structure. (D) Root induction from morphogenic structure. (E) Initiation of shoot induction from nodal segments. (F and G). Shoot multiplication after 45 days of incubation. (H) Root induction from regenerated shoots and (I) Hardening of rooted shoots.



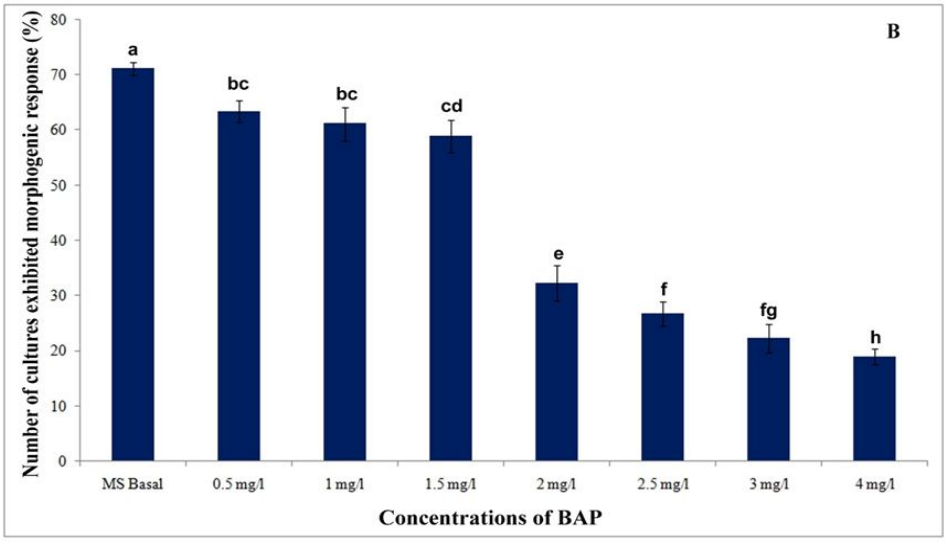


Figure 2: Callus induction and morphogcnic response obtained from leaf disc explants. (A) Callus induction in leaf disc explants in MS medium supplemented with different concentration of 2,4-D. (B) Morphoegenic response obtained in MS medium supplemented with different concentration of BAP from callus cultures.

Table 1: Effect of MS medium with different concentrations of BAP and kinetin on nodal explants of *C. aromaticus*

MS medium with cytokinins	Explants response (%)	Number of shoots/explant
Basal	23.33 ± 2.26^{de}	1.65 ± 0.14^{g}
1 mg/l BAP	42.22 ± 1.12^{a}	3.11 ± 0.05^{a}
2 mg/l BAP	33.33 ± 2.03^{b}	2.31 ± 0.11^{bc}
3 mg/l BAP	31.11 ± 1.98^{bc}	2.19 ± 0.25^{cd}
4 mg/l BAP	24.44 ± 1.27^{cd}	1.99 ± 0.37^{de}
5 mg/l BAP	15.56 ± 2.97^{gh}	1.86 ± 0.13^{ef}
1 mg/l Kinetin	12.22 ± 1.72^{i}	1.95 ± 0.08^{de}
2 mg/l Kinetin	17.78 ± 1.43^{f}	2.00 ± 0.14^{de}
3 mg/l Kinetin	26.67 ± 2.16^{cd}	2.54 ± 0.18^{bc}
4 mg/l Kinetin	22.22 ± 1.34^{de}	2.21 ± 0.33^{cd}
5 mg/l Kinetin	18.89 ± 1.43^{f}	1.88 ± 0.11^{ef}

Data are given as mean ± Standard deviation of three replicates; means followed by same letters are not significant at p=0.5 level. MS-Murashige and Skoog medium, BAP-6-Benzylamino purine.

Table 2: Effect of IBA and IAA on root induction in regenerated shoots of *C. aromaticus*

MS medium with auxins	Average root numbers/shoot	Average root length (cm)
Control	3.09 ± 0.10^{e}	2.31 ± 2.3^{e}
0.25 mg/l IBA	5.78 ± 0.10^{a}	5.35 ± 2.94^{a}
0. 50 mg/l IBA	4.17 ± 0.13^{c}	3.33 ± 1.71^{c}
0. 25 mg/l IAA	5.19 ± 0.19^{b}	3.80 ± 3.32^{b}
0.50 mg/l IAA	3.56 ± 0.14^{d}	2.94 ± 1.22^{d}

Data are given as mean ± Standard deviation of three replicates; means followed by same letters are not significant (p<0.05). IBA-Indole-3-butyric acid, IAA -Indole-3-acetic acid.

4. Discussion

Development of *in vitro* plant regeneration system in *Coleus* is essential for various objectives like large scale production of planting material as well as production of secondary metabolites. The techniques of plant tissue culture have been extensively used to enhance the number of desirable planting material as well as improvement of plant health (Brown and Thorpe, 1995). Present study utilized leaf disc explants for callus induction due to satisfactory availability of such explants from single plant. For callus induction, leaf discs were the explants of choice in several studies (Anbazhagan *et al.*, 2005; Srinivasan *et al.*, 2006; Sreedevi *et al.*, 2013). In present study, 2,4-D alone was found suitable for callus induction but several investigations used various combinations like BAP with 1-naphthaleneacetic acid (NAA) (Asamenew and Narayanaswamy, 2000; Anbazhagan *et al.*, 2005), BAP with IAA (Ibrahim *et al*., 1997), kinetin with IAA (Anbazhagan *et al.*, 2005) were used for callus induction in *C. forskolii* and *C. blumei*.

During present investigation, callus transferred on MS medium containing different concentrations of BAP induced globular structures, but failed to initiate shoot induction. As per the reports of (Vengadesan *et al.* 2002), addition of BAP into the medium enhances compactness of the callus which is essential for regeneration as we found in our study also, but failed to regenerate shoots. Hussain *et al.* (2013) also obtained green callus using leaf explant of *Taxus wallichiana*, but unable to regenerate shoot. Similar results were also obtained in *Pisum sativum* when callus obtained from leaves, cotyledons and root explants were failed to regenerate shoots on tested medium (El Sayed, 2011).

The cytokinin such as BAP and kinetin are known to enhance multiple shoot induction in various plants. We used BAP and kinetin in different concentrations with MS medium for direct shoot induction from nodal segment explants. BAP was fount better as compared to kinetin for initiation of multiple shoot induction. In *Coleus*, there are reports which employed BAP alone or along with any auxin were used to induce multiple shoot induction (Rajasekharan*et al.*, 2010; Sahoo *et al.*, 2019). MS medium with BAP and kinetin were also reported for multiple shoot induction from nodal segment explants of *C. aromaticus* (Govindaraju and Arulselvi, 2018).

All types of auxins were reported to induce rhizogenesis in lower concentrations. During this investigation, IAA and IBA in low concentrations in MS medium were used for root induction. IAA (0.25 mg/l) was found superior for roots per shoot as well as root length. In *Coleus*, NAA, IBA and IAA were employed for root induction in various studies (Khan and Jain, 2012; Sahai and Shahzad, 2013; Sahoo *et al.*, 2019).

5. Conclusion

To regenerate *C. aromaticus in vitro*, leaf and nodal explants were used for indirect and direct regeneration, respectively. In case of indirect regeneration, MS medium containing 3 mg/l 2,4-D exhibited maximum callus induction. These calluses further showed morphogenic changes in the form of globular structures but were failed to organogenesis. In another case, direct regeneration initiated using nodal segments showed maximum shoot induction on MS medium fortified with 1 mg/l BAP. These shoots were rooted on MS medium with IBA and successfully hardened to transfer in natural environment.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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