Regeneration and selection of root rot resistant *Coleus forskohlii* A threatened medicinal plant

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Abstract

Coleus forskohlii Briq. of the family Lamiaceae yields a valuable secondary metabolite known as forskolin which is a labdane diterpenoid.. *Coleus forskohlii* is the only known source of this compound. Forskolin is used in medicine for the treatment of glaucom a, congestive cardiomyopathy and asthma.

Morphogenic callus was induced from young leaves on MS medium augmented with NAA and BA. These calli, when subcultured on MS with KN alone gave rise to shoots. The regenerated shoot developed good root system on MS medium fortified with NAA. The fully grown plantlets were transferred to soil for acclimatization

Coleus plant is mainly infected by a fungi *Lasiodiplodia theobromae* which causes root rot disease. The fungal culture filterate (FCF) of *Lasiodiplodia theobromae*, has been used in regeneration media to find the MIC and further to select resistant plants to the pathogen. In the present study 40% FCF in the medium showed maximum inhibition and is there fore considered as the MIC level of *Coleus forskohlii*. This data could prove to be useful for the future for selecting a resistant *C.forskohlii* plant against the root disease caused by *L.theobromae*.

Introduction

The demand for the plant based herbal' medicines is increasing every day due to their fewer side effects in comparison to synthetic chemical drugs. It is therefore very important to conserve and propagate medicinal plants of any country.

Throughout history, plants have been the most important source of medicines for human health. About 1, 00, 000 compounds are now known from the plants with about, 4, 000 new ones being discovered every year (Verpoorte et al, 1999)

Medicinal and aromatic plants form an important group among the economic plants. Due to the increasing population, especially in the developing countries, many natural growing sites and ecosystems of important medicinal and aromatic plants are being destroyed. It therefore urgent to chalk out conservation measures for these invaluable natural resources.

Many modern medicines are either derived from the plants or artificially synthesized to copy plant chemical compounds. The World Health organization [WHO] estimates that up to 80% of the world's population rely on plants for their primary health care. India is one of the12 mega biodiversity centers where almost all known medicinal plants can be cultivated in some part of the country or the other. India has about 2, 000 species of medicinal plants and vast geographical area with high production potential and varied agro climatic conditions. Cultivation of medicinal plants offers considerable scope for rural employment and export for foreign exchange earnings.

India is already a major exporter of medicinal plants. The turn over of herbal medicines in India as over the counter products, ethical and classical formulations and home remedies of Ayurveda, Unani, and Siddha systems of medicine is about 1 million US $ with an export of about 80 million US $ (Khamboj , 2000)

Coleus forskohlii Briq. of the family Lamiaceae, is an aromatic herb 30-60 cm. high with dull orange tuberous roots. It grows wild in the sub-tropical Himalayas, distributed from the Kumaon hills to Nepal ascending up to 2000M, and in Bihar and Gujarat.(Anonymous, 1950). It is cultivated in Gujarat and Karnataka. The plant yields a valuable secondary metabolite known as forskolin which is a labdane

diterpenoid. Though forskolin is found in all parts of the plant, roots are the main source of compounds. Coleus forskohlii is the only known source of the compound.

Forskolin is used in medicine for the treatment of Glaucoma, Congestive cardiomyopathy and Asthma (Valdes etal.1987). It is characterized by hypertensive property, inhibitory action on thrombocyte aggregation and reduction of intra ocular pressure. In addition forskolin is used in purification of adenylate cyclase and in receptor binding assays (SukhDev, 1997).25)

Indiscriminate and large-scale collection of this plant from forest and the other natural sources and insufficient attempts to either allow its replenishment or its cultivation, have lead to the rapid depletion of its resource. Gupta (1988) has listed this plant as one of the plant species in India vulnerable to extinction. Moreover this plant is affected by root rot disease caused by a fungi *Lasiodiplodia theobrmae.* In severe case the entire plant dries up.

Lasiodiplodia theobromae belongs to the class-Deutromycotina, Order-Spheropsidales, and Family- Sphereopsidaceae. The fungus has a wide host range around the world. The fungus is found throughout the tropics and sub-tropics causing numerous kinds of diseases, especially rots of fruits and root crops during storage such as, stem and root in citrus, mango, fruit rot in papaya (Gupta and Nema, 1979), crown rot in banana, storage rot in sweet potato and also attacks crops of cassava. The fungus causes mycotic keratitis and oncomycosis in animals (Laverde etal, 1973 Mc-Ginnis, 1985). It forms pycnidia upto 5 mm in diameter. Conidia are initially hyaline and unicellular, sub ovoid to ellipsoidal with granular content. Mature conidia are two celled, cinnamon or light brown in color and often with longitudinal striations. Conidia are 18-30 x 10-15 micrometer with paraphyses up to 50 micrometer long. associated with a large number of rots (Sulton, 1980).

It is evident from the foregoing account that developing a method for the mass propagation of this economically useful, endangered plant species is highly essential. *In vitro* micropropagation technique offers a powerful tool for

plant germplasm conservation as well as obtaining disease resistant plants.

In any selection scheme, it is desirable that the trait of interest be selectable at cellular level and also expressed in the regenerated plants. In the past *in vitro* techniques have been employed successfully in the selection of resistant plants at cellular level (Jones, 1990). The basis of such selection is that some agents of known importance in the disease reaction should be employed as the selection criterion. The selection agents that can be used for selection can be (i) pathogen itself or (ii) pathogen metabolites.

Use of pathogen metabolite for selection is the most commonly employed method and seems to be reliable in obtaining disease resistant plants. Selection has been carried out with callus, or cell suspension cultures and also protoplast. Culture filtrates represents an easy, common and cheap method of selection by incorporation in the culture media at appropriate concentrations. In several selection experiments culture filtrates, both purified and partially purified have been successfully used. For many diseases, well-characterized toxins are not known, and culture filtrates in these exhibit phytotoxic activities (Larkin and Scowcraft, 1983).

The pathogen causing root rot disease, *Lasiodiplodia theobromae* has been isolated and identified (MTCC 3086). Establishment of host-pathogen interaction in vitro permits screening and selection for disease resistant at cellular level (Vidyasekharan, 1993). A number of workers have used fungal culture filtrate (FCF) as the selection agent, rather than purified toxin to select resistant plants through tissue culture. This approach has been adapted in the present study also. The FCF of *Lasiodiplodia theobromae* has been used in callus induction and regeneration media to select resistant plants to the pathogen.

The scope of the project undertaken includes development of reliable, efficient *in vitro* propagation methods for *Coleus forskohlii* plant as well as the establishment of the Maximum Inhibitory Concentration (MIC) for the fungal toxin. This information will be highly useful for mass cultivation of this threatened, medicinally useful plant as well as for obtaining disease free/resistant plant.

Materials and Methods

C.forskohli plants were grown in Sardar Patel University botanical Garden, Vallabh Vidyanagar. Young leaves were taken from field grown mature plants. The leaves were cut in to 1cm and were cultured in culture tube. The explants were sterilised using 0.1% HgCl2 for 3 minutes and thoroughly washed with sterile distilled water five times. For all the experiments, Murashige and Skoog (1962) medium supplemented with 3% sucrose (Hi Media, India) was used. The pH of the medium was adjusted to 5.8 followed by the addition of 0.8% agar (Hi Media, India) before autoclaving at 15 psi for 15-20 minutes (1230C). For callus induction and regeneration studies MS medium along with 2, 4-dichlorophenoxy acetic acid (2, 4-D), 1-naphthalene acetic acid (NAA), 3-indole butyric acid (IBA), 6-benzyl aminopurine (BA) and 6-furfuryl aminopurine (KN) were used as the growth hormones at varying concentrations. For shooting 150-200 mg green calli were subcultured on various concentrations of cytokinins alone or in combination with auxins. Cultures were raised in 15 mm X 150 mm culture tubes under cool white flourescent tubes emitting 50 mole m-2 s-1 for 14 hrs of photoperiod in a growth room maintained at 2520C. For hardening, the healthy plants were transferred to pro-trays containing sterile coco-peat and kept in a small poly house to maintain humidity (80-85%RH) for 15 days and later transferred to soil for acclimatization.

Isolation of the fungus

The fungi *Lasiodiplodia theobromae* was isolated from decaying roots of *Coleus forskohlii* on Potato dextrose Agar (PDA) medium and identified at IMTECH, Chandigarh (MTCC 3086). The pure cultures were transferred to PDA slants for subsequent maintenance of culture and bioassay. The fungus was grown on PD broth and the fungal culture filtrate was assayed for inhibitory/promotory effects by seed germination assay. The promotory effect of FCF from fresh subcultures was reconfirmed by wheat coleoptile elongation test.

The Maximum inhibitory concentration (MIC) of the FCF to *Coleus forskohlii* culture:-

The culture of *Lasiodiplodia theobromae* was inoculated in 1L flask having 250 ml PD broth (Himedia, Mumbai) and inoculated at 280C for 10 days. The FCF was first filtered with Whatman's filter paper and further filtered through a 0.45 m nylon membrane. The filtered crude toxin was added at different concentrations to MS medium supplemented with 1 mg/l BA, (viz.0%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%). The medium was then autoclaved at 1210C, 15 lbs. pressure for 15 minutes.

Calli weighing (~250 mg) were inoculated in these tubes and incubated in the growth room at 2420 with 14 hours of photoperiods using cool white fluorescent lamps of 3000 lux.

Results

MS medium augmented with IBA+BA, IBA+KN, IAA+KN, IBA +BA, NAA+KN at varying concentrations gave poor response with the leaf explants (Table.1) Out of the various combinations tried for callus induction with young leaf segments as explants. MS medium supplemented with NAA and BA showed better results. (Table.2)

The explants were placed in the medium with the abaxial and adaxial sides in contact with the medium. These explants placed with the abaxial surface in contact with the medium responded better. After 7 days of culture, the laminar portion as well as the petiolar portion showed callus initiation (Plate 1A).

MS medium augmented withl-2 mg/l NAA +1mg/l BA gave better results than other combinations of BA and NAA (Table.2)

The 50-60 days old greenish calli (Plate 2.B) were subcultured on either MS medium devoid of growth hormones or with KN or BA at varying concentrations (Table.3). The medium augmented with KN alone (0.5 to 2 mg/l) did not produce any shoot buds, though the calli remained green. But with BA (0.5 to 2 mg/l) shoot buds were initiated after 7-8 days of culture.

Out of these MS +1 mg/I BA produced maximum number of shoots (Plate.1C). Higher concentrations of BA have been found to be inhibitory for the differentiation of shoots. More than 15 shoots were produced per culture (Fig.1)

Rooting of healthy shoots was tried on half strength as well as MS basal medium or MS medium supplemented with growth hormones at varying concentrations (Table.4). MS medium augmented with IAA or IBA did not induce any roots or shoots, while with NAA at lower concentrations (0.1-0.4 mg/l), the shoots produced roots (Plate 1.D, E). At higher concentrations however the bases of shoots produced calli. In half strength and full strength medium devoid of any hormones also the shoots produced roots. More number of lengthy roots were found in half strength MS basal medium.

Three weeks old rooted plants weaned in a small polyhouse for 10-12 days in high *humid condition (70-80 %) were transferred to ambient conditions into cups filled* with 50% soil and 50% coco peat.(Plate, 1 F) Before transfer, the *in vitro* raised plantlets were dipped in 20% glycerol and then transferred to the soil, to reduce the rate of transpiration.

Determination of maximum inhibitory concentration (MIC):

Standardization of the Maximum Inhibitory Concentration (MIC) of the Fungal culture Filtrate (FCF) to the callus tissue of *Coleus forskohlii* was carried out by incorporating various concentrations of the FCF in the MS medium. It was observed that the callus retained its morphogenetic potential at 5% and 10% concentrations. At concentrations above 15% the growth and differentiation of the callus were inhibited. At 45% and 50% concentration of FCF the callus turned completely brown and eventually died.

Discussion

Callus initiation from leaf disc explants has been reported from various Lamiaceae members, such as *Coleus blumei* (Dodds and Roberts, 1982; Hervey and Robbins, 1978), *C.purriflorus* (Ashokan etal.1983), *Perilla frutescens*

(Tanimoto and Harada, 1980) *Mentha citrata* (Van Eck and Kitto, 1988;1992). Higher concentrations of BA (1-2 mg/l) has been reported to induce callus formation from leaf explants of *Curculigo orchioides* (Augustine and D'souza, 1997) whereas in another member of Lamiaceae, *Mentha piperita,* 0.5 mg/l each BA and IBA was enough for callus induction from leaf discs (Faure etal.1998). In the same species Jullien etal. (1998) induced calli in combination of 2, 4-D, NA, and BA.

The present observations with callus induction in *Coleus forskohlii* closely resemble the study on *Salvia officianalis,* another medicinal plant, where equimolar concentrations of auxin and cytokinin BA +NAA stimulated callus formation from leaf explants. (Kintziose etal.1999). In Pippermint another Lamiaceae member, callus was obtained with 2, 4-D with NAA and BA as growth regulators (Jullien etal, 1998). In the present study NAA+BA at equal concentrations was found to be good for callus induction.

In *Minthostachys* (Chebel etal.1988) MS +NAA and BA was found to induce higher number of shoots per nodal explants.

In earlier studies with *Pogostemon cablin* (Benth), another aromatic plant regenerating shoots were obtained from calli derived from leaf segments using MS +1 mg/l BA while Kinetin was found to be less effective for shooting (Misra, 1996). Similar results could be observed in the present study also. Sen and Sharma (1991) reported maximum number of shoots on MS medium augmented with 2 mg/l IBA within 20-25 days through shoot tip culture in *Coleus forskohlii* while Sharma et al.(1991) reported shoot induction from nodal segment culture of *C.forskohlii* on MS+ 1 mg/l KN + 1mg/l IAA. In the present study our results are similar to those of Sen and Sharma, 1991.
Sharma et al. (1991) induced roots in *in vitro* grown shoots of *Coleus forskohlii* on MS +1 mg/l NAA while Sen and Sharma (1991) obtained rooting on MS basal medium in a few plants. In the present study better and efficient rooting of shoots could be observed in half strength MS basal medium.

Acclimatization of *in vitro* plantlets is often difficult because they possess succulent stems and leaves due to high humidity with in the culture vessels

and free water in the medium besides poorly developed cuticle, reduced differentiation of palisade cells large intracellular spaces and defective due to ambient conditions (Thomas, 1998)

The transition from *in vitro* to *ex vitro* is a critical phase, required to make the large heterotropic plantlet completely autotrophic through photosynthesis and preparing it to withstand the unusual water loss by development of protective layers and regulation of stomatal functioning. The use of increased light intensity and gradual reduction in pH has been demonstrated to facilitate *ex vitro* establishment (Thomas, 1998; Mao etal, 2000).

Incorporation of an inhibitor or an anti metabolite at least two times the level of MIC, is one of the most common strategies used for the selection of plants resistant against diseases and various other environmental stresses (Suprasanna and Rao, 1998) A number of economically important plant species have been selected for disease resistance using the crude FCF of the pathogenic fungi as an inhibitor in the culture medium (Morpurgo etal, 1994; Venkatachalam etal, 1998) These include plant species such as Rice (Vidyasekaran etal, 1990), Tobacco (Thanutong etal, 1983), Potato (Behneke 1979) and Groundnut (Venkatachalam et al 1998).

Once the MIC is established the selection for disease resistance could be achieved using either a single step or a multi step selection method. In the single step selection procedure the inhibitor is added at least two to three times the level of MIC and cultures are maintained for several subculture regimes with the inhibitor (Gonzales, 1985) In the multi step selection procedure sub-lethal concentration (less than MIC) is added into the medium and subsequently a gradual increase in inhibitor level is maintained (McCoy, 1987; Miao etal, 1988).

In the present study 40% FCF in the medium showed maximum inhibition and is therefore considered as the MIC level of the FCF to *Coleus forskohlii*. This data could prove to be useful for the future for selecting a resistant *C.forskohlii* plant against the root disease caused by *L.theobromae*.

The protocol standardized for *in vitro* regeneration of *Coleus forskohlii* will be

a useful tool for mass propagation of this important medicinal plant. In addition, the MIC value determined for the FCF of *L.theobromae* can be employed for the selection of disease resistant plants at cellular level.

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Table.1 Media combinations tried for callus induction from leaf disc explants of *C.forskohlii*

Media combinations	Response
MS+0.5mg/l IBA+1mg/l BA	+
MS+1mg/l IBA+1mg/l BA	+
MS+1mg/l IBA+0.5mg/l BA	+
MS+0.5mg/l IBA+1mg/l KN	-
MS+1mg/l IBA+1mg/l KN	-
MS+1mg/l IBA+0.5mg/l KN	-
MS+0.5mg/l IAA+1mg/l BA	+
MS+1mg/l IAA+1mg/l BA	+
MS+1mg/l IAA+0.5mg/l BA	+
MS+0.5mg/l IAA+1mg/l KN	-
MS+1mg/l IAA+1mg/l KN	-
MS+1mg/l IAA+0.5mg/l KN	-
MS+0.5mg/l NAA+1mg/l BA	+++
MS+1mg/l NAA+1mg/l BA	+++
MS+1mg/l NAA+0.5mg/l BA	++
MS+0.5mg/l NAA+1mg/l KN	-
MS+1mg/l NAA+1mg/l KN	-
MS+1mg/l NAA+0.5mg/l KN	-

- no response + poor response

++ moderate +++ Good

Table.2 Media combinations tried for callus induction from leaf explants of *C.forskohlii*

Media Combinations	Response	% Response
MS+0.5mg/l NAA+0.5mg/l BAP	+	16
MS+1mg/l NAA+0.5mg/l BAP	+	20
MS+1.5mg/l NAA+0.5mg/l BAP	+	20
MS+2.0mg/l NAA+0.5mg/l BAP	+	25
MS+0.5mg/l NAA+1mg/l BAP	+++	85
MS+1mg/l NAA+1mg/l BAP	+++	83
MS+1.5mg/l NAA+1mg/l BAP	+++	66
MS+2.0mg/l NAA+1mg/l BAP	++++	100
MS+0.5mg/l NAA+1.5mg/l BAP	++	33
MS+1mg/l NAA+1.5mg/l BAP	++	33
MS+1.5mg/l NAA+1.5mg/l BAP	++	33
MS+2.0mg/l NAA+1.5mg/l BAP	++	50

Table. 3 Combination tried for shoot induction from leaf derived calli of *C.forskohlii*

Media Combination	Observation	Response
MS	Shoot initiation	+
MS+0.5mg/l BAP	Shoot initiation	+++
MS+1.0mg/l BAP	Shoots formed	++++
MS+1.5mg/l BAP	Greening of calli + shoots	++
MS+2.0mg/l BAP	Greening of calli + shoots	++
MS+0.5mg/l KN	Callus remained green	-
MS+1.0mg/l KN	Callus remained green	-
MS+1.5mg/l KN	Callus remained green	-
MS+2.0g/l KN	Callus remained green	-

- no response + Poor ++ moderate

+++ good ++++ profuse

Table 4. Media combinations tried for rhizogenesis in *C.forskohii*

Media combinations	**Response**
MS half strength	+++
MS full strength	++
MS+0.1mg/l NAA	+
MS+0.2mg/l NAA	+
MS+0.4mg/l NAA	+
MS+0.5mg/l NAA	Callus induction from base of the shoots
MS+1mg/l NAA	Callus induction from base of the shoots
MS+0.2mg/l IAA	-
MS+0.5mg/l IAA	-
MS+1mg/l IAA	-
MS+0.2mg/l IBA	-
MS+0.5mg/l IBA	-
MS+1mg/l IBA	-

- No response + poor

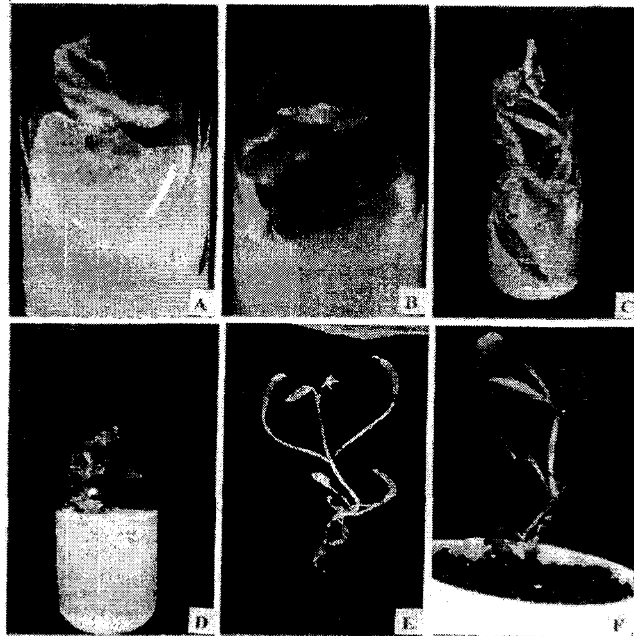
++ moderate +++ Good

Media combinations tried

1 = MS   2 = MS+0.5mg/l BA   3 = MS+1mg/l BA

4 = MS+1.5mg/l   5 = MS+2mg/l BA

Fig 1. Effect of BA on shoot induction from leaf derived calli in *C.forskohlii*



Explanation to **Plate 1.**

A. Callus induction from leaf explants
B. 40-50 days old calli in MS + 1mg/l each BA and NAA
C. Multiple shoots in MS + 1mg/l BA
D. Single shoot kept for rooting
E. Fully grown plantlet
F. Plants transferred to soil for acclimatisation