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Invitro studies in *Coleus forskholii* for induction of resistance against a fungal *toxin lasiodiplodia theobromae*

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ABSTRACT

*Coleus forskholii is a p*erennial plant with high medicinal value, made up of an active component called forskohlin extracted from roots. It shows anti cancer properties, promotes weight and fat loss, increases lean body mass, reduces allergies, asthma (anti-histamine effect), lowers blood pressure, stomach and menstrual cramps. Forskholin functions as a platelet aggregation inhibitor, relaxes vascular smooth muscle, decreases intraocular pressure due to glaucoma, and has an anti-allergy[9] potential since it inhibits IgE- mediated release of histamine and peptide leukotriene from human basophils and mast cells. *Coleus forskholii* is a very serious soil-borne disease caused by Lasiodiplodia theobromae. It is traditionally propagated by means of vegetable cuttings but it is time consuming and provides a limited number of propagules. Invitro propagation methods offer powerful tools for plant germplasm, conservation and multiplication. An attempt has been made to obtain invitro plants modified by mutation such that they are resistant to a high concentration of toxin produced by the fungus.

KEYWORDS

Benzyl Adenine (BA);
Kinetin (Kn);
Shoot induction;
Indole butyric acid (IBA);
Murashige & Skoog Medium;
Lasiodiplodia theobromae.

INTRODUCTION

This is a perennial herb with fleshy, fibrous roots that grows wild in the warm subtropical temperate areas in India, Burma and Thailand. It is one of the 150 coleus species, which are commonly cultivated as ornamental plants, because of their colorful foliage. The roots of C. forskohlii, unlike other coleus species, are used for health purpose. *Coleus forskholii* is an important ayurvedic herb that has been a part of Indian medicine for centuries. Its common name is makhandi. In the 1970s researchers isolated a chemically active ingredient in the herb and called it forskholin[5]. The species was discovered to have pharmacological activities of lowering blood pressure and producing positive inotropic activity. Forecasts of the requirements of forskolin for drug development indicated the need for a sustained supply of root material in quantities that would threaten the survival of the species in nature. Forskholin is a diterpene[3] from the roots of *Coleus forskholii*. The focus of the development studies was the increased yield of root tubers and forskolin. As a consequence of this development, *C. forskohlii* is now being cultivated as a source of forskolin.

This alkaloid has the unique property of activating all hormone–sensitive adenylate cyclase enzymes in bio-

logical systems. Activation of adenylate cyclase raises cyclic AMP levels in a variety of tissues. Cyclic AMP is an important cell regulating compound which once formed it activates many other enzymes involved in diverse cellular functions. Under normal situations cAMP is formed when a stimulatory hormone epinephrine[7] binds to receptor site on the cell membrane and stimulates the activation of adenylate cyclase. This enzyme is incorporated into all cellular membranes and only the specificity of the receptor determines which hormone will activate it in a particular cell. Forskholin bypass the need for direct hormonal activation of adenylate cyclase.

As a result of this direct activation of adenylate cyclase, intracellular cAMP levels rise.

Cyclic AMP is called the "second messenger" because it facilitates the action of "primary messengers", various hormonal and bioactive substances in the body. Based on its pharmacological actions, forskohlin appears to be well indicated in conditions, such as eczema (atopic dermatitis), asthma, psoriasis, cardiovascular disorders, and hypertension, where decreased intracellular cAMP levels is believed to be a major factor in the development of the disease process The physiological and biochemical effects of a raised intracellular cAMP level are manifold. Forskholin functions as a platelet aggregation inhibitor, relaxes vascular smooth muscle, decreases intraocular pressure due to glaucoma, and has anti-allergy[9].

Potential since it inhibits IgE- mediated release of histamine and peptide leukotriene from human basophils and mast cells. Forskholin has been shown to be a potent inhibitor of cancer metastasis in mice injected with malignant cells[1].

Forskholin content has been found to vary from 0.07 to 0.59% of dry tubers and just 1 g of forskholin costs $85, showing the importance of this crop. A major difficulty faced in obtaining forskholin is that the roots of the plant are faced with the root rot disease which decreases the forskholin content. The root-rot of *Coleus forskholii* is a very serious soil-borne disease caused by Lasiodiplodia theobromae. *Coleus forskholii* is traditionally propagated by means of vegetable cuttings but it is time consuming and provides a limited number of propagules[2]. Invitro propagation methods offer powerful tools for plant germplasm conservation and multiplication.

An attempt has been made to obtain invitro plants modified by mutation such that they are resistant to a high concentration of toxin produced by the fungus.

MATERIALS AND METHODS

Plant material

We have tried different explants like apical buds, shoot tips, nodal segments, internodes and leaf explants were excised from plants grown in the field. We found that leaf explant was found to be appropriate as it was responding well under invitro conditions[8] showing multiple shooting.

Leaf explant as ideal for our experimentation only young leaves were utilized for further studies on the effect of growth hormones BA and Kinetin. All explants were disinfected in sterile distilled water and then sterilized by immersing in 0.1% (w/v) $HgCl_2$ for 3 minutes. The leaves were trimmed into pieces of about 1 -2 cm and then inoculated into culture media. Only the petiolar portion of the leaf was used for inoculation and the leaves were placed with their dorsal side facing the medium.

Toxin extraction

The fungus *Lasiodiplodia theobromae*[0][10] which secretes the required toxin was grown in Potato Dextrose broth for 10 days, after which duration, the fungus was removed from the broth and the toxin obtained by filtering this broth.

Culture medium and conditions

The culture medium used for the explant selection was GR-free[3] medium supplemented with 0.8% (w/v) agar, 3% (w/v) sucrose. MS media[6] supplemented with 0.8% (w/v) agar, 3% (w/v) sucrose and enriched with varying BA & Kinetin concentration was used further on to determine optimum growth regulator levels. The concentrations tested for BA were 1.0, 1.5, 2.0 & 2.5 while those for Kinetin were 0.5, 1, 2.0, & 2.5 mg/lit.

The pH was adjusted to the range of 5.6 to 5.8 with 1 N NaOH or HCl before molten media were dispensed into petri plates (Borosil, India) and the media were autoclaved at 121° C at 15 p.s.i (1.04 kg cm^{-2}) pressure for 15min. The cultures were maintained at

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25±2 °C under a 16 hours photoperiod of 50μmol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes.

CALLUS INDUCED SHOOT REGENERATION

Callus formation

Various concentrations of Kinetin (0.5mg/lit – 2.0 mg/lit) either alone or in combination with 2, 4-D (0.5mg/lit – 2.0mg/lit) were used. Three pieces of leaf segment were inoculated in each petriplate. Growth was determined after 2 weeks (See Appendix A).

Appendix A

Growth Hormone	mg/l	No of Shoot Buds	Callus Induction
BA	0.5	25	
	1.0	40	
	1.5	55	
	2.0	75	
	2.5	90	
Kinetin	0.5	40	85
	1.0	60	70
	2.0	80	55
Kn +2,4-D	0.5		85
	1.0		75
	2.0		60

Effect of varying concentrations of Kinetin (Kn), 2, 4-D & BA: Shoot and Callus regeneration from leaf explants.

Shoot differentiation

Callus obtained from 0.5mg/lit Kn in 2 weeks was sub cultured on 0.5, 1, 2 & 2.5 mg/lit Kn. Efficient shooting was recorded from the calli buds after 10 days of inoculation (Figure A). Regenerated shootlets were rooted spontaneously on MS medium devoid of growth regulators.

Direct shoot regeneration

Leaf explants were inoculated on MS media enriched with BA (2.5mg/lit) was showing effective shooting in terms shoot length and breadth (Figure B). Leaf explants were inoculated on MS[6] media containing 2.5 mg/l BA and varying concentrations (0 – 100 % in steps of 10%) of the extracted toxin. Based on the results obtained after 1 week observation, the range was re-

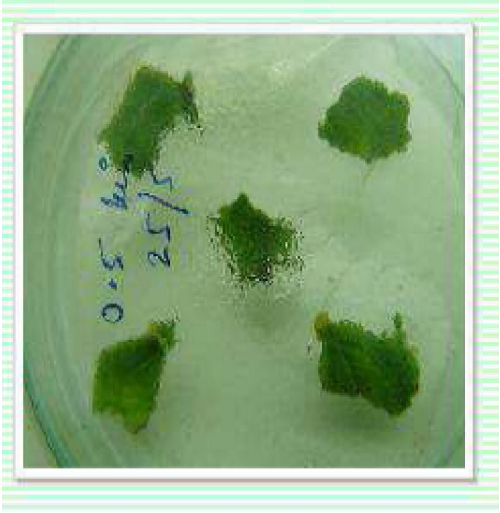

Figure A : Shoot Regeneration with Calli buds.

Figure B : Invitro Plantlet showing effective shooting.

Figure C : C-Leaf curling observed in 7.5% (v/v) Toxin concentration.

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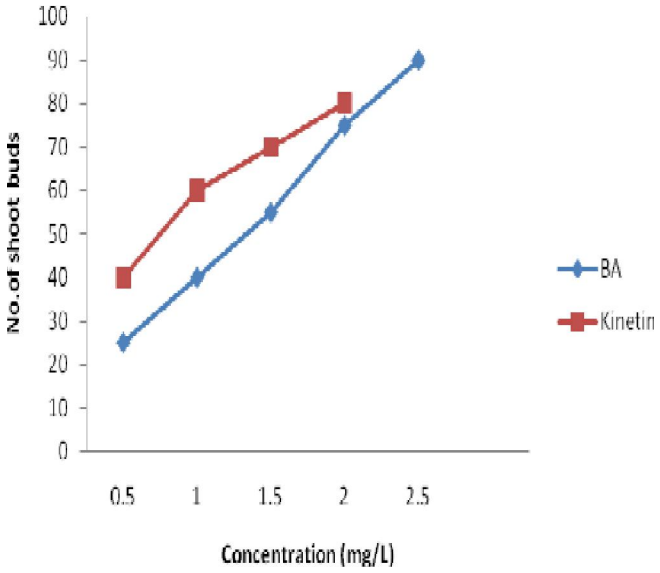
duced to 5 – 15 % in steps of 2.5%. Critical toxin concentration was noted based on observations after another one week.

RESULTS AND DISCUSSION

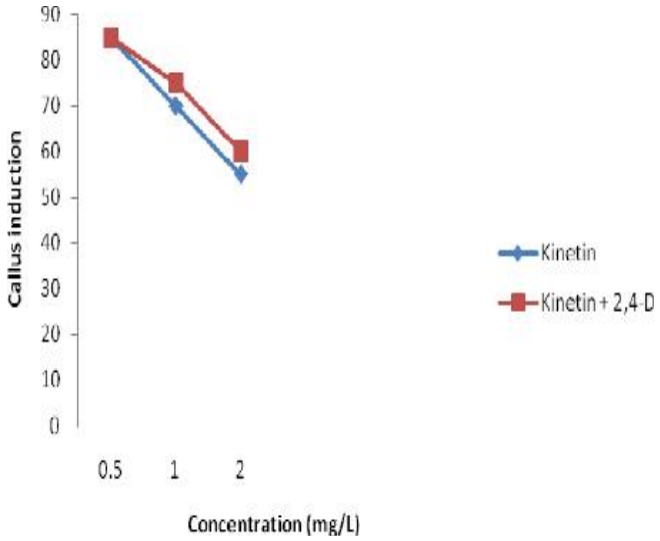
Leaf segments cultured on MS medium supplemented with Kinetin (0.5 mg/l – 5mg/l) induced callus formation from the cut ends of the leaves in 2 weeks. Addition of 2, 4-D (0.5 mg/l – 2.5mg/l) resulted in the formation of white cottony mass like callus. We studied the effects of various concentrations of plant growth substances on callus induction and plant regeneration from different explant sources. Callus was obtained from all four types of explant of the five investigated cultivars Morphology and quantity of callus varied with varying concentrations of Kn. Leaf explants produced highly proliferating light green colored, compact callus in the medium containing 0.5mg/L Kinetin. When these calli buds were sub cultured on MS media containing 2 mg/l Kn formation of adventitious shoot buds was noticed from the surface of the callus within two weeks of culture.

Leaf explants cultured on MS medium supplemented with BA (2.5mg/l) showed formation of shoot buds from the cut edges of the leaves within one week of culture. The frequency of direct shoot regeneration from leaf explants was found to be extremely high. It was also observed that the petiolar region of the leaf showed maximum regenerative capacity[4]. Leaf explants supplemented on MS medium contained perished toxin at a concentration higher than 7.5% (v/v), therefore this concentration was concluded to be the critical toxin concentration. Medium combination and explant source had an important influence on callus initiation and plant regeneration. This study used supplemented MS basal medium for callus induction and plantlet regeneration in *Coleus forkholii*. . The amount of forskolin in *in vitro*raised plants and wild plants was estimated and found that they produce comparable quantity of forskolin. This *in vitro* propagation protocol should be useful for conservation as well as mass propagation of this plant.

Invitro propagation protocol could be useful for mass propagation of this plant for use in commercialization, conservation and for further research like mutagenesis. Invitro plants after being subjected to mutagenesis could be grown on the obtained critical concentration of toxin (Lasiodiplodia theobromae) to isolate germplasm with higher toxin resistance. We can observe curled callus in the toxin concentration. Meanwhile, this protocol offers itself not only as a highly efficient method for mass clonal propagation of this species but also for its conservation. By this method we can isolate some agronomically or pharmaceutically improved clones of commercial value can be achieved[11].



Graph 1 : Effect of varying concentrations of BA, Kn on Shoot regeneration from leaf explants



Graph 2 : Effect of varying concentrations of Kn, and Kn + 2, 4-D on Callus regeneration from leaf explants

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CONCLUSION:

As it is difficult to propagate *Coleus forskholii* by traditional and vegetative methods, we have made an experimental attempt using invitro propagation methods for mass multiplication[5] of the plantlet[2] as it characterizes certain medicinal properties like anti cancer, anti emetic symptoms. Mass Propagation by means of cell and tissue culture techniques is a powerful tool for plant germplasm conservation and rapid clonal multiplication as well as for reforestation and tree improvement.

Therefore the need to raise this plant is essential for conservation of germplasm in order to synthesize better clones with potential agricultural and medicinal values. Our laboratory protocol was proved to be successful when we have induced with a particular toxin concentration of Lasiodiplodia theobromae.

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