CIBTech Journal of Biotechnology ISSN: 2319–3859 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/cjb.htm
2020 Vol. 9, pp.9-26/Manju

Research Article

TRANSFORMATION STUDIES IN *COLEUS FORSKOHLII* - AN IMPORTANT MEDICINAL PLANT

***Manju M. George**
Department of Botany, Union Christian College, Aluva,
Ernakulam Dt. Kerala – 683 102
**Author for Correspondence: manju@uccollege.edu.in*

ABSTRACT

Coleus forskohlii Briq. (syn. *Plectranthus barbatus* Andrews) It is an important medicinal plant of the family Lamiaceae, valued for its production of forskolin, a labdane diterpenoid with significant pharmaceutical applications. This study reports the development of micropropagation protocols and attempts at Agrobacterium-mediated transformation for this threatened medicinal species. Leaf explants cultured on Murashige and Skoog (MS) medium supplemented with various combinations of auxins (NAA, IAA, IBA) and cytokinins (BAP, kinetin) resulted in callus induction and subsequent shoot organogenesis. Optimal callus formation was observed on MS medium containing 2.0 mg/L NAA + 1.0 mg/L BAP, while shoot regeneration was most efficient on MS + 1.0 mg/L BAP. Direct organogenesis was also achieved using MS + 0.5 mg/L BA + 0.5 mg/L IBA. Rooting was successfully obtained on half-strength MS medium without growth regulators. Acclimatization of regenerated plantlets showed 55-60% survival. Transformation attempts using *Agrobacterium rhizogenes* strains R1000 and 15834 for hairy root induction, and *A. tumefaciens* LBA4404 harboring pCAMBIA 1305.1 for disease resistance, were unsuccessful. The developed micropropagation protocols provide a foundation for conservation and mass multiplication of this valuable medicinal plant, though further optimization and quantification of forskolin are needed for pharmaceutical applications.

Keywords: *Coleus forskohlii, Micropropagation, Tissue culture, Agrobacterium transformation, Hairy roots, Forskolin, Medicinal plant*

INTRODUCTION

Plant tissue culture has emerged as a potential tool and forms the backbone of plant biotechnology. The science of plant tissue culture is really not more than five decades old. It was conceived and enunciated by Haberlandt in 1902. He visualized growing plant cells in artificial media, hoping to rejuvenate a quiescent cell, trigger it into division and growth, and form a tissue that would eventually regenerate a new whole plant (Gottlieb, 1902). Tissue culture techniques are widely applied to improve field crops, forests, horticulture, and plantation crops, thereby increasing agricultural and forestry production (Bhojwani & Razdan, 1996). This technique has been commercialized worldwide and has significantly enhanced the production of high-quality planting material. Clonal propagation of selected phenotypes is an essential step in most plant breeding programs. It is a faster method of asexual reproduction in comparison to propagation through seeds (Thorpe, 2007).

Tissue culture also has wide importance in medicinal botany. The preparation of standardized formulations of the chemical constituents of plants and their parts requires uniformity in both qualitative and quantitative terms. Furthermore, an ever-increasing demand for uniform medicinal plant-based medicines warrants their mass cloning via plant tissue culture (Ramawat & Mérillon, 2008). A good number of medicinal plants have been reported to regenerate *in vitro* from their various parts (Hussain *et al.,* 2012; Karuppusamy, 2009). For some threatened or endangered species, conventional propagation methods are ineffective, and uncontrolled exploitation makes their situation even more critical. In such cases, tissue culture is more effective (George *et al.,* 2007).

After 1950, there was an immense advancement in the knowledge of the effect of PGR's on plant development. Later, it was shown that no single medium was suitable for the growth of all tissues, leading to the formulation of distinct media for specific tissues. Besides progress, scientists tried to improve culture media by varying their mineral content. In this direction, Murashige and Skoog (1962) prepared a medium by increasing the concentration of salts 25 times higher than Knop's (Murashige & Skoog, 1962). Even today, MS medium has immense commercial applications in tissue culture. According to Scowcroft (1987), tissue culture techniques can play a significant role in enriching genetic variability, giving rise to variations/mutations at an unexpectedly high rate, and may be a novel source of genetic variability in many plant species (Scowcroft, 1985). Therefore, the present investigation was conducted to determine a suitable protocol for callus induction and root-shoot development in the plant.

The preparation of standardized formulations of the chemical constituents from plants and their parts is required to be uniform both qualitatively and quantitatively. Furthermore, an ever-increasing demand for uniform medicinal plant-based medicines warrants their mass cloning via plant tissue culture. In the case of threatened or endangered species, conventional propagation methods are ineffective and more critical. In such cases, tissue culture is more effective (George *et al.,* 2007).

Coleus forskohlii: Botanical and Medicinal Significance

Coleus forskohlii Briq. (syn. *Plectranthus barbatus* Andrews) It is an important plant in Indian ayurvedic medicine (Hp & Fh, 1982; Kavitha *et al.,,* 2010). It produces the labdane diterpenoid forskolin in its tuberous roots (Bhat *et al.,,* 1977; Shah *et al.,,* 1980). *In vitro* studies in animal systems suggested that forskolin directly stimulates the catalytic subunit of adenylate cyclase, producing positive inotropic effects and lowering blood and intraocular pressure (Ammon & Müller, 1985; Seamon & Daly, 1981). In addition, it has been shown to have anti-inflammatory properties, and it is a good remedy for fever, burning sensation, hypertension, diabetes, cardiac debility, and allergic problems (Alasbahi & Melzig, 2010; Dubey *et al.,,* 1981). Standard *Coleus* extracts containing forskolin (17% in 50mg) are used in weight-loss programs (Godard *et al.,,* 2005). *C. forskohlii* is the only known source for this compound (Pateraki *et al.,* 2014; Rupp *et al.,* 1986).

C. forskohlii is traditionally propagated by means of seeds and vegetative cuttings, but it is time-consuming and provides a limited number of propagules (Hegde, 1997). Apparently, due to the unavailability of quality planting materials, commercial plantations of this aromatic plant species have not been attempted. At present, production of forskolin is completely dependent on the commercial collection of the wild and a few cultivated *C. forskohlii* plants in India (Tandon & Rane, 2008). Due to large-scale, indiscriminate collection of wild plants from the forest and insufficient attempts to allow their replenishment or cultivation, *C. forskohlii* is rapidly disappearing and is now listed as one of the plant species in India vulnerable to extinction (Ved & Goraya, 2007). It is necessary to develop conservation methods for this threatened species. *In vitro* propagation methods offer powerful tools for conserving and multiplying plant germplasm (Engelmann, 2011; Sarasan *et al.,,* 2006). Shoot organogenesis from callus cultures can be used as an effective method for the multiplication of medicinal plants (Ikeuchi *et al.,,* 2013; Sugimoto *et al.,,* 2010). In the present study, leaf-derived callus is used for shoot multiplication.

Coleus forskohlii Briq. of the family Lamiaceae yields a valuable secondary metabolite known as forskolin, which is a labdane diterpenoid (Shan & Tian, 2005; Valdés *et al.,,* 1987). *Coleus forskohlii* is the only known source of this compound. Forskolin is used in medicine for the treatment of glaucoma, congestive cardiomyopathy, and asthma (Caprioli & Sears, 1983; Lindner *et al.,,* 1978).

MATERIALS AND METHODS

Plant Collection

The plants were procured from the Department of Medicinal and Aromatic Plants, College of Horticulture, Tamil Nadu Agricultural University, Coimbatore, and Anamalai Hills, Madurai, and maintained in the Union Christian College Botanical Garden.

Systematic Position
Class: Dicotyledons
Subclass: Gamopetalae
Series: Bicarpellatae

Order: Lamiales
Family: Lamiaceae
Genus: *Coleus*
Species: *forskohlii*
Common Names
English: Coleus, Chebulic, Myrobalan
Hindi: Patharchur
Malayalam: Kadukka
Sanskrit: Pashan Bhedi, Makandi
Kannada: Makandiberu
Marathi: Maimnul

Botanical Description

Small, profusely branched, succulent, aromatic herb. Stems are greenish and square-shaped. Leaves are glandular and hairy, broadly ovate with dentate margins. Flowers are small and bluish in terminal racemes.

Explant Preparation and Surface Sterilization

Explants collected from the greenhouse are washed thoroughly in running tap water. Then it is treated with a 0.1% solution of mercuric chloride ($HgCl_2$) for 2 minutes. The leaves are then washed 3-4 times in sterile water. Murashige and Skoog medium (Murashige & Skoog, 1962) supplemented with 3% sucrose was used for culturing. The media was solidified with 0.8% agar, and the pH was adjusted to 5.8. Growth hormones are added to the medium before adjusting the pH. The media were autoclaved at 121°C for 15-20 minutes. The explants are then cut into a size of about 0.7 cm length and are inoculated in the media in such a way that the abaxial surface of the leaf touches the media in half of the tubes and vice versa in the other half. Inoculated tubes are maintained in the culture room at 23 ± 2°C under a 14-hour light cycle.

Culture Maintenance and Subculture

Weekly visual observation of culture was made. Greenish-brown calluses were formed from the cut ends of leaf-derived explants. The shoots were subcultured on the same medium that was used in the explant culture. The shootlets formed by organogenesis were cultured on MS medium for root formation.

Hardening and Transplantation

The *in vitro-developed* plantlets are acclimatized to the natural environment by gradually raising the temperature to atmospheric levels. Then, healthy plantlets with 3-4 cm long roots were removed individually from the culture tubes. After carefully washing the roots with sterile water, plantlets were transplanted into plastic pots containing autoclaved cocopeat. Plants were watered every 3 days with half-strength MS salt solution for 2 weeks. After one month, the plants were transferred to the greenhouse.

Methodology for Transformation Studies

A. Micropropagation Protocol

For transformation studies, the plants are to be produced by the micropropagation technique, and the work will be done as per the following steps:

1. Collection and establishment of *C. forskohlii* plants in greenhouse and natural conditions
2. Shoot multiplication and callus from suitable explants
3. Induction of multiple shoots/callus – subculture
4. Inoculation of callus in liquid MS media supplemented with various growth hormones and screening for secondary metabolites
5. The different factors involving growth and multiplication (hormonal, physical, and biological factors) will be studied at the laboratory scale
6. Subculture of healthy shoots to rooting media (½ MS or MS basal media ± IBA)
7. Hardening of tissue culture-raised plants in a greenhouse and transferring to soil and natural conditions

B. Hairy Root Induction

For the induction of hairy roots, the following *A. Rhizogenes* strains were tried: *Agrobacterium rhizogenes* R1000 and *A. rhizogenes* 15834 (Chilton *et al.,*, 1982; White *et al.,*, 1985). The explants were infected with

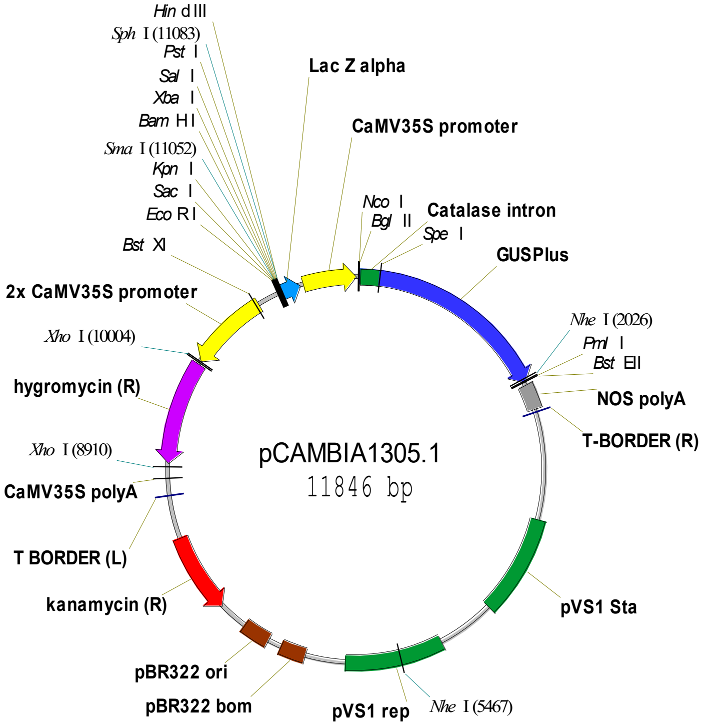
Agrobacterium, and after the appropriate co-cultivation time, they were incubated for hairy root induction, with excess bacteria removed by antibiotic treatment. Leaf explants were first rinsed with 70% Ethanol for 1 min, then sterilized with 0.1% mercuric chloride for 1-2 minutes, and further washed with sterile distilled water 3-4 times. These leaf explants were used for co-cultivation with different *Agrobacterium* strains.

Two types of wild *Agrobacterium* strains were used for the present study. They were R1000 and 15834 of *A. rhizogenes* (Guillon *et al.,,* 2006; Tepfer, 1984). The bacteria were grown in LB media without any antibiotics. Overnight-grown bacterial cultures were used for co-cultivation studies.

The excised leaf explants were kept in MS (Murashige and Skoog, 1962) basal medium for 24 hours as a preconditioning step and to remove any contaminated leaves, if present. The selected explants were dipped in bacterial cultures for 5-10 minutes, then dried on sterile filter paper and transferred to MS basal medium supplemented with 3% sucrose and 0.8% agar at 25°C under dark conditions. After 24 hours of cultivation to remove excess bacteria, the explants were transferred to MS media augmented with 250 ppm cefotaxime, without any growth regulators. After 2-4 days of culture in the antibiotic medium, the root discs were transferred to MS basal medium without any growth hormones for the induction of hairy roots or callus formation. The cultures were kept in dark conditions at 25 ± 2°C.

C. Agrobacterium-Mediated Transformation via *A. tumefaciens*

Streaked a single colony from agar plates to a 5 ml culture with Strep and Kan for 2 days, then 1 ml to a 50 ml culture from the above with Strep (25 mg/L) and Kan (50 mg/L) for overnight. 200 µM Acetosyringone (AS) was added to the media and incubated in a shaker for 4 hours before the experiment. Centrifuged the bacteria and resuspended them in 50 mL MS basal media. The leaf explants were dipped in the above 10 ml culture for 30 minutes. Later dried in sterile filter paper and 3 days in co-cultivation media (MS + AS-400 µM) in complete darkness. Later washed with sterile water 2-3 times, depending on the overgrowth, and with Cefotaxime (400 mg/L). Plated in Selection media first without any selection pressure, but only with Cefotaxime (250 mg/L) to kill the bacteria, and after two weeks in a lower concentration (2 mg/L) Hygromycin + 250 mg/L Cefotaxime. Subculture every week, depending on the Agro growth, and increase the Hygromycin (to 5 mg/L) concentration after two weeks.



pCAMBIA1305.1 Vector Map

Figure 1. Schematic representation of pCAMBIA1305.1 vector used for transformation studies.

Construct Used

pCAMBIA1305.1 (GenBank acc nr AF354045). This vector is based on pCAMBIA1300, in which the *E. coli gusA* gene has been replaced with GUSPlus. It is a compact binary vector with a pBR322 ori for high-copy replication in *E. coli* and a broad-host-range ori for low-copy, stable replication in Agrobacterium. It contains the hygromycin resistance gene (*hpt II*) for plant selection. The GUSPlus gene contains the intron from the castor bean catalase gene to ensure detection of plant-specific glucuronidase expression (Jefferson *et al.,,* 1987)

OBSERVATIONS

Morphogenic callus was induced from young leaves on MS medium augmented with NAA and BAP (Plate-1) (Gaspar *et al.,* 1996; George *et al.,* 2007). These calli, when subcultured on MS medium containing BAP alone, produced shoots (Plate 2) (George *et al.,,* 2008; Skoog & Miller, 1957). The regenerated shoot developed a good root system on half-strength MS medium without any auxins (Klerk *et al.,,* 1997). The fully grown plantlets were transferred to soil for acclimatization (Plate 2). This work is over, and the plants are in the greenhouse for further studies.

Table 1. Media Combinations Tried for Callus Induction from Leaf Explants of *C. forskohlii*

Media Combination	Response
MS + 0.5 mg/L NAA + 0.5 mg/L BAP	+
MS + 1.0 mg/L NAA + 0.5 mg/L BAP	+
MS + 1.5 mg/L NAA + 0.5 mg/L BAP	+
MS + 2.0 mg/L NAA + 0.5 mg/L BAP	+
MS + 0.5 mg/L NAA + 1.0 mg/L BAP	+++
MS + 1.0 mg/L NAA + 1.0 mg/L BAP	+++
MS + 1.5 mg/L NAA + 1.0 mg/L BAP	+++
MS + 2.0 mg/L NAA + 1.0 mg/L BAP	++++
MS + 0.5 mg/L NAA + 1.5 mg/L BAP	++
MS + 1.0 mg/L NAA + 1.5 mg/L BAP	++
MS + 1.5 mg/L NAA + 1.5 mg/L BAP	++
MS + 2.0 mg/L NAA + 1.5 mg/L BAP	++

Table 2. Media Combination Tried for Shoot Induction from Leaf Derived Calli of *C. forskohlii*

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

Disease Resistance Studies

In continuation, the *Coleus* plant is mainly infected by the fungus *Lasiodiplodia theobromae,* which causes root rot (Anandaraj & Sarma, 1995). Developing resistance to fungal diseases in plants remains a major challenge for breeders. An alternative strategy is to introduce genes encoding antifungal enzymes into the host plant genome (Collinge *et al.,* 2010; Punja, 2001). I had used *Agrobacterium tumefaciens* LBA4404 for cocultivation. pCAMBIA 1305.1 vector was introduced in both *Agrobacterium* and cocultivated.
Streaked a single colony from agar plates to a 5 ml culture with Strep and Kan for 2 days, then 1 ml to a 50 ml culture from the above with Strep (25 mg/L) and Kan (50 mg/L) for overnight. 200 µM Acetosyringone (AS) was added to the media and incubated in a shaker for 4 hours before the experiment. Centrifuged the bacteria and resuspended them in 50 mL MS basal media. The leaf explants were dipped in the above 10 ml culture for 30 minutes. Later dried in sterile filter paper and 3 days in co-cultivation media (MS + AS-400 µM) in complete darkness. Later washed with sterile water 2-3 times, depending on the overgrowth, and with Cefotaxime (400 mg/L). Plated in Selection media first without any selection pressure, but only with Cefotaxime (250 mg/L) to kill the bacteria, and after two weeks in a lower concentration (2 mg/L) Hygromycin + 250 mg/L Cefotaxime. Subculture every week depending on the Agro growth, and increase the Hygromycin (to 5 mg/L) concentration after two weeks. However, after 2 weeks, no growth was observed; the explants remained green but could not regenerate in the selection media.

Hairy Root Induction

I have used strains of *Agrobacterium rhizogenes, viz.,* R1000 and 15834 (Georgiev *et al.,,* 2012; Moore *et al.,* 1979). Streaked a single colony from agar plates to a 5 ml culture for 2 days, then 1 ml to a 50 ml culture from the above for overnight. 200 µM Acetosyringone (AS) was added to the media and incubated in a shaker for 4 hours before the experiment. Centrifuged the bacteria and resuspended them in 50 mL MS basal media. The leaf explants were dipped in the above 10 ml culture for 30 minutes. Later dried in sterile filter paper and 3 days in co-cultivation media (MS + AS-400 µM) in complete darkness. Later washed with sterile water 2-3 times, depending on the overgrowth, and with Cefotaxime (400 mg/L). Plated in Selection media only with Cefotaxime (250 mg/L) to kill the bacteria, and after two weeks in a lower concentration of 250 mg/L Cefotaxime. Subculture every week, or depending on the Agro growth. The explants remained green for 1-2 weeks but could not induce any roots.

Table 3. Media Combinations Tried for Rhizogenesis in *C. forskohlii*

Media Combinations	Response
MS half-strength	+++
MS full-strength	++
MS + 0.1 mg/L NAA	+
MS + 0.2 mg/L NAA	+
MS + 0.4 mg/L NAA	+
MS + 0.5 mg/L NAA	Callus induction from base of the shoots
MS + 1.0 mg/L NAA	Callus induction from base of the shoots
MS + 0.2 mg/L IAA	-
MS + 0.5 mg/L IAA	-
MS + 1.0 mg/L IAA	-
MS + 0.2 mg/L IBA	-
MS + 0.5 mg/L IBA	-
MS + 1.0 mg/L IBA	-

RESULTS

Leaf segments were cultured on MS medium supplemented with 0.2, 0.5, 1 mg/L of IBA, NAA, BA & KN in various combinations. Leaf segments cultured on MS medium supplemented with these growth hormones at various concentrations and combinations exhibit callus growth. MS medium supplemented with BA and IBA shows better callus growth (table). In which 0.5 mg/L BA, 0.5 mg/L IBA, and 0.2 mg/L BA & 0.5 mg/L IBA show moment growth of callus.

After 3 weeks of inoculation, some culture tubes of the above-mentioned medium (combination) show leaf-like growth from the cut, and after one week, a number of shoots directly from the cut end of the leaf segment. The MS medium containing 0.5 mg/L BA & IBA shows prominent shoot organogenesis. There are 4-5 shoots arising from the culture, each 0.5 cm long.

After 4 weeks of inoculation, the shoot organogenesis culture shows root formation in the same medium. There are 5-7 roots that arise in the culture, each measuring 3-3.5 cm. The shootlets formed from leaf-derived explants by direct organogenesis were subcultured on MS medium containing the same combination (0.5 mg/L BA & 0.5 mg/L IBA and 0.2 mg/L IBA & 0.2 mg/L IBA). After 3 weeks of subculture, the medium containing 0.5 mg/L BA & 0.5 mg/L IBA supports vigorous shootlet growth compared to the other medium. A number of shoots rise from the medium containing 0.5 mg/L BA & 0.5 mg/L IBA and nearly 9-12 roots. The medium containing 0.2 mg/L BA & 0.5 mg/L IBA shows 5-8 shootlets and 7-8 rootlets.

After 4 weeks of subculturing, the shootlets were separated and cultured on ½ MS medium alone, without growth hormones, to improve rooting. Plants are acclimatized to the environment within 20 days of transplantation. The plant shows about 55-60% survival capacity.

The leaf segments cultured on MS medium supplemented with 0.2, 0.5 & 1 mg/L of IBA, NAA, IAA (auxins) & BA, KN (cytokinins) were used in different combinations. The combination of MS with BA and IBA shows better callusing within 10 days of incubation. An earlier study of some medicinal plants shows BA has a great effect on shoot regeneration (Mok & Mok, 2001; Werner *et al.,* 2001). The culture shows prominent callusing. This is in accordance with the previous observation that an increase in BA concentration to 1 mg/L resulted in a significant increase in shoot growth. Cytokinin promotes *in vitro by* modifying apical dominance, thereby promoting axillary shoot formation at high concentrations (Wickson & Thimann, 1958). Cytokinins inhibit root formation and induce adventitious shoot formation. They usually promote cell division, especially when combined with auxin (Leyser, 2017; Zhao, 2010).

MS medium with 0.5 mg/L BA and 0.5 mg/L IBA shows leaf-like organs from the cut of the leaf after 3 weeks of inoculation. After 1 week, they grow to form a number of shootlets. This organogenesis appears more prominent with equal amounts of BA and IBA. Organogenesis is a process involving redifferentiation of meristematic cells present in the callus into shoot buds (Christianson & Warnick, 1983). The stimulation of shoot bud differentiation in plants depends on many factors, which differ for different plant species. In general, it is promoted by cytokinin and inhibited by auxins (Thorpe, 1980). The classical studies of Skoog and Miller (1957) demonstrated the nature of organogenesis in tobacco pith tissue (Skoog & Miller, 1957). Other factors affecting organogenesis include size, explant source, and plant genotype. The larger the explants (containing parenchyma, cambium & vascular tissue), the more likely of shoot bud formation (Ikeuchi *et al.,* 2016).

The medium is supplemented with auxin IBA, along with cytokinin BA. Auxins are generally required for the induction of callus from explants (Woodward, 2005). Applied auxins seem capable of fundamentally altering the genetically programmed physiology of whole plant tissues that have previously determined their differentiated state. Cells that respond to auxin revert to a dedifferentiated state and begin to divide (Sugiyama, 1999). It is found that auxins cause DNA to become more methylated than usual, suggesting that this might be necessary for the reprogramming of differentiated cells (Loschiavo *et al.,* 1989). The same medium shows root formation after 4 weeks of inoculation. The induction of rhizogenesis usually requires adjustments in auxin and cytokinin levels. Rhizogenesis is usually achieved by auxin treatment alone (Klerk *et al.,* 1999). Also, the development of lateral roots is stimulated by auxin, and IBA has been shown to be more effective than NAA (Hartmann *et al.,* 2010). Exogenous cytokinins are commonly inhibitory. Auxin-induced root formation is thought to require, or induce, the promotion of polyamine synthesis (Biondi *et al.,,* 1990).

The shootlets were subcultured on the same medium containing BA (0.5 mg/L) and IBA (0.5 mg/L). The culture shows vigorous growth after 3 weeks. The culture also shows prominent roots. A previous study showed that increasing BA concentration to 1 mg/L significantly increased the shoot number (Ramage & Williams, 2002). It has also been reported that auxin is required for root induction in *Coleus forskohlii* (Chandel & Sharma, 1997).

Transformation Studies

Earlier reports say that hairy root could be induced in *C. forskohlii* (Constabel, 1990; Sevón & Oksman-Caldentey, 2002). Here, no roots could be induced. This may be due to various factors. A similar result was observed with *A. tumefaciens*. Further work is needed for better results. An attempt has been made to transform the plants for hairy root induction with *Agrobacterium rhizogenes* R1000 and 15834, or with a disease resistance gene, since the plant roots are often infected by Fungi. *Agrobacterium tumefaciens* LBA4404 harboring pCAMBIA 1305.1 was used for transformation; however, technical issues prevented this from being achieved. Further research is needed for successful results; hence, work is in progress in the laboratory.

DISCUSSION

Micropropagation Protocol Development

The present study successfully established a micropropagation protocol for *Coleus forskohlii* using leaf explants. The optimal medium for callus induction was MS + 2.0 mg/L NAA + 1.0 mg/L BAP, while shoot regeneration was most efficient on MS + 1.0 mg/L BAP. These results are consistent with previous reports on *Coleus* tissue culture (Gaspar *et al.,* 1996; E. George *et al.,,* 2008; E. F. George *et al.,,* 2007; Ikeuchi *et al.,* 2013; Klerk *et al.,* 1997; Skoog & Miller, 1957; Sugimoto *et al.,,* 2010)the current protocol appears modest when compared with published benchmarks. Reddy *et al.,* (2001) reported >150 shoots per callus using kinetin-based protocols (Reddy *et al.,,* 2001), while Sreedevi *et al.,* (2013) achieved remarkable efficiency with >2000 shoots per callus using a two-stage protocol (B5 medium for callus induction followed by MS for shoot multiplication) (Sreedevi *et al.,,* 2013). Sahai and Shahzad (2010) demonstrated direct organogenesis producing 35 shoots per explant (Sahai & Shahzad, 2010). The current study reports “4-5 shoots” and “9-12 shoots” under different conditions, which are considerably lower than these benchmarks.

The finding that BAP was effective for shoot induction while kinetin was ineffective is interesting. This contrasts with Reddy *et al.,* (2001), who found kinetin superior to BAP (Reddy *et al.,,* 2001). Such differences may reflect genotype-specific responses, as *C. forskohlii* exhibits considerable genetic diversity across different geographical regions (Lukhoba *et al.,* 2005). The source of plant material (Tamil Nadu Agricultural University and Anamalai Hills) may represent different chemotypes or genotypes with distinct tissue culture responses.

Direct organogenesis was observed with MS + 0.5 mg/L BA + 0.5 mg/L IBA, which is valuable as it bypasses the callus phase and reduces the risk of somaclonal variation (Larkin & Scowcroft, 1981). This finding aligns with Sahai and Shahzad (2010), who also reported successful direct organogenesis (Sahai & Shahzad, 2010). However, the efficiency comparison is difficult due to the lack of quantitative data in the current study.

Rooting and Acclimatization

The finding that half-strength MS medium without auxins was optimal for rooting is consistent with many tissue culture studies (Bhojwani & Dantu, 2013; Pierik, 1987). Interestingly, IBA was completely ineffective in this study, which is unusual as IBA is typically the most effective auxin for rooting (Epstein & Ludwig-Müller, 1993). This unexpected result deserves further investigation - possibilities include degradation of IBA stock solution, genotype-specific responses, or interaction effects with residual cytokinins from the shoot multiplication stage.

The acclimatization success rate of 55-60% is modest compared to literature reports, which typically achieve 80-100% survival (Reddy *et al.,* 2001; Sahai & Shahzad, 2010; Sreedevi *et al.,* 2013)ggests that the acclimatization protocol could be optimized. Factors to consider include: (1) gradual humidity reduction using humidity chambers or plastic covers, (2) gradual light intensity increase, (3) appropriate substrate selection (cocopeat is good, but mixtures with perlite or vermiculite may improve drainage), (4) fungicide treatment to prevent damping-off, and (5) mycorrhizal inoculation to enhance establishment (Hazarika, 2006; Pospíšilová *et al.,,* 1999)

Transformation Attempts: Analysis of Failure

Both *Agrobacterium rhizogenes* and *A. tumefaciens* transformation attempts were unsuccessful in this study. This is particularly puzzling given that successful transformation protocols for *C. forskohlii* have been published using similar strains.

Hairy root induction: Krombholz *et al.,* (1992) successfully induced hairy roots using *A. rhizogenes* strain 15834, producing 4-5 mg/L forskolin in shake flasks and ~14 mg/L in 20-L bioreactors (Krombholz *et al.,,* 1992). Sasaki *et al.,* (1998) achieved high forskolin production (13-16 mg/L) in hairy root cultures using *A. rhizogenes* MAFF 03-01724 (Sasaki *et al.,* 1998). More recently, Pandey *et al.,* (2014) successfully established hairy roots using *A. rhizogenes* MTCC 2364 with PCR confirmation of *rolA* gene integration (Rajiv *et al.,,* 2014). provided comprehensive protocols for hairy root induction and metabolite analysis.

Possible reasons for transformation failure:

Bacterial viability and virulence: The *Agrobacterium* strains may have lost virulence through repeated subculturing or improper storage. Verification of virulence using positive control plants (e.g., tobacco) would be valuable (Gelvin, 2003). Explant competence: Leaf explants may not have been in the optimal physiological state for transformation. Younger, actively dividing tissues are generally more competent (Villemont *et al.,,* 1997). Pre-culture on a callus-inducing medium for 2-3 days before infection can enhance competence (Sangwan *et al.,* 1992).

Co-cultivation conditions: The protocol used 24 hours of co-cultivation, which may be insufficient. Successful protocols typically use 2-3 days of co-cultivation (Krombholz *et al.,,* 1992; Rajiv *et al.,,* 2014; Sasaki *et al.,,* 1998), acetosyringone concentration (200 µM during bacterial growth, 400 µM during co-cultivation) may need optimization - some studies use 100-200 µM throughout (Stachel *et al.,,* 1985).

Antibiotic selection: Cefotaxime at 250-400 mg/L may be too harsh, killing both bacteria and plant cells. Some protocols use lower concentrations (100-250 mg/L) or alternative antibiotics like carbenicillin (Cheng *et al.,,* 1997). For hygromycin selection with *A. tumefaciens*, the initial concentration (2 mg/L) may be too low to provide selection pressure, while 5 mg/L may be too high, killing non-transformed cells before they can regenerate (Miki & McHugh, 2004).

Lack of molecular confirmation: No PCR analysis for *rol* genes (*rolA*, *rolB*, *rolC*) or GUS assay was performed. These molecular tools are essential for: (a) confirming transient transformation during protocol optimization, (b) identifying transformed tissues, and (c) troubleshooting the protocol (Hamill *et al.,,* 1987; Vain *et al.,,* 1993).

Genotype-specific recalcitrance: Some *Coleus* genotypes may be recalcitrant to transformation. Testing multiple explant types (leaf, stem, petiole, root) and different wounding methods (scalpel, needle, sonication) could improve success (Birch, 1997).

Critical Omission: Forskolin Quantification

The most significant limitation of this study is the complete absence of forskolin quantification. *Coleus forskohlii* is valued exclusively for forskolin production, and tissue culture can significantly affect secondary metabolite content (Rao & Ravishankar, 2002; Verpoorte *et al.,,* 2002). All comparable studies in the literature include HPLC analysis of forskolin:

Krombholz *et al.,* (1992): 4-14 mg/L in hairy root cultures (Krombholz *et al.,,* 1992)
Sasaki *et al.,* (1998): 13-16 mg/L in hairy roots (Sasaki *et al.,,* 1998)
Mukherjee *et al.,* (2000): 2.3 mg/g DW with casein hydrolysate elicitation (Mukherjee *et al.,,* 2000)
Tripathi *et al.,* (1995): 0.075% of dry cells in callus cultures (Tripathi *et al.,,* 1995)
Sen *et al.,* (1992): quantitative forskolin in suspension cultures (Sen *et al.,,* 1992)

Balasubramanya *et al.,* (2012): forskolin analysis in morphogenic cultures (Balasubramanya *et al.,,* 2011)
Genetic Stability Considerations

The most significant limitation of this study is the complete absence of forskolin quantification. *Coleus forskohlii* is valued exclusively for forskolin production, and tissue culture can significantly affect secondary metabolite content (Rao & Ravishankar, 2002; Verpoorte *et al.,,* 2002). All comparable studies in the literature include HPLC analysis of forskolin:

Krombholz *et al.,* (1992): 4-14 mg/L in hairy root cultures (Krombholz *et al.,,* 1992)
Sasaki *et al.,* (1998): 13-16 mg/L in hairy roots (Sasaki *et al.,,* 1998)
Mukherjee *et al.,* (2000): 2.3 mg/g DW with casein hydrolysate elicitation (Mukherjee *et al.,,* 2000)
Tripathi *et al.,* (1995): 0.075% of dry cells in callus cultures (Tripathi *et al.,,* 1995)
Sen *et al.,* (1992): quantitative forskolin in suspension cultures (Sen *et al.,,* 1992)
Balasubramanya *et al.,* (2012): forskolin analysis in morphogenic cultures (Balasubramanya *et al.,,* 2011)

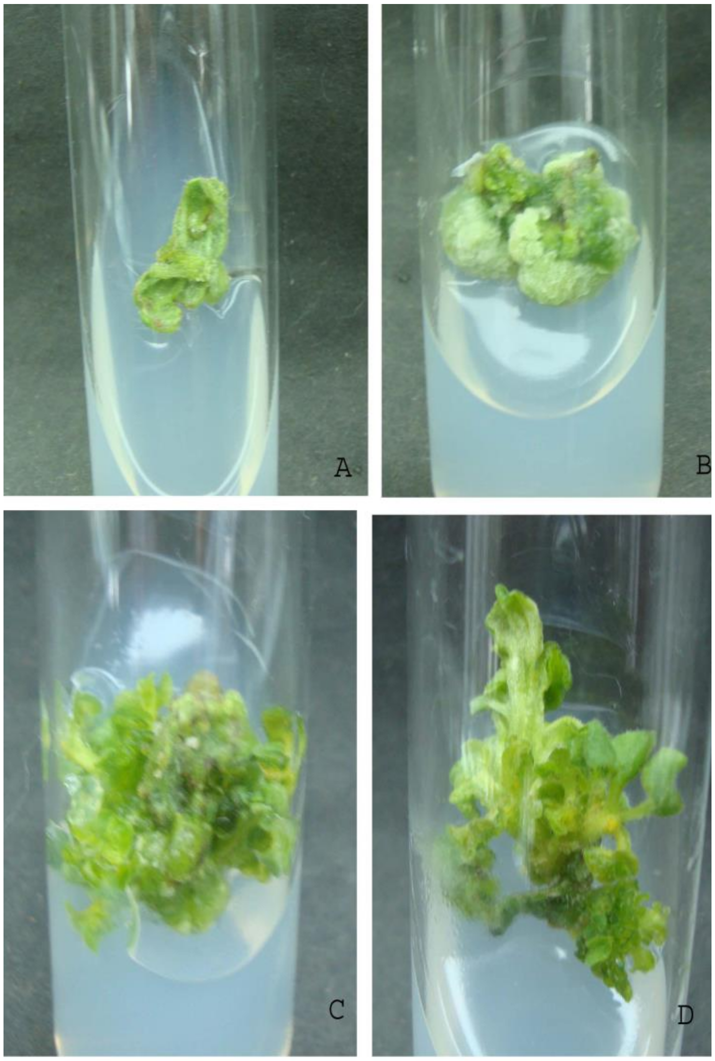


Plate 1. Stages of Micropropagation in *C. forskohlii*
A & B - Callus Initiation
C & D - Shoot initiation

Plate - 2

Plate 2. Stages of Micropropagation in *C. forskohlii*
A & B - In vitro Rooting
C - Acclimatized Plants

Genetic Stability Considerations

Another important consideration not addressed in this study is the genetic stability of regenerated plants. Prolonged callus culture can lead to somaclonal variation, potentially affecting both morphology and secondary metabolite production (Kaeppler *et al.,,* 2000; Miguel & Marum, 2011). Molecular marker analysis (RAPD, ISSR, SSR) and flow cytometry for ploidy assessment should be performed to ensure genetic fidelity (Bairu *et al.,,* 2010; Smýkal *et al.,,* 2007). This is particularly important for medicinal plants where phytochemical consistency is critical.

Comparison with Alternative Approaches

Recent literature has explored alternative approaches for *Coleus forskohlii* propagation and forskolin production:

Somatic embryogenesis: Gopi and Rose Mary (2014) reported 80% embryo induction and 80% plantlet conversion, which may offer advantages over organogenesis for large-scale propagation (GOPI & MARY, 2014).

Bioreactor systems: Krombholz *et al.,* (1992) demonstrated scale-up to 20-L bioreactors for hairy root culture, achieving higher forskolin yields than shake flasks (Krombholz *et al.,,* 1992). This approach is better suited to commercial production than agar-based cultures..

Elicitation strategies: Veeresham *et al.,* (2012) showed that methyl jasmonate (500 µM) + L-phenylalanine (1 mM) significantly enhanced forskolin production (Veeresham *et al.,,* 2012). Mukherjee *et al.,* (2000) demonstrated that casein hydrolysate (2.0 g/L) increased forskolin to 2.3 mg/g DW (Mukherjee *et al.,,* 2000). These elicitation strategies should be tested with the current protocol.

Endophyte co-cultivation: Recent studies have shown that fungal and bacterial endophytes can upregulate forskolin biosynthetic genes (*CfTPS*, *CfCYP76AH15*, *CfACT*) and enhance forskolin production (Bharadwaj, *et al.,,* 2019). This represents a promising avenue for future research

SUMMARY AND CONCLUSION

The present study yields an efficient protocol for large-scale mass propagation of *Coleus forskohlii* via tissue culture. This *in vitro* propagation procedure should be suitable for conservation and large-scale commercial cultivation of the threatened *C. forskohlii* (Fay, 1992; Pence, 2010). The tuberous roots of this plant yield a labdane diterpenoid, forskolin, which is widely used in medicinal preparations (Seamon *et al.,,* 1981; Shan & Tian, 2005). Murashige and Skoog's medium supplemented with different growth hormones was used for the culture studies. The major objective of the study is to develop a more standardized protocol for the production of *C. forskohlii* plants.

Various concentrations of growth hormones, such as BAP, Kinetin, IBA, NAA & IAA, were used in the study. The work aims to identify the optimal combination of growth hormones that improve callus formation, shoot growth, and rooting. MS medium supplemented with 0.5 mg/L BAP and 0.5 mg/L IBA shows shoot organogenesis after 3 weeks of inoculation. Rhizogenesis is also obtained from half-strength MS medium with or without IBA. BAP has a great effect on shoot regeneration, and IBA promotes rooting. An efficient plant regeneration system is essential for further manipulation of this medicinally important plant for beneficial purposes. Hence, these protocols will be enough for an efficient regeneration system in *Coleus forskohlii* plants.

An attempt has been made to transform the plants for hairy root induction with *Agrobacterium rhizogenes* R1000 and 15834, or with a disease resistance gene, since the plant roots are often infected by Fungi. *Agrobacterium tumefaciens* LBA4404 harboring pCAMBIA 1305.1 was used for transformation; however, technical issues prevented this. Further research is needed for successful results; hence, work is in progress in the laboratory.

Limitations and Future Perspectives

While this study establishes basic micropropagation protocols for *Coleus forskohlii*, several important limitations must be acknowledged:

No forskolin quantification: The absence of HPLC analysis of forskolin content is a critical omission that prevents assessment of whether regenerated plants maintain pharmaceutical value (Bourgaud *et al.,,* 2001; Ochoa-Villarreal *et al.,,* 2016).

Lack of statistical rigor: Subjective scoring without quantitative measurements and statistical analysis limits scientific validity and reproducibility (Kozai *et al.,,* 1997).

Modest efficiency: The reported shoot multiplication rates appear lower than published benchmarks, and acclimatization success (55-60%) is below typical standards (Reddy *et al.,,* 2001; Sahai & Shahzad, 2010; Sreedevi *et al,,* 2013)ed transformation: Both *A. rhizogenes* and *A. tumefaciens* transformation attempts were unsuccessful, and insufficient troubleshooting was performed to identify causes (Krombholz *et al.,,* 1992; Rajiv *et al.,,* 2014; Sasaki *et al.,,* 1998)enetic stability assessment: Molecular marker analysis is needed to ensure regenerated plants are genetically stable and true-to-type (Martins *et al.,,* 2004; Rani & Raina, 2000).

Limited scope: Only leaf explants were tested; other explant types may be more efficient. Only BAP and kinetin were tested; other cytokinins (TDZ, zeatin) may be superior (Murthy *et al.,* 1998)

Future work should prioritize quantification of forskolin, statistical validation, optimization of transformation conditions with molecular confirmation, and assessment of genetic stability. With these improvements, the protocols could serve as a valuable foundation for conservation, commercial cultivation, and biotechnological manipulation of this important medicinal plant.

ACKNOWLEDGMENTS

The author acknowledges the Department of Medicinal and Aromatic Plants, College of Horticulture, Tamil Nadu Agricultural University, Coimbatore for providing plant material. Thanks are due to Union Christian College, Aluva for providing laboratory facilities.

REFERENCES

Haberlandt G (1902) Culturversuche mit isolierten Pflanzenzellen. Sitzungsberichte der Kaiserlichen Akademie der Wissenschaften in Wien. Mathematisch-Naturwissenschaftliche Classe 111:69-92

Bhojwani SS, Razdan MK (1996) Plant Tissue Culture: Theory and Practice, a Revised Edition. Elsevier, Amsterdam

Thorpe TA (2007) History of plant tissue culture. Molecular Biotechnology 37:169-180. https://doi.org/10.1007/s12033-007-0031-3

Ramawat KG, Merillon JM (2008) Bioactive Molecules and Medicinal Plants. Springer, Berlin

Karuppusamy S (2009) A review on trends in production of secondary metabolites from higher plants by in vitro tissue, organ and cell cultures. Journal of Medicinal Plants Research 3:1222-1239

Hussain A, Qarshi IA, Nazir H, Ullah I (2012) Plant tissue culture: Current status and opportunities. In: Leva A, Rinaldi LMR (eds) Recent Advances in Plant in vitro Culture. InTech, Rijeka, pp 1-28

George EF, Hall MA, De Klerk GJ (2008) Plant Propagation by Tissue Culture, 3rd Edition, Vol 1. The Background. Springer, Dordrecht, The Netherlands. https://doi.org/10.1007/978-1-4020-5005-3

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15:473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Scowcroft WR (1987) Somaclonal variation: The myth of clonal uniformity. In: Bruening G, Harada J, Kosuge T, Hollaender A (eds) Tailoring Genes for Crop Improvement. Plenum Press, New York, pp 217-245

Ammon HP, Kemper FH (1982) Ayurveda: 3000 years of Indian traditional medicine. Medizinische Welt 33:148-153

Kavitha C, Rajamani K, Vadivel E (2010) *Coleus forskohlii*: A comprehensive review on morphology, phytochemistry and pharmacological aspects. Journal of Medicinal Plants Research 4:278-285. https://doi.org/10.5897/JMPR.9000372

Bhat SV, Bajwa BS, Dornauer H, de Souza NJ, Fehlhaber HW (1977) Structures and stereochemistry of new labdane diterpenoids from Coleus forskohlii Briq. Tetrahedron Letters 18:1669-1672. https://doi.org/10.1016/S0040-4039(01)93575-4

Shah V, Bhat SV, Bajwa BS, Dornauer H, de Souza NJ (1980) The occurrence of forskolin in the Labiatae. Planta Medica 39:183-185. https://doi.org/10.1055/s-2008-1074919

Ammon HP, Muller AB (1985) Forskolin: from an Ayurvedic remedy to a modern agent. Planta Medica 51:473-477. https://doi.org/10.1055/s-2007-969566

Seamon KB, Daly JW (1981) Forskolin: A unique diterpene activator of cyclic AMP-generating systems. Journal of Cyclic Nucleotide Research 7:201-224

Dubey MP, Srimal RC, Nityanand S, Dhawan BN (1981) Pharmacological studies on coleonol, a hypotensive diterpene from Coleus forskohlii. Journal of Ethnopharmacology 3:1-13. https://doi.org/10.1016/0378-8741(81)90010-6

Alasbahi RH, Melzig MF (2010) *Plectranthus barbatus*: A review of phytochemistry, ethnobotanical uses and pharmacology - Part 1. Planta Medica 76:653-661. https://doi.org/10.1055/s-0029-1240898

Godard MP, Johnson BA, Richmond SR (2005) Body composition and hormonal adaptations associated with forskolin consumption in overweight and obese men. Obesity Research 13:1335-1343. https://doi.org/10.1038/oby.2005.162

Rupp RH, de Souza NJ, Dohadwalla AN (1986) Forskolin - Is there a future for this compound? Journal of Ethnopharmacology 16:15-32. https://doi.org/10.1016/0378-8741(86)90065-1

Pateraki I, Andersen-Ranberg J, Hamberger B, Heskes AM, Martens HJ, Zerbe P, Bach SS, Møller BL, Bohlmann J, Hamberger B (2014) Manoyl oxide (13R), the biosynthetic precursor of forskolin, is synthesized in specialized root cork cells in Coleus forskohlii. Plant Physiology 164:1222-1236. https://doi.org/10.1104/pp.113.228429

Hegde S (1997) Vegetative propagation of *Coleus forskohlii* Briq. through stem cuttings. Journal of Medicinal and Aromatic Plant Sciences 19:1043-1045

Tandon S, Rane S (2008) Depletion of forskolin in *Coleus forskohlii* in India and strategies to overcome it. In: Ramawat KG, Merillon JM (eds) Bioactive Molecules and Medicinal Plants. Springer, Berlin, pp 247-257

Ved DK, Goraya GS (2007) Demand and Supply of Medicinal Plants in India. NMPB, New Delhi & FRLHT, Bangalore, India

Sarasan V, Cripps R, Ramsay MM, Atherton C, McMichen M, Prendergast G, Rowntree JK (2006) Conservation in vitro of threatened plants - Progress in the past decade. In Vitro Cellular & Developmental Biology - Plant 42:206-214. https://doi.org/10.1079/IVP2006769

Engelmann F (2011) Use of biotechnologies for the conservation of plant biodiversity. In Vitro Cellular & Developmental Biology - Plant 47:5-16. https://doi.org/10.1007/s11627-010-9327-2

Ikeuchi M, Sugimoto K, Iwase A (2013) Plant callus: Mechanisms of induction and repression. The Plant Cell 25:3159-3173. https://doi.org/10.1105/tpc.113.116053

Sugimoto K, Jiao Y, Meyerowitz EM (2010) Arabidopsis regeneration from multiple tissues occurs via a root development pathway. Developmental Cell 18:463-471. https://doi.org/10.1016/j.devcel.2010.02.004

Valdes LJ, Mislankar SG, Paul AG (1987) Coleus barbatus (*C. forskohlii*) (Lamiaceae) and the potential new drug forskolin (Coleonol). Economic Botany 41:474-483. https://doi.org/10.1007/BF02908139

Shan JJ, Tian GY (2005) Studies on chemical constituents in root of Coleus forskohlii. Zhongguo Zhong Yao Za Zhi 30:1753-1755

Caprioli J, Sears M (1983) Forskolin lowers intraocular pressure in rabbits, monkeys, and man. Lancet 1:958-960. https://doi.org/10.1016/S0140-6736(83)92195-1

Lindner E, Dohadwalla AN, Bhattacharya BK (1978) Positive inotropic and blood pressure lowering activity of a diterpene derivative isolated from Coleus forskohlii: Forskolin. Arzneimittel-Forschung 28:284-289

Chilton MD, Tepfer DA, Petit A, David C, Casse-Delbart F, Tempe J (1982) Agrobacterium rhizogenes inserts T-DNA into the genomes of the host plant root cells. Nature 295:432-434. https://doi.org/10.1038/295432a0

White FF, Taylor BH, Huffman GA, Gordon MP, Nester EW (1985) Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of Agrobacterium rhizogenes. *Journal of Bacteriology* 164:33-44

Tepfer D (1984) Transformation of several species of higher plants by Agrobacterium rhizogenes: Sexual transmission of the transformed genotype and phenotype. Cell 37:959-967. https://doi.org/10.1016/0092-8674(84)90430-6

Guillon S, Trémouillaux-Guiller J, Pati PK, Rideau M, Gantet P (2006) Hairy root research: Recent scenario and exciting prospects. Current Opinion in Plant Biology 9:341-346. https://doi.org/10.1016/j.pbi.2006.03.008

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO Journal 6:3901-3907

Gaspar T, Kevers C, Penel C, Greppin H, Reid DM, Thorpe TA (1996) Plant hormones and plant growth regulators in plant tissue culture. In Vitro Cellular & Developmental Biology - Plant 32:272-289. https://doi.org/10.1007/BF02822700

George EF, Hall MA, De Klerk GJ (2008) Plant growth regulators I: Introduction; Auxins, their analogues and inhibitors. In: George EF, Hall MA, De Klerk GJ (eds) Plant Propagation by Tissue Culture, 3rd Edition. Springer, Dordrecht, pp 175-204

George EF, Hall MA, De Klerk GJ (2008) Plant growth regulators II: Cytokinins, their analogues and antagonists. In: George EF, Hall MA, De Klerk GJ (eds) *Plant Propagation by Tissue Culture*, 3rd Edition. Springer, Dordrecht, pp 205-226

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symposia of the Society for Experimental Biology* 11:118-130

De Klerk GJ, Ter Brugge J, Marinova S (1997) Effectiveness of indoleacetic acid, indolebutyric acid and naphthaleneacetic acid during adventitious root formation in vitro in Malus 'Jork 9'. Plant Cell, Tissue and Organ Culture 49:39-44. https://doi.org/10.1023/A:1005850222973

Anandaraj M, Sarma YR (1995) Diseases of Coleus forskohlii and their management. Journal of Medicinal and Aromatic Plant Sciences 17:766-770

Punja ZK (2001) Genetic engineering of plants to enhance resistance to fungal pathogens - A review of progress and future prospects. Canadian Journal of Plant Pathology 23:216-235. https://doi.org/10.1080/07060660109506935

Collinge DB, Jørgensen HJL, Lund OS, Lyngkjær MF (2010) Engineering pathogen resistance in crop plants: Current trends and future prospects. Annual Review of Phytopathology 48:269-291. https://doi.org/10.1146/annurev-phyto-073009-114430

Moore L, Warren G, Strobel G (1979) Involvement of a plasmid in the hairy root disease of plants caused by Agrobacterium rhizogenes. Plasmid 2:617-626. https://doi.org/10.1016/0147-619X(79)90059-4

Georgiev MI, Agostini E, Ludwig-Müller J, Xu J (2012) Genetically transformed roots: From plant disease to biotechnological resource. Trends in Biotechnology 30:528-537. https://doi.org/10.1016/j.tibtech.2012.07.001

Mok MC, Mok DWS (2001) Cytokinin metabolism and action. Annual Review of Plant Physiology and Plant Molecular Biology 52:89-118. https://doi.org/10.1146/annurev.arplant.52.1.89

Werner T, Motyka V, Strnad M, Schmülling T (2001) Regulation of plant growth by cytokinin. Proceedings of the National Academy of Sciences USA 98:10487-10492. https://doi.org/10.1073/pnas.171304098

Wickson M, Thimann KV (1958) The antagonism of auxin and kinetin in apical dominance. Physiologia Plantarum 11:62-74. https://doi.org/10.1111/j.1399-3054.1958.tb08426.x

Leyser O (2018) Auxin signaling. Plant Physiology 176:465-479. https://doi.org/10.1104/pp.17.00765

Zhao Y (2010) Auxin biosynthesis and its role in plant development. Annual Review of Plant Biology 61:49-64. https://doi.org/10.1146/annurev-arplant-042809-112308

Christianson ML, Warnick DA (1983) Competence and determination in the process of in vitro shoot organogenesis. Developmental Biology 95:288-293. https://doi.org/10.1016/0012-1606(83)90031-5

Thorpe TA (1980) Organogenesis in vitro: Structural, physiological, and biochemical aspects. *International Review of Cytology Supplement* 11A:71-111

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symposia of the Society for Experimental Biology* 11:118-130

Ikeuchi M, Ogawa Y, Iwase A, Sugimoto K (2016) Plant regeneration: Cellular origins and molecular mechanisms. Development 143:1442-1451. https://doi.org/10.1242/dev.134668

Woodward AW, Bartel B (2005) Auxin: Regulation, action, and interaction. Annals of Botany 95:707-735. https://doi.org/10.1093/aob/mci083

Sugiyama M (1999) Organogenesis in vitro. Current Opinion in Plant Biology 2:61-64. https://doi.org/10.1016/S1369-5266(99)80012-0

LoSchiavo F, Pitto L, Giuliano G, Torti G, Nuti-Ronchi V, Marazziti D, Vergara R, Orselli S, Terzi M (1989) DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs. Theoretical and Applied Genetics 77:325-331. https://doi.org/10.1007/BF00305823

De Klerk GJ, Van der Krieken W, De Jong JC (1999) The formation of adventitious roots: New concepts, new possibilities. In Vitro Cellular & Developmental Biology - Plant 35:189-199. https://doi.org/10.1007/s11627-999-0076-z

Hartmann HT, Kester DE, Davies FT, Geneve RL (2010) Hartmann & Kester's Plant Propagation: Principles and Practices, 8th Edition. Prentice Hall, Upper Saddle River, NJ

Biondi S, Diaz T, Iglesias I, Gamberini G, Bagni N (1990) Polyamines and ethylene in relation to adventitious root formation in Prunus avium shoot cultures. Physiologia Plantarum 78:474-483. https://doi.org/10.1111/j.1399-3054.1990.tb09066.x

Ramage CM, Williams RR (2002) Mineral nutrition and plant morphogenesis. In Vitro Cellular & Developmental Biology - Plant 38:116-124. https://doi.org/10.1079/IVP2001269

Chandel KPS, Chaurasia OP, Paul A (1997) Micropropagation of Coleus forskohlii (Willd.) Briq. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, Vol 39. High-Tech and Micropropagation V. Springer, Berlin, pp 111-122. https://doi.org/10.1007/978-3-662-03354-8_6

Constabel F (1990) Medicinal plant biotechnology. Planta Medica 56:421-425. https://doi.org/10.1055/s-2006-961002

Sevón N, Oksman-Caldentey KM (2002) Agrobacterium rhizogenes-mediated transformation: Root cultures as a source of alkaloids. Planta Medica 68:859-868. https://doi.org/10.1055/s-2002-34924

Reddy PS, Rodrigues R, Rajasekharan R (2001) Shoot organogenesis and mass propagation of *Coleus forskohlii* from leaf derived callus. Plant Cell, Tissue and Organ Culture 66:183-188. https://doi.org/10.1023/A:1010697813852

Sreedevi E, Anuradha M, Pullaiah T (2013) Plant regeneration from leaf-derived callus in Plectranthus barbatus. African Journal of Biotechnology 12:1253-1258. https://doi.org/10.5897/AJB2013.11992

Sahai A, Shahzad A (2010) In vitro clonal propagation of Coleus forskohlii via direct shoot organogenesis from selected leaf explants. Journal of Plant Biochemistry and Biotechnology 19:151-155. https://doi.org/10.1007/BF03263344

Lukhoba CW, Simmonds MSJ, Paton AJ (2006) Plectranthus: A review of ethnobotanical uses. Journal of Ethnopharmacology 103:1-24. https://doi.org/10.1016/j.jep.2005.09.011

Larkin PJ, Scowcroft WR (1981) Somaclonal variation - A novel source of variability from cell cultures for plant improvement. Theoretical and Applied Genetics 60:197-214. https://doi.org/10.1007/BF02342540

Bhojwani SS, Dantu PK (2013) Plant Tissue Culture: An Introductory Text. Springer, New Delhi. https://doi.org/10.1007/978-81-322-1026-9

Pierik RLM (1987) In Vitro Culture of Higher Plants. Martinus Nijhoff Publishers, Dordrecht

Epstein E, Ludwig-Müller J (1993) Indole-3-butyric acid in plants: Occurrence, synthesis, metabolism and transport. Physiologia Plantarum 88:382-389. https://doi.org/10.1111/j.1399-3054.1993.tb05513.x

Hazarika BN (2006) Morpho-physiological disorders in in vitro culture of plants. Scientia Horticulturae 108:105-120. https://doi.org/10.1016/j.scienta.2006.01.038

Pospisilova J, Ticha I, Kadlecek P, Haisel D, Plzakova S (1999) Acclimatization of micropropagated plants to ex vitro conditions. Biologia Plantarum 42:481-497. https://doi.org/10.1023/A:1002688208758

Krombholz R, Mersinger R, Kreis W (1992) Production of forskolin by axenic Coleus forskohlii roots cultivated in shake flasks and 20-L glass jar bioreactors. Planta Medica 58:328-332. https://doi.org/10.1055/s-2006-961478

Sasaki K, Udagawa A, Ishimaru H, Hayashi T, Alfermann AW, Nakanishi F (1998) High forskolin production in hairy roots of Coleus forskohlii. Plant Cell Reports 17:457-459. https://doi.org/10.1007/s002990050425

Pandey R, Krishnasamy V, Kumaravadivel N, Manikanda Boopathi N (2014) Establishment of hairy root culture and production of secondary metabolites in Coleus (Coleus forskohlii Briq.). Journal of Medicinal Plants Research 8:1082-1088. https://doi.org/10.5897/JMPR12.1182

Gelvin SB (2003) Agrobacterium-mediated plant transformation: The biology behind the “gene-jockeying” tool. Microbiology and Molecular Biology Reviews 67:16-37. https://doi.org/10.1128/MMBR.67.1.16-37.2003

Villemont E, Dubois F, Sangwan RS, Vasseur G, Bourgeois Y, Sangwan-Norreel BS (1997) Role of the host cell cycle in the Agrobacterium-mediated genetic transformation of Petunia: Evidence of an S-phase control mechanism for T-DNA transfer. Planta 201:160-172. https://doi.org/10.1007/BF01007702

Sangwan RS, Bourgeois Y, Brown S, Vasseur G, Sangwan-Norreel BS (1992) Characterization of competent cells and early events of Agrobacterium-mediated genetic transformation in Arabidopsis thaliana. Planta 188:439-456. https://doi.org/10.1007/BF00192812

Stachel SE, Messens E, Van Montagu M, Zambryski P (1985) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in Agrobacterium tumefaciens. Nature 318:624-629. https://doi.org/10.1038/318624a0

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y (1997) Genetic transformation of wheat mediated by Agrobacterium tumefaciens. Plant Physiology 115:971-980. https://doi.org/10.1104/pp.115.3.971

Miki B, McHugh S (2004) Selectable marker genes in transgenic plants: Applications, alternatives and biosafety. Journal of Biotechnology 107:193-232. https://doi.org/10.1016/j.jbiotec.2003.10.011

Hamill JD, Parr AJ, Rhodes MJC, Robins RJ, Walton NJ (1987) New routes to plant secondary products. Bio/Technology 5:800-804. https://doi.org/10.1038/nbt0887-800

Vain P, Keen N, Murillo J, Rathus C, Nemes C, Finer JJ (1993) Development of the particle inflow gun. Plant Cell, Tissue and Organ Culture 33:237-246. https://doi.org/10.1007/BF02319007

Birch RG (1997) Plant transformation: Problems and strategies for practical application. Annual Review of Plant Physiology and Plant Molecular Biology 48:297-326. https://doi.org/10.1146/annurev.arplant.48.1.297

Ramachandra Rao S, Ravishankar GA (2002) Plant cell cultures: Chemical factories of secondary metabolites. Biotechnology Advances 20:101-153. https://doi.org/10.1016/S0734-9750(02)00007-1

Verpoorte R, Contin A, Memelink J (2002) Biotechnology for the production of plant secondary metabolites. Phytochemistry Reviews 1:13-25. https://doi.org/10.1023/A:1015871916833

Mukherjee S, Ghosh B, Jha S (2000) Enhanced forskolin production in genetically transformed cultures of Coleus forskohlii by casein hydrolysate. Biotechnology Letters 22:1071-1075. https://doi.org/10.1023/A:1005618307074

Tripathi CKM, Basu SK, Jain S, Upreti RK (1995) Production of coleonol (forskolin) by root callus cells of plant Coleus forskohlii. Biotechnology Letters 17:1287-1290. https://doi.org/10.1007/BF00130801

Sen J, Sharma A, Sahu NP, Mahato SB (1992) Production of forskolin in in vitro cultures of Coleus forskohlii. Planta Medica 58:324-327. https://doi.org/10.1055/s-2006-961477

Balasubramanya S, Rajanna L, Anuradha M (2012) Effect of plant growth regulators on morphogenesis and forskolin production in Plectranthus barbatus Andrews. In Vitro Cellular & Developmental Biology - Plant 48:90-95. https://doi.org/10.1007/s11627-011-9417-9

Yanagihara H, Sakata R, Minami H, Tanaka M, Shoyama Y, Murakami H (1996) Immunoaffinity column chromatography against forskolin using an anti-forskolin monoclonal antibody and its application. Analytical Chemistry 68:1459-1464. https://doi.org/10.1021/ac950824+

Patel MB, Mishra SM (2011) Hyphenated techniques for analysis of forskolin: An overview. Journal of Liquid Chromatography & Related Technologies 34:2231-2252. https://doi.org/10.1080/10826076.2011.587224

Kaeppler SM, Kaeppler HF, Rhee Y (2000) Epigenetic aspects of somaclonal variation in plants. Plant Molecular Biology 43:179-188. https://doi.org/10.1023/A:1006423110134

Miguel C, Marum L (2011) An epigenetic view of plant cells cultured in vitro: Somaclonal variation and beyond. Journal of Experimental Botany 62:3713-3725. https://doi.org/10.1093/jxb/err155

Bairu MW, Aremu AO, Van Staden J (2011) Somaclonal variation in plants: Causes and detection methods. Plant Growth Regulation 63:147-173. https://doi.org/10.1007/s10725-010-9554-x

Smýkal P, Valledor L, Rodríguez R, Griga M (2007) Assessment of genetic and epigenetic stability in long-term in vitro shoot culture of pea (Pisum sativum L.). Plant Cell Reports 26:1985-1998. https://doi.org/10.1007/s00299-007-0413-9

Gopi C, Rose Mary MD (2014) In vitro plant regeneration through somatic embryogenesis in medicinally important leaf explants of Coleus forskohlii Briq. IOSR Journal of Agriculture and Veterinary Science 7:1-6. https://doi.org/10.6084/m9.figshare.1226433

Veeresham C, Reddy CS, Praveena Ch (2012) Strategies to improve the production of forskolin from hairy root cultures of Coleus forskohlii Briq. International Journal of Pharmaceutical Sciences and Nanotechnology 5:1745-1752. https://doi.org/10.37285/ijpsn.2012.5.2.7

Pence VC (2011) Evaluating costs for the in vitro propagation and preservation of endangered plants. In Vitro Cellular & Developmental Biology - Plant 47:176-187. https://doi.org/10.1007/s11627-010-9330-7

Seamon KB, Padgett W, Daly JW (1981) Forskolin: Unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proceedings of the National Academy of Sciences USA 78:3363-3367. https://doi.org/10.1073/pnas.78.6.3363

Shan JJ, Tian GY (2005) Studies on chemical constituents in root of Coleus forskohlii. Zhongguo Zhong Yao Za Zhi 30:1753-1755

Ochoa-Villarreal M, Howat S, Hong S, Jang MO, Jin YW, Lee EK, Loake GJ (2016) Plant cell culture strategies for the production of natural products. BMB Reports 49:149-158. https://doi.org/10.5483/BMBRep.2016.49.3.264

Bourgaud F, Gravot A, Milesi S, Gontier E (2001) Production of plant secondary metabolites: A historical perspective. Plant Science 161:839-851. https://doi.org/10.1016/S0168-9452(01)00490-3

Kozai T, Kubota C, Jeong BR (1997) Environmental control for the large-scale production of plants through in vitro techniques. Plant Cell, Tissue and Organ Culture 51:49-56. https://doi.org/10.1023/A:1005809518371

Rani V, Raina SN (2000) Genetic fidelity of organized meristem-derived micropropagated plants: A critical reappraisal. In Vitro Cellular & Developmental Biology - Plant 36:319-330. https://doi.org/10.1007/s11627-000-0059-6

Martins M, Sarmento D, Oliveira MM (2004) Genetic stability of micropropagated almond plantlets, as assessed by RAPD and ISSR markers. Plant Cell Reports 23:492-496. https://doi.org/10.1007/s00299-004-0870-3

Murthy BNS, Murch SJ, Saxena PK (1998) Thidiazuron: A potent regulator of in vitro plant morphogenesis. In Vitro Cellular & Developmental Biology - Plant 34:267-275. https://doi.org/10.1007/BF02822732