J. Plant Biotechnology (2005) Vol. 7 (2), pp. 135~141

Journal of Plant Biotechnology

In vitro Conservation of *Coleus forskohlii*- an Endangered Medicinal Plant

P. E. Rajasekharan[1*], S. R. Ambika[2] and S. Ganeshan[1]

[1]In Vitro Conservation and Cryopreservation Laboratory, Division of Plant Genetic Resources, Indian Institute of Horticultural Research, Hessaraghatta Lake, Bangalore 560 089, India.; [2]Physiology laboratory, Department of Botany, Bangalore University, Jnanabharathi, Bangalore 560 056 India.

Abstract

Protocols for *in vitro* conservation was developed for *Coleus forskohlii*. Plants maintained both in field served as explant source. Shoot tips and single node cuttings were used to optimize protocols for *in vitro* multiplication. MS basal medium supplemented with 0.54 μM naphthalene acetic acid (NAA) and 8.87 μM benzyladenine (BA) induced multiple shoots in shoot tips and nodes. Shoot multiplication was amplified with a gradual decrease of BA concentration, leading to its final omission after 4 months. Concomitant rooting on multiplication media enabled successful establishment *extra vitrum*. For *in vitro* conservation studies, experiments were carried out with 2-3 week maintained in vitro plants under standard and reduced culture conditions (SCC, RCC). *In vitro* plants could be successfully conserved in full strength MS medium (FMS) under SCC for 6 months without subculture with full potential to regenerate, producing viable shoots and nodes. The root production remained unaffected due to conservation, showing high rooting activity in mannitol and low temperature treatments. Preset low temperature (15 and 10℃) and reduction in media constituents does not appear to favour conservation, although the former accomplished conservation levels equal to (FMS) under SCC.

Key words: *Coleus forskohlii*, *in vitro* conservation, endangered species, reduced culture condition, SCC

* Corresponding author, E-mail: pers@iihr.res.in
Received Jan. 8, 2005; Accepted Mar. 10, 2005

Introduction

Medicinal plants are increasingly recognized for their curative as well as healing properties. A spurt in their demand, which hitherto were largely collected from wild and not cultivated has made them most vulnerable in their natural habitats. Several of them are in the red list of IUCN (International Union for the Conservation of Nature) (Anonymous 1999). The fact that this class of species has played a significant role in the food and health security of tribal and rural populations has been over run by profit motives of commercial drug companies.

Coleus forskohlii has been used as an important component of folk medicine in India and has been found to be a potent activator of adenylate cyclase leading to increase of Cyclic AMP (Yangihara et al. 1996). The species is distributed over the tropical and subtropical regions of India, Pakistan, Sri Lanka, tropical regions of East Africa and Brazil, and grows in the Indian subcontinent as a herbaceous, pubescent, weakly aromatic species with annual stems and a perennial root stock. The roots, which are tuberous and fasciculate, are pickled and eaten. Indian ayurvedic preparations use roots, which produce the labdane diterpenoid, forskohlin. This has a positive inotropic effect on heart action, lowers blood and intra-ocular pressure with anti-inflammatory properties. As the commercial exploitation of this species from wild stands has increased in response to the growing demand for forskohlin, this indigenous species of India has become endangered (Vishwakarma et al. 1988; Gupta 1988).

Most investigations on *in vitro* culture of *C. forskohlii* discuss establishment of callus, suspension and root culture for the production of forskohlin and micropropagation.

Limited studies have been carried out on *in vitro* conservation. *In vitro* techniques applied to genetic conservation are now recognized as an ideal back-up to conventional field collections to safeguard against pests, pathogens and natural disasters (Engelmann 1997; Rajasekharan and Ganeshan 2002). *In vitro* conservation of genetic resources has advanced considerably during this decade. It facilitates the availability of planting material at any time, besides avoiding transfer of major pests and pathogens. In case of threatened species that are on decline, *in situ* conservation programs may not give adequate protection (Rajora and Mossier 2000). Tissue culture and cryopreservation provide excellent opportunities for *ex situ* conservation of these species. Low temperature storage of *in vitro* stock material is commonly used for conservation of plant germplasm (Ashmore 1997; Pruski et al. 2000). This method, if properly adjusted to specific genotypes, can substantially reduce labor and media costs.

Published reports cover mostly on micropropagation of *C. forskohlii* (Sharma and Chandel 1995; Suryanarayanan and Pai 1998; Sen and Sharma 1991), but most fail to focus on the importance and use of the technique for *in vitro* conservation. The present investigation was aimed to develop *in vitro* conservation protocols for *C. forskohlii*, which could emerge as a possible approach to conserve genetic diversity of this species.

Materials and Methods

Culture initiation and regeneration of vitroplantlets

Shoot tips and single node explants were used for optimizing *in vitro* regeneration protocols. Explants were derived from the field grown plants of *C. forskohlii* and brought to the laboratory, thoroughly washed in running water and with a mild detergent, 'Teepol' for 10 min to get rid of surface contaminants. Explants were then sterilized under a laminar flow by using 0.01 per cent mercuric chloride for 5 min and washed six times with sterile double distilled water to get rid of mercuric chloride traces retained in the explants. Then they were cut into pieces of 0.5 to 1.0 cm and inoculated on a semisolid culture medium.

Culture medium and conditions of storage

Murashige and Skoog (1962) basal medium was used for shoot and root proliferation. The medium was supplemented with 3% W/V sucrose and gelled with 6.5 % W/V bacteriological grade agar agar, (Hi Media, India). The pH of the medium was adjusted to 5.8 prior to autoclaving. 15 ml of molten medium dispensed into sterile 25 X 150 cm culture tubes. For each treatment, 10 replicates were used and all the experiments were repeated thrice. The cultures were incubated at 25±2℃ with 50 to 55 % RH and a 16 h photoperiod, provided by cool 'Philips' white fluorescent tubes with an intensity of 31.55 $\mu m^{-2}S^{-1}$. Details of Treatments imposed are given in Table 1. Cultures were initially kept at SCC (Standard Culture Conditions) for a contamination free establishment.

Parameters considered determining conservation gain

Conservation gain is defined as the period of conservation of a given species under *ex situ* conditions without any drastic alteration in survival, re-growth and capability to perform normal functions similar to that grown under natural conditions. The same can be measured in terms of : 1) Longer than expected duration of survival under imposed conservation treatments. 2) Sustainable post conservation recovery in terms of propagules (nodes and shoots). 3) Changes observed in the recovery of active ingredients post conservation from the targeted plant part (Root shoot ratio). 4) Reducing subculture frequency and economize on

Table 1. The following treatments were tested for optimizing *in vitro* storage conditions:

Medium	Culture conditions	Observations recorded monthly (small letter)
1.FMS	SCC (L+25℃)	PH, NOS, NOR, INTL, NN
2.HMS	SCC (L+25℃)	
3.QMS	SCC (L+25℃)	
4.FMS+M-1g	SCC (L+25℃)	
5.FMS	RCC (LL+10℃)	
6.FMS	RCC (LL+15℃)	

SCC: Standard Culture Conditions (16 h light, temperature 25±2℃ light intensity: 31.55 $\mu m^{-2}s^{-1}$) PH- plant height in cm; NOS- Number of Shoots; NOR- Number of Roots; INTL-Internodal Length in cm; NN- number of nodes; FMS-Full Strength MS HMS-Half Strength MS; QMS: Quarter Strength MS; LL-Low Light intensity (2.97 $\mu m^{-2}s^{-1}$). FMS+m-1g: full strength MS+ mannitol 1 g/l

material and human resources. 5) parameters to guide as conservation indices were identified for recording observation; to estimate growth reduction and conservation gain among the treatments induced. These parameters were chosen as per IPGRI guidelines (CIAT/ IPGRI 1994), modified to suit the species.

Hardening

Rooted *in vitro* plants were removed from culture tubes and washed, transferred to polybags with 'Soilrite' (a potting medium containing vermiculite), clipped and kept in a hardening chamber with 16 hours light and 8 hours darkness for one month. After this period, they were relocated and kept in glass house with the polybags open for another month and transferred to earthen pots.

Statistical analysis

The data were analyzed using factorial CRD and ANOVA was performed. (Compton 1996).

Results

Regeneration of *in vitro* plants

In vitro regeneration of shoots from nodal and shoot tip explants of *C. forskohlii* without callus formation was successfully developed. Shoot tips were found better than nodal explants for regeneration of *in vitro* plants, which ensured a fast regeneration. Increasing concentrations of auxins and cytokinins induced callusing around the root-shoot interface, at the expense of shoot formation. Root and shoot formation was simultaneous. The rate of multiplication decreased with decreasing hormonal concentrations and *in vitro* plants remained healthy in a basal hormone free medium, required for conservation treatments. Before imposing conservation treatments, cultures were kept at SCC for 2-3 weeks to establish without any contamination and only healthy cultures were selected for imposing conservation treatments. The hardened *in vitro* conserved plantlets have been observed to grow normally *extra vitrum*. 80% survival was recorded when these plants were transferred to earthen pots.

Response of *in vitro* plantlets to storage conditions

Temperature reduction from 25℃ to 15 or 10℃ slowed down growth. Since complete darkness with reduction in temperature was found harmful to the plantlets, low light intensity showed improvement with high survival rates.

Plantlet height

After six months, an average maximum height of 11.84 cm was recorded under FMS+SCC. When the plantlets were cultured and established in HMS+SCC and QMS+SCC, the average maximum height of 12.36 and 12.24 cm was recorded within 3 months after relocation to conservation treatments, showing faster growth compared to that under FMS+SCC (Fig. 1). This trend of fast growth was maintained even when cultures in FMS were relocated to 10 and 15℃ after 2 to 3 weeks of establishment under SCC. At 10℃, and average maximum height of 11.9 cm which was reduced to 10.68 at 15℃. The plantlets conserved in FMS + Mannitol also averaged to a height of 11.9 cm after 6 months.

Number of shoots

Under SCC, this variable averaged to 4.6 under FMS, which were reduced to 3.8 in HMS and 2.4 in QMS within 3 months of relocation, respectively. The average number of shoots formed was 1.4 at 10℃ and 1.2 at 15℃, while mannitol treatments produced an average of 1.4 shoots (Fig. 2). Mannitol in the medium produced etiolated and stunted shoots.

Number of Roots

In FMS+SCC, the number of roots averaged to 15.4 at the end of six months. In HMS+SCC by three months, the average root number was 21. Treatment FMS+SCC +M recorded the maximum number of roots, averaging to 26.2

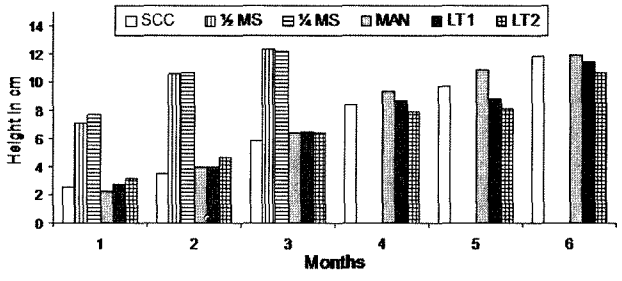

Figure 1. Plant height in *Coleus forskohlii* after different durations of storage (treatments ½ MS and ¼ MS terminated at the end of 3 months. X axis months Y axis plant height in cm. Treatments: 1. FMS, SCC (L+25℃) 2. HMS SCC+(L+25℃) 3. QMS SCC + (L+25℃) 4. FMS SCC M 1G (L+25℃) 5. FMS+RCC (L+10℃) 6. FMS+RCC (L+15℃).

per plantlet. FMS+RCC (10℃) treatment produced an average of 21.8 roots per plantlet. Treatment FMS+RCC (15℃) produced an average of 17.8 roots per plantlet (Fig. 3).

Internodal length

In FMS+SCC, Internodal length averaged to 0.5 cm at the end of six months. In HMS+SCC, average internodal length recorded was 3.04. In QMS+SCC, internodal length averaged to 2.6, while in FMS+SCC+M treatment, it was 0.6. Both low temperature treatments FMS+RCC (10℃ and 15℃) recorded internodal length averaging to Ca. 0.6 (Fig.4).

Number of Nodes

The average number of nodes in FMS+SCC was 7.8 after 6 months. In HMS+ SCC, the number of nodes averaged to 4 at the end of three months. QMS+ SCC treatment the average value for this parameter recorded was 6.6 at the end of third month, while FMS+SCC+M treatment produced an average nodal number of 6.2 nodes per plantlet. FMS+RCC (10℃) treatment produced an average of 4 nodes per plantlet, which was slightly higher in FMS+RCC (15℃)-4.8nodes per plantlet (Fig. 5)

The statistical analyses showed that all treatments were highly significant except in the case of one parameter root (4 month to 6th month).

Discussion

In context to plant regeneration, it has been reported that genetic variability occurs due to somaclonal variation increase with the number of multiplication cycles or with duration in culture (Ashmore 1977). Hence, the multiplication rate was restricted to minimum when *in vitro* conservation treatment is employed for this species. In the present study, the initial multiplication rate was restricted to generate

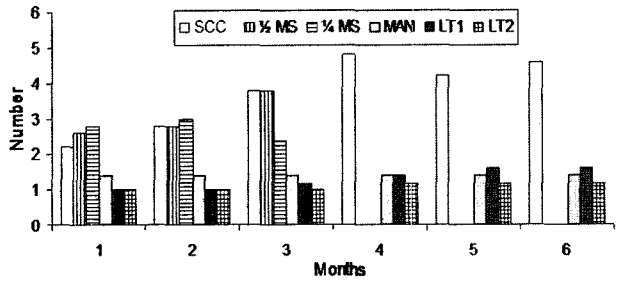

Figure 2. Number of plant in *Coleus forskohlii* after different durations of storage (treatments ½ MS and ¼ MS terminated at the end of 3 months. X axis months Y axis number vitroplant. Treatments: 1. FMS, SCC (L+25℃) 2. HMS SCC+(L+25℃) 3. QMS SCC + (L+25℃) 4. FMS SCC M 1G (L+25℃) 5. FMS+RCC (L+10℃) 6. FMS+RCC (L+15℃).

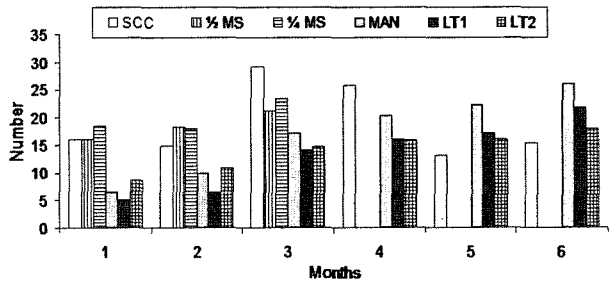

Figure 3. Number of roots in *Coleus forskohlii* after different durations of storage (treatments ½ MS and ¼ MS terminated at the end of 3 months. X axis months Y axis number of nodes. Treatments: 1. FMS, SCC (L+25℃) 2. HMS SCC+(L+25℃) 3. QMS SCC + (L+25℃) 4. FMS SCC M 1G (L+25℃) 5. FMS+RCC (L+10℃) 6. FMS+RCC (L+15℃).

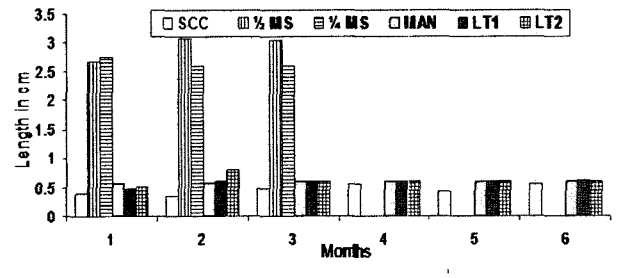

Figure 4. Internodal length in *Coleus forskohlii* after different durations of storage (treatments ½ MS and ¼ MS terminated at the end of 3 months. X axis months Y axis vitroplant internodal length in cm. Treatments: 1. FMS, SCC (L+25℃) 2. HMS SCC+(L+25℃) 3. QMS SCC + (L+25℃) 4. FMS SCC M 1G (L+25℃) 5. FMS+RCC (L+10℃) 6. FMS+RCC (L+15℃).

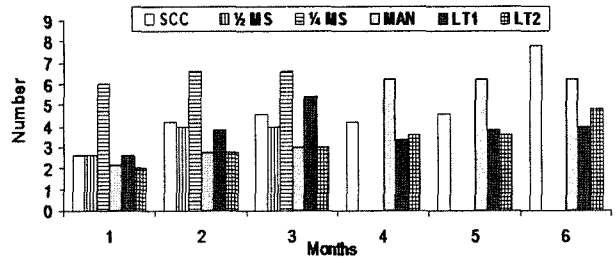

Figure 5. Number of nodes in *Coleus forskohlii* after different durations of storage (treatments ½ MS and ¼ MS terminated at the end of 3 months. X axis months Y axis No of nodes. Treatments: 1. FMS, SCC (L+25℃) 2. HMS SCC+(L+25℃) 3. QMS SCC + (L+25℃) 4. FMS SCC M 1G (L+25℃) 5. FMS+RCC (L+10℃) 6. FMS+RCC (L+15℃).

Table 2. Final conclusions inferred from the treatments and responses obtained for optimizing the most suitable method of in vitro conservation

Treatment (Medium and culture condition)	Plant Height	No of shoots	No of roots	Internodal length	No of Nodes
FMS SCC (L+25℃) 6M	+++	+++	+++	+++	+++
HMS SCC (L+25℃) 3M	*	*	++*	*	*
QMS SCC (L+25℃) 3M	*	*	++*	*	*
FMS 1G SCC (L+25℃) 6M	+	#	++*	++*	++*
FMS RCC (LL+10℃) 6M	#	#	++*	++	+
6.FMS RCC (LL+15℃) 6M	#	#	++*	++	+

+++ : Most recommended method of conservation with maximum conservation gain in terms of good shoot, root and node recovery and optimal internodal length.
* : Recommended for short conservation period
++* : Recommended for conservation with good root Recovery
++ : Moderately recommended for conservation, due to decreasing trend in node recovery
+ : Less priority for conservation due to poor shoot & node quality
# : Not recommended for conservation due to poor shoot quality & redundancy to conserve at low temperature

sufficient plantlets for *in vitro* conservation treatments.

From the results, it is evident that, conservation gain could be perceived in terms of plant height among treatments involving conservation for six months. Conservation under SCC (with full MS) resulted in survival of plantlets for extended periods, up to 6 months. Lowering of temperature and addition of osmoregultators had very little effect on plantlet height, surviving for the entire conservation period. Reduction of media components to Half MS and Quarter MS resulted in increased growth, culminating in plant heights equal to the ones kept under SCC+FMS within 3 months.

There is an increase in shoot number when vitroplants were conserved in FMS+SCC, providing scope for sustainable post conservation shoots recovery (i.e., harvest more shoots for regeneration after 6 months of conservation). Halving the media produced the same number of shoots after 3 months, which could be considered as a resource saving gain, if conservation is to be terminated at 3 months. A reduction trend in shoot number was noticed in treatments involving low temperature and mannitol, wherein extended duration of conservation may not yield optimal number of shoots for regeneration.

Although regeneration of *Coleus forskohlii* from root cultures is not reported, the need to investigate the conservation effect on root number was due to the fact that roots are the targeted part used in medicine. After 6 months, there has been a significant increase in root number in FMS+ SCC+ M treatment compared to other conservation treatments investigated. The results substantiate that the conservation treatments does not reduce root production, but a substantial increase in root number in mannitol treatment could be due to associated stress factors.

Mannitol is reported to reduce apical dominance. Generally, the presence of mannitol in the medium has a reducing effect on the growth and development of cultures (Jarret and Gawel 1991). In most cases a positive effect of this osmoticum on survival and regrowth has been reported at standard culture conditions (Roca et al 1989). The inclusion of mannitol with or without low temperature incubation has been used for prolonging the subculture periods (Mandal et al 2000).

Internodal length is inversely proportional to the number of nodes produced by a vitroplant during conservation. There was a marginal increase in the internodal length among conservation treatments involving 6 months, (lowest value recorded for FMS+ SCC), reducing the post conservation node recovery. In conservation treatments involving 3 months, half and quarter MS treatments increased the inter nodal length 4 to 6 fold, further reducing the post conservation nodal recovery, resulting in a low conservation gain. Conversely, Treatment FMS+ SCC recorded highest average number of nodes maximizing conservation gain, followed by mannitol and low temperature treatments. Half and quarter MS treatments resulted in a

lower average nodal number, compared to FMS+ SCC.

Rooting was simultaneous and occurred in the same medium; there was no need to use separate medium for rooting as reported by Sharma et al. (1991). Consequently, savings in culture medium was found to be an advantage in the present study. Chandel and Sharma 1992; Sharma and Chandel 1992 and Sharma 1992 reported conservation of shoot cultures of *C. forskohlii* for 12 to 20 months at 25℃ without requiring any sub culturing, with no apparent differences observed when explants were regenerated without growth regulators. These studies however, are deficient in terms of a detailed analysis of the parameters that affect conservation, which has been carried out in this study. From the results obtained, one can conclude that maximum conservation gain with extended storage duration of 6 months has been obtained with treatments involving FMS+ SCC, lowering the temperature or reducing media constituents appears less gainful for conservation.

Table 2 details the final conclusions inferred from the treatments and responses obtained for optimizing the most suitable method of *in vitro* conservation for *C. forskohlii*. In most studies, it is anticipated that incubation at temperatures lower than required for optimum growth would decrease the metabolic activity, thereby restricting the growth of plants. Tropical species are conserved in the range of 10 to 22℃ (Chandel et al. 1996). For *C. forskohlii* SCC appears to favor optimal conservation with high recovery of propagules without affecting root production. The method is very simple, easy to use and could be applied to wide range of genotypes. Reduced media concentration induced fast growth within one month, which remains more or less uniform up to a period of three months. Reducing the level of sucrose in the medium can facilitate growth by promoting autotrophy, decreasing expense of materials, and reducing biological contamination (Kozaki 1991). During conservation, photosynthetic and re-growth abilities would have to be maintained, but growth suppressed (Kubota and Kozai 1995).

Acknowledgement

The authors would like to thank Sri. V.R. Srinivasan (Principal Scientist and Former Head Economics and Statistics section) for statistical analysis.

References

Anonymous (1999) The key role of forestry sector in conserving India' s medicinal plants conceptual and operational features. FRLHT, Bangalore

Ashmore SE (1997) Status report on the development and application of *in vitro* techniques for the conservation and use of plant genetic resources. IPGRI, Rome.

Bhat SR, Chandel KPS (1993) *In Vitro* conservation of *Musa* germplasm. Effects of mannitol and temperature on growth and storage. J Horticultural Sci 68 841-846

Chandel KPS and Sharma N (1992) Conservation of diversity in medicinal plants species through *in vitro* technology. Abstr Symposium on Frontiers in Plant Biotechnology, Biotechnology center IARI, New Delhi

Chandel KPS, Shukla G, Sharma N (1996) Biodiversity in medicinal and aromatic plants in India. NBPGR, New Delhi, India

Compton ME (1996). Statistical analysis of plant tissue culture data. In: Trigeano RN, Gray DJ (eds), Plant Tissue Culture Concepts and Laboratory Exercises. CRC Press, Boca Raton

Engelmann F (1997) *In vitro* conservation methods. In: Ford-Lloyd BV, Newbury HJ, Callow JA (eds), Biotechnology and Plant Genetic Resources Conservation and use. CABI, Oxon, UK

Grout B (1995) Genetic Preservation of Plant Cells in vitro. Springer Verlag Laboratory Manual, Springer Verlag, Berlin

Gupta R (1988) Genetic resources of Indian Medicinal Plants J Plant Genet Resources 1:90-102

IPGRI/CIAT(1994) Establishment and operation of pilot *in vitro* active genebank. Report of a IPGRI/CIAT collaborative project using Cassava (*Manihot esculenta* Cranz) as a model IPGRI, Rome

Jarrret RL, Gawell N (1991) Chemical and environmental growth regeneration in sweet potato(*Ipomoea batatus* Lam.) *in vitro*. Plant Cell Tiss Org Cul 25: 153-160

Kozaki T (1991) Micropropagation under photoautotrophic conditions In: Debergh PE, Zimmerman RHZ (eds), Micropropagation Technology and application. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp447-469

Kubota C, Kozai T (1995) Low temperature storage of transplants at the light compensation point Air temperature and light intensity for growth suppression and quality preservation. Scientia Hort 64: 1-9

Mandal BB, Tyagi RK, Pandey R, Sharma N, Agrawal A (2000) Conservation of germplasm of agri-horticultural crops at NBPGR- An over view. In: Razdan MK, Cocking EC (eds) Conservation of PGR *in vitro*. Vol. 2, Application and Limitations. Oxford and IBH Publishing Co, Pvt Ltd, New Delhi

McCowen BH (2000) Recalcitrance of woody and herbaceous perennial plants: Dealing with genetic predeterminism. *In Vitro* Cell Dev Biol-Plant 36: 14

Murashige T, Skoog F(1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol Plant15:473-497

Pruski K, Kozai T, Lewis T, Astatkie T, Nowak J (2000)

Sucrose and light effects on *in vitro* cultures of potato, chokecherry and sakatoon berry during low temperature storage Plant Cell tiss Org Cult 63: 215-221

Rajasekharan PE, Ganeshan S (2002) Conservation of medicinal plant biodiversity - an Indian perspective. J Med Aroma Plant Sci 24: 132-147

Rajora OP, Mossier A (2000) Challenges and opportunities for conservation of forest genetic resources Euphytica 118: 197-21

Roca WM, Chavez R, Martin ML, Arias DI, Mafla G, Reyes R (1989). *In vitro* methods of germplasm conservation. Genome 31:813-817

Sen J, Sharma AK (1991) *In vitro* propagation of *Coleus forskohlii* Briq. For forkohlin synthesis. Plant Cell Rep 9:696-698

Sharma N, Chandel KPS (1995) *In vitro* conservation of threatened plants of Medicinal Importance. Indian Journal of Plant Genetic Resources 8:107-112

Sharma N, Chandel KPS, Srivatava VK (1991). *In vitro* propagation of *Coleus forskohlii* Briq. A threatened medicinal plant Plant Cell Rep 10:67-70

Sharma N, Chandel KPS (1992) Effects of ascorbic acid on axillary shoot induction on Tylophora indica (Burm. f.) Merill. Plant Cell Tiss Org cult 29:109-113

Suryanarayanan M, Pai JS (1998) Studies in micropropagation of *Coleus forskohlii* J Medicinal Aromatic Plant Sci 20:379-382

Vishwakarmakarma RA, Tyagi BR Ahmed B, Hussain A (1988) Variations in forkohlin content in roots of *Coleus forkohlii*. Planta Med 54: 471-472

Wilson SB, Rajapakse NC, Young RE (2000). Media composition and light affect storability and poststorage recovery of micropropagated *Hosta* plants. Hort Sci 35: 1159-1162

Yanagihara H, Sakata R, Shoyanna Y, Murakami H (1995)Relationship between the content of forskohlin and Growth environment in clonally propagated *Coleus forskohlii* Briq. Biotronics 24:1