SUMMARY AND CONCLUSION
6. SUMMARY AND CONCLUSION

*Coleus forskohlii* is a plant species of medicinal importance. This aromatic plant is herbaceous with perennial roots. The roots contain the economic important product forskolin, an alkaloid used in the production of drug for the treatment of glaucoma, congestive cardiomyopathy and asthma. The leaves are used as an expectorant and diuretic. *C. forskohlii* is the best known source for the commercial production of forskolin. However, the quantity of forskolin present in these plants was found to be very low, which makes it difficult to produce an economically viable product. Moreover, the growth rhythm of the plant is comparatively slow and the alkaloid accumulation pattern is influenced by environmental and/or geographical condition.

The low content of commercially important compounds in whole plants, the endangered status of medicinal plants due to their ruthless exploitation, commercially unfeasible chemical synthesis, increased anthropogenic activities, geographical and genotypic variations have resulted in development of alternate biotechnological means to produce these compounds of economic importance. A limited commercial success has been achieved to produce an extensive array of secondary metabolites by plant cell culture technology as alternative strategy. Plant–fungi interaction can be used as an alternative strategy to enhance accumulation of these phytochemicals as most of these secondary compounds are produced due to activation of defense related biosynthetic pathways.

Endophytes are microorganism, which lives inside a plant, in a parasitic or mutualistic relationship with the host. Endophytes including fungi play an important role in the fitness of the host and are present in diverse groups of plants. The endophytic fungi increase the drought resistance and nutrient uptake of the host plants besides many other beneficial roles. The majority of land plants live in mycorrhizal interaction with fungi, a symbiosis which has a healthy impact on ecosystem. The benefits of mycorrhizal associations arise from the nutrient transport between the plant roots and fungal hyphae. The carbon source is transported from the plant to the fungus, whereas fungal hyphae serve as a fine link between the roots and the rhizosphere and improve the supply of the plant with inorganic nutrients. Arbuscular mycorrhizal fungi (AMF) are thus the major model system to study mutualistic plant-fungus symbioses. Being an obligate symbiont commercial
application of AM has not been substantially successful due to the difficulty in production of its reliable mass inoculum.

*Piriformospora indica*, a root endosymbiotic fungus was isolated from the rhizosphere of *Prosopis juliflora* and *Zizyphus nummularia* in the Thar Desert in Rajasthan, India. It was named according to its characteristic pear-shaped chlamydomospores. Depending on the ultra structure of hyphae (presence of dolipore septa) and 18s rDNA sequence, *P. indica* was grouped in the class Hymenomycetes (Basidiomycota). The fungus *P. indica* was shown to possess at least six chromosomes and a genome size of about 15.4–24 Mb. Sequences of the genes encoding the elongation factor 1-a (TEF) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used for genome size estimation through real-time PCR analysis. This fungus mimics AMF in many morphological, functional and growth promotional aspects. Symbiotic fungus *P. indica* colonizes roots and increases the biomass of both monocot and dicot plants and interestingly, the host spectrum of *P. indica* is very much alike AM fungi. Like AM fungi, *P. indica* functions as bioregulator, biofertilizer and bioprotector against root pathogens; overcome the water stress (dehydration), acidity, desiccation and heavy metal toxicity; protection from pests delay the wilting of the leaves, prolong aging of callus tissues. In contrast to arbuscular mycorrhizal fungi, *P. indica* can be easily grown on synthetic media and hence allowing for large-scale propagation and a possible use in plant production.

The present study has been carried out to explore the comparative influence of introduced symbiotic fungus *P. indica* on the host *C. forskohlii* under *in vitro* and *in vivo* conditions. Research has been carried to study the growth, over all development and alteration in compounds of commercial interest of *C. forskohlii*. Existing study provides an easy and efficient protocol for *in vitro* co-culture of *C. forskohlii*, a threatened medicinal plant of commercial importance with symbiotic fungus. Enhanced shoot production and proliferation has been achieved on medium containing 2mg/L Kinetin and 1 mg/L IAA. The regenerated shoots of varied length (0.5 and 1.5 cm) were transferred to root induction medium comprising of MS with and without hormones. Hormones used were IBA, NAA and IAA (1mg/L) to determine the most suitable shoot length for proper root induction and best root induction medium. It was observed from the current research that when shoots of 1.5 cm was used along with rooting medium MS+IBA (1mg/L), resulted in 100% rooting
within 5 days. Highest number of roots with well-developed root system was noted in the aforesaid medium.

_P. indica_ was cultivated on four different synthetic solidified media. Among the tested media for fungus, Hill and Kaefer was found to be the best followed by combination of Murashige and Skoog with Hill and Kaefer medium (MS+HK). Good growth was observed on these media because glucose was used as a carbon source. All the above four media and along with a liquid medium were tested for co-culture studies for the symbiotic fungus and the _in vitro_ C. forskohlii. When _in vitro_ plants were placed in Hill and Kaefer medium, the medicinal plant failed to grow. It may be due to the fact that the Hill and Kaefer medium supports fungal growth and lacks the nutrients needed for the _in vitro_ growth of the medicinal plants. Based on various parameters studied _in vitro_, optimum medium for both the fungus and the _in vitro_ plants when co-cultured was found to be the combination medium of Murashige and Skoog with Hill and Kaefer medium (MS+HK) semisolid medium. In this medium both the fungus and the _in vitro_ plant had most favorable growth unlike other media where either one of the partners grew well. MS is a ‘high salt’ medium due to its contents of K and N in the medium and thus was highly suitable for plant growth where as fungus grows best on Hill and Kaefer medium. Plants growing on liquid MS+HK medium showed the problem of vitrification.

The symbiotic fungus promoted the growth of host C. forskohlii both in _in vitro_ and field conditions. Pattern of influence of _P. indica_ on the target plant under different growth conditions (_in vitro_, green house and field) was found to be same. Plant growth and biomass were strongly influenced by nutrients and environmental conditions when co-cultured with _P. indica_ under _in vitro_, green house and field conditions. Better root system in fungus treated plants helped in enhancement of nutrient uptake, which resulted in healthy plants with more shoot biomass, in all the investigated conditions. It was observed that treated plants had significantly higher photosynthetic rates in terms of leaf area and higher chlorophyll contents. It was observed that in field condition both aerial and underground growth parameters were significantly improved due to intervention of aforesaid fungus till four months of growth of the plants. At six months of growth although number and root length of the treated roots were superior to the control but roots of treated plants became fibrous. It was also noticed that the available phosphorus contents in the leaves were also
enhanced in the treated plants in both *in vitro* and field condition. Percent root colonization was greater in plants inoculated with *P. indica* compared to uninoculated plants although formation of many chlamydospores could not be visualized through microscope but profuse intense inter- and intracellular hyphal colonization was present.

In general, micropropagated plants exhibit high mortality rates upon their transfer to soil. The advantage of any *in vitro* system can be fully realized only by the successful transfer of plantlets from tissue culture vessels to the ambient conditions found *ex vitro*. In the current study treated *in vitro* plants exhibited better percent survivability when transferred to greenhouse condition. The treated plants were more green and healthy compared to the controls. Early endomycorrhizal inoculation of *P. indica* during *in vitro* condition ensured maximal survival and growth of micropropagated plants at acclimatization stage. Thus, *P. indica* acted as a biohardening agent and protected micropropagated young plantlets from ‘transplantation shock’.

Early and more vigorous flowering was observed in fungus treated plants compared to non-treated plants. Not only flowering was profuse, even length and number of inflorescence in the treated plants were more than the non-treated plants. Although the fungus promoted the growth and biomass of the aerial parts of the plants but in the underground parts the roots became fibrous instead of being tuberous. This phenomenon can be described as due to transfer of energy for early and plentiful flowering and higher aerial biomass. Hence, the root biomass and thickness of the non-treated plants were on the higher side compared to treated plants. As a result the forskolin content in the roots of the treated plants were also less than the control plants. But in case of essential oil content in the inflorescence some composition of essential oils were enhanced due to fungus intervention as compared to un-treated plants.

Inflorescences of *C. forskohlii* were subjected to GC-MS analysis. In the analysis a compound named 1, 4- Benzenediol which is a substrate for enzyme laccase (main lignin degrading enzyme in fungus) was detected. The amount of this substrate present in non-treated sample was significantly higher than in treated sample, which indicated the fungal colonization and more degradation of 1, 4- Benzenediol in treated ones. This can be inferred that presence of appreciably elevated laccase activity in treated sample. It was further observed that alongside laccase, action of other enzymes such as poly phenol oxidase, lignin
per oxidase, cellulase, xylanase and protease in root tissues of treated test plants were superior compared to non-treated plants. Cellulolytic enzymes and other fungal specific enzymes play a fundamental role in the penetration of phytopathogenic and mutualistic microorganisms into plant cells. Although root colonization by intense formation of chlamydospores could not be seen through microscope but enzymatic studies revealed that these enzymes can be used as marker for determining extent of root colonization by mycorrhizal fungi in various plants. The fact that more enzymatic activity was detected in colonized plants as compared to non colonized plants. It indicated the possible involvement of aforesaid enzymes in the colonization process.

In the present study, the effect of symbiosis of root endophyte with *C. forskohlii* induced increase in phenol concentration in the plant tissue. Phenolic compounds are widespread in all plants and are known to play a strong role in host defence mechanism against disease. Experimental evidence also suggests that phenolics function as signals in plant development and in plant-microbe interactions. Phenol is byproduct of laccase activity. Hence the study of phenol along with fungal specific enzymatic activities can indicate the extent of root colonization by the root endophyte *P. indica* on *C. forskohlii*.

From the existing study it can be concluded that the root endophyte *P. indica* has an overall beneficial effect on the medicinal plant *C. forskohlii*. In all the investigated experiments conducted in the existing study it was found that *P. indica* mimicked the properties of AM fungi. *In vitro* co-culture study can be developed as a powerful tool to study the nature of symbiosis without any interference from the other microbes. From the *in vitro* studies, it can be concluded that semisolid combination of Murashige and Skoog’s with Hill and Kaefer was found to be optimum medium for *in vitro* co-culturing of plant and fungus. Inoculation of *P. indica* resulted in development of better root system which enhanced the shoot growth along with higher photosynthetic activity that also resulted in early and copious flowering in field condition. There was enhancement of some components of essential oil in the inflorescence of treated plants compared to un-treated plants. It was found that *P. indica* inoculation enhanced the survival and post-transplantation performance of the *in vitro* raised plantlets of *C. forskohlii* and thus helped in sustainable utilization of the medicinal plant.
As the treated roots became fibrous due to transfer of energy to aerial parts, the forskolin content in the roots was however reduced. In order to harness the maximum benefits of the medicinal properties of the host plant still other symbiotic partners besides *P. indica* alone and in combination need to be assessed and optimized.
FUTURE
PROSPECTS
7. FUTURE PROSPECTS

Rhizosphere is the soil region which is subjected to the influence of plant roots and their associated microorganisms. This zone is chiefly the centre for all the mutualistic interactions between plants and microbes. Associations of plants and microbes can be friendly or hostile. Under harsh conditions, mutualistic association can be essential for plant survival. Microbes such as AM fungi associate with medicinal and aromatic plants and increase their productivity also. Coleus forskohlii is an important medicinal plant of commercial importance. Accordingly the future plan of work could be on the following lines:

1. Essential oil of C. forskohlii has very attractive and delicate odor with spicy note. The essential oil has potential uses in food flavoring industry and can be used as an antimicrobial agent also. Extraction of essential oils from the aerial parts of the plants can be attempted after the intervention of the fungus with the target plants and its commercial use can be exploited. P.indica alone or with combination with other symbiotic partners can be utilized to harness the maximum benefits from medicinally important plant.

2. P. indica promotes early and vigorous flowering in fungus treated plants. Inoculation with P. indica can increase productivity and quality of several commercially important ornamental plants.

3. Farmers are growing this medicinal plant because of its economic potential. But the plant is susceptible to many diseases of which root rot and wilt caused by the fungal pathogen Fusarium chlamydosporum and nematode disease by Meloidogyne incognita (root knot nematode) is most common. It is well documented that P.indica is a potential biocontrol agent. Effect of biocontrol agent P. indica can be studied in controlling the above plant disease in C. forskohlii.

4. Histochemical studies have indicated that the nucleus size got constructively enhanced due to interaction with P.indica. The explanation for this phenomenon needs to be explored.

5. Molecular aspects of the interaction process between the host plant and fungus need to be investigated in details. One should also make an attempt to understand the mechanism by which P.indica restricts the growth of pathogenic fungi. Efforts have to be made for mapping of the symbiotic fungus both in the positive and negative directions.
REFERENCES
8. REFERENCES


Barazani, O. and Baldwin, I.T (personal communication). A mixed bag: the plant growth-promoting *Sebacina vermifera* impairs defense mechanisms against herbivores


Camehl, I., Sherameti, I., Venus, Y., Bethke, G., Varma, A., Lee, J. and Oelmüller, R. (2010). Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and non beneficial traits in the symbiosis between the endophytic fungus Piriformospora indica and Arabidopsis thaliana. New Phytologist 185:1062-1073


Genes to Mycorrhiza Application (Gianinazzi, S., Schüepp, H., Barea, J.M. and Haselwandter, K. eds). Birkhauser Verlag, Switzerland, pp 137–149


9. APPENDIX I

Table 1
HILL AND KAEFER MEDIUM (2001)

<table>
<thead>
<tr>
<th>Composition</th>
<th>(g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>20.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>2.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.0</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>1.0</td>
</tr>
<tr>
<td>Vitamin stock solution</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Macro elements from stock</td>
<td>50.0 ml</td>
</tr>
<tr>
<td>Micro elements from stock</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>0.8 % (w/v)</td>
</tr>
<tr>
<td>CaCl$_2$ 0.1 M</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>FeCl$_3$ 0.1 M</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>pH</td>
<td>5.8</td>
</tr>
</tbody>
</table>

**Macro elements**

<table>
<thead>
<tr>
<th>Stock (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO$_3$</td>
</tr>
<tr>
<td>KCl</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
</tr>
</tbody>
</table>

**Minor elements**

<table>
<thead>
<tr>
<th>Stock (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn SO$_4$</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
</tr>
<tr>
<td>MnCl$_2$.4 H$_2$O</td>
</tr>
<tr>
<td>CoCl$_2$.6 H$_2$O</td>
</tr>
<tr>
<td>CuSO$_4$.5 H$_2$O</td>
</tr>
<tr>
<td>(NH$_4$)$_6$Mo$<em>7$O$</em>{24}$.7H$_2$O</td>
</tr>
<tr>
<td>Na$_2$EDTA</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
</tr>
<tr>
<td>Biotin</td>
</tr>
<tr>
<td>Nicotinamide</td>
</tr>
<tr>
<td>Pyridoxal phosphate</td>
</tr>
<tr>
<td>Amino benzoic acid</td>
</tr>
<tr>
<td>Riboflavin</td>
</tr>
</tbody>
</table>

The pH was adjusted to 5.8 with 1N HCl. All stocks were stored at 4°C except the vitamins which were stored at -20°C.
Table 2
MODIFIED MELIN-NORKRANS (Modified MMN)(Johnson et al. 1957)

<table>
<thead>
<tr>
<th>Composition</th>
<th>(g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.025</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.5</td>
</tr>
<tr>
<td>(NH₄)₂HPO₄</td>
<td>0.25</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.05</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.15</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>0.001</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>83µl</td>
</tr>
<tr>
<td>Tryticase peptone</td>
<td>0.1 %( w/v)</td>
</tr>
<tr>
<td>Glucose monohydrate</td>
<td>1.0 %( w/v)</td>
</tr>
<tr>
<td>Malt extracts</td>
<td>0.5 %( w/v)</td>
</tr>
<tr>
<td>Trace elements from stock</td>
<td>10ml</td>
</tr>
</tbody>
</table>

**Trace elements (stock)**

<table>
<thead>
<tr>
<th>Composition</th>
<th>(g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>3.73</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>1.55</td>
</tr>
<tr>
<td>MnSO₄.H₂O</td>
<td>0.85</td>
</tr>
<tr>
<td>ZnSO₄.7H₂O</td>
<td>0.56</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>0.13</td>
</tr>
</tbody>
</table>

pH adjusted to 5.8 with 1N HCl /NaOH. All stocks were stored at 4°C except thiamine hydrochloride which was stored at -20°C.
Table 3

MURASHIGE AND SKOOG (MS) MEDIUM (1962)

<table>
<thead>
<tr>
<th>Composition</th>
<th>(mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macro Elements</strong></td>
<td></td>
</tr>
<tr>
<td>NH$_4$NO$_3$</td>
<td>1650</td>
</tr>
<tr>
<td>KNO$_3$</td>
<td>1900</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>370</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>170</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>440</td>
</tr>
<tr>
<td><strong>Micro elements</strong></td>
<td></td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>6.20</td>
</tr>
<tr>
<td>MnSO$_4$.4H$_2$O</td>
<td>22.3</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>8.6</td>
</tr>
<tr>
<td>NaMoO$_4$.2H$_2$O</td>
<td>0.25</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>0.025</td>
</tr>
<tr>
<td>CoCl$_2$.H$_2$O</td>
<td>0.025</td>
</tr>
<tr>
<td><strong>Iron Source</strong></td>
<td></td>
</tr>
<tr>
<td>Na$_2$EDTA</td>
<td>37.3</td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>27.8</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>0.5</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.0</td>
</tr>
<tr>
<td>Myoinositol</td>
<td>100 (0.1g)</td>
</tr>
<tr>
<td>Agar</td>
<td>0.8 % (w/v)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3.0 % (30gm/Litre)</td>
</tr>
<tr>
<td>pH</td>
<td>5.8</td>
</tr>
</tbody>
</table>
Each chemical was dissolved in bidistilled water. The pH of the medium was adjusted using 1N HCl / NaOH before autoclaving at 121°C, for 20 mins. Stock solutions were stored 4°C at except organic supplements, which were stored at -20°C

Table 4

MURASHIGE AND SKOOG + HILL AND KAEFER MEDIUM (MS+HK)

The medium was developed which had nutrients of both Hill and Kaefer medium and MS medium. The medium contains ingredients of Murashige and Skoog Medium (1962) as well as of Hill & Kaefer (2001). It has Major, Minor, Iron source and Vitamins of Murashige and Skoog Medium along with some essential compounds of Hill & Kaefer are added (Glucose, Peptone, Yeast Extract, Casamino acid). The medium contains 0.8% Agar and pH is adjusted to 5.8. The detailed composition of Hill and Kaefer medium and MS basal medium is given in Table 1 and 3 of Appendix I.
APPENDIX II

REAGENTS AND BUFFERS

**Alkaline Copper Sulphate Reagent**
24 parts of Nelson Somogyii A and 1 part of Nelson Somogyii B

**Arsenomolybdate Color Reagent**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Molybdate</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Conc. Sulphuric acid</td>
<td>2.1 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>45 ml</td>
</tr>
<tr>
<td>Sodium arsenate</td>
<td>300 mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2.5 ml</td>
</tr>
</tbody>
</table>

Ammonium Molybdate in 45 ml of distilled water was strongly heated and then added Sulphuric acid to it. Sodium arsenate was dissolved in 2.5 ml of distilled water which was then added to Ammonium Molybdate solution. The solution was kept at 55°C for 25m and then stored in brown bottle.

**Buffers**

(i) **0.1 M Phosphate buffer (pH 6)**
For its preparation 87.7 ml of 0.2M solution of monobasic sodium phosphate was mixed with 12.3 ml of 0.2M sodium phosphate solution and diluted to 200 ml with distilled water.

(ii) **0.1M Acetate buffer (pH 5)**
To prepare 0.1M Acetate buffer (pH 5) 14.8 ml of 0.2M acetic acid was mixed with 35.2 ml of 0.2M sodium acetate solution and the solution was diluted to 100 ml with distilled water.

**Bradford Reagent (Bradford 1976)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie Brilliant Blue G250</td>
<td>100 mg</td>
</tr>
<tr>
<td>90 percent Ethanol</td>
<td>50 ml</td>
</tr>
<tr>
<td>85 percent o-phosphoric acid</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Diluted to 1 liter with distilled water.
**Ethidium Bromide Stain**

1X PBS buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>2.9 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
</tbody>
</table>

Ethidium bromide of 1 mg was dissolved in 20ml of 1XPBS Buffer. Container containing the dye was wrapped in aluminium foil and stored at room temperature in dark bottle.

**Lactophenol Aniline Blue Stain** (Phillips and Hayman, 1970)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol crystals</td>
<td>20 g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>20 ml</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>20 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>40 ml</td>
</tr>
<tr>
<td>Aniline blue</td>
<td>0.05 g</td>
</tr>
</tbody>
</table>

Dissolved aniline blue in distilled water, left overnight to eliminate insoluble dye. Next day phenol crystal was added to lactic acid in a glass beaker and with the help of magnetic stirrer, phenol was dissolved.

**Lactophenol**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic acid</td>
<td>20 ml</td>
</tr>
<tr>
<td>Phenol crystals</td>
<td>20 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>40 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>20 ml</td>
</tr>
</tbody>
</table>

Added lactic acid and glycerol to the distilled water and mixed thoroughly. Added phenol crystals and heat gently in hot water bath with frequent agitation until the crystals completely dissolved.
Glassware Cleaning Solution:
Potassium di-chromate : 40g
Distilled water : 150ml
Conc. Sulphuric acid : 230ml
Potassium di-chromate was dissolved in distilled water and to it Conc. Sulphuric acid was then added slowly.

Martin’s Rose Bengal Streptomycin Agar
KH$_2$PO$_4$ : 1.0 g  MgSO$_4$.7H$_2$O : 0.5 g
Peptone : 5 g  Dextrose : 10g
Rose Bengal : 30 mg  Agar : 20 g
Distilled Water : 1000 ml  Streptomycin : 30 mg
pH : 6.5

Nutrient Agar
Beef extract : 3.0 g  Peptone : 5.0 g
NaCl : 8.0 g  Agar : 7.3 g
Distilled Water : 1000 ml  pH : 7.3

Ringer’s Solution
NaCl : 2.25 g  KCl : 0.105 g
CaCl$_2$ : 0.12 g  NaHCO$_3$ : 0.05 g
Distilled Water : 1000 ml

Starch Casein Agar
Starch : 10 g  Casein : 0.3 g
KNO$_3$ : 2.0 g  NaCl : 2.0 g
K$_2$HPO$_4$ : 2.0 g  MgSO$_4$.7H$_2$O : 0.05 g
CaCO$_3$ : 0.02 g  FeSO$_4$.7H$_2$O : 0.01 g
Water : 1000 ml  Agar : 18 g
pH : 7.2
**Nelson Somogyii A**

Sodium carbonate : 25 g  
Sodium potassium tartarate : 25 g  
Sodium bicarbonate : 20 g  
Sodium sulphate : 200 g  
Distilled water : 1000 ml  

Mixed and filtered through Whatmann filter paper no. 1

**Nelson Somogyii B**

Copper sulphate : 15%  
Conc. Sulphuric acid : 2 drops  
Distilled water : 100 ml  

The solution was strongly heated
APPENDIX III

PUBLICATIONS


10. Das, A., Kamal, S. and Varma A. Growth responses of the medicinal plant Coleus forskohlii to inoculation by Piriformospora indica in a field trial. (under communication)

11. Das, A., Kamal, S. and Varma A. Root colonization and growth enhancement of micropropagated Coleus forskohlii by Piriformospora indica. (under communication)