Poster presentation

MOLECULAR CHARACTERIZATION AND IMPLEMENTATION OF INDOLE 3-ACETIC ACID FROM COLLETOTRICHIUM GLOEOSPORIOIDES – A FIRST REPORT

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Background and Research questions
Microbial contamination is ineradicable from the Plant tissue culture. However, intensive studies for the occurrence of microbial contaminants are available only for bacteria, but fungal contaminants have not been studied much.
The cost component of tissue culture medium has been another concern for most commercial laboratories.
• Are the contaminants taxonomically diverse?
• Whether these contaminants are endophytes that are residing in healthy plants?
• Does the fungal IAA effective for plant development in tissue culture?

Materials and Methods
The explants were treated with 1% Tween 80 (8 min), 1% HgCl2 (5 min) and 70% ethanol (1 min)
For identification of contaminant fungi morphological standard keys and fungal 18S rRNA gene sequencing were used
The IAA responsive gene CgOpt1 from C. gloeosporioides PTC03 was amplified according to Veronique et al., (2009)
Fungi were inoculated in Czapek Dox medium with 100 µg/ml tryptophan for IAA production
IAA was extracted with ethyl acetate and analysed by thin-layer chromatography (TLC)

Results
◆ Simultaneous regeneration of shoots and roots were developed from nodal explants of S. dulcis in MS medium amended with 2.0 mg/l IAA and 0.5 mg/l of kinetin (KIN) (Figure 1)
The total fungal contamination rate is 4.5 % which belong to 3 different strains i.e. Penicillium citrinum (2.4 %), C. gloeosporioides (1.4 %) and Alternaria alternata (0.6 %)
Partial 18S rRNA gene of A. alternata PTC04, C. gloeosporioides PTC03 and P. citrinum PTC07 was sequenced and stored in NCBI-GenBank (Figure 2-4).
C. gloeosporioides PTC03 produced the maximum level (53.2 µg/ml) of IAA (Graph 1).
C. gloeosporioides PTC03 is confirmed for the presence of the CgOpt1 gene by PCR amplification
Morphologically similar results in plant formation were observed with the fungal IAA as like authentic IAA (Sigma-Aldrich, India) (Figure 5; Table 1)

Conclusions
◆ Only few reports are available to reveal the taxonomic groups of fungal contaminants. It is the first report on molecular taxonomy of fungal contaminants
P. citrinum, A. alternata and C. gloeosporioides are common endophytes of tropical medicinal plants. This proves the possibility of occurrences of endophytes as a contaminant fungi
The similar morphological results against authentic IAA reveals the potential implementation of fungal IAA in plant tissue culture

Future directions
◆ Although fungi are capable of producing IAA, its purpose, if any, is unclear
◆ For successful commercial exploitation of fungal derived IAA, more trials are required
◆ Both endophytic and contaminant diversity should be investigated to ensure the occurrence of endophytes as contaminant fungi

Acknowledgements and References
We are grateful to VELS University for infrastructural facilities and Department of Science and Technology, Government of India for travel support
Direct regeneration and *in vitro* flowering of *Scoparia dulcis* L.

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**Abstract:** This study reports a simple micropropagation protocol and thereby rapid multiplication of the useful medicinal plant *Scoparia dulcis* L. Single node explants were inoculated on basal MS medium containing 3% (w/v) sucrose, supplemented with different concentrations and combinations of 6-benzylaminopurine (BAP), kinetin (KN), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and Naphthalene acetic acid (NAA) for direct plant regeneration. Maximum numbers of shoot (~22) were observed on the medium containing 0.5 mg/l BAP and 0.25 mg/l IAA after four weeks of culture. Regenerated shoots were separated and rooted on half strength MS medium supplemented with 0.5 mg/l of IBA alone for three weeks. Simultaneous regeneration of shoots and roots *in vitro* flowering were achieved from the nodal explants on MS medium supplemented with 0.5 mg/l KN and 2.0 mg/l IAA. Well-developed complete plantlets were transferred on to specially made plastic cup containing soil rite. Acclimatized plantlets were successfully grown in garden soil.

**Keywords:** *Scoparia dulcis*, nodal explants, micropropagation, plant tissue culture.

**Introduction**

*S. dulcis* is a perennial multi purpose medicinal herb distributed throughout tropical and subtropical regions which belongs to the family Scrophulariaceae (Riel et al., 2002, Latha et al., 2004). Traditionally the fresh or dried plant has been used as a remedy for treating stomach ailments, hypertension, diabetes, inflammation, bronchitis, hemorrhoids, hepatitis, an analgesic and antipyretic agent (Riel et al., 2002, Ratnasooriya et al., 2005). Extracts of the plant contains antidiabetic activity (Pari et al., 2004), anticancer activity (Nishino et al., 1993), antimalarial activity (Riel et al., 2002), antiviral activity (Hayashi et al., 1988), neurotrophic activity (Li et al., 2004) and anti-inflammatory activity (Ahmed et al., 2001).

Conventional vegetative propagation on commercial scale has limitations (Thakur et al., 1998). Therefore, micropropagation offers a reliable method for mass production of plants in a shorter time without seasonal constraints. So far, there is no report on *in vitro* method of propagation for this plant in order to improve its cultivation. Therefore this first report on *in vitro* multiplication of *S. dulcis* through direct plant regeneration technique offers an effective alternative method of propagation for this important multipurpose medicinal plant.

**Materials and methods**

Explants were collected from moist deciduous forest, cut into nodal segments and used for induction of multiple shoots. The explants were washed with soap (soap powder) in running tap water for 1 hour. This is necessary to remove the exudates (phenolics, tannins and mucillages) present within the tissues. The explants were washed with Tween 20 (2%, w/v) and rinsed until traces of soap were removed. Later these explants were surface sterilized with 0.1% mercuric chloride (w/v) for 30 seconds and washed thrice using sterilized distilled water. Under aseptic conditions, explants were inoculated on basal MS (Murashige & Skoog, 1962) medium containing 6% (w/v) sucrose, supplemented with different concentrations and combinations of indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) or Naphthalene acetic acid (0.0, 0.25, 0.5 and 1.0 mg/l) either with 6- benzylaminopurine (BA: 0.0, 0.5, 1.0 and 2.0 mg/l) or kinetin (KN: 0.0, 0.5, 1.0 and 2.0 mg/l) for direct shoot regeneration and root induction. The pH was adjusted to 5.7 prior to the addition of 0.8% agar and autoclaved at 121°C (1.06 kg/cm²) for 15 min. Cultures were then incubated at 26±2°C with a 16-h photoperiod by cool white fluorescent tubes (Das et al., 1996) and 70-75% relative humidity (Mukherjee et al., 2002, Latha et al., 2004).

<table>
<thead>
<tr>
<th>Growth regulators (mg/l)</th>
<th>% of explant showing response</th>
<th>No. of shoots</th>
<th>Average length of shoots (cm)</th>
</tr>
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<tbody>
<tr>
<td>BAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>60.0</td>
<td>5.4 ± 0.14</td>
<td>1.4 ± 0.23</td>
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<tr>
<td>1.0</td>
<td>68.0</td>
<td>7.2 ± 0.16</td>
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<td>1.5</td>
<td>82.0</td>
<td>12.3 ± 0.12</td>
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<tr>
<td>2.0</td>
<td>95.0</td>
<td>14.6 ± 0.09</td>
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<tr>
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<td>4.6 ± 0.32</td>
<td>4.8 ± 0.46</td>
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<td>79.0</td>
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<td>5.1 ± 0.25</td>
</tr>
<tr>
<td>1.5</td>
<td>91.0</td>
<td>8.1 ± 0.34</td>
<td>5.4 ± 0.28</td>
</tr>
<tr>
<td>2.0</td>
<td>97.0</td>
<td>8.8 ± 0.12</td>
<td>5.7 ± 0.18</td>
</tr>
<tr>
<td>BAP+NAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0+0.25</td>
<td>89.0</td>
<td>20.5 ± 0.18</td>
<td>1.7 ± 0.51</td>
</tr>
<tr>
<td>2.0+0.50</td>
<td>96.0</td>
<td>22.1 ± 0.26</td>
<td>2.0 ± 0.26</td>
</tr>
<tr>
<td>2.0+0.75</td>
<td>90.0</td>
<td>20.9 ± 0.15</td>
<td>1.7 ± 0.20</td>
</tr>
<tr>
<td>2.0+1.00</td>
<td>81.0</td>
<td>18.8 ± 0.13</td>
<td>1.2 ± 0.22</td>
</tr>
<tr>
<td>BAP+IBA</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2.0+0.25</td>
<td>95.0</td>
<td>16.6 ± 0.24</td>
<td>1.9 ± 0.24</td>
</tr>
<tr>
<td>2.0+0.50</td>
<td>97.0</td>
<td>16.2 ± 0.17</td>
<td>1.5 ± 0.32</td>
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<tr>
<td>2.0+0.25</td>
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<td>1.4 ± 0.21</td>
<td>5.94 ± 0.28</td>
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<td>90.0</td>
<td>1.2 ± 0.52</td>
<td>5.22 ± 0.14</td>
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<tr>
<td>2.0+1.00</td>
<td>83.0</td>
<td>1.2 ± 0.14</td>
<td>5.08 ± 0.16</td>
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20 explants and culture were maintained in each treatment and data (SE) were recorded up to four weeks of culture.
Table 2: Root induction at different concentrations of IBA and IAA in MS medium from shoots of S. dulcis

<table>
<thead>
<tr>
<th>Growth regulators (mg/l)</th>
<th>% of root response</th>
<th>No. of roots</th>
<th>Average length of roots (cm)</th>
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<tr>
<td>IAA</td>
<td>0.25</td>
<td>95.0</td>
<td>3.8 ± 0.14</td>
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<td>100.0</td>
<td>4.2 ± 0.23</td>
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<td></td>
<td>1.00</td>
<td>97.0</td>
<td>4.0 ± 0.08</td>
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<td></td>
<td>2.00</td>
<td>98.0</td>
<td>3.7 ± 0.12</td>
</tr>
<tr>
<td>IBA</td>
<td>0.25</td>
<td>96.0</td>
<td>3.7 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>100.0</td>
<td>4.1 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>100.0</td>
<td>4.3 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>92.0</td>
<td>3.8 ± 0.16</td>
</tr>
</tbody>
</table>

20 explants and culture were maintained in each treatment and data (SE) were recorded up to three weeks of culture 1991. For root induction, separated shoots were transferred to half strength MS basal medium supplemented with different concentrations of neither IBA nor IAA (0.0, 0.25, 0.50 and 1.0 mg/l) and 3% (w/v) sucrose. For hardening, the rooted plants were removed from the culture tubes, washed with sterile distilled water, and transferred to protrays with sterile cow dung: coco peat: sand (1:1:1 v/v/v). The plantlets were placed at 70% to 80 % humidity, 25 ± 2 °C under a 12-hours photoperiod for acclimatization. After the plants get acclimatized, the plants were transferred to pot with farmyard mixture: sand (1: 1 v/v) and placed in green house. After three weeks of development, these hardened plants were transferred to the field and the survival rate was recorded. Twenty cultures were used per treatment and each experiment was repeated at least three times. Percentage of success was scored four weeks after culture. Data collected were statistically analyzed and results presented in the tables.

Results and discussion

The nodal explants inoculated in MS medium individually supplemented with both BAP and KN showed remarkable response. In order to evaluate the synergistic effect different hormone combinations were tested in MS medium. Data on the effect of different concentrations of BAP, KN in combination with IBA and IAA in MS medium for multiple shoot induction of S. dulcis are presented in Table 1. The maximum number of shoot induction from the nodes of the explants was exhibited in a combination of BAP and IAA. Normally, other species like S. monteviendis shows adequate response towards shoot regeneration in MS medium in the presence of BAP (Escandon et al., 2005). The maximum average shoot length resulted when KN and IBA were used at a concentration of 2.0 and 0.5 mg/l respectively (Fig. 1).

The mean values of root induction from shoots of S. dulcis cultured in MS medium with different concentrations of IBA and IAA are given in Table 2. In the case of IAA, maximum root induction was noticed at a concentration of 0.5mg/l (Fig. 2), whereas in MS medium with IBA showed maximum root induction at a concentration of 0.5mg/l. Regarding root length, there was not much difference between IAA and IBA treatments.

The mean values of simultaneous shoot and root induction of tissue cultured S. dulcis in MS medium with different concentrations of KN and IAA combinations are depicted in Table 3. The MS medium with 0.5 and 2.0 mg/l of KN and IAA respectively gave better result in respect of number of shoots, roots and flowers (Fig. 3).

During the process acclimatization and hardening, about 95% survival in chamber culture (Fig. 4) and about 100% survival in both greenhouse (Fig. 5) and field were noticed. The regenerated plants were phenotypically normal. Direct shoot multiplication is preferred for generating true-to-type plants than callus regeneration. This study reports a simple micropropagation protocol and the rapid multiplication of the useful medicinal plant- S. dulcis L by in vitro conditions. Shoots can be easily derived from node cultures on BAP containing medium and subsequently rooted on IBA containing medium. Both shoot and root can be derived from node cultures on KN and IAA.
containing medium. This approach offers a means for producing more identical plantlets from node explants of S. dulcis.

Acknowledgement
Authors are thankful to the Management of Vel’s Educational Trust, Chennai, Tamilnadu, India, for providing the infrastructure for the present study.

References

Table 3: Effects of different concentrations of KN combination with IAA in MS medium for simultaneous shoot and root regeneration and in vitro flowering from node explants of S. dulcis

<table>
<thead>
<tr>
<th>Growth regulators KN-HAA (mg/l)</th>
<th>explant response (%)</th>
<th>No. of shoots</th>
<th>Average shoot length (cm)</th>
<th>No. of roots</th>
<th>Average root length (cm)</th>
<th>No. of flowers</th>
</tr>
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<tr>
<td>0.25+2.0</td>
<td>92.0</td>
<td>1.5 ± 0.34</td>
<td>5.45 ± 0.28</td>
<td>4.1 ± 0.22</td>
<td>1.81 ± 0.20</td>
<td>4.1 ± 0.18</td>
</tr>
<tr>
<td>0.50+2.0</td>
<td>97.0</td>
<td>1.8 ± 0.14</td>
<td>5.88 ± 0.10</td>
<td>4.4 ± 0.16</td>
<td>2.08 ± 0.10</td>
<td>4.8 ± 0.12</td>
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<td>0.75+2.0</td>
<td>90.0</td>
<td>1.2 ± 0.28</td>
<td>4.64 ± 0.20</td>
<td>3.7 ± 0.26</td>
<td>1.47 ± 0.18</td>
<td>2.3 ± 0.24</td>
</tr>
</tbody>
</table>
Diversity of Fungal Endophytes in Few Medicinal Herbs of South India

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2Department of Botany, Bishop Heber college, Tiruchirappalli-620 017, India
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ABSTRACT
Endophytic fungi cause symptomless infections in healthy tissues of plants. This crypto guild of fungi is regarded as benchmark for estimating fungal biodiversity. We studied endophyte distribution and diversity in four medicinal herbs Coleus aromaticus, Lycium asperum, Ocimum basilicum, Ocimum sanctumand Tridax procumbens of South India. Five hundred lamina and petiole segments from each herb were screened. More endophytes would be isolated from petiole. Coelomycetes and Ascomycetes were the most dominant group than Hyphomycetes. Sterile forms were isolated from herbs. Some endophytes were common to all the host and few appeared to be host specific.

Key words: Ascomycetes fungi, Ascomycetes, Coelomycetes, lamina, petiole

INTRODUCTION
Fungi are heterotrophic eukaryotes with unique characteristics that set them well apart from both plants and animals. Fungi are so versatile in adapting themselves that they could occupy a variety of ecological habitats, so that as a group they can be isolated from unusual habitats such as salterns [1], peat soil [2], petroleum polluted soils [3], inner tissues of plants and animals [4]. Fungi are also known to establish themselves inside healthy plant tissues without causing any “injury or overt symptom to their host”. Such an association is generally termed as endophytic association [5]. Carroll (1986) restricts the use of the term endophyte to organisms that cause asymptomatic infections within plant tissues excluding pathogenic fungi and mutualists such as mycorrhizal fungi [6]. Only few tropical trees have been studied for the fungal endophytes such as Azadirachta indica [7], mangrove trees [8] and palm trees [9]. Tropical areas are hyper diverse in their plant species as well as in their fungal diversity than in the temperate regions [2] that South India is one among the tropics, very little work has been carried out with endophytyc diversity. This study reveals the diversity of fungal endophytes of tropical medicinal herbs.

MATERIALS AND METHODS
Collection of plants
Five species of medicinal herbs were surveyed for foliar fungal endophytes. Leaves (petiole and lamina) of Coleus aromaticus, Lycium asperum, Ocimum basilicum, Ocimum sanctum and Tridax procumbens were collected from the University Herbal Garden, Tamil Nadu, India. Fifty matured leaves were collected from each herb. Samples were transported in closed sterile polythene bags and processed within 24 hrs of collection [10].

Sterilization of glassware and chemicals
Media and glasswares other than petri dishes were sterilized in an autoclave at a pressure of 105 Kpa for 30 min. Petri dishes were sterilized in a hot air oven at 160°C for 3 hrs.

Surface sterilization of plant material
Midrib portion of lamina segments (0.5 cm²) cut from the middle portion of healthy leaves and segments (0.5 cm²) cut from the basal part of the petiole, were dipped in 70 % ethanol for 5 seconds, immersed in 4 % NaOCl for 1 minute and rinsed in sterile distilled water for 10 seconds [11]. Five hundred segments of lamina and petiole from each plant species were placed on Potato Dextrose Agar
media (In 1000 ml distilled water, Potato – 200 g, Dextrose – 20 g and Agar – 20 g were added, pH 6.5) amended with Chloramphenicol (150 mg l⁻¹).

**Culture condition and isolation**

Ten segments were placed on 20 ml PDA medium in a Petri dish and incubated [6]. The incubation temperature was 27 °C ± 1 °C. The fungi that grew out from the plant tissues were periodically observed through a microscope. The endophytic fungi were identified with the help of keys provided by Subramanian [11]. The sterile Mycelia that grew out from the tissue were subcultured and exposed to light to induce sporulation [12]. Those fungi which failed to sporulate or produce other features that aid in the preliminary identification were given code numbers.

**Data reduction**

The colonization frequency (CF) of single endophyte species in plant tissues was calculated by the method of Hata & Futai (1995). \( CF = \frac{N_{col}}{N_i} \times 100 \) where \( N_{col} \) and \( N_i \) are the number of segments colonized by each endophyte and total number of segments examined respectively.

**RESULT AND DISCUSSION**

The total of eighteen species of endophytic fungi were isolated from five different species of medicinal herbs. Of the eighteen endophytic fungi, six belonged to Ascomycetes; four belonged to Coelomycetes; three belonged to Hyphomycetes and rest of them were sterile forms. Occurrence of sterile mycelium as endophytes is not unusual [5]. In this study Ascomycetes was the most dominant one. Similar results were obtained in other endophytic studies [7, 13]. Of the five medicinal herbs studied, a greater number of endophytes could be seen in Ocimum basilicum and O. sanctum. Coleus aromaticus and Tridax procumbens had the least number of endophytic fungi (Table 1). Of the eighteen endophytes isolated, C. indicum presents only in the lamina of Leucas aspera, Phyllotisic спе., Xylaria sp. and Pestalotiopsis sp. present in petiole region. All the other endophytes could be seen in both lamina and petiole (Table 1). Petiole region had more endophyte than lamina. This finding is in consonance with earlier reports in Azadirachta indica [7] and palm tree [9]. Such variation within leaf of endophytes is attributed to differential leaf expansion and leaf chemistry [5]. Another reason for this difference may be:

<table>
<thead>
<tr>
<th>S. No</th>
<th>Endophytes</th>
<th>Tridax procumbens</th>
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<th>O. basilicum</th>
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<td>P L</td>
<td>P L</td>
<td>P L</td>
<td>P L</td>
<td>P L</td>
</tr>
<tr>
<td>14</td>
<td>Sterile I</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>15</td>
<td>Sterile II</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>16</td>
<td>Sterile III</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>17</td>
<td>Sterile IV</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>18</td>
<td>Sterile V</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
</tbody>
</table>

P – Petiole. L – Lamina
be due to differential maturation of leaf. The petiole region expands less than the lamina portion hence infections by endophytes established before leaf expansion may become “diluted” as the leaf expands [14]. Further higher number of endophytes recovered from petiole may be due to movement of water and food through the petiole and this is conducive for fungal growth. Sterile mycelium I, II, III, IV had wider host range as they could be isolated from all the five hosts studied. Occurrence of more sterile mycelium has been reported in other hosts also Azadirachta indica [7] and mangrove trees [8]. Sporormiella minima, Alternaria alternata, Phoma sp., Phomopsis sp., Chaetomium sp., Chaetomium globosum and Talaromyces sp., are present in three hosts. Phyllosticta sp., Pestalotiopsis sp., Chaetomium indicum, Curvularia lunata, Fusarium oxysporum, Xylaria sp. and sterile mycelium V could isolated from two or one host studied. Alternaria alternata and Phomopsis are reported as endophytes in wide range of plant species [7]. Normally Alternaria alternata and Curvularia lunata occur as phyloplane fungi but they are capable of penetrating the superficial layers of leaf and grow as endophyte, suggesting that phyloplane fungi has report to an endophytic mode of life to overcome adverse environmental conditions [15]. Fungi like Alternaria and Curvularia have been found to well adopted for endophytic mode of life in wide varieties of plants [5]. It was true in the present study also. Petri (1992) grouped endophytic fungi into Xylariaceae, Coprophilous, epiphytic and true endophytic forms [15]. The present study representatives from endophytic Xylariaceae, epiphytic and Coprophilous fungi were present. In addition non-sporulation forms accounted for 36 % of the endophytic population. Some of them were host specific Phyllosticta sp., Xyloria sp. and Pestalotiopsis sp. were present only in petiole region of the herb. C. indicum presents only in lamina of Lecos ospera. Thus, the difference in species composition of endophytes indicated that host specificity was exhibited by certain fungal endophytes [5]. Generally a large number of endophytic fungal genera can be isolated from tropical trees, forest trees and palm [7, 9]. However, sampling of these medicinal herbs yields only eighteen endophytes. Of these only few were dominant others showed low colonization frequency. Presence of few endophytes in this host could be due to the presence of antifungal compounds. As most of the host studied used for ayurvedic medicine preparation. Thus it appears that the occurrence of fungal endophytes are influenced by the type of host tissue and chemicals present in the medicinal herb. The endophytic genera such as Phoma, Phomopsis, Phyllosticta and Xylaria that are ubiquitous and are commonly isolated from the leaves of other host including many tropical trees were also present in the leaves of medicinal plants screened in this study.

ACKNOWLEDGEMENTS
Authors thanks Department of Plant Biology and Biotechnology, Bishop Heber college and VEALS Educational trust for funding and support.

REFERENCES
Thidiazuron Induced Direct Regeneration from Leaf Explants of *Scoparia dulcis* L. – A Pharmaceutical Plant

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**ABSTRACT:**
Leaf explants of *Scoparia dulcis* L. gave rise to multiple shoots when cultured on MS medium supplemented with different concentrations of TDZ and IAA. The highest rate of shoot multiplication was obtained in MS medium containing 4.0 µM TDZ and 1.0 µM IAA (26.6 ± 0.98). Differentiated shoot buds elongated to 5.8 cm in 21 days in 9 µM KIN amended medium. The regenerated shoots were rooted on half-strength MS basal medium with different concentrations of IBA and IAA. The maximum number of roots was achieved on the medium containing 2.8 µM IBA. *In vitro* regenerated plantlets were transferred to plastic pots containing coco peat as a potting mix and were thereafter successfully established under *ex vitro* conditions. The survival percentage of transplanted plantlets was 90.6%.

**KEYWORDS:** *Scoparia dulcis*, medicinal plant, leaf explants, micropropagation, Thidiazuron.

**INTRODUCTION:**
Medicinal plants are of great interest to the researchers in the field of biotechnology as most of the drug industries depend, in part, on plants for the production of pharmaceutical compounds³. Among the world’s 25 best selling pharmaceutical medicines, 12 are plant derived⁴. *Scoparia dulcis* L. commonly known as ‘Sarkari Vembu’ in Tamil and ‘sweet broom’ in English (Family: Scrophulariaceae) a small erect, slender, rigid, perennial herb with three serratate-margined, ovate-elliptic leaves at each node, small white, axillary, solitary flowers and small coriander-like fruits⁵⁶. Traditionally *Scoparia dulcis* has been used as a remedy for treating stomach ailments, hypertension, diabetes, inflammation, bronchitis, hemorrhoids, hepatitis, an analgesic and antipyretic agent⁷. Hot water infusion and decoction of the leaves or whole plant is used medicinally by indigenous tribes of Nicaragua to treat malaria, stomach disorders, menstrual disorders, insect bites, fevers, heart problems, liver disorders, and venereal disease also for blood cleansing⁸. Moreover the plant is reported in Siddha system of medicine to treat diabetes and to treat any poisonous bite⁹. Mucilage is released when the whole plant is soaked in water, thus helping to protect and regenerate normal cells; it may also act as an immunostimulator. The plant has been used to treat skin rashes in Martinique and Trinidad for irritated skin in Brazil, as a multi-ingredient preparation for treating burns in eastern Nicaragua and to kill lice and fleas and used against vermin in Paraguay¹⁰. The dried roots and aerial parts of *Scoparia dulcis* contain hydroxamic acids which provide insect, fungal, and bacterial resistance¹¹. The scientific research reveals numerous chemical studies on isolated chemicals include coumarins, phenols, saponins, tannins, amino acids, flavonoids, terpenoids and catecholamines¹². The terpenoids are responsible for numerous medicinal effects. Scoparic acid A, Scoparic acid B, Scopadulcic acid A, Scopadulcic acid B, Scopadulcin and Scopadulin are all biologically active. These chemical compounds have various biological activities, including inhibition of the replication of herpes simplex virus (The mechanism of action is unknown but does not involve a direct virucidal effect or inhibition of virus attachment), inhibition of proton pumps, potassium adenosine triphosphate (ATP)ase activator and antitumor promoting activity. The acetylated flavone glycosides from broomweed have nerve growth factor (NGF)-potentiating activity or neurotrophic activity that may be useful in treating neurological disorders. The flavone glycosides including isovitexin also inhibit β-glucuronidase¹³. The plant has been proved for its antidiabetic and antioxidant activity¹⁴. The over exploitation of medicinal plant leads to habitat loss, extinction and reduces species size. Besides that they
are prone to environmental catastrophe, demographic or loss of genetic variations and accumulations of deleterious mutations. Mass propagation of plant species through in vitro culture is one of the best and most successful examples of commercial application of plant tissue culture technology. Recently, there has been much progress in this technology for some pharmaceutically important medicinal plants. Thidiazuron (TDZ), a urea-derived cytokinin, is a potent cytokinin for plant tissue culture and is extensively used for the induction of shoot regeneration in several plant species. While micropropagation through auxiliary shoot proliferation of Scoparia dulcis has been reported, the study using other explant sources have not been reported. Hence, the present investigation reveals the invitro regeneration from leaf explant of Scoparia dulcis.

 MATERIALS AND METHODS:
Plant materials:
Scoparia dulcis was freshly harvested from Madras Christian College campus, Chennai, India and their leaves were used as explant source. The explants were washed with soap (soap powder) in running tap water for 1 hour. This is necessary to remove the exudates present within the tissues. The explants washed with Tween 20 (2%, v/v) and rinsed until traces of soap was removed. Then the explants were transferred to a sterile laminar flow and surface sterilized the explants using mercuric chloride (0.1%, w/v) followed by three washes with sterile distilled water. The leaves were trimmed into pieces of about 0.5 cm² and inoculated on to culture medium.

Culture medium and conditions:
Murashige and Skoog medium (1962) basal medium was supplemented with various plant growth regulators, 3.0% sucrose and 0.8% agar. The pH was adjusted to 5.8 and autoclaved at 121°C under 15 lb pressure for 15-20 minutes. Explants were placed in culture tube and incubated at 24±2°C and 60% relative humidity under the light 16 hour-day photoperiod, provided light intensity of 2000 lux using a white fluorescent light.

Shoot Proliferation media:
MS medium supplemented with TDZ (1.0, 2.0, 3.0, 4.0 and 5.0 μM) was used for microshoots formation from leaf explants and the cultures. Number of new shoot proliferation of each culture was recorded after every week of inoculation.

Root induction:
For in vitro rooting, individual shoots (3 - 6 cm) were excised from the proliferated shoot cultures and implanted onto half strength of MS with different concentrations and combinations of IBA (Indole butyric acid) and IAA (Indole acetic acid) (Table 1).

Acclimatization and hardening:
The formation of healthy shoots and roots make sure that this is ready to harden. The rooted plants were removed from the culture tubes, washed with sterile distilled water, and transferred to protrays with sterile cow dung: coca peat: sand (1:1:1 v/v/v). The plantlets were placed at 70 to 80% humidity, 25 ± 2°C under a 12-hours photoperiod for acclimatization.

For hardening, the rooted plants were removed from protrays, washed with sterile distilled water and transferred to green house. These hardened plants were transferred to the field and the survival rate was recorded. Twenty cultures were used per treatment and each experiment was repeated at least three times. Percentage of success was scored four weeks after culture. The effects of different treatments were quantified and the data subjected to statistical analysis.

RESULT:
Within seven to 15 days of culture microshoots were formed from leaf explant on MS supplemented with 2 – 5 μM TDZ either alone or in combination with 0.5 – 2 μM IAA (Table 1). Maximum number of microshoots was 26.6 ± 0.98 per culture developed on MS with 4 μM TDZ + 1 μM IAA after four weeks (Fig. 1). Elongation of shoots was observed from the microshoots when subcultured on MS + 6 -12) μM KIN after four weeks (Table 1). The highest length of shoot was 5.8 ± 0.78 developed on MS with 9 μM KIN (Table 2, Fig. 2). The mean values of root induction from shoots of S. dulcis cultured in half strength MS medium with different concentrations of IBA and IAA are given in Table 3. In the case of IAA, maximum root induction was noticed at a concentration of 2.8 μM (Fig. 3), whereas in half strength MS medium with IBA showed maximum root induction at a concentration of 2.4 μM. Though root length was not much differ between IAA and IBA treatments, IBA produced better results than IAA. During the process acclimatization and hardening, about 85 % survival in chamber culture and about 90% survival in both greenhouse (Fig. 4) and field were noticed. The regenerated plants were phenotypically normal. 20 explants and culture were maintained in each treatment and data (SE) were recorded up to four weeks of culture.
Table 1. Effect of different concentrations and combinations of growth regulators on MS for microshoots from the leaf explants of \textit{S. dulcis}.

<table>
<thead>
<tr>
<th>Growth regulator (µM)</th>
<th>TDZ IAA</th>
<th>% of explants producing microshoots</th>
<th>Mean No. of shoots/culture (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>22.6</td>
<td>5.7 ± 0.83</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>44.8</td>
<td>11.3 ± 0.75</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>86.2</td>
<td>20.6 ± 0.96</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>66.6</td>
<td>12.2 ± 0.62</td>
<td></td>
</tr>
<tr>
<td>4.0 0.5</td>
<td>86.8</td>
<td>23.7 ± 0.86</td>
<td></td>
</tr>
<tr>
<td>4.0 1.0</td>
<td>88.6</td>
<td>26.6 ± 0.98</td>
<td></td>
</tr>
<tr>
<td>4.0 1.5</td>
<td>64.2</td>
<td>14.2 ± 0.73</td>
<td></td>
</tr>
<tr>
<td>4.0 2.0</td>
<td>38.4</td>
<td>6.7 ± 0.74</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Effect of different concentrations KIN on MS for elongated shoots from the microshoots of \textit{S. dulcis}.

<table>
<thead>
<tr>
<th>Growth regular KIN (µM)</th>
<th>% of microshoots producing elongated shoots</th>
<th>Average length (cm) of shoots (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>36.8</td>
<td>2.7 ± 0.92</td>
</tr>
<tr>
<td>7.0</td>
<td>48.4</td>
<td>4.3 ± 0.77</td>
</tr>
<tr>
<td>8.0</td>
<td>86.4</td>
<td>4.6 ± 0.86</td>
</tr>
<tr>
<td>9.0</td>
<td>94.2</td>
<td>5.8 ± 0.78</td>
</tr>
<tr>
<td>10.0</td>
<td>76.6</td>
<td>4.2 ± 0.32</td>
</tr>
<tr>
<td>11.0</td>
<td>54.8</td>
<td>3.3 ± 0.77</td>
</tr>
<tr>
<td>12.0</td>
<td>32.0</td>
<td>2.4 ± 0.61</td>
</tr>
</tbody>
</table>

DISCUSSION:

TDZ, a synthetic phenylurea, is considered one of the most active cytokinins for shoot induction in plant tissue culture \cite{5,21}. TDZ-induced shoot regeneration from different explants of many recalcitrant species as well as from medicinal plants has been reported \cite{22,23,18,26,27}, suggesting that TDZ results in shoot regeneration better than other cytokinins \cite{28,27}. TDZ-induced morphogenesis probably depends on the levels of endogenous growth regulators, and TDZ modulates endogenous auxin levels \cite{29,30}. Direct shoot multiplication is preferred for generating true-to-type plants compared with callus regeneration. The results also correlate with the KIN-induced regeneration of \textit{S. dulcis} from nodal explants \cite{9}. IBA is the most commonly used auxin for root induction; its superior role in this function has been reported in several plants \cite{31,32,27,30}. This study reports a simple micropropagation protocol and the rapid \textit{in vitro} multiplication from leaf segments of the useful medicinal plant \textit{S. dulcis}. Shoots can be easily derived from node cultures on TDZ containing medium and subsequently elongated with KIN and rooted on IBA containing medium. Both shoot and root can be derived from node cultures on KN and IAA containing medium. This investigation offers a means for producing more identical plantlets from leaf explants of \textit{S. dulcis}.

ACKNOWLEDGEMENT:

Authors are thankful to the Management of Vel’s Educational Trust, Chennai, Tamilnadu, India, for providing the infrastructure for the present study.

REFERENCES:

Isolation and Characterization of Disease Resistant Cell Lines of *Cleome viscosa* Using Fungal Elicitor

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**ABSTRACT:**

Two independent experiments were performed to isolate diseases cell lines of *Cleome viscosa* from leaf segments. In the first experiment, callus induction was obtained on Murashige and Skoog medium with different concentrations of Thidiazuron (TDZ). Best dedifferentiation rates of 2.08 g fresh weight occurred in the presence of 2.0 mg/l. In the second experiment, *Aspergillus niger* and *Aspergillus flavus* were prominently isolated from garden soil and infected leaves respectively. Biochemical studies of anthocyanin accumulation at the different elicitor treated callus tissue revealed isolation and characterization of diseases resistant cell lines. Among different concentration, 0.5 gm/l mycelia of both fungal strains elucitate the maximum accumulation of anthocyanin. The best accumulation of anthocyanin was achieved with eliciters of *A. flavus*. Hence, pathogenic fungi are more effective for elicitation and to develop diseases resistant traits.

**KEYWORDS:** callus, *Cleome viscosa*, anthocyanin, *Aspergillus*, medicinal plant

**INTRODUCTION:**

*Cleome* is a large genus included in the Capparaceae family, which comprises 427 species occurring in tropical and subtropical regions of the world\(^1\). Many species of the genus were investigated for medicinal properties and showed several important activities. *Cleome viscosa* L. is a widely distributed sticky herb with yellow flowers and long slender pods containing seeds, which resemble those of mustard. Nayikkadugu (Tamil) found throughout the greater part of India\(^2\). In Ayurvedic system of medicine, this plant is used in fever, inflammations, liver diseases, bronchitis, diarrhea and infantile convulsions. *Cleome viscosa* possess various biological activities such as anthelmintic, analgesic, antiinflammatory, immunomodulatory, antiscorbutic, sudorific, febrifuge, cardiac stimulant, antipyretic, psychopharmacological, anti diarrheal, and hepatoprotective activities. *Cleome viscosa* is a good source of vitamin-C and iron\(^3,4\). Methanolic extract of the whole plant showed the presence of steroids, triterpenoids, flavonoids and tannins\(^5\). The main prerequisite for the development of high-quality medicinal products is a consistent source of high-quality plant material\(^6\). In this context, research in the area of plant tissue culture technology has contributed for the production of some important plant pharmaceuticals\(^1\). Anthocyanin pigments and derivatives are flavonoid and their significant role is in plant resistance kingdom. Anthocyanin is also known for anticancer properties\(^7\). Fungal cell walls and fragments thereof (biotic elicitors) trigger this defense response\(^2\). It is the first report on *in vitro* cell culture of Capparaceae species when a cytokinin was used alone. Hence, the main objective of the work was to improve the disease resistance of cell lines of *Cleome viscosa* through the application of different fungal elicitors.
MATERIALS AND METHODS:
Standardization of Hormone concentration for callus induction:
Fresh healthy leaf explants of C. viscosa plants collected from the herbal garden of the VELS University, Chennai, India. The explants were first washed with detergent several times and rinsed under running tap water, then immersed in 70% (v/v) ethanol for ten seconds before surface-disinfection in 0.1 % HgCl₂ solution under continuous agitation for 3 min and were subsequently rinsed three times with sterile distilled water. After surface sterilization, the leaf explants were cultured in Murashige and Skoog basal medium (MS) supplemented with 30 g l⁻¹ Analytical grade sucrose (Hi-Media, India) and 8.0 g l⁻¹ agar (Hi-Media, India) supplemented with different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0) of TDZ to obtain callus through dedifferentiation. The cultures were incubated at 24°C to 27°C and 60% relative humidity under light 18 hour/day photoperiod.

Isolation of fungi:
Soil fungi:
Soil sample were collected from the herbal garden of the VELS University. Potato Dextrose Agar (PDA) medium (ref) was prepared amended with chloramphenicol (25mg/100 ml). About 0.1 ml of each aliquot from 10⁻³, 10⁻⁴ and 10⁻⁵ soil dilutions was spread evenly on surface of PDA medium. Each dilution was plated in duplicates. The plates were sealed and incubated at room temperature.

Plant pathogenic fungi:
Young leaves with lesions were selected, because the fungus will be at its most active. The selected segments should have both infected and healthy part, 80 segments were collected. Surface sterilization is usually needed for leaf material with 0.1% mercuric chloride for one minute, followed by a rinse in sterile tap water to remove surface soil, dust and other contaminants. Eight segments were placed on PDA medium and incubated at 27 ± 1°C. The fungi that grew out from the leaf segments were periodically observed through a microscope.

Identification of fungi:
Pure cultures of soil and pathogenic fungus can be obtained from the primary isolation plates by colonies initiated from single spores culture. The fungal species were identified using standard characters used for fungal identification. The sterile mycelia that grew out from the tissue were subcultured and exposed to light to induce sporulation.

Fungal elicitor preparation:
The fungal cultures were established in liquid potato dextrose medium and kept at room temperature. The mycelia were separated for the cultures by filtration and washed four times in sterile distilled water. The mycelia were homogenized and the homogenate was used as elicitor.

Effect of Elicitors in Callus induction:
The fungal extracts at different concentration (0, 0.1, 0.25, 0.5, 0.75, 1.0) were added individually to the standardized medium for callus induction i.e. MS medium amended with 2 mg/l TDZ and a piece of callus was subcultured. The growth index was calculated as:-
Growth index = (Final weight-Initial weight) / Initial weight.

Estimation of Anthocyanin:
Acidic methanol (Methanol - 80ml, Distilled water - 20ml and Hydrochloric acid - 1ml) is used as solvent for anthocyanin extraction from wild, elicitor treated callus and control i.e. callus without elicitor treatment. 1 g of sample was taken in 1 ml of acetic methanol and kept 20 hours incubation, followed by centrifugation at 10000 rpm for 5 minutes. The optical density values were absorbed at 525 nm and calculated as described in Teha et al.

RESULTS:
The leaf tissue of C. viscosa was inoculated in MS medium containing different concentrations of TDZ. The callus tissues developed from the explants were studied and tabulated (Table 1). Growth of callus was found to be more (2.08 g) in 2.0 mg/l TDZ amended medium from leaf explants.

Isolated soil and pathogenic fungal strains were identified based on macroscopic and microscopic characteristics. Aspergillus niger was prominently isolated from soil whereas Aspergillus flavus from infected leaves. Hence these two strains were taken from elicitor preparation.

In elicitor-treated cells, changes in anthocyanin accumulation have been observed. Different concentrations of mycelia used from cultures of A. niger and A. flavus, show different values of growth index and anthocyanin accumulation (Table 3) (Plate 7, 8). Among these 0.5 g/ml mycelia of both fungal strains elicitate the maximum accumulation of anthocyanin. Least accumulation of anthocyanin was observed in 0.1 g/ml and of A. niger and 0.75 g/ml of A. flavus. Although elicitors of A. niger and A. flavus show more or less similar results A. flavus show significantly higher accumulation of anthocyanin (Table 2).

DISCUSSION:
This study has demonstrated that best callus induction rate could be achieved on the MS medium containing 2.0 mg/l TDZ. Promoting role of TDZ in callus induction has been observed in Leucaena leucocephala, Vitex negundo and Achras sapota. In elicitor-treated cells, changes in the phosphorylation status of proteins have been observed. Exposure of cell suspension cultures of Rauvolfia canescens to a yeast cell wall elicitor leads to the rapid transient induction of endogenous jasmonic acid and methyl jasmonate. The effect of different concentrations of L-phenylalanine and calcium chloride in MS medium to increase the anthocyanin content (µg/gm callus fresh weight) was investigated.
Table 1. Effect of TDZ in the growth of callus from the leaf tissue of C. viscosa on MS medium. This data was obtained after 25 days of inoculation.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Growth regulator (TDZ) mg/ml</th>
<th>Survival Rate %</th>
<th>Fresh weight (g)</th>
<th>Dry weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>60</td>
<td>0.98</td>
<td>0.20</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>65</td>
<td>1.11</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>68</td>
<td>1.31</td>
<td>0.29</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
<td>86</td>
<td>2.08</td>
<td>0.49</td>
</tr>
<tr>
<td>5</td>
<td>2.5</td>
<td>72</td>
<td>1.49</td>
<td>0.32</td>
</tr>
<tr>
<td>6</td>
<td>3.0</td>
<td>74</td>
<td>1.56</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Table 2. Effect of elicitors in the resistant cell line from the callus of C. viscosa on MS medium with 2 mg/l TDZ. This data was obtained after 25 days of inoculation.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Fungus</th>
<th>Concentration of elicitor (gm/l)</th>
<th>Survival rate %</th>
<th>Fresh weight (gms)</th>
<th>Dry weight (gms)</th>
<th>Growth index</th>
<th>Anthocyanin Content (µg/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aspergillus niger</td>
<td>0</td>
<td>86</td>
<td>2.08</td>
<td>0.49</td>
<td>5.30</td>
<td>56.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>60</td>
<td>1.04</td>
<td>0.23</td>
<td>2.15</td>
<td>74.5</td>
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<td></td>
<td></td>
<td>0.25</td>
<td>65</td>
<td>1.26</td>
<td>0.28</td>
<td>2.81</td>
<td>80.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>80</td>
<td>1.68</td>
<td>0.39</td>
<td>4.09</td>
<td>105.4</td>
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<tr>
<td></td>
<td></td>
<td>0.75</td>
<td>72</td>
<td>1.32</td>
<td>0.31</td>
<td>3.00</td>
<td>92.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>63</td>
<td>1.24</td>
<td>0.27</td>
<td>2.75</td>
<td>75.7</td>
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<tr>
<td>2</td>
<td>Aspergillus flavus</td>
<td>0</td>
<td>86</td>
<td>2.08</td>
<td>0.49</td>
<td>5.30</td>
<td>56.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>63</td>
<td>1.29</td>
<td>0.28</td>
<td>2.90</td>
<td>84.6</td>
</tr>
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<td>0.24</td>
<td>2.60</td>
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<tr>
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<td>1.0</td>
<td>67</td>
<td>1.34</td>
<td>0.31</td>
<td>3.06</td>
<td>90.0</td>
</tr>
</tbody>
</table>

In the present investigation anthocyanin content is high when biotic elicitors i.e. fungal mycelia were implemented in MS medium. Cheaper alternatives of gelling agents, carbon source are being used by various workers. In this way this work is a novel economical and also an effective method of using fungal strains from the plant’s surrounding environment such as an infected plant and garden soil. Dietrich et al. have observed elicitor from pathogenic fungus is influenced higher anthocyanin accumulation in callus tissue of M. charantia. Similar results were observed in this experiment with the pathogenic fungus, A. flavus.

ACKNOWLEDGEMENT

Authors are thankful to the Management of Vael’s Educational Trust, Chennai, Tamilnadu, India, for providing the infrastructure for the present study.

REFERENCE:

Research Article

In vitro development of Salt Tolerant Plants in Scoparia dulcis L.

Karthikeyan Subbarayan¹, Sarasnya Viswanathan¹, Gowri Karthik¹, “Rajasekaran Murugan” and Gomathe Ravichandran¹

Abstract

An effective tissue culture system to regenerate Scoparia dulcis (Scrophulariaceae), an important multipurpose - medicinal herb was established. The optimized protocol resulted in plant formation from nodal explants via organogenesis. Callus induction was obtained on MS basal medium supplemented with 2.0 µM TDZ and 3% sucrose. Proliferation and elongation of shoots - derived from callus - was achieved on medium supplemented with 4.0µM TDZ and 1.0 µM IBA. Rooted shoots were successfully acclimatized and transferred to soil. Callus cultures were also screened for salt tolerance using direct selection methods and their growth responses were examined. Despite the presence of salt in culture medium resulting in the loss of regeneration potential in callus, plantlets were obtained from culture media with 17.2 mM, 34.4 mM and 51.6 mM NaCl and subsequently regenerated in salinity soil. The salt tolerant callus was showed comparatively lesser fresh weight, dry weight and protein content. The development of salinity tolerant plants was achieved from 17.2 mM NaCl containing medium with 64% survival rate.

Key words: Scoparia dulcis, salt tolerant plant, NaCl, thidiazuron, callus

Introduction

Callus and cell culture possess an enormous potential for the plant improvement and can also be used as a tool for various in vitro analysis (Silva et al., 2005). Scoparia dulcis has been used as a remedy for treating diabetes mellitus in India and hypertension in Taiwan. It has been revealed that numerous chemical studies on isolated chemicals from S. dulcis that includes coumarins, phenols, saponins, tannins, amino acids, flavonoids, terpenoids and catecholamines (Hayashi et al., 1996, Hayashi et al., 1997, Hayashi et al., 1999, Ratnasooriya et al., 2005). Most plant species are particularly sensitive to a low concentration of sodium. The potential of physiological responses to differentiate between salt sensitive and salt tolerant species or individuals is now being examined. Or considerable use for the development of salt tolerance in S. dulcis depends on the availability of a technique that could identify salt tolerance within a species. Looking at such a response in vitro may assist in providing information about the salt tolerance mechanisms, leading to further capacity to increase salt tolerance (Sharry and Abedini, 2001). Although callus induction of S. dulcis has been already investigated (Nkembo et al., 2005; Saitoh et al., 2007), there are no reports on the effects of salt on plant development. The aim of the first part of this work was to develop an efficient and reproducible protocol for in vitro propagation via organogenesis of S. dulcis in order to obtain vigorous plants that might be used for commercial purpose. Second was to examine the possibility of obtaining NaCl tolerance in S. dulcis through the in vitro selection of callus, and their subsequent regeneration. Comparative growth characteristics of the callus culture and to follow the dynamics of the proteins synthesis during growth were determined.

Materials and Methods

Collection of explants and Surface sterilization

S. dulcis were collected freshly from Madras Christian College campus, Chennai, India. Nodal segments were used as the explant source. Explants were surface-sterilized in 0.1 % mercuric chloride, and rinsed three times in sterile distilled water.

Culture media and growth conditions

Murashige and Skoog (1962) vitamins, macro and micronutrient at full concentration with 3% (w/v) sucrose gelled with 8 g/L agar was used throughout the experiment. The pH of
all media was adjusted to 5.8-6.0 and sterilized by autoclaving for 17 min at 121°C at 15 lbs. The cultures were maintained at 25±2°C under a 16/8 h (day/night) photoperiod with light supplied at an intensity of 3000 lux. Plantlets were grown under continuous cool, white light fluorescent tubes (General Electric) at 25°C in a plant tissue culture chamber before their transplantation into the greenhouse.

Calium induction and shoots proliferation

The freshly harvested leaf and nodal explants was cultured on MS medium supplemented with 2.0 μM thidiazuron (TDZ). The freshly formed calium was subcultured after 21 days for shoot proliferation on basal MS medium supplemented with 4.0 μM TDZ and 1.0 μM indole butyric acid (IBA) (Karshikeyan and Prasad, 2009).

Rooting and gardening

Elongated shoots - 4-5 cm in length with 4 or 5 compound leaves were transferred to the MS media supplemented with 2.5 μM IBA (Karshikeyan et al., 2009). The formation of healthy shoots and roots made sure that this is ready to harden. The rooted plants were removed from the culture tubes, washed with sterile distilled water, and transferred to pots with sterile cow dung: coco peat: sand (1:1:1 v/v). The plantlets were placed at 70% to 80% humidity, 25 ± 2°C under a 12-hours photoperiod for acclimatization. After the plants get acclimatized, the plants were transferred to pots with farmyard mixture: sand (1:1 v/v) and placed in the green house (Karshikeyan et al., 2009). After three weeks of development, these hardened plants were transferred to the field, and the survival rate was recorded. Twenty cultures were used per treatment and each experiment was repeated at least three times. Percentage of success was scored four weeks after culture.

In vitro selection of salt tolerance

In vitro selection of salt tolerant plants of S. dulcis has been accomplished by screening highly morphogenic explants cultured on high NaCl media. Media were prepared with different concentrations of NaCl (17.2, 34.4, 51.6, 68.8 mM). The treatments in which calium survived were subcultured for shoot elongation. The shoots were put on rooting medium with the addition of NaCl. Data on survival, organogenesis and fresh weight were recorded after each week of culture. Regenerated plants were transferred to saline soil for acclimatization and hardening.

Determination of calium growth and protein determination

The development of calium was monitored at 4 days intervals up to 32 days and a growth curve was determined based in fresh and dry weight of the calium. The calium obtained after 28 days were lyophilized, macerated and submitted to extraction with 0.1 M glycine-HCl pH 2.6, 0.1 M sodium acetate (Na2OAc) pH 4.0, 0.1 M Na2OAc, pH 6.0, 0.1 M Tris-HCl, pH 8.0 and 0.1 M Na-borate pH 10.0 buffers, while all the buffers contains 0.15 M NaCl. The samples were sonicated for 1 hour and clarified by centrifugation at 12,000 x g for 20 min at 4°C. The clear supernatants were used for determination of soluble protein content. The protein content in the different fractions were performed according to Bradford (1976), using bovine serum albumine (BSA) as standard.

Results and Discussion

Calium induction and shoot proliferation

The induction of calium from nodal explants were effective on MS medium supplemented with 2.0 μM TDZ (Fig. 1A). When portions of these calium were subcultured for shoot elongation. The elongation of shoots were effective on medium supplemented with 0.8 mg/L TDZ and 0.2 mg/L IBA (Fig. 1B). As reported in Glycine max (L) Merr., (Kaneda et al., 1997), Rosa damascena (Kumar et al., 2001) and Artemisia judaica L. (Liu et al., 2003), the presence of TDZ in the culture medium proved vastly superior to the treatment with BAP in case of S. dulcis (Karshikeyan et al., 2009), and the rate of proliferation was significantly higher in TDZ-containing media. Maximum of 24 shoots/g of fresh weight of calium was obtained.

In vitro selection of salt tolerance

Explant necrosis was observed at 51.6 and 68.8 mM of NaCl. At 17.2 mM NaCl the response was slightly similar to the control. The gradual decline in the growth was recorded at 34.4 and 51.6 mM NaCl. In vitro selection of salt tolerant plants of Brassica juncea L. (Indian mustard) cv. Prakash has been accomplished by screening highly morphogenic cotyledon explants cultures on high NaCl media (Jain et al. 1991). In our work, high NaCl media also resulted in explant necrosis. The

Figures

1A – Nodular calium
1B – Multiple shoots
1C – Regenerated sapling
1D – Hardening in Green house
2A – Callus
2B – Multiple shoots
2C – Regenerated sapling
2D – Hardening in Green house

Control i.e. without Salt stress
17.2 mM NaCl (salt stress)
callus on 17.2 mM NaCl gave a higher growth mass and was significantly higher than other treatments (Fig. 2A). Work with callus adapted to salinity is characterized by their poor regeneration (Binh et al. 1992). The presence of high concentration of salt in the culture medium produced significant loss in regeneration potential at 68.8 mM. The loss of growth potential may be due to increased osmotic potential of the saline medium; increased osmotic potential affects water and nutrient uptake, which may in turn inhibit the metabolic activities necessary for shoot initiation and growth (Vijayan et al. 2003). Callus did not lose its regeneration capacity when a portion was subcultured onto medium with NaCl for shoot proliferation.

Comparison of callus growth and protein content

Growth curves of callus were plot based on fresh and dry weights. Callus presented sigmoid growth curves with lag, exponential, linear and stationary phases. The adaptive period (lag phase), in which the fresh and dry weight of calli increases slowly, occurred up to 3rd day in control and 5th day in 17.2 mM NaCl. According to Sragg and Allan (1993), the lag phase is considered an energy producing period. The fast growth phases (exponential and linear phases) occurred between 3rd and 15th day in control while 5th to 19th day of treatment with 17.2 mM NaCl. The exponential or biosynthetic phase is the period of maximum cellular division and greatest growth rate of the callus, while the linear phase is the period in which the cells grow but the cellular division decreases (Sragg and Allan, 1993). In stationary phase, the rate of cellular division is gradually reduced and then remains constant. According to Smith (2000), in the stationary phase occurs deprivation of nutrients in culture medium and a reduction of the O2 amounts inside the cells.

The protein content was analyzed during the callus growth. The results showed that between 0 to 10th day and 0 to 15m day the protein content was decreased in control and in treatment with 17.2 mM NaCl respectively. This reduction possibly occurred due to an adaptation period of callus in the culture medium. The increase in the protein levels between 10th and 16th day in control and 15 to 20th day in 17.2 mM NaCl of culture could be related to mitotic activity during the exponential and linear growth phases. But Gutmann et al. (1996) observed an increment in the protein levels in the cells of Larix leptolepis during the first 2 weeks of culture, followed by a decrease of this content during the following weeks. The decrease of the protein levels during to stationary phase in control and NaCl treatments correlates with the physiological stress related to diminishing nutrition in the medium, ageing and cell degeneration.

Rooting and acclimatization

Rooting of S. dulcis and its subsequent establishment in outdoor was better achieved with a low concentration of auxins. Of the various auxins tested for root induction, IBA proved to be the most effective (Tanakur et al. 1998; Karthikeyan et al., 2009) (Fig. 1C). Whole plants were grown at 27°C and 50±5% relative humidity in the culture chamber. In agreement with Tanakur et al. (1998), we observed that high relative humidity is necessary for plantlet survival. As opposed to Tanakur et al. 1998, in the current experiment, a maintenance period of high humidity was not necessary for some time after transplantation to the greenhouse. Plants were subsequently transferred to larger pots and gradually acclimated under greenhouse conditions with variable relative humidity (35-60%). Plants were phenotypically normal (Fig. 1D) and had a good growth rate. All plants transferred to soil conditions survived.

Whole plants from NaCl medium were grown under greenhouse conditions in salinity soil (Fig 2B). Their performance on saline soil was carried out to ascertain if their salinity tolerance that was expressed under in vitro conditions was also observed in saline soil.

Conclusions

This protocol established the potential to produce plantlets from leaf and nodal explants through indirect organogenesis. The saplings under salt stress were showed comparatively lesser survival and larger duration of development than the saplings under control. The callus under salt stress was showed comparatively lesser fresh weight also protein content than the control. Plant regeneration with a high percentage conversion of explants to plants will allow adjustment of the methodology to use in vitro selection for abiotic stress resistance. In addition, successful differentiation and multiplication of shoots and subsequent rooting of S. dulcis shows the feasibility of this technique for large-scale production of planting stock. It is very important to note that high shoot proliferation rate, conversion to plants and survival under field conditions was observed in this present study. There has been much interest in the development of medicinal plants tolerant to a biotic stress, primarily salinity. This study provides an understanding of the response of callus to salinity, which is important for future studies aimed at developing strategies for selecting and characterizing somaclonal variants tolerant to salt stress. The overall conclusion that can be drawn from this work is that in vitro screening is clearly effective for developing salinity-adapted regenerated plants of S. dulcis.

<table>
<thead>
<tr>
<th>NaCl mM</th>
<th>Fresh weight g</th>
<th>Dry weight g</th>
<th>Protein mg/g</th>
<th>In vitro plant regeneration (days)</th>
<th>Hardening (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.9±0.73</td>
<td>0.52±0.94</td>
<td>18.2±0.88</td>
<td>63</td>
<td>42</td>
</tr>
<tr>
<td>17.2</td>
<td>1.6±0.45</td>
<td>0.45±0.64</td>
<td>16.8±0.62</td>
<td>89</td>
<td>54</td>
</tr>
<tr>
<td>34.4</td>
<td>0.9±0.86</td>
<td>0.27±0.58</td>
<td>15.6±0.48</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>51.6</td>
<td>0.7±0.56</td>
<td>0.15±0.66</td>
<td>15.1±0.76</td>
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</tr>
</tbody>
</table>

Table: Effect of NaCl on callus growth, protein content and plant development
Acknowledgement

Authors are thankful to the Management of Vel’s Educational Trust, Chennai, Tamilnadu, India, for providing the infrastructure for the present study.

Reference

Low-Cost Alternatives for Accelerating the Plant Cell Suspension Culture of *Momordica charantia* L.

Karthikeyan Subbarayan, Rajagopal Kalyanaraman and Shobana Vijayanand

Dept. of Biotechnology, School of Life Sciences, VELS University, Velan Nagar, Pallavaram, Chennai- 600 0117, Tamil Nadu, India

**ABSTRACT:**

The carbon source in plant cell suspension culture is a very important factor for growth and development. In the present investigation the role of different carbon sources such as analytical grade sucrose (Hi-Media, India), commercial grade sugar, sugarcubes and jaggery (both purchased from local market) were studied in cell growth of *Momordica charantia* in an effort to reduce the cost instead of using high cost carbon source. Studies have revealed that significant improvement was observed in cell growth on a medium containing sugarcubes. More callus could be induced successfully from leaf and shoot tissues when cultured on MS medium supplemented with two combinations of growth regulators influence (Kin+IAA and BA+IAA). More fresh weight and the best friability of callus was obtained from leaf explants on MS medium containing 1.0 mg/l IAA and 0.5 mg/l Kin with semi-solid medium (0.6% agar). The cell suspension culture promoted the best cell growth in medium containing sugarcubes with biomass of 8.245 g fresh weight and 0.298 g dry weight in 24 days of inoculation. This is the first report using four different carbon sources in plant cell suspension cultures. The results showed that out of four carbon sources tested, sugarcubes could be the best source of carbon and it potentially reduces the cost of using costly carbon sources in cell suspension cultures.

**KEYWORDS:** callus, carbon source, kinetin, sugarcubes,

**INTRODUCTION:**

Micropropagation and Cell culture technology have been widely applied for the production of large number of economically important plants and secondary metabolites. Despite advancements in synthetic chemistry, we still depend upon biological sources for a number of secondary metabolites including pharmaceuticals. Commercialization of a large number of such technologies has been hampered by high production cost. The cost of components of tissue culture medium has been another concern for most commercial laboratories. Agar and sucrose are the chief constituents which play a significant role in cost of production. Attempts have been made for the use of cheaper alternatives of agar and sucrose. Commercial grade sugar could replace analytical grade sucrose, with no significant change in the frequency of shoot formation in banana. Plant cell cultures are typically grown as cell suspension cultures in liquid medium without agar or as callus cultures on solid medium. *Momordica charantia* L., native of Indo-Malayan region is an important pharmaceutical medicinal plant of Cucurbitaceae family, due to the high content of biologically active substances. In various systems of traditional medicines, *M. charantia*, named bitter melon, is used for several ailments: anti-diabetic, jaundice, contraceptive, piles, pneumonia, abdominal pain and anti-cancerous.
The fruits of bitter gourd contain very high amount of vitamins A and C, iron and minerals \(^{13}\). The aim of the present investigation was to test low cost alternatives of sucrose in cell suspension culture. Till date, there is hardly any report on the usage of commercial grade in plant cell suspension cultures.

**MATERIALS AND METHODS:**

**Standardization of Hormone concentration for callus induction:**

*M. charantia* plants collected from the herbal garden of the VELS University, Chennai, India. Fresh healthy plants were used as explants for the establishment of callus culture. The explants were first washed with detergent several times and rinsed under running tap water. They were then immersed in 70% (v/v) ethanol for five seconds before surface-disinfection in 0.1 % HgCl\(_2\) solution under continuous agitation for 2 min. They were subsequently rinsed three times with sterile distilled water and then surface-disinfected again with 20 % (v/v) with the addition of three drops of Tween-20 (polyoxyethylene sorbitan monolaurate) for 20min. After three rinses with sterile distilled water, they were cultured in Murashige and Skoog basal medium (MS) supplemented with 30 g \(^{-1}\) Analytical grade sucrose (Hi-Media, India) and 7.5 g \(\text{L}^{-1}\) agar (Hi-Media, India) for 4 weeks. A total of 16 combinations of IAA and BAP/Kin were tested for each type of explant.

**Effect of carbon source and agar concentrations for callus induction:**

Fresh explants were surface sterilized in the same manner as described in the previous section. The leaf explants were inoculated in MS medium supplemented with 1.0 mg L\(^{-1}\) IAA and 0.5 mg L\(^{-1}\) Kin, the best growth regulator combination determined for callus induction that is, standard induction (SI) medium. Different carbon sources such as analytical grade sucrose (Hi-Media, India), commercial sugar, sugarscubes and jaggery (both purchased from local market) were added to medium at 3% concentration. With the efficient carbon source detected, experiments were also conducted with semi-solid medium (0.6% agar). The experiment was repeated three times. The average fresh weight of callus formed from each medium was recorded after 6 weeks. The number of explants that produce compact type or friable type of callus was also determined.

**Effect of carbon source for cell culture establishment and study of the growth kinetics of the cell culture:**

Friable callus induced from leaf explants were inoculated into 100 ml Erlenmeyer flasks containing 20 ml of standard induction (SI) medium with sugar cubes. Analytical grade sucrose was taken as control. All the cultures were placed in a culture room with temperature regulated at 25±2°C and maintained under 24 h light provided with cool white fluorescent lamps at 3000 lux. All the cell cultures were placed on a rotary shaker with a speed of 125 rpm. The fresh weight of the cell biomass was determined from each flask. Three culture flasks were taken randomly every 3 days over a 27 days period to determine the cell growth pattern and the optimum inoculum density for best cell growth. The cells were harvested by filtering the cell suspension cultures through filter paper (Whatman No. 1, diameter 90 mm) using a filter funnel (90 mm) connected to a vacuum pump. The average fresh weights of the cell biomass from three cell suspension culture flasks were taken every 3 days over a 30 days period. The dry weights of harvested cell were recorded after 10 days of air-drying until a constant weight was obtained.

**RESULTS:**

**Standardization of Hormone concentration for callus induction:**

Our preliminary test indicated that the leaf explants of *M. charantia* showed good response towards friable callus formation when cultured on MS medium supplemented with 1.0 mg L\(^{-1}\) IAA plus 0.5 mg L\(^{-1}\) Kin. Formation of compact callus was observed in case of shoot explants. Since friable callus is finely dispersed in suspension cultures, it was used for subsequent experiments. Thus, this study indicated that the type of explants did influence the formation of callus in *M. charantia* in agreement with many other studies\(^{11,12}\). Based on the fresh weight of callus formed from each type of explant in 6 weeks, the leaf explants were found to promote the most callus tissue than stem explants. In Table 1, some of the data from this study was described that is, only the best five media for the induction of callus for each type of explant. The results showed that MS medium supplemented with 1.0 mg L\(^{-1}\) IAA plus 0.5 mg L\(^{-1}\) Kin induced the most calluses growth (2.24 g) from leaf explants in 6 weeks (Table 1). For the leaf explants, culture medium without IAA resulted in less friable callus.

**Table 1. Callus formation from different explants of *M. charantia* after 6 weeks culturing on MS medium supplemented with different concentrations of BAP/Kin (0.0–2.0 mgL\(^{-1}\)) and IAA (0.0–4.0 mgL\(^{-1}\)) with 3.0% Sucrose AR grade and 0.8% Agar**

<table>
<thead>
<tr>
<th>Explant</th>
<th>Hormone concentration (mgL(^{-1}))</th>
<th>Fresh weight (g) ±SE</th>
<th>Average percentage (%) ± S.E. (texture)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leaf</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 IAA</td>
<td>1.55 ± 0.04</td>
<td>31.6 ± 0.32</td>
<td>68.4 ± 0.28</td>
</tr>
<tr>
<td>1.0 IAA</td>
<td>2.05 ± 0.11</td>
<td>10.8 ± 0.38</td>
<td>89.2 ± 0.22</td>
</tr>
<tr>
<td>1.0 IAA +0.5 Kin</td>
<td>2.24 ± 0.08</td>
<td>7.2 ± 0.28</td>
<td>92.8 ± 0.26</td>
</tr>
<tr>
<td>1.0 IAA +1.0 Kin</td>
<td>1.95 ± 0.14</td>
<td>14.8 ± 0.26</td>
<td>85.2 ± 0.21</td>
</tr>
<tr>
<td>1.0 IAA +0.5 BAP</td>
<td>1.92 ± 0.09</td>
<td>13.6 ± 0.36</td>
<td>86.4 ± 0.32</td>
</tr>
<tr>
<td>1.0 IAA +0.5 BAP</td>
<td>1.92 ± 0.15</td>
<td>38.4 ± 0.34</td>
<td>61.6 ± 0.34</td>
</tr>
<tr>
<td>1.0 IAA +1.0 Kin</td>
<td>1.76 ± 0.06</td>
<td>33.6 ± 0.31</td>
<td>66.4 ± 0.23</td>
</tr>
<tr>
<td>1.0 IAA +0.5 BAP</td>
<td>1.78 ± 0.12</td>
<td>82.4 ± 0.34</td>
<td>17.6 ± 0.31</td>
</tr>
<tr>
<td>1.0 IAA +0.5 BAP</td>
<td>1.78 ± 0.12</td>
<td>82.8 ± 0.41</td>
<td>17.2 ± 0.24</td>
</tr>
<tr>
<td><strong>Stem</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 IAA</td>
<td>1.36 ± 0.13</td>
<td>31.6 ± 0.32</td>
<td>68.4 ± 0.28</td>
</tr>
<tr>
<td>1.0 IAA</td>
<td>1.88 ± 0.08</td>
<td>38.4 ± 0.34</td>
<td>61.6 ± 0.34</td>
</tr>
<tr>
<td>1.0 IAA +0.5 Kin</td>
<td>1.92 ± 0.15</td>
<td>33.6 ± 0.31</td>
<td>66.4 ± 0.23</td>
</tr>
<tr>
<td>1.0 IAA +1.0 Kin</td>
<td>1.76 ± 0.06</td>
<td>82.8 ± 0.41</td>
<td>17.2 ± 0.24</td>
</tr>
<tr>
<td>1.0 IAA +0.5 BAP</td>
<td>1.78 ± 0.12</td>
<td>73.2 ± 0.35</td>
<td>26.8 ± 0.25</td>
</tr>
</tbody>
</table>
Most of the leaf explants (92.6%) that were cultured on MS + 1.0 mgl⁻¹ IAA + 0.5 mgl⁻¹ Kin produced friable callus; hence it was considered as the best medium for callus induction from the leaf explants. But this medium was not the best medium for the induction of friable callus using stem explants, 1.92 g of compact callus was produced instead (Table 1).

Effect of carbon source and agar concentrations for callus induction:
The standard induction (SI) medium containing 3.0% AR grade sucrose and 0.8% agar was used as control for callus induction from leaf explants. All the carbon sources (AR grade sucrose, commercial sugar, sugarcubes and jaggery) at their 3.0% concentration evoked almost similar response during 1st subculture. However, a marked difference was noticed when the cultures were maintained for three consecutive passages on same fresh medium. The type of carbon source and number of subculture showed a significant interaction. A higher induction rate than the control was obtained on medium containing sugarcubes. The other carbon sources showed a decline in the rate of induction; at least one is by jaggery (Table 2). But the callus cultures on all the four carbon sources were apparently green without any sign of deterioration even after three passages. Reducing agar concentration with semi-solid agar (0.6%) in the SI medium did not cause any reduction in induction rate. Interestingly, a combination of semi-solid medium with sugarcubes caused increment in the rate of induction (Table 3). Both semi-solid medium and sugarcubes showed no adverse effect on the callus cultures. Hence, based on biomass and friable type of callus formed from leaf explants on MS medium supplemented with 1.0 mgl⁻¹ IAA + 0.5 mgl⁻¹ Kin (SI medium) with 3.0% AR grade sucrose/sugarcubes in semi-solid medium was found to be the most suitable culture medium for the production of callus that could be used as material source for the preparation of cell suspension culture of M. charantia.

Effect of carbon source on cell culture establishment and the growth kinetics of the cell culture:
The cells of M. charantia were found to grow well in liquid MS medium that contained the same combination of plant growth regulators (1.0 mgl⁻¹ IAA + 0.5 mgl⁻¹ Kin) as in solid/semi-solid medium for callus induction and maintenance. A higher induction rate than the control was obtained on medium containing sugarcubes. The results indicated that 0.75 g inoculum in 20 ml culture medium gave the best and typical growth kinetics. The culture reached the growth peak on the 24th day and declined after that. The maximum increase of cell biomass with sugarcubes was almost 8.245 g after 24 days of culture. AR grade sucrose produced only 7.825 g of cell biomass within the same period (Table 4). The lag phases were short (6 days) for both fresh weight and dried weight growth curve but the following log phase were different. Fresh weight of the cell culture reached its maximum biomass (7.985 g) with sugarcubes after 18 days of culture followed by the stationary growth phase. The maximum dry weight (0.255 g) of the culture occurred 15 days after inoculation followed by a gradual decline of growth.

### Table 2. Effect of different C-sources on callus induction from leaf explants of M. charantia grown on Standard Induction (SI) medium and 0.8% agar

<table>
<thead>
<tr>
<th>Standard Induction (SI) medium + different C-sources at 3% concentration</th>
<th>Fresh weight (g) ±SE</th>
<th>I subculture</th>
<th>II subculture</th>
<th>III subculture</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose AR</td>
<td>2.24 ± 0.05</td>
<td>2.28 ± 0.11</td>
<td>2.22 ± 0.12</td>
<td>2.25 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Sugarcubes</td>
<td>2.42 ± 0.04</td>
<td>2.38 ± 0.09</td>
<td>2.44 ± 0.08</td>
<td>2.41 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Commercial sugar</td>
<td>2.02 ± 0.07</td>
<td>1.97 ± 0.12</td>
<td>1.94 ± 0.11</td>
<td>1.98 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Jaggery</td>
<td>1.84 ± 0.09</td>
<td>1.81 ± 0.14</td>
<td>1.72 ± 0.13</td>
<td>1.79 ± 0.06</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. Effect of different agar concentrations on callus induction from leaf explants of M. charantia grown on Standard Induction (SI) medium with 3.0% sucrose AR/sugarcubes

<table>
<thead>
<tr>
<th>Standard Induction (SI) medium + different concentrations of agar (%)</th>
<th>Fresh weight (g) ±SE after subculture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sucrose AR</td>
</tr>
<tr>
<td>0.8</td>
<td>2.24 ± 0.08</td>
</tr>
<tr>
<td>0.75</td>
<td>2.25 ± 0.07</td>
</tr>
<tr>
<td>0.7</td>
<td>2.29 ± 0.12</td>
</tr>
<tr>
<td>0.65</td>
<td>2.29 ± 0.05</td>
</tr>
<tr>
<td>0.6</td>
<td>2.32 ± 0.14</td>
</tr>
</tbody>
</table>

### Table 4. Cell biomass of M. charantia after 24 days culture in liquid MS medium supplemented with 1.0 mgl⁻¹ IAA + 0.5 mgl⁻¹ Kin and 3.0% sucrose AR/sugarcubes

<table>
<thead>
<tr>
<th>Carbon sources</th>
<th>Cell biomass (g)</th>
<th>Fresh weight</th>
<th>Dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose AR</td>
<td>7.985 ± 0.06</td>
<td>0.291 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Sugarcubes</td>
<td>8.245 ± 0.08</td>
<td>0.298 ± 0.06</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION:**
This study has demonstrated that best callus induction rate could be achieved on the medium containing sugarcubes as carbon sources, replacing analytical grade sucrose used in the control experiments. Promoting role of sugarcubes in tissue culture has been observed in Leucaena leucocephala, Vitex negundo and Achras sapota. However, Use of LR grade sucrose and sugarcubes in Curcuma longa cultures was recommended. Incorporation of jaggery in the medium during the present study was not useful. Similar results like this in micropropagation of Wrightia tomentosa.

Cheaper alternatives of gelling agents used by various workers have suffered from one or the other drawback limiting their use on commercial scale. In the present study, semi-solid (0.6%) agar similar response as that of control which is 0.8% agar in standard induction medium at the same concentration. Combination of semi-solid medium with sugarcubes in SI medium in the
The present study has also demonstrated that the cost of plant cell culture of M. charantia could be reduced significantly by the incorporation of sugarcubes and semi-solid agar. The prepared cell suspension cultures will be used as the material source for the production of secondary metabolites study in the forthcoming research activity.

ACKNOWLEDGEMENT:
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REFERENCE:
Homology Based Inhibitor Design For Naja melanoleuca Short Neurotoxin Using Plant Based Drugs

*Gowri Karthik*, Vidya Venkatasubramanian, Gomathi Ravichandran, Karthikeyan Subbarayan, and Deepa Nagarajan

Abstract

Plants are one of the most widely used medicines in the world today due to its less side effects over synthetic drugs. Anabasine derived from Anabasis sp. and Papaverine derived from Papaver somniferum were investigated for skeletal muscle relaxant activity against Naja melanoleuca short neurotoxin. This neurotoxin produces peripheral paralysis by blocking neuromuscular transmission at the post synaptic site and binds to the muscular nicotinic acetylcholine receptor which was chosen for structure prediction using homology modeling. The sequence was retrieved from snake neurotoxin database and was modeled using MODELLER9-2. The quality of the modeled structure was validated using Structural Analysis and Verification Server. The interaction between the predicted structure of short neurotoxin and the inhibitors were analysed in silico by ARGUSLAB. The investigation reveals that Anabasine has maximum inhibitor activity of energy value (-5.98 KJ/Mol) against the targeted protein.

Keywords: Short neurotoxin, Naja melanoleuca, MODELLER9-2, ARGUSLAB, Anabasine.

Introduction

Snake envenomation is a socio-medical problem of considerable magnitude. About 2.5 million people are bitten by snakes annually, more than 100,000 fatally (Koh et al., 2006). Snake venoms are complex mixtures of proteins, nucleotides and inorganic ions. These combinations confer a formidable array of toxic properties on the venom, the peptides and polytoxins being responsible for a variety of toxic properties. The number of venom components in venomous animals like snake, scorpion or cone snail ranges from 50-200 toxins (Tan et al., 2003). Snake venoms are important tools in toxicology, neuroscience, and pharmacology. The venom components are highly variable and functionally complex and they offer many research opportunities (Joyce et al., 2004). Naja melanoleuca inhabited mainly primary and secondary forest patches, but also plantations and suburbs (Luiselli et al., 2000).

The main toxins from snake venom that affect the CNS are neurotoxins. Neurotoxins form one of the largest families of proteins with established primary structures. Snake neurotoxin is a toxic agent or substance that inhibits damages or destroys the tissues of the nervous system and neurons. Neurotoxic proteins isolated from various snake venoms have high affinity for a particular target site and are used extensively as pharmacological tools to gain insights into the function of the nervous system. The vast majority of snake venom neurotoxic peptides competitively bind to the nicotinic acetylcholine receptor. The potency of these molecules lies in their affinities towards the biomolecules involved in the functioning of neuromuscular transmission. Nicotinic acetylcholine receptors are prototypes for the pharmacologically important family of pentameric ligand-gated ion channels (Celie et al., 2004). Among the best studied snake neurotoxins are the α-neurotoxins that bind to nicotinic acetylcholine receptors (nAChR). They are capable of reversibly blocking nerve transmission by competitively binding to the nAChR located at the postsynaptic membranes of skeletal muscles and neurons, preventing neuromuscular transmission and thereby leading to death by asphyxiation (Teetin et al., 2004). Short- and long-chain neurotoxins from snake venoms are potent blockers of nicotinic acetylcholine receptors (nAChR). Short neurotoxin from Naja melanoleuca belongs to snake toxin family. It belongs
to the type 1 alpha neurotoxin super family. The neurotoxin produces peripheral paralysis by blocking neuromuscular transmission at the post synaptic site and binds to the muscular nicotinic acetylcholine receptor. Short neurotoxins consist of 60–62 amino acid residues and include 4 disulfide bridges, whereas long neurotoxins have 66–75 residues and 5 disulfides (Mordvintsev et al., 2006). Sequence annotations, functional and structural data on snake venom neurotoxins (svNTx) are scattered across multiple databases and literature sources. Sequence annotations and structural data are available in public molecular databases, while functional data are almost exclusively available in published articles (Joyce et al., 2004).

Fossil records revealed that the human use of plants as traditional medicine date back to middle Paleolithic age, approximately 60,000 years ago (Solecki et al., 1975). At present, natural products (and their derivatives and analogs) represent over 50% of all drugs in clinical use, in which natural products derived from higher plants represent ca. 25% of the total. The World Health Organization estimated that over 80% of the people in developing countries rely on traditional remedies such as herbs for their daily needs and about 85% traditional medicines include crude plant extracts. This means that about 3.5 to 4 billion of the global population rely on plants resources for drugs (Farnsworth, 1988). Medicinal plants have provided a good source of a wide variety of compounds, such as phenolic compounds, nitrogen compounds, vitamins, terpenoids and some other secondary metabolites, which are rich in valuable biochemical activities (Mariadass et al., 2008). In this study two medicinal plant compound Anabasin derived from Anabaena sp. and Papaverine derived from Papaver somniferum investigated for skeletal muscle relaxant activity against Naja melanoleuca short neurotoxin. The minor tobacco alkaloid anabasine has high affinity for nAChRs and act as agonists in most receptor assays (Dwojak et al., 1995). Papaverine is an opium alkaloid used primarily in the treatment of visceral spasm. Papaverine used as a smooth muscle relaxant in microsurgery where it is applied directly to blood vessels.

Materials and methods

Snake venom neurotoxin database (svNTxB)

The svNTxB provides a unique compilation of these toxins collected from public databases and literature sources. Each entry had been analyzed for possible errors and inconsistencies, and annotated with functional information. Short neurotoxin sequence of database Entry ID 6773 was retrieved through querying searchable on-line database of NTX proteins sequences.

Template identification for the target molecule

The template may be a predefined layout to give an idea about the unknown structure of the query molecule (Brinda et al., 2009). The NCBI BLAST was used to identify the template for modeling the three dimensional structure of short neurotoxin. The sequence of the target molecule in FASTA format was submitted for blastp against pdb database which yields that alpha toxin from Naja nigricollis (PDB ID: 1NEA) as a suitable template. The target and the template have 53% of residues identical with an E-value of 4e-18.

Homology modeling

Among all current theoretical approaches, comparative modeling is the only method that can reliably generate a 3D model of a protein from its amino acid sequence. Modeling of protein structures usually requires extensive expertise in structural biology and the use of highly specialized computer programs for each of the individual steps of the modeling process (Tramontano et al., 2001). The method of homology modeling is based on the observation that protein tertiary structure is better conserved than amino acid sequence. (Renom et al., 2000). The three dimensional structure of short neurotoxin has been predicted using MODELLER9v2 (http://www.salilab.org/modeller/).

Model refinement and evaluation

The model generated by MODELLER9v2 was subjected to energy minimization using the steepest descent technique to eliminate bad contacts between protein atoms using swiss pdb viewer. Validation of modeled structure was carried out using Structure Analysis and Validation Server. It performs structure validation calculations using PROCHECK, PROVE, Vary3D, ERRAT and WHAT IF programs. The validated result of the modeled protein from the server is an important part of comparative modeling process.

Active site prediction

After obtaining the final model, the possible binding sites of short neurotoxin were searched using Computed Atlas of Surface Topography of Proteins (CASTp) (http://cast.engr.uiuc.edu/). These include pockets located on protein surfaces and voids buried in the interior of proteins. CASTp includes a graphical user interface, flexible interactive visualization, as well as on-the-fly calculation for user uploaded structures. (Binkowski et al., 2003)

Docking the inhibitors against the active site of the Short Neurotoxin

Docking is a computational technique that samples conformations of small molecules in protein binding sites; scoring functions are used to assess which of these conformations best complements the protein binding site (Warren et al., 2006). The inhibitor and target protein was geometrically optimized and docked using docking engine ArgusDock (http://www.arguslab.com/).
Results and Discussion

The homology-based inhibitor design for the target short neurotoxin involved in paralysis is as follows.

Sequence retrieval of short neurotoxin

The protein sequence was retrieved from snake neurotoxin database and has database entry ID of 6773 was shown in Tab. 1.

<table>
<thead>
<tr>
<th>Virulence Factor Sequence Info ID</th>
<th>Sequence Name</th>
<th>Protein or Nucleic</th>
<th>Virulence Factor ID</th>
<th>Reference ID</th>
<th>Database Entry ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>12266</td>
<td>Short neurotoxin 1 (Neurotoxin ID)</td>
<td>Protein</td>
<td>10685</td>
<td>1</td>
<td>6773</td>
</tr>
</tbody>
</table>

Table 1: Short neurotoxin protein details

Homology modeling of the target protein

The absence of the three-dimensional structure for Naja melanoleuca short neurotoxin in PDB prompted us to construct the 3D model of the protein. The three-dimensional structure provides information into the function and also helps us to analyses of its interactions with the suitable inhibitors. 1IQ9 alpha toxin from Naja nigricollis was chosen as template. The alignment of template and the target protein was performed using the script „align2d.py“ which was shown in Fig. 1.

Among the five models generated using the script „model single.py“, the thermodynamically stable model was chosen for further refinement and validation. After the final refinement the modeled protein shows the energy minimization value of -3452.636 KJ/Mol. The energy minimization value suggests that the protein was well refined. The final 3D structure of short neurotoxin obtained after energy minimization was shown in Fig. 2.

Validation of the predicted structure

The refined structure was submitted in Structure validation and analysis server for validation. Ramachandran plot was used to visualize dihedral angles \( \Psi \) against \( \Phi \) of amino acid residues in protein structure (Ramachandran et al., 1993). From the Fig. 3, it was concluded that there was no presence of disabled amino acids.

Active site prediction

Among the nineteen binding sites obtained from Castp server site 1 was chosen as best site for the inhibitor binding. The binding pocket containing the residues Met1, Gln2, Cys3, Cys17, Thr21, Asn22, Cys23, Cys54, Thr55, Thr56 which are shown in Fig. 4 and this was chosen for docking analysis.

![Image of alignment between the target protein and the template](image1)

![Image of Ramachandran plot showing the position of amino acids](image2)

![Image of Green color indicating the active site chosen for docking analysis](image3)
Docking between the target protein and the drug molecule:

Owing to the growing number of identified snake venom neurotoxin sequences, it is increasingly difficult to study them by experimentation alone. Detailed bioinformatics analysis offers a convenient methodology for efficient in silico preliminary analysis of possible function of new toxins (Joyce et al., 2004). The high specificity of neurotoxins for nACHRs has been utilized as a tool in understanding the structure and function of the nervous system (Keh et al., 2006). The two inhibitors Anabasine derived from Anabasis sphyila and Paevarine derived from Papaver somniferum were shown in Fig. 5.

![Fig. 5 Structure of the inhibitors](image)

The scoring function takes a pose as input and returns the number indicating the likelihood that the pose represents a favourable binding interaction. A low (negative) energy indicates a stable system and thus a likely binding interaction (Kitchen et al., 2007). Fig. 6 shows the binding of ligand with the receptor molecule. The docking energy value of Anabasine and paevarine was -5.9846 KJ/Mol and -2.6015 KJ/Mol respectively. The RMS score value is 0.40.

Anabasine has formed one hydrogen bond interaction with Thr 21 and two bonds with Thr 55(2.6, 2.8, 2.8), of the active site of the target molecule. Paevarine has hydrogen bond interaction with of Glu 2 and Thr 55(2.4, 2.7) of the target protein, which was shown in Fig. 7.

![Fig. 6 shows the binding of the ligand with the receptor molecule.](image)

![Fig. 7 Hydrogen bond formation of the inhibitor to the target molecule.](image)

The specificity of the drug and target protein varies depending upon their binding sites. The docking results show that the compound anabasine has higher specificity and efficiency towards the target protein short neurotoxin, which causes paralysis.

Reference


