CHAPTER-I

ISOLATION AND IDENTIFICATION OF FUNGAL ENDOPHYTES FROM RHYNCOSIA BEDDOMEI AND THEIR SCREENING FOR IAA (INDOLE ACETICACID) AND GIBBERELLIN PRODUCTION

RESULTS

In this study a total of 222 fungal endophytes were isolated from 300 leaf and stem segments (150 each) of the R. beddomei an endemic plant to Tirumala Hills. Total 222 endophytic fungal isolates obtained 123 endophytes were isolated from leaf and the remainder (99) from stems segments.

All the isolated endophytic fungi were assigned to 15 species in 15 genera, of these, 8 sporulating strains (designated as IS1, IS2, IS3, IS4, IS5, IS6, IS7 and IS8) were identified based on their morphological characteristics and the other non-sporulating strains were grouped into seven morphotypes (these fungal isolates were designated as ENT9, ENT10, ENT11, ENT12, ENT13, ENT14, ENT15). Among the morphologically identified fungi, 8 isolates belonged to 8 different taxa (Alternaria alternata, Aspergillus aculeatus, Colletotrichum gloeosporioides, Pestalotiopsis maculans, Phyllosticta elongata, Xylaria, Phomopsis and Penicillium corylophilum)

All the morphotypes isolated were then identified based on the 18S rRNA gene sequence analysis. The PCR amplification of 18S rRNA gene was done by using universal ITS1 and ITS4 primers. An amplification product obtained for all the isolates is shown. The fungal rDNA-ITS sequences of amplified products obtained in this study were deposited in GenBank (Accession numbers: KF493862, KF493865, KF493866, KF493867, KJ542650, KJ542652, KJ542653).

Molecular identification using ITS rRNA sequences obtained from the non sporulating isolates revealed seven taxa of endophytic fungi: Aspergillus japonicus, Cladosporium delictes, Sordariomycetes sp, Fusarium equiseti, Neotyphodium sp, Phaemoniells sp, Phylostica sp. The
phylogenetic tree based on ITS-rRNA sequences of endophytic fungi associated with \textit{R. beddomei} were also shown.

**Table 1. Molecular identification of non-sporulating endophytic fungal isolates**

<table>
<thead>
<tr>
<th>Colonization rate of fungal endophytes</th>
<th>Leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total No. of samples</td>
<td>200</td>
</tr>
<tr>
<td>No. of samples yielding endophytic fungi</td>
<td>123</td>
</tr>
<tr>
<td>Colonization rate (%)</td>
<td>61.5</td>
</tr>
</tbody>
</table>

The Table 1 shows the total number of samples, number of samples yielding endophytic fungi, Colonization rates (CR %) of fungal endophytes isolated from leaf and stem segments of \textit{R. beddomei}. The Colonization rate was found to be (61.5%).

**Table 2. Molecular identification of non-sporulating endophytic fungal isolates**

<table>
<thead>
<tr>
<th>Colonization rate of fungal endophytes</th>
<th>Stem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total No. of samples</td>
<td>200</td>
</tr>
<tr>
<td>No. of samples yielding endophytic fungi</td>
<td>99</td>
</tr>
<tr>
<td>Colonization rate (%)</td>
<td>49.5</td>
</tr>
</tbody>
</table>

The table 2 shows the total number of samples 200 from the leaf and stem segments of \textit{R. beddomei} number of samples yielding endophytic fungi, Colonization rates (CR %) of fungal endophytes was found to be (49.5%).
Figure 1. Isolation frequency of endophytic fungi associated with the leaves and stems of *R. beddomei*
Significant difference in the colonization frequency (%) of endophytic fungi in both the plant tissues. *Alternaria alternate*, *Aspergillus japonicus*, *Sordariomycetes*, *Xylaria*, *Phomopsis* and *Penicillium corylophilum*,*Aspergillus aculeatus*, *Cladosporium*, *Pestalotiopsis maculans* were dominant in leaf segments. *P.corylophilum*, *C.gloeosporioides*, *A.chrysogenum*, were isolated frequently in stem segments.The endophytes *Phyllosticta elongate*, *Neotyphodium*sp were found commonly associated with both leaf and stem tissue of *R.beddomei*.

Isolation frequency on both the tissues indicates.*Alternaria alternate*, *Aspergillus japonicus*, *Sordariomycetes*, *Xylaria*, *Phomopsis* and *Penicillium corylophilum*, *Aspergillus aculeatus*, *Cladosporium*, *Pestalotiopsis maculans* were the common isolates, whereas *Phyllosticta elongate* were restricted to leaf and only *Phaemoniella sp* was found associated with the stem tissues.

*Xylaria* and *Alternaria alternate*, *Aspergillus japonicus* had the highest isolation frequencies of 8.0 and 16.14.5% respectively. whereas *Sordariomycetes*, *Penicillium corylophilum*, *Phomopsis*,*Cladosporium*, *Phyllosticta elongate* *Neotyphodium*sp, had frequencies of 14.0%,10.0%,8.1%,8.3%,4.1% respectively. *Fusarium oxysporum* had the lowest relative frequency of 1.2%.

**DISCUSSION**

Fungal endophytes live within their host plants without causing any apparent disease symptoms (Zhang *et al.*, 2006), but still this area is underexplored. Endophytes offer plethora of unknown advantages to the host with immense applications in agriculture (Clay *et al.*, 2005). Recently,their role in enhancing biomass production and stress resistance (Ghimire *et al.*, 2011) and as components of tropical community ecology (Arnold, 2007; Hyde and Soytong, 2008) have emerged. A perusal of the literature over the past decades indicated the plant species sampled from unique ecological niches species are known to harbor potential endophytic microbes.

There has been an increasing surge of interest among the research groups for the isolation of endophytes from the tropical plant species (Arnold and Lutzoni, 2007; Suryanarayanan *et al.*, 2011).One such region represents the Tirumala of Seshachalam Hills in
Eastern Ghats, India. The Tirumala hills represent rich flora with enormous species diversity as well as endemic taxa and are therefore recognized as Seshachalam biosphere reserve (Krishnaveni and Sirinivasa Rao, 2000; Arokiyaraj et al., 2008). Despite the reports of ethnomedicinal plants of this region, the biodiversity and the endophytic microbes of this region remain unexplored. Therefore, in the present investigation, *R. beddomei* representing endemic medicinal taxa was subjected to diversity studies on fungal endophytes.

In the present study, when different methods of isolation of endophytic fungi were employed, few common fungal endophytes were isolated from *R. beddomei*, an endemic medicinal plant to Tirumala Hills of Seshachalam Biosphere Reserve. The observation indicates that treatment - 3 was superior as compared to others with respect to recovery of endophytic fungi for both tissues of the plant. During the course of study it was observed that appearance of fungi from sterilized tissues decreased with increase in treatment time of surface sterilant (sodium hypochlorite). Among them, maximum fungi were recovered from tissues sterilized by sodium hypochlorite when compared to segments treated with formaldehyde. Although surface sterilant was similar in treatments - 2 and 4, treatment 4 yielded least endophytes since ethanol concentration in treatment - 4 (96%) was higher than 2 (70%). Though concentration of sodium hypochlorite was similar in treatments 2 and 4, less species of endophytes was recovered in treatment- 4. This might be attributed to the enhanced toxicity of the sodium hypochlorite because of increase in treatment time and sometimes it may be because of wetting agents (ethanol in this case), although ethanol has limited penetrating and antibiotic activity (Bills and Polishook, 1994). Adding some alcohol seems to enhance the wetting, penetrating and killing properties of NaOCl (Verma et al., 2011). Finally, the efficacy of 3% NaOCl treatment (for 4 min) for destruction of epiphytes was reaffirmed by the results. Many other reports also support this conclusion as they find the treatment-3 as most effective treatment protocol (Christine and Jonathan, 2008; Shankar and Krishnamurthy, 2013). The selection of isolation method also increases the probability of obtaining non-sporulating fungi (Gaikwad, 2011). Hence, the treatment 3 was employed for further isolation of endophytes since it yielded maximum number of endophytic fungi with no epiphytic bacteria detected.
Morphological and microscopic characterization for definitive identification of the isolates was carried out on the basis of morphological characters and growth pattern on growth media.

The colonies of isolate IS1 produced profuse mycelial growth on PDA. Mycelium was hyaline that turned to grey brownish, multicelled, septate and irregularly branched. Conidiophores arised singly and in clusters, usually long or short. They were pale olivaceous to olivaceous brown, straight or curved, geniculate, slightly swollen at apex having terminal scars indicating the point of attachment of conidia. Conidia were born in chains on conidiophores. They were light olivaceous to dark brown in colour, varied in shape from obclavate to mostly ellipsoidal, muriform having tapered apex with longitudinal and transverse septa. The chlamydospores were formed in the old culture and were intercallary, thick walled, roundish to oval in shape, dark brown in colour (Dipak et al., 2013). On the basis of these characteristics, the isolate IS8 were identified as *Alternaria alternata* respectively. *Alternaria* are well adapted to endophytic mode of life, and have been well documented in many hosts (Tian et al., 2006; Li et al., 2007; Hormazabal and Piontelli, 2009). The isolate IS1 were also further confirmed by the National Fungal Culture Collection of India, a National Facility established by Department of Science and Technology, New Delhi at Agharkar Research Institute, Pune.

In the present study isolate IS2 showed rapid growth on PDA medium and the colonies are wooly and have become compact in time. The surface colony color is grey colour. The hyphae appeared septate and hyaline. Conidiophores are hyaline and branched. Furthermore, the conidia are unicellular, round to ellipsoidal, green in color, smooth walled and are grouped in sticky heads at the tips of the phialides. Phialides are hyaline, branched, flask shaped, inflated at the base, solitary appeared in clusters, and are attached to the conidiophores at right angles (Samuels et al., 2010). However, these clusters got disrupted during slide preparation procedure for microscopic examination. The above characteristic features of the isolate IS2 have revealed that it is close in proximity to *Aspergillus aculeatus*.

The isolate IS3 shows dense aerial, initially white or cream white, becoming gray and then turning dark gray, as the cultures aged on PDA (Figure 3. IS3). Colony reverse was white to white gray. Black colour acervuli around the centre of the colony. Mycelia were branched,
septate, and hyaline. Conidia were hyaline, aseptate, and cylindrical with obtuse ends (Baxter, *et al*., 1983; Smith and Black, 1990). The characteristics appressoria have also been used for taxonomy of the genus *Colletotrichum* (Svetlana *et al*., 2010). Appressoria were also observed on the underside petri plate arising from vegetative hyphae, smooth, simple, variable, irregular to ovate, and varied from light to dark brown (Sutton, 1992). Thus it was identified as *Colletotrichum gloeosporioides*. The fast growing, cottony, whitish to grey colored colony with orange conidial mass of the *Colletotrichum gloeosporioides* was observed by Photita *et al*., (2004).

In the present study isolate IS4 conidial morphology (Figure 3.IS4) is the most widely used taxonomic character for the genus *Pestalotiopsis* (Hu *et al*., 2007). *Pestalotiopsis* research is based on endophytic isolates (Liu *et al*., 2006; Wei *et al*., 2007). Most endophytic studies have used morphological characters and either gene sequence data (Hu *et al*., 2007; Wei *et al*., 2007). The distribution of the endophytic species of *Pestalotiopsis* is ubiquitous and is not largely influenced by geographical factors (Wei *et al*., 2007; Tejesvi *et al*., 2009). The colonization frequency of species of *Pestalotiopsis* increased with the increasing the age of the host plant and colonization frequency was variable (Wei *et al*., 2007). Association of *Pestalotiopsis* was also prevalent in most of the endophytic research studies (Tejesvi *et al*., 2009; Srinivasan and Muthumary, 2009; Watanabe *et al*., 2010).

Isolate IS5 had morphological traits very similar to the morphology of *Phyllosticta elongate*. The colony on PDA had a cottony texture and the surface was brown colour. (Figure 3.IS5). The hyphae were hyaline, septate and narrow. The conidiogenous cells on hyphae were typically flask shaped with inflation at the base and narrow zigzag filaments at the apex. Laterally from the filaments conidia were produced from each bending point. Conidia were hyaline, one celled globose to ovoid in shape. Conidiogenous cell tends to form dense clusters (Figure 3 IS5). Several authors made similar observations on morphological characteristics, growth, colony characteristics, etc. (Samson *et al*., 1988; Fernandes *et al*., 2009). *Phyllosticta elongata* is reported as a natural endophyte in *Coffea arabica*.

Isolate IS6 was suggested to be *Penicillium corylophilum*. The thallus (mycelium) of *P.corylophilum* typically consisted of a highly branched network of multinucleate, septate,
colorless hyphae. Many branched conidiophores sprouted on the mycelia, bearing individually constricted conidiospores (Kirk et al., 2008). Previously, this fungus was reported as a common endophyte to other plant species (Rezwana et al., 2010; Zhang et al., 2006). Conidial morphology (IS6) is the most widely used taxonomic character for the genus Xylaria (Hu et al., 2007).

The colonization frequency of Xylaria were increased in stem of R. beddomei (Wei et al., 2007). Association of Xylaria was also prevalent in most of the endophytic research studies (Tejesvi et al., 2009; Srinivasan and Muthumary, 2009; Watanabe et al., 2010).

In case of isolate IS7 the microscopic analysis showed the hyphae, conidiophores, and conidia. Hyphae are septate. Conidiophores are brown to dark brown, erect, parallel walled, and ceasing to elongate when the terminal conidium is formed. Conidia are multicellular, large, solitary, club shaped, and pale to dark brown in color. They are located along the sides of the conidiophores and their wider end is towards the conidiophore (Figure 3.IS5). Similar results were reported for Phomopsis by Chhillar, (2013), thus isolates IS5 could be predicted as Phomopsis.

Present study shows that isolates IS8 was suggested to be Penicillium corylophilum. The thallus (mycelium) of P. corylophilum typically consisted of a highly branched network of multinucleate, septate, colorless hyphae. Many branched conidiophores sprouted on the mycelia, bearing individually constricted conidiospores (Kirk et al., 2008). Previously, this fungus was reported as a common endophyte to other plant species (Rezwana et al., 2010; Selim et al., 2011; Zhang et al., 2006).

The nuclear small subunit ribosomal DNA (18S rDNA) was selected for characterization and identification of endophytes, because established universal fungal primers are available based on the conserved regions of 18S rDNA, making it possible to obtain the PCR products from most of the fungal endophytes. Secondly, the large numbers of 18S rDNA sequences are available in GenBank which makes similarity searches convenient. Several workers have also reported characterization of fungi based on 18S rRNA gene sequence analysis (Smit et al., 1999; Borneman and Hardin, 2000).
In this study, Seven (7) fungal endophytes of non-sporulating strains were identified and confirmed using their ITS-rDNA sequences. On the basis of 18S rRNA gene similarity isolates ENT9, *Aspergillus japonicus* with Accession number (KF493865) showed 100% and ENT10 shows 100% sequence homology with *Cladosporium* at BLAST (Supaphon et al., 2013), *Sordariomycetes*, ENT11 (KF493865) showed 100% sequence homology and *Fusarium oxysporum* (KF493867) ENT12 showed 100% sequence homology respectively (Rubini et al., 2005).

The 18S rRNA partial sequence with the genera/species *Neotyphodium sp*, (KJ542650) ENT13,*Phaemoniells sp* (KJ542652),ENT14 *Phillosticta*, (KJ542653) ENT15. Partial 18S rRNA sequences of all the isolates were submitted to NCBI GenBank. The sequences of close relatives were obtained from Gen Bank to reconstruct the phylogenetic tree.

Many studies have traditionally used sequence data from the ITS region to identify non-sporulating cultures and evaluate morphotaxon boundaries (Lacap et al., 2003). ITS data are considered useful for these purposes due to the rapid evolution of the ITS. However, most fungi are not represented in GenBank, and some GenBank records are misidentified or lack taxonomic information (Arnold et al., 2007).

Therefore, BLAST and phylogenetic analyses of the genomic regions should be combined with those of the ITS region to improve the accuracy of identification. In this study, we selected representative isolates from morphotypes, and then conducted phylogenetic analysis based on ITS sequences. However, three out of seven taxa could not be identified at the species level, indicating that these isolates might be new fungal endophytes.

The present study provides first hand information on the colonization frequencies of endophytic fungi from different plant parts of *R.beddomei*. The role of fungal endophytes in enhancing the plant growth and natural bioactive products, persistence and stress tolerances is well established (Ghimire et al., 2011), it is important to explore these fungal endophytes. This broad range of uses and medicinal values reflects about the idea that, in future, pharmaceutical and drug manufacturing sector mainly relies on plants to obtain life saving therapeutics and drugs.
### Table 3. Different sterilization methods used for Isolation of Fungal endophytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chemicals</th>
<th>Concentration</th>
<th>Time</th>
<th>Reference</th>
<th>Fungal endophytes Isolated</th>
<th>% removal of epiphytic microflora</th>
<th>Leaf</th>
<th>Stem</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Formaldehyde Sterile Distilled water</td>
<td>40% -</td>
<td>1 min 3 min × 4 times</td>
<td>Schulz et al., 1993</td>
<td>76 P.corylophilum A.chrysogenum Phomopsis sp. A.aculaetinus C.gloeosporioides</td>
<td>Nil</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Ethanol Sterile Distilled water NaOCl Sterile Distilled water Ethanol Sterile Distilled water</td>
<td>70% 4% 70%</td>
<td>1 min 3 min × 4 times 30 sec 3 min × 4 times</td>
<td>Suryanarayanan &amp; Vijaykrishna (2001)</td>
<td>85 P.corylophilum A.chrysogenum Phomopsis sp. A.aculaetinus C.gloeosporioides</td>
<td>100 P.corylophilum P.maculans Helminthosporium sp. Fusarium sp. A.alternata Phyllosticta sp. T.harzianum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Ethanol Sterile Distilled water NaOCl Sterile Distilled water Ethanol Sterile Distilled water</td>
<td>70% 3% 96%</td>
<td>1 min 3 min × 4 times 30 sec 3 min × 4 times</td>
<td>Arnold et al., 2000</td>
<td>100 P.corylophilum P.maculans Helminthosporium sp. Fusarium sp. A.alternata Phyllosticta sp. T.harzianum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Ethanol Sterile Distilled water NaOCl Sterile Distilled water Ethanol Sterile Distilled water</td>
<td>96% 4% 96%</td>
<td>1 min 3 min × 4 times 5 min 3 min × 4 times 30 sec</td>
<td>Crous et al., 1995</td>
<td>80 P.corylophilum F.oxysporum A.alternata</td>
<td>100 P.corylophilum P.maculans Helminthosporium sp. Fusarium sp. A.alternata Phyllosticta sp. T.harzianum</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Colony morphology of fungal endophytes; IS1: Alternaria alternata, IS2: Aspergillus aculeatus, IS3: Colletotrichum gloesporioides, IS4: Pestalotiopsis maculans, IS5: Phyllosticta elongata sp., IS6: Xylaria sp., IS7: Phomopsis and IS8: Penicillium corylophilum

Figure 4. Non-sporulating fungal endophytes isolated from leaf and stem segments of *R. beddomei*

Aspergillus japonicus  Cladosporium  Sordariomycetes  Fusarium oxysporum

Neotyphodium sp  Phaemoniells sp  Phyllosticta sp

colony, conidia, and hyphal features of non-sporulating endophytic fungi *Aspergillus japonicus, Cladosporium delicatus, Sordariomycetes* sp. *Fusarium equiseti*, *Neotyphodium sp, Phaemoniells sp, Phillosticta*
Figure 5. PCR amplification of 18S rRNA gene using ITS1 and ITS4 primers for non-sporulating endophytic fungal isolates

Lane L: DNA Ladder; Lane 1-7: (designated as ENT9-ENT15)
Table 4. Molecular identification of endophytic fungal isolates

<table>
<thead>
<tr>
<th>S.No</th>
<th>GenBank accession number</th>
<th>Code for endophytic fungal isolate</th>
<th>Identified species</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KF493865</td>
<td>ENT1</td>
<td><em>Sordariomycetes sp</em></td>
<td>100%</td>
</tr>
<tr>
<td>2</td>
<td>KF493862</td>
<td>ENT9</td>
<td><em>Asperigillus japonicus</em></td>
<td>100%</td>
</tr>
<tr>
<td>3</td>
<td>KF493866</td>
<td>ENT10</td>
<td><em>Cladosporium delicatus</em></td>
<td>100%</td>
</tr>
<tr>
<td>4</td>
<td>KF493867</td>
<td>ENT12</td>
<td><em>Fusarium equiseti</em></td>
<td>100%</td>
</tr>
<tr>
<td>5</td>
<td>KJ542650</td>
<td>ENT13</td>
<td><em>Neotyphodium sp</em></td>
<td>100%</td>
</tr>
<tr>
<td>6</td>
<td>KJ542652</td>
<td>ENT14</td>
<td><em>Phaemoniells sp</em></td>
<td>100%</td>
</tr>
<tr>
<td>7</td>
<td>KJ542653</td>
<td>ENT15</td>
<td><em>Phillosticta</em></td>
<td>100%</td>
</tr>
</tbody>
</table>

Accession numbers in the parenthesis indicates the closest identified by BLAST search
Figure 6. Phylogenetic tree based on ITS-rDNA sequences of endophytic fungi associated with *R. beddomei* and the closest identified relatives from GenBank

![Phylogenetic Tree Image]

**GenBank Accession Numbers:**
- gi|557882015|gb|KF493862.1|
- gi|557882019|gb|KF493866.1|
- gi|635335114|gb|KJ542650.1|
- gi|635335117|gb|KJ542653.1|
- gi|557882020|gb|KF493867.1|
- gi|557882018|gb|KF493865.1|
- gi|635335116|gb|KJ542652.1|
Chapter 1

---GATTGATACCAATCGTAAACTTCCAAAATGATTGCATCTCTTGGT 270
---AATCT-TAATTAAAATAATTTTTTAACACCGGATCCTTGGT 238
---GATTACATGCAAATAATCGTAAACTTCCAAAATGATTGCATCTCTTGGT 288
AAAAGGAAAAAAAATAAATGAACTTTATTTAACAACCGGATCCTTGGT 218
-----AAATTTAATATAAATTTTTTAACACCGGATCCTTGGT 232
-----AAATTTAATATAAATTTTTTAACACCGGATCCTTGGT 281
-----AAATTTAATATAAATTTTTTAACACCGGATCCTTGGT 231

-----ATTATATAATATAAATTTTTTAACACCGGATCCTTGGT 231

** ************ ************ ************ ************

---CTG----GTTGTTGGG----CCGCGC---- 436
---CTT----GTTGTTGGG----CCGCGC---- 406
---TCCGGACTTTGTTGTTGGG----CCGCGC---- 395
---TCTCTACGAGGAAGCGGTGTTGTTGGG----CCGCGC---- 415
---TTTACGAGGAAGCGGTGTTGTTGGG----CCGCGC---- 399
---TTTACGAGGAAGCGGTGTTGTTGGG----CCGCGC---- 454
---TTTACGAGGAAGCGGTGTTGTTGGG----CCGCGC---- 401

---CTT----GTTGTTGGG----CCGCGC---- 436
---CTT----GTTGTTGGG----CCGCGC---- 406
---TCCGGACTTTGTTGTTGGG----CCGCGC---- 395
---TCTCTACGAGGAAGCGGTGTTGTTGGG----CCGCGC---- 415
---TTTACGAGGAAGCGGTGTTGTTGGG----CCGCGC---- 399
---TTTACGAGGAAGCGGTGTTGTTGGG----CCGCGC---- 454
---TTTACGAGGAAGCGGTGTTGTTGGG----CCGCGC---- 401

---CTG----GTTGTTGGG----CCGCGC---- 436
---CTT----GTTGTTGGG----CCGCGC---- 406
---TCCGGACTTTGTTGTTGGG----CCGCGC---- 395
---TCTCTACGAGGAAGCGGTGTTGTTGGG----CCGCGC---- 415
---TTTACGAGGAAGCGGTGTTGTTGGG----CCGCGC---- 399
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---TTTACGAGGAAGCGGTGTTGTTGGG----CCGCGC---- 401
Chapter 1

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    gi|557882808|gb|KF493867.1| GAAACC---CCGGCTCCCAATCGAGTGGGCTGCGAGTACGAGGAGGGCGGAGAG 445
    gi|557882801|gb|KF493865.1| CCTGCTG---TAGGTCCTCTAACAGTTAGTGGGCGGAGGTTGCTTCCACATCGAG 500
    gi|635335116|gb|KJ542652.1| -TAAAGG---ATAGGCAGGGAAAGATATAAGTCGCGTCACAAATGACCGAAGCC 447

    *  *  *  *  *  *
    gi|557882015|gb|KF493862.1| GA---GCGTATGGGCTCTGTGACACCGTCTATGG-GCCCGGCGCGGC 529
    gi|557882019|gb|KF493866.1| AA---GCGTTGGAGACT---ATTCCGCTAAAGGGTGCTCGG---GGGGG 491
    gi|635335114|gb|KJ542650.1| TT---GCGTAG-TCACCATACATCACTCCGCAACCCGAGGAGCCGCGGGCCGCC 487
    gi|635335117|gb|KJ542653.1| CT---GCGTAG-TCACCTA---ACGCTGACA-CTGAGACGCGAAGCAGCGG 501
    gi|557882020|gb|KF493867.1| TA---GCGTAG-TCACCATACACCTCGTTACTGCTGTAAT-CGTCGCGGCC 489
    gi|557882018|gb|KF493865.1| AG---ACGTAGTAAATCTTTATCTGCTGCTATAGTAGATGAGCG---GCCGCC 544
    gi|635335116|gb|KJ542652.1| GATGCAGCAGCTTTATACAGCATAACATGAAAGGTTTTGTGCGCGGCGCC 497

    **:  *
    gi|557882015|gb|KF493862.1| TT--------------GCCTGGA----------------------------- 538
    gi|557882019|gb|KF493866.1| TA---------------C----------------------------------- 494
    gi|635335114|gb|KJ542650.1| ACTGCGGTAA--AACGCCCAACTTCTCTCAAGATGGACTGCGAATCCAGG-- 534
    gi|635335117|gb|KJ542653.1| AC-GCGGTAA--AACGCCCAAGCTTTTTTTAAG-GTTGACCTGGAATCGGT 547
    gi|557882020|gb|KF493867.1| AC-GCGGTAA--AAC-GCCCAACTTCTGAGG---TGAC---------------- 520
    gi|557882018|gb|KF493865.1| TT-GCCCGTAA--AACCCCAAACATCTTTCTACACAGGTTGCGCGGATGCT 591
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    :  
    gi|557882015|gb|KF493862.1| --------------------------- 556
    gi|557882019|gb|KF493866.1| --------------------------- 556
    gi|635335114|gb|KJ542650.1| --------------------------- 556
    gi|635335117|gb|KJ542653.1| AGGACTACC------------------ 556
    gi|557882020|gb|KF493867.1| --------------------------- 556
    gi|557882018|gb|KF493865.1| AGGAATACCGCGTGAATTTA 611
    gi|635335116|gb|KJ542652.1| AGGAATACCGCGTGAATTTA 646
```

105
This is a Neighbour joining tree without distance corrections.

(gi|635335114|gb|KJ542650.1|:0.06780, gi|635335117|gb|KJ542653.1|:0.09983,  
(gi|557882020|gb|KF493867.1|:0.07462, (gi|557882018|gb|KF493865.1|:0.13949,  
gi|635335116|gb|KJ542652.1|:0.13838):0.04125, (gi|557882015|gb|KF493862.1|:0.10910,  
gi|557882019|gb|KF493866.1|:0.09048) :0.05085) :0.01937) :0.00816)

SCREENING OF FUNGAL ENDOPHYTES FOR IAA AND GIBBERELLIN PRODUCTION

RESULTS

Beneficial effects of the introduction of specific microorganisms on plant growth have been reported for numerous crops including *G. Sylvestre* were grown under green house conditions . (Gagne 2003). The culture filtrate of fungal endophytes were isolated were initially subjected for the screening experiments. It helped us in accurate identification of endophytic fungi having potential to produce plant growth promoting hormones. In the present study, A total of 15 fungal endophytes were screened, two fungal endophytes showed high production of phytoharmones when compared to others. Viz *Aspergillus japonicus, Sordariomycetes*. The culture filtrate of both strains produce varying levels of IAA. The range of IAA production with or without tryptophan was found to be (25.2μg/ml) in *Aspergillus japonicus*, while *Sordariomycetes* shows a significantly higher amount of IAA (27.8μg/ml) when compared to control. Similarly the range of gibberellins production with or without tryptophan was found to be (0.08 μg/25ml) *Aspergillus japonicus*, while *Sordariomycetes* shows a significantly higher amount of gibberellins (0.13 μg /25ml) When
compared to control. The detection wavelengths used were 530 nm for auxins and 254 nm for gibberellins.

**DISCUSSION**

Secondary metabolites are involved in plant defense reactions and fungal–host interactions. The concentrations of the secondary metabolites differ as a result of endophytic infections. Elicitation of plant cells in culture represents one of the useful biotechnological tools to improve the production of valuable secondary metabolites. Plants respond to other biotic and abiotic stresses by activating an array of defense mechanisms including induction of biosynthesis of secondary metabolites. *G. sylvestre* is an important medicinal plant used in different systems of medicine as a remedy for the treatment of diabetes (Kanetkar, 2004). In this study the effect of two endophytic fungi *Sordariomycetes sp*, *Aspergillus japonicus* on *G. Sylvestre* under greenhouse conditions were investigated. Based on morphological and biochemical methods representative isolates were grouped into thirteen presumptive genera and the sequencing results of the 18S rRNA gene of isolates were grouped into seven presumptive genera were all consistent with the phenotypic characterization. All the representative isolates of *Sordariomycetes spp*, *Aspergillus japonicus* relatively 18S rRNA gene sequence homology (100%) with sequences available on the NCBI server. Although 97% similarity value for the 18S rRNA sequence is often considered to be a novel species, strains that are more than 97% similar in their 18S rRNA gene sequences can still be different species (Rossello-Mora and Amann, 2001). In our study all isolates of genus *Sordariomycetes spp*, *Aspergillus japonicus* showed rather high IAA production ability although no such report for this is available to date. Therefore these isolates might represent new species in their respective genera and more intensive studies should be conducted to deepen the understanding of their genetic characters and to ascertain the potential of this genus. Our study appears to be the first report on IAA production of *Sordariomycetes spp*, *Aspergillus japonicus*. However based on our study the endophytic *Sordariomycetes spp*, *Aspergillus japonicus* showed a very high ability to produce IAA compared with endophytic fungi. In our study the inoculation of most of the representative isolates capable of IAA production resulted in significantly higher than the control.
Among plant growth regulators indole-3-acetic acid (IAA) is the most common natural auxin found in plants and its positive effect on root growth and morphology is believed to increase the access to more nutrients in the soil (Vessey, 2003). The involvement of IAA in the complex interaction between the rhizosphere microflora and the host plant, which relies on a constant exchange of materials and signals (Antoun and Pre’vost, 2005) has been the focus of numerous works (Costacurta and Vanderleyden, 1995; Patten and Glick, 1996; Persello-Cartieaux et al., 2003). Such beneficial microorganisms referred as PGPF (plant-growth promoting fungi) enhance plant growth through numerous mechanisms including the protection of roots against infection by minor and major pathogens (Whipps, 1997, 2001) enhancing the availability of nutrients to the host plant lowering of the ethylene level within the plant or by the enhanced production of stimulatory compounds such as plant growth regulators (Antoun and Pre’vost, 2005).

The production of plant growth regulators by the microorganisms is another important mechanism often associated with growth stimulation (Vessey, 2003). The balance between vegetative and reproductive growth is controlled by hormone signalling within the plant and can therefore be highly influenced by it (Taiz and Zeiger, 1991). At relatively high concentrations, natural auxins, such as IAA, Gibberellins stimulate shoot elongation and root induction while reducing root elongation (Tanimoto, 2005). IAA, Gibberellins is also involved in plant growth (Srivastava and Handa, 2005). Previous works have reported that the synthesis of IAA, Gibberellins is often associated with plant growth stimulation by endophytic fungi Sordariomycetes, Aspergillus japonicus (Xie et al., 1996; Patten and Glick, 2002). In this study, results showed that Sordariomycetes, Aspergillus japonicus are able to synthesize IAA, Gibberellins from different precursors in vitro which supports the theory that microbial IAA and Gibberellins involved in the growth stimulation observed in our greenhouse assay. Of particular interest, the results showed that the growth of G.Sylvestre inoculated with Sordariomycetes, Aspergillus japonicus increased as the concentration of L-tryptophan increased in the pouches.

This suggests that the synthesis of IAA and Gibberellins through tryptophan-dependent pathways by Sordariomycetes, Aspergillus japonicus affected the growth of the G.Sylvestre. Tryptophan is naturally secreted in root exudates of G.Sylvestre plants and most
of the auxin found in the rhizosphere is believed to come from the biosynthesis by microorganisms (Kamilova et al., 2006). Exogenous sources of IAA, Gibberellins such as produced by microorganisms are known to cause changes in the morphology of the root, shoot system which influence the uptake of nutrients by the plant (Arteca, 1996).

In this regard, San-Francisco et al. (2005) showed that exogenous applications of IAA, Gibberellins increased the amount of P in root and shoot of G. Sylvestre grown under hydroponic conditions. It is therefore possible that the increase in the level of P in the leaves of plant grown in the organic medium might also be related at least partially to the production of IAA by Sordariomycetes, Aspergillus japonicus. In addition to having a stimulating effect on plant growth, exogenous IAA, Gibberellins in the rhizosphere can also have a detrimental effect on the elongation of root and shoot over a wide range of concentrations. Such an effect has been associated with an increase in the level of ethylene in the plant (Glick et al., 1997, 1998). IAA can increase the activity of ACC synthase, which catalyses the conversion of Sadenosyl methionine to ACC, the precursor of ethylene in the plant.

The results from this study suggest the involvement of two possible mechanisms. First, both Sordariomycetes and Aspergillus japonicus were able to partially degrade IAA and Gibberellins in vitro. (Leveau and Lindow, 2005) Such degradation could have reduced the concentration of IAA and Gibberellins in the vicinity of the root and shoot to a level which was not detrimental to the elongation. Also, previous studies have shown that ACC deaminase activity in PGPR, which hydrolyses ACC into ammonia and a-ketobutyrate, prevents the synthesis of inhibiting levels of ethylene (Penrose et al., 2001). This reduction in the level of ACC in the rhizosphere increases the exudation of ACC by the plant to maintain equilibrium, reducing the potential synthesis of ethylene since ACC is the immediate precursor of this compound in the plant (Glick et al., 1998). This study demonstrated that both Sordariomycetes and Aspergillus japonicus possess ACC deaminase activity when grown in vitro, suggesting that these microorganisms could also regulate the concentration of ethylene within the plant by reducing the amount of its precursor present. ACC deaminase has previously been reported for Pseudomonas spp. and its activity has been associated with an increase in root elongation due to the reduced inhibition caused by ethylene (Glick et al., 1997; Wang et al., 2000; Safronova et al., 2006). The effect of
microbial production or degradation of IAA in the rhizosphere is likely indirect through an effect on the overall growth of the plant. Indeed, IAA, including microbial can greatly influence the growth of the root system depending on the amount found in the rhizosphere through root elongation and the formation of lateral or adventitious roots (Scott, 1972; Patten and Glick, 2002). Gibberellins combined with their ACC deaminase activity may have promoted an optimal development of the root system which could have resulted in the stimulation of the reproductive growth observed in this study. Microbial production of IAA, Gibberellins is known to result from different pathways (Persello-Cartieaux et al., 2003). Although tryptophan-independent biosynthesis pathways have been identified in numerous microorganisms, tryptophan remains the most common precursor of microbial IAA, Gibberellins (Patten and Glick, 1996). This research demonstrated the capacity of two endophytic microorganisms Sordariomycetes, Aspergillus japonicus to promote the reproductive growth of G. Sylvestre under typical hydroponic growing conditions.

The plant growth stimulation reported in this study is most likely the synergic result of numerous modes of action exhibited by each microorganism tested including a regulation in the concentration of IAA in the rhizosphere and a regulation of the concentration of ethylene within the roots. This study showed that Sordariomycetes and Aspergillus japonicus could be used as plant growth-promoting microorganisms to improve the productivity under hydroponic conditions. More specific works are however needed to further study the specific mechanisms involved in the growth stimulation by Sordariomycetes and Aspergillus japonicus as well as to better understand the close interaction between the host plant of these two endophytic fungi.

In the present study the application of endophytic fungi Sordariomycetes, Aspergillus japonicus enhances the plant growth. The IAA, Gibberellin production potential of the isolates from Sordariomycetes, Aspergillus japonicus shows a significantly higher amount when compared to others. Our results indicated that the plant growth promotion by Sordariomycetes and Aspergillus japonicus was most likely due to the ability of the isolates to produce IAA and Gibberellins.