Chapter VI
Isolation of Endophytic Mycoflora Associated with *Achyranthes aspera* L. for Studying their Effect on 20-hydroxyecdysone Production

ABSTRACT

*Achyranthes aspera* Linn. is a widely used medicinal herb in Indian sub-continent, but with limited information on endophytic fungal associations. A total of 513 isolates belonging to ten different species of fungi was obtained from asymptomatic, surface sterilised segments of leaf, stem and root of *A. aspera* collected from ten districts of Kerala, India. Among the isolates Ascomycetes were most prevalent, but a fraction of Basidiomycetes yeast species were also recovered. Colonisation rate was high for leaf tissue (95%) followed by stem (77.75%) and root segments (33.33%). The most frequent and dominant coloniser of the host were *Colletotrichum* sp., which was isolated from all ten locations. Morphological and phylogenetic analyses using internal transcribed spacer (ITS) sequences revealed that the fungi recovered belongs to different fungal lineages of Sordariomycetes, Dothideomycetes, Eurotiomycetes and Tremellomycetes. A maximum likelihood tree revealed the relationship between the obtained sequence data and the closest sequences retrieved from the GenBank. Further, colonization of endophytes in the host plant was detected by using Light and Scanning Electron Microscopy which demonstrated the presence of hyphae in inter and intra cellular spaces. In the current study, addition of endophytic fungal extract as elicitor to the cell suspension culture and adventitious root culture medium failed to enhance the production of 20-hydroxyecdysone.

**Key Words:** *Achyranthes aspera* Linn., Endophytic fungi, Internal transcribed spacer (ITS) sequences, Scanning Electron Microscopy.
6.1 INTRODUCTION

Plants are inhabited by numerous microorganisms from phyllosphere to rhizosphere and epiphytic microorganisms are located on the surfaces whereas endophytes colonize the inner tissues of the host organ. Endophytic fungi are commonly plant-associated fungal groups, which colonizes inner plant tissues, persisting for the whole or part of the life cycle without causing disease symptoms in the host (Petrini, 1996). They have been found to be associated with every plant species so far investigated and are important components of plant micro-ecosystems. Medicinal plants are reported to harbor endophytes (Strobel, 2002), which produce numerous bioactive compounds and facilitate the host plants to persist in the external biotic as well as abiotic stresses and promotes the host growth in return (Silvia et al. 2007).

Plants synthesize an array of structurally novel bioactive molecules, therefore, making them a source for diverse types of medicines. It is expected that certain relatively rare bioorganic molecules synthesised by specific higher plants may be produced by some endophytes residing in it. This is demonstrated with the case of anticancer compound taxol isolated from the bark of the Pacific Yew (*Taxus brevifolia*). Moreover, taxol is being produced by a series of endophytes from yews as well as from other plants (Stierle and Strobel, 1995; Gangadevi and Muthumary, 2007). It has also been reported that secondary metabolite production in certain plant species is enhanced through application of various fungal elicitors (including cell wall fragments, polysaccharides, oligosaccharides and glycoproteins) (Holkova et al. 2010; Esmaeilzadeh et al. 2012). For instance, fungal elicitors were reported to induce taxol synthesis in cell suspension cultures of *Taxus chinensis* (Yu et al. 2002).
Elicitation is an effective approach adopted for enhancing the secondary metabolite production in the cell culture system, which may lead to improved yields and reduce culture times (Zhou et al. 2007; Zhao et al. 2010). Elicitors of biological origin comprise of proteins, glycoproteins, polysaccharides or cell-wall fragments obtained from fungi, bacteria and plants. Currently, pathogenic and non-pathogenic fungal preparations as elicitors have been developed as one of the most significant and successful approaches to enhance secondary metabolite production in plant cell culture (Baldi et al. 2009). Furthermore, in these procedures fungal elicitors applied are fairly undefined mixtures, like autoclaved fungal homogenate (Yoshikawa et al. 1993) or fungal culture filtrates (Veersham et al. 1995).

Exploration of endophytic fungi residing in plants has turned into a hotspot of research studies in current years, for the valuable production of metabolites with multiple biological actions having uses in the field of agriculture, medicine and food industry (Strobel et al. 2004). In this study, an effort was made to investigate the endophytic mycobiota associated with the traditional medicinal plant A. aspera. Despite its medicinal importance, there are a few fragmentary reports on endophytic communities from, A. aspera and no comprehensive work has been done on biology and distribution of endophytes within the plant and plants from several locations. Here we assessed the diversity and colonisation frequency of endophytic fungi in four different plant tissues (leaves, stems, roots and seeds) of A. aspera collected from ten districts of Kerala, India. To the best of our knowledge, there are no reports on the effects of endophytic fungi on 20-hydroxyecdysone production in in vitro cultures of A. aspera.
6.2 SPECIFIC OBJECTIVES

The study was designed with the following objectives

1. To isolate the endophytic fungi from different plant tissues of *A. aspera* collected from ten districts of Kerala.

2. To identify the endophytic fungi based on rDNA ITS sequences analysis.

3. To study the role of isolated endophytic fungi in 20-hydroxyecdysone production from *in vitro* cultures of *A. aspera*.

6.3 REVIEW OF LITERATURE

Endophytes are generally regarded as symbiotic or mutualistic, benefiting the host by producing plant growth regulatory, antimicrobial, antiviral and insecticidal substances (Carroll, 1988). Previous studies report that the endophyte-host interaction is a balanced antagonism where the non-pathogenic relationship is based on equilibrium between fungal virulence and plant defences, which may change depending on the environmental conditions or variations in the plants physiological state (Schulz et al. 1999). In addition to their ecological significance, endophytes have been recognized as a good source of novel natural products for pharmaceutical, agricultural and industrial uses, especially those unique secondary metabolites produced by fungal endophytes residing in medicinal plants (Strobel and Daisy, 2003). Many authors suggested that the endophytes harboured by medicinal plants are excellent producers of strong fungicidal, bactericidal and cytotoxic metabolites (Radu and Kqueen, 2002; Wang et al. 2007).

Endophytic organisms colonize all plants, and are isolated from nearly all plants. Histological studies of the endophytes are conducted
using light or electron microscopy, which helps to study structural characteristics of endophytes, their infection process, and their interaction with the host plant (Hinton and Bacon, 1985). Endophytic assemblages are influenced by various features such as geographic location, specificity and the age of the tissue of colonization (Collado et al. 1999; Ganley and Newcombe, 2006). Often endophytes are studied at the morphological level and usually, several endophytes either fail to sporulate or some of them are rare and difficult to identify. So, molecular methods based on DNA sequences are documented as the reliable technique for revealing genetic relationship between the strains. These can be unambiguously used to identify and evaluate the taxonomic rank of the isolates (Bruns et al. 1992).

Fungal elicitors are reported to stimulate taxol synthesis in cell suspension cultures of *Taxus chinensis* (Yu et al. 2002). Fungal strain *Phomopsis* isolated from the inner bark of *Betula platyphylla* improves the triterpenoid production in *B. platyphylla* cell cultures (Zhai et al. 2011). Various concentrations of fungal extracts (*Aspergillus niger* and *Penicillium notatum*), yeast extracts and chitosan increases the production of psoralen in suspension cultures of *Psoralea corylifolia* (Ahmed and Baig, 2014). Namdeo et al. (2000) reported enhanced accumulation of ajmalicine in *Catharanthus roseus* cultures when they were elicited with various concentrations of extracts of *Trichoderma viride*, *Aspergillus niger* and *Fusarium moniliforme*.

**6.4 MATERIALS AND METHODS**

**6.4.1 Sampling site and plant material**

Different parts (leaves, stems, root and seeds) from the traditional medicinal plant *A. aspera* were sampled for the isolation of endophytic fungi. Four to five asymptomatic plants were collected from ten districts of
Kerala, India (Fig 6.1). The samples were collected, labelled and transported to the laboratory. They were stored at 4 °C and processed within 2 days.

![Map of Kerala showing ten districts](image)

**Fig. 6.1** Map of Kerala, Grey coloured regions shows ten districts of Kerala (Thiruvananthapuram, Kollam, Pathanamthitta, Alapuzha, Kottayam, Idukki, Palakkad, Kozhikode, Wayanad, Kasarcode) from which *A. aspera* was sampled for the isolation of endophytic fungi.

### 6.4.2 Isolation and Identification of fungal endophytes

The plant material was washed in running tap water, immersed in 70% ethanol for 1 min and surface sterilized in 0.1% mercuric chloride solution for 3 min, followed by several rinses in sterile distilled water. The surface-sterilised samples were dried by placing in pre-sterilized petri dishes lined with sterile blotting paper. Stem, root and leaves were cut into small segments (5mm, 5mm×5mm) and seeds were removed from the seed coat and placed on plates with potato dextrose agar medium (PDA, Himedia, India). The antibiotic streptomycin sulphate (250 mg L⁻¹) (Himedia, India)
was added to the media above to suppress bacterial growth. Leaf imprint control was taken on the plates to check the efficacy of adopted surface sterilisation method. Four surface-sterilized segments were evenly placed in each petri dish. The petri dishes were sealed by parafilm, incubated at 28 °C and observed every day for fungal growth. Emerging hyphal tips were transplanted to fresh PDA plates to obtain pure isolates.

Morphological identification was done by using the characters such as growth pattern, colony appearance, surface texture, margin characters, mycelium color and pigmentation. For microscopic identification slide culture techniques were adopted, where 10 mm squares of PDA blocks were excised from the sterile agar plates and placed on a slide in pre-sterilised petri dishes. The edge of the agar was inoculated with the fungus; a sterile cover slip was placed over the agar block and incubated at room temperature. The coverslip containing the fungus was stained with lacto phenol cotton blue (Himedia, India) and observed under the bright field microscope (Olympus, Model BX43, Japan) with 100X and 400X objectives.

6.4.3 Data Analysis

\[
\text{Colonization rate} = \left( \frac{\text{Total number of plant tissue segments infected by fungi}}{\text{Total number of segments incubated}} \right) \times 100
\]

\[
\text{Isolation rate} = \left( \frac{\text{Number of isolates obtained from plant segments}}{\text{total number of segments incubated}} \right)
\]

\[
\text{Colonization frequency (\% CF)} = \left( \frac{\text{Number of segments colonized by each fungus}}{\text{Total number of segments studied}} \right) \times 100
\]

(Hata and Futai, 1995)
6.4.4 Histological analysis of fungal endophytes in *A. aspera*

Anatomical analysis of plant samples was done for demonstrating the presence of endophytic fungi in the interiors of plant tissues. Leaves and stem segments were collected, surface sterilized and incubated in PDA for 48 h at 28 °C for stimulating the growth of endophytic fungi. Leaves and stem were removed from the medium and immersed in Carnoy’s solution for 24 h, and then placed in 70% ethyl alcohol for 24 h to facilitate chlorophyll removal (Hignight et al. 1993). Samples were inserted between small blocks of stiff Styrofoam to provide support and fine sections were cut by hand with a sharp razor blade. Sections were placed on the slide and stained with trypan blue and observed under a bright field microscope (Olympus, Model BX43, Japan) with 100X and 400X objectives.

6.4.5 Scanning Electron Microscopy (SEM)

The fungal and the plant specimens were fixed with 2.5% gluteraldehyde in 0.1M phosphate buffer for 12 h and then washed three times in the phosphate buffer. The specimens were dehydrated by a graded series of ethanol (25, 50, 70, 80, 90 and 100%) for about 15-20 min. Samples were mounted on metal stubs and covered with a fine platinum layer coated (45s, 20MA, at 27 °C) using a metallic covering apparatus and they were analysed in SEM emission field. Images were recorded with JEOL 1600 (Japan) scanning electron microscope in high-vacuum mode.

6.4.6 DNA extraction and PCR amplification

100 mg of mycelium was ground with 750 µL of STE buffer along with 50 mg of PolyVinyl Pyrrolidone and 1.0g of silica powder and transferred to a microfuge tube of 1.5 mL and incubated 65 °C for 1 h. The sample was centrifuged at 13,000 rpm for 10 min. To the supernatant equal volume of Chloroform- Isoamyl alcohol (24:1) was added and centrifugation was repeated.
The aqueous layer was transferred to fresh 1.5 mL microfuge tube and added with twice the volume of icecold isopropanol and 1/10th volume Sodium acetate and centrifuged. The pellet was washed with 70% ethanol, dried and dissolved in 50µL TE buffer. Quality of DNA was estimated by agarose Gel Electrophoresis (0.8%) and quantity was estimated by UV absorbance. ITS fragment was amplified by PCR from fungal genomic DNA using ITS - PCR universal primers ITS1:5’ TCCGTAGGTGAACCTGCGG-3’ and ITS4: 5’TCCCTCCGCTTATTGATATGC-3’. PCR (Agilent Sure Cycler, 8800,USA) was carried out in a final reaction volume of 25 µL in 200 µL capacity thin wall PCR tube which contained deionised water 17.1 µL, Taq buffer(10x) 2.5 µL, MgCl₂ (25mM) 0.6 µL, Primer forward ITS1 (10pm/ µL) 0.5 µL, Primer reverse ITS4 (10pm/ µL) 0.5 µL, dNTP mix (2.5mM) 2 µL, Taq (3u/ µL) 0.8 µL, Template DNA (25µg/µL) 1 µL. A negative DNA control was also kept. The PCR programme was designed for 35 cycles, 5 min initial denaturation at 94°C followed by denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec, extension at 72 °C for 1 min and final extension 72°C for 10 min. The amplified product was visualised in 1.5% agarose gel with 100bp ladder and it was then purified and subjected to sequencing. Sequencing of amplicon with forward and reverse primers was carried out in ABI 3730xl cycle sequencer in Scigenome labs, Cochin, Kerala, India.

6.4.7 Phylogenetic analyses

Sequence similarity searches were performed for obtained fungal sequences and compared with ITS sequence data from strains available at the public databases GenBank (http://www.ncbi.nlm.nih.gov) by using the BLAST sequence match routines. The closest hit sequences were downloaded in FASTA format and were multiple aligned using the
Chapter VI

CLUSTAL W (1.6) (Thompson et al. 1994) program and adjusted manually to maximise alignment using BioEdit 7.0.0 (Hall, 1999).

The datasets were analysed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian (BI) methods. MP analyses were conducted using Paup 4.0B (Swofford, 2002). Heuristic searches with 10 replicates of random addition sequences (RAS) and tree bisection and reconnection (TBR) branch swapping were carried out. Gaps were treated as a fifth character. A Bootstrap test with 1000 replicates was used for testing the tree reliability. ML analyses were conducted using RAxML 7.2.5 (Silvestro and Michalak, 2012) by using default parameters. Support for the nodes was assessed from 1000 bootstrap replicates (Felsenstein, 1985). BI analyses based on a Markov Chain Monte Carlo (MCMC) were conducted using the program MrBayes version 3.1.2 (Ronquist et al. 2012). Two independent MCMC chains were run for five million generations using the best fitting model chosen through the AIC in J Modeltest v2 (Darriba et al. 2012). Model selected was TRN+G. Trees were sampled every 100 generations resulting in 50000 trees from both runs. From these, the first 25% of trees were discarded as burn-in. The remaining trees were used to construct a majority rule consensus tree.

6.4.8 Nucleotide sequence accession number

Sequences obtained from this study were deposited in GenBank with accession numbers, KF709446, KF841604, KF841605, KF841606, KF841607, KF841608, KF841609, KF841610 and KF841611.

6.4.9 Fungal elicitor preparation

The isolated endophytic fungi were maintained on potato dextrose agar (Himedia, India) slants. They were transferred to 250 mL Erlenmeyer flask containing 100 mL of liquid PDA medium and were shaken at 110 rpm
on a rotary shaker at 30°C for 7 days. After growth for 7 days, the fungal suspension cultures were autoclaved at 121 °C and 1.2 kg/cm² for 20 min. The autoclaved cultures were filtered through Whatman no.1 filter paper to obtain culture filtrates of the respective fungi. The fungal mat was washed several times with sterile distilled water and dried at 60°C for 48 h in oven, and ground with sterile mortar and pestle. The preparation so obtained was denoted as dried cell powder. The culture filtrates and dried cell powder were stored at 4-8°C until further use. Dried cell powder and culture filtrates from the endophytic fungi were tested at 0.1, 0.25, and 0.5% (w/v) and 5, 7.5, and 10% (v/v) concentrations. The endophytic fungal extract was aseptically added to the 15 day old cell suspension and adventitious root culture medium. The cultures were harvested after, 3, 6 and 9 days after addition of endophytic fungal extract and suitable control culture was kept for each experiment.

6.5 RESULTS
6.5.1 Isolation of fungal endophytes

Healthy and symptomless leaves, stem, roots and seeds of A. aspera were used to evaluate the biodiversity of endophytic fungi residing in it. A total of 513 fungal isolates belonging to 10 different taxa were recovered from 950 segments of A. aspera collected from 10 districts of Kerala, India. In this study, culture dependent method was employed for isolation of endophytic fungi. The adopted surface sterilization method was appropriate and no fungal growth was recorded from the imprints of surface sterilized leaf pieces. Fig.6.2 shows the emergence of fungal hyphae from leaves, stem and root segments of A. aspera. No fungal endophytes were isolated from the seeds of A. aspera.
Fig. 6.2 Emergence of fungal endophytes from A. aspera when placed on Potato Dextrose Agar (PDA) supplemented with streptomycin sulphate (250mgL$^{-1}$). (a) leaf (b) stem (c) root segments (d) seeds.

6.5.2 Colonisation and isolation rates from different tissues of A. aspera

The colonization rates of endophytes from A. aspera were 95% (leaves), 77.7% (stems) and 33.3% (roots) respectively (Table 6.1). The isolation rates of the leaves and stem tissue were found to be higher than that of the root segments (Table 6.1). No fungal endophytes were isolated from the seeds of A. aspera. Fig 6.3 describe the pattern of distribution of endophytic fungi from different segments of A. aspera. Low colonization of fungal endophytes was observed in the root segments of A. aspera showing that there might be a difference in microbial and physiological conditions in different plant parts. The most commonly isolated Ascomycetes were Sordariomycetes, followed by Dothideomycetes and Eurotiomycetes. Tremellomycetes (Basidiomycetes) were also isolated as endophytes from
A. aspera (Fig. 6.4). The prominent fungal populations observed from leaves and stem segments in all ten locations were *Colletotrichum* sp.

**Table 6.1** Colonisation and isolation rates of fungal endophytes in *A. aspera*.

<table>
<thead>
<tr>
<th></th>
<th>Leaves</th>
<th>Stem</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>400</td>
<td>400</td>
<td>150</td>
</tr>
<tr>
<td>No. of samples yielding fungi</td>
<td>380</td>
<td>311</td>
<td>50</td>
</tr>
<tr>
<td>No. of Isolates</td>
<td>244</td>
<td>220</td>
<td>40</td>
</tr>
<tr>
<td>Colonisation rate</td>
<td>95%</td>
<td>77.77%</td>
<td>33.33%</td>
</tr>
<tr>
<td>Isolation rate</td>
<td>0.61</td>
<td>0.55</td>
<td>0.26</td>
</tr>
</tbody>
</table>

**Fig. 6.3** Colonisation frequency of fungal endophytes in *A. aspera*. 
6.5.3  Light Microscopy

Histological examinations of the stem and leaf tissues of *A. aspera* were done to determine the location of fungal endophytes. In the leaf tissue, hyphae were seen to exit tissue and grow externally and the sections showed the presence of blue coloured hyphae on the surface of leaf tissues (Fig. 6.5a). Transverse sections of stem showed Trypan blue-stained inter and intracellular hyphae of endophytic fungi located below the epidermis (Fig. 6.5b).

6.5.4  Scanning Electron Microscopy

Leaves and stem segments of *A. aspera* incubated after 48 h were analysed by scanning electron microscopy revealing the presence of endophytic hyphae inside the cells and intracellular spaces. Fig. 6.6 shows the emergence of endophytic hyphae from the interiors of leaf and stem tissue.
**Fig. 6.5** Light micrographs of *A. aspera* leaf and stem tissue stained with Trypan blue after 48 h of incubation on PDA. (a) Images of leaf tissue showing blue coloured stained superficial hyphae on the host surface. (b) Images showing the transverse sections of stem, the arrow indicates endophytic mycelium coloured blue in the inter and intracellular spaces.

**Fig. 6.6** SEM micrographs of *A. aspera* leaves and stem after 48 h of incubation on PDA. (a) emergence of endophytic fungal hyphae from leaf surface (b) Fungal hyphae in mats on the surface of leaf tissues. (c) transverse sections of stem, showing the colonization of endophytic fungi.
6.5.5 Molecular Phylogeny of endophytes based on ITS analysis

Amplification of the ITS sequences of the isolated endophytes generated a single fragment of 550-600 bp in size. Sequencing of the PCR product yielded 502-623 bp of informative sequences. The ITS1-5.8S-ITS2 sequences from the isolated endophytic strains were compared with the published GenBank sequences for identification. Results from BLAST categorized the isolated endophytes under Ascomycota and Basidiomycota, which coincides with the morphological identification. Species *Gongronella butleri* and *Cunninghamella bertholletiae* were selected as outgroups (Fig. 6.7). Bayesian analysis produced trees which were consistent in topology with ML, MP and NJ analysis illustrating the phylogenetic placement of the endophytes associated with *A. aspera*. For Bayesian analysis best fitting substitution model selected were TRN+G.

In the phylogenetic tree Order Eurotiales was represented by the strain AASBS.110. Isolate AASBS.110, formed a cluster supported by 100% bootstrap value with *Eurotium rubrum* and *Aspergillus ruber*. The sequence showed 100% similarity and 99% coverage to *Aspergillus ruber* (DQ778909, AY373891), *Eurotium rubrum* (U18357) and 99% similarity to *Eurotium* sp. (KM197171) in the BLAST searches. Another isolate AASBS.117 which was morphologically identified as *Aspergillus niger*, but the ITS sequencing data obtained were not good enough so it is not represented in the phylogenetic tree.

One of the major clade of the tree was represented by seven isolates belonging to the family Glomerellaceae (Hypocreomycetidae) which was morphologically identified as *Colletotrichum* sp. and their BLAST search showed 97-100% sequence similarity to numerous *Colletotrichum* sequences in the GenBank database. Strain AASBS.106 was placed in the clade of *Glomerella cingulate* (AB042317) and *Colletotrichum siamense* (JX010159). Isolates AASBS.101, 102, 103, 105, 104 were closely related and grouped with
Colletotrichum gloeosporioides, Colletotrichum kahawae and Colletotrichum siamense species. Strain AASBS.107 was morphologically different from other isolates, was sister to the Colletotrichum crassipes and Glomerella septospora in all the phylogenetic analysis. AASBS.107 was most likely to be C.crassipes. The phylogenetic placement of AASBS.107 was consistent on MP, ML and Bayesian analysis. Two closely related isolates (AASBS.115 and AASBS.116) showing sequence similarity with, Phomopsis sp. belonging to the order Diaporthales were nested together in MP, ML and Bayesian analysis, with bootstrap support of 100% and Bayesian posterior probability (BPP) of 1.00.

A considerable number of Basidiomycets yeasts were sampled during this study and the strains (AASBS.108, AASBS.109) were distributed in the family Tremellomycetes. Isolates AASBS.108 and AASBS.109 showed the highest similarity (99%) with Cryptococcus sequences in the GenBank database and clustered with Cryptococcus liquefaciens, Cryptococcus albidosimilis, Cryptococcus albidus with high bootstrap values in the phylogenetic tree.

Four isolates belonging to the class Dothideomycetes, order pleosporales were recognised. The first species AASBS. 112, was similar to Setophoma terrestris and were separated into a clearly distinct lineage, with strong bootstrap support (100%) and Bayesian posterior probability. Strain AASBS.113 had very similar ITS sequences in comparison with the reference isolate of Rhizopycnis vagum (100% identity and 100% query cover) from the GenBank database. AASBS.113 was clustered close to Rhizopycnis vagum and formed a well support clade as they also belong to same family. This molecular identification was consistent with its morphological identification. Sequences of AASBS.114 were placed together with Phaeosphaeria sp (DQ092510) and Ascomycete sp. (DQ092533), and they formed a sister clade to AASBS.112 and AASBS. 113 in MP, ML and Bayesian analysis. The strain AASBS.111 formed a cluster supported by 100% bootstrap value with Dothideomycetes sp.
Fig. 6.7 Results of phylogenetic analyses of endophytes isolated from *A. aspera*. Topology resulted from maximum likelihood analyses of the ITS region. The tree is rooted with *Gongronella butleri* (KM083049) and *Cunninghamella bertholletiae* (DQ155288). Scale bar indicates the nucleotide substitutions per site. Thickened lines indicate bootstrap value >70%.
6.5.6 Effect of fungal elicitor on 20-hydroxyecdysone production

Fungal elicitor induced accumulation of secondary metabolites is an extensively adopted method due to its ability to enhance the productivity of the plant cell culture systems. In the present study, addition of endophytic culture filtrate and dried cell powder did not elicit the accumulation of 20-hydroxyecdysone in cell suspension cultures and adventitious root cultures of *A. aspera*.

6.6 DISCUSSION

Endophytic fungi are ubiquitous organisms living within healthy, asymptomatic plant tissues, and constitute an underexplored dimension of fungal diversity and plant ecology (Saikkonen et al. 1998). One of the critical needs for isolating and studying endophytes is to ensure the sterility of the plant surface (Hallmann et al. 1997) and absence of colonies from the imprint plate indicates that the sterilisation is effective (Schulz et al. 1998). In this study, we adopted traditional culture dependent method for the isolation of fungal endophytes (Kumar and Hyde, 2004). The diversity of isolated fungal endophytes is largely dependent on the isolation methods (Hyde and Soytong, 2008). The present study confirmed the presence of endophytic fungal colonisation in different parts of medicinal plant *A. aspera*. Frequency of fungal colonization was highest in the leaves and lowest in the roots. Leaves get colonized by air born spores from distant sources but the uniform and stable environmental condition in the rhizosphere may be responsible for high evenness and low species richness in roots. No endophytes were isolated from the seeds of *A. aspera*.

In this present study 513 fungal isolates belonging to 10 different taxa was recovered from 950 segments of *A. aspera* and a combination of traditional and molecular methods was used for their identification. Preliminary morphological identification of the isolated strains in this study
showed that they comprised both Ascomycetes and Basidiomycetes. The majority of the isolated taxa were members of Ascomycota but one species from the collected isolates belonged to the Basidiomycota. High relative abundance of ascomycetes appears characteristic of endophytic mycota (Stone et al. 2004). Nevertheless in diverse plant species basidiomycetes also seem to be normal components of the endophytic mycota, although limited in numbers (Crozier et al. 2006). Analysis of endophyte assemblage in different tissue types led to the observation that some endophytes were commonly found in different tissues. On comparing the endophytes from leaf and stem, *Colletotrichum* sp., *Cryptococcus* sp. and *Phomopsis* sp. were consistent isolates from these tissues and were absent in root segments.

To confirm the reliability of morphological identification, the isolates were subjected to phylogenetic analysis using ITS1-5.8S-ITS2 sequences. Here the results obtained from morphological and ITS sequence based molecular methods were in good agreement. The clade represented by class Sordariomycetes included seven isolates belonging to the *Colletotrichum* species which was the most frequent and dominant endophytic fungi isolated from the medicinal plant *A.aspera*. Genus *Colletotrichum* consists of morphologically similar taxa which are commonly found as endophytic, saprobic and plant pathogenic fungi (Photita et al. 2004).

In this study, the endophytic yeast *Cryptococcus* sp. was isolated from leaves and stems segments of *A.aspera*. Different plants are reported to harbour endophytic yeast populations and some of them have the ability to produce plant growth hormones like auxin that stimulate plant growth (Nassar et al. 2005; Xin et al. 2009). A prevalence of yeast assemblages belongs to *Sporobolomyces*, *Rhodotorula*, *Debaryomyces*
and Cryptococcus were reported endophytes in apple (Camatti-Sartori et al. 2005).

Two fungal isolates from A. aspera was molecularly identified as species of Phomopsis. Phomopsis species are considered to be the asexual phases of Diaporthe species, a teleomorphic genus in the family Valsaceae (Girlanda et al. 2002). Phomopsis sp. are not host specific and their variabilities in host range are wide, their assemblage is in numerous plant hosts which are taxonomically irrelevant (Siebe, 2007).

Previous evidences suggested that genera and species that are capable of causing diseases are regularly isolated as endophytes and the difference between a pathogen and endophyte is not always clear (Sinclair and Cerkauskas, 1996). In this study, the endophytic community isolated from the roots of A.aspera showed similarity with, Setophoma terrestris (synonym Phoma terrestris, Pyrenochaeta terrestris), Rhizopycnis vagum and Phaeosphaeria sp. Rhizopycnis vagum belongs to the complex of root rot pathogens which contribute root rot and associated with the vine decline (collapse) of cucurbits in different parts of the world. The fungus is also reported as an endophytic associate of mycorrhizal roots of wild, asymptomatic Pinus halepensis and Rosmarinus officinalis plants in Italy (Ghignone et al. 2003). S. terrestris was isolated in the ginseng root. P. terrestris are associated with pink root disease on onions (Allium cepa) (Biles et al. 1992). This indicates that the status of the interaction between endophyte and host may vary; many pathogens of economically important crops may be endophytic or latent in weeds (Sinclair and Cerkauskas, 1996).

Addition of fungal elicitor to the cell suspension and adventitious root culture medium does not increase the production of
20-hydroxyecdysone in *A. aspera*. It coincides with the previous data which suggest that in *Spinacia oleracea*, root inoculations with two different fungal pathogens (*Pythium aphanidermatum* and *Phytophthom capsici*) failed to elicit the accumulation of 20-hydroxyecdysone (Schmelz et al. 1998).

To summarise, the work was conducted to estimate the diversity and phylogenetic relationship of the endophytic mycobiotas associated with *A. aspera* and the isolated endophytic fungal strains did not elicit 20-hydroxyecdysone production in *in vitro* cultures of *A.aspera*. 