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SHORT REPORTS

Endophytic fungal assemblage in *Achyranthes aspera* Linn. revealed by internal transcribed spacer region of nuclear ribosomal RNA genes

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Abstract Only scanty reports are available on endophytic fungal associations in *Achyranthes aspera* Linn. Hence in this study a total of 504 isolates belonging to ten different species of fungi were isolated from asymptomatic, surface sterilised segments of leaf, stem and root of *A. aspera* collected from different locations of Kerala, India. Among the isolates ascomycetes were most prevalent. Colonisation rate of fungal endophytes was high in leaf tissue (95%) followed by stem (77.75%) and root segments (33.33%). The most frequent and dominant coloniser of the host plant were *Colletotrichum* sp., which was isolated from all locations. Scanning electron microscopy demonstrated the presence of hyphae in the intra and intercellular spaces of the plant tissue. Morphological and phylogenetic analyses using internal transcribed spacer (ITS) sequences of nuclear rRNA genes showed that the fungi recovered belonged to the 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.

Keywords *Achyranthes aspera* Linn. · Endophytic fungi · Internal transcribed spacer (ITS) sequences · Scanning Electron Microscopy

Introduction

Endophytic fungi are microorganisms that invade and colonise plant tissues internally without causing any undesirable effects on host plant species (Aly et al. 2011). They are associated with numerous plant species and isolated from nearly all organs of every plant sampled (Currie et al. 2014). Endophytes enhance plant growth by the production of plant hormones such as indole-3-acetic acid (IAA), gibberellins (GAs) and cytokinins (Khan et al. 2014). Antimicrobial metabolites like colletotric acid (Zou et al. 2000), griseofulvin (Park et al. 2005) and glucosidase inhibitor 3*S*, 4*R*-(+)-4-hydroxymellein (Rivera-Chavez et al. 2015) were reported from endophytic fungi. Endophytic fungi are ubiquitous as well as extremely diverse in plants. Only a small fraction of it has been described and explored till date (Chen et al. 2013).

Present study investigates the endophytic mycobiota associated with the medicinal plant *A. aspera*. No comprehensive work has been done on biology and distribution of endophytes within this plant. 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 different locations of Kerala, India.

Materials and methods

Plant material and sampling

Different parts (leaves, stems, root and seeds) from the plant *A. aspera* were sampled for the isolation of endophytic fungi. Four to five asymptomatic plants were collected from different locations of Kerala, India. The

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samples were collected, labelled and transported to the laboratory. They were stored at 4 °C and processed within 48 h. A voucher herbarium specimen of the plant was deposited at Regional Herbarium Kerala, India, with field no: 6302 and Accession no: 7565.

Isolation and morphological identification of fungal endophytes

The plant materials (stem, leaves, seeds and roots) were washed in running tap water, immersed in 70% ethanol for 1 min and surface sterilized in 0.1% mercuric chloride solution for 5 min. Thereafter, the samples were rinsed 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 (5 mm, 5 mm × 5 mm). Four surface-sterilized segments were evenly placed in each of the petri dishes containing potato dextrose agar medium (PDA, Himedia, India). The antibiotic streptomycin sulphate (250 mg L^{-1}) (Himedia, India) was added to the media to suppress bacterial growth. The surface sterilized plant segments were pressed on to the surface of PDA medium to check the efficacy of the adopted surface sterilization method. The petri dishes were sealed with parafilm, incubated at 28 °C and observed every day for fungal growth. Emerging hyphal tips were transferred to fresh PDA plates to obtain pure isolates. Morphological identification was done by growth pattern, colony appearance, surface texture, margin characters, mycelium color and pigmentation.

Data analysis

The colonization rate (CR) was calculated as the total number of segments in a sample yielding ≥1 isolates divided by the total number of segments incubated. The isolation rate (IR) was calculated as, IR = Total number of isolates yielded in a sample divided by the number of segments incubated in the sample. The colonization frequency (CF %) of each endophyte was calculated as the number of segments colonized by each endophyte divided by the total number of segments observed.

DNA extraction and PCR amplification of ITS region of nuclear ribosomal RNA genes

The endophytic fungi were inoculated into liquid potato dextrose broth (PDB) medium and incubated at 28 °C, for 7 days. The fungal mycelia were used for DNA extraction. Fungal DNA from the samples was isolated as per Cenis (1992). Quality of DNA was estimated by agarose gel electrophoresis (0.8%) and quantity was estimated by UV absorbance. ITS fragment was amplified from fungal genomic DNA using primers ITS1:5′-TCCGTAGGT GAACCTGCGG-3′ and ITS4: 5′-TCCTCCGCTTATT GATATGC-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 (10×) 2.5 µL, $MgCl_2$ (25 mM) 0.6 µL, Primer forward ITS1 (10 pmol/µL) 0.5 µL, primer reverse ITS4 (10 pmol/µL) 0.5 µL, dNTP mix (2.5 mM) 2 µL, *Taq* (3U/µL) 0.8 µL, template DNA (25 ng/µL) 1 µL. The PCR protocol was designed for 35 cycles, 5 min initial denaturation at 94 °C followed by denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, extension at 72 °C for 1 min and final extension 72 °C for 10 min. Sequencing of amplicon with forward and reverse primers was carried out in ABI 3730xl cycle sequencer (SciGenome labs, Cochin, Kerala, India).

Phylogenetic analyses

Sequence similarity searches were performed for obtained fungal sequences and compared with ITS sequence data from strains available in the public database GenBank (http://www.ncbi.nlm.nih.gov) by using the BLAST sequence match routines. The closest hits were downloaded in FASTA format and were multiple aligned using the 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 Paup4.0B (Swofford 2002). 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 by MrBayes version 3.1.2 (Ronquist et al. 2012) using the best fitting model chosen through the AIC in jModel Test v2 (Darriba et al. 2012). All Trees were visualized by Fig Tree, Tree Figure drawing tool version 1.4.2 (Rambaut 2014).

Sequences obtained from this study were deposited in GenBank with Accession numbers, KF709446, KF841604, KF841605, KF841606, KF841607, KF841608, KF841609, KF841610 KF841611, KX664305, KX664306, KY924822, KY924823 and KY924824.

Histological analysis of endophytic colonization in *A. aspera* by scanning electron microscopy (SEM)

Histological analysis of plant samples was done for ascertaining the presence of endophytic fungi in the internal plant tissues. For SEM analysis the plant specimens

were fixed using 2.5% gluteraldehyde in 0.1 M phosphate buffer for 12 h and then washed thrice in 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 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.

Results and discussion

Isolation and histological analysis of fungal endophytes in *A. aspera*

Explorations of the endophytic fungi residing in plants are essential for the assessment of global fungal diversity, distribution and also for the discovery of new species (Siqueira et al. 2008). In the present investigation scanning electron microscopic studies clearly visualized the emergence of fungal endophytic mycelium in *A. aspera* leaf and stem tissues (Fig. 1). Histological analysis 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).

A total of 504 fungal isolates belonging to 10 different species were recovered from 950 segments of *A. aspera* collected from different localities of Kerala, India. Morphological identification of the isolated strains showed that majority of them comprised of ascomycetes with only one genus of basidiomycetes. The colonization rates of endophytes from *A. aspera* were 95% (leaves), 77.7% (stems) and 33.3% (roots) respectively (Table 1). Frequency of fungal colonization was highest in leaves and lowest in roots. Leaves may get colonized by air born spores from distant sources. However, the uniform and stable environmental condition in the rhizosphere could be responsible for high evenness and low species richness in roots (Mishra et al. 2012). The most commonly isolated ascomycetes were Sordariomycetes, followed by Dothideomycetes and Eurotiomycetes. Tremellomycetes (basidiomycetes) were also isolated as endophytes from *A. aspera*. The prominent fungal populations observed from leaves and stem segments in all locations were *Colletotrichum* species. High relative abundance of ascomycetes appears characteristic of endophytic mycota (Stone et al. 2004). Nevertheless, in many plant species; a limited number of basidiomycetes are also found as normal components of the endophytic mycota (Crozier et al. 2006). In this study it is observed that some endophytes were of common occurrence in different tissues. On leaf and stem, *Colletotrichum* sp., *Cryptococcus* sp. and *Phomopsis* sp. were consistent isolates, but they were absent in root segments. Literature states that the dominant endophytic species and the species composition of the endophytes may vary among tissue types (Arnold et al. 2007).

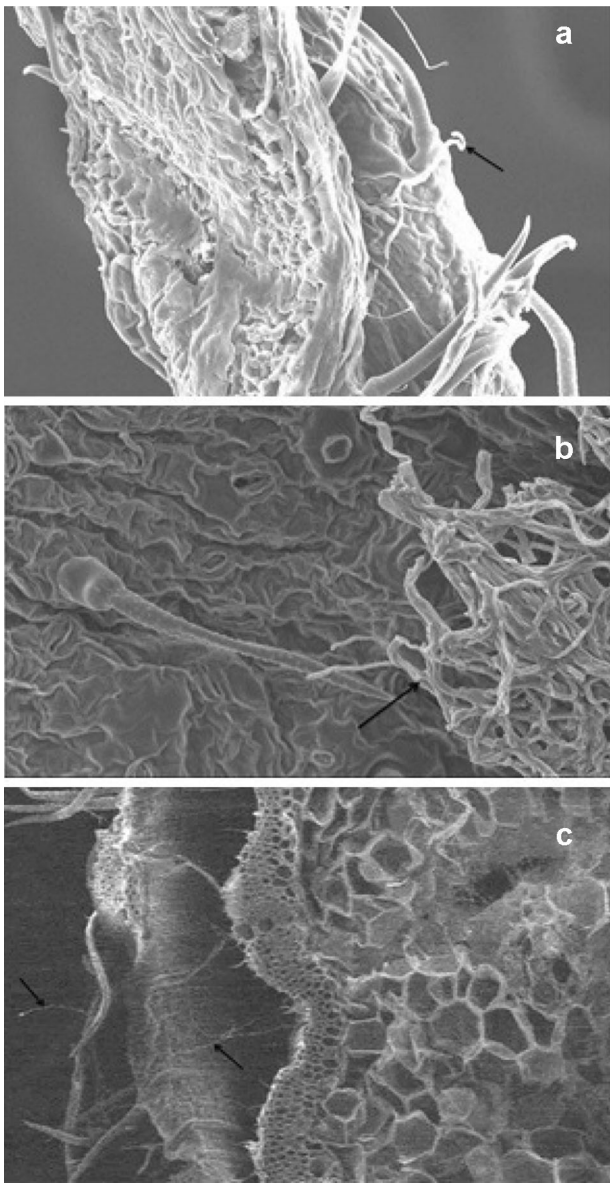

Fig. 1 SEM micrographs of *A. aspera* leaf and stem after 48 h of incubation on PDA. **a** Emergence of endophytic fungal hyphae from leaf surface. **b** Fungal hyphae in mats on the surface of leaf tissues. **c** Transverse sections of stem, showing the colonization of endophytic fungi

Molecular identification of endophytes based on ITS analysis

To confirm the morphological identification, the isolates were subjected to a molecular analysis using nuclear

Table 1 Colonisation and isolation rates of fungal endophytes in *A. aspera*

	Leaves	Stem	Roots
No. of samples	400	400	150
No. of samples yielding fungi	380	311	50
No. of isolates	244	220	40
Colonisation rate (%)	95	77.77	33.33
Isolation rate (%)	61	55	26

ribosomal RNA ITS1-5.8S-ITS2 gene sequences. Amplification of the ITS sequences of the isolated endophytes generated a single fragment of 550–650 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 coincided with the morphological identification. For Bayesian analysis best fitting substitution model selected was TRN + G. Bayesian analysis produced trees (Fig. S1) which were consistent in topology with ML and MP (Fig. S2) analyses, confirming the phylogenetic placement of the endophytes associated with *A. aspera.* A maximum likelihood tree based on the ITS sequences of the fungal endophytes with *Gongronella butleri* and *Cunninghamella bertholletiae* as outgroup taxa is shown in Fig. 2.

The clade represented by class Sordariomycetes included seven isolates belonging to *Colletotrichum* species [KF709446 (AASBS.101), KF841604 (AASBS.103), KF841605 (AASBS.102), KF841606 (AASBS.104), KF841607 (AASBS.105), KF841608 (AASBS.106), and KF841609 (AASBS.107)]. These were the most frequent and dominant endophytic fungi isolated from *A. aspera*. Genus *Colletotrichum* consists of morphologically similar taxa which are commonly found as endophytes, saprobes and plant pathogens (Photita et al. 2004). In the phylogram the fungal species belonging to order Eurotiales was represented by the strain AASBS.110 (KY924823). Isolate AASBS.110, formed a cluster supported by 100% bootstrap value with *Eurotium rubrum* and *Aspergillus ruber*. Endophytic fungus *Eurotium rubrum* has been isolated from the stem segments of *Hibiscus tiliaceus* (Li et al. 2008).

Two fungal isolates from *A. aspera* were identified as species of *Phomopsis. Phomopsis* sp. is the asexual phases of *Diaporthe* species, a teleomorphic genus in the family Valsaceae. *Phomopsis* sp. is not host specific and has a wide range of taxonomically unrelated host plants (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 was not always clear (Sinclair and Cerkauskas 1996). In this study, four isolates belonging to the class Dothideomycetes were isolated from the roots of *A. aspera* which showed similarity with, *Setophoma terrestris* [KX664305 (AASBS.112)] (synonym *Phoma terrestris*, *Pyrenochaeta terrestris*), *Rhizopycnis vagum* [KX664306 (AASBS.113)], *Phaeosphaeria* sp. [KY924824 (AASBS.114)] and Dothideomycetes sp. [KY924822 (AASBS.111)]. 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). In the present study, the endophytic yeast *Cryptococcus* sp. [KF841610 (AASBS.108), KF841611 (AASBS.109)] 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 auxins that stimulate plant growth (Xin et al. 2009).

Conclusion

The present study contributed to the knowledge about the biodiversity and phylogenetic relationship of the endophytic mycobiota associated with *A. aspera*. The isolated endophytic fungi were grouped according to their morphology and subjected to molecular analysis using ribosomal ITS region. Endophytic fungal species obtained throughout the study were *Colletotrichum* sp., *Cryptococcus* sp., *Phomopsis* sp., *Eurotium* sp., Dothideomycetes sp., *Setophoma terrestris*, *Rhizopycnis vagum*, and *Phaeosphaeria* sp. Histological methods with scanning electron microscopy demonstrated the presence of fungal hyphae in inter and intra cellular spaces. Further studies are needed to expand our knowledge about the biotechnological potential of these endophytic fungi for the production of bioactive compounds.

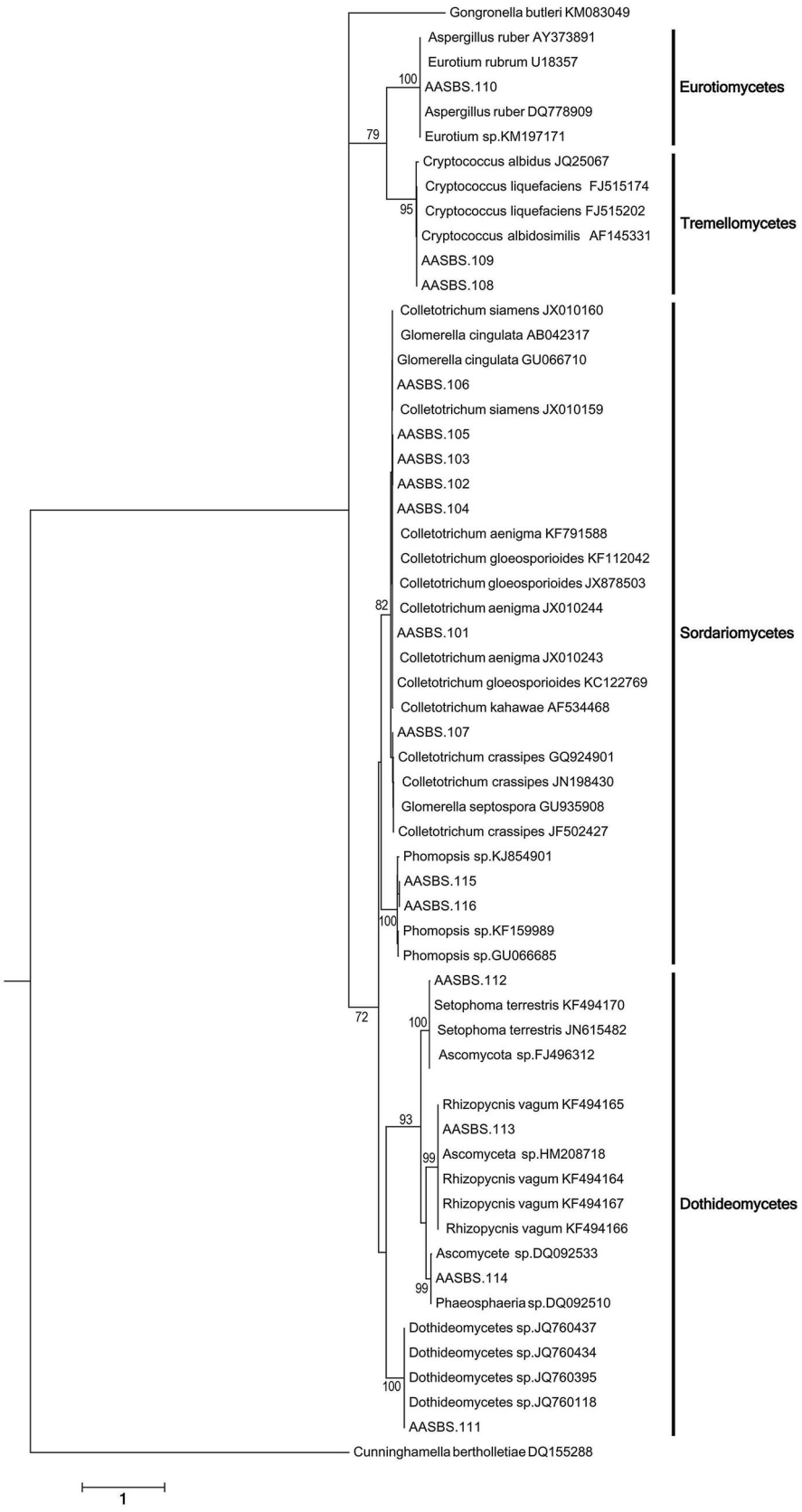

Fig. 2 Results of phylogenetic analyses of endophytes isolated from *A. aspera*. Topology resulted from maximum likelihood analyses of the ITS region. The tree is rooted with *Gongronella butleri* (KM083049) and *Cunninghamella bertholletiae* (DQ155288). *Scale bar* indicates the nucleotide substitutions per site

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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