Fungal Diversity In Leaves And Stems Of Neem (Azadirachta Indica)

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Abstract— A study was conducted to examine fungal endophytes present in leaf and stem tissues of Azadirachta indica. A total of 65 fungal isolates were recovered from 144 neem plant segments (leaf and stem tissues) showing no disease symptoms or physical damage. The samples were collected from 8 different locations in Oman during the year 2017. The isolates were classified into 15 different morphotypes according to culture characteristics and were identified based on rDNA ITS sequence analysis. In total, 23 taxa belonging to 15 genera were identified, all belonging to the ascomycetes classes Dothideomycetes, Sordariomycetes and Eurotiomycetes. Class Dothideomycetes was dominant and was represented by six families: Cladosporiaceae, Saccotheciaceae, Botryosphaeriaceae, Didymellaceae and Pleosporaceae, followed by Sordariomycetes (Chaetomiaceae, Microascaceae and Nectriaceae) and Eurotiomycetes (Trichocomaceae and Aspergillaceae). The most frequently isolated taxa were Cladosporium sphaerospermum, Alternaria spp and Aspergillus niger. Leaf samples yielded more fugal taxa compared to stem, and our data show that neem contains taxonomically diverse fungal endophytes. Furthermore, this is the first report of Aspergillus caespitosus, Curvularia geniculate, Curvularia subpapendorfii, Leptosphaerulina australis and Microascus cinereus from Oman.

Index Terms— Endophytic fungi, fungal diversity, Medicinal plants, endophytes

1 Introduction

Azadirachta indica A. Juss, known as neem, belongs to the family Meliaceae. Due to its medicinal importance, it has long been for improving human health [1, 2]. Neem is the most studied tree in the world and has been declared worldwide due to its medicinal importance as the "Tree of the 21st century" by the United Nations [3]. Besides these uses, other plant parts such as fruits, seeds, leaves, bark and roots were proven to contain compounds with biological actions such as antiseptic, antipyretic, antiulcer and antifungal uses [3, 4]. A number of studies have been conducted on the endophytic fungi isolated from neem. Kusari, Verma [2] isolated the endophytic fungus Eupenicillium parvum from A. indica that produces the natural insecticide azadirachtin, which is also produced by the host plant. Lactones were produced by an endophytic Phomopsis sp. fungus obtained from neem stems and were used against some plant pathogens such as Botrytis cinerea [5]. The endophytic fungu Chloridium sp. isolated from root tissues of the neem plant was able to produce an antibacterial nephthaquinone 'Javanicin' against Pseudomonas spp. [6]. Limited studies are available on fungi from neem in Oman. The aim of the present study was to isolate endophytes from neem trees and to assess the phylogenetic diversity of the isolated species.

2. Materials and methods

2.1 Isolation of neem endophytes

Healthy mature leaves and stems of neem plants were collected from eight different locations in Oman during 2017 (Table 1). Samples developing disease symptoms or with physical damage excluded from analysis. The samples were cut into 0.5 cm pieces and sterilized in 1% sodium hypochlorite solution. Isolation was done in Potato Dextrose Agar (PDA) the samples were incubated at (24-26°C) for 1-3 days. Fungal colonies were transferred to PDA slants, and stored at Sultan Qaboos University Culture Collection (SQUCC) at 4°C for further studies.

2.2. PCR and sequencing

The above method resulted in 65 fungal isolates. Total genomic DNA was extracted from those fresh cultures using a modified protocol of Al-Sadi, Al-Jabri [7]. Polymerase chain reaction (PCR) was used to amplify the Internal Transcribed Spacer region (ITS) using primer pairs ITS5/ITS4 [8]. The PCR amplification was performed with an initial denaturing step of 94 °C for 3 min, followed by 40 amplification cycles of 94 °C for 45 s, 55 °C for 50 s and 72 °C for 1 min and a final extension step of 72 °C for 10 min. Sequences generated from the study were lodged in GenBank (Table 1).

2.3. Phylogenetic analyses

Sequence alignment was generated with MEGA v.5.2.2 (Kumar et al. 2012) and the alignment was visually improved where necessary. Phylogenetic analyses were performed for maximum likelihood in RAxMl GUI v. 1.3 [9]. Parameters of the RAxMl GUI v. 1.3 were set to rapid bootstrapping and the analysis carried out using 1000 replicates and GTRGAMMA model of nucleotide substitution. The resulting trees were printed with FigTree v. 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

3. Results

3.1 Fungal isolates

In our study, a total of 65 fungal strains have been recovered from leaves (37) and stems (28) of 144 neem plant segments (Fig. 1). These mycelia sterilia were separated into 15 morphological groups based on the similarity of their culture characteristics (Bills 1996; Fróhlich et al. 2000; Lacap et al. 2003).

TABLE 1
ISOLATES, COLLECTION DETAILS AND THEIR GENBANK ACCESSION NUMBERS OF TAXA GENERATED DURING THIS STUDY

Species name	Culture collection (SQUCC)	Plant parts	GenBank accession
Alternaria sp.	13668	leaves	MH368583
Alternaria sp.	13671	leaves	MH368584
Alternaria sp.	13672	leaves	MH368563
Alternaria sp.	13599	leaves	MH368568
Alternaria sp.	13600	leaves	MH368569
Alternaria sp.	13656	leaves	MH368578
Alternaria sp.	13727	stems	MH368602
Alternaria sp.	13728	stems	MH368603
Aspergillus	13804	leaves	MH368613

caespitosus			
Aspergillus niger	13605	leaves	MH368571
Aspergillus niger	13669	stems	MH368612
Aspergillus niger	13673	leaves	MH368585
Aspergillus niger	13679	stems	MH368587
Aspergillus niger	13680	leaves	MH368588
Aspergillus niger	13726	leaves	MH368601
Aspergillus sp.	13561	leaves	MH368564
Cladosporium sp.	13563	stems	MH368566
Cladosporium sp.	13652	leaves	MH368575
Cladosporium sp.	13654	leaves	MH368577
Cladosporium sp.	13663	stems	MH368581
Cladosporium sp.	13667	leaves	MH368582
Cladosporium sp.	13677	leaves	MH368586
Cladosporium sp.	13706	leaves	MH368590
Cladosporium sp.	13707	leaves	MH368591
Cladosporium sp.	13708	stem	MH368592
Cladosporium sp.	13710	stem	MH368593
Cladosporium sp.	13779	stem	MH368554
Cladosporium sphaerospermum	13572	leaves	MH368617
Cladosporium sphaerospermum	13606	leaves	MH368572
Cladosporium sphaerospermum	13659	leaves	MH368579
Cladosporium sphaerospermum	13660	leaves	MH368580
Cladosporium sphaerospermum	13666	leaves	MH368562
Cladosporium sphaerospermum	13778	stems	MH368553
Cladosporium sphaerospermum	13783	leaves	MH368555
Cladosporium sphaerospermum	13800	leaves	MH368556
Cladosporium sphaerospermum	13802	stems	MH368558
Cladosporium sphaerospermum	13803	leaves	MH368559
Cladosporium sphaerospermum	13806	stems	MH368614
Cladosporium sphaerospermum	13807	stems	MH368560
Curvularia geniculata	13722	stems	MH368599
Curvularia sp.	13725	stems	MH368600
Curvularia subpapendorfii	13602	leaves	MH368570
Curvularia subpapendorfii	13653	leaves	MH368576
Fusarium brachygibbosum	13484	stems	MH368604
Fusarium chlamydosporum	13488	stems	MH368606
Fusarium equiseti	13711	stems	MH368594
Fusarium solani	13562	leaves	MH368565
Fusarium solani	13566	stems	MH368567
Fusarium solani	13567	stems	MH368615
Fusarium solani	13721	leaves	MH368598
Fusarium sp.	13564	stems	MH368605
Kabatiella sp.	13481	stems	MH368561
Lasiodiplodia sp.	13485	stems	MH368607
Leptosphaerulina australis	13607	leaves	MH368573
Leptosphaerulina australis	13685	leaves	MH368610
Leptosphaerulina australis	13610	leaves	MH368574
Microascus cinereus	13681	leaves	MH368589
Nigrospora sp.	13601	leaves	MH368608
Penicillium citrinum	13801	stems	MH368557
Peyronellaea sp.	13612	stems	MH368609
Phoma sp.	13714	stems	MH368595
Phoma sp.	13715	stems	MH368596
Phoma sp.	13716	stems	MH368597
Talaromyces pinophilus	13486	stems	MH368611
Thielavia sp.	13571	leaves	MH368616

3.1 Phylogenetic analyses

The alignment comprised 143 strains with an alignment length of 648 characters, including the outgroup taxon Dissophora decumbens and 65 newly generated sequences in this study. The majority of fungal isolates were recovered from the samples collected from the SQU botanical garden. The phylogenetic analysis indicated that strains were mainly affiliated to class Dothideomycetes (44 isolates) with a small fraction associated to Sordariomycetes (11 isolates) and Eurotiomycetes (10 isolates) (Fig. 2). Fungal isolates obtained are in six families of class Dothideomycetes (Cladosporiaceae, Saccotheciaceae, Botryosphaeriaceae, Didymellaceae and Pleosporaceae), two families of class Eurotiomycetes (Trichocomaceae, Aspergillaceae) and three families of class Sordariomycetes (Chaetomiaceae, Microascaceae, Nectriaceae). In the present study, the fungal endophytes could be assigned to 15 genera: Alternaria, Aspergillus, Cladosporium, Curvularia, Fusarium, Kabatiella, Lasiodiplodia, Leptosphaerulina, Peyronellaea, Microascus, Nigrospora, Penicillium, Phoma, Talaromyces and Thielavia. Talaromyces pinophilus, Penicillium citrinum, Kabatiella sp., Lasiodiplodia sp. and Phoma spp. Only were present in the stems of neem plants. On the other hand Leptosphaerulina australis, Nigrospora sp., Thielavia sp. and Microascus cinereus were detected only from the leaves of neem plants. Cladosporium sphaerospermum was the most isolated species and Aspergillus niger, Alternaria sp. and Cladosporium sp are the other commonly isolated fungi.

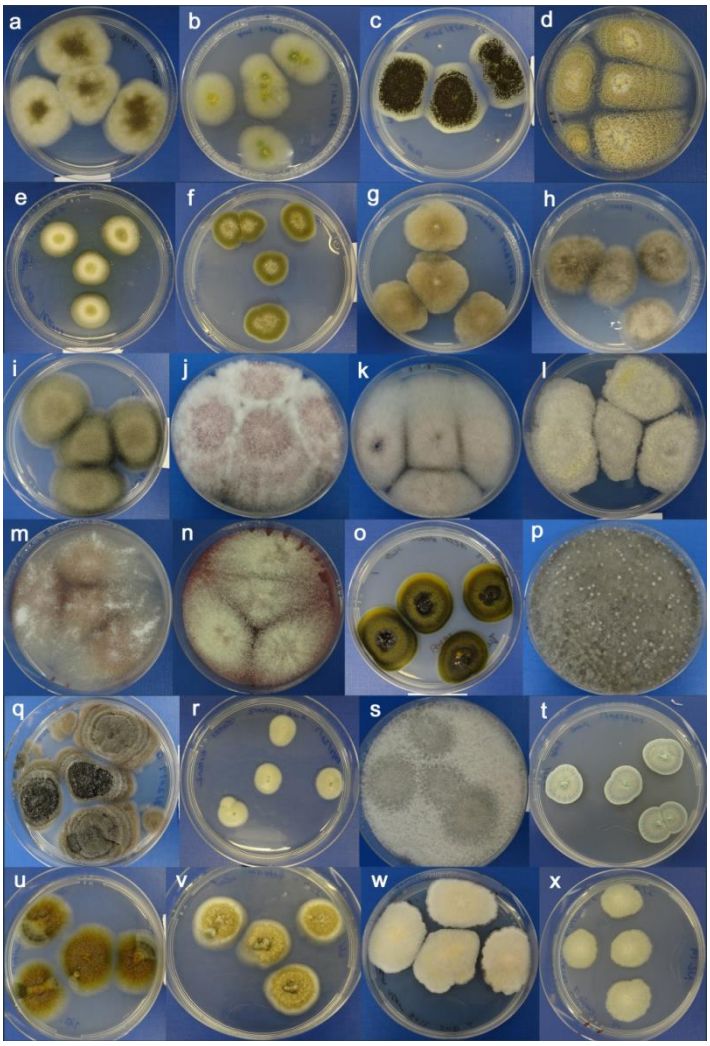
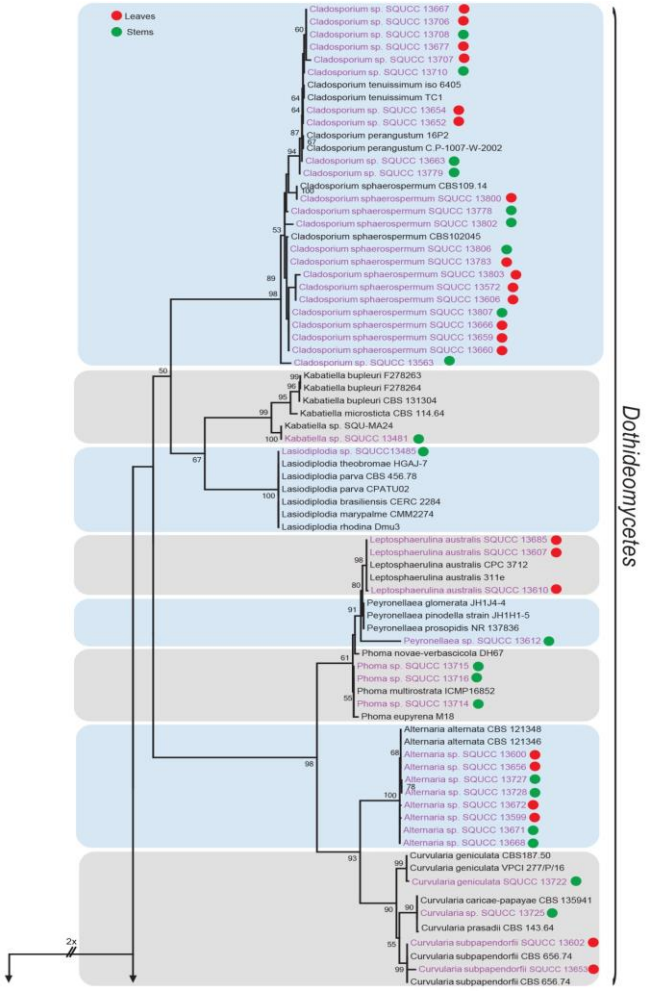


Fig 1. *Fungal species isolated during the study. a. Alternaria sp. (SQUCC 13672) b. Aspergillus caespitosus (SQUCC 13804) c. Aspergillus niger (SQUCC 13605) d. Aspergillus sp. (SQUCC 13561) e. Cladosporium sp. (SQUCC 13677) f. Cladosporium sphaerospermum (SQUCC 13659) g. Curvularia geniculata (SQUCC 13722) h. Curvularia sp. (SQUCC 13725) i. Curvularia subpapendorfii (SQUCC 13602) j. Fusarium brachygibbosum (SQUCC 13484) k. Fusarium chlamydosporum (SQUCC 13488) l. Fusarium equiseti (SQUCC 13711) m. Fusarium solani (SQUCC 13562) n. Fusarium sp. (SQUCC 13564) o. Kabatiella sp. (SQUCC 13481) p. Lasiodiplodia sp. (SQUCC 13485) q. Leptosphaerulina australis (SQUCC 13607) r. Microascus cinereus (SQUCC 13681) s. Nigrospora sp. (SQUCC 13601) t. Penicillium citrinum (SQUCC 13801) u. Peyronellaea sp. (SQUCC 13612) v. Phoma sp. (SQUCC13715) w. Talaromyces pinophilus (SQUCC 13486) x. Thielavia sp. (SQUCC 13571).*



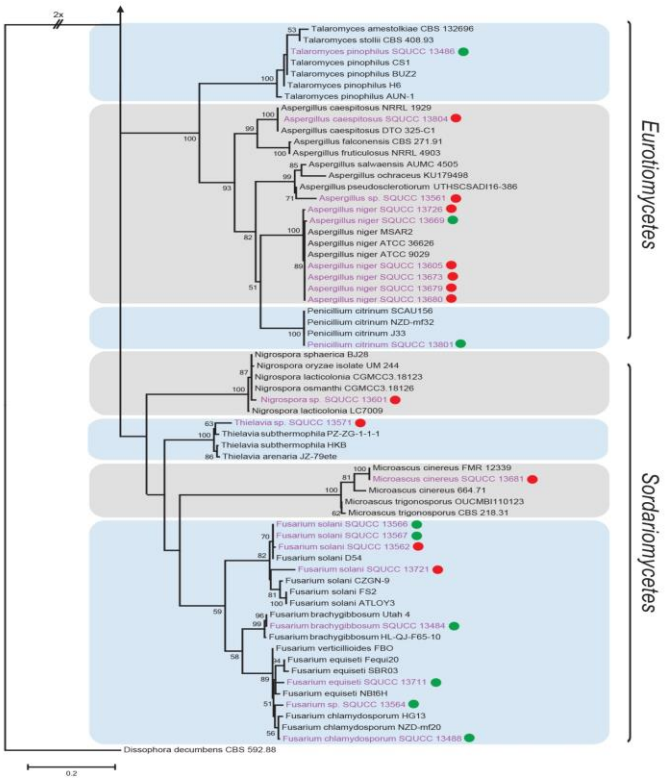


Fig 2. Phylogram generated from maximum likelihood analysis of a ITS sequence data generated in this study and related taxa obtained from GenBank. Dissophora decumbens (CBS 592.88) is used as the outgroup taxa. Bootstrap support values for maximum likelihood equal to or greater than 50% is given at the nodes. Newly generated sequences are in purple.

4. Discussion

Our study showed that several fungal species inhibit as endophytes within the stems and leaves of neem plants. The occurrence of these fungi varied from one location to the other, with some fungal species being common in most of the surveyed regions and plant samples. Most of the fungal species were recovered from the leaf samples. This is in an agreement with previous studies in which endophytes were isolated from different plant parts, with leaves having higher species diversity compared to stems [10, 11]. Our study demonstrated that the class Dothideomycetes is the most dominant fungal group in neem plants. Previous studies have indicated the dominance of endophytic Dothideomycetes in vascular plants species [12]. Aspergillus caespitosus, Curvularia geniculate, Curvularia subpapendorfii, Leptosphaerulina australis and Microascus cinereus are reported for the first time from Oman. Furthermore, to our knowledge this is the first report that Curvularia geniculata, Curvularia subpapendorfii, Leptosphaerulina australis and Microascus cinereus as endophytes on neem plants. Endophytic fungi have been isolated from many tree species throughout the world and these fungi establish mutualistic interactions with their host and therefore both parties benefit from each other [13]. Verma, Gond [11] found Phomopsis oblonga, Cladosporium cladosporioides, other endophytes. Cladosporium was the most dominant endophytic fungus isolated during the present study. Members of genus Cladosporium are generally considered the most common outdoor fungi, as well as being frequently isolated as endophytes [14]. Most of the fungi isolated during this study are known to produce a wide range of chemically diverse metabolites. Cladosporium sphaerospermum, the dominant fungal endophyte isolated during this study, is well known to promote plant growth by producing Gibberellin [15]. The endophytic Curvularia geniculata has been shown to produce hybrid peptide-polyketides, curvularides A–E which show an antifungal activity against Candida albicans [16]. Aspergillus niger is a core microorganism used in biotechnology to produce extracellular enzymes and citric acid [17]. Many known plant pathogenic genera; Alternaria, Cladosporium, Fusarium, Lasiodiplodia and Phoma were detected in this study. Studies have shown that some endophytes may remain dormant symptomless inhabitants of plants, but may cause symptoms in plants under stress [18]. The ITS region has a high sequence and PCR success rate and it is the universal barcode for fungi [19]. However, it sometimes does not have a high resolving power within some fungal lineages. Some fungal genera isolated during this study could not be discriminated to the species level. Previous studies have shown that the combination of multi genes can give better species resolution than a single gene [20, 21]. For an example the combination of ITS, Calmodulin and β-tubulin genes gave better species resolution to fungal genera in Eurotiomycetes such as Aspergillus, Penicillium and Talaromyces [22]. Therefore, future studies with multilocus data may need to address the identity of the isolates recovered during this study.

5. CONCLUSIONS

The study provides a detailed description of fungal endophytes in leaves and stems of neem grown under arid conditions. To our knowledge this is the first report that Curvularia geniculata, Curvularia subpapendorfii, Leptosphaerulina australis and Microascus cinereus as endophytes on neem plants. Future studies should investigate the roles of these endophytes in neem and the potential bioactive compounds that may be produced by the endophytes.

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