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Molecular Characterization and Analysis of Antimicrobial Activity of Endophytic - From Medicinal Plants in Saudi Arabia

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Abstract

Background: Endophytic fungi, which have been reported in numerous plant species, are important components of the forest community and contribute significantly to the diversity of natural ecosystems.

Objectives: The current study aimed to evaluate and characterize, at the molecular level, the diversity and antimicrobial activities of endophytic fungi from medicinal plants in Saudi Arabia.

Materials and Methods: Fungi growing on plant segments were isolated and identified based on morphological and molecular characteristics. The isolates were grouped into 35 distinct operational taxonomic units, based on the sequence of the internal transcribed spacer regions in the *rRNA* gene. The colonization frequency and the dominant fungi percentage of these endophytic fungi were calculated. A dual culture technique was adopted to investigate the antifungal activity of these endophytes.

Results: *Tamarix nilotica* showed the highest endophytic diversity with a relative frequency of 27.27%, followed by *Cressa cretica* with a relative frequency of 19.27%. The most frequently isolated species was *Penicillium chrysogenum* with an overall colonization rate of 98.57%. Seven out of 35 endophytic fungi exhibited strong antifungal activity to all plant fungal pathogens tested. *P. chrysogenum*, *Fusarium oxysporum*, and *F. nygamai* exhibited the highest inhibition against the human pathogenic bacteria *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. *Aspergillus sydowii*, *P. chrysogenum*, and *Eupenicillium crustaceum* showed strong antimicrobial activity against *Enterococcus faecalis*.

Conclusions: The antimicrobial activity of these endophytic microorganisms could be exploited in biotechnology, medicine, and agriculture.

Keywords: Endophytic Fungi, Medicinal Plants, Antimicrobial Activity

1. Background

Epiphytic or endophytic fungi spend a part of their lifecycle outside or inside leaf tissues, with no negative impact on the host (1). Endophytic fungi, which have been reported in numerous plant species, are important components of the forest community and significantly contribute to the diversity of natural ecosystems (2). It has also been shown that some fungal endophytes can produce various bioactive chemicals and have potential applications in biocontrol and resistance (3). They play important roles in recycling nutrients in natural ecosystems (4, 5). Medicinal plants represent an important health and economic component of biodiversity; therefore, it is essential to make a complete inventory of the medicinal components of each country's flora, in order to facilitate conservation and sustainable use (6). According to Mossa et al. (7), the Kingdom of Saudi Arabia possesses a wide range of flora, consisting of a large number of medicinal herbs, shrubs, and trees; folk medicine has been practiced since time immemorial.

Previous studies have reported the isolation and identification of endophytic fungi and bacteria from medicinal plants such as *Cressa cretica*, *Achillea fragrantissima*, and *Artemisia* species (8-10). A few other studies have evaluated the antimicrobial activity of the fungal endophytes against plant pathogenic fungi such as *Neurospora* sp. (11), *Magnaporthe grise*a, *Corticium sasakii*, *Botrytis cinerea*, *Phytophthora infestans*, *Puccinia recondita*, *Blumeria graminis* (12), *Alternaria alternata*, *Fusarium oxysporum*, *B. cinerea*, and *Pythium ultimum* (13).

2. Objectives

In the present investigation, we evaluated the diversity of endophytic fungi colonizing seven species of medicinal plants (*Alhagi graecorum*, *C. cretica*, *Citrullus colocynthis*, *Tamarix nilotica*, *A. fragrantissima*, *Artemisia sieberi*, and *Neurospora retusa*), collected from salt marshes of the Al-Gouf governorate in North Saudi Arabia. The molecular characterization and antimicrobial activity of these isolates were investigated.

Table 1. List of Medicinal Plants Utilized in This Study

S/NO	Scientific Name	Family	Common Name	Part of Plant Used	Collection Site
1	*Achillea fragrantissima*	*Asteraceae*	Lavender cotton	Stem and roots	Al-Gouf Governorate
2	*Alhagi graecorum*	*Fabaceae*	Camel thorn	Stem and roots	Al-Gouf Governorate
3	*Artemisia sieberi*	*Asteraceae*	Desert wormwood	Stem and roots	Al-Gouf Governorate
4	Citrullus *colocynthis*	*Cucurbitaceae*	Bitter apple	Stem and roots	Al-Gouf Governorate
5	*Cressa cretica*	*Convolvulaceae*	Rudravanti	Stem and roots	Al-Gouf Governorate
6	*Neurospora retusa*	*Nitrariaceae*	Salt tree	Stem and roots	Al-Gouf Governorate
7	*Tamarix nilotica*	*Tamaricaceae*	Nile tamarisk	Stem and roots	Al-Gouf Governorate

3. Materials and Methods

3.1. Collection of Plant Samples

A total of 70 stem and roots samples of seven disease-free medicinal plants, namely *A. graecorum*, *C. cretica*, *C. colocynthis*, *T. nilotica*, *A. fragrantissima*, *A. sieberi*, and *N. retusa*, were randomly collected from the salt marshes of Al-Gouf Governorate, Kingdom of Saudi Arabia, during the period from July to September 2013. The selected plants that belonged to different families are listed in Table 1.

3.2. Isolation of Fungal Endophytes

The samples were rinsed gently in running tap water to remove dust and debris. The stem and roots samples were cut into three segments measuring 0.5 to 1 cm. The samples were surface sterilized according to the modified method of Dobranic et al. (14), immersed in 70% ethanol for five seconds, in 4% sodium hypochlorite for 90 seconds, and then rinsed in sterile distilled water for 10 seconds. Excess moisture was blotted using sterile filter paper. The sterilized segments were placed in Petri dishes containing PDA medium (HiMedia, Mumbai, India); these were sealed with parafilm, and incubated at 26 ± 1°C in 12-hours light/12-hours dark cycles for two to four weeks. The petri dishes were monitored daily to check the growth of fungal endophytic colonies from the segments. Fungi growing out of the plant segments were isolated and identified using morphological characteristics, according to the established procedure (15).

The colonization frequency (CF) and the percentage of the dominant endophytic fungi were calculated (13).

$$\text{(1)} \quad CF = \frac{\text{Number of segments colonized by endophyte}}{\text{Total number of segments analysis}} \times 100$$

3.3. Antimicrobial Activity of Endophytic Fungi

Three plant pathogenic fungi obtained from the college of agriculture, King Saud University (*F. oxysporum*, *F. solani*, and *Alternaria*) and five human pathogenic bacteria obtained from the military hospital in Riyadh City (*Enterococcus faecalis, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*) were used as target fungal and bacterial pathogens in this study. A dual culture technique was adopted to investigate the antimicrobial activity of fungal endophytes isolated from *A. graecorum*, *C. cretica*, *C. colocynthis*, *T. nilotica*, *A. fragrantissima*, *A. sieberi*, and *N. retusa* against the selected fungal and bacterial pathogens. The conidial suspension and assay plates of plant pathogenic fungi were prepared according to the method described by Gong et al. (16). The inocula and assay plates for bacterial strains were prepared as described by Pelaez et al. (17).

Endophytic fungi were grown on PDA plates. Five-day-old discs (5 mm in diameter) of endophytes were placed on three points in petri plates containing PDA medium. The target pathogens were inoculated in the center of the PDA plates. All petri dishes were incubated in the dark, and were randomly distributed. After incubation of the fungi at 25°C for 10 days, and of the bacteria at 37°C for 48 hours, the diameters (in mm) of the inhibition zones were measured. The level of inhibition was calculated by subtracting the distance (mm) of fungal and bacterial growth in the direction of an antagonist colony from the fungal growth radius. The widths of inhibition zones between the pathogen and the endophytes were grouped as follows: 10 mm (+++, strong inhibition), 2 to 10 mm (++, moderate inhibition), 1 mm (+, weak inhibition), and less than 1 mm (-, no activity determined) (18).

3.4. DNA Extraction, Amplification, and Sequencing

Two microliters of potato dextrose broth (PDB) (HiMedia, Mumbai, India) were poured into PDA tubes and vortexed to disperse the spores, and the spores-PDB mixtures were poured into flasks containing 100 mL PDB. The flasks were kept undisturbed at room temperature for two to three days. The mycelium was harvested by filtration, frozen at -80°C for 30 minutes, lyophilized, and stored at -80°C. The mycelium was ground in liquid nitrogen with a sterile mortar to obtain mycelium powder. DNA was extracted from 20 mg of mycelium powder using a DNeasy plant mini kit. The DNA quantity and quality were checked by electrophoresis on a 0.8% agarose gel, and visualized with ethidium bromide under UV trans-illumination (13).

The ITS region of the ribosomal DNA was amplified by PCR with the primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (19). PCR amplifications were carried out in a final volume of 50 μL,

containing 2 μL of DNA, 0.5 mM of each primer, 150 mM of dNTP, 1 U of Taq DNA polymerase (Promega), and PCR reaction buffer. Amplification was carried out in a thermal cycler with an initial denaturation of three minutes at 94°C, followed by 35 cycles of one minute at 94°C, one minute at 50°C, one minute at 72°C, and a final extension of 10 minutes at 72°C. The amplified products were checked by electrophoresis on a 1% agarose gel, and visualized with ethidium bromide under UV trans-illumination. The PCR products were purified using an ExoSAPIT kit (USB Corporation, Amersham Place, UK, under license from GE Healthcare), based on the manufacturer's instructions. The purified products were sequenced in an automated DNA sequencer (ABI PRISM 3700) using the BigDye Deoxy Terminator cycle-sequencing kit (Applied Biosystems, Darmstadt, Germany), following the manufacturer's instructions. Sequences were submitted to GenBank, NCBI (http://www.ncbi.nl m.nih.gov). Sequences obtained in this study were compared with the previously deposited sequences in the GenBank database, using BLAST, on the NCBI website (http://www. ncbi.nlm.nih.gov/BLAST/).

3.5. ITS Sequence and Phylogenetic Analysis

DNA sequences were initially aligned with Clustal X 1.81 (20). TREECON (21) for Windows (version 1.3b, 1998) was used to construct a neighbor-joining tree using the Jukes-Cantor model.

4. Results

This is probably the first report to describe the endophytic fungi that colonize the medicinal plants *A. graecorum*, *C. cretica*, *C. colocynthis*, and *N. retusa* in the northern desert of Saudi Arabia. A total of 70 stem and root samples were derived from seven medicinal plants, and were screened for the presence of endophytic fungi. 275 isolates belonging to 23 species and 14 genera were obtained from stem and root segments of *A. graecorum*, *C. cretica*, *C. colocynthis*, *T. nilotica*, *A. fragrantissima*, *A. sieberi*, and *N. retusa*. The isolates were identified as follows: one species of *Alternaria* from 23 isolates, seven species of *Aspergillus* from 68 isolates, one species of *Chaetomium* from two isolates, one species of *Drechslera* from two isolates, one species of *Emericella* from one isolate, one species of *Eupenicillium* from three isolates, four species of *Fusarium* from 14 isolates, one species of *Gibberell*a from four isolates, one species of *Monilia* from one isolate, one species of *Mucor* from four isolates, one species of *Mycelia* from 65 isolates, one species of *Penicillium* from 81 isolates, one species of *Scopulariopsis* from three isolates, and one species of *Ulocladium* from four isolates. The most commonly isolated species were *P. chrysogenum* with an overall colonization frequency of 98.57%, followed by *Mycelia sterilia* with a colonization frequency of 92.86% (Table 2).

Table 2. Colonization Frequency of Endophytic Fungi Isolated From Stems and Roots of Medicinal Plants on PDA Medium at 26 ± 1°C

Fungal Endophyte	Isolate Numbers	CF[a]	Dominant Fungi[a]
Alternaria alternata	23	32.86	8.4
Aspergillus candidus	2	2.86	0.73
A. flavus	1	1.43	0.36
A. niger	19	27.14	6.91
A. ochraceous	30	42.86	10.91
A. oryzae	1	1.43	0.36
A. sydowii	4	5.71	1.45
A. terreus	11	15.71	4
Chaetomium subaffine	2	2.86	0.73
Drechsleria indica	2	2.86	0.73
Emericella quadrilineata	1	1.43	0.36
E. crustaceum	3	4.3	1.1
Fusarium brachygibbosum	1	1.43	0.36
F. nygamai	1	1.43	0.36
F. oxysporum	11	15.71	4
F. cf. solani	1	1.43	0.36
Gibberella moniliformis	4	5.71	1.45
Monnilia sp.	1	1.43	0.36
Mucor sp.	4	5.71	1.45
Mycelia sterilia	65	92.86	23.64
Penicillium chrysogenum	69	98.57	29.45
***Scopulariopsis* sp.**	3	4.3	1.1
Ulocladium atrum	4	5.71	1.45
Total	263	NA	NA

Abbreviation: NA, not available.
[a]Value's unit is %.

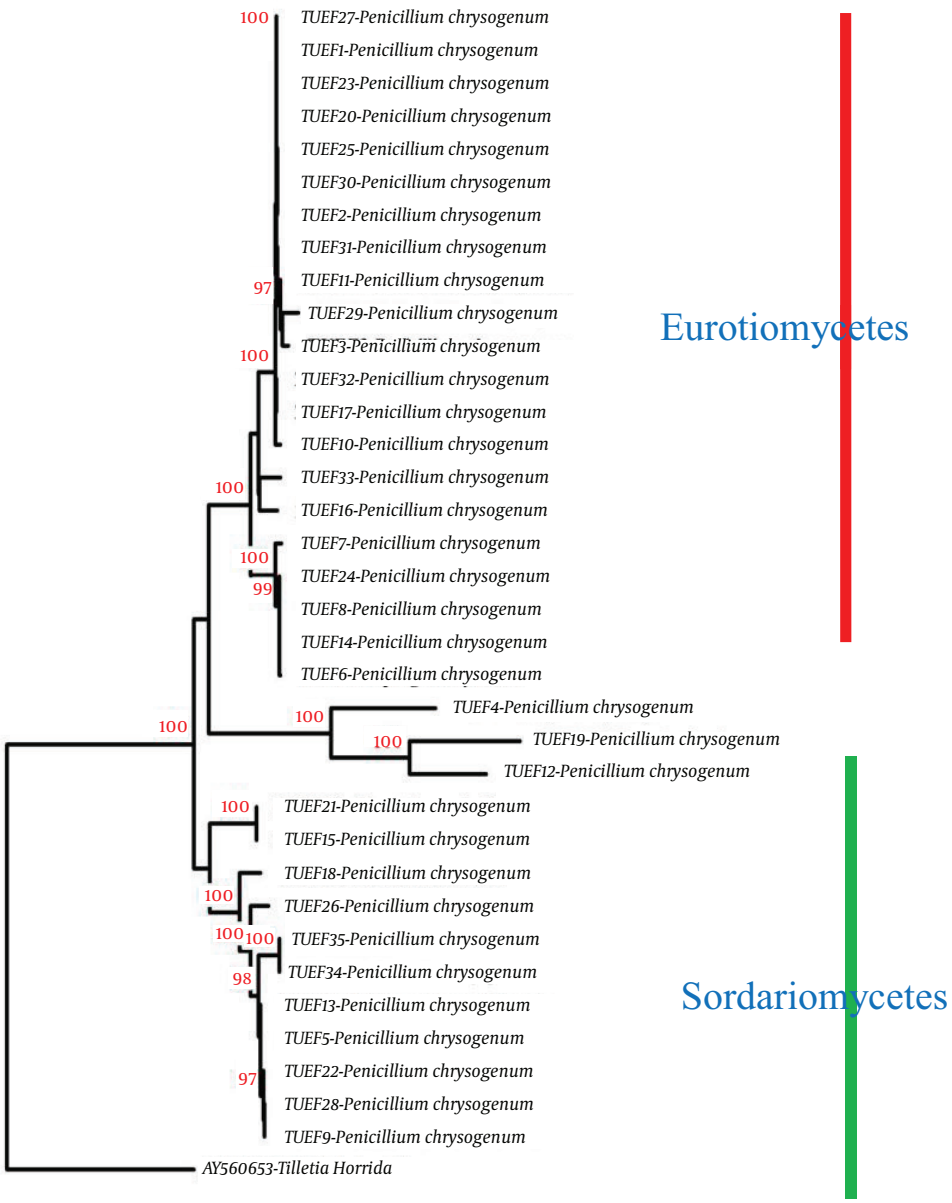
Table 3. Endophytic Fungi Isolated From Stems and Roots of Medicinal Plants in Saudi Arabia

NO.	Isolate Codes	Accession Numbers	The Closet Genbank Taxa	Similarity, %
1	TUEF1	HF546360	*Penicillium chrysogenum* FJ613114	99
2	TUEF2	HF546361	*P. chrysogenum* EF200090	99
3	TUEF3	HF546362	*P. chrysogenum* FJ613114	98
4	TUEF4	HF546363	*Scopulariopsis flava* AB627114	93
5	TUEF5	HF546364	*Gibberella moniliformis* AB587011	100
6	TUEF6	HF546365	*Aspergillus sydowii* EF652473	99
7	TUEF7	HF546366	*Emericella quadrilineata* EF652433	100
8	TUEF8	HF546367	*A. sydowii* EF652473	99
9	TUEF9	HF546368	*Gibberella moniliformis* AB587011	99
10	TUEF10	HF546369	*Eupenicillium crustaceum* AF033466	99
11	TUEF11	HF546370	*P. chrysogenum* AF034451	99
12	TUEF12	HF546371	*Scopulariopsis* sp. EU821474	87
13	TUEF13	HF546372	*G. moniliformis* AB587011	99
14	TUEF14	HF546373	*A. sydowii* EF652473	99
15	TUEF15	HF546374	*Chaetomium subaffine* HM365247	100
16	TUEF16	HF546375	*A. terreus* EF669586	100
17	TUEF17	HF546376	*E. crustaceum* AF033466	99
18	TUEF18	HF546377	*Fusarium cf. solani* JN235174	99
19	TUEF19	HF546378	*Scopulariopsis* sp. EU821474	86
20	TUEF20	HF546379	*P. chrysogenum* AF033465	100
21	TUEF21	HF546380	*C. subaffine* HM365247	99
22	TUEF22	HF546381	*F. nygamai* X94174	100
23	TUEF23	HF546382	*P. chrysogenum* FJ613114	99
24	TUEF24	HF546383	*A. sydowii* EF652450	99
25	TUEF25	HF546384	*P. chrysogenum* GQ241341	99
26	TUEF26	HF546385	*F. brachygibbosum* GQ505450	99
27	TUEF27	HF546386	*P. chrysogenum* EF200090	99
28	TUEF28	HF546387	*G. moniliformis* AB587011	99
29	TUEF29	HF546388	*P. chrysogenum* AF033465	95
30	TUEF30	HF546389	*P. chrysogenum* FJ613114	99
31	TUEF31	HF546390	*P. chrysogenum* AF034451	99
32	TUEF32	HF546391	*E. crustaceum* AF033466	99
33	TUEF33	HF546392	*A. oryzae* EF591304	100
34	TUEF34	HF546393	*F. oxysporum* EU214567	99
35	TUEF35	HF546394	*F. oxysporum* EU214567	99

A total of 263 endophytic fungal isolates were obtained from 70 stem and root fragments. Thirty five isolates were selected based on their antimicrobial activity, and identified based on the sequencing of the ITS region of rDNA (Table 3).

The sequence results corroborated with the morphological identification of the isolated fungal endophytes (Figure 1). Most of the isolates belonged to the taxa Ascomycota (58.54%), *Deuteromycota* (39.27%), or *Zygomycota* (1.45%). All seven medicinal plants were found to host one or more endophytes. Different endophytic taxa showed different relative frequencies on different plants (Table 4). *T. nilotica* had the highest endophytic diversity (relative frequency 27.27%), followed by *C. cretica* (relative frequency 19.27%), and *C. colocynthis* had the lowest endophytic diversity (relative frequency 5.82%) among the seven medicinal plants used in this study.

Figure 1. Phylogenetic Tree Based on the ITS Region of *rDNA*



It is showing the closest relatives of fungal endophytes isolated from medicinal plants. The tree was constructed by neighbor-joining algorithm, using the maximum composite likelihood model. Bootstrap percentages from 100 replicates are shown.

Table 4. Relative Frequency of Endophytic Fungi Isolated From Medicinal Plants

Medicinal Plants	Number of Isolates	CF [a]	Relative Frequency [a]
Achillea fragrantissima	38	54.29	13.82
Alhagi graecorum	22	31.43	8.00
Artemisia seiberi	42	60.00	15.27
Citrullus *colocynthis*	16	22.86	5.82
Cressa cretica	53	75.71	19.27
Neurospora retusa	29	41.43	10.55
Tamarix nilotica	63	90	27.27
Total	263	NA	100

Abbreviation: NA, not available.
[a]Value's unit is %.

4.1. Antimicrobial Activity of Endophytic Fungi

All endophytic fungi exhibited significant inhibition against a wide range of plant pathogenic fungi and human pathogenic bacteria. The isolates, namely *P. chrysogenum* (TUEF2), *E. crustaceum* (TUEF10), *A. sydowii* (TUEF14), *F. brachygibbosum* (TUEF26), *P. chrysogenum* (TUEF30), *P. chrysogenum* (TUEF31), and *E. crustaceum* (TUEF32) showed strong inhibition towards plant pathogenic fungi.

thirty one isolates in this work showed promising growth-inhibitory activity against at least one of the test human pathogenic bacteria, but no endophyte had antimicrobial activity against all five pathogenic microbes. A high proportion of fungi (54.3%) had activity against *K. pneumonia*. The numbers of fungal isolates displaying antimicrobial activity against *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa*, and *K. pneumonia* were 10, 13, 6, 18, and 19, respectively. The isolates *P. chrysogenum* (TUEF27), *P. chrysogenum* (TUEF23), *F. oxysporum* (TUEF35), and *F. nygamai* (TUEF22) displayed the highest inhibition against the human pathogenic bacteria *S. aureus*, *E. coli*, *P. aeruginosa*, and *K. pneumonia*, respectively. The isolates *A. sydowii* (TUEF6), *P. chrysogenum* (TUEF23), and *E. crustaceum* (TUEF32) showed strong activity against *E. faecalis* (Table 5).

Table 5. Antimicrobial Spectra of Endophytic Fungi[a]

Fungal Endophyte	Isolate Number	Plant Pathogenic fungi[b]			Human Pathogenic Bacteria[b]				
		A	B	C	D	E	F	G	H
Penicillium chrysogenum	TUEF1	+++	++	+++	-	-	-	++	-
P. chrysogenum	TUEF2	+++	+++	+++	-	-	-	-	-
P. chrysogenum	TUEF3	+++	++	+++	-	-	-	-	++
Scopulariopsis flava	TUEF4	++	+++	+++	-	++	-	-	++
Gibberella moniliformis	TUEF5	++	+++	++	-	-	-	-	-
Aspergillus sydowii	TUEF6	++	+++	++	-	+++	-	-	++
Emericella quadrilineata	TUEF7	+++	+++	-	++	++	++	-	++
A. sydowii	TUEF8	+++	+++	-	-	-	-	++	-
G. moniliformis	TUEF9	+	++	+++	-	-	-	++	-
E. crustaceum	TUEF10	+++	+++	+++	-	-	-	-	-
P. chrysogenum	TUEF11	+++	+++	-	-	-	-	++	-
***Scopulariopsis* sp.**	TUEF12	+++	++	+++	-	-	-	-	-
G. moniliformis	TUEF13	+++	+++	-	-	-	++	-	++
A. sydowii	TUEF14	+++	+++	+++	-	-	-	++	-
Chaetomium subaffine	TUEF15	+++	+++	++	-	++	-	-	++
A. terreus	TUEF16	+++	+++	-	++	-	-	-	-
E. crustaceum	TUEF17	+++	+++	-	-	++	-	++	++
Fusarium cf. solani	TUEF18	+++	+++	++	++	-	-	++	++
***Scopulariopsis* sp.**	TUEF19	+++	+++	+	-	++	-	++	++
P. chrysogenum	TUEF20	+++	+	+++	++	++	-	-	++
Chaetomium subaffine	TUEF21	+++	++	++	-	-	-	++	-
F. nygamai	TUEF22	++	+++	++	++	-	++	++	+++
P. chrysogenum	TUEF23	++	+++	++	-	+++	+++	++	++
A. sydowii	TUEF24	+++	+++	++	++	-	-	-	+++
P. chrysogenum	TUEF25	+++	+++	-	-	++	-	-	-
F. brachygibbosum	TUEF26	+++	+++	+++	++	-	-	++	++
P. chrysogenum	TUEF27	+++	+++	-	+++	-	-	++	++
G. moniliformis	TUEF28	+++	+++	-	++	-	-	-	++
P. chrysogenum	TUEF29	++	+++	++	-	++	-	-	++
P. chrysogenum	TUEF30	+++	+++	+++	-	++	-	++	-
P. chrysogenum	TUEF31	+++	+++	+++	-	-	-	-	-
E. crustaceum	TUEF32	+++	+++	+++	-	+++	-	++	-
A. oryzae	TUEF33	+++	+++	-	++	-	++	++	-
F. oxysporum	TUEF34	+++	+++	-	-	-	-	++	++
F. oxysporum	TUEF35	++	+++	++	-	++	++	+++	++

[a] Inhibition Zone, (-) No activity determined; +, > 2 mm; ++, 2 - 10 mm; +++, < 10 mm.
[b] F. oxysporum

5. Discussion

275 isolates, belonging to 23 species and 14 genera, were recovered from the stem and root segments of seven medicinal plants collected from salt marshes in North Saudi Arabia. *Penicillium chrysogenum* was the most frequently isolated species with a colonization frequency of 98.57%, followed by *Mycelia sterilia* with a colonization frequency of 92.86%. High colonization rates, ranging from 81 - 89%, were reported in palms in Brunei and Australia (22), and up to 95 - 98% in leaf fragments of *Guarea guidonia* (*Meliaceae*) in Puerto Rico (23). Fisher et al. (24) found that *Camarosporium* spp. (85.3% in stems) and *Colletotrichum gloeosporioides* (30% in leaves) endophytically colonized *Suaeda fruticosa* in England. Crabtree et al. (25) found that the salt marsh fungus Camarosporium roumeguerii, occurring on plants belonging to Chenopodiaceae, produced a dark green pigment. Other fungi such as *Pleospora* spp., *Stemphylium* spp., *Cladosporium* spp., and *Camarosporium* spp. were always isolated from plants in deserts and saline lands (26, 27). A possible explanation for the relatively low overall colonization rate noted in this study could be the high salinity of salt marshes at the collection site, and the desert nature of Saudi Arabia.

Molecular analysis of fungal *rDNA* sequences provides a powerful technique for assessing fungal diversity at the genus level. Most fungal isolates described in this study belong to *Ascomycota* (58.54%), *Deuteromycota* (39.27%), and *Zygomycota* (1.45%), confirming the previous reports by Huang et al. (10) and other reports on the fungal endophytes of *T. nilotica*, *A. fragrantis*sima, and *Artemisia* plants from various locations (9, 28). The ITS sequences of the isolated species submitted to EMBL-EBI were compared with the previously deposited sequences using BLAST. Isolates used for the sequencing analysis, their codes, and GenBank accession numbers are listed in Table 3. Most of the isolates belonged to *Ascomycota*, which confirms Petrini's findings (29) that fungal endophytes are mainly ascomycetes. Khan et al. (30) reported that all the endophytic fungi, collected from *Calotropis procera* and situated at different locations within Karachi university campus, belonged to Ascomycetes. Similar results were also obtained by Gherbawy et al. (13) during their research on *C. procera* from the Taif region of Saudi Arabia.

Some of the fungi isolated in this study (*A. alternata*, *G. moniliformis*, and some species of *M. sterilia*) are well-known plant pathogens. It is known that an endophyte in one plant may act as a pathogen in another plant, depending on the balance between the pathogenicity and endophytism of the microorganism in different hosts. The fungi isolated during this study have previously been isolated as endophytes from a wide range of different host plants such as *Pinus tabulaeformis* (31), *Avicennia marina* (32), *ginseng leaves* (33), *Thymus decussatus* (34), and *C. procera* (13). Endophytic fungi were tested for antifungal activity using a dual culture method. The results clearly showed that the endophytic fungi have stronger inhibition against plant pathogenic fungi, compared to human pathogenic bacteria. This could be because the endophytic fungi and plant pathogenic fungi naturally exist in the same habitat.

The antibacterial activity of the endophytic isolates from medicinal plants shown in this work against human pathogenic bacteria indicates the probable role of endophytes in the medicinal activity of these plants, partially agreeing with Jahanpour et al. (6), who demonstrated that the ethanolic extract of *P. harmala* seed and *P. granatum* peel exhibited potential activity against all *Mycobacterium tuberculosis* isolates with a mean inhibitory zone of 18.7 and 18.8 mm, at a 200 mg/mL concentration. Several groups (11, 12, 18) have previously reported the antifungal activities of plant endophytic fungi and actinomycetes. Bernardi-Wenzel et al. (35) evaluated the antibacterial activity of nine endophytic fungi isolated from leaves of Luehea divaricata against phytopathogens and the human pathogenic bacteria *E. coli* and *S. aureus*. The endophytes had varied effects on *A. alternata*. One isolate produced an inhibition halo against *Moniliophthora perniciosa* and *E. coli*.

Cui et al. (36) isolated 28 fungal endophytes from agarwood and found that 13 (46.4%) showed antimicrobial activity against at least one of the test human pathogenic bacteria. The diameters of the inhibition zones of YNAS07, YNAS14, HNAS04, HNAS05, HNAS08, and HNAS11 were equal to or higher than 14.0 mm against *S. aureus*, *E. coli*, *Bacillus subtilis*, *B. subtilis*, *A. fumigatus*, and *B. subtilis*, respectively. Production of active metabolites by endophytes may be related to the characters of the host plants and to a genetic recombination of the endophyte with the host that may have occurred in evolutionary time.

This is probably the first study that demonstrates the diversity of molecular identification of endophytic fungi from the stems and roots of medicinal plants, collected from salt marshes in Al-Gouf Governorate, Saudi Arabia. This is also perhaps the first study that has examined these host plants. The successful colonization of these plants by such fungi suggests that they can be utilized in future applications, such as delivery of degradative enzymes for controlling certain plant diseases. Meanwhile, the use of endophytes as producers of bioactive agents will help in the conservation of medicinal plants and the maintenance of environmental biodiversity.

Footnotes

Authors' Contribution: Rukaia Gashgari: isolation of fungi, identification of fungi, and writing; Youssuf Gherbawy: molecular identification of fungi, analysis of data, design of experiments, and writing; Fuad Ameen: analysis of data and writing; Salam Alsharari: collection of samples, selection of medicinal plants, and writing.

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References

1. Arnold AE. Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. *Fungal Biol Rev.* 2007;**21**(2-3):51–66. doi: 10.1016/j.fbr.2007.05.003.
2. Suryanarayanan TS, Murali TS, Thirunavukkarasu N, Govinda Rajulu MB, Venkatesan G, Sukumar R. Endophytic fungal communities in woody perennials of three tropical forest types of the Western Ghats, southern India. *Biodivers Conserv.* 2011;**20**(5):913–28. doi: 10.1007/s10531-011-0004-5.
3. Liu L, Li Y, Liu S, Zheng Z, Chen X, Zhang H, et al. Chloropestolide A, an antitumor metabolite with an unprecedented spiroketal skeleton from Pestalotiopsis fici. *Org Lett.* 2009;**11**(13):2836–9. doi: 10.1021/ol901039m. [PubMed: 19496604]
4. Sun X, Ding Q, Hyde KD, Guo LD. Community structure and preference of endophytic fungi of three woody plants in a mixed forest. *Fungal Ecol.* 2012;**5**(5):624–32. doi: 10.1016/j.funeco.2012.04.001.
5. Purahong W, Hyde KD. Effects of fungal endophytes on grass and non-grass litter decomposition rates. *Fungal Diversity.* 2010;**47**(1):1–7. doi: 10.1007/s13225-010-0083-8.
6. Jahanpour S, Ghazisaidi K, Davoodi H, Mazandarani M, Samet M, Jahanpour N, et al. Antimicrobial Effects of Folk Medicinal Plants From the North of Iran Against Mycobacterium tuberculosis. *Arch Pediatr Infect Dis.* 2015;**3**(1 TB):e18098.
7. Mossa JS, Al-Yahya MA, Al-Meshal IA. *Medicinal Plants of Saudi Arabia.* Saudi Arabia: King Saud University libraries; 1987.
8. Sanjay A, Purvi NP, Meghna JV, G GR. Isolation and characterization of endophytic bacteria colonizing halophyte and other salt tolerant plant species from coastal Gujarat. *Africa J Microbiol Res.* 2014;**8**(17):1779–88. doi: 10.5897/ajmr2013.5557.
9. Najjar A, Abdullah N, Saad WZ, Ahmad S, Oskoueian E, Abas F, et al. Detoxification of toxic phorbol esters from Malaysian Jatropha curcas Linn. kernel by Trichoderma spp. and endophytic fungi. *Int J Mol Sci.* 2014;**15**(2):2274–88. doi: 10.3390/ijms15022274. [PubMed: 24504029]
10. Huang WY, Cai YZ, Surveswaran S, Hyde KD, Corke H, Sun M. Molecular phylogenetic identification of endophytic fungi isolated from three Artemisia species. *Fungal Divers.* 2009;**36**(6988.16).
11. Huang Y, Wang J, Li G, Zheng Z, Su W. Antitumor and antifungal activities in endophytic fungi isolated from pharmaceutical plantsTaxus mairei,Cephalataxus fortuneiandTorreya grandis. *FEMS Immunol Med Microbiol.* 2001;**31**(2):163–7. doi: 10.1111/j.1574-695X.2001.tb00513.x. [PubMed: 11549424]
12. Park J, Park JH, Choi GJ, Lee S, Jang KS, Choi YH, et al. Screening for Antifungal Endophytic Fungi Against Six Plant Pathogenic Fungi. *Mycobiol.* 2003;**31**(3):179. doi: 10.4489/myco.2003.31.3.179.
13. Gherbawy YA, Gashgari RM. Molecular characterization of fungal endophytes from Calotropis procera plants in Taif region (Saudi Arabia) and their antifungal activities. *Plant Biosystems.* 2014;**148**(6):1085–92.
14. Dobranic JK, Johnson JA, Alikhan QR. Isolation of endophytic fungi from eastern larch (Larix lancina) leaves from New Brunswick, Canada. *Canada J Microbiol.* 1995;**41**(2):194–8. doi: 10.1139/m95-026.
15. Domsch KH, Gams W, Anderson T. *Compendium of soil fungi.* Soc General Microbiol; 1980.
16. Gong L, Guo S. Endophytic fungi from Dracaena cambodiana and Aquilaria sinensis and their antimicrobial activity. *Africa J Biotechnol.* 2009;**8**(5):731–6.
17. Peláez F, Collado J, Arenal F, Basilio A, Cabello A, Matas MTD, et al. Endophytic fungi from plants living on gypsum soils as a source of secondary metabolites with antimicrobial activity. *Mycol Res.* 1998;**102**(6):755–61. doi: 10.1017/s0953756297005662.
18. Paul NC, Kim W, Woo S, Park M, Yu S. Fungal Endophytes in Roots of Aralia Species and Their Antifungal Activity. *Plant Pathol J.* 2007;**23**(4):287–94. doi: 10.5423/ppj.2007.23.4.287.
19. White TJ, Bruns T, Lee S, Taylor J. Amplification and Direct Sequencing of Fungal Ribosomal Rna Genes for Phylogenetics. *inoculum.* 1990;**64**(1):315–22. doi: 10.1016/b978-0-12-372180-8.50042-1.
20. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 1997;**25**(24):4876–82. [PubMed: 9396791]
21. Van de Peer Y, De Wachter R. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Appl Biosci.* 1994;**10**(5):569–70. [PubMed: 7828077]
22. Fróhlich J, Hyde KD, Petrini O. Endophytic fungi associated with palms. *Mycol Res.* 2000;**104**(10):1202–12. doi: 10.1017/s095375620000263x.
23. Gamboa MA, Bayman P. Communities of Endophytic Fungi in Leaves of a Tropical Timber Tree (Guarea guidonia: Meliaceae)1. *Biotropica.* 2001;**33**(2):352–60. doi: 10.1111/j.1744-7429.2001.tb00187.x.
24. Fisher PJ, Petrini O. Location of fungal endophytes in tissues of Suaeda fruticosa: A preliminary study. *Transact Brit Mycol Soc.* 1987;**89**(2):246–9. doi: 10.1016/s0007-1536(87)80161-4.
25. Crabtree SL, Gessner RV. Growth and Nutrition of the Salt-Marsh Fungi Pleospora gaudefroyi and Camarosporium roumeguerii. *Mycologia.* 1982;**74**(4):640. doi: 10.2307/3792753.
26. Petrini O, Fisher PJ. Fungal endophytes in Salicornia perennis. *Transact Brit Mycol Soc.* 1986;**87**(4):647–51. doi: 10.1016/s0007-1536(86)80109-7.
27. Muhsin TM, Booth T. Fungi associated with halophytes of an inland salt marsh, Manitoba, Canada. *Canada J Botany.* 1987;**65**(6):1137–51. doi: 10.1139/b87-159.
28. El-Morsy EM. Fungi isolated from the endorhizosphere of halophytic plants from the Red Sea Coast of Egypt. *Fungal Divers.* 2000;**5**:43–54.
29. Petrini O. Taxonomy of endophytic fungi of aerial plant tissues. In: Fokkema NJ, Heuvel J, editors. *Microbiology of the phyllosphere.* London: Cambridge University Press; 1986.
30. Khan R, Shahzad S, Choudhary M, Khan SA, Ahmad A. Biodiversity of endophytic fungi isolated from Calotropis procera (Ait.) R. Br. *Pakistan J Botany.* 2007;(39):2233–9.
31. Wang Y, Guo L, Hyde KD. Taxonomic placement of sterile morphotypes of endophytic fungi from Pinus tabulaeformis (Pinaceae) in northeast China based on rDNA sequences. *Fungal Divers.* 2005;**20**:235–60.
32. Bharathidasan R, Panneerselvam A. Panneerselvam, Pelagia Research Library. *Euro J Exp Biol.* 2011;**1**(3):31–6.
33. Eo JK, Choi MS, Eom AH. Diversity of endophytic fungi isolated from korean ginseng leaves. *Mycobiology.* 2014;**42**(2):147–51. doi: 10.5941/MYCO.2014.42.2.147. [PubMed: 25071383]
34. Selim KA. Biodiversity and antimicrobial activity of endophytes associated with Egyptian medicinal plants. *Mycosphere.* 2011;**2**(6):669–78. doi: 10.5943/mycosphere/2/6/7.
35. Bernardi-Wenzel J, Garcia A, Azevedo JL, Pamphile JA. Molecular characterization by amplified ribosomal DNA restriction analysis and antimicrobial potential of endophytic fungi isolated from Luehea divaricata (Malvaceae) against plant pathogenic fungi and pathogenic bacteria. *Genet Mol Res.* 2013;**12**(4):5072–84. doi: 10.4238/2013.October.29.2. [PubMed: 24301768]
36. Cui JL, Guo SX, Xiao PG. Antitumor and antimicrobial activities of endophytic fungi from medicinal parts of Aquilaria sinensis. *J Zhejiang Univ Sci B.* 2011;**12**(5):385–92. doi: 10.1631/jzus.B1000330. [PubMed: 21528493]