Molecular Characterization of Endophytic Fungi Associated with High-Altitude Juniperus Trees and Their Antimicrobial Activities

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Abstract: Fungal endophytes were isolated from twigs of *Juniperus procera* (Cupressaceae) collected from Taif region, Saudi Arabia). Twenty six different taxa were recovered. The overall foliar colonization rate was 36%. A total of 144 isolates were obtained and identified into 6 distinct operational taxonomic units (OTUs) based on the sequencing of the ITS regions of the rRNA gene. The most prevalent fungi were *Aspergillus fumigates*, *Penicillium oxalicum*, *Preussia* sp., *Peyronellaea eucalyptica*, *Peyronellaea sancta* and *Alternaria tenuissima*. A total of 144 isolates were tested for antibacterial and antifungal activities against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Candida albicans* and *Fusarium solani* 52 isolates showed antimicrobial activity against at least one of the tested microbes. *Aspergillus fumigates* (7 isolates), *Hypocrea lutea* (4), *Penicillium oxalicum* (10) and *Preussia* sp. (5) presented the strongest antimicrobial activity. This study confirmed the variation of different isolates of the same species in the term of antibacterial activity. Also, it indicates that the endophytic fungi of *Juniperus procera* plants should be another potential source of bioactive antimicrobial agents.

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1.Introduction

Taif region is situated in the central foothills of the western mountains of Saudi Arabia at an altitude of an approximately 2000 m above sea level (21^0 16` N - $40^0$ 25` E). This area characterized by several unique plant species that can adapt with the natural forests of Saudi Arabia in general and the high altitude conditions. *Juniperus procera* L., a gymnosperm, belonging to the family Cupressaceae, common name-Juniper) is a high altitude shrub which occurs at 2295 to 2592 m in the mountainous region of Taif. It is well documented for its medicinal value for urinary tract and bladder infections and inflammations (**Moore, 2003**), the treatment of hyperglycemia, tuberculosis, bronchitis, pneumonia, ulcers, intestinal worms, to heal wounds and cure liver diseases (**Burits *et al.*, 2001; Loizzo *et al*., 2007**).

Plant endophytic fungi are defined as the fungi which spend the whole or part of their Lifecycle colonizing inter-and/or intra-cellularly inside the healthy tissues of the host plants, typically causing no apparent symptoms of disease (**Carroll, 1988**). Endophytic fungi have been examined in conifers (**Petrini *et al.*, 1992**), including *Taxus* spp. (**Fisher and Petrini, 1987**), and *Juniperus* spp. (**Petrini and Müller, 1979; Petrini and Carroll, 1981**), and they are presumed to be ubiquitous (**Wang *et al.*, 2008**). Endophytes comprise a large but little explored portion of fungal diversity (**Fröhlich and Hyde, 1999; Hawksworth, 2001**).

Different works carried out so far regarding the role of endophytes in host plants indicate that they can stimulate plant growth, increase disease resistance, improve the plant's ability to withstand environmental stresses and recycle nutrient (**Sturz and Nowak, 2000**). Endophytic fungi are of biotechnological interest due to their potential as a source of secondary metabolites have proven useful for novel drug discovery (**Guo *et al.*, 2008; Yan *et al*., 2011)** and a biological control agent **(Clay, 1989; Bacon, 1990; Schardl *et al.*, 1991; Dorworth and Callan, 1996**). Antifungal and antibacterial activities of plant endophytic fungi have been reported by a several groups (**Fisher *et al.*, 1984; Radu and Kqueen, 2002; Park *et al.*, 2003; Phongpaichit *et al.*, 2006; Raviraja *et al.*, 2006; Gangadevi *et al.*, 2008; Gherbawy and Gashgari, 2013; Liang *et al*., 2012, Idris *et al*., 2013**).

Several endophytic sterile mycelia were isolated many hosts, because sterile cultures lack the taxonomic characters needed for identification, morphotaxa, based on gross colony features, are used frequently as functional taxonomic units (**Arnold *et al.*, 2000, 2003; Guo *et al.*, 2000, 2003)**. In some cases molecular sequence data from the nuclear ribosomal internal transcribed spacer region (ITS) have been used to identify sterile cultures and to evaluate morphotaxon boundaries (**Arnold, 2002; Lacap *et al*., 2003**).

As the medicinal plants are known to harbor endophytic fungi that are believed to be associated with the production of pharmaceutical products (**Zhang *et al*., 2006**), in this context, the aims of this work were to characterize the fungal endophytes community associated with *Juniperus procera* from Taif region and to detect antimicrobial activities of these fungi against some pathogenic microbes.

2.Material and Methods

2.1.Sampling

One hundred twig samples of fifty *Juniperus procera* plants were collected from different locations at Al-Hada and Al-Shafa regions, in Taif, Saudi Arabia. Healthy and mature plants were carefully chosen for sampling.

2.1.1.Isolation of endophytic fungi

The twigs were thoroughly washed in running tap water followed by DI water and small fragments of twigs (4) of approximately 10 mm (length) containing about 5–10 needles were cut with the aid of a flame-sterilized razor blade (**Kusari *et al.*, 2009**). Then the small twig fragments were surface sterilized by sequential immersion in 70% ethanol for 2 min, 1.3 mol l^{1} sodium hypochlorite (3–5% available chlorine) for 3 min and then 70% ethanol for 1 min. Finally, these surfaces sterilized twigs were rinsed three times in sterile double distilled water for 1 min each, to remove excess surface sterilants. The excess moisture was blotted with a sterile filter paper. Surface sterilized twigs, thus obtained, were evenly spaced in Petri dishes containing PDA (HiMedia, India) medium amended with streptomycin 100 mg l) 1) to eliminate any bacterial growth. Petri dishes were sealed using Parafilm and incubated at 26 ± 2°C in an incubator until fungal growth started. The cultures were monitored every day to check the growth of endophytic fungal colonies from the twig segments. The hyphal tips, which grew out from twigs over 4–6 weeks were isolated and subcultured onto PDA plates, and brought into pure culture. Fungi growing out of the twigs segments were isolated and identified after reference to **Domsch *et al.*, (2007**). The Colonization Frequency (CF) percentage and the dominant fungi percentage of the endophytyic fungi were calculated (**Petrini and Fisher 1988; Kumar and Hyde 2004**). Colonization frequency (%) (Number of segments colonized by endophytes / Total number of segments analyzed) X 100.

2.2.Molecular identification of endophytic fungal isolates

2.2.1.DNA isolation

Two ml of potato dextrose broth (PDB) was poured into PDA tubes and vortexed to disperse the spores, and the spores-PDB mix were poured into flasks containing 100 ml of PDB. Flasks were incubated at room temperature without shaking for 2 to 3 days. The mycelium was harvested by filtration, frozen at −80°C during 30 min, lyophilized and stored at −80°C. The mycelium was ground in liquid nitrogen in a sterile mortar to obtain a mycelium powder. The DNA was extracted from 20 mg of mycelium powder using the DNeasy plant mini kit. The DNA quantity and quality were checked by electrophoresis on a 0.8% agarose gel, revealed with ethidium bromide and visualized by UV trans-illumination. **2.2.2.ITS region sequencing**

The internal transcribed spacer.ITS) region of the ribosomal DNA (rDNA) was amplified by PCR with the primers ITS1-F(CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) (**White *et al*., 1990; Gardes and Bruns, 1993**). PCR amplifications were done in a final volume of 50 µl by mixing 2 µl of DNA with 0.5 µM of each primer, 150 µM of dNTP, 1 U of Taq DNA polymerase (Promega) and PCR reaction buffer. Amplification conducted in a thermal cycler with an initial denaturation of 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, and a final extension of 10 min at 72°C. Aliquots of PCR products checked by electrophoresis on a 1% agarose gel revealed with ethidium bromide and visualized by UV trans-illumination. The PCR products were purified by ExoSAP-IT.USB Corporation, under license from GE Healthcare) based one manufacturer's instructions. The purified products were sequenced using an automated DNA sequencer (ABI PRISM 3700) using the BigDye Deoxy Terminator cycle-sequencing kit (Applied Biosystems) following manufacturer's instructions. Sequences were submitted to GenBank on the NCBI website.http://www.ncbi.nlm.nih.gov). Sequences obtained in this study were compared with the GenBank database using the BLAST software on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). The sequence results of collected strains deposited in GenBank with accession numbers from HG798712 to HG798745.

2.2.3.ITS sequence and phylogenetic analysis

DNA sequences were aligned first with Clustal X 1.81 (**Thompson *et al*., 1997**). TREECON (**Van de Peer and Wachter, 1994**) for Windows (version 1.3b, 1998) was used to construct neighbor-joining tree using Jukes-Cantor model (**Jukes and Cantor, 1969**).

2.3.Antimicrobial potential of endophytic fungi

2.3.1.Culture media

Yeast extract sucrose broth (Yeast extract, 20 g/L; sucrose 40 g/L; Magnesium sulfate 0.5 g/L) was used with a water extract of *Juniperus* to cultivate the endophytic isolates in a shake-flask system. The plant extracts were prepared by boiling 5 g of the dried plant materials in 500 ml distilled water for 30 min. The extracts were filtered and mixed with freshly prepared culture media and autoclaved at 121°C for 15 min (**Tong *et al*., 2011**).

3.3.2. Cultivation and extraction.

The inoculum was prepared by introducing two mycelial agar plugs into 250 ml Erlenmeyer flask containing 100 ml of the broth medium. Both agar plugs were 1 cm in diameter and excised from the periphery of 7-days-old fungal culture. The cultures were cultivated at 30°C with rotational speed of 120 rpm. After 20 days of incubation, the fermented broth and fungal biomass were separated out by centrifugation at 5311 g (**Tong *et al*., 2011**). Freeze-dried fungal biomass were extracted by soaking in methanol (1:50, w/v) overnight. Supernatant was then extracted thrice with equal volume of ethyl acetate (1:1, v/v). The upper organic phase was concentrated to dryness under reduced pressure to obtain the crude broth paste. 3.2.3. Pathogenic microbes The test microorganisms were used in the study included Gram positive bacteria (*Staphylococcus aureus*), Gram negative bacteria (*Klebsiella pneumoniae*), yeasts (*Candida albicans*) and fungi (*Fusarium solani*). The bacterial cultures were subculturing every two weeks on fresh nutrient agar (NA) slants and incubated at 37°C, whereas the yeasts and fungal cultures were subculturing every four weeks on the fresh potato dextrose agar (PDA) slants and incubated at 37°C for yeasts and 30°C for fungi. All the cultures were then kept at 4°C until further use. The inoculum was prepared by adding 4 ml of sterile physiological saline to the agar slant, and shake vigorously to get the cell or spore suspension (**Tong *et al*., 2011**).

2.3.2.Disc diffusion assay

The antimicrobial potential of the isolated endophytic fungi was determined by the method described by **Jorgensen and Turnidge (2007**). Briefly, the crude extracts were dissolved in 50% dimethyl sulfoxide, DMSO). The test organisms with the inoculum size of 10^5 colony-forming units (CFU) / ml for bacteria or 5 CFU/ml of yeast cells or fungal spores were streaked on the surface of the media using sterile cotton swab. Muller-Hinton agar (Hi-media) was used for test bacteria, whereas PDA was used for yeasts and fungi. Sterile Whatman antibiotic disc, impregnated with 20 µL of each extract of 20 mg/ml concentration, was then placed on the surface of inoculated medium. Twenty percent DMSO was applied as a negative control to detect the solvent effects whereas 30 µg/ml chloramphenicol was used as the positive controls for bacteria, 30 µg/ml ketoconazole for fungi and yeasts, respectively. The plates were incubated at 30°C for 48 to 96 h for fungi, and at 37°C for 24 h for bacteria and yeasts. The diameter of the clear zones surrounding the disc was measured.

3.Results and Discussion

3.1.Endophytic fungi associated with *Juniperus procera* twigs

The present study is the first one about the fungal endophytes of *Juniperus procera* plant found in Saudi Arabia. A total of 400 twig segments obtained from 50 different plants were screened for the presence of endophytic fungi. Mycelium emerged since 120 out of 400 segments, yielding an overall colonization rate of 30%. In most cases (100 twig segments out of 120), a single fungal strain emerged from a leaf segment. Overall, 144 isolates were recovered and identified as 26 distinct operational taxonomic groups OTUs) according to morphological characters (Table 1). Thirty one isolates of endophytic fungi were recovered in culture from 90 tissue samples of *Juniperus virginiana* in Arizona (**Hoffman and Arnold, 2008**). In Arizona also, 22 isolates of endophytic fungi were collected from 144 leave segments of *Juniperus deppeana* (**U'Ren *et al.*, 2010**). In Korea, total 59 isolates and 19 species of endophytic fungi were isolated from the leaves of *Juniperus rigida, Larix kaempferi and Pinus densiflora* and identified using morphological and molecular characteristics (**Kim *et al*., 2013**). A possible explanation of the relatively low overall colonization rate noted in the present study could be due desert nature for Saudi Arabia as previously reported (**Gherbawy and Gashgari, 2013**).

A total of 144 fungal endophytes isolates were obtained from 400 twig fragments. Twenty six distinct operational taxonomic groups (OTUs) were identified based on the sequencing of the ITS region of rRNA (Table 2). The sequence results indicated in full correspondence between the molecular identification of the isolated fungal endophytes and the morphological identification. The Majority of the recovered taxa belong to the Ascomycota. Fungal endophytes are especially common among the Ascomycota, representing at least five classes, dozens of families, and large numbers of previously unknown species (**Clay, 1989; Pepeljnjak *et al*., 2005; Gehlot *et al*., 2008**). Only one species from the collected isolates in this study belong to the Basidiomycota. Generally Basidiomycota are relatively rare as cultured endophytes but have been recorded previously from coniferous hosts such as *Juniperus communis* and *Pinus cembra* (**Petrini and Müller, 1979; Petrini, 1986**).

The collected fungi were classified into 3 classes: Eurotimycetes Dothideomycetes and Sordariomycetes belonged to the Ascomycota and Agaricomycetes belonged to Basidiomycota (Fig. 1). Most endophytes of conifer leaves are filamentous Ascomycota (**Petrini, 1986**). This finding is in harmony with those mentioned by **Hoffman and Arnold (2008)**. They mentioned that the majority of endophytes recovered from *Cupressus arizonica*, *Juniperus virginiana* and *Platycladus orientalis* were placed to support in the Dothideomycetes (46 isolates) and Sordariomycetes (15 isolates). Only one endophyte.from *Platycladus*) was recovered as a member of the Eurotiomycetes (**Hoffman and Arnold, 2008**). **Gehlot *et al.* (2008)** reported that the fungus composition included 13.6% zygomycetes, 5.6% ascomycetes, 72.8% hyphomycetes, 4% coelomycetes and 4% sterile fungi have been found as endophytic fungi of inner bark of *Prosopis cineraria*.

Teleomorphs were produced in 32 isolates in culture and represented 7 distinct taxonomic groups belonging to 6 genera: *Cochliobolus, Emericella Eupenicillium, Hypocrea, Peniophora* and *Peyronellaea.* Anamorphs were encountered in 80 isolates, representing eleven different taxa in seven genera (*Alternaria, Aspergillus, Melanops, Penicillium, Phoma, Preussia* and *Ulocladium*). Out of the 166 collected isolates, 34 isolates were classified as fungal sp. Using morphological criteria and molecular techniques they were classified into 8 species (Fungal sp. 1-8). Fungal sp. 7 (TUEF40) was clustered with *Emericella fruticulosa* with a 100 % bootstrap factor, so this species may be belonged to Eurotiales. Fungal sp.1 (TUEF1), 3 (TUEF23), 4 (TUEF26), 5 (TUEF27), 6 (TUEF32) and 8 (TUEF41) were grouped with *Melanops* sp. that belonged to Botryosphaeriales. On the other hand fungal sp. 2 (TUEF7) was clustered with *Pleosporales* species (Fig. 1). These results indicated not all endophytic fungi could be identified to the species or genus level using the data available in the GenBank. In accordance with this finding, **U'Ren *et al.* (2010)** reported that all endophytic fungal species from Mosses and Lichens including *Juniperus deppeana* are members of the Pezizomycotina (Ascomycota; n=939 sequences with defined taxonomy; the remaining 21 isolates were classified as either "uncultured fungus" or "fungal endophyte").

The most prevalent fungi were *Aspergillus fumigates* (19 CF), *Penicillium oxalicum* (15), *Preussia* sp. (15), *Peyronellaea eucalyptica* (10), *Peyronellaea sancta* (8) and *Alternaria tenuissima* (7) as shown in table (1). Fungi occurring at ≥10 % frequency are referred to as 'core group fungi' (**Alias *et al.*, 1995; Sarma and Hyde, 2001**) and such dominant endophytic fungi may play a major role in plant fitness. The fungal species *Aspergillus fumigates*, *Penicillium oxalicum* and *Preussia* sp. were found to be the core-group fungi with the colonization frequency ranged from 3.8 to 4.8% (Table 1). The most frequently endophytic fungi that have been isolated from the medicinal plants were *Alternaria, Aspergillus, Curvularia, Fusarium, Nigrospora, Colletotrichum, Papulospora Pestalotiopsis, Phoma, Phomopsis, Penicillium, Leptosphaerulin, Mycelia, Trichoderma* (**Raviraja *et al.*, 2006; Wang *et al.*, 2008; Romina *et al.*, 2010; Srimathi *et al.*, 2011**). *Aspergillus fumigatus* Fresenius, an endophytic fungus from *Juniperus communis* was isolated in Germany (**Kusari *et al.*, 2009**). Endophytic *Penicillium oxalicum* was isolated from coffee plants (**Vega *et al.*, 2006**). *Preussia* sp. endophytes isolated from Australian dry rainforests (**Mapperson *et al.*, 2013**). Endophytic *Peyronellaea* species were isolated from *Pinus koraiensis* in Korea (**Deng *et al.*, 2001**). Endophytic fungus *Alternaria tenuissima* was previously isolated from the bark of *Erythrophleum fordii* Oliver (**Fang *et al.*, 2012**).

3.2.Antimicrobial activity screening

The antimicrobial activities of fungal endophytes isolated from *Juniperus* plants were shown in Table 3. In total, 38.2 and 57.6% of the endophytic fungal isolates exhibited antibacterial and antifungal activities, respectively (Table 1). The percentage of antifungal activity of endophytic fungi isolated from Chinese medicinal plants was 30.0% (**Li *et al.*, 2005**). All endophytic fungi isolated from the medicinal plant Kigelia Africana in Sudan showed antibacterial activities against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* (**Idris *et al.*, 2013**). Many works have been reported different percentages of the antifungal activities obtained from fungal endophytes, ranging about 12.7 to 52.3% (**Huang *et al.*, 2001; Aghighi *et al.*,2004; Li *et al.*, 2005; Paul *et al.*, 2007**).

From the 26 endophytic fungi used in this experiment, Fungal sp. 1 (1 isolates), Fungal sp.2 (1), *Hypocrea lutea* (4) and *Peniophora lycii* (1) exhibited 100% antimicrobial activities (Table 3). Isolates of *Aspergillus fumigates*, *Hypocrea lutea Penicillium oxalicum* and *Preussia* sp. showed strongest inhibition of testing microbe growth (Tables 3&4). *Penicillium oxalicum* and *Trichoderma harzianum*.*Hypocrea lutea*) were the most effective fungi against *Pseudomonas solanacearum, Xanthomonas campestris, Agrobacterium tumefaciens, Eschericchia coli* and *Serratia marcescens* (**Santamarina *et al.*, 2002**). The antibacterial activity of *Aspergillus fumigates* against *Staphylococcus aureus, Candida albicans* and *Micrococcus luteus* was reported (**Furtado *et al.*, 2002**). Fumifungin and synerazol, new antifungal antibiotics, were isolated from the culture broth of *A. fumigates* (**Mukhopadhyay *et al*., 1987; Ando *et al*., 1991**). Another antibiotic, fumagillin, is produced by the fermentation of certain strains of *A. fumigatus* and since it was reported as an angiogenesis inhibitor, many semisynthetic fumagillin analogues have been synthesized (**Han *et al*., 2000**). Also, novel diketopiperazine derivatives were isolated from the fermentation broth of *A. fumigatus* BM939 (**Cui *et al*., 1997**) as new cell cycle inhibitors of microbial origin. The most active metabolite isolated from *Trichoderma harzianum* and *Trichoderma longibrachiatum* was 6-n-pentyl- α -pyrone, which showed the highest antifungal and antibacterial activity against *Nematospora corylii*, *Paecilomyces variotii*, *Bacillus subtilis*, *Bacillus brevis*, *Sarcina lutea* and *Enterobacter dissolvens* (**Traus *et al*., 2003**). *Penicillium oxalicum* strain PY-1 produces antifungal substances that suppress the mycelial growth of *Sclerotinia sclerotiorum* and many other plant pathogenic fungi tested (**Yang *et al*., 2008**). Thirteen isolates of endophytic *Preussia* sp. out of 18 isolates exhibited antimicrobial activity against at least one of *B. cereus*, *E. Cole*, *E. faecalis*, *P. aeruginosa*, *S. marcescens*, methicillin-resistant *S. aureus* (MRSA) and *C. albicans* (**Yang *et al*., 2008**). The results of this study indicated that the antibacterial activity of endophytic fungi isolated from *Juniperus* was more effective against Gram positive bacteria (*Staphylococcus aureus*) than Gram negative bacteria (*Klebsiella pneumoniae*) as shown in table (4). The Gram-positive bacteria appeared to be more susceptible to the inhibitory effect of the crude extracts of endophytic fungi isolated from leaves of *Mitragyna javanica* than Gram-negative bacteria and the yeast (**Pharamat *et al*., 2013**). The extracts of endophytic fungi isolated from wheat (*Triticum durum*) in Algeria were more effective on Gram positive bacteria and fungi compared to Gram negative bacteria (**Sadrati *et al*., 2013**).

The isolates endophytes showed variable degrees of inhibition against pathogenic microbes. Table 4). Several prior studies also reported differences in the biological activities among fungal isolates for the same species (**Peláez *et al.*, 1998; Vaz *et al*., 2012**). In addition, these results suggested that more than one strain per species should be tested when examining biological activities (**Park *et al*., 2003**).

Table 1. Frequency of endophytic fungi isolated from 400 twig segments of *Juniperus procera* plants on PDA medium at 26±2° C.

Fungal endophytes	Isolate numbers	Colonization frequency.%)	Dominant fungi.%)
Alternaria citrimacularis	2	0.5	1.4
Alternaria tenuissima	7	1.8	4.9
Aspergillus fumigates	19	4.8	13.2
Aspergillus niger	6	1.5	4.2
Cochliobolus australiensis	2	0.5	1.4
Emericella fruticulosa	5	1.3	3.5
Eupenicillium rubidurum	2	0.5	1.4
Fungal sp.1	1	0.3	0.7
Fungal sp.2	2	0.5	1.4
Fungal sp.3	3	0.7	2.1
Fungal sp.4	6	1.5	4.2
Fungal sp.5	2	0.5	1.4
Fungal sp.6	5	1.3	3.5
Fungal sp.*7*	7	1.8	4.9
Fungal sp.8	6	1.5	4.2
Hypocrea lutea	4	1	2.8
Melanops sp.	2	0.5	1.6
Penicillium crustosum	6	1.5	4.2
Penicillium expansum	2	0.5	1.6
Penicillium oxalicum	15	3.8	10.4
Peniophora lycii	1	0.3	0.7
Peyronellaea eucalyptica	10	2.5	6.9
Peyronellaea sancta	8	2	5.6
Phoma sp.	3	0.8	2.1
Preussia sp.	15	3.8	10.4
Ulocladium consortiale	3	0.8	2.1
Total	144		

Table 2. Isolated and identified endophytes from *Juniperus procera* with relationship to the genus or species and the identity percentage found in the CBS.The Centraalbureau voor Schimmelcultures) website.

No	Isolate Codes	Accession Numbers	Closely related fungal sequence	Max. identity %	Identification
1	TUEF1	HG798712	Fungal sp.(FJ612670)	100	Fungal sp. 1
2	TUEF 2	HG798713	*Ulocladium consortiale* (FJ266482)	99	*Ulocladium consortiale*
3	TUEF3	HG798714	*Peyronellaea sancta* (FJ427065)	100	*Peyronellaea sancta*
4	TUEF4	HG798715	*Peyronellaea sancta* (FJ427063)	100	*Peyronellaea sancta*
5	TUEF6	HG798716	*Peyronellaea eucalyptica* (GU237846)	100	*Peyronellaea eucalyptica*
6	TUEF7	HG798717	Fungal sp. (GQ249889)	100	Fungal sp. 2
7	TUEF8	HG798718	*Peniophora lycii* (CBS 262.56)	98.96	*Peniophora lycii*
8	TUEF9	HG798719	*Preussia* sp. (JN418769)	99.5	*Preussia* sp.
9	TUEF10	HG798720	*Emericella fruticulosa* (FJ755267)	97.98	*Emericella fruticulosa*
10	TUEF11	HG798721	*Alternaria tenuissima* (DTO 069-G3)	100	*Alternaria tenuissima*
11	TUEF12	HG798722	*Peyronellaea eucalyptica* (CBS 377.91)	99.97	*Peyronellaea eucalyptica*
12	TUEF13	HG798723	*Phoma* sp.(JX164068)	100	*Phoma* sp.
13	TUEF14	HG798724	*Alternaria citrimacularis* (JX418334)	100	*Alternaria citrimacularis*
14	TUEF15	HG798725	*Penicillium oxalicum* (EF103461)	100	*Penicillium oxalicum*
15	TUEF18	HG798726	*Penicillium oxalicum* (HQ732138)	100	*Penicillium oxalicum*
16	TUEF19	HG798727	*Penicillium crustosum* (DAOM 215345)	99	*Penicillium crustosum*
17	TUEF20	HG798728	*Penicillium expansum* (HQ732138)	100	*Penicillium expansum*
18	TUEF21	HG798729	*Hypocrea lutea* (HE649478)	98.19	*Hypocrea lutea*
19	TUEF22	HG798730	*Peyronellaea sancta* (FJ427063)	100	*Peyronellaea sancta*
20	TUEF23	HG798731	Fungal sp. (HM123390)	92	Fungal sp.3
21	TUEF24	HG798732	*Penicillium oxalicum* (GQ376104)	99.8	*Penicillium oxalicum*
22	TUEF25	HG798733	*Penicillium oxalicum* (GQ376104)	99.8	*Penicillium oxalicum*
23	TUEF26	HG798734	Fungal sp. (HM123390)	88	Fungal sp. 4
24	TUEF27	HG798735	Fungal sp.(HM123046)	92	Fungal sp. 5
25	TUEF29	HG798736	*Eupenicillium rubidurum* (HQ607978)	98.64	*Eupenicillium rubidurum*
26	TUEF30	HG798737	*Peyronellaea eucalyptica* (GU237846)	99.78	*Peyronellaea eucalyptica*
27	TUEF32	HG798738	Fungal sp.(HM123390)	87.03	Fungal sp. 6
28	TUEF35	HG798739	*Peyronellaea eucalyptica* (GU237846)	99.78	*Peyronellaea eucalyptica*
29	TUEF36	HG798740	*Cochliobolus australiensis* (JX310570)	99.80	*Cochliobolus australiensis*
30	TUEF37	HG798741	*Aspergillus niger* (IHEM17892)	100	*Aspergillus niger*
31	TUEF38	HG798742	*Aspergillus fumigates* (JX231005)	100	*Aspergillus fumigates*
32	TUEF39	HG798743	*Melanops* sp.(FJ824771)	90.64	*Melanops* sp.
33	TUEF40	HG798744	Fungal sp. (AY546021)	96	Fungal sp. 7
34	TUEF41	HG798745	Fungal sp. HM123390)	92	Fungal sp. 8

Table 3. The number and percentage of the endophytic fungi with antibacterial and antifungal activity.

No	Genus & species	Number of strains tested	Number of strains with Antibacterial activity.%)	Number of strains with antifungal activity.%)
1	*Alternaria citrimacularis*	2	1 (50)	0 (0)
2	*Alternaria tenuissima*	7	3 (42.9)	2 (28.6)
3	*Aspergillus fumigates*	19	8 (42.1)	12 (63.2)
4	*Aspergillus niger*	6	1 (16.6)	3 (50)
5	*Cochliobolus australiensis*	2	1 (50)	1 (50)
6	*Emericella fruticulosa*	5	0 (0)	3 (60)
7	*Eupenicillium rubidurum*	2	1 (50)	1 (50)
8	Fungal sp.1	1	1 (100)	1 (100)
9	Fungal sp.2	2	1 (100)	1 (100)
10	Fungal sp.3	3	1 (33.3)	1 (33.3)
11	Fungal sp.4	6	2 (33.3)	1 (16.7)
12	Fungal sp.5	2	1 (50)	1 (50)
13	Fungal sp.6	5	1 (20)	3 (60)
14	Fungal sp.7	7	2 (28.6)	3 (42.9)
15	Fungal sp.8	6	2 (33.3)	2 (33.3)
16	*Hypocrea lutea*	4	4 (100)	4 (100)
17	*Melanops* sp.	2	0 (0)	0 (0)
18	*Penicillium crustosum*	6	2 (33.3)	4 (66.7)
19	*Penicillium expansum*	2	1 (50)	1 (50)
20	*Penicillium oxalicum*	15	12 (80)	14 (93.3)
21	*Peniophora lycii*	1	1 (100)	1 (100)
22	*Peyronellaea eucalyptica*	10	1 (10)	8 (80)
23	*Peyronellaea sancta*	8	1 (12.5)	6 (75)
24	*Phoma* sp.	3	2 (66.7)	2 (66.7)
25	*Preussia* sp.	14	5 (35.7)	7 (50)
26	*Ulocladium consortiale*	4	2 (50)	1.25)
Total		144	55 (38.2)	83 (57.6)

Table 4. Antibacterial and antifungal activities of selected fungal endophytes from *Juniperus procera* against *Staphylococcus aureus, Klebsiella pneumoniae, Candida albicans,* and *Fusarium solani*.

Fungal endophytes	Isolate number	Pathogenic Microbes [a]			
		A	B	C	D
Alternaria tenuissima	TUEF1	+++[b]	-[c]	+++	++
Aspergillus fumigates	TUEF38	+++	+++	+++	+++
Aspergillus fumigates	TUEF42	+++	+++	+++	++
Aspergillus fumigates	TUEF44	+++	++	+++	+++
Aspergillus fumigates	TUEF45	+++	+++	+++	++
Aspergillus fumigates	TUEF47	+++	++	+++	+++
Aspergillus fumigates	TUEF48	+++	+++	+++	+++
Aspergillus fumigates	TUEF50	+++	++	+++	+++
Aspergillus niger	TUEF37	+++	+	+	++
Cochliobolus australiensis	TUEF36	+	-	+++	++
Emericella fruticulosa	TUEF10	++	-	+++	++
Eupenicillium rubidurum	TUEF29	+	+	+++	+++
Fungal sp.2	TUEF7	++	+	+++	+++
Fungal sp.3	TUEF23	++	+	++	++
Fungal sp.4	TUEF26	+	-	++	++
Fungal sp.4	TUEF51	++	+	++	++
Fungal sp.5	TUEF27	++	-	++	++
Fungal sp.6	TUEF32	++	-	+	+
Fungal sp.6	TUEF52	++	-	+	+
Fungal sp.*7*	TUEF40	++	-	+	++
Fungal sp.*7*	TUEF53	++	+	+	++
Fungal sp.8	TUEF41	++	++	+	++
Fungal sp.8	TUEF53	++	+	-	++
Fungal sp.8	TUEF54	++	-	+	++
Hypocrea lutea	TUEF21	++	+	+++	+++
Hypocrea lutea	TUEF56	++	+	+++	+++
Hypocrea lutea	TUEF57	++	++	+++	+++
Hypocrea lutea	TUEF58	++	++	+++	+++
Penicillium crustosum	TUEF19	+++	+	++	++
Penicillium expansum	TUEF20	++	++	++	++
Penicillium oxalicum	TUEF15	+++	++	++	++
Penicillium oxalicum	TUEF18	+++	+++	+++	++
Penicillium oxalicum	TUEF24	+++	++	+++	++
Penicillium oxalicum	TUEF25	+++	++	+++	++
Penicillium oxalicum	TUEF59	+++	+++	+++	++
Penicillium oxalicum	TUEF60	+++	++	+++	++
Penicillium oxalicum	TUE611	+++	+++	+++	++
Penicillium oxalicum	TUEF62	+++	+++	+++	++
Penicillium oxalicum	TUEF63	+++	++	+++	++
Penicillium oxalicum	TUEF65	+++	+++	+++	+++
Peniophora lycii	TUEF8	+++	+++	+++	+++
Peyronellaea eucalyptica	TUEF6	+	+	+++	++
Peyronellaea eucalyptica	TUEF12	++	+	+	++
Peyronellaea sancta	TUEF3	++	+	++	++
Peyronellaea sancta	TUEF22	++	+	++	+
Phoma sp.	TUEF13	+++	-	++	+
Preussia sp.	TUEF9	+++	-	++	++
Preussia sp.	TUEF66	++	++	+++	++
Preussia sp.	TUEF67	+++	++	+++	++
Preussia sp.	TUEF70	+++	++	+++	++
Preussia sp.	TUEF71	+++	+	+++	+
Ulocladium consortiale	TUEF 2	+++	+++	+++	+++

[a] A; *Staphylococcus aureus,* B; *Klebsiella pneumoniae,* C; *Candida albicans,* and D ; *Fusarium solani.*
[b]Inhibition zone. +, < 2 mm; ++, 2-10 mm; +++, >10 mm.
[c] No effect.

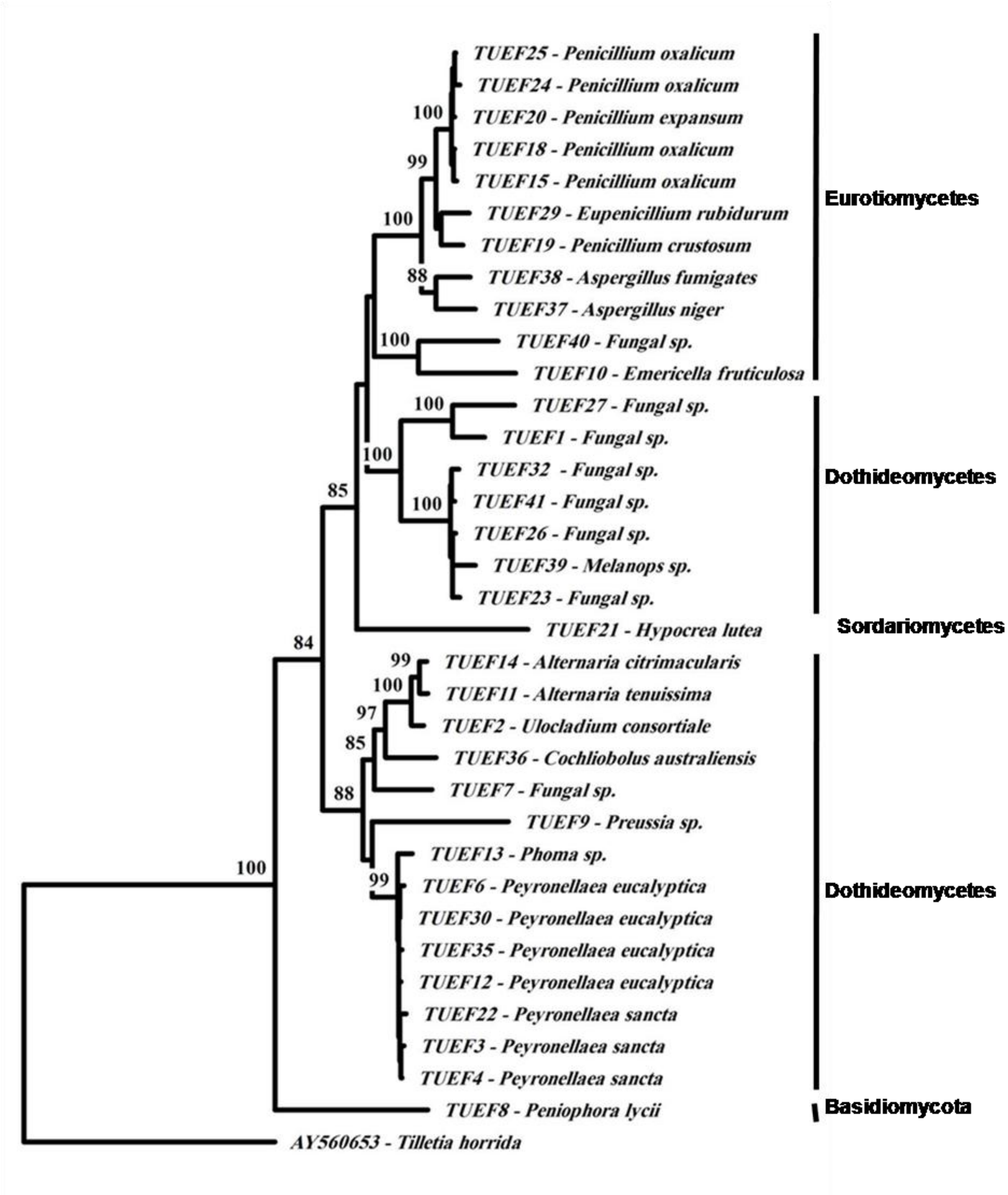
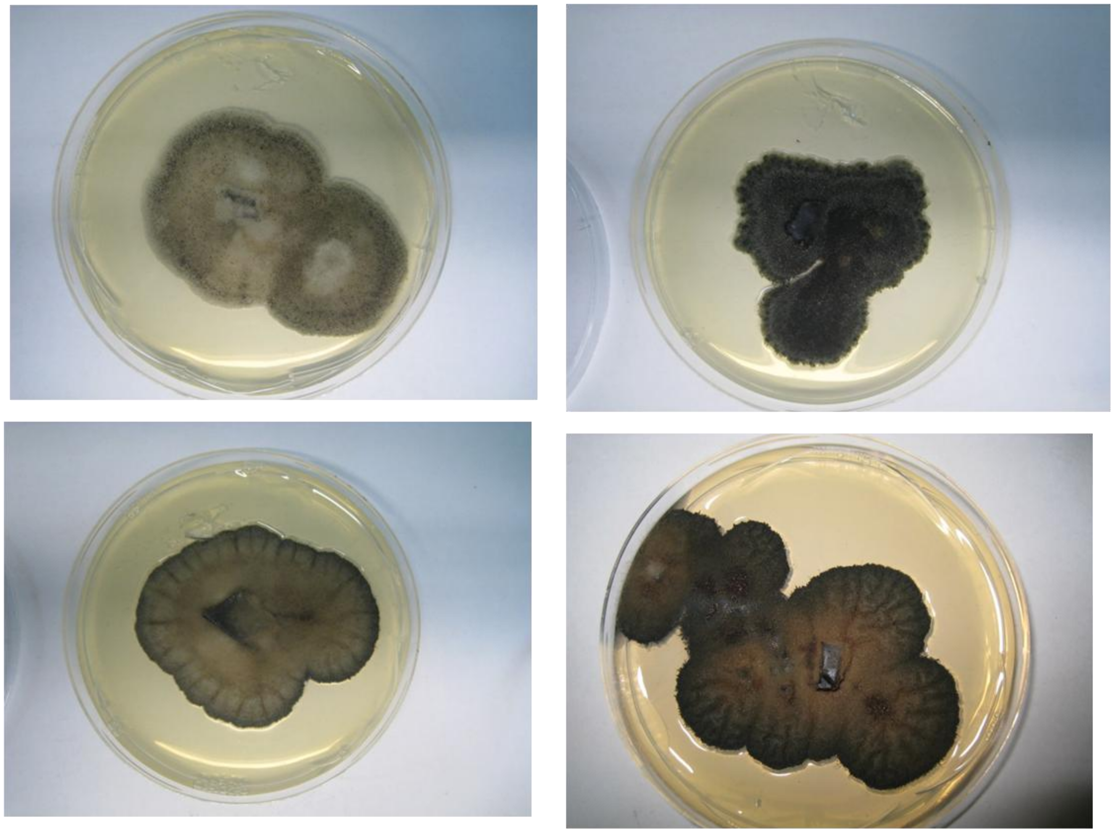
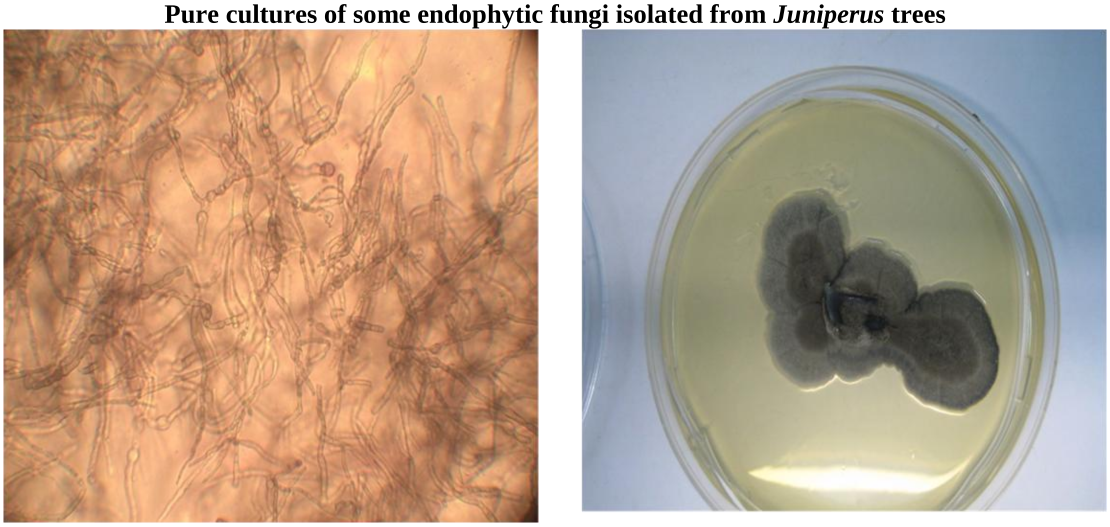


Figure 1. Phylogenetic tree based on the internal transcribed spacer.ITS) region of rRNA showing closest relatives of fungal endophytes isolated from *Juniperus procera*. The tree was constructed by neighbour-joining algorithm using maximum composite likelihood model. Bootstrap percentages from 100 replicates are shown. The tree was rooted with *Tilletia horrida* [AY560653] as the out-group.



Pure cultures of some endophytic fungi isolated from *Juniperus* trees



Pure culture and microscopic slid of Fungal sp. 7

4.Conclusions

In conclusion, *Juniperus* twigs harbored several endophytic fungal species with 33 different operational taxonomic units, of which the *Aspergillus fumigates, Penicillium oxalicum, Preussia* sp., *Peyronellaea eucalyptica*, *Peyronellaea sancta* and *Alternaria tenuissima* were the most frequently isolated species. Moreover, about 36.1% of the endophytic fungi isolated from *Juniperus procera* twigs have the potential in producing antimicrobial metabolites, and the *Aspergillus fumigates*, *Hypocrea lutea*, *Penicillium oxalicum* and *Preussia* sp. show broad inhibition against the growth of all the 4 microbes. The results obtained in the current study indicated that the endophytic fungi isolated from

Juniperus procera plants could be used as a potential source of bioactive antimicrobial agents.

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References

1. Aghighi S, Bonjar GHS, Rawashdeh R, Batayneh S, Saadou I. 2004. First report of antifungal spectra of activity of Iranian actinomycetes strains against *Alernaria solani*, *Alternaria alternata*, *Fusarium solani*, *Phytophthora megasperma*, *Verticillium dahliae* and *Saccharomyces cerevisiae*. Asian J Plant Sci 3:463-471.
2. Alias SA, Kuthubutheen AJ, Jones EBG. 1995. Frequency of occurrence of fungi on wood in Malaysian mangroves. Hydrobiologia 295: 97–106.
3. Ando O, Satake H, Nakajima M, Sato A, Nakamura T, Kinoshita T, Furuya K, Haneishi T.1991. Synerazol, a new antifungal antibiotic. J Antibiot 44 : 382–389.
4. Arnold AE, Maynard Z, Gilbert GS, Coley PD, Kursar TA.2000. Are tropical fungal endophytes hyperdiverse? Ecol Lett 3:267–274.
5. Arnold AE, Mejía LC, Kyllo D, Rojas E, Maynard Z, Robbins NA, Herre EA.2003. Fungal endophytes limit pathogen damage in a tropical tree. Proc Nat Acad Sci USA 100:15649–15654.
6. Arnold AE.2002. Neotropical fungal endophytes: diversity and ecology (Doctoral dissertation). Tucson: University of Arizona. 337 p.
7. Bacon CW.1990. Isolation, culture and maintenance of endophytic fungi of grasses In: Labeda DP (ed) Isolation of biotechnological organisms from nature. McGraw-Hill, New York.
8. Burits M, Asres K, Bucar F.2001. The antioxidant activity of the essential oils of Artemisia afra, Artemisia abyssinica and Juniperus procera. Phytother Res 15:103-108.
9. Carroll G.1988. Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. Ecology 69:2-9.
10. Clay K.1989. Clavicipitaceous endophytes of grasses: their potential as biocontrol agents. Mycol. Res. 92:1–12.
11. Cui C-B, Kakeya H, Osada H.1997. Novel mammalian cell cycle inhibitors, cyclotryprostatins A-D, produced by *Aspergillus fumigatus*, which inhibit mammalian cell cycle at G2/M phase. *Tetrahedrom* 53 :59–72.
12. Deng JX, Paul NC, Li MJ, Seol EY, Sung GH, Yu SH.2001. Molecular Characterization and Morphology of Two Endophytic Peyronellaea Species from *Pinus koraiensis* in Korea. Mycobiology 39: 266-271.
13. Domsch KH, Gams W, Anderson T-H.2007. Compendium of Soil Fungi, Second Edition.
14. Dorworth CE, Callan BE.1996. Manipulation of endophytic fungi to promote their utility as vegetation biocontrol agents In: Reddlin SC, Carris LM (eds) Endophytic fungi in grasses and woody plants. APS Press, St. Paul.
15. Fang ZF, Yu SS, Zhou WQ, Chen XG, Ma SG, Li Y, Qu JA.2012. New isocoumarin from metabolites of the endophytic fungus *Alternaria tenuissima* (Nee & T. Nee: Fr) Wiltshire. Chin Chem Lett 23, 317–320.
16. Fisher PJ, Anson AE, Petrini O.1984. Antibiotic activity of some endophytic fungi from ericaceous plants. Botanica Helvetica 94: 249-253.
17. Fisher PJ, Petrini O.1987. Location of fungal endophytes in tissues of *Suaeda fruticosa*: a preliminary study. Trans. British Mycol. Soc. 89: 246-249.
18. Fröhlich J, Hyde KD.1999. Biodiversity of palm fungi in the tropics: are global fungal diversity estimates realistic? Biodivers Conser 8:977–1004.
19. Furtado NAJC, Said S, Ito IY, Bastos JK.2002. "The antimicrobial activity of *Aspergillus fumigatus* is enhanced by a pool of bacteria." Microbiological Research 157: 207-211.
20. Gangadevi V, Sethumeenal S, Yogeswari S, Rani G.2008. Screening Endophytic Fungi Isolated from a Medicinal Plant, *Acalypha Indica* L. for Antibacterial Activity. Indian journal of science and technology 5:1-8.
21. Gardes M, Bruns TD.1993. ITS primers with enhanced specificity for Basidiomycetes - Application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113-118.
22. Gehlot P, Bohra NK, Purohit DK.2008. Endophytic Mycoflora of Inner Bark of *Prosopis cineraria* - a Key Stone Tree Species of Indian Desert. American-Eurasian Journal of Botany 1: 01-04.
23. Gherbawy Y, Gashgari R.2013. Molecular characterization of endophytic fungi from *Calotropis procera* plants in Taif region (Saudi Arabia) and their antifungal activities. *Plant Biosystems*.in press).
24. Guo B, Wang Y, Sun X, Tang K.2008. Bioactive natural products from endophytes: a review. Appl. Biochem. Microbiol. 44:136-142.
25. Guo LD, Hyde KD, Liew ECY.2000. Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA sequences. New Phytolog 147:617–630.

26. Han SK, Choi ANS, Hong RK, Moon SK, Chun HS, Lee SJ, Kim JW, Hong CI, Kim D, Yoon JH, No KT. 2000. Design and synthesis of highly potent fumagillin analogues from homology modeling for a human metAP-2. Bioorg Med Chem Lett 10: 39 – 43.
27. Hawksworth DL.2001. The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycol Res 105:1422–1432.
28. Hoffman MT, Arnold AE.2008. Geographic locality and host identity shape fungal endophyte communities in cupressaceous trees. Mycological Research. 112: 331–344.
29. Huang YJ, Wang JF, Li GL, Zheng ZH, Su, WJ.2001. Antitumour and antifungal activities in endophytic fungi isolated from pharmaceutical plants *Taxus mairei*, *Cephalataxus fortune* and *Torreya grandis*. FEMS Immunology and Medical Microbiology 31, 163–167.
30. Idris A, Al-tahir I, Idris E.2013. Antibacterial activity of endophytic fungi extracts from the medicinal plant *Kigelia Africana*. Egypt Acad J Biolog Sci 5: 1-9.
31. Jorgensen JH, Turnidge JD.2007. Susceptibility test methods: dilution and disk diffusion methods. In: Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA (eds) Manual of Clinical Microbiology. ASM Press, Washington 1152-1172.
32. Jukes TH, Cantor CR.1969. Evolution of protein molecules. In:Munro HN, editor. Mammalian protein metabolism. Vo1. 3. New York: Academic Press. pp. 21–32.
33. Kim C-K, Eo J-K, Eom A-H.2013. Diversity and Seasonal Variation of Endophytic Fungi Isolated from Three Conifers in Mt.Taehwa, Korea. Mycobiology 41: 82–85.
34. Kumar D. Hyde KD.2004. Biodiversity and tissue-recurrence of endophytic fungi in *Tripterygium wilfordii*. Fungal Diversity 17: 69-90.
35. Kusari S, Lamshöft M, Spiteller M.2009. *Aspergillus fumigatus* Fresenius, an endophytic fungus from *Juniperus communis* L. Horstmann as a novel source of the anticancer pro-drug deoxypodophyllotoxin. J Appl Microbiol 107:1019-30.
36. Lacap DC, Hyde KD, Liew ECY.2003. An evaluation of the fungal ‘morphotype’ concept based on ribosomal DNA sequences. Fung Divers 12:53–66.
37. Li H, Qing C, Zhang Y, Zhao Z.2005. Screening for endophytic fungi with antitumour and antifungal activities from Chinese medicinal plants. World Journal of Microbiology & Biotechnology 21:1515–1519.
38. Liang H, Xing Y, Chen J, Zhang D, Guo S, Wang C.2012. Antimicrobial activities of endophytic fungi isolated from*Ophiopogon japonicus* (Liliaceae). BMC Complementary and Alternative Medicine 12:238.
39. Loizzo MR, Tundis R, Conforti F, Saab AM, Statti GA, Menichini F.2007. Comparative chemical composition, antioxidant and hypoglycaemic activities of *Juniperus oxycedrus* ssp. *oxycedrus* L. berry and wood oils from Lebanon. Food Chem**.** 105:572-8.
40. Mapperson RR, Kotiw M, Davis RA, Dearnaley JD.2013. The Diversity and Antimicrobial Activity of Preussia sp. Endophytes Isolated from Australian Dry Rainforests. Curr Microbiol; (Epub ahead of print).
41. Moore M.2003. Medicinal Plants of the Mountain West. 351 pp. ISBN 0-89013-454-5. Museum of New Mexico; Rev Exp edition.
42. Mukhopadhyay T, Roy K, Coutinho L, Rupp RH, Ganguli BN.1987. Fumifungin, a new antifungal antibiotic from *Aspergillus fumigatus* Fresenius 1863. J. Antibiot. 40:1050–1052.
43. Park J-H, Park JH, Choi GJ, Lee SW, Jang KS, Choi YH, Cho KY, Kim JC.2003. Screening for Antifungal Endophytic Fungi Against Six Plant Pathogenic Fungi. Mycobiology 31:179–182.
44. Paul NC, Kim WK, Woo SK, Park MS, Yu SH.2007. Fungal Endophytes in Roots of Aralia Species and Their Antifungal Activity. Plant Pathol J 23: 287-294.
45. Peláez F, Collado J, Arenal F, Basilio A, Cabello MA, Díez MT, García JB, González del Val A, González V, Gorrochategui J, Hernández P, Martín I, Platas G, Vicente F.1998. Endophytic fungi from plants living on gypsum soils as a source of secondary metabolites with antimicrobial activity. Mycological Research 102: 755-761.
46. Pepeljnjak S, Kosalec V, Kaloera Z, Blazevic N.2005. Antimicrobial activity of juniper berry essential oil (*Juniperus communis* L., *Cupressaceae*) Acta Pharm 55:417–422.
47. Petrini O, Fisher PJA.1988. Comparative study of fungal endophytes in xylem and whole stem of *Pinus sylvestris* and *Fagus sylvatica*. Trans British Mycol Soc 91:233-238.
48. Petrini O, Carroll GC.1981. Endophytic fungi in foliage of some Cupressaceae in Oregon. Can. J. Bot. 59: 629-636.
49. Petrini O, Müller E.1979. Pilzliche Endophyten, am Beispiel von *Juniperus communis* L. *Sydowia* 32: 224-251.
50. Petrini O.1986. Taxonomy of endophytic fungi of aerial plant tissues. In: Fokkema NJ, van den Huevel J, eds.Microbiology of the Phyllosphere. Cambridge, UK:Cambridge University Press. 175–187.
51. Petrini O.1986. Taxonomy of endophytic fungi of aerial plant tissues. In: Fokkema NJ, van den Huevel J, eds. Microbiology of the Phyllosphere. Cambridge, UK: Cambridge University Press. 175–187.
52. Petrini, O, Fisher PJ, Petrini LE.1992. Fungal endophytes of bracken (*Pteridium aquilinum*), with

some reflections on their use in biological control. Sydowia 44: 282-293.
53. Pharamat T, Palaga T, Piapukiew J, Whalley AJS: Sihanonth, P.2013. Antimicrobial and anticancer activities of endophytic fungi from *Mitrajyna javanica* Koord and Val. African Journal of Microbiology Research 7: 5565-5572.
54. Phongpaichit S, Rungjindama N, Rukachaisirikul V, Sakayaroj J.2006. Antimicrobial activity in cultures of endophytic fungi isolated from *Garcinia* species. FEMS Immunol Med Microbiol 48:367-372.
55. Radu S, Kqueen CY.2002. Preliminary Screening of Endophytic Fungi From Medicinal Plants in Malaysia for Antimicrobial and Antitumor Activity. Malaysian J. Medical Sci 9, 23-33.
56. Raviraja NS, Maria GL, Sridhar KR.2006. Antimicrobial Evaluation of Endophytic Fungi Inhabiting Medicinal Plants of the Western Ghats of India. Eng. Life Sci. 6: 515–520.
57. Romina G, Priscila C.2010. Diversity of fungal endophytes in leaves and stems of wild rubber trees (*Hevea brasiliensis*) in Peru. Fungal Ecology 3: 240-254.
58. Sadrati N, Daoud H, Zerroug A, Dahamna S, Bouharat S.2013. Screening of antimicrobial and antioxidant secondary metabolites from endophytic fungi isolated from wheat (*Triticum durum*). J Plant Protec Research 53: 128.
59. Santamarina M, Roselló J, Llacer R, Sanchis V.2002. Antagonistic activity of *Penicillium oxalicum* Corrie and Thom, *Penicillium decumbens* Thom and *Trichoderma harzianum* Rifai isolates against fungi, bacteria and insects *in vitro.* Rev Iberoam Micol 19: 99-103.
60. Sarma VV, Hyde KD.2001. A review on frequently occurring fungi in mangroves. Fungal Diversity 8: 1–34.
61. Schardl CL, Liu J, White JK, Finkel RA, An Z, Siegel M.1991. Molecular phylogenetic relationship of non-pathogenic grass mycosymbionts and clavicipitaceous plant pathogens. Plant Syst Evol. 178:27–41.
62. Srimathi S, Indrakumar I, Johnpaul M.2011. Biodiversity Of The Endophytic Fungi Isolated From *Calotropis Gigantea* (L.) R.Br. Recent Research in Science and Technology 3: 94-100**.**
63. Sturz AV Nowak J.2000. Endophytic communities of Rhizobacteria and the strategies required creating yield-enhancing associations with crops. Applied Soil Ecology 15: 183.
64. Tarus PK, Langat-Thoruwa CC, Wanyonyi AW, Chhabra SC.2003. Bioactive metabolites from *Trichoderma harzianum* and *Trichoderma longibrachiatum*. Bull Chem Soc Ethiop 17:185-190.
65. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG.1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876-4882.
66. Tong WY, Darah I, Latiffah Z.2011. Antimicrobial activities of endophytic fungal isolates from medicinal herb *Orthosiphon stamineus* Benth. Journal of Medicinal Plants Research 5: 831-836.
67. U'Ren JM, Lutzoni F, Miadlikowska J, Arnold AE.2010. Community Analysis Reveals Close Affinities Between Endophytic and Endolichenic Fungi in Mosses and Lichens. Microb Ecol 60:340-353.
68. Van de Peer Y, De Wachter R.1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Computer Applications in the Biosciences (CABIOS) 10:569–570.
69. Vaz ABM, Brandão LR, Vieira MLA, Pimenta RS, Morais PB, Sorbral MEG, Rosa LH, Rosa CA.2012. Diversity and antimicrobial activity of fungal endophyte communities associated with plants of Brazilian savanna ecosystems. African J Microbio Resea 6:3173-3185.
70. Vega FE, Posada F, Peterson SW, Gianfagna TJ, Chaves F.2006. *Penicillium* species endophytic in coffee plants and ochratoxin A production. Mycologia 98:31-42.
71. Wang Y-T, Lo H-S, Wang P-H.2008. Endophytic fungi from Taxus mairei in Taiwan: first report of *Colletotrichum gloeosporioides* as an endophyte of *Taxus mairei.* Botanical Studies 49: 39-43.
72. White TJ, Bruns T, Lee S, Taylor J.1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M. A., Gelfand D. H., Sninsky J. J. and White T. J., eds. *PCR protocols*. A Guide to Methods and Applications. San Diego : Academic Press: ed., 315-322.
73. Yan XN, Sikora IR, Zheng JW.2011. Potential use of cucumber (*Cucumis sativus* L.) endophytic fungi as seed treatment agents against root-knot nematode *Meloidogyne incognita*. J. Zhejiang Univ.-Sci. B. (Biomed. & Biotechnol)., 12:219-225.
74. Yang L, Xie J, Jiang, D, Fu Y, Li G, Lin F.2008. Antifungal substances produced by *Penicillium oxalicum* strain PY-1—potential antibiotics against plant pathogenic fungi. World J Microbiol Biotechnol 24:909–915.
75. Zhang HW, Song YC, Tan RX.2006. Biology and chemistry of endophytes. Natural Product Reports. 23: 753-771.

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