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Research article

Identification and distribution of endophytic fungi associated with the stem and bark of old and young plane trees (*Platanus orientalis* L.) in the urban landscape

Soheila Khorsandy[1], Ali Nikbakht[1], Mohammad R. Sabzalian[2]

1. Department of Horticulture, College of Agriculture, Isfahan University of Technology, 84156-83111, Isfahan
2. Department of Agronomy and Plant Breeding, College of Agriculture, Isfahan University of Technology, 84156-83111, Isfahan

✉ anikbakht@iut.ac.ir

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Abstract

Microorganisms associated with healthy plant tissues that endure for some or all of their life cycle without causing the host to exhibit disease symptoms are endophytes. One of Iran's most popular ornamental plants is the plane tree (*Platanus orientalis* L.), which can live up to 1000 years. We looked for fungal endophytes in the sapwood and bark of young and old plane trees in three cities in central Iran: Mahallat, Natanz, and Isfahan. According to a gridded sampling strategy, samples were taken from young (less than 100 years old) and old (200-1000 years old) trees that were randomly selected as replications. The findings showed that Mahallat had a higher density of fungal endophytes. Mahallat, which has higher rainfall and a lower yearly temperature, had the largest species richness, according to the Shannon-Wiener diversity index. Over all sites, older trees considerably outperformed younger ones in terms of endophyte frequency and the Shannon-Wiener diversity index. Our findings also imply that the tissue type has a discernible impact on the frequency of fungal endophytes and species diversity; higher isolate frequency was observed in bark tissue, but higher species diversity was discovered in stems. According to the identification of endophytic fungi based on morphology and rDNA (ITS1-5.8S-ITS2) sequences, the most prevalent fungal endophytes were *Penicillium sp*., *Fusarium sp*., *Alternaria sp*., and *Ulocladium sp.*

Keywords: Diversity, Endophyte, *Platanus orientalis*, Taxonomy.

Introduction

Endophytes are defined as microorganisms that spend at least a portion of their lives asymptomatically inside healthy plant tissues (Arnold, 2007; Sánchez Márquez *et al.*, 2010). Fungi, bacteria, yeasts, and even cyanobacteria have all been discovered to behave as endophytes (Krings *et al.*, 2007). These demonstrate a variety of symbiotic lives, each of which is distinguished by the host plants' health benefits (Rodriguez & Redman, 2008).

Although it is unknown whether these are universal observations, several evidence indicate these symbioses are essential for plant survival under high-stress situations (Rodriguez *et al.*, 2004). For instance, endophytes help plants resist diseases, fend off insect pests, and improve their fitness by increasing their ability to withstand abiotic stresses (Suryanarayanan *et al.*,

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2009). According to a large group of scholars, endophytes are an excellent source of secondary metabolites, which have antibacterial and antifungal activity (Mishra *et al.*, 2012). However, the plant host must pay the price for the endophytes that support them (Rodriguez *et al.*, 2004, 2008). Wali *et al*. (2006) discovered that seedlings carrying *Epichloë festucae* were smaller than endophyte-free seedlings in wild populations of sheep fescue (*Festuca ovina*). Additionally, compared to endophyte-free plants, an endophyte-containing cultivar of tall fescue (*F. arundinaceae*) had increased crown and root rot disease brought on by *Pythium graminicola*. Although most endophytes' biology and ecology are unknown, their advantages are thought to differ depending on the host and environment (Mishra *et al.*, 2012).

Endophytic fungi's rates of colonization and isolation in trees are influenced by a number of variables, including the type of tissue, tree age, and location. In *Terminalia arjuna* trees, the incidence of endophytic colonization was higher in the bark than in the twigs (Tejesvi *et al.*, 2005). Seasons and tissue types affected the frequency of endophyte colonization in the Indian medicinal plant *Tinospora cordifolia* (Mishra *et al.*, 2012). Regarding the impact of tree age on endophyte colonization, it appears that older needles of the Chinese oil pine, *Pinus tabulaeformis* Carr, have a much higher rate of endophyte infection than younger ones (Guo *et al.*, 2008). Additionally, this species' colonization and isolation rates considerably rose as needle age increased (Wang & Guo, 2007).

There is proof that the incidence of endophyte colonization varies significantly among collection sites. Changes in climatic and ecological conditions are likely to be the cause of this variation in colonization frequency (Sokolski *et al.*, 2007). Fungal populations are directly impacted by toxic metals, air pollution, and acid rains, which decrease their diversity and abundance before reaching plant tissue (Helander *et al.*, 2007).

One of the most popular ornamental trees in urban landscapes in Iran and other Mediterranean regions is the plane tree (*Platanus orientalis* L.) (Diamandis, 2004). A few 500–1000-year-old plane trees can reach heights of 30–40 meters and stem diameters of up to 5 meters (Tello *et al.*, 2000). They typically withstand biotic (pathogens, pests) and abiotic (cold, nutrient deficiency, heat, and drought) conditions. Old-aged plane trees may develop symbiotic connections with specific fungal populations, impacting their persistence in these situations. A detailed investigation of this relationship between symbiotic fungi and plane trees has not yet been done. Accordingly, the objectives of this study are (i) to identify endophytic fungi that may have infected plane trees using morphology and internal transcript spacer (ITS) sequence data, and (ii) to determine the frequency and diversity of endophyte assemblages as a function of tree location, tree age, tissue type, and tissue surface sterilization method.

Materials and Methods

Studied sites and sampling

Plane trees were sampled from three central Iranian cities of Mahallat, Natanz, and Isfahan, renowned for their old plane trees. These cities have moderate yearly temperatures and low precipitation (Table 1). For the study, 18 young (less than 100 years old) and 18 older (200-1000 years old) trees were sampled at random. To calculate the age of the young trees, the circumference at shoulder height was measured in centimeters (cm), divided by the growth factor of plane trees (2.75), and rounded to the nearest whole number. Records on the plane tree's planting history held in the area or by locals were examined to determine the age of old

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trees. Each site was divided into six portions, and one old and one young tree placed in the center of each area with the shortest distance were chosen to select trees randomly.

Table 1. Geographic characteristics of the three locations used in the study.

Location	Latitude	Longitude	Elevation (m)	Mean annual temperature (°C)	Mean Jan. temp. (°C)	Mean Jul. temp. (°C)	Mean annual Precipitation (mm)
Isfahan	32° 38′ N	51° 39′ E	1575	18.7	5.3	34.6	120.2
Natanz	33° 31′ N	51° 54′ E	1650	16.4	3.6	30.1	139.4
Mahallat	33° 54′ N	50° 27′ E	1780	15.9	1.4	29.3	266.1

From June through September, healthy tissues of plane trees were harvested. Samples were taken from the trees' stem and bark (not the leaves) to identify endophytes that are more persistent and not occasional and temporary. Around the circumference, samples of bark encompassing both outer and interior layers were taken around 2–3 meters above the ground. In addition, 1-2 m long, 1-2 cm in diameter stems were gathered from tree branches 5–10 m above the ground. Labeled samples were packed in sterile polythene bags and transported to the laboratory.

Endophyte isolation and identification

Stem and bark pieces were surface sterilized by sequential washing in 70% (v/v) ethanol (1 min) and 10% (v/v) NaOCl (15 min), followed by rinsing with sterile water and drying under sterile conditions (Schulz *et al.*, 1993; 1999). The second technique consisted of ethanol 70 percent (v/v) for one minute and flame (30 s). Under sterile conditions, stem samples were cut into small pieces (approximately 10 mm in length and 10-15 mm in diameter) and placed in 9 cm diameter Petri dishes containing sterile PDA (potato-dextrose-agar) media containing antibiotics (Penicillin, Streptomycin, and Tetracycline, 100 g L^{-1}) to inhibit bacterial growth (Wang *et al.*, 2007; Sun *et al.*, 2011). Outer (dead) tissues were removed from the bark samples using a sterile blade, and fresh inner bark (about 10 mm in length) containing cambium and phloem was used for culture.

In total, two thousand five hundred ninety-two fragments (stem and bark) were incubated at 25 ºC in dark and monitored every other day (Oses *et al.*, 2008). The samples' newly developing hyphae were sub-cultured on new plates until pure cultures were obtained. Using conventional fungal taxonomy guides, pure cultures of sporulating endophytic strains were identified based on their morphological traits (colony margin, elevation, color, growth pattern, conidiogenous cells, and conidia) (Carmichael *et al.*, 1980; Crous *et al.*, 2007). Based on internal transcript spacer (ITS) sequencing data, pure cultures of non-sporulating strains were categorized into morphotypes and then classified at the genus level. Morphotypes were also distinguished visually based on their shared morphological traits of colony margin, color, and growth pattern.

Molecular phylogenetic analyses

Using the AccuPrep® Genomic DNA Extraction Kit, DNA was extracted from 10 mg of crushed, dried mycelia to isolate fungal DNA (BIONEER, Korea). The gene encoding ITS1-5.8S-ITS2 rDNA was amplified by PCR with ITS1 and ITS4 universal primers (White *et al.*,

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1990). The amplified nucleotide product was sequenced using the dideoxynucleotide chain termination method (Sanger *et al.,* 1977) by Bioneer Sequencing Service (Bioneer, South Korea). Similar sequences were then identified using online BLAST in the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The dataset included ITS sequences from tested fungal endophytes as well as database sequences from other fungi. Initially, separate alignments were done for each major group of fungi; however, the findings of phylogenetic studies in separate and non-separate alignments were identical and confirmed each other. Consequently, combined and multiple alignments were performed, and the maximum likelihood phylogenetic tree was constructed using the software CLUSTAL X 2.0 (Larkin *et al.,* 2007) and MEGA 4. (Kumar *et al.,* 2008).

Statistical Analysis

ANOVA was utilized to determine if there were statistically significant differences in endophytes' number (frequency) between various locations, tree ages, tissue types, and surface sterilizing techniques. To accomplish this, trees served as the level of replication (r=6) for each tree age as a treatment in each site as a separate habitat. Consequently, combined data analysis from three locations was conducted using SAS V.9 and a completely randomized experimental design with tissue types and surface sterilization procedures as split plots (SAS Institute, Cary, NC). Endophyte species diversity was also determined using the Shannon–Wiener Index according to the equation H= $-\sum_{i=1}^{s} pi(LnPi)$ where s is the number of species and pi is the relative contribution of each endophyte species to all isolates. Species accumulation curves were also estimated using EstimateS v 9.1.0 (Colwell, 2013, http://viceroy.eeb.uconn.edu/Estimates).

Results

Endophyte isolation and identification

A total of 2592 tissue segments, comprising 1296 stem and 1296 bark segments of plane trees from three locations, were processed, resulting in the recovery of 1591 fungal isolates classified into 16 taxa. Using morphological characteristics, 1059 sporulating strains belonging to 9 taxa were identified, with *Penicillium sp*., *Rhizopus sp*., and *Alternaria sp.* being the most prevalent (Data not shown). The remaining 532 strains isolated from plane trees could not be identified morphologically since they grew solely as vegetative mycelium in vitro; therefore, they were classified into seven morphotypes (taxa) and subjected to ITS sequencing data analysis for identification. The nucleotide sequences were submitted to GenBank and assigned as *Fusarium sp.*1 (accession number KM878572), *Fusarium sp.*2 (accession number KM878573), *Fusarium sp.*3 (accession number KM878574), *Fusarium sp*.4 (accession number KM878575), *Fusarium sp*.5 ((accession no. KM878577). Additionally, the Maximum Likelihood (ML) phylogenetic tree demonstrated that newly found taxa are closely related to other *Fusarium*, *Chaetomium*, and *Cochlobolus* species (Fig 1).

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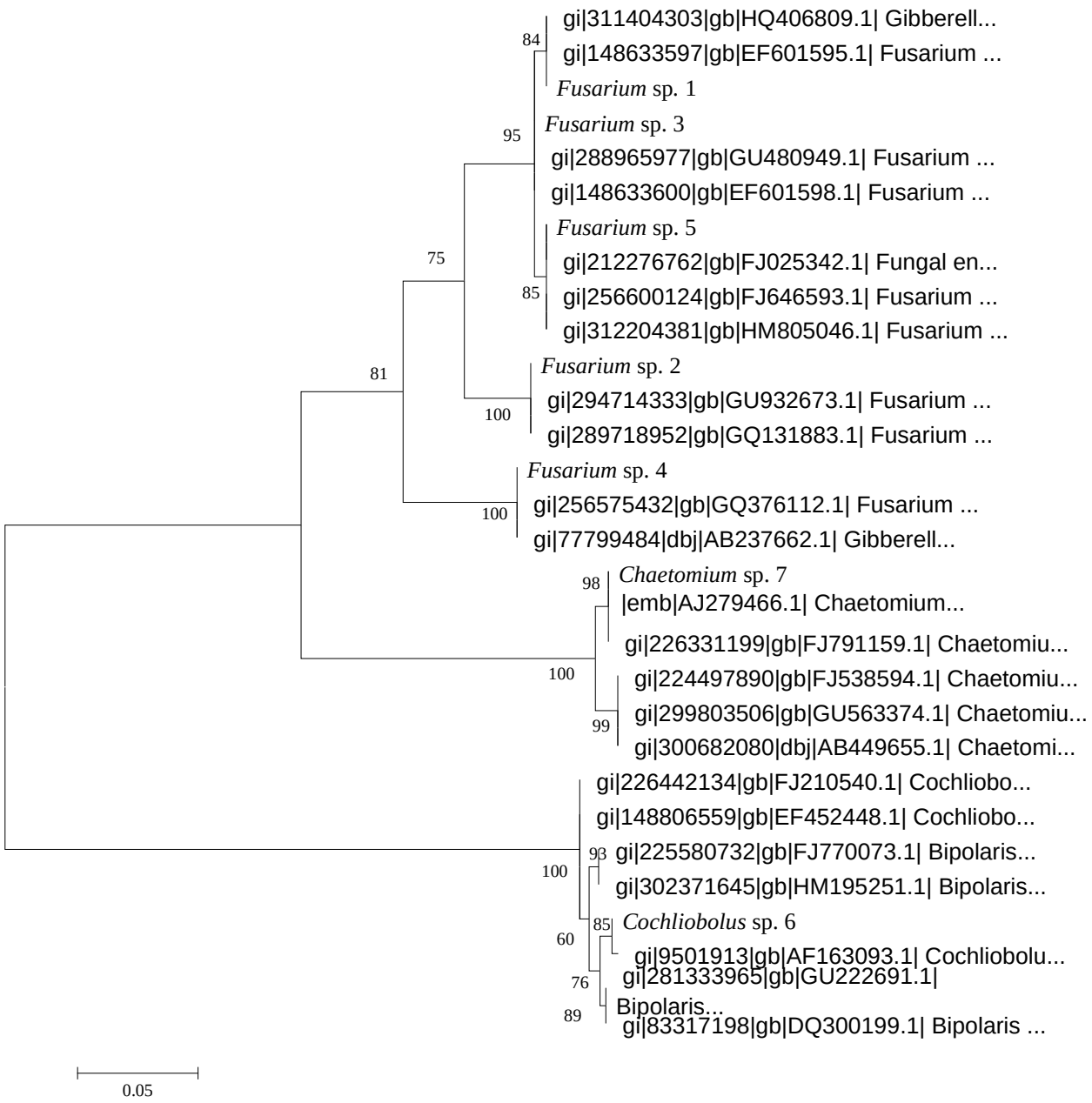

Figure 1. Phylogenetic tree generated by the Maximum Likelihood method on the basis of ITS1-5.8S-ITS2 rDNA sequence showing the evolutionary position of some unknown endophytic fungi isolates (in red) among related fungal species retrieved from GenBank. Bootstrap values (expressed as percentages of 1000 replications) are shown at major branching points.

Tree location effect

The ANOVA (Table 2) revealed that the position of plane trees significantly affected the quantity of fungal endophytes ($p<0.001$). The infection rate of plane trees in Mahallat was the greatest, whereas in Isfahan, it was the lowest (Table 4). Based on the analysis, 20.42 % of isolated endophytes were obtained from Isfahan, 25.20 % from Natanz, and 54.30 % from Mahallat (Table 4). In Isfahan, Natanz, and Mahallat, *Rhizopus sp.* (123 isolates), *Alternaria sp.* (78 isolates), and *Penicillium sp.* (161 isolates) were the predominant endophyte species

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(Data not shown). The locations with the greatest species variety, as measured by the Shannon-Wiener index, are Mahallat and Natanz (Table 4), which have greater precipitation and altitude but lower annual temperature than Isfahan (Table 1).

Table 2. Analysis of variance on the number of fungal endophytes as a function of various parameters.

Source	DF	Mean Square	F Value	Pr> F
Location	2	1780.111111	5274.40	<.0001
Tree (Location)*	15	0.337500	---	---
Age	1	370.562500	1919.46	<.0001
Location × Age	2	2.583333	13.38	0.0005
Tree (Location Age)*	15	0.193056	---	---
Tissue	1	7.562500	32.60	<.0001
Location × Tissue	2	9.083333	39.16	<.0001
Age ×Tissue	1	3.062500	13.20	0.0010
Location × Age × Tissue	2	0.750000	3.23	0.0535
Tree (Location Age Tissue)*	30	0.231944	---	---
Surface sterilization	1	10.562500	96.27	<.0001
Location × Surface sterilization	2	7.750000	70.63	<.0001
Age × Surface sterilization	1	0.173611	1.58	0.2133
Location × Age × Surface sterilization	2	2.111111	19.24	<.0001
Tissue × Surface sterilization	1	1.562500	14.24	0.0004
Location × Tissue × Surface sterilization	2	5.083333	46.33	<.0001
Age × Tissue × Surface sterilization	1	0.562500	5.13	0.0272
Location × Age × Tissue × Surface sterilization	2	6.083333	55.44	<.0001
Error*	60	0.109722	--	--

*used as error term for testing variation sources.

Tree age effect

The analysis of variance revealed that the number and species variety of fungal endophytes are affected by tree age (P<0.001) (Tables 2 and 3). Endophytes were more prevalent in older trees (57.25 %) than in younger trees (42.74 percent). *Rhizopus sp.* (25.73 %) and Penicillium sp. (13.17 %) were the species with the highest frequency. In contrast, *Humicola sp.* (0.29 %) and *Cladosporium sp.* (2.74 %) were the species with the lowest frequency isolated from the young and old trees, respectively (Data not shown). rDNA sequences were used to identify isolates that were largely categorized as morphotypes. These isolates accounted for most of the distinction between old and young trees. In many instances, such as *Fusarium sp.*1 and *Chaetomium sp.*7, the number of isolates on old trees was 3 and 4 times higher than on young trees (Data not shown).

Tissue type effect

The number of isolates was found to be bigger in bark (812 isolates) than in stems (779 isolates) (Table 4). The influence of tissue type on the number of fungi was not as statistically significant as that of the sample collecting site and tree age (Tables 2 and 3). Similarly, the Shannon-Wiener index was impacted by tissue type (Table 4), but unlike the frequency of fungal endophytes, species diversity was greater in stems than in bark. *Rhizopus sp.* (22.90%) and *Penicillium sp.* (16.04%) were the most abundant species of endophytes isolated from the bark and stems, respectively. *Cladosporium sp.* (1.23%) and *Fusarium sp.* 2 (1.54%) were the

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species with the lowest frequency (Data not shown).

Table 3. ANOVA results for Shannon-Wiener diversity index (H').

Source	DF	Mean Square	F Value	Pr> F
Location	2	0.54434444	36.08	0.0005
Age	1	0.26041667	244.14	<.0001
Tissue	1	0.12906667	21.27	0.0099
Surface sterilization	1	0.01306667	6.17	0.0679

Table 4. Fungal isolates number in plane trees and Shannon-Wiener diversity index based on different locations, age, tissue and surface sterilization methods.

Treatment	Location			Age		Tissue		Surface sterilization	
Category	Isfahan	Mahallat	Natanz	Old	Young	Stem	Bark	Ethanol and flame	Ethanol and NaOCl
No. samples	864	864	864	1296	1296	1296	1296	1296	1296
No. isolates obtained	325	865	401	911	680	779	812	776	815
The number of isolates per tree	6.77^{c}*	18.02^{a}	8.35^{b}	12.65^{a}	9.44^{b}	10.81^{b}	11.27^{a}	10.77^{b}	11.31^{a}
Shannon-Wiener (H')	1.68^{b}	2.52^{a}	2.25^{a}	2.67^{a}	2.26^{b}	2.64^{a}	2.35^{b}	2.45^{a}	2.55^{a}

*Means followed by the same letters in each treatment are not significantly different based on Tukey test (P<0.05)

The effect of surface sterilization methods

The efficiency of two standard surface sterilization methods in isolating endophytes from plane trees was examined. The isolation frequencies of fungal endophytes differed significantly between the two methods of surface sterilizing (Table 4). Comparing the two approaches, the isolation frequency of *Penicillium sp*. (153 isolates) and *Rhizopus sp*. (155 isolates) was the highest when employing ethanol and NaOCl and ethanol and flame, respectively. *Cladosporium* sp. was absent in the sterilization ethanol and flame method, but its isolation rate in the ethanol and NaOCl method was 4.29 % (Data not shown). The ANOVA (Table 3) revealed that surface sterilization procedures did not affect the species diversity of endophytic fungi. ANOVA table also showed that all interaction effects between variation sources were significant except for the tree age surface sterilization technique (Table 2).

Species accumulation curves

The species accumulation curves of fungal endophytes from the entire pieces investigated from the old and young trees of *P. orientalis* at three sites are shown in Figs. 2 and 3. It is evident from the species accumulation curve in Fig. 2 that Mahallat had a greater diversity of fungal endophytes in trees (almost 94% of expected endophyte species) than Natanz and Isfahan. These curves appear asymptotic in three locations, indicating that if more plant samples had been studied, fewer fungal endophytic species would have been detected. As the

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curve in Fig. 3 has not yet reached its asymptote, the number of species retrieved from young trees should increase. Consequently, the sampling effort among young trees was insufficient to recover most of the expected biodiversity. In contrast, the species accumulation curve found in old trees (Fig. 3) is nearly saturated at n=15 trees (n18), indicating that additional sampling would not identify more fungal species. Similar results were reported in species accumulation curves for two surface sterilization procedures and tissue types, and sampling saturation occurred with the number of trees analyzed in this study (curves not shown).

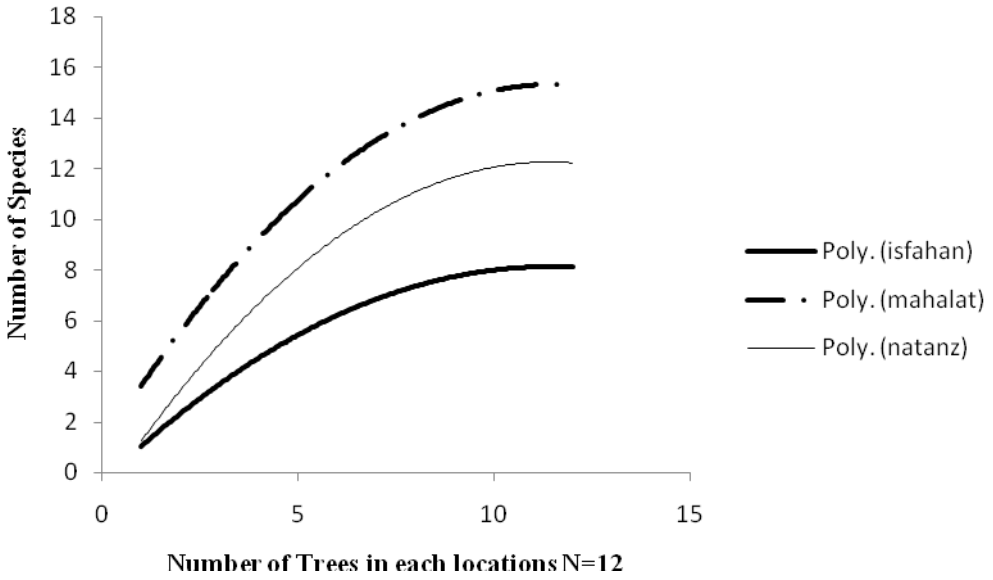

Figure 2. Species accumulation curves for fungal endophytes isolated from *P. orientalis* trees in three locations. Individuals were shuffled among samples and 100 randomizations were made.

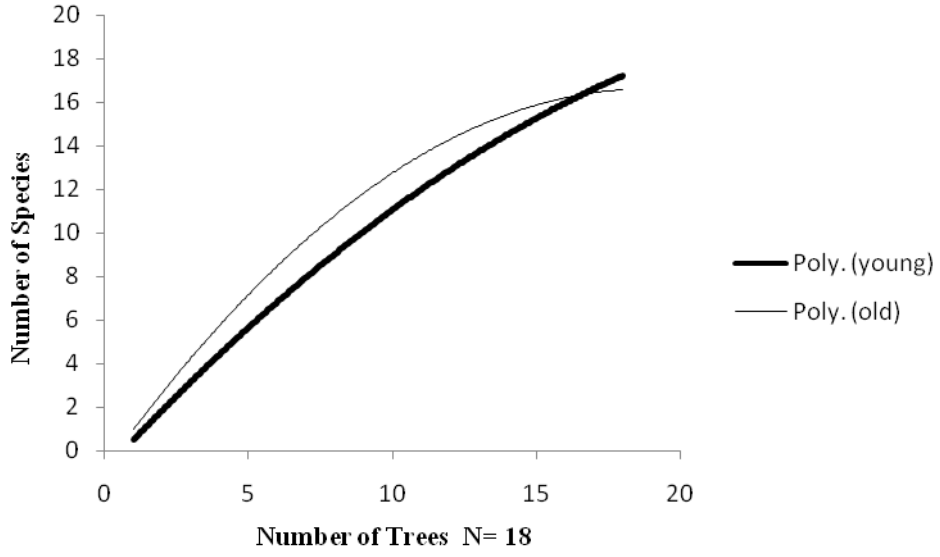

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Figure 3- Species accumulation curves for fungal endophytes isolated from old and young *Platanus orientalis* trees. Individuals were shuffled among samples and 100 randomizations were made.

Discussion

Airborne fungi with significant spore production, such as *Penicillium* sp., *Rhizopus* sp., *Alternaria* sp., and *Aspergillus* sp., were among the most commonly isolated endophytes from plane trees. They are important components of numerous endophyte communities (Huang *et al.*, 2009; Sun *et al.*, 2011), and it appears that their prevalence as endophytes may be attributable to their high environmental activity and sporulation, which statistically increases their likelihood of becoming established as permanent endophytes (Mishra *et al.*, 2012).

In the present study, the highest endophyte frequency was observed in Mahallat, where precipitation, latitude, and temperature were all greater. There was a high positive association between fungal infection rate and elevation (r=0.97) and precipitation rate (r=0.99) among collecting sites, which supports this result. In contrast, there was a negative association between infection rate and temperature (r = -0.73). The diversity of species was also greater in Mahallat and Natanz, which shared environments that were largely identical yet distinct from those of Isfahan. Diversity and number of endophyte infections are influenced by site characteristics, climate, humidity, and abiotic and geographic structures (Terhonen *et al.*, 2011; Langenfeld *et al.*, 2013).

Furthermore, the distribution and abundance of fungus vary according to latitude and yearly precipitation (Arnold & Lutzoni, 2007; Botella & Diez, 2011). According to Guo & Wang (2008), heavy precipitation can facilitate the spread of fungal spores, leading to greater species diversity. Zamora *et al*. (2007) also suggested that an increase in precipitation boosted the diversity of fungi in the needles of *Pinus* species. Higgins *et al*. (2007) similarly observed high diversity of endophytic fungi at higher altitudes, supporting our findings. Mahallat and Natanz have greater vegetation densities than Isfahan due to more significant precipitation and cooler temperatures. This effect may also protect the leaf and stem tissues of plane trees from external stress factors that contribute to greater fungus populations in host plants (Gore & Bucak, 2007).

The ITS sequencing data could be used to differentiate and identify seven unidentified morphotypes. Nevertheless, due to limitations in the taxonomic resolution of ITS data, we limited our phylogenetic analysis to separating morphotypes at the genus level. The ITS region has significant potential for the survey of fungal diversity and identification of closely related fungal populations (Mello *et al.*, 2011); however, it is thought that ITS does not provide the direct identification of all fungal species (Ko *et al.*, 2011; Liu *et al.*, 2015). In future investigations, ITS paired with TEF (translation elongation factor) and -tubulin as the common genetic markers may distinguish species within the recognized genera.

The frequency of morphotype groups separated from older trees was greater than younger trees, which may indicate a long-term symbiosis between old-plane trees and endophytes. It indicates that when the tissues of older trees expand, the fungal mycelia growth of morphotypes spreads in older plane tissues at a higher density (Wang & Guo, 2007). It is anticipated that older trees are more likely to be colonized by these fungi due to their extended exposure to a small number of conidia and spores produced by the morphotypes, which could repeatedly attack older plane tissues (Terhonen *et al.*, 2011). In addition, greater leachates from older plane tissue may probably promote the germination and infection of

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morphotype group spores (Miller & Roy, 1982). To prove the prospective effect of these microbes on the longevity of some old plane trees and their resistance to stress, however, additional and more elaborate trials are required.

A greater frequency of fungal endophytes was observed in the bark of plane trees than in their stems. However, stems had a greater species diversity than bark. Collado *et al*. (2001) demonstrated that the colonization rates of endophytic fungi on *Quercus ilex* bark (65%) were higher than on its leaves (25%). This difference in endophyte infection between the main stem (bark) and twigs may be due to tissue texture, physiology, chemistry, and tissue substrates. It influences the colonization of endophytic fungi, or the bark of the main stems has had more time to accumulate fungal propagules. Stems of dicots, such as plane trees, consist of three types: epidermal tissue, vascular tissue (xylem and phloem), and the cortex. Xylem and cortex are predominantly lignified and may be composed of cellulose and hemicellulose, which are non-nutritive and ineffective for most endophytic fungi (Guo *et al.,* 2008). Bark, which is predominantly composed of phloem that transports sugars from leaves to other parts of the tree, maybe a better location for endophytic fungi to reside and accumulate for a more extended period than stems, which contain a large proportion of lignified tissues.

There was a significant distinction between the two surface sterilization methods. It may suggest that isolated endophytes emerge from different parts (depth) of the bark and stem that have been sterilized on the surface. Schulz *et al*. (1993) compared the efficacy of seven surface sterilization methods on the isolation of endophytes in herbaceous and shrubby plants. They discovered that one method was distinct from the others. The species diversity was unaffected by surface sterilization techniques. However, the lack of isolation of *Cladosporium* sp. under ethanol and flame methods of surface sterilization suggests its sensitivity to heat treatment (Kawai *et al.,* 1990).

Out of 1591 isolated endophytes in this study, six common taxa were consistently isolated from each sample collection: *Penicillium* sp., *Alternaria* sp., *Ulocladium* sp., *Mucor* sp., *Rhizopus* sp., and *Fusarium* sp. The most widespread species in Isfahan, Natanz, and Mahallat, respectively, were *Rhizopus* sp. (37.84 %), *Alternaria* sp. (19.45 %), and *Penicillium* sp. (18.61 %). Only *Cochliobolus* sp.6, *Cladosporium* sp. and *Humicola* sp. could be isolated from Mahallat, while all other fungi were found in at least two locations. It indicates that a small number of dominant species were identified from many isolates. The region chosen for sampling is located in a low-rainfall, high-temperature, and low-humidity region of the globe. It may drastically reduce the likelihood of fungal spores penetrating the stem surface or surviving for an extended period within the plane tree. It could explain why diversity richness was most remarkable in Mahallat (with greater annual precipitation) and lowest in Isfahan (with lower annual rainfall). Also, since all of the methods utilized in this study were culture-based, it is possible that non-cultivable endophytes were neglected. However, climate and the availability of resources appear to be the primary factors determining the geographical distribution and abundance of fungi. Before generalizing, additional reports from this region or other parts of the world should be presented. In addition, the interaction between plane trees and their endophytic communities, especially those classified as morphotypes, is unknown, and future research is required to clarify the applied consequences of this symbiotic relationship for both partners.

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