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Biodiversity of phaeohyphomycotic agents in public gardens in Cairo vicinities, Egypt

S. M. Zaki*, S. S. Mohamed, A. A. EL-Mehalawy and K. A. Youssef
Microbiology Department, Faculty of Science, Ain Shams University, Abbassia 11566, Cairo, Egypt
*Corresponding author: E-mail: shzaki@yahoo.com
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Abstract: The biodiversity of phaeohyphomycotic agents in public garden habitats located in four governorates near Cairo (Faiyoum, Ismailia, Minufia, and Qalyubia) was analyzed. A total of 120 samples of soil and plant debris were collected seasonally during the period 2006-2007 and screened for the occurrence of phaeohyphomycotic agents. Dichloran chloramphenicol peptone agar (DCPA) medium was used as a selective medium for isolation by dilution plate method. Nine fungal species belonging to 5 genera, all previously implicated as agents causing phaeohyphomycosis were identified and are as follows: *Alternaria alternata*, *A. infectoria*, *A. tenuissima*, *Cladosporium cladosporioides*, *C. oxysporum*, *C. sphaerospermum*, *Cochliobolus lunatus*, *Phialophora sp.*, and *Veronaea botryosa*. Molecular typing of the recovered species based on ITS1-5.8S-ITS2 region sequences were carried out and the obtained sequence data confirmed the morphological identification. The diversity analysis showed that the most prevalent species was *C. sphaerospermum* representing (56.3%) of the total recovered isolates, followed by *C. oxysporum* (16.31%), *C. cladosporioides* (14.0%), *A. alternata* (5.2%), *C. lunatus* (2.5%), *Phialophora sp.* (1.9%), *A. infectoria* and *A. tenuissima* (1.6% each) and finally *V. botryosa* representing 0.4% of the total recovered isolates.

Key words: Phaeohyphomycosis, ITS1-5.8S-ITS2, biodiversity, natural environments, Egypt.

Introduction

Phaeohyphomycosis is a clinical term used to describe fungal infections caused by various dematiaceous hyphomycetes which are characterized by the production of brown-pigmented yeast like cells and hyphae in culture and infected tissue (Ajello 1986 and Kwon-Chung and Bennett 1992).

Phaeohyphomycosis includes a diverse group of dematiaceous fungal infections which can be classified as superficial, cutaneous, corneal, subcutaneous and systemic forms (Rinaldi, 1996).

This group of fungi can cause systemic dissemination in immunosuppressed patients (Hauck et al., 2008) and were reported recently in immunocompetent individuals (Albaz et al., 2009). Moreover, the mortality rate due to dematiaceous hyphomycetes pathogens have been significantly increased especially in cases of solid organ transplant recipients, diabetes mellitus, lymphoma, eye trauma, intravenous drug abuse and long term peritoneal dialysis (Ben-Ami *et al.* 2009 and Taj-Aldeen *et al.* 2010).

About 130 species in 70 genera of dematiaceous fungi have been implicated as etiologic agents of phaeohyphomycosis (Kondo *et al.* 2007).

Many of these species have been rarely reported, while others such as *Bipolaris*, *Curvularia*, *Exserohilum*, *Exophiala* and *Alternaria* species have been frequently involved in human infections (Schell 1995 and Harris *et al*. 2009).

Dematiaceous hyphomycetes are ubiquitous microorganisms. They exist in almost every conceivable habitat: in soil (Yue-Li *et al.* 2008), litter, desert soil (El-Said 1994), in living plants and decaying remains of plants (Iwamoto and Tokumasu 2001), in fresh water and sea water (Krik and Gordon 1988).

Humans are exposed constantly to fungi through inhalation or traumatic implantation of fungal elements. The accidental or permanent presence of fungi in animals, plants, soils and watercourses is considered an important issue in the investigations focused on public health because they constitute the source where potential pathogens will be contracted. The determinations of the fungal habitats that impose the highest risk of exposure and also the seasonal variation in the production of infectious propagules are of great importance for avoiding human infections (Restrepo *et al.* 2000).

Although the great importance of phaeohyphomcotic agents allover the world, the incidence of phaeohyphomycosis infection in immunosuppressed and/or immunocompetent patients have not taken the consideration from the medical mycologists and physicians in Egypt. Accordingly, the aim of this investigation is to analyze the biodiversity of the phaeohyphomycotic agents in public gardens located at highly populated areas in Cairo vicinities, Egypt in order to draw the attention for the importance of such group as etiologic agents for some complications in patients.

Materials and Methods

Collection sites and sampling

A total of 120 samples of soil and plant debris were collected from public gardens located in four governorates near Cairo (Faiyoum, Ismailia, Minufia, and Qalyubia). From each governorate, 15 soil samples were collected 2-3 cm of soil surface around perennial trees and 15 samples of rotted leaves and twigs underlying surface layer of soil around perennial trees. Sampling was carried out during the period 2006-2007 in winter, spring, summer and autumn seasons. The collected samples were placed

in polyethylene bags, taken to the laboratory and stored at 15°C until mycological analysis.

Isolation of dematiaceous hyphomycetes

Dilution plate method was used. Twenty five grams of soil, rotted leaves or twigs were placed in 500 ml Erlenmeyer flasks containing 250 ml sterile distilled water to which penicillin and streptomycin were added and then serially diluted (Gugnani and Shrivastav 1971). Dichloran chloramphenicol peptone agar (DCPA) medium was used as a selective medium for isolation of dematiaceous hyphomycetes (Andrews and Pitt 1986). Aliquots of 0.5 ml of 10^{-2} and 10^{-3} of sample suspension were placed in 10 cm Petri-dishes prior to addition of DCPA medium. Three replicate plates were prepared for each dilution (Johnson and Curl 1972). Plates were incubated at 25°C for 2 weeks and observed daily.

Identification of phaeohyphomycotic agents

Selected dematiaceous colonies were picked and transferred onto fresh Potato Dextrose Agar (PDA) medium for purification and identified according to their morphological characteristics by recognition of their macroscopic and microscopic features following the manuals of Ellis (1971 & 1976), Moubasher (1993) and de Hoog *et al.* (2009).

Molecular identification

Extraction of DNA

A small amount of mycelium grown on potato dextrose agar was suspended in 200 µl of TE buffer (100 mM Tris-HCl, pH 8.0, 1mM EDTA) in an Eppendorf tube (1.5 ml). DNA extraction was carried out according to the procedure described by Sandhu *et al*. (1995). Briefly, 250 µl of GPT reagent (6M guanidine thiocyanate dissolved in 50 mM Tris [pH 8.3]) and 700 µl of phenol-buffered in Tris (pH 8.0) were added to a washed fungal inoculum in a screw-capped tube and boiled for 15 min; 250 µl of chloroform-isoamyl alcohol was then added, and the aqueous phase was separated by centrifugation at 14,000 x g, mixed with an equal amount of 100% isopropanol and 1/10 volume of 3 M ammonium acetate, and placed at 20 °C for 1h. Samples were centrifuged at 14,000 x g for 20 min, and the nucleic acid pellet was washed with ice-cold 70% ethanol, dried, and re-suspended in sterile TE-buffer at a concentration of 5 µg/ml.

Oligonucleotides

The oligonucleotide primers used for amplification and sequencing of the ITS regions were those described by White *et al*. (1990). ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were made by Pharmacia Biotech CO., LTD. (Tokyo, Japan).

PCR and DNA sequencing of ITS1-5.8S-ITS2 region rDNA of fungal strains

Amplification reactions were performed in 25 µl of distilled water containing 2.5 µl of each primer (20 pm), 2.5 µl of genomic DNA (5 µg/ml) and one PCR bead. PCR was performed using the initial denaturation at 94 °C for 4 min, followed by 35 cycles at 94°C for 2 min, 55°C for 2 min and 72 °C for 2 min, and a final extention at 72 °C for 10 min. The PCR reaction products were sequenced directly using a big Dye terminator reagent kit including *Taq* polymerase and the protocol recommended by the manufacturer (Model 3010 automated DNA sequencer, Perkin-Elmer/Applied Biosystems, Japan).

Data analysis

The following parameters were used to analyze the diversity of the recovered species of dematiaceous hyphomycetes

- Species richness (S): the total number of species in the community
- Relative Risk Ratio (RRR): the incidence of the recovered species in soil or plant debris samples collected from the four governorates in the four seasons,
  (RRR) = Odd's ratio of plant debris samples (P) / Odd's ratio of soil samples (S).
  Odd's ratio = Number of isolates of certain species recovered from certain site / Total number of isolates of this species recovered from all sites.
- Relative density (RD, %): the distribution and frequency of occurrence of each recovered species
- $\text{RD (\%)} = \frac{\text{Number of isolates of certain species}}{\text{Total number of isolates}} \times 100$
- Statistical analysis:

Echosoft Spps statistical software package, V.13, corp., USA 2003 was used for data analysis. Data were expressed as both number and percentage for categorized variables. Chi-square test was done to define the categorized variables. The probability of error (*P* values) at or less than 0.05 was considered significant, while at 0.01 and 0.001 are highly significant.

Results

Isolation and identification of phaeohyphomycotic agents

A total of 478 of dematiaceous hyphomycetes isolates were obtained. Twenty species belonging to 9 genera of which 9 species belonging to 5 genera, all previously implicated as agents of phaeohyphomycosis were identified and are as follows: *Alternaria alternata*, *A. infectoria*, *A. tenuissima*, *Cladosporium cladosperoides*, *C. oxysporum*, *C. sphaerospermum*, *Curvularia lunata* (teleomorph: *Cochliobolus lunatus*), *Phialophora* sp., and *Veronaea botryosa* (Table 1).

Molecular typing of the isolated phaeohyphomycotic agents

NCBI GenBank was accessed to identify the isolated species using the BLAST homology search with the obtained ITS data of the 9 strains. The results obtained revealed that the isolates *A. alternata, A. infectoria, A tenuissima, C. cladosporioides, C. oxysporum, C. sphaerospermum, C. lunata, Phialophora* sp. and *V. botryosa* were identical to the ITS data of *A. alternata* (GenBank Accession Number EU594567), *A. infectoria* (AF397239), *A. tenuissima* (EU326185), *C. cladosporioides* (EF577236), *C. oxysporum* (DQ875018), *C. sphaerospermum* (AM182171), *C. lunata* (AF071339), *Phialophora* sp. (AM176752), and *V. botryosa* (AB369905) respectively. The complete data of molecular identification of the obtained phaeohyphomycotic agents were covered in a separate publication (Zaki, 2008). The phaeohyphomycotic agents obtained in this study were deposited in the culture collection of Medical Mycology Research Center, Chiba University, Japan. The databank accession numbers and IFM numbers of the recovered species are listed in Table (1).

Diversity analysis of the recovered phaeohyphomycotic agents
Species richness

The highest number of species was recorded from soil samples collected from Qalyoubia governorate in the four seasons (7 species) followed by Ismailia governorate (6 species). While the lowest number of species was recorded from soil samples collected from Minufia and Faiyum Governorates (4 species each).

Winter season showed the highest variation in species recovered from soil samples collected from the four governorates (8 species) followed by spring (6 species) and summer (4 species). The lowest number of species (3 species) was recovered from soil samples collected during autumn season.

The highest number of species was recorded from samples of plant debris collected from Faiyum governorate in the four seasons (6 species) followed by Minufia and Ismailia Governorates (4 species each), while only 3 species were recovered from samples collected from Qalyubia Governorate.

The highest variation of species was recorded from plant debris samples collected during summer and autumn seasons from the four governorates (4 species each), followed by winter and spring seasons (3 species each).

Incidence of the recovered species in soil and /or plant debris samples

As shown in Table (2), *C. sphaerospermum*, *C. oxysporum*, *C. cladosporioides*, and *Phialophora* sp. were the dominant species in both soil and plant debris samples. The incidence of *C. sphaerospermum* and *C. oxysporum* was higher on plant debris than in soil samples (RRR>1), while the incidence of *C. cladosporioides* and *Phialophora* sp. was higher in soil samples than on plant debris samples (RRR<1). The remaining five species including *A. alternata*, *A. infectoria*, *A. tennuissima*, *C. lunata*, and *V. botryosa* were isolated from soil samples only.

Distribution and frequency of occurrence

The highest number of isolates representing all the identified species were obtained from Qalyubia Governorate (166 isolates) representing 34.2% of the total isolates, whereas the lowest number of isolates was recovered from Faiyum Governorate (90 isolates) representing 18.8% of the total recovered isolates. A total of 116 isolates were recovered from Minufia governorate representing 24.3% of the total recovered isolates and a total of 106 isolates representing 22.2% were recovered from Ismailia Governorate as shown in table (3).

Regarding the different seasons, the highest number of isolates representing all the identified species was obtained in the winter season (169 isolates) contributing 35.4% of the total recovered isolates, while the lowest number of isolates was obtained in summer season (67 isolates) representing 14% of the total recovered isolates. A total of 122 isolates representing 25.5% of the total isolates was recovered in autumn season and a total of 120 isolates representing 25% was obtained during spring season (Table 4).

Cladosporium was the most frequently isolated genus represented by 414 isolates matching 86.6% of the total recovered isolates, followed by *Alternaria* (41 isolates matching 8.6%), and *Curvularia* (12 isolates accounting for 2.5%). The most prevalent species was *C. sphaerospermum* representing 56 % of the total isolates, followed by *C. oxysporum* (16.3%), *C. cladosporioides* (14%), *A. alternata* (5.2%), *C. lunata* (2.5%), *Phialophora* sp. (1.9%), *A. infectoria* and *A. tenuissima* (1.6% each) and finally *V. botryosa* which shared only with 0.4% of the total isolates.

Within the genus *Cladosporium*, *C. sphaerospermum* was the most frequently isolated species representing 65% of the total *Cladosporium* isolates followed by *C. oxysporum* (18.8%). The occurrence of *C. sphaerospermum* differed significantly among the different governorates ($P<0.005$). The occurrence of *C. sphaerospermum* was also significantly different along different seasons ($P<0.01$), the highest number of isolates was recorded in autumn season (96 isolates).

Alternaria alternata was the most predominant species among the three recovered *Alternaria* species representing 61% of the total recovered isolates. The occurrence of *Alternaria* species differed significantly among different governorates ($P<0.001$). *A. alternata* was isolated from Qalyubia Governorate, while *A. infectoria* and *A. tenuissima* were isolated only from Ismailia Governorate. Also the occurrence of *Alternaria* species was

significantly different along different seasons (P<0.001); *A. alternata* was recovered in winter and spring, while *A. infectoria*, and *A. tenuissima* were confined to the winter season.

The occurrence of *Curvularia lunata* differed significantly among different governorates (P<0.001) as it was isolated only from Qalyubia governorate. Along different seasons the occurrence of *C. lunata* also differed significantly (P<0.001) where it was recovered only in winter season.

The occurrence of *Phialophora* sp. was not significantly different among different governorates or along different seasons (P>0.05). It was isolated from all the four governorates in all seasons except autumn. The occurrence of *Veronaea botryosa* differed significantly among different governorates and along different seasons (P<0.05). It was only isolated from Qalyubia Governorate in spring season (Tables 3 & 4).

In view of seasonal distribution and species richness, winter season showed the highest richness index of fungal species (8 species) among all studied sites. It was followed by spring (6 species) and summer seasons (4 species).

Discussion

Phaeohyphomycotic etiologic agents comprise fungal species with diverse ecology and different growth styles as they comprise saprophytes, endophytes or epiphytes of living plants and animals in addition to human pathogens (Wilhelmus, 2005 and Schoch *et al*. 2006). They also can occur in extreme environments and under poor nutrient conditions (Sterflinger *et al*. 1999).

In this study, the highest density of phaeohyphomycosis agents [Colony Forming Unit (CFU)] was obtained from plant debris samples (25148 CFU). This may be attributed to the high organic matter content in plant remains that enhance sporulation and conidial production by these species (Pratt, 2003). Moreover, many species such as *Alternaria*, *Cladosporium* and *Curvularia* are considered plant-inhabiting fungi that occur as phytopathogens (Chao *et al.* 2001) or saprobes on various living, senescing and dead leaves, needles, litter and stems of herbaceous and woody plants (Iwamoto and Tokumasu 2001 and Shirouzu and Harada 2004).

In view of the influence of seasonal variation on the density of the recovered species, the highest density was obtained in winter season followed by spring season, while the lowest density was obtained in summer season. This was consistent with Nayak *et al*. (1998) who found that the maximum load of spores was observed in January and November and the minimum was observed in June and July. The higher population during winter and spring seasons can be attributed to the moderate temperatures (15-25^{o}C) and humidity (60-80%) and mild rainfall which favor the fungal sporulation (Nayak *et al.* 1998 and Rowan *et al.* 1999). The lower population of dematiaceous hyphomycetes during summer season may be due to the deficiency of soil moisture and high temperature which inhibit growth and spore germination (Harrison *et al.* 1994). It was also reported that fungi exhibit higher activity in decomposition of litter and other plant remains in winter season than in summer season (Lipson *et al.* 2002).

The highest number of species was obtained from soil samples collected from Qalyubia Governorate in the four seasons (7 species). This may be associated with the high activities of human and animals which led to the increased amount of nutrient contents in the remains of plant, animal and man in this area providing the optimal conditions for microbial growth (Vitousek *et al.* 1997).

Based on frequency of occurrence, *Cladosporium* was the most frequently isolated genus, followed by *Alternaria*. These genera have been reported among the predominant soil dematiaceus hyphomycetes in Eastern Tibet (Tian-Yu *et al.* 2008). The prevalence of *Cladosporium* over *Alternaria* might be due to the higher sensitivity of *Alternaria* to variation in relative humidity and temperature than *Cladosporium* (Hjlemroos 1993). It may be also attributed to size and nature of *Cladosporium* conidia (small, dry & carried in long chains) which facilitate their dispersal by air (Di Giorgio *et al.* 1996).

The dominant species recovered during this study were *Cladosporium sphaerospermum*, *C. oxysporum*, *C. cladosporioides* and *A. alternata*. These species were also the predominant species in soil samples from the hypersaline Dead Sea coastal area (Grishkan *et al.* 2003).

Interestingly, *Cladosporium* was the only genus that was distributed in all governorates and in all seasons, indicating that it adapts to different environments (Hjlemroos 1993 and Pan *et al.* 2008). The isolated species which were recovered from soil and /or plant debris have been increasingly reported recently to be the etiologic agents of serious diseases in human through traumatic inoculation, these included *Alternaria* species (Gallelli *et al.* 2006), *Cladosporium* species (Tamsikar *et al.* 2006), *Curvularia lunata* (Smith *et al.* 2007), *Phialophora* sp. (Brandt and Warnock 2003) and *V. botryosa* (Yukiko *et al.* 2007).

Table 1: List of the recovered phaeohyphomycotic agents

No.	Fungal species	IFM number	Accession number
1	*Alternaria alternate* (Fr.) Keissl.	IFM 56391	EU759974
2	*Alternaria infectoria* Simmons	IFM 56389	EU759975
3	*Alternaria tenuissima* (Kunze: Pers.) Wiltsh.	IFM 56393	EU759976
4	*Cladosporium cladosporioides* (Fres.) de Vries	IFM 56395	EU759977
5	*Cladosporium oxysporum* Berk. & Curt.	IFM 56396	EU759978
6	*Cladosporium sphaerospermum* Penz.	IFM 56397	EU759979
7	*Curvularia lunata* (Wakker) Boedijn	IFM 56386	EU759980
8	*Phialophora* sp.	IFM 56398	EU759981
9	*Veronaea botryose* Ciferri & Montemartini	IFM 56385	EU759982

IFM = Medical Mycology Research Center, Chiba University, Chiba, Japan

Table 2: Incidence of phaeohyphomycotic agents recovered from soil and plant debris samples collected from the four governorates in the four seasons.

Fungal species	Number of isolates		Total	Odd's Ratio		RRR
	Soil	Plant debris		Soil	Plant debris	
Cladosporium sphaerospermum	119	150	269	0.44	0.56	1.27
Cladosporium oxysporum	30	48	78	0.38	0.62	1.63
Cladosporium cladosporioides	41	26	67	0.61	0.39	0.64
Alternaria alternata	25	0	25	1	0	-
Alternaria infectoria	8	0	8	1	0	-
Alternaria tenuissima	8	0	8	1	0	-
Curvularia lunata	12	0	12	1	0	-
Phialophora sp.	6	3	9	0.66	0.33	0.50
Veronaea botryosa	2	0	2	1	0	-
Total	251	227	478	0.53	0.47	0.89

Table 3: Frequency of occurrence of phaeohyphomycotic agents recovered from soil and plant debris samples collected from the four governorates in the four seasons.

Fungal species	Number of isolates						
	Faiyum	Ismailia	Minufia	Qalyubia	Total	%	*P* value
Cladosporium sphaerospermum	56	72	67	74	269	56.3	<0.005
Cladosporium oxysporum	17	12	23	26	78	16.3	>0.05
Cladosporium cladosporioides	13	15	14	25	67	14.0	>0.05
Alternaria alternata	0	0	0	25	25	5.2	<0.001
Alternaria infectoria	0	8	0	0	8	1.6	<0.001
Alternaria tenuissima	0	8	0	0	8	1.6	<0.001
Curvularia lunata	0	0	0	12	12	2.5	<0.001
Phialophora sp.	4	1	2	2	9	1.9	>0.05
Veronaea botryosa	0	0	0	2	2	0.4	>0.05
Total	90	116	106	166	478	100	

Table 4: Seasonal variation of phaeohyphomycotic agents recovered from soil and plant debris samples collected in the four seasons from the four governorates.

Fungal species	Number of isolates						
	Winter	Autumn	Summer	Spring	Total	%	*P* value
Cladosporium sphaerospermum	74	96	36	63	269	56.3	<0.01
Cladosporium oxysporum	27	14	13	24	78	16.3	>0.05
Cladosporium cladosporioides	21	12	16	18	67	14.0	>0.05
Alternaria alternata	17	0	0	8	25	5.2	<0.001
Alternaria infectoria	8	0	0	0	8	1.7	<0.001
Alternaria tenuissima	8	0	0	0	8	1.7	<0.001
Curvularia lunata	12	0	0	0	12	2.5	<0.01
Phialophora sp.	2	0	2	5	9	1.9	<0.05
Veronaea botryosa	0	0	0	2	2	0.4	<0.05
Total	169	122	67	120	478	100	

There have been many reports of infection caused by black molds in healthy individuals and in immunocopromised patients, including an outbreak of fungemia in hospitalized patients (Silveira and Nucci 2001). For instance, *Veronaea botryosa*, was reported to be the causal agent of cutaneous phaeohyphomycosis in a heart transplant recipient in USA and in a liver transplant recipient in India (Sutton *et al*. 2004 and Foulet *et al*. 1999).

Also skin infections due to *Alternaria* in kidney transplant recipient have been reported by some investigators (Gallelli *et al*. 2006 and Farina *et al*. 2007).

The prevalent clinical features caused by *C. herbarum, C. sphaerospermum* and *C. cladosporioides* included, infection of skin (Tamsikar *et al*. 2006), corneal ulcer (Gugnani *et al*. 1978), brain abscesses (Kantarcioglu *et al*. 2002), pulmonary ball (Kwon Chung *et al*. 1975) and dental granuloma (Pepe and Bertolotto 1991).

Several dessiminated infections due to *Curvularia lunata* have been reported including, endocarditis, brain abscesses, skin infections, onychomycosis, keratitis, pneumonia, allergic bronchopulmonary, sinusitis (Rinaldi *et al*. 1987), peritionitis (Vachharajani *et al*. 2005) and optic atrophy (Smith *et al*. 2007).

In conclusion, there was a significant difference in the occurrence and diversity of phaeohyphomycotic agents recovered from soil and plant debris samples collected from public gardens located in the four studied governorates. The higher number of species was obtained from plant debris samples, while a higher diversity of the recovered species was observed in soil samples.

The distribution of these fungi in such habitats which are in close association with human activities supports that contact with such habitats is a risk factor for the infections caused by these fungal species. Therefore careful contact with such habitats especially for immunocompromised individuals must be controlled to avoid fungal infections.

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