JJBS

Jordan Journal of Biological Sciences

Volume 13, Number 2, June 2020
ISSN 1995-6673
Pages 197 - 205

Diversity and Antimicrobial Activity of Endophytic Fungi from the Medicinal Plant *Pelargonium graveolens* (geranium) in Middle Egypt

Manal M. Yasser, Marym A. Marzouk, Nadia M. El-Shafey and Salwa A. Shaban*

Botany and Microbiology Department, Faculty of Science, Beni-Suef University, Egypt

Received June 13, 2019; Revised July 25, 2019; Accepted July 28, 2019

Abstract

This study assesses the diversity and antimicrobial activity of the *Pelargonium graveolens*-associated endophytic fungi in Beni-Suef, Egypt. *P. graveolens* is a medicinal plant very common in Beni-Suef area and is used basically as a source of the expensive geranium oil. A total of 280 isolates belonging to 20 morphologically different fungal species have been isolated from root, leaf, and stem of *P. graveolens*. Identification of the isolates has been carried out by combining the morphological and molecular methods. Also, antifungal activity against plant and human pathogens has been tested using a dual culture method.

Results show that *P. graveolens* is extensively colonized by fungi that live inside it systematically at least during the test. The root exhibits the highest colonization frequency CF% and isolation rate IR% (96% and 99% respectively). All the isolates belong to the phylum ascomycetes, and phylogenetic analysis shows that they are relatively related genetically. The most occurring genera are *Aspergillus* and its teleomorph *Emericella* (CF=28%). Most of the isolates exhibit antifungal activity against one or more of the tested pathogens. The maximum inhibition rate is for *Emericella nidulans* (E6658) against *Microsporum audouinii* (80%). *Aspergillus niger* (E6657) and *Penicillium* sp. (E6651) show very strong activity against all the tested pathogenic fungi.

Keywords; Endophytic Fungi, Medicinal Plant, Molecular Identification, Phylogenetic Tree, *Pelargonium Graveolens*. Antifungal Activity.

1. Introduction

Endophytic microorganisms live inside plant tissues imperceptibly for at least part of their life cycle without any negative effect on their host (Kusari et al., 2012). Fungi are considered the most occurring endophytes (Staniek et al., 2008). They are highly diverse and polyphyletic and exist in all parts of the plants in any environment investigated till now (Yan et al., 2015). All plants form an endophytism relation with one or more fungi (Haddadderafshi, 2015).

Despite the new techniques, identification of fungi still depends on morphological features using light microscope examination. However, this may not be very accurate as it depends on the examiner taxonomic expertise. Besides, many endophytes grow in culture media as sterile mycelia (Ginting et al., 2013). Molecular identification is an effective way to identify the most related species. For fungi, this technique basically depends on the analysis of specific fragments of the ribosomal rDNA like 18S rDNA sequence or internal transcribed spacer (ITS), 28S subunit, and 5.8S rDNA sequence. ITS sequence analysis is widely used for the identification of endophytic fungi (Diaz et al., 2012). It was reported that molecular analysis of morphologically different isolates proved belonging them to the same species (Ginting et al., 2013). Endophytic fungi, therefore, are usually identified using a combination of morphological and molecular techniques (Zhang et al., 2006).

Plant-endophyte association is dependent on both partners (fungi and host plant) phylogeny and environmental factors. It was reported that endophytes from related host species are relatively phylogenetically related than those from unrelated plants. Moreover, endophytes from related hosts from different environments are found to be unrelated and unrelated plants from the same environment incorporate related species of endophytes (Arnold, 2007).

Endophytes have been reported to help plant in many ways. They may help to adapt environmental stress (adaptation benefits) like drought and salts stress or may help their host to resist invading by pathogens, insects, weeds or even herbivore (Rodriguez et al., 2009).

Endophytic fungi can produce wide varieties of bioactive secondary metabolites such as phenols, alkaloids, steroids, peptides, flavonoids, quinones, and terpenoids which may stimulate plant growth or used as therapeutic agents against plant and human diseases (Zhao et al., 2019). They also have been reported to produce one or

* Corresponding author e-mail: micro_sa2010@yahoo.com.

more of their host's metabolites. For example, endophytes isolated from the medicinal plant *Salacia oblonga*, which is known for its production of taxol (anticancer agents), was found to produce taxol by using genomic mining approach to detect two genes incorporated in the production of taxol (Roopa et al., 2015) and other several examples have been reported. Such studies led to discover new compounds and commercial production of these therapeutic compounds in large scale by the cheap and easy fermentation process.

The endophytic *Curvularia pallescens* (URM 6048) isolated from *Calotropis procera* has shown antagonistic effect against the plant pathogenic fungus *Colletotrichum dematium*, and against human pathogens *Staphylococcus aureus* and *Streptococcus pyogenes* (Nascimento et al., 2015). In a study on the diversity of endophytes isolated from *Opuntia humifusa* in the United states, six of the isolates was found to inhibit the plant pathogenic fungus *Phomopsis obscurans*

Studies on endophytic fungi and their antimicrobial activity help understanding their diversity, interaction with their host, and metabolites production. The diversity and antimicrobial activity of endophytic fungi associated with *P. graveolens* from Egypt environment have never been studied before.

Pelargonium graveolens (geranium) is a medicinal, aromatic plant dominant in Egypt, especially in Beni-Suef governorate. Its genera belong to the family Geraniaceae. *Pelargonium graveolens* has an abundance of positive benefits. These benefits include antibacterial, antifungal and antioxidant activity and others. Traditionally, the plant has been used to treat a variety of symptoms including: nephritis, wounds, fever, colds and sore throats, inflammation, heavy menstrual flow, hemorrhoids, dysentery, cancer gastrointestinal diseases, hyperglycemia, insomnia, heart disease, asthma, nausea and vomiting, fever and tuberculosis (Ćavar and Maksimović, 2012; Hsouna and Hamdi, 2012). It is mainly used to produce geranium oil. As only about 1 kg of geranium oil can be produced from 500 kgs of fresh weight (Centre, 2015), its production is very expensive. Studying its mutualistic communities may help in improving its production.

The aims of this study are to 1) assess the diversity of fungi isolated from the medicinal plant *P. graveolens* collected from Beni-Suef, middle Egypt, 2) investigate the genetic relation between the isolates and 3) evaluate the antifungal activity of the isolated endophytes against plant and human pathogenic fungi.

2. Materials and methods

2.1. Collection of the samples

Pelargonium graveolens samples were collected from 7 different fields in Beni-Suef governorate, Egypt. Sampled plants that were mature enough, healthy, and have no disease symptoms, based on the visual investigation of plant organs (leaves, stem, flowers and root), were chosen. Every sampled plant placed in a sterile bag and sealed well then labeled. Samples were preserved in the refrigerator until the next step for at most four days to avoid contamination.

2.2. Preparation of segments for isolation

Plants were washed carefully with tap water to remove mud and debris and let to dry on towels at room temperature. In the laminar air hood, the plant parts were cut into small pieces (about 1 cm) using sterile scalpels and numbered.

2.3. Sterilization of the samples' surfaces

The samples were surface sterilized using 75% ethanol for 1-3 minutes to remove wax and kill epiphytes followed by immersion in 4% sodium hypochlorite for 30-60 seconds for further sterilization depending on the structure of the tissues of the samples. Then the segments were rinsed with distilled sterile water three times and allowed to dry on sterile petri dishes containing sterile towels or filter papers.

2.4. Culturing

1-4 segments of a single part of the geranium plant (leaf, root, stem) were placed in a plate containing PDA media supplemented with 0.5 ml/l chloramphenicol and incubated at 25°C for 10 -20 days in the dark.

The effectiveness of the surface sterilization protocol was tested by imprint method of Schulz *et al.*, (Schulz, 1993) where a surface sterilized leaf segment was pressed and placed on the surface of a sterile PDA plate for 5 minutes then removed and the plate incubated with the other plates. Any appearing of growth indicate that the surface sterilization was not enough to eliminate epiphytes, and more sterilization period would be required.

Fungi that appeared to grow slowly from the inside plant tissues only were considered endophytes. While plates containing fast growing fungi and surface contaminates were discarded.

The isolated fungi were purified on antibiotic-free PDA media. A single spore of each fungal isolate was isolated and stored for further investigations.

2.5. Single spore isolation

Two drops of tween 80 were added to a tube containing 10 ml sterile distilled water and inoculated with the aerial mycelium of fungi from pure culture and shaken well. The suspension was then streaked by a sterile cotton swap on a thin film of PDA media in a petri dish and incubated at 25°C. After 24 h, the plates were examined for tiny hyphal growth arises from a single spore. The chosen germinating spore was cut off and transferred into another sterile PDA plate and incubated at 25°C for 5-10 days and used for preservation and subculturing for identification and further investigations. The isolates were stored in slants containing Czapecks media at 4°C.

2.6. Diversity of the isolated fungi

A number of segments colonized by endophytic fungi from each distinct tissue was recoded. Colonization frequency (CF%) and isolation rate (IR%) for each tissue also were calculated using the following equations (Wang et al., 2015) :

$$CF\% = \frac{\text{Number of segments colonized with isolated fungi}}{\text{Totall number of segments investigated}} \times 100 \quad (1)$$

$$IR\% = \frac{\text{Number of isolated fungi recovered from a tissue}}{\text{Totall number of the tissue segments investigated}} \times 100 \quad (2)$$

2.7. Microscopic identification of fungal endophytes

Isolates were identified based on the morphology of the fungi in the plates and direct microscopic examination of mycelium, conidia, fruiting bodies and spores using standard manual Moubasher, 1993 (Moubasher, 1993).

2.8. Molecular identification of fungal endophytes

The molecular identification was conducted in Microbiology and Applied Genomics Group, Institute of Chemical, Environmental & Bioscience Engineering Vienna, University of Technology, Austria. Genomic DNA was extracted from mycelia grown on 3% MEA (Malt Extract Agar) and incubated at 28°C then harvested after 2 days with the Plant DNeasy Minikit (QIAgen GmbH, Hilden, Germany) according to the manufacturer's instructions. A region of nuclear DNA containing the ITS1 and two regions of the rRNA gene cluster was amplified by PCR using the primer combinations SR6R and LR1 (White et al., 2014) as described by Kullnig-Gradinger et al.,(Kullnig-Gradinger et al., 2002). PCR products were sequenced. ITS sequences of the isolates were subjected to sequence similarity comparison of the sequences from the NCBI GenBank database (www.ncbi.nlm.nih.gov) using blast search. Sequences were aligned using the Clustal W program. The phylogenetic tree was built using MEGA X software (Kumar et al., 2018).

2.9. Calculation of the colonization frequency (CF%) of the isolates

$CF\%$ indicates the abundance of the isolates in their host plant and has been calculated for the isolates on the level of genera as described in equation (1).

2.10. Antifungal activity of the isolates

A total of 20 isolated endophytic fungi were screened for their antimicrobial activity against three plant pathogenic fungi (*Rhizoctonia solani*., *sclerotium rolfsii*, *Fusarium solani*) and against a human pathogen (*Microsporum audouinii*). Dual culture method presented by Morton and Stroube (1955) was used where seven-day-old pure cultures of endophytes and pathogens grown on PDA media were used in the experiment. A 5 mm disc from endophyte plate was cut by a sterile cork poorer and moved to one side of a sterile plate containing SDA (Sabouraud dextrose agar) media. Another disc of the pathogen was placed on the opposite side of the test plate. The control plate was done by replacing the endophyte disc by a sterile PDA disc (without mycelia). Plates were incubated in the dark at 27°C for seven days, then the radius of the growth of the pathogen has been measured at three dimensions, and their average was recorded as the radius of the growth. The activity of endophytes to inhibit the pathogenic fungal growth was expressed as the inhibition rate which was calculated as;

RP; is the radius of pathogen growth in the test plate.

RC; is the radius of pathogen growth in the control plate.

2.11. Statistical analysis of data

The results of inhibition rate were compared using one-way ANOVA and a Post Hoc-Tukey HSD test ($P<0.05$). The analyses were conducted using SPSS v.19.

3. Results

3.1. Isolation of endophytes

A total of 50 plants of Pelargonium graveolens were collected for the isolation of endophytic fungi from Beni-Seuf governorate in Egypt. The collection and isolation have done on 10 separated experiments. A total of 435 segments (150 leaf segments,150 stem segments, and 135 root segments) were inoculated on PDA media. Twenty morphologically different fungi have been successfully isolated and purified, as shown in Figure 1.

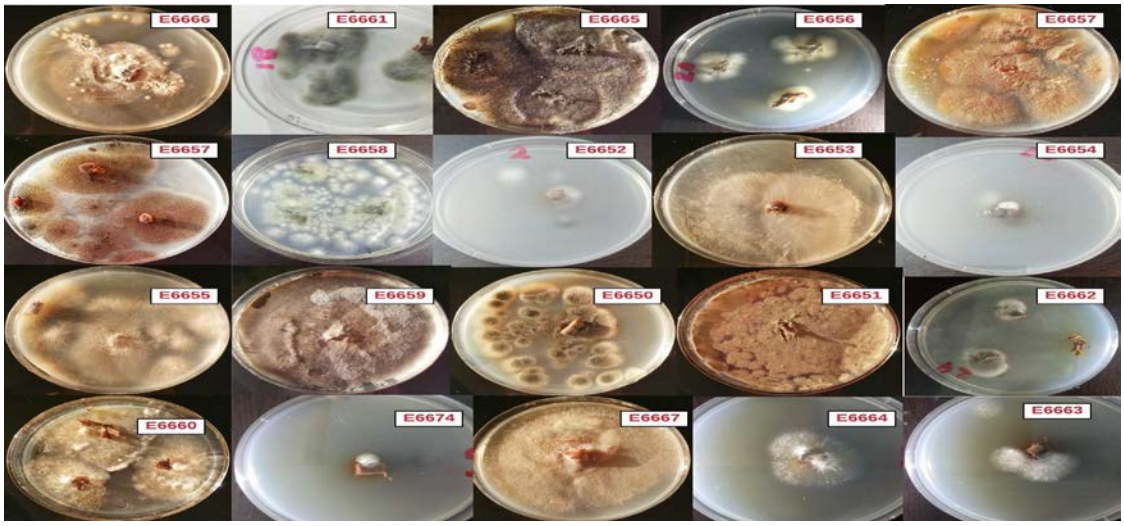

Figure 1. Different morphologically fungi isolated on PDA media

3.2. Morphological identification of the isolates

Twenty different taxa were identified depending on macro and micromorphology, including characteristics of colonies on plates, reverse colony color, pigment diffusion, sporophore and spore chain characteristics as presented in Table 1.

Table.1 List of the morphologically identified fungal species with their morphological features and the tissue source of isolation.

Code	Name	Morphological structure	Tissue
E6666	*Acremonium* sp.	White to cream- conidia one- celled	Stem
E6661	*Alternaria* sp.	Dark olive to black-conidia globose	Leaf
E6665	*Alternaria* sp.	Dark gray to black-conidia straight, elliptical- septate	Leaf
E6656	*Asperigillus* sp.	Golden yellow-coninial heat columner- smooth – conidia globose	Leaf-stem
E6675	*Asperigillus terrus*	Golden yellow to brown-conidial heads large-conidia globose rough	Root
E6657	*Aspergillus niger*	Dark black- conidia globose- conidiophores smooth	Root-stem-leaf
E6658	*Emericella nidulans*	Faint green- reverse grayed-orange-ascospores with no equatorial ridges	Leaf
E6652	*Fusarium* sp.	Pale pink-microconidia-globose	Root
E6653	*Fusarium* sp.	White to cream-microconidia-fusiform	Root
E6654	*Fusarium* sp.	Pale purple-macroconidia-	Root
E6655	*Fusarium* sp.	White buff -micro and macroconidia	Root
E6659	*Stemphylium* sp.	Dark grayed olive- smooth rounded conidia-conidiophore nodose-transverse and longitudinal septa	Root-stem
E6650	*Peicillium duclauxii*	Green- white margin-conidia elliptical-symmetrical	Root
E6651	*Penicillium corylophilum*	diffuse Dark green-conidia globose - asymmetrical- red pigment in the media	Stem
E6662	Black sterile mycelium	Dark black gray hyphal tip	Leaf
E6660	Sterile mycelium	Pale yellow white margin-sterile white mycelium	Leaf
E6664	Sterile mycelium	Cream color- sterile hypha	Root
E6674	Sterile mycelium	Light gray- white margin	Leaf
E6667	Sterile mycelium	Buff to cream	Leaf
E6663	*Ulocladium botrytis*	Dark gray, light gray margin- conidia verruculose-transverse septa	Root

* (the code of the isolates "E and numbers": E refers to endophyte, the numbers refer to the TUCIM No. [Collection of Industrially Important Microorganisms, Vienna University of Technology]).

Most of the isolates have recovered from a single organ except for *Stemphylium* sp. (E6659) which has been isolated from both leaf and stem of the plant and *Aspergillus* sp. (E6656) Which has been isolated from root and stem. *Aspergillus niger* (E6657) was found to recover from all plant tissues (leaf, stem, and root). All Fusaria species have been isolated only from the root of *P. graveolens*. 15% of the isolates have failed to produce spores in culture media.

Table.2 The colonization frequency (CR%) and isolation rate (IR%) of different tissues of Pelargonium graveolens.

Parameter	Leaves	Stem	Root	Total
No. of segments	150	150	135	435
No. of segments with isolates	70	58	129	257
No. of isolated fungi	87	72	133	292
colonization frequency (CF%)	47%	39%	96%	59%
Isolation rate (IR%)	58%	48%	99%	67%

Total of 257 isolates have been recovered from the investigated plants. Data in table. 2 show that root harbors most of the isolates with colonization frequency (CF)=96% and isolation rate (IR)=99%. Leaf tissues exhibit CF=47% and IR=58%. The stem shows the lowest CF% and IR% (39% and 48% respectively). Total plant tissues exhibit relatively high frequency (CF=59%) and richness of isolates (IR=67%). These results indicate that, *P. graveolens* is extensively colonized by endophytic fungi, specially the root of the plant.

3.3. Molecular identification of strains

Molecular analysis of the isolates has been carried out using ITS rDNA sequences to confirm the microscopic identification and study the phylogenetic relation between related species and the relation between all the isolates that colonize *p. graveolens* as endophytes. The most related taxa from GenBank and their accession number are listed in table 3. The score, E value, and query coverage and identity percentage are also displayed. Sequences that exhibited the highest identity and query coverage percentage have been chosen. From table 3, all chosen sequences exhibit at least 99% identity and 95% query coverage with one or more species or genera in the GenBank.

3.4. Phylogenetic analysis of the molecular data

Alignment of the sequences and construction of the phylogenetic tree have been carried out for choosing the most genetically related taxa to the isolates and understanding the genetic relation between the isolates. The sequences of the isolates and sequences chosen from the GenBank have been aligned using the Clustal W program. MEGA X software has been used to build the tree, using Neighbor- joining method (Saitou and Nei, 1987) with p-distance model and pairwise deletion of gaps.

A number of taxa that exposed to the analysis are 48 (20 query sequences of the isolates and 28 taxa with the closet sequences from GenBank) with a bootstrap of 500 replicates. The produced tree has 1822 sites and sum of branch length 1.1397. Branches with bootstrap value $\leq$ 50% is considered supported. The phylogenetic tree placed the isolated fungi in to 4 clades as shown in fig. 2.

All belong to the phylum Ascomycota. Clade (A) contains 6 query sequences and 9 from GenBank. All belong to the order Hypocreales with high branch support (100%) and divided into two subclades. One includes 5 fungi from the family Nectriaceae (E6652, E6654, E6653, E6655 and E6664) and all identified as *Fusarium* sp. with 100% support.

Table.3 Molecular identification of the isolates and the closest similar taxa from GenBank.

Isolates	Most closely related taxa from GenBank	Max score	Total score	Query cover (%)	E value	Identities (%)	Accession	Length
E6650	*Talaromyces* sp.	1016	1016	98	0	99.64	MG745311.1	579
	Talaromyces flavus var. *flavus* strain	1013	1013	100	0	99.12	MH860587.1	1124
	Penicillium pinophilum	1002	1002	98	0	99.11	HQ392503.1	588
E6651	*Talaromyces flavus var. flavus* strain	1020	1020	99	0	99.12	MH860587.1	1124
	Penicillium sp.	1007	1007	95	0	100.0	KX008642.1	560
E6652	*Fusarium oxysporum*	976	976	99	0	99.81	LT970803.1	1419
	Fusarium oxysporum	963	1073	97	0	100.0	KY587331.1	616
E6653	*Fusarium oxysporum*	970	970	99	0	99.44	LT746252.1	1407
E6654	*Fusarium oxysporum*	961	961	99	0	100.0	KX421425.1	1419
E6655	*Fusarium verticillioides*	983	983	97	0	100.0	MF682356.1	572
	Fusarium oxysporum	998	998	99	0	99.82	KU872849.1	1052
	Fusarium nygamai	998	998	99	0	99.82	HF546381.1	963
	Fusarium nygamai	983	983	97	0	100.00	KY039301.1	534
E6656	*Aspergillus terreus*	1086	1086	99	0	99.50	JX188057.1	1341
E6675	*Aspergillus terreus*	1079	1079	97	0	100.0	KY200574.1	608
E6657	*Aspergillus niger*	1062	1062	97	0	100.0	FJ668837.1	625
E6658	*Aspergillus sublatus*	1011	1011	99	0	99.64	KU866668.1	748
	Aspergillus quadrilineatus	1011	1011	99	0	99.64	NR_131289.1	587
	Emericella rugulosa	1011	1011	99	0	99.64	AB244780.1	590
	Aspergillus nidulans	1000	1000	96	0	100.0	MG459155.1	553
	Emericella nidulans	1000	1000	96	0	100.0	KC466534.1	567
	Aspergillus floriformis	998	998	96	0	100.0	KU866568.1	556
E6659	*Stemphylium vesicarium*	1026	1026	100	0	99.64	MG065799.1	1005
	Pleospora herbarum	1016	1016	98	0	100.0	KP334719.1	582
E6660	*Chaetomium madrasense*	985	985	95	0	100.0	MH864195.1	597
	Chaetomium ascotrichoides	985	985	95	0	100.0	MH861550.1	586
E6661	*Alternaria alternate*	1007	1007	100	0	100.0	MH221088.1	596
	Dothideomycetes sp.	1007	1007	100	0	100.0	KX908431.1	1074
	Alternaria tenuissima	1007	1007	100	0	100.0	KX664335.1	1110
E6662	*Alternaria alternate*	1003	1003	100	0	99.82	KY676196.1	865
	Dothideomycetes sp.	1003	1003	100	0	99.82	KX908365.1	1074
	Alternaria tenuissima	1003	1003	100	0	99.82	KX664322.1	1117
E6663	*Gilmaniella humicola*	904	904	94	0	100.0	MH855915.1	534
	Zopfiella longicaudata	878	878	92	0	100.0	KF811038.1	475
E6664	*Fusarium verticillioides*	983	983	97	0	100.0	MF682356.1	572
	Fusarium nygamai	983	983	97	0	100.0	KY039301.1	534
	Hypocreales sp.	981	981	97	0	100.0	GQ923973.1	558
	Fusarium oxysporum	998	998	99	0	99.82	KU872849.1	1052
E6665	*Alternaria alternate*	1009	1009	100	0	99.82	KX115416.1	678
	Dothideomycetes sp.	1009	1009	100	0	99.82	KX909052.1	1070
	Alternaria tenuissima	1009	1009	100	0	99.82	KX664335.1	1110
E6666	*Acremonium sclerotigenum*	990	990	97	0	100.0	MG980070.1	576
	Scopulariopsis sp.	989	989	97	0	100.0	KU523862.1	574
	Acremonium alternatum	989	989	97	0	100.0	KT192193.1	575
	Peniophora sp.	989	989	97	0	100.0	HQ607928.1	598
E6667	*Chaetomium subaffine*	1007	1007	97	0	100.0	MG770272.1	559
E6674	*Botryotrichum murorum*	1000	1000	97	0	100.0	MG770259.1	548

In the other subclade E666 fungus is placed near 2 different species of *Acremonium* (*A. alternatum* and *A. sclerotigenum*) with very strong bootstrap value (100%), E666 is defined as *Acremonium* sp. by combining the microscopic examination with the genetic analysis. Clade (B) includes 4 fungi belong to the family Caetomiaceae. E6663 is located near a sister clade containing two different genera (*Gilmaniella humicola* and *Zopfiella longicaudata*) with a very strong bootstrap value (98%) which has been denoted as unidentified genus as the

microscopic examination does not match with any of them. E6674 is defined as *Botryotrichum murorum* with 100% support. E6667 is *C. subaffine* with 89% support. E6660 locates in the same subclade with 2 different *Chaetomium* sp. so it is defined on the level of genera as *Chaetomium* sp. Cluster (c) involves 6 isolated fungi. All belong to Aspergillaceae family with 100% bootstrap value. In Clade (D), 4 fungal species belonging to the family Pleosporaceae, and are divided in to 2 subclades with 100% support. E6659 fungus is in the same subclade with 2 different genera (*Stemphylium vesicarium* and *Pleospora herbarum*), but it clustered in a sister clade with *Stemphylium vesicarium* with 60% bootstrap value which supports the morphological examination. E6665, E6662 and E6661 are in the same subclade with more than one Alternaria species. E6665 and E6662 are identified as *Alternaria alternate* with 63% and 75% support respectively, while E6661 fungus is identified as *Alternaria tenuissima* with 73% bootstrap value.

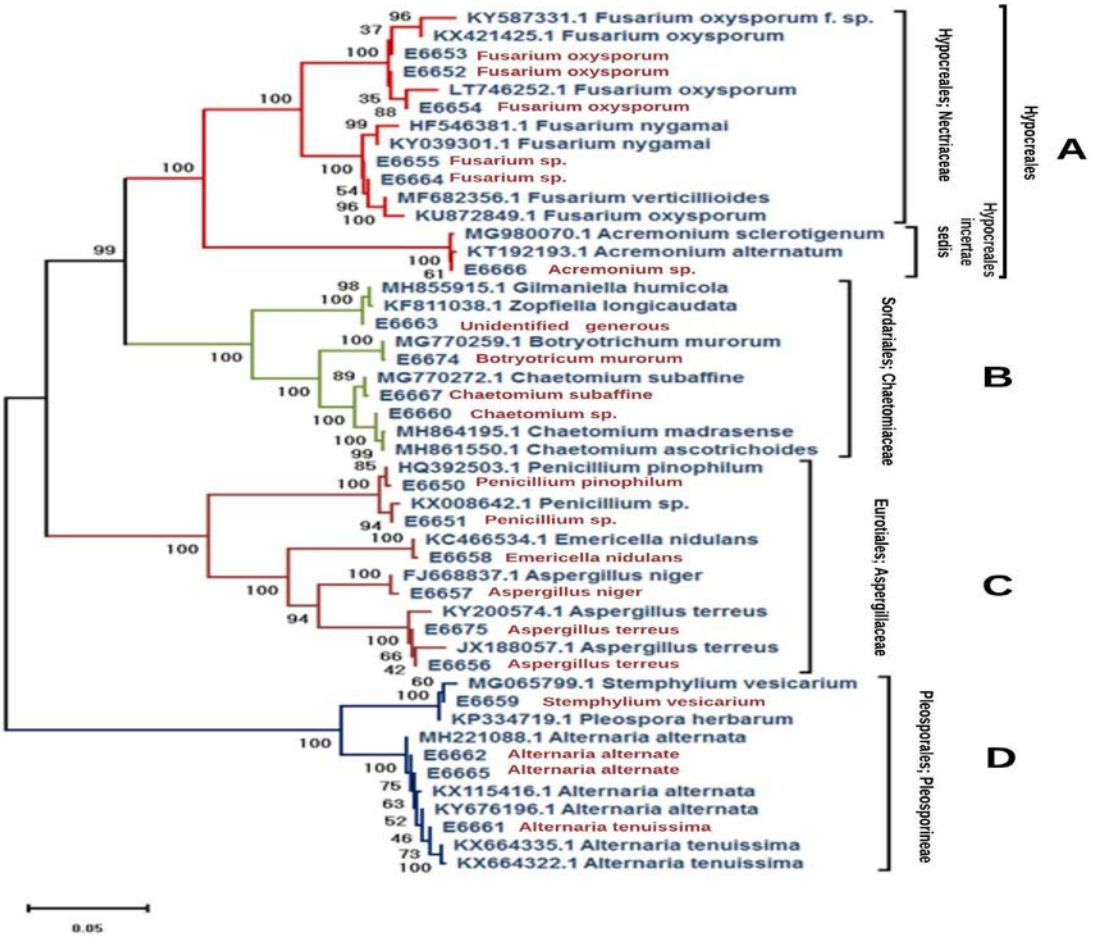

Figure 2. neighbor joining phylogenetic tree of the isolates with 500 replicates bootstrap

3.5. Colonization frequency of the isolated fungi

The colonization frequency indicates the abundance of the endophytes in their host plant. As shown in fig.3, Aspergillus and its teleomorph (Emericella) are the most occurring isolated genera with 28% colonization frequency followed by Fusarium 21%, then Penicillium 16%. Stemphlium and Alternaria have the same frequency 12%, whereas Chaetomium, Acremonium and botyotrichum sp. show low colonization abundance with frequency 4%, 3%, and 2% respectively. An isolate (E6663) could not be able to determine its genera and is represented as unidentified genus with low CF% (2%).

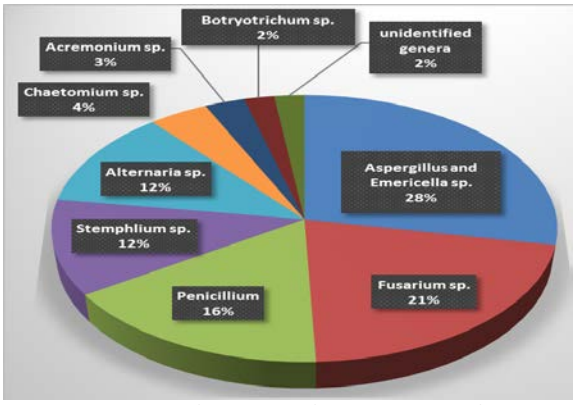

Figure 3. The CF% of the isolated fungi on the level of genera.

3.6. Antimicrobial activity of the isolates

Most of the fungi exhibit antagonistic activity against *Fusarium solani* with inhibition rate ranging from 60% for *Emericella nidulans* (E6658) to 4% for *Alternaria*

sp.(E6662). *Penicillium* sp.(E6651), *Aspergillus terreus* (E6675) and *Aspergillus niger* (E6657) are, also, very active against *F.solani* with inhibition rate 45%, 47 and 28% respectively. Five fungal isolates (*Alternaria alternate* (E6665), *Alternaria tenuissima* (E6661), *Fusarium* sp. (E6664), *Acremonium* sp. (E6666) and *Chaetomium subaffine* (E6674)) have negative inhibition rate indicating that they stimulate the growth of the tested pathogen fig.4(a)

Out of the 20 isolated fungi, only 4 fungal isolates exhibit antifungal activity against *sclerotium rolfsii*. *Aspergillus niger* (E6657) has a maximum inhibition rate (72%). Both *Penicillium* sp. (E6651) and *Chaetomium subaffine* (E6667) inhibit *S. rolfsii* by 50%, and *Emericella nidulans* (E6658) exhibits 31% inhibition rate against *S. rolfsii* fig.4(b).

Six fungal species exhibit antifungal activity against *Rhizoctonia solani*. The highest inhibition rate is recorded for *Aspergillus niger* (E6657), and the lowest rate is for *Aspergillus terreus* (E6656) (27%), and the rest of the tested fungi show no antimicrobial activity Fig.4(c).

As shown in Fig.4(d), most of the isolates exhibit antagonistic activity against *Microsporum audouinii*. *Emericella nidulans* (E6658) exhibits the highest rate of inhibition (80%). *Acremonium* sp. (E6666) shows no effect on the tested pathogen and *Chaetomium subaffine* (E6667) has negative inhibition rate, which reveals that it stimulated the growth of the pathogen.

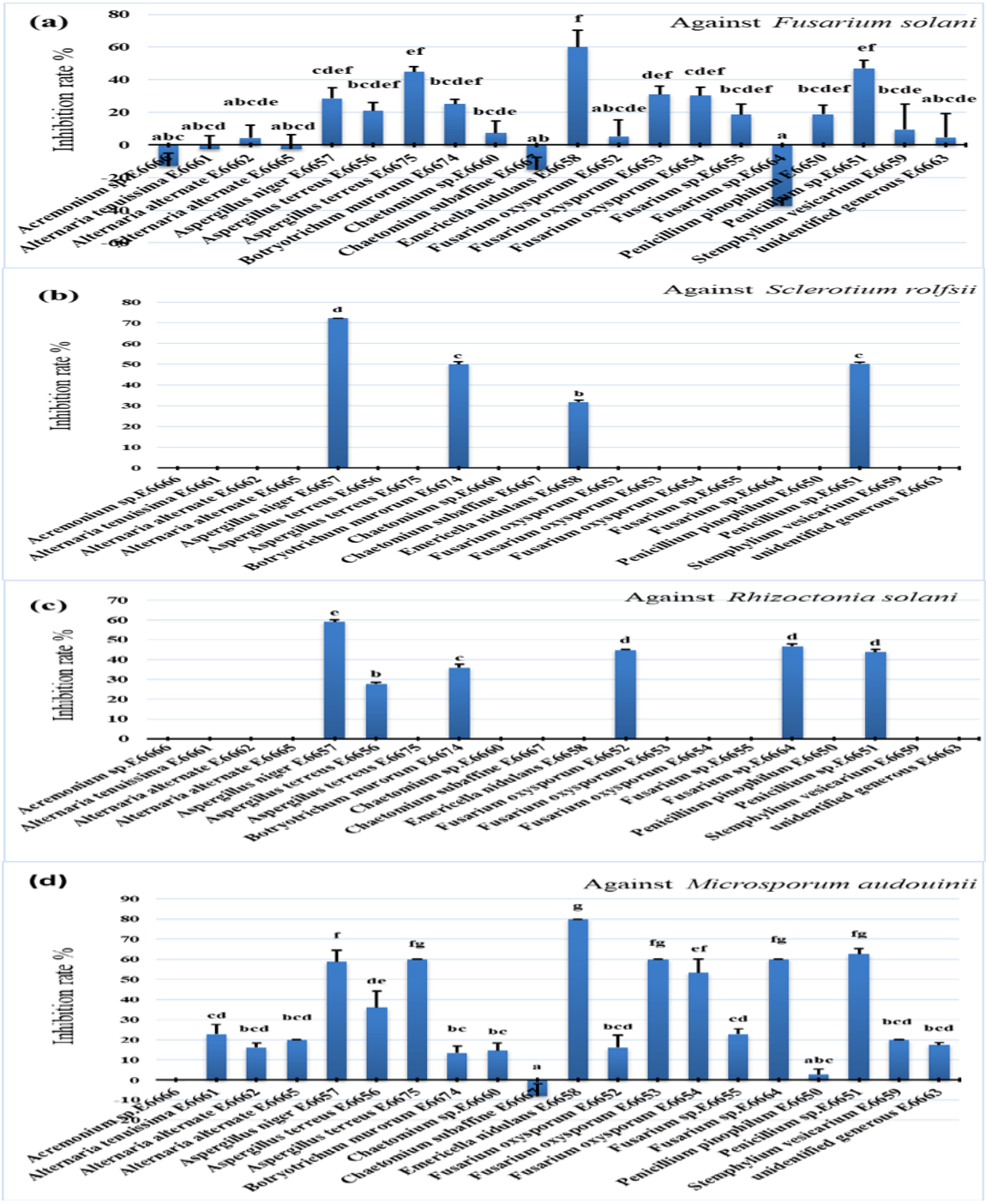

Figure 4. Inhibition rate IR% values of the isolated fungi against pathogenic fungi. Error bars represent the standard error SE± of three replicates. Significant differences between the mean values are represented as different letters on the bars (P ≤0.05) by Tukey HSD test.

4. Discussion

In the current study, a total of 274 fungi have been isolated from leaf, root, and stem of *P. graveolens* plant. Plant root exhibits the highest CF% (96%) and IR% (99%) than leaf and stem. These results match with the study of M. Manganyi, et al., who reported higher colonization rate of endophytic fungi in the root (28%) of Pelargonium sidoides than the leaf (25%) (Manganyi et al., 2018). This may be due to the exposure of the root to rich microbial community from the surrounding soil. Stem harbors the lowest number of isolates (CF=39% and IR=48%). The variation in the isolates diversity among plant organs may also be due to differences in the chemical composition of these organs (Huang et al., 2008). Composition of endophytic fungi community from different organs display a tissue-specific manner as most of the isolates were recovered from a specific organ while *Stemphylium* sp. (E6659) has been isolated from the leaf and stem of the plant. *Aspergillus* sp. (E6656) has been isolated from the root and stem. *Aspergillus niger* (E6657), also, was found to recover from all plant tissues (leaf, stem and root). All *Fusarium* species have been isolated only from the root of *P. graveolens*. This indicates that the isolated fungi may develop a specific relationship with their host plant organ. This finding is supported by many studies on endophytes. From a previous study on *Angelica sinensis*-associated endophytes, many fungi exhibited specificity to certain organ such as *Alternaria* sp. that recovered only from the leaf (JIANG et al., 2013).

The isolated fungi were grouped into 20 morphologically different fungi. Morphological identification has been able to determine most of the isolates genera and detect the sexual phase (teleomorph) if exist. Microscopic examination, also, were able to identify the species of some genera. As many isolated fungi were reported to fail to sporulate in culture media, Non-sporulated isolates have hampered their identification and classified as Mycelia sterilia. The identities of the isolated fungi have been confirmed by combining morphological with molecular identification. ITS region is one of the most frequently utilized tool to identify fungi at the species level. However, it has complications and cannot serve as the universal barcode of fungi (Diaz et al., 2012). In this study, ITS sequences of the isolates have been analyzed and subjected to sequence similarity comparison using blast search. In our study, molecular identification effectively identifies 9 fungi on the level of species with at least 99% identity and 95% query coverage, including fungi that are identified as Mycelia sterilia in microscopic examination.

In many cases, fungi exhibit similarity with more than one species or even genera or with the sexual and asexual phase of a fungus strain. A study on endophytic fungi isolated from *Glycine max* has faced the same complication (Fernandes et al., 2015). These cases were confirmed by microscopic examination. Some fungi that exhibit different morphological feature were identified as the same species in molecular analysis. Similar results were reported (Ginting et al., 2013). Therefore these isolates may differ at the sub- species level. Only one fungus could not be identified on the level of genera as it exhibits similarity with two different genera from GenBank and microscopic examination did not match one of them.

Calculation of the phylogenetic tree illustrates the close phylogenetic relation between the fungi exposed to the analysis. They are clustered into 4 clades. All belong to the phylum Ascomycetes and 4 orders Hypocreales (30%), Sordariales (20%), Eurotiales (30%) and Pleasporales (20%) and five families. All the isolate represents 10 genera: Fusarium, Aspergillus, Emericella, Acremonium, Chaetomium, Penicillium, Stemphylium, Alternaria, Botryoticum, and an unidentified genus. In many studies, most of these fungi isolated in high frequency as endophytes, especially from medicinal plants (Nair and Padmavathy, 2014).

Aspergillus and its telemorph Emericella are the most abundant isolated genera (CF=28%) followed by Fusarium (CF=21%) and Penicillium (CF=16%). Stemphylium and Alternaria exhibit the same CF value (12%). Other isolated genera show frequency values equal to or less than 4%. These results, however, cannot be compared with other studies as it depends on many factors including host and endophyte progeny, their chemical composition, other microbial communities associated with the plant and the environmental factors (Schulz and Boyle, 2006).

In the current study, all the isolated fungi exhibit antifungal activity against at least one of the tested pathogens except for *Acremonium* sp. (E6666) and *Chaetomium subaffine* (E6667). The maximum inhibition rate is for *Emericella nidulans* (E6658) (IR=80%) against *M. audouinii*. Many of the isolates exhibit strong activity against more than one of the tested pathogenic fungi. For example, *A. niger* (E6657) and *Penicillium* sp. (E6651) show very strong activity against the four pathogenic fungi. These results reveal that endophytic fungi isolated from *P. gravoelons* can be a source of bioactive metabolites, involving those able to control plant and human diseases(Chi et al., 2019).

5. Conclusion

The current study suggests that *P. gravoelons* plant is symbiotically associated with rich endophytic fungi community. Identification of these fungi is best done by combining morphological and molecular analysis. The diversity of these fungi varied among different organs. Some of the isolates exhibit a manner of specificity than the others. The isolated fungi are closely related, and all belong to Ascomycetes. Most of the isolates exhibit antifungal activity against some tested pathogens, which reveal that they may be a source of bioactive compounds against plant and human diseases.

Although using the ITS marker was valuable for species identification in this study other markers are recommended to offer more resolution to ITS and avoid the limitations of a single-marker barcoding system. These markers such as the largest (RPB1) and second largest (RPB2) subunits of RNA polymerase, translation elongation factor 1-alpha (tef1), beta-tubulin (tub2) and partial calmodulin (CaM).

Acknowledgments

Authors thank Marica Grujic and Dr. I. S. Druzhinina (the leader of the Microbiology and Applied Genomics Group), Microbiology and Applied Genomics Group, Institute of Chemical, Environmental & Bioscience Engineering Vienna University of Technology, Austria for their help during the molecular identification of isolates.

References

Arnold, A.E. 2007. Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. *Fungal Biology Reviews* **21 (2–3)**: 51–66.

Ćavar, S., and Maksimović, M. 2012. Antioxidant activity of essential oil and aqueous extract of Pelargonium graveolens L'Her. *Food Control* **23 (1)**: 263–267.

Centre, I. trade. 2015. The IFEAT socio-economic study of geranium oil. *IFEAT World http://www.intracen.org/uploadedFiles/intracenorg/Content/Exporters/Market_Data_and_Information/Market_information/Market_Insider/Essential_Oils/Geranium%20oil.pdf* (July): .

Chi, W.C., Pang, K.L., Chen, W.L., Wang, G.J., and Lee, T.H. 2019. Antimicrobial and iNOS inhibitory activities of the endophytic fungi isolated from the mangrove plant Acanthus ilicifolius var. xiamenensis. *Botanical Studies* **60 (1)**: .

Diaz, P.L., Hennell, J.R., and Sucher, N.J. 2012. Genomic DNA Extraction and Barcoding of Endophytic Fungi. *Springer Science+Business Media* **862 (862)**: 171–179.

Fernandes, E.G., Pereira, O.L., Silva, C.C. da, Bento, C.B.P., and Queiroz, M.V. de. 2015. Diversity of endophytic fungi in Glycine max. *Microbiological Research* **181**: 84–92.

Ginting, rohani cinta badia, Sukarno, N., Widyastuti, U., Darusman, latifah kosim, and Kanaya, S. 2013. Diversity of Endophytic Fungi from Red Ginger (Zingiber officinale Rosc.) Plant and Their Inhibitory Effect to Fusarium oxysporum Plant Pathogenic Fungi. *HAYATI Journal of Biosciences* **20 (3)**: 127–137.

Haddadderafshi, N. 2015. Diversity and antagonistic activity of endophytic fungi from sweet cherry and pepper. .

Hsouna, A. Ben, and Hamdi, N. 2012. Phytochemical composition and antimicrobial activities of the essential oils and organic extracts from pelargonium graveolens growing in Tunisia. *Lipids in Health and Disease* **11 (1)**: 1.

Huang, H., Rong, H., Li, X., Tong, S., Zhu, Z., Niu, L., and Teng, M. 2008. The crystal structure and identification of NQM1/YGR043C, a transaldolase from Saccharomyces cerevisiae. *Proteins* **73 (4)**: 1076–1081.

JIANG, S., QIAN, D., YANG, N., TAO, J., and DUAN, J. 2013. Biodiversity and Antimicrobial Activity of Endophytic Fungi in Angelica sinensis. *Chinese Herbal Medicines* **5 (4)**: 264–271.

Kullnig-Gradinger, C.M., Szakacs, G., and Kubicek, C.P. 2002. Phylogeny and evolution of the genus Trichoderma: A multigene approach. *Mycological Research* **106 (7)**: 757–767.

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. 2018. MEGA X : Molecular Evolutionary Genetics Analysis across Computing Platforms. **35 (6)**: 1547–1549.

Kusari, S., Hertweck, C., and Spiteller, M. 2012. Chemical ecology of endophytic fungi: Origins of secondary metabolites. *Chemistry and Biology* **19 (7)**: 792–798.

Manganyi, M.C., Regnier, T., Kumar, A., Bezuidenhout, C.C., and Ateba, C.N. 2018. Biodiversity and antibacterial screening of endophytic fungi isolated from Pelargonium sidoides. *South African Journal of Botany* **116**: 192–199.

Moubasher, A.H.. e. al. (1993). *Soil fungi in Qatar and other Arab countries*. The Centre for Scientific and Applied Research, University of Qatar.

Nair, D.N., and Padmavathy, S. 2014. Impact of endophytic microorganisms on plants, environment and humans. *The Scientific World Journal*.

Nascimento, T.L., Oki, Y., Lima, D.M.M., Almeida-Cortez, J.S., Fernandes, G.W., and Souza-Motta, C.M. 2015. Biodiversity of endophytic fungi in different leaf ages of Calotropis procera and their antimicrobial activity. *Fungal Ecology* **14**: 79–86.

Rodriguez, R.J., White Jr, J.F., Arnold, A.E., and Redman, R.S. 2009. Fungal endophytes: diversity and functional roles. .

Roopa, G., Madhusudhan, M.C., Sunil, K.C.R., Lisa, N., Calvin, R., Poornima, R., Zeinab, N., Kini, K.R., Prakash, H.S., and Geetha, N. 2015. Identification of Taxol-producing endophytic fungi isolated from Salacia oblonga through genomic mining approach. *Journal of Genetic Engineering and Biotechnology* **13 (2)**: 119–127.

Saitou, N., and Nei, M. 1987. The Neighbor-joining Method : A New Method for Reconstructing Phylogenetic Trees '. **4 (4)**: 406–425.

Schulz, B., and Boyle, C. 2006. What are Endophytes? 9: 1–14.

Schulz, H.J. 1993. HJ Schulz, Phys. Rev. Lett. 71, 1864 (1993). *Phys. Rev. Lett.* **71**: 1864.

Staniek, A., Woerdenbag, H.J., Kayser, O., Staniek, A., Woerdenbag, H.J., and Kayser, O. 2008. Endophytes : exploiting biodiversity for the improvement of natural product-based drug discovery Endophytes : exploiting biodiversity for the improvement of natural product-based drug discovery. 9145 (May): .

Wang, Y., Gao, B.L., Li, X.X., Zhang, Z. Bin, Yan, R.M., Yang, H.L., and Zhu, D. 2015. Phylogenetic diversity of culturable endophytic fungi in Dongxiang wild rice (Oryza rufipogon Griff), detection of polyketide synthase gene and their antagonistic activity analysis. *Fungal Biology* **119 (11)**: 1032–1045.

White, T.J., Bruns, T., Lee, S., and Taylor, J. 2014. Amplification and Direct Sequencing of Fungal Ribosomal Rna Genes for Phylogenetics. *PCR Protocols* (**January**): 315–322.

Yan, J.F., Broughton, S.J., Yang, S.L., and Gange, A.C. 2015. Do endophytic fungi grow through their hosts systemically? *Fungal Ecology* **13**: 53–59.

Zhang, H.W., Song, Y.C., and Tan, R.X. 2006. Biology and chemistry of endophytes. *Natural Product Reports* **23 (5)**: 753–771.

Zhao, X., Zhang, Y., Shi, J., Liu, Y., Lu, Y., and Lian, Z. 2019. Biosynthesis of antibacterial compound against multidrug resistant foodborne pathogens by Phomopsis sp. XP-8. *Food Control* 95 (April 2018): 223–231.