J. Plant Prot. and Path., Mansoura Univ., Vol.9 (9): 543 – 546, 2018

Isolation, Identification and Biological Activities of Endophytic Fungi from Egyptian Medicinal and Aromatic plants

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ABSTRACT

Eight endophytic fungi were isolated from three Egyptian plants (*Chenopodium album, Pelargonium graveolens* and *Malva parviflora*). The fungi endophytes were identified by morphological and molecular methods as *Fusarium chlamydosporum* MG786540, *Fusarium oxysporum* MG786541, *Alternaria alternata* MG786542, *Alternaria solani* MG786543, *Fusarium equiseti* MG786544, *Alternaria alternata* MG786545, *Stemphylium sp* and *Phoms* sp. The endophytic fungi isolates exhibited antagonistic activities to phytopathogenic fungi by dual culture bioassay. *A. alternata* (MG786545) caused 32.58% and 44.68% growth inhibition of *Fusarium oxysporum* and *Phytophthora infestance*, respectively. *F. equiseti* (MG786544) caused 34.64% growth inhibition of *Alternaria solani*. Spore suspension of *A. solani* (MG786543), *A. alternata* (MG786545) and *Stemphylium* sp endophytes were tested for their herbicidal activity and plant growth promotion. *A. solani* (MG786543) at10^8 spore/ ml inhibited 33.1% of seed germination and reduced 31.3%, 23.1% of shoot and root growth of *Lolium temulentum*, respectively. *Stemphylium sp* at10^8 spore/ ml had the ability to enhance *Triticum aestivum* growth by increasing shoot and root lengths, and fresh and dry weights comparing to control.

Keywords: Antagonism; Plant growth promotion; herbicidal; *Lolium temulentum*; *Triticum aestivum*

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INTRODUCTION

The expression endophyte is derived from the Greek language, "endon" means within and "phyton" means plant. There are many definitions of the term endophyte since De Bary (1866) defined endophytes for the first time as those microbes which reside inside the living healthy tissues of plants. But one of the most conclusive and widely accepted definitions of endophyte as 'microbes that colonize living, internal tissues of plant without causing any immediate overt negative effect' was given by Bacon and White (2000).

Almost all examined plants family contain endophytes in all parts of the plant, roots, stems, leaves, flowers and fruits (Arnold *et al.* 2000). Research studies on endophytes have increased during the last 30 years and demonstrated the beneficial of endophytes to plants by direct or indirect mechanism such as antagonistic activities against different microorganisms or by induction of plant defense mechanisms. Many endophytic fungi exhibited antagonistic effects against phytopathogenic organisms, increased plant resistance to diseases, promoted plant growth, increased nutrients uptakes and increased plant resistance to cold, to drought and to environmental stresses (Brem and Leuchtmann 2001, Gond *et al.* 2010, Redman *et al.* 2002 and Sturz *et al.* 2000). The use of microorganisms as biological control agents has a definite potential due to their different modes of actions from traditional chemical treatments.

Therefore, the objective of the present study is to isolate some endophytic fungi from Egyptian medicinal and aromatic plants that rich of bioactive compounds. The role of the isolated endophytic fungi in plant defense against phytopathogenic fungi and weeds and their capability to enhance host plant growth is also considered.

MATERIALS AND METHODS

Isolation of endophytic fungi

Healthy leaves of *Pelargonium graveolens* and *Chenopodium album* and roots of *Malva parviflora*collected from Abees farm, Alexandria, Egypt were washed by tape water and rinsed with distilled water. l3.0 % sodium hypochlorite (NaOCl) for 3 min. Sterilized samples were rinsed three times by sterile distilled water for 1 min each. Each sterilized sample was cut into small pieces (approximately 5 mm) and placed on potato dextrose agar Petri dishes containing 50 mg/l of ampicillin and incubated at 25± 2 °C. The tips of the endophytic fungi that grew out from the leaves were sub-cultured into other PDA plates to obtain pure culture examined by light microscope.

Morphological characterization and molecular Identification of endophytic isolates

The endophytic isolates were identified on the basis of morphological characters, for spore shape and type and by molecular identification using 18S rDNA. The isolated endophytic fungi were subjected into DNA extraction, using the Qiagen DNA extraction kit (Qiagen, Germany). These fungi were identified by amplification of ITS1-4 gene using universal primers. ITS 1 (Forward primer: 5` TCC GTA GGT GAA CCT GCG G 3`) and ITS 4 (Reverse primer: 5` TCC TCC GCT TAT TGA TAT GC 3`). 25µl of PCR reaction mixture contained 5µl master mix, 1µl forward primer, 1µl reverse primer, 1µl DNA Template and 17µl d. water. The PCR amplified products were analyzed by gel electrophoresis at approximately 600–700 bp Figure 1. The PCR amplicones were sequenced by LGC group (Berlin, Germany). The nucleotide sequences were deposited in National Center for Biotechnology Information database (NCBI nucleotide sequence databases). For the identification of the isolates, the nucleotide sequences obtained were compared with those sequences already deposited in the data bank of the National Center for Biotechnology and Information (NCBI) using the nucleotide Basic Local Alignment Search Tool (BLAST) to find the most closely related sequences. The identification of the species was determined based on the best sequence alignment score.

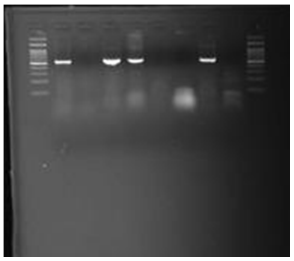

Fig.1. Gel electrophoresis shows (from right to left) DNA Ladder fragments of endophytic fungi; *F. equiseti, F. chlamydosporum, A. alternata* and *A. solani* respectively

***In vitro* antagonistic activity of endophytic fungi in dual culture**

Primary antagonism between endophytic isolates and phytopathogenic fungi (*Alternaria solani, Phytophthora infestance* and *Fusarium oxysporum*) were carried out by dual culture. All plates were incubated at 28±1ºCfor 5 to10 days until control plates were fully grown. The percent of growth inhibition (PGI) was calculated using the formula: PGI % = (KR-R1)/KR ×100, where KR is the growth average of three rays on the control dishes, and R1 is the ray from the point of inoculation to the colony margin in the direction of the antagonist (Korsten *et al.* 1995). The distance between the two fungal growths known as inhibition zone was noticed and recorded after 7 days.

Effects of endophytes on growth of *Lolium temulentum* and *Triticum aestivum* in pot experiment.

The endophytic fungi grown in PDA plates were flooded with sterile distilled water containing 0.05% Triton X- 100, scrapped with surface sterilized spatula and filtered through cheese cloth to remove mycelial debris. Three concentrations (10^4, 10^6, 10^8 spore/ ml) of spore suspension of each endophytic fungi were prepared and adjusted by hemocytometer. Seeds of *Triticum aestivum* and *Lolium temulentum* were sterilized by 0.1% sodium hypochlorite rinsed by distilled water and were sown in pots. Soil was treated with spore suspension concentrations by drenching 10 ml of each concentration for each pot with four replicates for each concentration. Plants were irrigated as needed and after 30 days the plants were uprooted to record germination, shoot and root lengths, fresh and dry weights.

Table 1. Antagonistic activities of endophytic fungi to phytopathogenic fungi

Endophytic fungi isolates	*F. oxysporum* PGI%	*P. infestance* PGI%	*A. solani* PGI%
A. alternata (MG786545)	32.58	44.68	10.25
A. alternata (MG786542)	14.28	14.28	18.03
F. chlamydosporum (MG786540)	23.27	26.82	29
F. oxysporum (MG786541)	11.11	32.60	-9.28
Phoma sp	20.23	26.17	16.96
Stemphylium sp	27.22	40.90	15.83
F. equiseti (MG786544)	18.98	30.23	34.64
A. solani (MG786543)	4.04	31.70	-5.36

PGI %: The percent of growth inhibition.

Statistical Analysis

Root, shoot lengths of tested plants were subjected to one-way analysis of variance followed by Student–Newman–Keuls test Costat (Cohort Software Inc. 1985) to determine significant differences between mean values at the probability level of 0.05.

RESULTS AND DISCUSSION

Morphological and molecular Identification of endophytic isolates

Endophytic isolates were identified under light microscope by their sporulation structures on PDA growth medium as *Alternaria* sp and *Fusarium* sp. The molecular identification of the species was determined based on the best sequence alignment score and is available under the accession numbers *Fusarium chlamydosporum* MG786540,*Fusarium oxysporum* MG786541, *Alternaria alternata* MG786542, *Alternaria alternata* MG786545 and *Phoma* sp isolated from *Chenopodium album*. *Fusarium equiseti* MG786544 and *Stemphylium* sp from *Malva parviflora*. *Alternaria solani* MG786543 from *Pelargonium graveolens*. The Phylogenetic tree of the isolated endophytic fungi showed in Figure 2.

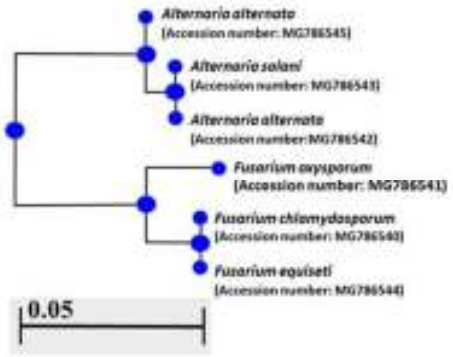

Fig. 2. Phylogenetic tree of endophytic fungi isolated from leaves of *Chenopodium album* and *Pelargonium graveolens* and root of *Malva parviflora*

Antagonistic activities of endophytic fungi by dual culture

Antagonistic activity between endophytic fungi and phytopathogenic microorganism might due to competition for space or nutrients as demonstrated through dual culture experiments. Our results revealed that all endophytic isolates exhibited moderate to weak antagonistic activity against phytopathogenic fungi *A. alternata, F. oxysporum* and *Phytophthora infestance* by dual culture bioassay. Isolate MG786545: *A. alternata* was the most antagonistic isolate to pathogenic fungus; *F. oxysporum* and *P. infestance* causing 32.58 and 44.68 % growth inhibition respectively. Isolate MG786544: *F. equiseti* had the most antagonist effect against *A. solani* causing 34.6% inhibition and it was characterized by inhibition zone as shown in figure 3. Ravely *et al* (2015) stated that *Lasiodiplodia theobromae* JF766989, endophytic fungi which isolated from *Piper hispidum* Sw reduced the growth of *A. alternata*, *Colletotrichum* sp., *Phyllosticta citricarpa and Moniliophthora perniciosa* by dual bioassay. Grazia *et al* (2007) found that *F.tricinctum* and *Trichoderma viride* showed significant antagonistic activity by 54 to 65% to *Drechslera corticola*.

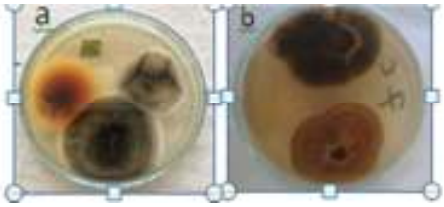

Fig. 3. Endophytic fungi emerging from leave tips (a), antagonism of *F. equiseti* (MG786544) against phytopathogenic fungi *A. solani* (b)

Effects of endophytic fungi on growth of *Lolium temulentum* and *Triticum aestivum* planted in pots.

Tables 2 and 3 showed the effects of the endophytic isolates on plant growth of both *L. temulentum* weed and *T. aestivum* crop. Endophytic isolates *A. alternata*-MG786545 and *A. solani*-MG786543 had the ability to reduce weed growth of *L. temulentum* but it had no significant effects on the growth of *T. aestivum*. *A. alternata*-MG786545 caused significant reduction in root growth of *L. temulentum* weed by 30.5 % growth inhibition using 10^8 spore/ ml. *A. solani*-MG786543 caused significant reduction in shoot growth of the weed by 34.3 % growth inhibition at 10^8 spore/ ml. Endophytic isolate *Stemphylium* sp significantly enhanced plant growth of wheat crop *T. aestivum* at 10^8 spore/ ml. But it had no significant effects on *L. temulentum* weed except for germination that was reduced to 31.1% at all tested concentrations. Fungi have been used as bio-herbicides in North America such as formulations of *Colletotrichum gloeosporioides* f.sp. malvae, used to control round leaf mallow (Mortensen 1988 and PMRA 2006, 2010).

Alternaria destruens, *Phytophthora palmivora* and *Phoma* sp are another example of registered fungus used as bioherbicide formulations (Kenney 1986, Neumann and Boland 1999, 2002 and Bailey 2014). Many researchers have been reported that endophytic fungi have the ability to improve growth of their host plant by various growth mods. Ernst *et al.* (2003) concluded that when three endophytic species of *Stagonospora* isolated from *Mentha piperita* were re-inoculated into axenic host seedlings of *Phragmites australis*, all increased plant growth significantly. This improvement of host plant growth might be due to synthesis of plant growth hormones by endophytes or solubilizing or mobilizing insoluble minerals to their host plants or to their hydrolytic capabilities which enable endophytes to penetrate plant tissue and establish symbiotic relationship with host plant (Mucciarelli *et al* 2002, 2003, Lin *et al* 2013, Khan *et al* 2015 and Saad El-Din Hassan 2017). In conclusion, the present study revealed that endophytic fungi can have numerous benefits to host plants. *F. equiseti* MG786544 had antagonistic activity against phytopathogenic fungi while *A. alternata* MG786545 and *A. solani* MG786543 showed herbicidal activity and *Stemphyllium* sp promoted wheat growth;. So endophytic fungi represent a group of microorganisms that might be utilize as bio-control agent to control plant pathogens and/or to improve plant growth.

Table2. Herbicidal activity of endophytes on *Lolium temulentum* (Avena sativa) 30 d after sowing[a]

Fungal Isolates	Conc. (spores/ ml)	GI (%)[c]	Shoot length (cm)	GR (%)[d]	Root length (cm)	GR (%)	Fresh weight (gm)	Dry weight (gm)
Control	-	-	11.1±.95b[b]	-	4.03±0.46b	-	0.023	0.003
A. alternata (MG786545)	10^4	1.8	10.5±1.8b	2.8	3.3±1b	18.1	0.018	0.002
	10^6	-1.1	11.3±0.5b	-4.6	3.3±0.44b	18.1	0.025	0.003
	10^8	1.1	9.7±0.97b	10.2	2.8±0.17a	30.5	0.023	0.003
Stemphylium sp	10^4	31.1	11.3±0.88b	-4.6	6.2±1b	-54.1	0.017	0.003
	10^6	31.1	10.0±0.57b	2.8	4.5±0.17	-56.3	0.018	0.003
	10^8	31.1	12.8±1.3b	-18.5	4.8±0.23b	-19.1	0.023	0.003
A. solani (MG786543)	10^4	0	11.7±1.14b	-8.3	4.7±0.6b	-20.1	0.026	0.003
	10^6	22.1	9.1±1.24b	15.7	3.1±0.47b	23.1	0.025	0.003
	10^8	33.1	7.1±0.38a	34.3	3.1±0.46b	23.1	0.021	0.003

[a] Data are expressed as means ±SE from experiments with four replicates of 4 plants each. [b] Means within a column with the same letter are not significantly different at the 0.05 probability level. [c] Germination inhibition percent. [d] Growth reduction percent

Table 3. Effect of endophytic fungi on *Triticum aestivum* germination, shoot growth, root growth, fresh weight and dry weight 30 d after sowing[a]

Fungi Isolates	Conc. (spores/ ml)	% Germination	Shoot length (cm)	Root length (cm)	Fresh weight (gm)	Dry weight (gm)
	Control	87.5	26.7±0.9b[b]	12.6±0.29b	0.331	0.034
A. alternata (MG786545)	10^4	87.5	28.4±1b	11.1±0.8b	0.471	0.043
	10^6	83.5	28.7±1b	11.7±0.17b	0.408	0.043
	10^8	81.5	28.9±0.55b	13.1±0.32b	0.467	0.044
Stemphylium sp	10^4	90.0	26.6±1.1b	11.5±0.65b	0.394	0.038
	10^6	88.3	26.8±0.73b	11.7±1b	0.414	0.043
	10^8	89.3	29.8±0.5a	15.6±0.9a	0.496	0.051
A. solani (MG786543)	10^4	81.25	27.1±2b	13.3±0.6b	0.461	0.047
	10^6	81.25	26.7±1.9b	12.4±1.3b	0.456	0.047
	10^8	93.75	29.0±0.8b	15.0±0.8b	0.479	0.050

[a] Data are expressed as means ±SE from experiments with four replicates of 4 plants each.
[b] Means within a column with the same letter are not significantly different at the 0.05 probability level

ACKNOWLEDGMENTS

I am very grateful to Dr. Doa Komeil and Mr. Ali A. Saeed for their helpful and kind support during this work.

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عزل والتعرف والفعالية البيولوجية للفطريات الداخلية بالنبات Endophytic Fungi المعزولة من نباتات طبية وعطرية بمصر

منى منصور جبريل سعد ، قسمة مسعود علي وأدهم السيد قرنه

قسم كيمياء المبيدات – كلية الزراعة (الشاطبى)- جامعة الأسكندرية – الأسكندرية – مصر

تم عزل ثمانية من الفطريات المتواجدة داخل النبات والمعروفة بـ endophytic fungi من ثلاث نباتات طبية وعطرية من البيئة المصرية وهي العتر البلدي, الزربيح والخبيزة. وتم التعرف علي العزلات بالطرق المورفولوجية morphological باستخدام الميكرسكوب الضوئي ودراسة شكل وحجم الجراثيم المتكونة كما تم التعرف لـ6 فقط علي المستوى الجزيئي molecular بعمل DNA sequencing لناتج purified PCR وتم تسجيله في GenBank واخذ رقم accession number وهي كالتالي: *Fusarium chlamydosporum* MG786540, *Fusarium oxysporum* MG786541, *Alternaria alternata*MG786542, *Alternaria solani* MG786543, *Fusarium equiseti* MG786544, *Alternaria alternata* MG786545, *Stemphylium* sp and *Phoms* sp المعزولة ضد الفطريات endophytic fungi تم إجراء التجارب المعملية *in vitro* لدراسة الفعلية البيولوجية لـ الممرضة للنبات وكذلك تجارب بالصوبة لدراسة تأثيرها لتحسين نمو المحاصيل وكذلك نشاطها كمبيدات حشائش . أظهرت نتائج تجارب التضاد antagonism لهذه العزلات بطريقة dual culture لنمو الفطريات الممرضة *Alternaria solani, Phytophthora infestance* and *Fusarium oxysporum* تثبيطها لنمو الفطريات الممرضة بدرجات متفاوته حيث سببت عزلة *A. alternata* (MG786545) تثبيطا بنسبة 32.58% و 44.68% لنمو كلا من *F. oxysporum* و *P. infestance* على الترتيب. أما عزلة *F. equiseti* (MG786544) فهي الوحيدة التي أظهرت قدرة على تثبيط نمو *A. solani* بنسبة 34.64%. أما نتائج تجارب تأثير معلق الجراثيم على انبات ونمو الحشائش (حشيشة الصامة *Lolium temulentum*) وكذلك تحسين نمو المحاصيل (القمح *Triticum aestivum*) تم اجرائها لـ 3 فقط من العزلات التي أظهرت قدرة عالية على تكوين الجراثيم. ب تم زراعة البذوربتربة معقمة والمعاملة بتركيزات مختلفة لمعلق جراثيم *Stemphylium sp* (MG786543), *A. alternata* (MG786545) and *A.solani* أظهرت النتائج تثبيط عزلة *A. solani* (MG786543)عند تركيز spore/ ml 10^8النمو الخضري والجذري لحشيشة الصامة بنسبة 23.1%, 31.3% على الترتيب. أظهرت عزلة *Stemphylium* sp عند تركيز spore/ ml 10^8تأثير ا ايجابيا على نمو المجموع الخضري والجذري لنبات القمح بنسبة 10% و 19% وكذلك زيادة الوزن الرطب والجاف بنسبة 33% مقارنة بالكونترول.وتؤكد هذه الدراسة الأولية على أهمية هذه الفطريات الداخلية endophytes للنبات العائل ومدي تنوعها وكيفية اختلاف دور وفائدة كل منها. تعتبر هذه الدراسة تمهيدا لدراسات متخصصة والمتمثلة في الميكانيكيات البيوكيميائية التي تقوم بها الفطريات الداخلية endophytes والمفيدة للنبات العائل. fungi