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Endophytic fungi of three leguminous plant roots in Egypt

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Abstract: Seventy-eight species, in addition to six varieties belonging to twenty-one genera of endophytic fungi were isolated and identified from the roots of three leguminous plants (peanut, alfalfa and broad bean) on PDA and water agar at 28±2˚C. *Fusarium* (15 species), *Penicillium* (16 species) and *Aspergillus* (12 species and 5 varieties) were the dominant genera of which *F. solani, F. subglutinans, F. oxysporum, P. duclauxii, P. funiculosum, A. tubingensis* and *A. flavus* were the most prevalent. The endophytes of peanut and alfalfa were rich in fungal counts (229 & 209 and 230 & 188 colonies/20 samples on PDA and water agar respectively) compared with broad bean (138 & 102 colonies). All isolated fungi belong to Ascomycota and Deuteromycetes.

Key words: Endophytes, peanut, alfalfa, broad bean, roots.

Introduction

The legumes are second to cereal crops in agricultural importance based on area harvested and total production. The legume family (Fabaceae) is the third largest family of higher plants (Anuradha *et al*. 2006). Seeds of legumes provide about one third of all dietary protein nitrogen and one-third of processed vegetable oil for human consumption (Graham and Vance 2003).

Peanut (*Arachis hypogaea* L.) is an important cash crop of farmers particularly in the semiarid tropics (Iqbal *et al*. 2011). In Egypt, it is grown in 589740 ha mainly for direct consumption than for oil extraction (Ahmad and Mohamed 2009). In addition, peanuts are a rich source of edible oil (43-55%), and protein (25-28%) (Gohari and Niyaki 2010). Also, it contains 20% carbohydrate and 5% fiber and ash which make a substantial contribution to human nutrition (Ahmad and Rahim 2007).

Alfalfa (*Medicago sativa* L.) is a widely cultivated, environmentally tolerant forage crop. It is grown in Egypt in arid and semiarid regions, and provides high quality forage and green manure (Kamel and Shoukry 2001). It is famous for its excellent nutritive value, high digestibility and a high biomass yield (Stajkovic-Srbinović *et al*. 2012). Alfalfa contributes to the incorporation of nitrogen in agriculture systems, with a consequent economic benefit, helping to reduce the application of synthetic N fertilizers (Jensen and Hauggaard-Nielsen 2003).

Broad bean (*Vicia faba* L.) sometimes referred to as horse bean or field bean. It is the fourth important pulse crop in Egypt and many countries. It occupies the greatest area planted to legume crops in the Arab countries (Amin 1988). Nutritionally, mature seeds of broad bean are a good source of protein (about 25% in dried seeds), starch, cellulose, vitamin C and minerals (FAO 1995 and Hamilton 2005). Also, it is an excellent candidate crop to provide nitrogen input into temperate agricultural systems; moreover, it makes a significant contribution to soil fertility restoration as a suitable rotation crop that fixes atmospheric nitrogen (Samuel *et al*. 2008).

Fungal endophytes are microfungi that colonize living tissues of plants without producing any apparent symptoms or obvious negative effects (Hirsch and Braun 1992). Many endophytes produce unusual secondary metabolites of industrial importance (Hawksworth *et al*. 1995). They have a protective role against insect herbivory and many are producer of novel antimicrobial secondary metabolites (Arnold *et al*. 2003, Malinowski and Belesky 2006 and Shiomi *et al*. 2006). Additionally, endophytic fungi are a fascinating source of new natural products which are of great potential for medicinal and agricultural applications (Aly *et al.* 2010 & 2011). Also, endophytic fungi represent an important and quantified component of fungal biodiversity, and are known to affect on diversity and structure of plant community (Gonthier *et al.* 2006 and Krings *et al*. 2007).

The current work aimed for studying the diversity (frequency and counts) of endophytic fungi associated with roots of three leguminous plants in Egypt.

Materials and Methods

Collection of plant samples

Roots of three economical plants (*Arachis hypogaea*, *Medicago sativa* and *Vicia faba*) were collected during the flowering stage of each plant, from different locations in Sohag Governorate. The plants (20 samples each) were chosen to isolate endophytic fungi. The collected plant materials were stored in separate plastic bags at 4°C in an ice box until isolation of fungi (Strobel and Daisy 2003).

Fungal isolation and identification

Isolation of endophytic fungi was done according to the method described by Hallmann *et al.* (2007) with minor modifications. The plant roots were rinsed gently in running water to remove adhered dust and debris. Samples were surface sterilized with 75% ethyl alcohol for 1 min, soaked in 5% sodium hypochlorite solution for 3 min, and then rinsed with 75% ethyl alcohol for 30 sec. They were finally rinsed with sterile distilled water and dried between sterilized filter papers under laminar air flow chamber and the roots were cut into segments (1 cm).

Forty sterilized segments from each root were placed on both PDA (potato extract, 200 ml; glucose, 20.0 g; agar, 15.0 g; distilled water, to 1.0 L) and WA (agar, 15.0 g; distilled water, to 1.0 L) media. The plates (4 plates for each medium type, 5 segments for each) were incubated at 28°C for 15 days and were periodically observed for fungal growth. The growing fungi were then sub-cultured on PDA and glucose-Czapek's agar medium plates (glucose, l0.0 g; $NaNO_3$, 2.0 g; KH_2P0_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; KC1, 0.5 g; $FeSO_4.7H_2O$, 0.01 g; agar, 15.0 g; distilled water, to 1.0 L; pH, 6.5-7) for purification and identification purposes. The endophytic fungal isolates were identified microscopically on the basis of their critical morphological structures such as hyphal features, arrangement of spores and reproductive structures (Ellis 1971 & 1976, Raper and Fennell 1965, Pitt 1979, Nelson *et al*. 1983, Moubasher 1993 and Leslie and Summerell 2006). Isolates that failed to produce reproductive structures after 3-4 weeks of incubation were referred to as sterile mycelium, and divided into their color (Lacap *et al*. 2003).

Results and Discussion

In the current study, endophytic fungi were isolated from roots of three economic plants. Most isolates were recorded in the first two weeks of incubation. These results correspond with other results obtained for the rate of isolation of endophytic fungi from other hosts (Abdel-Wahab 2000 and Shebany 2008).

Seventy-eight species and six varieties which belong to twenty-one genera were isolated and identified from 60 plant samples of peanut, alfalfa and broad bean roots (each, 20 samples) on PDA and WA media at 28±2˚C (Tables, 1 & 2). Most species isolated belong to genera which have already been described as endophytes (Abdel-Wahab 2000, Shebany 2008 and Nath *et al*. 2012). The endophytic fungi belong to Ascomycetes and Deuteromycetes. The authors who worked in this field indicated that members of the Ascomycotina and Deuteromycotina have been isolated as endophytes (Petrini 1986, Siegal *et al*. 1987, Clay 1991 and Abd-Elaah and Soliman 2005).

A total of 1096 isolates were isolated from the three plants using PDA (597 isolates) and WA (499 isolates); (229 & 209 from peanut, 230 & 188 isolates from alfalfa and 138 & 102 from broad bean on PDA and WA respectively). The differences in the number of isolates rely on the nature, age and other factors of the plants. Hoff *et al*. (2004) mentioned that endophytic fungi usually occur in above ground plant tissues but, are also found in root. Unlike mycorrhizal fungi, fungal endophytes of roots lack extra radical (outside the root) hyphal networks and mantles (sheaths around the roots).

Of the three economic plants studied, the most frequently-occurring genera were *Fusarium*, *Aspergillus* and *Penicillium* in the counts (7.25-44.35%, 8.5- 22.5% and 8.5- 45% of total fungi) and in the number of cases of isolation (30- 95%, 30-80% and 40-90% of the total samples in both PDA and WA media, respectively). These genera were previously isolated as endophytic fungi by several researches from different plants such as *Fraxinus excelsior*, *Gossypium* sp., *Gynoxis oleifolia*, *Manilkara bidentata*, *Picea abies* and *Taxus* sp. (Fisher *et al*. 1995, Redlin and Carris 1996, Strobel *et al*. 1997, Caruso *et al*. 2000 and Wang *et al*. 2007), twigs of *Kandelia candel* and *Avicennia marina* (Abdel-Wahab 2000), different parts of *Althea rosea*, *Calotropis procera* and *Nerium oleander* (Shebany 2008) and roots, stems and leaves of *Hyoscyamus muticus* (Abdel-Motaal *et al*. 2010).

Fusarium (13 species) was the most common genus regarding the number of cases of isolation and total fungal count from peanut and alfalfa, (each, 95% of the samples and 28.4 and 44.35% of total fungi respectively) on PDA, and 75 and 80% of the samples and 22.5 and 34.6% of total fungi, respectively on WA medium (Table 1). These results are in agreement with those obtained by Shebany (2008) who reported that *Fusarium* was the most common genus based on number of cases of isolation and fungal count from *Althea rosea*, *Calotropis procera* and *Nerium oleander*. *Fusarium* spp. have been recorded as endophytes from *Amomum siamense* (Bussaban *et al*. 2001) and was the most dominant genus in *Dracaena cambodiana* and *Aquilaria sinensis* (Jiang *et al*. 1995 and Tian *et al*. 2004). In the present investigation, *F. solani*, *F. subglutinans*, *F. oxysporum*, *F. nygamai* and *F. anthophilum* were the dominant species recovered from the three economical plants (5-55% and 5-45% of the samples, and 0.48-13.4% of total fungi on both PDA and WA media respectively). Most of these species were recovered by Shebany (2008) from *Althea rosea* and *Nerium oleander*. Weber *et al*., (2007) isolated *F. solani* from healthy leaves of *Quercus ilex* as endophytic fungus on 2% malt extract agar medium and used extract of this fungus against pathogenic and non-pathogenic yeasts and filamentous fungi.

Aspergillus was the second most prevalent genus based on the counts from the three plant roots (Table 1). Caruso *et al*. (2000) recovered *Aspergillus* spp. from woody and herbaceous tissues of *Taxus* sp. The genus was represented by 12 species in addition to 5 varieties of which the most dominant species were *A. tubingensis* and *A. terreus* in the 3 plants. They accounted for 0.87-5% and 2.7-4.9% on PDA and WA respectively. The previous species were recorded in high quality and quantity from *Citrus limon*, *Ocimum basilicum*, *Morus rubra* and *Psidium guajava* (Mohammed 2010).

Penicillium was isolated from the three plants and ranked first in frequency of occurrence (90% of the samples) in broad bean comprising 45% of total fungi and third in peanut and alfalfa (70 and 55% of the samples and 17.9 and 11% of total fungi respectively). The above genus was previously isolated by several researchers (Sinclair and Backman 1989, Fisher *et al*. 1995, Caruso *et al*. 2000, Ananda and Sridhar 2002 and Sasaki *et al*. 2005). *Penicillium* spp. have been commonly recorded as endophytes from leaves and roots of various hosts such as soybean leaves (Larran *et al*. 2002), roots of *Alnus glutinosa* (Cappellano *et al*. 1987 and Fisher *et al*. 1991), *Picea marina*, *Sorbus* spp. (Suryanarayanan *et al*. 2000) and from *Amomum siamense* (Bussaban *et al*. 2001). Sinclair and Backman (1989) recovered *Penicillium* sp. from surface-disinfested soybean seeds. Caruso *et al*. (2000) isolated *Penicillium* sp. from herbaceous tissues of *Taxus* sp. Of the genus, 16 species were collected and identified of which *P. funiculosum* and *P. duclauxii* were the most predominant species in the 3 plant roots (Table 1). The former species was recovered from three medicinal plants as an endophyte (Shebany 2008). Also, Mohammed (2010) isolated *P. duclauxii* from leaves of *Ocimum basilicum* and *Psidium guajava* which comprised 15.6 and 3.3% of total fungi and 15 and 5% of the samples, respectively.

Four genera, namely *Cylindrocarpon* (2 species), *Humicola* (2), *Drechslera* (6) and *Scopulariopsis* (2) were recorded in the 3 tested plants in low counts (0.72-14% of total fungi) and frequencies (5-50% of the samples). Also, four genera were observed in two plants in low counts (0.43-3.9%) and frequencies (5-20%) and these were *Cladosporium* (2 species), *Alternaria* (4), *Macrophomina* (1) and *Curvularia* (4) on PDA (Table 1).

On the other hand, *Cylindrocarpon* was recorded in all plants tested with moderate counts (9.8-19.6% of total fungi) and frequencies (20-45% of the samples), while, *Humicola*, *Drechslera* and *Macrophomina* were observed in two plants with low counts (0.96-9% of total fungi) and frequencies (5-40% of the samples) on WA medium (Table 1). These species were recovered as endophytic fungi from many plants (Rubini *et al*. 2005, Ganley and Newcombe 2006 and Weber *et al*. 2007). *Cladosporium* sp. was collected from 30 trees samples throughout the trees limited range in Northern Florida (Lee *et al*. 1995). Caruso *et al*. (2000) isolated *Alternaria* from woody tissues and herbaceous tissues of *Taxus* sp. In particular, *Alternaria* was isolated from all the analysed plant materials and can be considered a resident genus of *Taxus* tissues.

Sterile mycelia were observed in high diversity of colour (1.3- 8.2% and 3.4-10.1% of counts on PDA and WA respectively) from the three plant roots tested, where alfalfa had the best frequencies and counts (65 and 55% of the samples and 8.2 and 10.1% of the counts on PDA and WA respectively) as shown in Table (1). Brown or blackish sterile fungi isolated from conifer roots were referred to as *Mycelium radicis atrovirens* Melin (MRA) (Melin, 1922 & 1923), but very little is known what comprises MRA, because the name has since been applied to any sterile, dark and septate fungus isolated from roots or soil (Jumpponen and Trappe 1998). Shebany (2008) recovered sterile mycelia from different organs of *Althea rosea*, *Calotropis procera* and *Nerium oleander* with low counts (10.8% of total fungi). Also, Caruso *et al*. (2000) isolated sterile mycelium from woody and herbaceous tissues of a *Taxus* sp. Moreover, dark septate endophytes may indeed function physiologically as mycorrhizas in natural conditions, since some dark septate endophytes have been found to enhance host mineral nutrition and growth (Shivanna *et al*. 1994, Fernando and Currah 1996 and Jumpponen *et al*. 1998).

In conclusion: There are no specific fungal genera and species for each plant tested (*Arachis hypogaea, Medicago sativa* and *Vicia faba*) and most of the species were isolated and identified from Egyptian sources. Fusaria were the most prevalent based on frequency and count. Sterile mycelia had varieties of colour and varied in counts in different plants tested.

Table 1: Total counts (TC, calculated per 400 root segments) and number of case of isolation (NCI, out of 20 samples) of fungal genera and species isolated from peanut, alfalfa and broad bean recovered on potato dextrose agar (PDA) and water agar (WA) at 28±2˚C.

Genera and species	Peanut				Alfaalfa				Broad bean			
	PDA		WA		PDA		WA		PDA		WA	
	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI
Fusarium	65	19	47	15	102	19	65	16	10	6	27	9
F. solani (Mart.) Sacc.	32	9	28	9	23	9	13	7			5	2
F. subglutinans (Wollenweber & Reinking) Nelson, Toussoun & Marasas	12	6			24	11	1	1				
F. oxysporum Schlecht.	7	6	8	4	9	3	11	4	3	2		
F. udum Butler					11	6	7	4				
F. nygamai L. W. Burgess & Trimboli	6	3	1	1	6	3	6	4	3	1	4	2
F. semitectum Berkeley & Ravenel	3	3			4	4			1	1		
F. xylarioides Steyaert					4	3	3	1				
F. decemcellulare Brick					3	2						
F. anthophilum (A. Braun) Wollenweber	2	2	2	1	2	2	1	1	1	1	2	2
F. thapsinum Klittich, Leslie, Nelson & Marasas					2	2	8	2	1	1	1	1
F. chlamydosporum Wollenweber & Reinking					3	1	6	4			11	5
F. camptoceras Wollenweber & Reinking	2	1										
F. verticillioides (Saccardo) Nirenberg	1	1			11	5	8	5	1	1	4	2
F. poae (Peck) Wollenweber			8	3								
F. fusarioides (Gonz, Fragoso & Ciferri) C. Booth							1	1				
Aspergillus	44	14	39	13	33	11	16	6	31	16	15	7
A. eburneo-cremeus Sappa	8	6	4	2								
A. tubingensis (Schöber) Moss	11	5	10	5	6	4	5	2	7	6	5	5
A. flavus link	6	4	7	5	7	3			11	5	6	3
A. sulphureus (Fres.) Thom & Church					5	4	2	1				
A. ficuum (Reich.) Hennings	7	3			2	1			1	1		
A. terreus Thom	3	2	8	2	2	2	7	3	2	2	3	2
A. flavus var. *columnaris* Fennell & Raper					4	2						
A. flavo-furcatis Batista & Maia					4	1	1	1				
A. terreus var. *aureus* Thom & Raper	5	1	6	3	2	2					1	1
A. ochraceus Wilhelm					1	1	1	1				
A. terricola var. *americana* Marchal	2	1	3	2								

Table 1: Continued

Genera and species	Peanut				Alfaalfa				Broad bean			
	PDA		WA		PDA		WA		PDA		WA	
	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI
A. terricola var. *indicus* (Mehrotra & Agnihotri) Raper & Fennell			1	1								
A. niveus Blochwitz	1	1										
A. speluneus Thom & Raper	1	1										
A. phoenicis (Cda.) Thom									8	7		
A. janus Raper & Thom									1	1		
A. oryzae (Ahlb.) cohn var. *effuses* (Tiraboschi) Ohara									1	1		
Emericella nidulans var. *echinulata* (Fennell & Raper) Subramanian	2	2	4	2								
Penicillium	41	14	29	10	25	11	16	8	62	18	34	13
P. duclauxii Delacroix	26	7	14	6	9	4	9	4	16	10	2	1
P. piscarium Westling	7	3	3	1								
P. funiculosum Thom	3	3	6	4	15	9	6	4	33	17	10	7
P. baarnense van Beyma	2	1	5	2								
P. kapuscinskii Zaleski	2	1										
P. verruculosum Peyronel	1	1										
P. resticulosum Birkinshaw, Raistrick & Smith					1	1						
P. lavendulum Raper & Fennell									4	4		
P. rubrum Stoll									3	2	2	1
P. chrysogenum Thom									2	1		
P. citrinum Thom									1	1	2	1
P. corylophilum Dierckx									1	1	1	1
P. pulvillorum Turfitt									1	1		
P. variabile Sopp			1	1			1	1	1	1		
P. islandicum Sopp											13	5
P. pulvillorum Turfitt											4	2
Cylindrocarpon	32	10	41	6	13	6	28	9	2	1	10	4
C. radicicola Wollenweber	14	6	21	4	8	4			2	1	1	1
C. didymum (Hartung) Wollenweber	18	5	20	5	5	2	28	9			9	4
Humicola	11	5	5	2	7	3	2	2	3	2		
H. grisea Traaen	10	6	5	2	6	2			3	2		
H. fuscoatra Traaen	1	1			1	1	2	2				

Table 1: Continued

Genera and species	Peanut				Alfaalfa				Broad bean			
	PDA		WA		PDA		PDA		WA		PDA	
	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI
Cladosporium					3	2			3	3	3	3
C. cladosporioides (Fresen.) de Vries					3	2			1	1	2	2
C. oxysporum Berk. & Curt									2	2	1	1
Alternaria	5	4	15	4	1	1						
A. tenuissima (Kunze: ex Pers.) Wilshire	3	2										
A. sonchi J. J. Davis & apud J. A. Elliott					1	1						
A. alternata (Fr.) Keissler	1	1	15	4								
A. radicina Meier, Drechsler & Eddy	1	1										
Drechslera	4	4	3	1	7	3	17	8	2	2		
D. biseptata (Sacc. & Roum.) Richardson & Fraser	1	1					1	1	1	1		
D. dematioidea (Bubák & Wróblewski) Subram & Jain	1	1										
D. hawaiiensis (Bugnicourt) Subram. & Jain ex Ellis, Subram. & Jain	1	1					2	1				
D. rostrata (Drechsler) Richardson & Fraser	1	1										
D. halodes (Drechsler) Subram. & Jain			3	1	3	2	1	1	1	1		
D. bicolor Paul & Parbery					4	1	11	5				
D. papendorfii (van der Aa) M. B. Ellis							2	1				
Macrophomina phaseolina (Maublanc) Ashby	8	3	2	1	3	1	15	6				
Trichoderma ghanense Doi, Y. Abe & J. Sugiyama									9	3		
Curvularia	5	3	5	4	9	4						
C. clavata Jain	3	2	3	2								
C. brachyspora Boedijn	1	1	1	1								
C. lunata (Wakker) Boedijn	1	1	1	1	7	4						
C. ovoidea (Hiroe & Watan.) Muntañola					2	1						
Scopulariopsis	5	2	2	2	2	2			1	1		
S. brumptii Salvanet-Duval	4	1	2	2					1	1		
S. brevicaulis (Sacc.) Bainier	1	1			2	2						
Botryotrichum	1	1									5	2
Botryotrichum atrogriseum van Beyma	1	1										
Botryotrichum piluliferum Saccardo & Marchal											5	2
Stachybotrys atra Corda					1	1						

Table 1: Continued

Genera and species	Peanut				Alfaalfa				Broad bean			
	PDA		WA		PDA		WA		PDA		WA	
	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI
Torula			1	1	1	1			1	1	2	2
Torula graminis Desm			1	1	1	1			1	1		
T. herbarum (Pers.) Link ex Fries											2	2
Mucor hiemalis Wehmer	1	1							2	1		
Paecilomyces variotii Bainier									2	1	1	1
Trimmatostroma salicis Corda	1	1										
Paecilomyces marquandii (Massee) S. Hughes			9	3								
Gilmaniella humicola Barron							2	1				
Pleospora infectoria Fuckel							1	1				
Unknown 1	1	1										
Unknown 2									3	1		
Unknown 3							1	1				
Unknown 4					3	2						
Unknown 5					1	1	6	1				
Sterile mycelium (white)	2	1	6	2	3	2	2	1	1	1		
Sterile mycelium (buff)			1	1			2	1			1	1
Sterile mycelium (white-gray)	1	1			1	1			1	1		
Sterile mycelium (gray)					2	2					3	2
Sterile mycelium (gray red)							1	1				
Sterile mycelium (white-brown)					3	1			2	1		
Sterile mycelium (white beige)					3	1						
Sterile mycelium (white gray orange)					2	1						
Sterile mycelium (white red)							1	1				
Sterile mycelium (olive-brown)					1	1	13	7	1	1	1	1
Sterile mycelium (gray green)					1	1						
Sterile mycelium (white gray blue)					1	1			2	1		
Sterile mycelium (violet gray)					1	1						
Sterile mycelium (versicolor)					1	1						
Total count	229		209		230		188		138		102	
No. of genera	14		13		13		9		12		8	
No. of species + varieties	41+3		26+4		36+2		29		32+1		23+1	

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