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MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF ECTOMYCORRHIZAL FUNGI ISOLATED FROM EGYPTIAN ENVIRONMENT

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ABSTRACT

In a trial to isolate and identify ectomycorrhizal fungi for the first time in Egypt 13 sporocarpes associated with rang of plants grown on Egyptian soils were collected, to be used for this purpose. Fungal isolates were obtained from collected sporocarpes and tested for ectomycorrhiza formation with *Bauhinia* sp. and *Pinus* seedlings. The paper sandwich technique was used for simultaneous inoculation of root apices *Bauhinia* to produce synchronously developing ectomycorrhizas. Typical ectomycorrhizal roots were obtained within 14 days after inoculation with the collected strains. The isolates tested for ectomycorrhiza formation with *Pinus* sp. to ensure their identity by formation of distinct root characteristics on this host. Root colonization levels varied markedly among the tested fungal strains with respect to growth enhancement and NPK uptake of *Pinus* shoots and roots.

INTRODUCTION

Ectomycorrhizae (ECM) represents a complex interaction between fungi and plant roots. Fungi forming ECM are diverse in morphological and physiological characteristics (**Allen *et al* 1995 and Molina *et al* 1992)** and are mostly belonging to Basidiomycota, Ascomycota, Zygomycota or one specie of imperfect fungi **(Read, 1991 and Molina *et al* 1992).**

They are functionally important in temperate forest ecosystems for plant nutrient and water uptake as well as protection from root pathogens **(Smith & Read, 1997).**

ECM fungi are usually isolated from fruiting body tissue, sexual spores, and bulky substrates such as rhizomorphs, mycelial cords, sclerotia and fungal sheath. Fruiting bodies provide the majority of diagnostic characters used for ECM fungi identification, while non fruiting isolates derived from vegetative materials require more taxonomic and culture experience. Isolation is more easily achieved from bulky fruiting bodies than from other structures **(Hutchinson, 1991 and True & Agerer, 1990).**

Ectomycorrhizal inoculation is particularly important because native soil population of this group may be low in both afforested and disturbed areas **(Perry *et al* 1995)** or incompatible with the introduced species. In pouch cases, nursery inoculations with selected ectomycorrhizal fungi adapted to the ecological conditions of the planting area may by necessary **(Marx, 1980).**

This study aimed to isolate and identify ECM isolates fungi collected from different sites in Egypt along with evaluation the infectiveness and effectiveness on *Pinus* plants.

MATERIALS AND METHODS

1. Sources of Sporocarpes

Young sporocarpes free of rot and insect damage and fully matured Sporocarpes were collected from *Macharium tipu*, Doranta, Grape, Citrus and

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weeds. The sporocarpes were placed into waxed paper bag and kept in low temperature in the refrigerator until examination.

2. Seeds of host plants

Seeds of *Bauhinia* and *Pinus* were kindly obtained from forestry and woody tree Department, Horticulture research Institute, ARC. Giza.

3. Identification of ECM fungi

3.1. Making spore prints

In the case of mushrooms, the stem was cut from a mature sporocarpes, the cap were placed over a card and covered with waxed paper, caps were laid flat over the card to obtain a spore print. After a period of one hour to overnight, the white or colored spores deposit on the paper was obtained. Spore prints were used for microscopic and macroscopic examination according to **Brundrett *et al* (1996)**.

3.2. Sporocarpes and spores characteristics

Macroscopic and microscopic examinations of the fruiting bodies or spores morphology were based on following characteristics (Cap-Gills-Step-Universal veil- Partial veil- Spore print color, spore size, spore shape, spore well structure and ornamentation) as described by **Brundrett *et al* (1996) and Agerer, (1991).**

4. Isolation of ECM fungi from Sporocarpes

Adhering debris were brushed especially from the step base. For the mushroom, a shallow slit (1-2 mm) was cut across the middle of the cap surface and along the length of a side of the step.

To expose interior tissue, the sporocarpes were gently pulled apart along the initial shallow cuts, using fingertip pressure. Quickly the exposed interior surface for areas of tissue free from contact with obvious contaminating sources was scanned. A small amount of tissue was removed with a fine forceps and placed on MMN agar medium **(Marx, 1969)** contained Malt extract 3g& d-glucose 10g& $(NH4)_2HPO_4$, 25 g & KH_2PO_4 ,5 & $MgSO_4$,15 & $CaCl_2$ .05 g & $FeCl_3$ 1% solution (1,2 ml) & thiamin HCl 100 ug & distilled H_2O 1000 ml &pH 5,8 in a Petri dish. After3 to 4 days observation for initial fungus growth and contamination was examined under a stereomicroscope. Fungi which are easily isolated and grow well in culture produce visible mycelial growth 4 to7 days after isolation. Other fungi may take 2 to 6 weeks to show any sign of growth (the tissue cultures are usually incubated at 25 to 28 ^{0}C). After ectomycorrhizal fungus established on the medium with no visible contaminants, mycelia from the developed colony edge were aseptically transferred with a needle onto fresh MMN medium to set up a stock culture.

5. Infection test

Healthy, uniformly sized seeds of *Bauhinia* sp host plants were selected, and surface sterilized with 5% sodium hypoclorite (NaOCl) for 20 min. Sterilized seeds were washed with three changes of sterile water, and were individually placed on agar in plates for germination and checked for contaminating microbes.

Petri dishes (140-20mm) were filled with 30 ml of complete nutrient agar based on solution for plant nutrient **(Brundrett *et al* 1996)** culture that maximizes mycorrhizal development

On *Bauhinia* seedling. The agar media was overlain with cellophane disks that have been washed, autoclaved before use. Discs of hyphal plug (5mm in diameter) was taken by cork borer from the edge of 14 day old colonies of ECM fungi growing in agar media. These plugs were placed approximately 1.5cm apart in 2 rows in the center of petri dish. Plates were incubated at 25-28 ^{0}C in the dark, until sufficient hyphal growth has occurred. Axenically germinating seeds with a short radical were placed in a row 1-2 cm above the growing hyphae. The plates were incubated on a slant, so the seedling roots grow toward the fungus and adhere to the cellophane surface. Plates with fungi and seedling were incubated at 25 ^{0}C with the presence of adequate light for seedling growth (**Burgess *et al* 1996**).

On *Pinus* seedlings. A pot experiment was conducted in the Unit of Biofertelizers greenhouse, Faculty of Agriculture, Ain shams University. *Pinus* plants infection techniques were made to test the potent of ECM strains. For this purpose polyethylene bags filled with 500 g of clay and sand soil (2:1) and inoculated with 10 g of a colonized ECM inoculum (each strain separately). Seeds of *Pinus* were planted and then watered when necessary. Four replicates were used for each treatment. After 3 months from planting under greenhouse conditions, the plants were collected to observation and photographs.

6. Evaluation infectiveness and effectiveness of ECM fungal strains on *Pinus* seedlings

a) Preparation of ECM fungal inoculum

A 10 disk (0,5 cm) of the 4 weeks old colony of each one of ECM fungi isolates were aseptically transfer into bottles containing of 100 gram of cooked and sterilized wheat grain. Bottles were incubated at 25^0C for 2 months and mixed well periodically.

b) Experimental technique

The enffectiveness and effectiveness of the 4 ECM strains on growth and N, P, K uptake of *Pinus* seedling were evaluated under greenhouse conditions. The soil samples were mixed sieved and sterilized by autoclaving, and placed into polyethylene bag (10x20 cm) and packed at rat of 1kg. The pages were inoculated with 10 g ECM inoculum before seeds were planted. The developed seedlings were watered once weekly.

d) Parameter measured

Five months old seedling were carefully collected to record shoot and root length (cm/plant), shoot and root dry weight (g/plant), dichotomous/plant.

Total nitrogen, phosphorus and potassium uptake of *Pinus* seedlings shoots and roots were determined according to the methods described by **Jackson, (1973).**

7) Determination of similarity index of identified ECM fungi using the Inter-Simple Sequence repeat Polymerase Chain Reaction (ISSR-PCR)

For this purpose DNA were extracted from axenic mycelia of all tested isolates using the methods of **Jin *et al* (2004).** The inter-simple sequence repeat PCR reactions (ISSR- PSR) were performed using the degenerate primers 844A (CT8AC); HB11 (GT) 6CC; HB9 (GT) 6GG; HB12 (CAC) 3GC; HB13 (GAG) 3GC; HB14 (CTC)3GC**;** HB15 (GTG)3GC. DNA amplification was performed in a model 2400 Perkin-Elmer DNA thermal cycler. PCR reactions were performed in a 25 µl template DNA solution containing approximately 100 ng genomic DNA 200 µl of dATP, dTTP, dCTP, and dGTP,50 mM KCl, 2.0mM $MgCl_2$, 10mM Tris – HCl (pH 9.0) ,0.1% Triton X -100 and 1.5 unit *Taq* DNA polymerase (Bioron- Germany). The PCR amplification products were separated by electrophoresis in 1.8 % agarose gel in 0.5 x TBE (Tris-boric acid-EDTA) buffer at low voltage over night. After electrophoresis gels were stained with ethidium bromide and photographed on a UV transilluminator. The DNA size marker used for electrophoresis was the 100bp plus Ladder (GIBCO-BRL, NY) **(Zhou *et al* 2001).**

RESULTS

Isolation and identification of EMC fungi

Thirteen fungal isolates were obtained from fruiting bodies collected from weeds, Citrus, Grapes, Doranta and *Macharium tipu* plants, respectively

Based on macroscopic examination of cap, cap surface, stip surface, gill attachment, ring, cap cuticle and cortina Step, Universal veil, partial veil, spore print color, spore wall and spore shape as characteristics proposed by **Brundrett *et al* (1996)** for ECM identification. Four out of the Thirteen sporocarpes were found to belong to ectomycorrhizal genera *Leccinum* sp., *Hebloma* sp., *Amanita* sp. and *Cortinus* sp. (See **Figs. 1-4**). The characteristics and results are given in **Table (1).**

Confirmatory test for ectomycorrhizal formation by identified fungi.

The four identified strains were tested for their ability to form association with *Bauhinia* and *Pinus* roots (See **Fig.5 a&b** and **Fig. 6**).

Bauhinia

Bauhinia plantlets grown on sterile MMN medium and inoculated with either of the 4 tested. fungi, within 2 weeks. The hyphae were sparse and the mantel was unrecognized in the first 4.5 days. But the hyphae were completely surrounded the main root and the mantel was developed and recognized at day 14. Microscopic examination of the roots showed totally enveloped root system and the newly emerging lateral roots were completely enveloped by the fungal hyphae which form distinct mantel (**Fig. 5 a, b**)

Pinus

Roots of *Pinus* seedlings showed the distinguished characteristics known for ectomycorrhizal formation. Hence, plants harvested after 3 months of inoculation showed a distinct dichotomously branched roots (**see Fig. 6**).

Fig 1. Fruiting body (mature stage *Leccinum* sp. (F8).
Fig 2. Fruiting bodies *Hebloma* sp. (F9) a) Bottom stage, b) mature stage, c) spore print, d) primordia on plat.
Fig 3. Fruiting bodies *Amanita* sp. (F10) a) bottom stage; b) mature stage, c) spore print, d) primordia on plat.
Fig 4. Fruiting bodies *Cortinus* sp. (F11) a, b) bottom stage; c) mature stage, d) spore print.
Fig 5. Ectomycorrhizal synthesis on roots of *Bauhinia* grown in sterile MMN medium.
Fig 6. Ectomycorrhizal synthesis on roots of *Pinus*
Fig 7. a,b) Characteristics of Ectomycorrhizal roots on *Pinus*.

Table 1. Characteristics of ECM fungi isolated from sporocarpes

Isolate code / characteristics	F(8)	F(9)	F(10)	F(11)
Host	*Macharium tipu*	Weeds	Weeds	Weeds
Spore wall	Smooth	Smooth	Smooth	Smooth
Spore shape	Fusoid elongate	Fusoid	Global to ellipsoid	Global
Cap surface	Smooth sponge	Smooth	Smooth	Smooth
Mushroom cap	Tubular	With gills	With gills	With gills
Veil	Partial veil	Absent	Partial veil	Partial veil
Stip surface	With dots	smooth	smooth	smooth
Stip long	Long	Short	Long	Long
Gill attachment	Pores	Free	Free	Free
Ring	Absent	Absent	Present	Present
Cap cuticle	-	Filamentous	Filamentous	Filamentous
Cortina	Absent	Absent	Absent	Present
Genus	***Leccinum* sp.**	***Hebloma* sp.**	***Amanita* sp.**	***Cortinus* sp.**
Family	**Boletaceae**	**Cortinariaceae**	**Amanitaceae**	**Cortinariaceae**

ISSR analysis

Highly purified DNA extracts of the four ECM strains were used as templates for ISSR-PCR. Data revealed that no amplified fragments were observed in any of the negative controls (PCR mixture without any DNA templates), which indicates that reaction mixtures were free from any contamination DNA. Data in **Table (2)** showed that the number of amplified fragments differed with different primers, which is expected. On the other hand, the number and sizes of amplified fragments differed from one strain to another for the same primer. This is clear since as they differed in their DNA sequences. Data in **Table (3)** showed that a total number of 70 amplified fragments were obtained using the seven primers used. In addition, 40, 48.6, 58.8 and 57.1% of the 70 fragments were amplified from the DNA of the four ECM strains (F9, F11, F8 and F10), respectively. Results in **Table (4)** showed that out of the 70 fragments, 16 unique fragments distributed as follows: 4, 2, 5, and 5 for F9, F11, F8 and F10 ECM strains, respectively, were found. Results in **Table (5)** revealed that the similarity between the DNA of the four ECM strains in this investigation ranged from 49.3% to 64.5%. Results in **Fig. (8)** showed the phylogenetic tree of the four tested strains. The first cluster included strains F9 & F11 with similarity of 64.5 %. While, strains F11 & F8 laid in the second cluster with 61.3% similarity.

Application of ECM strains on *Pinus* growth parameter.

After 5 months of cultivation, growth of *Pinus* seedlings were evaluated as affected by inoculation with 4 strains of ECM. Data recorded in **Table (6)** show that inoculation with ECM gave the higher growth performance of shoot, root length (cm/ plant) and shoot, root dry weight (g/plant) compared with uninoculated (control) plants (**Fig. 7 a&b**). Inoculating the four ECM strains onto *Pinus* seedling showed that all ECM strains were able to form mycorrhizal dichotomous roots. However there were differences among in this ECM strains in their ability to being 64.17, 43, 37.5 and 26 dichotomous root/plant with stains F10, F11, F9 and F8, respectively. The maximum *Pinus* root colonization reached to 64.17 and 43 mycorrhizal dichotomous roots/plant with strains F10 and F11, respectively, **(Table 6).**

Table 2. ISSR analysis of seven ectomycorrhizal fungi isolates shows the DNA polymorphisms produced using 7 primers

NAF	MW (bp)	Ectomycorrhizal fungi isolates F9	F11	F8	F10
8448b Primer					
1	1.884	0	0	1	0
2	1.614	0	1	0	0
3	0.991	0	0	1	0
4	0.743	1	1	1	1
5	0.583	1	1	1	1
6	0.466	0	0	1	0
7	0.413	1	1	0	0
8	0.377	0	0	1	0
9	0.311	0	1	0	0
10	0.26	1	1	1	0
11	0.181	1	1	1	0
HB-09 Primer					
12	3.396	1	0	0	0
13	2.646	1	0	0	0
14	1.94	1	1	1	1
15	1.206	0	0	0	0
16	1.097	1	1	1	1
17	0.848	1	1	1	1
18	0.547	0	1	1	1
HB-11 Primer					
19	1.428	0	0	1	0
20	1.206	0	0	0	0
21	1.114	0	0	1	0
22	0.97	0	0	1	0
23	0.732	1	1	1	0
24	0.607	1	1	1	1
25	0.518	1	1	0	0
26	0.421	1	1	1	1
27	0.365	0	0	1	0
HB-12 Primer					
28	2.093	1	1	1	1
29	1.238	1	1	1	1
30	0.839	0	1	0	1
31	0.553	0	0	0	1
32	0.346	1	1	1	1
HB-13 Primer					
33	1.939	0	0	1	1
34	1.594	0	1	1	1
35	1.467	1	0	0	1
36	1.341	0	1	0	1
37	1.228	1	0	1	1
38	1.158	0	0	0	0
39	1.079	1	1	0	0
40	0.963	0	1	1	1
41	0.868	1	0	0	1
42	0.795	0	1	1	0

Table 2. Continue.

NAF	MW (bp)	Ectomycorrhizal fungi isolates F9	F11	F8	F10
43	0.699	0	0	0	1
44	0.6	0	0	0	1
HB-14 Primer					
45	1.939	0	0	1	1
46	1.594	0	1	1	1
47	1.467	1	0	0	1
48	1.341	0	1	0	1
49	1.228	1	0	1	1
50	1.158	0	0	0	0
51	1.079	1	1	0	0
52	0.963	0	1	1	1
53	0.868	1	0	0	1
54	0.795	0	1	1	0
55	0.699	0	0	0	1
56	0.6	0	0	0	1
HB-15 Primer					
57	1.974	0	0	1	0
58	1.747	0	0	1	0
59	1.576	0	0	0	1
60	1.488	0	0	1	0
61	1.319	1	1	0	1
62	1.235	1	1	1	0
63	1.04	0	1	0	1
64	0.939	0	0	1	0
65	0.846	1	1	1	1
66	0.713	0	0	1	1
67	0.642	0	0	0	1
68	0.514	0	0	1	1
69	0.447	0	0	0	1
70	0.394	0	1	1	1

Table 3. Total amplified fragments of seven ectomycorrhizal fungi isolates based on ISSR analysis using 7 primers.

NAF	TAF	Ectomycorrhizal fungi isolates F9	F11	F8	F10
8448b	11	5	7	8	2
HB-09	7	5	4	4	4
HB-11	9	4	4	7	2
HB-12	5	3	4	3	5
HB-13	12	4	5	6	11
HB-14	12	4	5	4	7
HB-15	14	3	5	9	9
Total No.	70	28	34	41	40
%	100	40	48.6	58.8	57.1

NAF: Number of amplified fragments.
TAF: Total amplified fragments.

Table 4. Unique fragments of seven ectomycorrhizal fungi isolates based on ISSR analysis using 7 primers.

NAF	TAF	Ectomycorrhizal fungi isolates			
		F9	F11	F8	F10
8448b	11	0	1	4	2
HB-09	7	2	0	0	2
HB-11	9	0	0	1	0
HB-12	5	0	0	0	1
HB-13	12	1	0	0	0
HB-14	12	0	1	0	0
HB-15	14	1	0	0	1
Total	70	4	2	5	5

NAF: Number of amplified fragments.
TAF: Total amplified fragments.

Table 5. Similarity between four identified ECM fungi based on ISSR-PCR analysis using Dice Measure

ECM isolates	%of similarity between the ECM strains			
	F9	F11	F8	F10
F 9	100			
F11	64.5	100		
F8	49.3	61.3	100	
F10	52.9	59.5	56.8	100

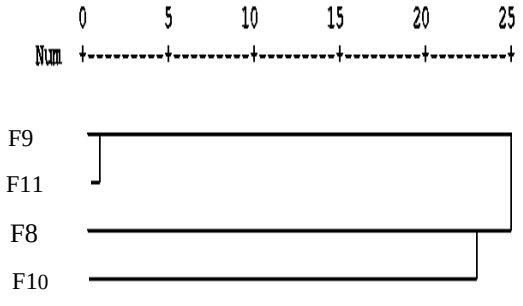

Fig. 8. Dendrogram showing molecular relationship between the four ECM strains (F8, F9, F10 and F11) based on ISSR analysis using Dice Measure

Data also showed that, there were no differences in root length between treated and untreated but there were clear differences in shoot length plants with the same treatment. On the other hand, *Pinus* seedling inoculated with ECM strains increased the root and shoot dry weight compared with uninoculated treatment (control).

Data of NPK uptake of *Pinus* seedling inoculated with ECM isolates and uninoculated were recorded in **Table (7).** Results showed that, inoculation with ECM strains increased the NPK uptake in root and shoot compared with the uninoculated plants (control).The highest records of N and K uptake were obtained from strain F8 being, 0.6, 0.1 and 1.19, 0.18 gm/plant of shoot and root, respectively but P uptake of the shoot and root obtained being 0.02 and 0.12 respectively.

DISCUSSION

Ectomycorrhizal (ECM) fungi are functionally important in forest ecosystem for plant nutrient and water uptake as well as protection from root pathogens **(Smith & Read, 1997).**

EMC fungi are usually isolated from fruiting bodies, sexually spores, rhizomorphs, mycelial cords, sclerotia and ECM sheath **(True & Agerer, 1990).** Members of Boletaceae, Cortinariaceae, Amanitaceae, and Cortinariaceae see **Table (1);** have been cited as ectomycorrhizal with *Pinus* and other conifers (**Malajczuk *et al* 1982).** All genera are considered to be mainly associated with adult trees **(Fleming *et al* 1986 and Mason *et al* 1983).** Differences in root colonization capacity and different mycorrhizal dichotomous roots were detected among fungal isolates **(Pera& Alvares 1995).**

Inoculation of *Pinus* seedling with ECM isolates (F8, F9, F10 and F11) were associated with increased growth parameters (root, shoot length and root shoot dry weight) and nutrient uptake (NPK) compared with control. The enhancement of *Pinus* seedlings growth and nutrient uptake could be attributed to the ability of the ectomycorrhizal fungi to produce plant- growth promoting factors that affect the root and shoot growth and branching. The same trend of results was also reported by (**Le Tacon *et al* 1992; Villeneuve *et al* 1991; Tonkin *et al* 1989; Castellano & Trappe 1985 and Stenstrom *et al* 1985).**

Table 6. Effect of inoculation with four Ectomycorrhizal strains on growth parameters of *Pinus* seedlings

ECM strains	Number of Mycorrhizal dichotomous roots/plant	Shoot length (cm/plant)	Root length (cm/plant)	Root dry weight (gm/ plant)	Shoot dry weight (gm/ plant)
F8	26.00	13.00	33.20	0.45	0.69
F9	37.50	10.50	34.17	0.26	0.33
F10	64.17	11.17	33.50	0.27	0.2
F11	43.00	10.80	34.00	0.17	0.35
Control	-	8.67	33.00	0.12	0.15

Table 7. Effect of inoculation with four ectomycorrhizal strains on N & P & K uptake of *Pinus* seedling

ECM Strains	Root			Shoot		
	N	P	K	N	P	K
F8	0.61	0.02	0.18	1.19	0.12	0.18
F9	0.36	0.02	0.10	0.66	0.04	0.18
F10	0.24	0.02	0.06	0.39	0.02	0.09
F11	0.17	0.02	0.05	0.88	0.08	0.09
control	0.08	0.01	0.04	0.16	0.02	0.07

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