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Differentiation Between *Glomus* Species in Egyptian Soil Using Fatty Acid Methyl Ester Profiles

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ABSTRACT

The present study was to investigate the variation among *Glomus* species. Standard method used for Extraction, purification of Vesicular Arbuscular Mycorrhizal (VAM) fungi, during summer and winter season. Study Effect of some factors on number of spores collected from the rhizosphere of various plants in Egypt. Used single spores technique inoculated on maize root to purified and identified isolates of *Glomus* spp. The highest number of spores was found in summer season while the lowest number was found in winter season. Variation of *Glomus* spp. isolates among polymorphic DNA markers was studied. Species 2, 3, 4 and 7 identified by VANS1/VALETC primer as *Glomus etunicatum*. Fatty Acid Methyl Ester (FAME) profiles were analyzed to assess the diversity and quantity of fatty acids in 8 isolates of *Glomus* species. Spores and endomycorrhizal roots of Sudan grass (*Sorghum sudanense*) were examined. Analysis of lipid for species found that affinity specific and generic levels. Differentiation on the genus level using lipid profiles and content which was carried out by evaluating the methyl esters of fatty acids, revealed convergence between studied isolates. Palmitic acid (16:0), Oleic acid (cis18:1n9) and Stearic acid (18:0) were the dominant fatty acid of the *Glomus* spp., while the dominant fatty acids of *Glomus etunicatum* isolates were myristic acid (14:0) palmitoleic acid (16:1n7 *cis*), archidic acid (20:0), palmitic acid (16:0), oleic acid (cis18:1n9) and stearic acid (18:0). This study indicates that using lipids as biochemical markers to identify the different genus of AM fungi and differentiate between the species.

Key words: Endomycorrhizae, *Glomus* spp., DNA markers, VANS1, VALETC, fatty acids

INTRODUCTION

Soil fungi are not always infectious to plants or causing deleterious effects for them but some fungi could be very beneficial for plants. The majority of plants species on earth can form a symbiotic relationship with some kind of fungi, known as mycorrhizae, this symbiotic relationship is a beneficial association for both of them. In such a relationship mycorrhizae are able to capture and deliver elements, mainly phosphorus and zinc, to the plants and in turn plants supply mycorrhizae with readymade food essential for them. Therefore, plants living in association with mycorrhizae can show vigorous growth which is due to the easy access to the elements from soil (Sanders *et al*., 1977; Smith and Read, 1997). Hence, the more mycorrhizae hyphae spreading into the soil and attached to the plant roots, the more efficient the association would be and the plant will be amended extensively with phosphorus.

As Eukaryotes, fungi possess phospholipids in their membranes which are considered a core component of the membranes. In addition, lipids are present in eukaryotic cells as source of energy. However, they mostly differ in the types of fatty acids forming the lipid components in the cells which could be used as biochemical markers to be used for identification. Therefore, phospholipid fatty acids are used to identify the different genus of AM fungi and, in some cases, indentifying to the species level. In addition, they also indicate the fungal biomass in association with plant roots which is also an indication for the efficiency of the AM relations with the plants (Tunlid and White, 1992; Sakamoto *et al*., 2004). Together with the morphological characters, researchers are using the biochemical and molecular features to distinguish between different mychorhizae species. However, the tendency to use lipids as biochemical markers in identification of AM fungi is due accuracy and to avoid the obstacles of handling and staining the AM hayphae and spores associated with plant roots in order to distinguish morphological characters (Walker and Trappe, 1993).

In previous investigations some phospholipid fatty acids (PLFA) were found to be distinguishing for some AM genus. For instance, *Glomus* species were found to possess 18:w7, 18:1w9, 20:3, 20:4 and 20:5 in the spores and hyphae associated with plant roots. However, some of these fatty acids were common between *Glomus* spp and other fungi such as *Acaulospora laevis* (Beilby, 1980, Beilby and Kidby, 1980; Graham *et al*., 1995; Jansa *et al*., 1999; Sakamoto *et al*., 2004).

On the other hand, some phospholipid fatty acids could be used as markers for AM fungi to the species level. It was detected that PLFA 20:1w9 was used as a marker to detect the presence of *Gigaspora rosea* (Sakamoto *et al*., 2004).

Class Glomermycota is a major mycorrhiae fungi which consists of genus commonly found in the Egyptian soil (Fares, 1986).

This study aimed to investigate the survey of vesicular arbuscular mycorrhizal fungi in Egypt, study some factors affecting AM fungi sporulation and to differentiate between the isolated *Glomus* species using lipids as biochemical markers.

MATERIALS AND METHODS

Extraction, purification and preservation of vesicular arbuscular mycorrhizal (VAM) fungi: Vesicular arbuscular mycorrhizal fungi were collected from the rhizosphere of various plants, through summer and winter months grown in four governorates namely; Beni Suef, Ismailia, El-Menofia and El-Behera. Samples of rhizosphere soil (100 g) was carefully collected from roots of healthy plants and placed in polyethylene bag. Extraction of VAM propagules from soil was carried out under laboratory conditions as soon as possible using wet-sieving and decanting technique described by Gerdemann and Nicolson (1963), Gerdemann and Trappe (1974) and Trappe (1982). *Sclerotium rolfsii* isolates were obtained from Mycology Research and Diseases Survey Dept., Plant Pathology Research Institute, A.R.C.

Mycorrhizal fungi spores will breed as many as single spores inoculated on maize root, where VAM propagation were multiplied and maintained in open pot cultures of Sudan grass as a host on *in vivo* medium.

Effect of some factors on number of spores collected from the rhizosphere of various plants-Measuring some factors i.e. electrical conductivity (EC), total dissolved solids (TDS) and pH of soil: Soil-water equilibrium was carried out using the procedure of Negrin *et al*. (1996) as follows: 10 g of air dried soil (<2 mm fraction) was shaken in 25 mL distilled water on reciprocal shaker for 30 min before equilibration at 4°C were used to prevent biological activity

(Hedley and Stewart, 1982) and to favor sedimentation of colloidal material (Schoenau and Huang, 1991). When the equilibration time was attained, the suspensions were centrifuged for 15 min at 2000 Xg and then filtered through a 0.45 Mm Millipore (Millipore corp., Milford, MA) cellulose ester filter. Electrical Conductivity (EC), Total Dissolved Solids (TDS) and pH of the filtrate were measured.

Variation of *Glomus* spp. isolates among polymorphic DNA markers

DNA isolation technique: DNA of spores of each isolate (approximately 200 spores/isolate) of Eight *Glomus* spp. previously isolated from different governorates ground to fine powder using a pestle and mortar in liquid nitrogen then placed in a lable 1.5 mL tube then mixed with 600 µL preheat extraction buffer (50 mM EDTA, 100 mM Tris pH 8, 500 mM NaCl) to 65°C in a water bath. 45 µL 10% SDS and 7 µL β merecapto ethanol were adding into each tube and mix them thoroughly by inversion. The samples were incubated at 65°C for 30-40 minutes and mix every 15 min. the samples were cooled down to room temperature before adding 220 µL 5 M potassium acetate into each tube mix thoroughly by inversion then incubate sample on ice or in a freezer for 15 min. the plate were centrifuged for 10 minutes at 5700 rpm. The supernatant was Transferred into a labeled new tubes containing 300 µL of cold isopropanol incubate in -20°C for 24 h, the tube was inverted gently 10-15 times to precipitate DNA for 5 min.

The samples were centrifuged for 10 minutes at 5700 rpm in order to pellet the DNA. The supernatant was discarded by quickly and smoothly inverting the tubes. plate was air dried for 5 min, washed in the two volumes of 70% ethanol (room temperature) were added. The DNA was then pelleted by centrifugation for 12 min (room temperature, 15 000 g). The brown-colored pellet containing the DNA was resuspended in 30 mL TE (10 mM Tris pH 8, 1 mM EDTA). (Dellaporta *et al*., 1983).

Amplification protocol: The following oligonucleotides used as primers review for the three primers used in this study Simon *et al*. (1993) was obtained commercially. Their location relative to the SSU gene the sequences of used primers were as shown:

- VANS1, 5'GTCTAGTATAATCGTTATACAGG
- NS21, 5'AATATACGOCTATTGGAGCTGG
- VALETC, 5'ATCACCAAGGTTTAGTTGGTTGC
- VAGLO, 5'CAAGGGAATCGGTTGCCCGAT

The amplification reaction include approximately 50 ng of fungal genomic DNA as template, 1 unit of *Taq* DNA polymerase, o.2 mM each dNTPase, 1x PCR buffer, 3 mM $MgCl_2$ and 10 pmol of primer. The temperature profile was programmed on a DNA Thermal Cycler (Perkin-Elmer) to repeat 25 to 35 times a cycle of denaturation (60 sec at 94°C), annealing (45 sec at 50°C) and polymerization (60 sec at 72°C), followed by a final extension step of 10 min at 72°C. Hold at 4°C 7 µL of 6 X tracking buffer (manufactured by Qiagen Kit) were added to 25 µL of the amplification product.

The amplified product were separated on 1.5% agarose gel to check for product size and purity, using 1X TBE buffer followed by staining in ethidium bromide solution (1 ug mL^{-1}), at 75 constant volt and determine with UV transilluminator.

The total volume was completed to 25 µL using sterile distilled water. The amplification protocol was carried out as follows using PCR unit II biometra.

Gel analysis: DNA gels were scanned for band RF using gel documentation system (AAB Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6 C, Fullerton CA 92631). The different M W of bands was determined against PCR marker promega G 317A by unweighted pair-group method based on arithmetic mean (UPGMA).

Lipid differentiation by evaluation lipid on root of grass: The root samples (75 mg) were dissolved in toluene (1 mL) in a test tube fitted with a condenser, then H_2SO_4 in methanol (2 mL, 1%) was added. The mixtures were left overnight in a stoppered tube at 50°C then sodium chloride solution (5 mL, 5%) was added and the required esters were extracted with hexane (2x, 5 mL), then the organic layer was separated using Pasteur pipettes for both samples. The hexane layers were washed with potassium bicarbonate solution (4 mL, 2%) and dried over anhydrous Na_2SO_4 and filtered. The organic solvent was removed under reduced pressure on a rotary evaporator to give FAMEs and other lipids. GC-MS Conditions: GC-MS analysis was performed in a Fisons MD800 mass (quadrupole) -GC8000 series instrument equipped with a flame ionization detector. A silica column (30 m×0.25 mm I.D.) coated with OV1 was utilized. The initial temperature was 40°C for 4 min after injection and then increased to 280°C (8°C min^{-1}) with a final hold at 280 °C for 20 min. The injector and detector temperature were maintained at 270 and 250°C, respectively. Helium was used as the carrier gas at a flow-rate of 0.8 mL min^{-1} (Yayli *et al*., 2001).

Statistical analysis: Data were statistically analyzed according to the standard procedure in completely random design as mentioned by Snedecor and Cochran (1982).

RESULTS

Collection, Isolation and Purification of Vesicular Arbuscular Mycorrhizal (VAM) fungi

Extraction of VAM spores from some Egyptian governorates during summer season: Data in Table 1 show that the highest numbers i.e., 107, 101, 81 and 60 of VAM fungal spores g^{-1} soil isolated from maize sown after winter crops i.e., wheat and clover were found at El-Behira, Ismailia, Beni-Suef and El-Menofia governorates respectively. On the other hand, three crops sown

Table 1: Number of VAM fungi spores extracted from some Egyptian governorates during summer season

Governorate	Previous crop	The current crop	Soil type	pH of soil	EC (µmhos/cm) of soil	TDS (µmhos/cm)	No. of spore/g soil
El-Behera	Wheat	Cabbage	Sand	7.3	200	90	30
	Wheat	Maize	Sand	7.2	100	60	67
	Wheat	Sunflower	Sand	7.2	400	180	27
	Barley	Peanut	Sand	7.2	400	240	9
	Barley	Watermelon	Sand	7.2	200	90	98
	Barley	Pepper	Sand	7.5	200	90	65
	Wheat	Maize	Sand	7.3	100	50	107
	Sugarbeet	Sugarbeet	Sand	7.2	1000	530	0
El-Menofia	-	pear	Clay	7.0	250	140	51
	Wheat	Maize	Clay	7.0	300	150	60
Beni-Suef	Sunflower	Sunflower	Clay	7.2	100	80	39
	Clover	Sesame	Clay	7.3	300	180	25
	Wheat	Maize	Clay	7.2	400	190	81
	Wheat	Cabbage	Clay	7.3	300	130	29
Ismailia	Barley	Peanut	Sand	6.8	300	170	93
	Clover	Maize	Sand	6.8	200	150	101

after barley i.e. water melon (El-Behira), peanut (Ismailia) and pepper (El-Behira) recorded high rates (98,93 and 65 of VAM spores g^{-1} soil, respectively).

Type of soil had an effect on survival of VAM fungal spores. It is noticed in Table 1 that sand soil was the best medium for survival of VAM spores. On the other hand pH of Egyptian soil had no effect on the existence of VAM fungal spores. It's worth to note that there is no VAM fungal spores recorded at EC 1000 µmhos/cm and 530 µmhos/cm of Total Dissolved Solids concentration (TDS) of Egyptian soil.

Extraction of VAM spores from some Egyptian governorates during winter season: Data in Table 2 show that the highest numbers i.e., 45, 26 and 17 VAM fungal spores g^{-1} soil isolated from onion, lentil and sugar beet respectively sown after maize at Beni-Suef governorate .The average of VAM spores isolated from soil sown with wheat after maize was 23 spores g^{-1} soil at Elbehera governorate. At El-Menofia, three crops were sown after maize i.e. wheat (20 spores g^{-1} soil), onion (18 spores g^{-1} soil) and bean (15 spores g^{-1} soil); whereas two crops were sown at Ismailia after maize i.e., Barley (16 spore/g soil) and clover (16 spores g^{-1} soil). Some crops couraged VAM spores existence in the next crops i.e. pear before clover (28 spores g^{-1} soil) at El-Menofia, soybean before pepper (17 spore/g soil).

It is noticed in that the type of soil had an effect on survival of VAM spores.

Clay soil revealed a good medium for survival of VAM spores.

On the other hand pH of Egyptian soil had no effect on the VAM spores its worth to note that there is no effect at EC 100-400 µmhos/cm and (40-260) TDS of Egyptian soil.

Table 2: Number of VAM fungi spores extracted from some Egyptian governorates during winter season

Governorate	The current crop	Ph of soil	Soil type	EC (µmhos/cm)	TDS (µmhos/cm)	The previous crop	No. of spore/g soil
El-behera	Maize	Wheat	Clay	7.2	300	180	23
	Maize	Lentil	Clay	7.0	400	230	5
	Maize	Clover	Clay	7.0	400	240	12
	Maize	Bean	Clay	7.2	400	210	8
El-menofia	Maize	Wheat	Clay	7.2	200	130	20
	Pear	Clover	Clay	7.0	300	160	28
	Maize	Bean	Clay	7.2	200	130	15
	Maize	Onion	Clay	7.0	200	150	18
Beni-Suef	Maize	Wheat	Clay	7.2	400	190	13
	Maize	Bean	Clay	7.3	400	200	4
	Maize	Potato	Clay	7.2	100	80	12
	Maize	Clover	Clay	7.3	300	130	7
	Maize	Onion	Clay	7.2	100	70	45
	Maize	Lentil	Clay	7.2	100	80	26
	Maize	Beets	Clay	7.2	200	150	17
Ismailia	Maize	Wheat	Sandy	7.2	300	160	10
	Soybean	Lupine	Sandy	7.2	300	170	5
	Maize	Barley	Sandy	7.2	200	160	16
	Maize	Clover	Sandy	6.8	100	60	15
	Soybean	Onion	Sandy	6.8	150	90	12
	Soybean	Pepper	Sandy	6.8	200	95	17
	Sesame	Eggplant	Sandy	7.0	200	90	17
	Sesame	Tomato	Sandy	6.8	200	85	16

Isolation of VAM spores

Percentage of genera of mycorrhizal fungi in different type soil mixtures: Natural occurrence of VAM fungal species, belonging to four genera such as; *Acaulospora*, *Glomus*, *Gigaspora* and *Sclerocystis* were observed in the rhizosphere soils of four governorates of Egypt (Behira, El-Menofia, Beni Suef and Ismailia. Frequency percentages of aforementioned genera in 10 g soil were *Glomus* (57-100%) in aforementioned governorates, *Acaulospora* (14-22%) in Behira and Ismailia, *Gigaspora* (21-26%) in Behira and Ismailia and *Sclerocystis* (32%) in El-menofia. *Glomus* was the dominant genus in all inspected governments with the highest frequencies as it shown in Table 3.

Purification and Identification of *Glomus* spp.: Examined spores were identified according to the morphological characteristics using keys of Gerdemann and Trappe (1974), Trappe (1982). Data in Table 4 show the main characteristics (color, shape and size) to determine the differentiation between species of genus *Glomus* the shape was not critical character where all species had globose and subglobose shape but some spores of species *G. mosseae* and *G. coronatum* had irregular shape. It is worth to note that the identification was also made according to the size of *Glomus* spp. *G. intraradicesis* was the smallest (50-67 μm) compared with other *Glomus* spp. While *G. mosseae* and *G. etunicatum* 3 was large (<100 μm) but they were different in color (*G. mosseae* Hyaline-dark orange to brown and *G. etunicatum* 3 was Pale orange brown to dark orange brown).

Variation of *Glomus* spp. isolates among polymorphic DNA markers: This analysis was carried out on 8 isolates. The eight isolates were isolated from different Egyptian governorates.

Table 3: Frequency Percentages of genera of mycorrhizal fungi in 100 g soil of four governorate of Egypt

Governorates	Genus	Percentages
El-Behira	*Glomus* sp.	57BC*
	Gigaspora sp	21D
	Acaulospora sp.	22D
El-Menofia	*Glomus* sp.	68B
	Sclerocystis sp.	32CD
Beni Suef	*Glomus* sp.	100A
Ismailia	*Glomus* sp.	60BC
	Gigaspora sp.	26D
	Acaulospora sp.	14D

*Data in the same column followed by the same letter are not significantly different ($p<0.05$)

Table 4: Characteristics of *Glomus* spp., isolated from four governorates in Egypt

Governorates	Species	Color	Shape	Size (μm)
Behira	*Glomus intraradices*	White, cream, yellow brown	Globose, subglobose	52-67
	G. etunicatum 1	White to yellow brown	Globose, subglobose	58-100
	G. etunicatum 2	White to yellow brown	Globose, subglobose	100-167
Beni suef	*G. etunicatum* 3	yellow brown	Globose, subglobose	60-160
Ismailia	*G. mosseae*	hyaline to dark orange- brown	Globose, subglobose some irregular	100-150
	G. diaphanum	Hyaline to white	Globose, subglobose	60-85
El-Menofia	*G. etunicatum* 4	White, Pale yellow to dark yellow	Globose, subglobose	50-84
	G. coronatum	Pal orange brown to dark orange brown	Globose to some globose, some irregular	80-140

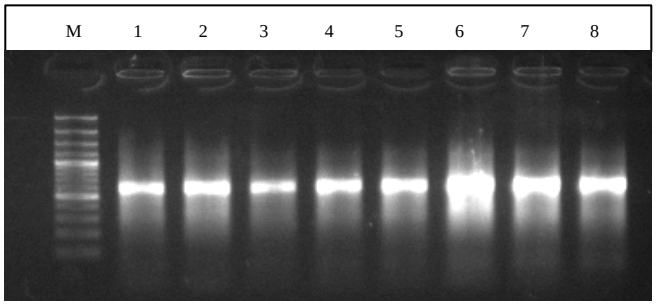

Fig. 1: (1%) gel electrophoresis of DNA extracted from 8 strains of VAM fungi to detect the presence of 550-bp ribosomal DNA fragments with VANS1/NS21. M DNA marker, Lanes (1-8) presenting isolates of *Glomus G. intraradices*, *G. etunicatum* 1, *G. etunicatum* 2, *G. etunicatum* 3, *G. mosseae*, *G. diaphanum*, *G. etunicatum* 4 and *G. coronatum* respectively, show the presence of 550-bp in all lanes

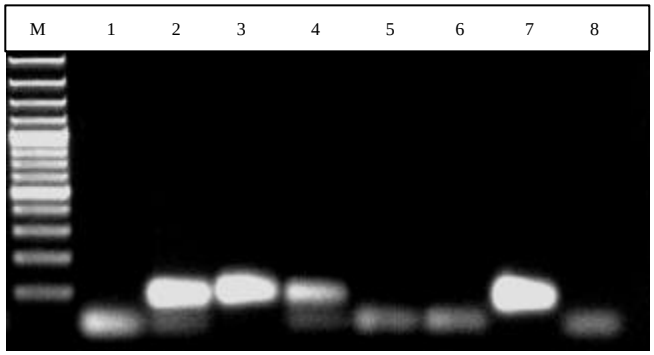

Fig. 2: (1%) gel electrophoresis of DNA extracted from 8 strains of VAM fungi to detect the presence of 100-bp ribosomal DNA fragments with VANS1/VALETC primer. M DNA marker, Lanes (1-8) presenting isolates of *Glomus G. intraradices*, *G. etunicatum*1, *G. etunicatum* 2, *G. etunicatum* 3, *G. mosseae*, *G. diaphanum*, *G. etunicatum* 4 and *G. coronatum* respectively, show the presence of 100-bp in lanes 2, 3, 4, 7

The VANS1/NS21 primer pair was used to amplify 550-bp ribosomal (r) DNA fragment from vesicular arbuscular mycorrhizal fungi, directly from spores and colonized root extracts. A procedure to detect these obligatory biotrophs rapidly, based on competitive PCR, was developed by constructing a suitable internal standard to be used with these primers. DNA fragment appeared identical in all studded *Glomus* spp. (Fig. 1).

The VANS1/VALETC primer pair was used to amplifying a 100bp ribosomal (r) DNA fragment would be classified as belonging to *Glomus etunicatum*, directly from spores and colonized root extracts. A procedure to detect these obligatory biotrophs rapidly, based on competitive PCR, was developed by constructing a suitable internal standard to be used with these primers. DNA fragment appeared identical in isolates 2,3,4,7 *Glomus etunicatum* as shown in Fig. 2.

The lipid differentiation by evaluation lipid on root of grass: Data in Table 5 the total Methyl Esters Fatty Acids in all 8 *Glomus* isolates on root of grass which illustrated that Palmitic acid (16:0), Oleic acid (cis18:1n9) and Stearic acid (18:0) were the dominant fatty acid of the

Table 5: Total methyl esters fatty acids in *glomus* isolates on root of grass

		Isolates*							
Lipid name	Lipid numbers	1	2	3	4	5	6	7	8
Myristic acid	14:00	0.00	2.21	2.02	1.62	2.30	17.70	1.36	0.96
Nonahexacontanoic acid	-	0.00	1.00	2.18	0.60	0.00	0.11	0.00	0.00
Pentadecanoic acid	15:00	0.00	0.68	0.48	0.49	0.00	0.26	0.00	0.78
Palmitoleic acid	16:1n7	0.00	1.67	2.83	0.68	0.00	0.47	1.72	17.10
Linoleic acid	18:2n6	0.00	1.92	0.00	0.00	0.00	0.00	0.00	13.20
Ecosanoic acid(arachidic acid)	20:00	0.00	1.96	2.34	3.01	0.00	1.55	2.82	1.75
Dodecanoic acid (lauric acid)	12:00	0.00	0.00	0.00	1.50	0.00	13.50	13.30	0.00
Nonanoic acid	9:00	0.00	3.46	0.40	0.38	0.00	0.00	0.00	0.00
Docosanoic acid	22:00	0.00	1.08	1.86	2.22	0.00	1.23	0.00	2.20
Palmitic acid	16:00	17.90	41.50	40.89	25.90	31.40	16.20	33.10	24.70
Oleic acid	cis18:1n9	2.03	3.51	5.18	3.12	6.03	3.98	4.85	12.30
Stearic acid	18:00	7.72	25.40	19.30	31.00	17.50	14.70	18.40	9.20
Elaidic acid	Trans18:1n9	1.76	2.54	0.00	3.12	0.00	0.00	0.00	11.20
Margaric acid (heptadecanoic)	17:00	0.81	3.51	0.00	0.00	0.00	3.98	0.86	0.00
Phthalic acid	8:00	69.70	9.34	22.45	26.20	42.70	26.10	23.50	1.40
Erucic acid	22:1n9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.06
Lignocceric acid	24:00:00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.42
Cerotic acid	26:00:00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.30
Pentacosanoic acid	25:00:00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.49
Ecosatrienoic acid	20:3n3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.83

Glomus spp. Saturated fatty acid, 16:0 was present in all isolates and constituted 16.2 to 41.5% of the total profile. One unsaturated fatty acid, cis18:1n9 was present in all isolates and constituted 2.03-12.3% of the total profile. A high proportion of 18:1n9 *cis* also occurred in (isolate 8) *G. coronatum* was unique in possessing most of fatty acid. Conversely, 18:1n9 Trans accounted for 1.76 to 11.2% of the total fatty acids in *Glomus* isolates 1,2,4,8.

The Myristic acids (14:0), ranging from 0.96 to 17.7%, palmitoleic acid (16:1n7 *cis*) 0.47 to 17.1, archidic acid (20:0) 1.75 to 3.01, palmitic acid (16:0)16.2 to 41.5, oleic acid (cis18:1n9) 2.03 to 12.3 and stearic acid (18:0) 7.72 to 25.4 were present in all isolates of *Glomus etunicatum* isolates 2,3,4,7, also Linoleic acid (18:2n6) was present only in *Glomus etunicatum* isolates (2) and *G. coronatum* (isolate 8).

DISCUSSION

In summer season it was found that the highest numbers of vesicular arbuscular mycorrhizal (VAM) fung l spores g^{-1} soil were recorded on maize grown after winter crops i.e. wheat and clover at El-Behira, Ismailia, Beni-Suef and El-Menofia governorates respectively. On the other hand, three crops grown after barley, i.e., water melon (El-Behira), peanut (Ismailia) and pepper (El-Behira) recorded high numbers of spores. In winter season the highest VAM fungi spores numbers were extracted from onion, lentil and sugar beet rhizosphere respectively grown after maize at Beni-Suef governorate. Sand soil was the best medium for survival of VAM spores in summer season but Clay soil revealed a good medium for survival of VAM spores in winter season. On the other hand pH of Egyptian soil had no effect on the VAM spores. Fares (1986) examined soil samples taken from around the roots of 23 different hosts grown in 5 sites in Egypt, i.e., El- Kaliobia, Ismailia, Alexandria, El-Minia and Sinai desert.

Schultz *et al.* (1999) and Bever *et al.* (2001) also found evidence that AM fungi differ in their seasonality, with some fungi sporulating in late spring and others sporulating at the end of summer. As the spores represent the dormant state of the fungus, the physiologically active state is most likely the mirror image of the seasonal spore counts. Therefore, *Gigaspora gigantea* which sporulates most abundantly in the fall and appears to overwinter as spores is likely to be physiologically active during the warm season. Similar patterns have been seen for *Gi. gigantea* in a sand dune on the coast of Rhode Island (Gemma *et al.*, 1989; Lee and Koske 1994). On the other hand, *Acaulospora colossica* which sporulates most profusely at the beginning of summer and over summers as spores, is physiologically active with the cool season plant community (e.g., *Allium vineale*). Hayman (1982) indicated that variation in the populations of the fungi and their symbiosis with plant roots is related to both soil properties and host plants. In addition, species and isolates of AMF differ in their tolerance to adverse physical and chemical conditions in soil (Juniper and Abbott 1993).

Arbuscular mycorrhizal fungi are also known to vary in their response to the mineral environment of the soil. About 30% of the variation in the spatial distribution of AM fungi could be explained by variation in aspects of the mineral soil (Schultz 1996; Bever *et al.*, 2001). Individual fungi showed opposite associations with certain soil parameters. For example, the distribution and abundance of *A. colossica* was negatively associated with soil phosphorus concentration, while the reverse was true for *G. gigantea* (Schultz, 1996). This dependence of fungal spatial distributions on edaphic factors is consistent with observations in other communities, including tall grass prairie (Johnson *et al.* 1992) and sand dunes (Koske 1981). However, previous study on AM fungi sporulation in different seasons showed that heat can reduce AM fungal spore production in arid and semiarid areas (Camargo-Ricalde and Esperon-Rodriguez 2005). Lugo *et al.*, 2005 found plant-specific and AM fungi-specific variations in the formation of intraradical AM fungal structures and spores in the moist and dry part of the year in an Argentinean dry climate with no seasonal variation in temperature.

In this study natural occurrence of VAM fung, belonged to four genera such as; *Acaulospora*, *Glomus*, *Gigaspora* and *Sclerocystis* were observed in the rhizosphere soils of four governorates in Egypt Behira, El-Menofia, Beni Suef and Ismailia . Percentages of aforementioned genera in 10 g soil were *Glomus* (57-100%) in all governorates, *Acaulospora* (14-22%) in Behira and Ismailia, *Gigaspora* (21-26%) in Behira and Ismailia and *Sclerocystis* (32%) in El-menofia. *Glomus* was the dominant genus in all inspected governments with the highest frequencies. This result agrees with that of Fares (1986) showed that morphological characteristics revealed that the tested spores belonged to the genera *Glomus*, *Gigaspora* and *Sclerocystis*. Spores belonged to genus *Glomus* were the most dominant. These spores were identified as *G. mosseae*, *G. clarum* and *G. fasciculatum*. A good number of spores was recorded in the rhizosphere soil of non-colonized plant species in saline depression habitat. Tressner and Hayes (1971), Hirrel (1981) suggested that AMF sporulation is stimulated under salt stress conditions, but In this study arbuscular mycorrhizal fungi were found to be present in almost all samples and show that AMF and their symbiosis with plants are widely spread in Egypt.

Simon *et al.* (1993) designed primers to identify AMF in colonized roots by PCR fragment amplification of the 18S rDNA combined with the SSCP analysis. The VALETC, VAGLO, VAACAU and VAGIGA primers were designed to discriminate among four distinct groups of endomycorrhizal species. Furthermore they designed the VANS22 and VANS32 which were able to amplify a 150-bp informative fragment from any endomycorrhizal fungi. These primers were not designed specifically for Glomales and Simon *et al.* (1993) mentioned the possibility that they could also be

useful for other fungi or eukaryotes. Later, Simon and Lalonde (1995) designed and patented the VANS1 primer which amplified part of the 18S rDNA from AMF (*Glomus intraradices* and *Gigaspora margarita*) directly from colonized roots. However, Simon (1996) concluded that primers pairs VANS22/VANS32 and NS71/SSU1492 can only detect AMF genus differences. Furthermore, he reported that those primers are not AMF specific and samples must be treated with a nested PCR amplified firstly by the primers VANS1 (Glomalean specific) and VANS22 and then the amplicons amplified by the VANS22/VANS32 primers.

The results of the present study show that VANS1/NS21 primer pair is useful for specifically amplifying use as review for the three primers used in this study Simon *et al.* (1993) 550-bp ribosomal (r) DNA fragment from arbuscular endomycorrhizal fungi, directly from spores and colonized root extracts. A procedure to these obligatory biotrophs rapidly, based on competitive PCR, was developed by constructing a suitable internal standard to be used with these primers. The taxon-specific primers, VANS1 and NS21. DNA fragment showed identical in all arbuscular endomycorrhizal fungi. These results are similar to those obtained by Simon *et al* (1992) who indicated that a portion of the small subunit rRNA gene (rDNA) specific for AM fungi can be amplified when a taxon-specific primer (VANS1) is paired with a universal primer (NS21). And examined the specificity of the VANS1 primer, when paired it with a downstream universal primer (NS21) and tested its ability to amplify a 550-bp portion of the SSU sequences from DNAs of other arbuscular endomycorrhizal fungi as well as a variety of other fungi and plants. The fungi selected for testing spanned a broad phylogenetic range and included putative relatives of arbuscular endomycorrhizal fungi as well as other rhizosphere associates such as ectomycorrhizal species and root pathogens. Also Di Bonito *et al.* (1995) concluded that PCR was used with the primer pair VANS1-NS21 to detect the arbuscular mycorrhizal fungus *Glomus intraradices* (commercial inoculum source) on roots of lettuce, zinnia, leek, pepper and endive plants and during the application of these methods a number of problems have been identified. Specificity is limited to the level of genus and may not be absolute. The studies of PCR application for AM fungi have been carried out with spores or purified DNA from colonized leek roots (Wyss and Bonfante, 1993) the AM taxon-specific primer VANS1 was used for detection of the AM fungus *Glomus intraradices* on roots of five host species colonized by this fungus. This was accomplished without extraction of DNA from colonized roots by simply boiling root tissue in a buffer (Henson *et al.*, 1993).

The VANS1/VALETC primer pair is useful for specifically amplifying use as review for the three primers used in this study Simon *et al.* (1993) 100bp ribosomal (r) DNA fragment would be classified as belonging to *Glomus etunicatum*, directly from spores and colonized root extracts. A procedure to these obligatory biotrophs rapidly, based on competitive PCR, was developed by constructing a suitable internal standard to be used with these primers. The taxon-specific primers, VANS1 and VALETC. DNA fragment showed identical in isolates 2, 3, 4, 7 *Glomus etunicatum*. SSCP analysis is a technique that could detect sequence variation, sometimes as small as a single-base substitution, between DNA fragments ranging from 100 to 450 bp Simon *et al.* (1992). By using SSCP analysis, fragments amplified using a single primer pair could be further characterized without the need for other specific primers or probes. This was illustrated by the analysis of fragments amplified with primer pair VANS1-VAACAU, with which discrimination between related species was achieved.

In this study Palmitic acid (16:0), Oleic acid (cis18:1w9) and Stearic acid (18:0) were the dominant fatty acid of the *Glomus* spp. Saturated fatty acid, 16:0 was present in all isolates and constituted 2.65 to 20% of the total profile. As Graham *et al.* (1995) reported that in spores, 16:0 was present in all isolates of *Glomus* spp. and constituted 10 to 38% of the total profile. Also some

fatty acids have been known as specific markers for AM fungi. In the case of *Glomus* species, fatty acids 16:0, 16:1n5, 18:1n7, 18:1n9, 20:3, 20:4 and 20:5 have been detected in *Glomus* spores and the roots of plants colonized by *Glomus* spp. (Beilby and Kidby, 1980; Jabaji-Hare, 1988; Pacovsky and Fuller, 1988; Pacovsky, 1989; Graham *et al*., 1995; Jansa *et al*., 1999; Madan *et al*., 2002). On the other hand (Sakamoto *et al*., 2004) conclude that 18:1n9 and 20:1n9 can be used as a specific marker for identifying and quantifying the external hyphae of *Gigaspora* species in soil (Bentivenga and Morton, 1994, 1996; Graham *et al*., 1995; Madan *et al*., 2002).

One unsaturated fatty acid, cis18:1n9 was present in all isolates and constituted 0.30 to 6.84% of the total profile. A high proportion of 18:1n9 *cis* also occurred in (isolate 8) *G. coronatum* was unique in possessing most of fatty acid. Conversely, 18:1n9 Trans accounted for 0.26 to 6.18% of the total fatty acids in *Glomus* isolates 1,2,4,8. As Graham *et al*. (1995) reported that 18:1n9 *cis* accounted for 38 to 48% of the total fatty acids in all *Gigaspora* isolates tested. A high proportion of 18:1n9 *cis* also occurred in *G. leptotichum* and *Scutellospora persica*. Isolates of *G. leptotichum* and *G. occultum* were unique in possessing 16:1n7 *cis*. These results are in a harmony with those reported by Peng *et al*. (1993) where he stated that 16:1n7 *cis* was erroneously reported to be the principal fatty acid present in citrus roots infected by *G. intraradices* . Many authors have reported the presence of 18:1n9 in the spore of *Glomus,Gigaspora, Scutellospora* and *Acaulospora* species (Graham *et al*., 1995; Bentivenga and Morton, 1996; Madan *et al*., 2002) and in the soybean root colonized by *Glomus fasciculatum* (Pacovsky and Fuller, 1988).

The Myristic acids (14:0), ranging from 0.4 to 1.06%, palmitoleic acid (16:1n7 *cis*) 0.18 to 1.05, archidic acid (20:0) 0.8 to 1.08, palmitic acid (16:0)6.9 to 12.65, oleic acid (cis18:1n9) 0.83 to 1.68 and stearic acid (18:0) 7.06 to 12.15 were present in all isolates of *Glomus etunicatum* isolates 2, 3, 4, 7 also Linoleic acid (18:2n6) was present only in *Glomus etunicatum* isolates (2, 3, 4, 7) and *G. coronatum* (isolate 8).

Morton (1985) stated that Profiles of *G. etunicatum* were distinct in that 20:0 iso was unique to these isolates and the one isolate of *G. claroideum* studied. *G. leptotichum* and *G. occultum* isolates were distinguished from all other glomalean fungi by the high levels of 16:1n7 *cis* (almost 50% in two *G. occultum* isolates).

The end product of fungal Fatty Acid Synthesis (FAS) is palmitic acid (16:0) which can then be elongated and desaturated to produce all of the other fatty acids (Williams *et al*., 1987). Trepanier *et al*. (2005) reported that in germinating spores and the extraradical hyphae of *G. intraradices* supplied with [14C] acetate, only fatty acids longer than 16 carbons were labeled which suggests that no de novo fatty acid synthesis by FAS was occurring in these two fungal cell domains. Previous work (Phillips and Hayman, 1970) also found no labeling of 16:1n5 when extraradical hyphae were supplied with 13C-acetate. In other study, germinating spores and the extraradical mycelium of *G. intraradices* could elongate pre-existing 16-carbon fatty acids (16:0 or 16:1) with labeled acetate to form 18- and 20-carbon fatty acids. An equivalent result was obtained with *G. rosea*, demonstrating that this fungus also lacks FAS activity in germinating spores. These two phylogenetically distant AM fungi share the same lack of enzymatic activity suggests that the lack of FAS syntheses might be a general feature of the early development of AM fungi. 16-Carbon fatty acids of *G. intraradices* were labeled only when [14C] acetate or [14C] sucrose was added to colonized roots. In the root compartment, plant fatty acids were highly labeled and mixed with the fungal fatty acids. Carrot roots contain only minute levels of 16:1, so this strongly labeled fatty acid was almost exclusively of fungal origin. Both organisms synthesize the other fatty acids and their origins cannot be distinguished. When radioactive precursors were included in the root compartment, all fungal compartment fatty acids also became highly labeled, especially the most abundant one: 16:1.

CONCLUSION

The previous study showed that there were many factors affecting the activity of mycorrhizal fungi such as season. Palmitic acid (16:0), Oleic acid (cis18: 1w9) and Stearic acid (18:0) were found in all isolates that were under investigation. However, other fatty acid was found to be specific for the species.

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