

USE OF FATTY ACID PROFILES TO DIFFERENTIATE BETWEEN ISOLATES OF *Fusarium oxysporum* F.SP. *Vasinfecum*, THE COTTON WILT PATHOGEN AND OTHER ISOLATES OF *Fusarium oxysporum*



Eman A. Osman

Plant Pathology Research Institute, Agriculture Research Center, Giza, Egypt.

ABSTRACT

Twenty three fatty acid profiles were qualitatively and quantitatively determined by using fatty acid methyl esterification (FAME) to differentiate between three isolates of *Fusarium oxysporum* f.sp. *vasinfecum*, the cotton wilt pathogen, and seven isolates of *Fusarium oxysporum* from other hosts. Cluster analysis by the unweighted group method with arithmetic mean (UPGMA) demonstrated that although qualitative profiles were simpler than the quantitative ones, they were more useful in differentiation between *Fusarium oxysporum* f. sp. *vasinfecolates* and the other isolates of *Fusarium oxysporum*.

INTRODUCTION

Fusarium oxysporum Schlechtend: Fr. plays the role of a silent assassin – the pathogenic strains of this fungus can be dormant for thirty years before resuming virulence and infecting a plant (Smith and Smedley, 2011). *F. oxysporum* is famous for causing a disease called Fusarium wilt which is lethal to plants. By time the plant shows any outward signs of infection, it is already too late, and the plant may die. Additionally, isolates of *F. oxysporum* are not discriminating, they can cause disease in nearly every agriculturally important plant (Smith and Smedley, 2011). Not only is bad enough for farmers to sustain the loss of one rotation of crops to Fusarium wilt, but as a whole *F. oxysporum* proves to be incredibly tough to eradicate. To combat this scourge, scientists developed wilt-resistant varieties of crops. Usage of resistant varieties is the most effective and practical mean of control. *F. oxysporum* has many formae speciales (special forms) that exist as plant pathogen, which are differentiated by host range. *F.oxysporum* and its various formae speciales have been characterized as causing: vascular wilt, yellow, crown rot, root rot, and damping off. The most important of these is vascular wilt (Gonsalves and Ferreira, 1993). In general, the fungus manage to infect the vascular system of plant, where it cause their deleterious effects. This infiltration to the vascular system affects the plant's water supply greatly. A lack of water will induce the leaves' stomata to close and the leaves to wilt. The leaves turn yellow or brown before falling off completely.

Early attempts to apply cellular fatty acid (CFA) analysis to bacterial identification were made in 1950s (James and Martin, 1952). In 1963, Abel *et al.*, were the first to present evidence suggesting that CFA analysis by Gas-Liquid Chromatography (GLC) could successfully identify bacteria. Analysis of CFA composition routinely is used to characterize, differentiate and identify

genera, species and strains of bacteria and yeast (Augustyn, *et al.*, 1990; Bronz, *et al.*, 1989; Sasser, 1990; Sasser, 1991; Veys, *et al.*, 1989; and Welch, 1991). Taxa are distinguishable by the types of fatty acids produced and the relative concentrations of individual fatty acids (Larkin and Groves, 2003). Although fungi and related taxa generally produce few types and lower quantities of fatty acids than bacteria, there is growing evidence that fatty acid profiles may also be useful for the identification and characterization of fungi such as species and sub specific groups of *Penicillium* and *Rhizoctonia* (Lopes, *et al.*, 1998; Müller, *et al.*, 1994; Rueset *et al.*, 2002; Stahl and Klug, 1996; Stevens and Jones, 1994; Stevens and Jones, 2001).

As part of an effort to develop methods to facilitate the analysis of microbial communities in soil, Stahl and Klug (1996) have been evaluating the use of cellular fatty acid profiles as a method to rapidly characterize and differentiate fungal isolates. When compared with standard morphological methods, this approach requires significantly less time and taxonomic expertise to characterize unknown isolates.

Fusarium spp. is an example of fungi that has been analyzed by CFA content (Staratt and Madhosingh, 1967) through mycelium fatty acid methyl esterification (FAME). The technology of CFA analysis has gained applicability for several purposes. It offers considerable power as a tool for fungal identification because characteristic patterns of CFAs can be defined for several fungi at the species level and results are achievable rapidly (Larkin and Groves, 2003). FAME profile proved to be useful in differentiation between isolates of *F. oxysporum* f.sp. *lycopersici* and *F. oxysporum* f. sp. *radices-lycopersici* (Matsumoto, 2006). Lanoiselet *et al.*, (2005) reported that the total cellular fatty acid analysis from *Rhizoctonia spp.* permitted differentiation between isolates of *R. oryzae* and *R. oryzae-sativae*.

In this study, FAME was used to differentiate between isolates of *Fusarium oxysporum* f.sp. *vasinfectum* and isolates of *Fusarium oxysporum* from other hosts.

MATERIALS AND METHODS

Pathogenicity test:

Before using *F. oxysporum* isolates to conduct fatty acids analysis, greenhouse pathogenicity test was performed on cotton line 260/2014, which is highly susceptible to *Fusarium* wilt, by using ten isolates of *F. oxysporum* (Table 1). Substrate for growth of each isolate was prepared in 500-ml glass bottles. Each bottle contained 50 gm of sorghum grains and 40 ml of tap water. Contents of bottles were autoclaved for 30 minutes. Inoculum was obtained from one-week-old culture on Potato Dextrose Agar medium (PDA), this inoculum was aseptically introduced into the bottle and allowed to colonize sorghum grains for three weeks. Fungus-sorghum mixture was used to infest soil (50 gm/kg soil). Infested soil was dispensed in a 15-cm-diameter clay pots (three replicates for each isolate) and each pot was planted with 10 seeds of line 260/2014. Pots were placed on greenhouse benches.

Temperature of the greenhouse ranged from 28 to 35°C. The final results were recorded after 45 days from planting date. Seedlings which

showed cotyledonary yellowing and death were determined. Apparently healthy seedlings were cut diagonally across root and stem to examine the vascular discoloration.

Preparation of fungal cultures for fatty acids analysis:

The fungal isolates were grown in 100 ml Potato Dextrose Broth in 250-ml Erlenmeyer flasks. Flasks (10 flasks for each isolate) were inoculated with 15-mm-diameter disks of mycelia of each isolate, taken from one-week-old culture on PDA. After 21 days the growth of each isolate was harvested on filter paper then washed in sterile deionized water and dried in oven for 3 days on 50°C.

Extraction and analysis of fatty acids:

Extraction of fatty acids and analysis were conducted at the Regional Center of Food and Feedstuff (Agriculture Research Center, Giza- Cairo). Fatty acid profile of *F. oxysporum* isolates were trans esterified into their corresponding fatty acids methyl esterases (FAMES) using methanolic sodium hydroxide (NaOH) and boron trifluoride (BF₃) with methanol described by AOAC (2012). The FAME were quantified by Shematizu Gas Chromatograph (GC) Series 2010 equipped with a 2010 + Sautosampler (Japan) and interfaced with a Flame Ionization Detector (FID). The GC was equipped with a temperature programmable column. The column phase was *Supplco* DB-Wax (carbowax) with the following dimensions: 30 m long, 0.25 mm i.e. (internal diameter) with a 0.25 μ phase thickness. Helium was used as a carrier gas with flow 40 ml/min. One microliter was injected using the inlet a split mode. The head pressure was set at 2 psi, and the split vent flow was 7ml/min. The injector temperature was 250°C. the column flow rate at 2 psi was 0.68 ml/min. The column temperature was maintained at 200°C for 10°C/s and was held at 260°C for 80 min. The detector was operated in the selected ion monitoring mode. Fatty acids were identified by retention times obtained from the FAME standard (Sigma Company, St. Louis, MO). The determined was conducted in twice GC injections per extraction. The fatty acid contained was reported as percentage of total fatty acids.

Cluster analysis:

Pearson correlation coefficient (r) was calculated to measure the degree of association between fatty acid profiles for each pair of isolates. Based on these data, a correlation matrix was constructed and from this matrix isolates were clustered by the unweighted pair group method based on arithmetic mean (UPGMA). Cluster analysis was performed using SPSS 6.0 software package.

RESULTS AND DISCUSSION

When ten isolates of *F. oxysporum* tested on the highly susceptible cotton line 260/2014, three isolates no_s. 1, 2 and 3 exhibited the typical external and internal wilt symptoms, while the remaining isolates did not show any symptoms on this line (Table 1). The external symptoms were vein discoloration in the cotyledonary leaves usually began at the margin, then turned yellow or brown and eventually the entire leaf wilted and death of

seedlings occurs. The internal symptoms were discoloration of xylem vessels, which was observed by cutting plants diagonally across the root. Based on the observed external and internal symptoms it was concluded that isolates no_s. 1,2 and 3 belong to *F. oxysporum* f.sp. *vasinfectum*, while the other isolates belong to *F. oxysporum*. Fusarium wilt of cotton caused by *F. oxysporum* f.sp. *vasinfectum* is a soil inhabitant fungus which causes vascular wilt in susceptible cotton cultivars (Watkins 1981, Hillocks 1992). The first report of Fusarium wilt in Egypt dates back to the 1920s (Fahmy, 1927). Occurrence of Fusarium wilt in Egypt reported in the Nile Valley, where it remains one of the most damaging pathogen on *Gossypium barbadense* cultivars (Watkins, 1981; and Abd El-Salam *et al*, 2004).

Regarding fatty acid content of *F. oxysporum* isolates, twenty three fatty acids were detected (Table 2). This number is much less than the fatty acid profiles of bacteria, which are as high as 184 (Sasser, 1990). Palmitic acid, stearic acid, oleic acid, linoleic acid and lineolenic acid were found in all isolates but in different quantities. This result is in agreement with Starrat and Madhosingh (1967) who reported that palmitic, stearic, oleic and linoleic acids were identified by gas-liquid chromatography as the principal fatty acids of *F. oxysporum*. On the other hand, some fatty acids were found in only one isolate such as Gamma linolenic acid, which was only found in isolate no 4 and Cis-9-eicosaenoic acid which was only found in isolate no 6 (Table 3). This result is also in agreement with the previous reports, which indicated that fungi have only a small number of fatty acids (Collins and Kalnins, 1968; and Rambo and Bean, 1974) which can be used for differentiation.

The dendrogram shown in figure 1 classified the isolates quantitatively into four groups. The first group (the largest group) included isolates no_s. 6, 7, 1 and 5. The second group included isolates no_s. 8, 9 and 10. The third group included isolates no_s. 3 and 4. Isolates no 2 of FOV constituted a unique group unrelated to the remaining isolates. The level of similarity within isolates of each group was greater than those between isolates from different groups. For example, the level of similarity between isolates no 6 and no 5 was 0.992 while the level of similarity between isolates no 6 and no 3 was 0.596 (Table 4). Isolate no. 2 showed the lowest levels of similarity with the other isolates ranging from 0.063 to 0.598. Dendrogram of figure 1 showed that isolates 1,2 and 3 of FOV were widely separated.

The dendrogram shown in figure 2 divided the isolates qualitatively into six groups. Isolates no_s. 8, 10 and 9 were placed in the first group. Isolates no_s. 1,2 and 3 were included in the second group. Each of isolates no. 5, no. 6, no.7 and no. 4 constituted separate group (Fig. 2). It is worth noting that all isolates of *F. oxysporum* f. sp. *vasinfectum* (isolates no_s. 1, 2 and 3) were included in one group (Distance = 10). Within this group the similarity levels ranged from 0.607 to 0.704. It is noticeable that the isolates of *F. oxysporum* f. sp. *vasinfectum* were placed in one group. This result indicates that the qualitative fatty acid profiles can be used to differentiate between isolates of *F. oxysporum* f. sp. *vasinfectum* and other isolates of *F. oxysporum*. However, this conclusion needs to be critically tested by analyzing a more comprehensive collection of isolates.

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Figure 1. Phenogram based on average linkage cluster analysis of quantitative fatty acid profiles obtained from ten isolates of *Fusarium oxysporum*. The tested isolates are: *Fusarium oxysporum* f.sp. *vasinfectum* from cotton (S1), *Fusarium oxysporum* f.sp. *vasinfectum* from cotton (S2), *Fusarium oxysporum* f.sp. *vasinfectum* from cotton (S3), *Fusarium oxysporum* from pepper (S4), *Fusarium oxysporum* from pepper (S5), *Fusarium oxysporum* from cotton (S6), *Fusarium oxysporum* from cotton (S7), *Fusarium oxysporum* from cotton (S8), *Fusarium oxysporum* from onion (S9) and *Fusarium oxysporum* from potato (S10)

Figure 2. Phenogram based on average linkage cluster analysis of qualitative fatty acid profiles obtained from ten isolates of *Fusarium oxysporum*. The tested isolates are: *Fusarium oxysporum* f.sp. *vasinfectum* from cotton (S1), *Fusarium oxysporum* f.sp. *vasinfectum* from cotton (S2), *Fusarium oxysporum* f.sp. *vasinfectum* from cotton (S3), *Fusarium oxysporum* from pepper (S4), *Fusarium oxysporum* from pepper (S5), *Fusarium oxysporum* from cotton (S6), *Fusarium oxysporum* from cotton (S7), *Fusarium oxysporum* from cotton (S8), *Fusarium oxysporum* from onion (S9) and *Fusarium oxysporum* from potato (S10)

These results were partially in agreement with Hering *et al*, 1999 who found that both qualitative and quantitative analyses of cellular fatty acids have been successfully used to characterize isolates of *F. oxysporum* f. sp. *vasinfectum*. Hering *et al*, 1999 also reported high correspondence between infection tests, morphological and random amplified polymorphic DNA (RAPD) studies with fatty acid profiles, which points out that this technique can be used for characterizing closely related fungal groups within a species.

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استخدام انماط الأحماض الدهنية للترقية بين عزلات فطر فيوزاريوم اوكسيسبورم
طراز فازينفيكتوم المسبب لذبول القطن و العزلات الأخرى لفطر فيوزاريوم
اوكسيسبورم

ايمان امين محمد عثمان

معهد بحوث أمراض النباتات- مركز البحوث الزراعية- الجيزة - مصر

عند تقدير الاحماض الدهنيه فى عزلات فطر فيوزاريوم اوكسيسبورم من عوائل مختلفه امكن الحصول على ٢٣ حمض دهنى. قدرت هذه الأحماض الدهنية نوعيا" و كميًا" بعد تحويلها الى مشتقات لأسترات الميثيل للترقية بين ثلاث عزلات لفطر فيوزاريوم اوكسيسبورم طراز متخصص فازينفيكتوم ، المسبب لذبول القطن ، و سبع عزلات من فطر فيوزاريوم اوكسيسبورم من عوائل مختلفة. أظهر التحليل العنقودى للنتائج أنه بالرغم من أن الأنماط النوعية للأحماض الدهنية أبسط من الانماط الكمية إلا أنها الأكثر جدوى فى الترقية بين عزلات فطر فيوزارم المسبب لذبول القطن و باقى عزلات الفيوزاريوم من العوائل المختلفة.

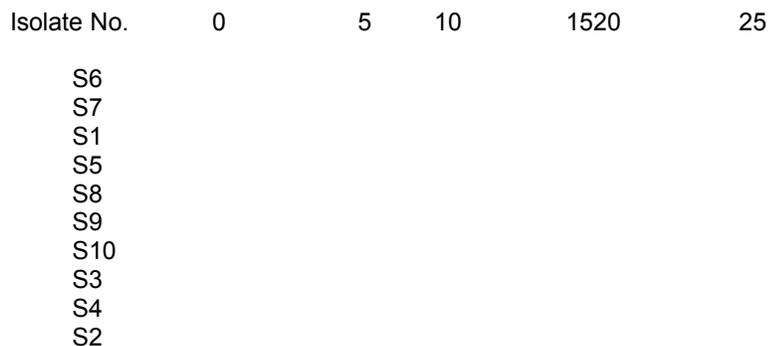


Figure 1. Phenogram based on average linkage cluster analysis of quantitative fatty acid profiles obtained from ten isolates of *Fusarium oxysporum*. The tested isolates are: *Fusarium oxysporum* f.sp. *vasinfectum* from cotton (S1), *Fusarium oxysporum* f.sp. *vasinfectum* from cotton (S2), *Fusarium oxysporum* f.sp. *vasinfectum* from cotton (S3), *Fusarium oxysporum* from pepper (S4), *Fusarium oxysporum* from pepper (S5), *Fusarium oxysporum* from cotton (S6), *Fusarium oxysporum* from cotton (S7), *Fusarium oxysporum* from cotton (S8), *Fusarium oxysporum* from onion (S9) and *Fusarium oxysporum* from potato (S10)

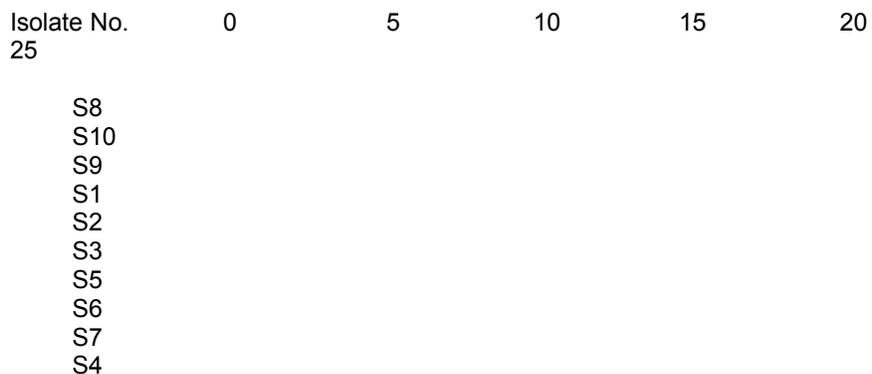


Figure 2. Phenogram based on average linkage cluster analysis of qualitative fatty acid profiles obtained from ten isolates of *Fusarium oxysporum*. The tested isolates are: *Fusarium oxysporum* f.sp. *vasinfectum* from cotton (S1), *Fusarium oxysporum* f.sp. *vasinfectum* from cotton (S2), *Fusarium oxysporum* f.sp. *vasinfectum* from cotton (S3), *Fusarium oxysporum* from pepper (S4), *Fusarium oxysporum* from pepper

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(S5), Fusariumoxysporum from cotton (S6), *Fusariumoxysporum* from cotton (S7), *Fusariumoxysporum* from cotton (S8), *Fusariumoxysporum* from onion (S9) and *Fusariumoxysporum* from potato (S10)

Table (5): Similarity among qualitative profiles of fatty acid from *Fusarium oxysporum* isolates

Isolate	Isolate									
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
S1	0.704									
S2	0.691	0.607								
S3	0.302	0.257	0.244							
S4	0.652	0.514	0.502	0.302						
S5	0.589	0.541	0.530	0.087	0.411					
S6	0.439	0.309	0.303	0.102	0.210	0.337				
S7	0.568	0.646	0.444	0.233	0.394	0.302	0.479			
S8	0.565	0.577	0.373	0.195	0.389	0.344	0.402	0.840		
S9	0.652	0.704	0.502	0.302	0.477	0.411	0.439	0.917	0.916	
S10										

^a Identification of isolates shown in Table 1

^b Linear correlation coefficient

Table (1): Pathogenicity of *Fusarium oxysporum* isolates on cotton (Line 260/2014) under greenhouse conditions

Isolate no. ^a	Classification	Host used in isolation	Wilt symptoms	
			External Symptoms (cotyledonary yellowing and/or dead seedlings)	External Symptoms (vascular discoloration)
S1	<i>Fusarium oxysporum</i> f.sp. <i>vasinfectum</i>	Cotton	+ ^b	+
S2	<i>Fusarium oxysporum</i> f.sp. <i>vasinfectum</i>	Cotton	+	+
S3	<i>Fusarium oxysporum</i> f.sp. <i>vasinfectum</i>	Cotton	+	+
S4	<i>Fusarium oxysporum</i> f.sp. <i>capsici</i>	Pepper	- ^c	-
S5	<i>Fusarium oxysporum</i> f.sp. <i>capsici</i>	Pepper	-	-
S6	<i>Fusarium oxysporum</i>	Cotton	-	-
S7	<i>Fusarium oxysporum</i>	Cotton	-	-
S8	<i>Fusarium oxysporum</i>	Cotton	-	-
S9	<i>Fusarium oxysporum</i> f. sp. <i>cepae</i>	Onion	-	-
S10	<i>Fusarium oxysporum</i> f. sp. <i>batatas</i>	Potato	-	-

^a Isolates of *F. oxysporum* f.sp. *vasinfectum* and other isolates of *F. oxysporum* from cotton were obtained from fungal culture collection of cotton and fiber crops diseases research Section, Plant Pathology Research Institute, Agric. Res. Cent. Giza, Egypt, while the other isolates of *F. oxysporum* were obtained from different sections of Plant Pathology Research Institute, Agric. Res. Cent. Giza, Egypt

^b Symptoms are present

^c Symptoms are absent

Table (2): Quantitative profiles of fatty acids extracted from different isolates of *Fusarium oxysporum*

Fatty acid	S1 ^a	S2	S3	S4	S5	S6	S7	S8	S9	S10
Myristic acid	0.52 ^b	0.51	0	0.10	0.47	0.53	0.69	0.34	0.41	0.53
Pentadecanoic acid	0.44	0.54	0	0	0	0.17	0.20	0.30	0.46	0.93
Palmitic acid	31.36	26.08	17.41	9.91	27.00	29.98	31.51	29.64	27.34	32.60
Palmitoleic acid	2.24	1.58	1.62	0.10	0.56	0.40	0.34	1.14	0	2.85
Stearic acid	11.54	8.46	4.93	4.75	8.16	9.73	11.16	8.34	7.39	10.17
Oleic acid	38.08	33.20	25.84	21.36	43.52	46.20	41.87	35.61	28.98	23.41
Vaccenic acid	1.43	0	0	0	2.32	2.52	2.08	0.19	1.23	0.36
Linoleic acid	10.41	28.52	46.63	52.79	11.52	5.67	2.61	21.67	29.40	22.55
Linolenic acid	0.42	1.11	2.06	7.36	0.62	0.10	0.10	1.17	1.79	6.17
Eicosaenoic acid	0.71	0	1.51	0	0	0.17	0.72	0	0	0
Gladoleic acid	0.60	0	0	0.20	0.28	0	0.42	0	0	0
Gamma linolenic acid	0	0	0	0.46	0	0	0	0	0	0
Octadecatetraenoic acid	0	0	0	0.40	0	0	0	0	0	0
Behenic acid	0	0	0	0.37	0	0	0.14	0.84	0.20	0.27
Hexagonic acid	0	0	0	0	0.15	0.13	0.18	0	0	0
Arachidonic acid	0	0	0	0	1.23	0	0	0	0	0
Hepladecanoic acid	0	0	0	0	0	0.18	0.42	0	0	0
Octadecosaenoic acid	0	0	0	0	0	0.18	0.13	0	0	0
Cis-9-eicosaenoic acid	0	0	0	0	0	0.30	0	0	0	0
10-pentadecanoic acid	0	0	0	0	0	0	0.35	0.15	0	0
Arachidic acid	0	0	0	0	0	0	0.10	0.60	0.22	0.11
9-eicosaenoic acid	0	0	0	0	0	0	3.12	0	0	0
11-docosenoic acid	0	0	0	0	0	0	0.19	0	0	0

^aIdentification of isolates is shown in Table 1

^b Amount of fatty acid (%)

Table (3): Qualitative profiles of fatty acids extracted from different isolates of *Fusarium oxysporum*

Fatty acid	S1 ^a	S2	S3	S4	S5	S6	S7	S8	S9	S10
Myristic acid	+	+	-	+	+	+	+	+	+	+
Pentadecanoic acid	+	+	-	-	-	+	+	+	+	+
Palmitic acid	+	+	+	+	+	+	+	+	+	+
Palmitoleic acid	+	+	+	+	+	+	+	+	-	+
Stearic acid	+	+	+	+	+	+	+	+	+	+
Oleic acid	+	+	+	+	+	+	+	+	+	+
Vaccenic acid	+	-	-	-	+	+	+	+	+	+
Linoleic acid	+	+	+	+	+	+	+	+	+	+
Linolenic acid	+	+	+	+	+	+	+	+	+	+
Eicosaenoic acid	+	-	+	-	-	+	+	-	-	-
Gladoleic acid	+	-	-	+	+	-	+	-	-	-
Gamma linolenic acid	-	-	-	+	-	-	-	-	-	-
Octadecatetraenoic acid	-	-	-	+	-	-	-	-	-	-
Behenic acid	-	-	-	+	-	-	+	+	+	+
Hexagonic acid	-	-	-	-	+	+	+	-	-	-
Arachidonic acid	-	-	-	-	+	-	-	-	-	-
Hepladecanoic acid	-	-	-	-	-	+	+	-	-	-
Octadecosaenoic acid	-	-	-	-	-	+	+	-	-	-
Cis-9-eicosaenoic acid	-	-	-	-	-	+	-	-	-	-
10-pentadecanoic acid	-	-	-	-	-	-	+	+	-	-
Arachidic acid	-	-	-	-	-	-	+	+	+	+
9-eicosaenoic acid	-	-	-	-	-	-	+	-	-	-
11-docosenoic acid	-	-	-	-	-	-	+	-	-	-

^aIdentification of isolates shown in Table 1

^bThe fatty acid is present

^cThe fatty acid is absent

Table (4): Similarity among quantitative profiles of fatty acid from *Fusarium oxysporum* isolates

Isolate	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
S1										
S2	0.184 ^b									
S3	0.674	0.591								
S4	0.519	0.598	0.976							
S5	0.984	0.182	0.689	0.544						
S6	0.983	0.105	0.596	0.437	0.992					
S7	0.980	0.063	0.546	0.378	0.976	0.993				
S8	0.967	0.311	0.834	0.710	0.960	0.929	0.911			
S9	0.893	0.400	0.924	0.831	0.887	0.832	0.806	0.975		
S10	0.910	0.334	0.834	0.726	0.868	0.833	0.829	0.960	0.966	

^a Identification of isolates shown in Table 1

^b Linear correlation coefficient