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Identification of some *Fusarium* spp. using Molecular Biology Techniques

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Electrophoretic detection of protein banding patterns and esterase isozymes in polyacrylamide gel electrophoresis were used for the identification of some Fusarium isolates. Seven Fusarium isolates which were identified by their morphological and pathological characteristics as *F. semitectum*, *F. culmorum*, *F. moniliforme*, *F. solani*, *F. graminearum*, *F. oxysporum* f.sp. *lycopersici* and *F. oxysporum* f.sp. *vasinfectum* were used in this study. Results showed that, each of the studied Fusarium isolates were unique in protein banding patterns. The phylogeny tree proved that *F. oxysporum* f.sp. *lycopersici* isolate showed gene expression pattern differing from that of the rest tested isolates. The dendrogram exhibited 93.8% similarity between the studied Fusarium isolates. On the other hand, the studied Fusarium isolates exhibited three different features and esterase activities. The first category included *F. culmorum*, *F. solani* and *F. graminearum* since each of them exhibited the highest activity of esterases. The second group includes *F. oxysporum* f.sp. *lycopersici* and *F. oxysporum* f.sp. *vasinfectum* followed by the third category which includes *F. moniliforme*. *F. semitectum* exhibited different pattern than all the other isolates. This latter group proved to have three different esterase isozymes with the lowest activity. In conclusion, cluster analysis of the protein banding patterns by SDS-PAGE, and electrophoretic detection of esterase banding patterns were useful tools for differentiating between species and formae speciales of the genus *Fusarium* either alternatively or complementary to those methods based upon morphological and pathological characteristics.

Keywords: *Fusarium* spp., identification and molecular biology.

Fusarium spp. are a widespread cosmopolitan group of fungi and commonly colonize aerial and subterranean plant parts, either as primary or secondary invaders. Some species are common in soil and it is rare to find necrotic root of a plant in most agricultural soils that is not colonized by at least one *Fusarium* sp. (Nelson *et al.*, 1983). Of all diseases caused by Fusarium, probably the most important are the vascular wilt diseases caused by formae speciales of *Fusarium oxysporum*. These fungi attack a diverse group of plants including crops, ornamentals and trees (Nelson *et al.*, 1981). Classical taxonomy, based upon morphological characteristics and pathogenicity were used for identification of different *Fusarium* spp. (Wendy and Lynne, 1998, Liu Weicheng *et al.*, 2002 and Abd El-Salam *et. al.*, 2003). They concluded that, because of the instability in morphology, pathogenicity, culture

property and other characters of Fusarium isolates, molecular biology techniques might be important for the identification of species, subdivision of intraspecific strains and analysis of population composition of the pathogen. Although virulence has been an extremely useful characteristic for differentiating isolates of *F. oxysporum,* it is still only a single trait. Moreover, virulence has been shown to be influenced by a number of variables, including temperature, (Williams, 1981), host age, (Hart and Endo, 1981) and method of inoculation, (Kraft and Haglund, 1978). Recently, molecular biology techniques such as cluster analysis of the protein banding patterns by SDS- PAGE, and electrophoretic detection of esterase banding patterns overcome all limitations and provide additional information for fungal characterization (Ouellett and Seifert, 1993).

It was found that electrophoretic analysis of esterase is a useful tool for differentiating between *Fusarium* spp. as well as different formae speciales of *F. oxysporum* collected from various geographic regions (Cao and Ye, 2001; Lugo *et al.*, 2001; Patel and Anahosur, 2001 and Aly *et al.*, 2003).

Therefore, the present work has been undertaken to explore the possible utilization of molecular biology techniques for identifying *Fusarium* spp., either alternatively or complementary to those based upon morphological characteristics and pathogenicity. However, besides using the morphological and pathological characteristics, the present work aims to employ electrophoretic detection of protein banding patterns by SDS-PAGE and esterase isozymes electrophoresis.

Materials and Methods

1. Isolation and identification on the basis of morphological characteristics: Isolation trials were carried out on different diseased host plants showing symptoms of damping- off, root rot and wilt of tomato, sweet pepper, eggplant, squash, cucumber, peas, maize, wheat, soybean, broad bean, sugar beet and cotton plants collected from different locations of Kafr EL-Sheikh Governorate according to the method described by Khalifa (1991). Identification was carried on the basis of morphological characteristics described by Burgess *et al.* (1994).

2. Pathogenicity tests:

Thirty three Fusarium isolates which were isolated from the diseased host plants mentioned before were tested for their pathogenicity using the soil infestation technique (Khalifa, 1991).

3. Disease assessment:

Disease incidence was recorded as percent of pre-emergence damping-off of seedlings throughout two weeks from sowing. Post emergence damping-off and healthy survival plants were counted up to 45 days from sowing according to Khalifa (1987).

4. Identification of Fusarium spp. and formae speciales of F. oxysporum using molecular biology techniques:

In the present study, seven Fusarium isolates which were identified according to the morphological and pathological characteristics were used.

4.1. Total protein extraction and electrophoresis methods:

Total protein banding patterns were determined electrophoretically using SDS discontinuous gel as slabs (4% and 7.5% for stacking and separating gels, respectively) according to Stegmann *et al.* (1989).

4.2. Isozymes electrophoresis:

The collected mycelium was crushed in prechilled mortar and pestle with liquid nitrogen. Four ml of 20% sucrose was added to one gram of the mycelium. The homogenate was centrifuged at 12.000 rpm for 13 min. at 4°C.

4.2.1. Esterases isozymes:

The method adopted by Stegmann *et al.* (1989) was followed.

Results

Isolation and identification on the basis of morphological characteristics:

Thirty three Fusarium isolates were isolated from twelve different plant species. These isolates were divided into six groups based upon morphological and microscopical characteristics as follows:

- Group (1): Isolates of this group forms abundant uniform mycelium on PDA, initially white to salmon becoming beige with age. A pale to dark brown pigment develops in the agar. Abundant, straight to curved macroconidia usually 5 septa formed from polyphialides. A few 1 to 2-celled microconidia. Chlamydospores formation is variable.

- Group (2): Isolates of this group grows rapidly on PDA forming floccose mycelium which usually light yellow around the central spore mass. Greyish rose to pink mycelium with greyish rose to burgundy pigment in the agar. The macroconidia are formed from monophialides and the microconidia are absent, while Chlamydospores develops singly, in chains or clumps.

- Group (3): forms white floccose mycelium on PDA medium which may become greyish violet with age. Pigmentation in the agar is quite variable ranging from no pigmentation or greyish orange to violet grey, or dark violet. The macroconidia are produced from monophialides on branched conidiophores. Microconidia are formed abundantly in chains. Chlamydospores are absent.

- Group (4): Produces white to cream usually spare, floccose mycelium with pale to dark violet pigment in the agar. Abundant macroconidia are produced in confluent cream or bluish green formed from monophialides. The microconidia are 1 or 2 celled and are oval formed abundantly in false-heads on very long monophialides.

- Group (5): The mycelium of this isolates is predominantly light yellow, greyish rose, white to pale orange forms greyish rose to burgundy pigment in the agar. The macroconidia are relatively slender, falcate to almost straight, usually 5 to 6 septa produced from monophialides. Microconidia are absent. Chlamydospores formation is variable.

- Group (6): This group is highly variable in respect to colony morphology. It produces floccose, sparce or abundant mycelium ranging in colour from white to pale violet with pale to dark violet or dark magenta pigment. The macroconidia are usually non septate and are oval formed from monophialides on branched conidiophores. Microconidia are usually formed abundantly in false-heads. Chlamydospores are formed abundantly in most isolates.

Accordingly, these Fusarium isolates representing the sex groups were typically identical to *F. semitectum*, *F. culmorum*, *F. moniliforme*, *F. solani*, *F. graminearum* and *F. oxysporum*, respectively.

2. Pathogenicity tests:

Pathogenicity tests showed that two isolates of *Fusarium oxysporum*, one isolated from tomato and the other from cotton were highly pathogenic to their host plants producing typical wilt symptoms. Consequently, such isolates were identified as *F. oxysporum* f.sp. *lycopersici* and *F. oxysporum* f.sp. *vasinfectum*, respectively. On the other hand, all isolates of other different *Fusarium* species were pathogenic causing various diseases on different economic plants with variable degrees (Tables 1, 2, 3, 4 and 5).

Table 1. Pathogenicity test of different isolates of *Fusarium semitectum* obtained from different host plants on various commercial cultivars under greenhouse conditions during 2001-2002 season

Isolate No.	Host plant	Tested plant cultivar	Pathogenicity assessment (%)		
			pre-emergence damping-off	post-emergence damping-off	survival plants
14	Tomato	Super strain B	5.27 a*	2.40 a	92.33 h
182	S. pepper	California W	27.40 e	21.73 f	50.87 d
1	Eggplant	Black beauty	17.47 c	15.27 e	67.26 f
15	Squash	Al-Eskandrani	35.33 f	21.50 e	43.17 c
4	Cucumber	Prince	7.47 b	7.30 b	85.50 g
6	Soybean	Clark	36.30 g	26.67 g	43.03 b
7	Sugar beet	Kawmera	26.60 d	20.27 d	53.13 e
3	Cotton	Giza 75	40.10 h	32.97 h	26.93 a

* Values with the same letter are not significantly different at 5% level by DMRT.

Table 2. Pathogenicity test of different isolates of *Fusarium culmorum* obtained from different host plants on various commercial cultivars under greenhouse conditions during 2001-2002 season

Isolate No.	Host plant	Tested plant cultivar	Pathogenicity assessment (%)		
			pre-emergence damping-off	post-emergence damping-off	survival plants
16	Tomato	Super strain B	13.23 b*	6.13 a	80.64 c
113	Wheat	Sakha 69	11.60 a	13.33 c	75.37 b
30	Sugar beet	Kawmera	17.57 c	9.43 b	73.00 a

* As described in footnote of Table (1).

Table 3. Pathogenicity tests of different isolates of *Fusarium moniliforme* obtained from different host plants on various commercial cultivars under greenhouse conditions during 2001-2002 season

Isolate No.	Host plant	Tested plant cultivar	Pathogenicity assessment (%)		
			pre-emergence damping-off	post-emergence damping-off	survival plants
3	Tomato	Super strain B	33.20 f *	20.40 f	46.96 d
6	Cucumber	Prince	6.83 a	5.34 c	87.60 h
1	Peas	Master B	40.47 g	30.33 i	29.20 c
10	Maize	Giza 2	14.17 c	3.27 b	83.56 g
169	Wheat	Sakha 69	7.77 b	2.02 a	90.86 i
5	Soybean	Clark	49.70 i	25.63 g	24.64 a
4	Broad bean	Sakha 1	19.73 e	17.97 e	62.30 e
7	Sugar beet	Kawmera	16.60 d	10.33 d	73.07 f
2	Cotton	Giza 75	43.07 h	30.17 h	26.76 b

* As described in footnote of Table (1).

Table 4. Pathogenicity tests of different isolates of *Fusarium solani* obtained from different host plants on various commercial cultivars under greenhouse conditions during 2001-2002 season

Isolate No.	Host plant	Tested plant cultivar	Pathogenicity assessment (%)		
			pre-emergence damping-off	post-emergence damping-off	survival plants
4	Tomato	Super strain B	26.60 e *	14.67 c	60.73 g
1	S. pepper	C. wonder	25.87 d	20.93 e	53.20 e
2	Eggplant	B. beauty	30.67 f	20.83 e	48.50 d
3	Peas	Master B	24.97 c	20.30 d	54.75 f
5	Maize	Giza 2	13.10 a	2.53 a	83.37 i
8	Soybean	Clark	46.27 h	30.80 g	22.93 a
15	Broad bean	Sakha 1	35.27 g	30.40 f	34.33 b
36	Sugar beet	Kawmera	20.27 b	13.40 b	66.33 h
11	Cotton	Giza 75	26.53 e	30.33 f	43.14 c

* As described in footnote of Table (1).

Table 5. Pathogenicity tests of different isolates of *Fusarium graminearum* obtained from different host plants on various commercial cultivars under greenhouse conditions during 2001-2002 season

Isolate No.	Host plant	Tested plant cultivar	Pathogenicity assessment (%)		
			pre-emergence damping-off	post-emergence damping-off	survival plants
7	Tomato	Super strain B	23.07 c *	11.33 c	65.33 a
3	Maize	Giza 2	16.20 b	10.50 b	73.30 b
112	Wheat	Sakha 69	11.77 a	1.37 a	88.21 c

* As described in footnote of Table (1).

3. Identification of Fusarium spp. and formae speciales of F. oxysporum using molecular biology techniques:

3.1. Electrophoretic detection of protein banding patterns:

The relationships between the six studied *Fusarium* spp. including two formae speciales of *F. oxysporum* were determined according to the protein banding patterns as a result of genes expression for each isolate. Figure (1) show that each of the studied Fusarium isolates was unique in protein banding patterns category. The phylogeny tree (Fig. 2) proved that the *F. oxysporum* f.sp. *lycopersici* isolate showed gene expression pattern which differ from that of the rest tested isolates. The dendrogram exhibited 93.8% similarity between the studied Fusarium isolates.

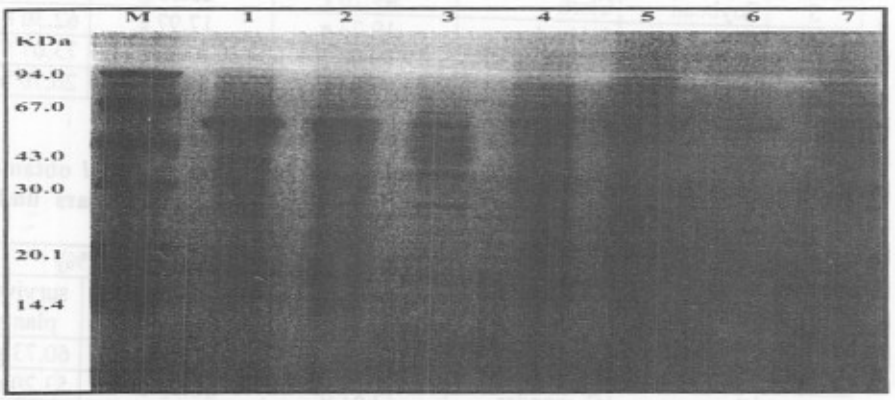

Fig. 1. Photograph of protein banding patterns of 1= *F. semitectum*; 2= *F. culmorum*; 3= *F. moniliforme*; 4= *F. solani*; 5= *F. graminearum*; 6= *F. oxysporum* f.sp. *lycopersici*; 7= *F. oxysporum* f.sp. *vasinfectum* and M= Molecular marker.

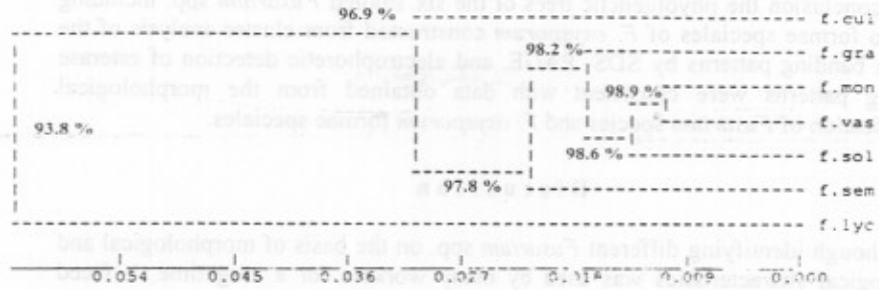

Fig. 2. Dendrogram showing polymorphism of protein profiles of *F. semitectum* (f.sem), *F. culmorum* (f.cul), *F. moniliforme* (f.mon) *F. solani* (f.sol) *F. graminearum* (f.gra), *F. oxysporum* f.sp. *lycopersici* (f.lyc) and *F. oxysporum* f.sp. *vasinfectum* (f.vas) revealed by UPGMA cluster analysis of Jaccard genetic similarity coefficients.

4.3. Electrophoretic detection of esterase banding patterns:

Figure (3) illustrates the esterases isozyme activities for the six tested *Fusarium* spp. including two formae speciales of *F. oxysporum*. It was clearly noticed that the studied isolates exhibited three different features and esterases activity. The first category included *F. culmorum, F. solani* and *F. graminearum* since each of them exhibited the highest activity of esterases. The second group includes *F. oxysporum* f.sp. *lycopersici* and *F. oxysporum* f.sp. *vasinfectum* followed by the third category which contains *F. moniliforme*. *F. semitectum* exhibited so different patterns than all of the other isolates; since it proved have three different esterases isozymes with the lowest activity.

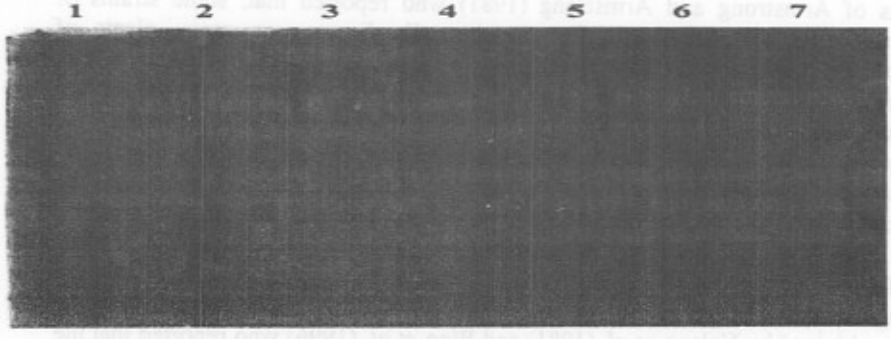

Fig.3. Electrophoretic of esterases isozyme of 1= *F. semitectum*; 2= *F. culmorum;* 3= *F. moniliforme;* 4= *F. solani;* 5= *F. graminearum;* 6= *F. oxysporum* f.sp. *lycopersici*; 7= *F. oxysporum* f.sp. *vasinfectum.*

In conclusion the phyolgenctic trees of the six studied *Fusarium* spp. including the two formae speciales of *F. oxysporum* constructed from cluster analysis of the protein banding patterns by SDS- PAGE, and electrophoretic detection of esterase banding patterns were consistent with data obtained from the morphological identification of *Fusarium* species and *F. oxysporum* formae speciales.

Discussion

Although identifying different *Fusarium* spp. on the basis of morphological and pathological characteristics was used by many workers for a long time, it faced problems in strain identification and also caused confusion in the use of names in literature (Brayford, 1989).

In the present study thirty three Fusarium isolates isolated from twelve host plants showing various symptoms including damping- off, root rot and wilt were classified on the basis of morphological characteristics described by Burgess *et al.* (1994) because they added some consistent characters, *i.e.* shape and mode of formation of microconidia, presence or absence of chlamydospores, colony morphology and growth rates on PDA medium. Accordingly, all obtained Fusarium isolates were identified as six groups, *i.e. F. semitectum, F. culmorum, F. moniliforme, F. solani, F. graminearum* and *F. oxysporum*. These results agree with those obtained by Nelson *et al.* (1981), Niemberg (1989), Leslie *et al.* (1997), Sabo *et al.* (2002) and Abd EL-Salam *et al.* (2003).

The pathogenic properties of isolated *Fusarium* species indicated that, two isolates of *F. oxysporum* isolated from tomato and cotton plants were identified as *F. oxysporum* f.sp. *lycopersici* and *F. oxysporum* f.sp. *vasinfectum*, respectively. While, the remaining isolates of *F. oxysporum* isolated from other plant hosts were pathogenic to their host plants producing only damping- off symptoms. Therefore, they were identified as *F. oxysporum* only. Such results were in parallel with the results of Armstrong and Armstrong (1981) who reported that, some strains of *F. oxysporum* are responsible for vascular wilt diseases on many plants of economical importance. These pathogenic strains show a high level of host specificity and are classified on this basis into formae speciales and races. Also, Gordon and Martyn (1997) and Corbiere and Bouznad (1998) reported that *F. oxysporum* has about 80 formae speciales (*i.e.* pathotypes specific to species), and several are subdivided into races (specific to cultivar within a species). The traditional criteria used to differentiate these genera and their species are based on plant hosts, symptoms, colony appearance, morphologic characterization of their conidia and telemorph. The rest isolates of other different *Fusarium* species were pathogenic causing various diseases to different hosts. This finding coincide with the results obtained by Nelson *et al.* (1981) and Woo *et al.* (1996) who reported that the genus *Fusarium* is one of the most economically important among fungus genera since it includes many pathogenic species which cause a wide range of plant diseases.

Identification of an organism based on morphology is a difficult and tedious process. This is further complicated by that the morphology of spores is influenced

to a great extent by cultural and environmental factors (Williams, 1981 and Woo *et al.*, 1996). Therefore, molecular biology techniques were used in an attempt for identifying relationships of the studied *Fusarium* species either alternatively of complementary to those methods based upon morphological characteristics.

Biochemical and molecular markers are being increasingly used to characterize fungal plant pathogen populations. They are versatile and highly informative tools for fungal pathogen identification and diagnosis (Majer *et al.*, 1996) and for population genetics studies (McDonald and McDermott, 1993 and McDonald *et al.*, 1999). They can be used to evaluate levels for genetic diversity and phenotypic relationships within and between species, and to identify particular races and pathotypes (Brown, 1996). In the present study, results showed that each of *Fusarium* spp. and formae speciales had its own unique protein profiles. In the same time their different protein banding patterns of the studied Fusarium isolates were obtained. The phylogeny tree proved that *F. oxysporum* f.sp. *lycopersici* isolate showed a gene expression pattern which differ from that of the rest tested isolates. Consequently, protein profile data can clearly separate *Fusarium* spp. and formae speciales. These results agree with those obtained by Mandeel *et al.* (1994) who compared SDS-PAGE patterns from eight isolates belonging to three *Fusarium* species. These results coincide with the finding of Aly *et al.* (2003) who reported that protein profiles data can clearly separate *Fusarium* spp. isolates with a few exceptions. On the contrary, Belisario *et al.* (1998) found no differences when comparing total mycelium protein profiles (SDS- PAGE) of different species and formae speciales of *F. oxysporum, F. solani* and *F. culmorum*.

The present results suggested that esterase patterns clearly separated *Fusarium* spp. isolates with a few exceptions. These results are similar to those obtained by Ye and Wu (1985). However, no relationship was observed between isozyme patterns and geographic origin, phenotypic distance or virulence of fungal isolates (Bosland and Williams, 1987 and Etebarian *et al.*, 1996)

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تعريف بعض أنواع الفيوزاريوم باستخدام تقنيات البيولوجيا الجزيئية

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إستخدمت طريقة الفصل الكهربى في بيئة جيل البولي اكريلاميد لدراسة أنماط البروتين والمشابهات الانزيمية لإنزيم الاستيريز وذلك لتعريف بعض عزلات الفيوزاريوم. إستخدم فى الدراسة سبعة عزلات فيوزاريوم تم تعريفها طبقا لخصائصها المورفولوجية والمرضية على أنها فيوزاريوم سيميتكتم *Fusarium semitectum* ، فيوزاريوم كولمورم *F. culmorum* ، فيوزاريوم مونيليفورم *F. moniliforme* ، فيوزاريوم سولانى *F. solani* ، فيوزاريوم جراميناريم *F. graminearum* ، وفيوزاريوم أوكسيسبورم ف. ليكوبرسيسى *F. oxysporum* f.sp. *lycopersici* وفيوزاريوم أوكسيسبورم ف. فازنفكتم *F. oxysporum* f.sp. *vasinfectum*.

إتضح من النتائج أن كل عزلة من الفيوزاريوم تحت الدراسة تتميز بنمط متفرد من البروتين. أظهر الطراز المتخصص *lycopersici* للنوع فيوزاريوم اوكسيسبورم نوع مختلف من التعبير الجينى كبروتين عن جميع العزلات المختبرة. كما أظهر تحليل مجاميع الفينوجرامات للبروتين وجود اختلافات بين الأنواع المختلفة لجنس الفيوزاريوم تحت الدراسة. وكانت درجة التشابه بينهم ٩٣.٨%.

من ناحية أخرى فقد أظهرت الدراسة أن كل عزلات الفيوزاريوم التي اشتملت عليها الدراسة كونت ثلاثة أنماط مختلفة من المشابهات الانزيمية والنشاط الإنزيمي الاستيريز وكان الطراز الأول يضم النوع فيوزاريوم كولمورم *F. culmorum* ، فيوزاريوم سولانى *F. solani* وفيوزاريوم جراميناريم *F. graminearum* وأظهروا أعلى نشاط لإنزيم الاستيريز. وضمت المجموعة الأخرى كلا من فيوزاريوم أوكسيسبورم ف. ليكوبرسيسى *F. oxysporum* f.sp. *lycopersici* وفيوزاريوم أوكسيسبورم ف. فازنفكتم *F. oxysporum* f.sp. *vasinfectum* متبوعا بالطراز الثالث الذي يمثل فيوزاريوم مونيليفورم *F. moniliforme* . بينما النوع فيوزاريوم سيميتكتم *F. semitectum* فقد أظهر طراز مختلف عن كل الأنواع الأخرى حيث أنه أعطى ثلاثة مشابهات إنزيمية لإنزيم الاستيريزذات نشاط لهذا الإنزيم.

وبصفة عامة يمكن القول بأن استخدام تقنيات البيولوجيا الجزيئية مثل التكنيك التى تعتمد على الفصل الكهربى فى بيئة جل البولى اكريلاميد لدراسة أنماط البروتين والمشابهات الإنزيمية لإنزيم الاستيريز هى وسائل مفيدة ودقيقة فى التفريق بين الأنواع المختلفة لجنس الفيوزاريوم وكذلك الطرز المتخصصة للنوع (formae speciales) للنوع فيوزاريوم أوكسيسبورم *F. oxysporum* كطريقة مكملة أو بديلة للطرق المعتمدة على استخدام الخصائص المورفولوجية والمرضية.