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FLAX SEED-BORNE MYCOFLORA, PATHOGENICITY OF *Fusarium oxysorum* ISOLATES AND THEIR CHARACTERIZATION BY PROTEIN ELECTROPHORESIS.

El-Wakil, D.A. [*]; M. R. Omar [*]; M. A. Mahmoud [*] and Rania Z. Ahmed[**]

[*] **Plant Pathology Research Institute, Agric. Res. Center, Giza, Egypt**

[**] **Faculty of Science and Humanity Studies, Al-Kharj University, Kingdom of Saudi Arabia**

ABSTRACT

Seed samples of 7 flax cultivars (Giza-8, Giza-7,Giza-6,Giza-5,Sakha-1, Sakha-2 and Marline), were screened for the associated seed-borne fungi,15 fungal species were isolated belonging to 11 genera .The isolated fungi were *Aspergillus niger, Aspergillus flavus , Aspergillus ochraceous, Alternaria altrenata, Cladosporium* sp.,*Chatomium* sp., *Drechslera* sp., *Epicoccum* sp., *Fusarium oxysporum, Fusarium solani, Fusarium moniliforme,Penicillium* sp., *Stemphyllium* sp.,*Phoma* sp., *Trichoderma* sp. The genus *Aspergillus* recorded the highest percentage of seed colonization between the seven tested flax cultivars, ranged from (2-20%). Pathogenicity test for 14 isolates of *F. oxysporum* were studied for the levels of pathogenicity on seven flax cultivars under greenhouse conditions.Percentage of infection was used as a criteria to evaluate the pathogenicity of *F. oxysporum* isolates some of the tested isolates *i.e.* isolate no. 8 showed (85.0%), followed by isolate no.13 at 73.33% on flax cultivar (Giza-8). Moreover, cluster analysis was a reliable method to differentiate between 14 isolates belonging to the closely related *F. oxysporum.* Similarity coefficient matrix of *F.oxysporum* ,isolates ,involved in flax seedling damping-off showed a few significant correlations between the tested isolates of *F. oxysporum,* based on their pathogenicity on 7 flax cultivars.

Keywords: Seed-borne mycoflora, flax seeds, electrophoresis*,F. oxysporum* isolates.

INTRODUCTION

Several fungi usually attack flax seeds through capsule formation during the ripening stage for many flax cultivars; the capsules may be split to favor the fungal infection. Pathogen aggressiveness, moisture content on infected surface, cultivar genotype, sowing date, preceeding crop, and crop density are also, important factors for flax seed infection by mycoflora (Elvyra *et al.,* 2006).The diversity of fungi occurring on flax seed dependens on the growing conditions. On the other hand, some of fungal species of the genera *Colletotrichum,Fusarium,Rhizoctonia,Alternaria,Aspergillus,* and *Penicillium* sp., occur in all flax growing countries (Paul *et al*.,1991 and Kumud *et al*.,1997).In Egypt the mycoflora isolated from flax seed included *Penicillium chrysogenum, Alternaria tenuis ,A.linicola,stemphylium* sp.,*Botrytis cinerea ,Drechslera tetramera, Sclerotium bataticola,Aspergillus niger, A.ochraceous, Curvularia lunata, Mucor* sp.,*Phoma* sp.,*Cladosporium herbarum, Fusarium moniliforme,F.oxysporum,Pythium* sp.,and *Cephalosporium* sp.(Mahdi *et al*.,1973).The above-mentioned fungi of flax seeds, were considered as

associated fungi, one of the most important genus of these fungi is *F.oxysporum,* which play an important role in wilt disease on numerous field crops (Mahdi *et al*.1973 and Ali *et al*.,2004).

The main objective of this investigation was to identify the associated fungi with seeds of 7 flax cultivars, investigating the correlation among pathogenicity of 14 isolates of *F.oxysporum* on 7 flax cultivars*,* within cluster analysis and to compare this with the protein patterns obtained by SDS-PAGE from 14 *F.oxysporum* isolates.

MATERIALS AND METHODS

Isolation of flax seed borne fungi:

Seed samples of seven flax cultivars were obtained from Cotton Research Department, Agric. Res. Center, Giza, Egypt. A random subsample of 100 flax seeds from each cultivar was surface sterilized in 2.5% Clorox solution for 2 minutes, and washed several times in sterilized water. The surface sterilized seeds were then blotted between dry sterilized filter paper. Seed borne fungi were determined by the standard blotter method (Nyvall, 1981 and ISTA,1983).Twenty five nonsterilized flax seeds of each cultivar were randomly selected and placed on five layers of 9-cm Whatman no.1 filter paper in Petri dishes, each was replicated 4 times, plates were incubated for 12-hr darkness at 20±2 ^{O}C for 7 days .After incubation each colony was examined macroscopically or microscopically for identification the genus or species level according to Gilman (1966), Booth (1971) or Barnet and Hunter (1979).Isolation frequency of each fungus was expressed as the percentage of seeds from which the fungus grew.

Pathogenicity test of *Fusarium oxysporum* isolates on seven flax cultivars:

Substrates for growth of each *Fusarium oxysporum,* isolates, previously isolated from flax seeds were prepared in 500-ml glass bottles; each bottle contained 50 gm of sorghum grains and 40 ml of tap water. The bottles were autoclaved for 30 min. isolate inocula, taken from one-week-old culture on PDA were aseptically introduced into the bottle and allowed to colonize sorghum for 3 weeks. Batches of autoclaved clay loam soil were inoculated separately with inoculums of each isolate at the rate of 50 g/kg of soil. Infested soil was dispended in 15-cm-diameter clay pots and these were planted with 20 seeds per pot for each flax cultivar. In the control treatment sterilized sorghum grains were mixed thoroughly with soil at a rate of 50g/kg of soil. Pots were randomly distributed on a greenhouse bench under a temperature regime ranged from 20±2 ^{O}C to 24±2 ^{O}C. Percentage of infection was recorded after 45 days of planting (Seem, 1984).

Extraction of fungal proteins:

Protein extracts from 14 *Fusarium oxysporum,* isolates were prepared according to Hussein *et al.* (2000); Sammons *et al*.(1981) and Nelson and Campbell. (1992) in the following way: Fungal isolates were grown for 22 days at 22-30˚ C on liquid Czapek medium. The mycelium was harvested by filtration through cheesecloth, washed with distilled water several times, and freeze- dried. This frozen mycelium suspended in phosphate buffer PH 8.3

(1-3 ml/g mycelium), mixed thoroughly with glass beads, and ground in liquid nitrogen to a fine powder. The ground mycelium was centrifuged at 19,000 rpm. for 30 minutes at 0˚C. The protein content in the supernatant was estimated according to Bradford (1976) by using bovine serum albumin as a standard protein. If protein concentration was low, protein was precipitated from the clarified supernatant by adding ammonium sulphate at 70% of saturation (60g/100 ml) then kept in the refrigerator for 30 hr. pellets, collected by centrifugation at 11,000 rpm for 30 minutes, were resuspended in phosphate buffer PH 8.3 and subjected to dialysis for 24 hrs against the buffer and centrifugation at 11,000 rpm., for 30 minutes. Protein was estimated in the obtained supernatant.

Electrophoresis of dissociated protein (SDS-PAGE):

Each supernatant was mixed with an equal volume of a solution consisting of (by volume) 64% buffer (0.15 M Tris HCl, PH6.8), 20% glycerol, 6% SDS, 10% 2-6 mercaptoethanol, and 0.1% bromophenol blue, before boiling in a water bath for 3 minutes. Twenty-microliters samples (40 µg of protein) were subjected to electrophoresis in a 7.5% polycarylamide gel prepared 0.1% SDS with a 3.5% stacking gel (Laemmli, 1970). Electrophoresis was conducted at 10˚ C for 4 hr at 15 and 30 mA for the stacking and the separating gels, respectively, until dye reached the bottom of the separating gel. Electrophoresis was performed in vertical slab mold (16x18x0.15 cm). Gels were stained with silver nitrate for the detection of protein bands (Sammons *et al*., 1981).

Gel analysis:

A gel documentation system (Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6C, Fullerton CA 92631) was used to document the results of PAGE and to cluster the electrophoretic patterns by the unweighted pair- group method based on arithmetic mean (UPGMA).

Statistical analysis:

The experimental design of pathogenicity test was a randomized complete block with four replicates. Analysis of variance (ANOVA) of the data was performed with the MSTAT-C package (A Micro-computer Program for the Design, Management, and Analysis of Agronomic Research Experiments, Michigan State Univ., USA). Least significant difference (LSD) was used to compare isolate means within cultivars. Percentage data were transformed into arc sine angles before carrying out the ANOVA, to produce approximately constant variance. Cluster analysis of *Fusarium oxysporum,* isolates was performed with the software package SPSS6.0.

RESULTS AND DISCUSSION

Isolation of flax seed-borne fungi:

A total of 15 fungal species were isolated from 7 flax cultivars in Table (1), in this survey all flax cultivars yielded different species of fungi. Giza- 8 yielded the highest number of fungi, while the Marline yielded the lowest number of fungi. The other flax cultivars yielded a number of fungi ranged from 1-20%. The occurrence of pathogen species are of central importance in the ecology of host-pathogen interactions in complex pathosystems, *i.e.*, those with

multiple pathogens in a single or multiple hosts. Within such pathosystems, biotic and abiotic factors influence the distribution and abundance of such pathogen species (Mahdi *et al*., 1973;Aly *et al*., 2004, 2008 and Elvyra *et al*.,2006). Subsequently, patterns of association result from interrelationships among organisms and from environmental factors. Such theses patterns depends on whether or not organisms selected or avoid the same habitat, have the same mutual attraction or repulsion, or have no interaction (Bradford ,1976; Barnet and Hunter,1979**;** Hussein *et al*,2000 and Anuja and Aneja,2001). Therefore, pathogen species (e.g.seedborne pathogens) were in a competition for a single resource (e.g., a seed) tends to occupy the same niche. Such niche overlap generates affinity (or lack affinity) for coexistence among species, known as inter specific associations (Walter *et al,* 1974 and Mansour *et al*, 2008).Data presented in Table(2),Anova analysis for the isolated fungi shows that there is a remarkable significance between the isolated fungi in the percentage of occurrence.

Table (1): Frequencies of fungi isolated from nonsterilized flax seeds of seven flax cultivars

Cultivars / Fungi	Giza-8		Giza-7		Giza-6		Giza-5		Sakha-1		Sakha-2		Marline	
	%	T[a]	%	T[a]	%	T[a]	%	T[a]	%	T[a]	%	T[a]	%	T[a]
Aspergillus niger	13	(3.48)	0.0	(0.71)	16	(3.90)	7	(2.72)	15	(3.38)	13	(2.96)	11	(2.67)
Aspergillus flavus	4	(1.77)	4	(1.77)	15	(3.76)	20	(4.47)	4	(1.55)	15	(3.90)	0.0	(0.71)
A. ochraceous	2	(1.41)	6	(2.32)	5	(2.17)	10	(3.16)	4	(1.77)	4	(1.55)	16	(3.63)
Alternaria alternata	1	(1.06)	10	(2.57)	7	(2.87)	2	(1.41)	3	(1.61)	15	(4.55)	17	(4.54)
Cladosporium sp.	4	(1.96)	7	(2.42)	6	(2.25)	0.0	(0.71)	0.0	(0.71)	8	(2.22)	4	(1.41)
Chatomium sp.	1	(1.06)	8	(2.87)	5	(1.97)	0.0	(0.71)	0.0	(0.71)	0.0	(0.73)	0.0	(0.71)
Drechslera sp.	18	(4.25)	9	(2.96)	4	(1.77)	3	(1.76)	5	(2.32)	0.0	(0.71)	14	(2.75)
Epicoccoum sp.	10	(3.23)	3	(1.61)	0.0	(0.71)	0.0	(0.71)	0.0	(0.71)	0.0	(0.71)	2	(1.78)
F. oxysporum	3	(1.76)	4	(1.96)	8	(2.55)	11	(3.26)	8	(3.02)	7	(2.67)	5	(2.83)
F. solani	3	(1.61)	5	(2.12)	2	(1.26)	0.0	(0.71)	3	(1.41)	1	(1.06)	0.0	(0.71)
F.moniliforme	2	(1.42)	0.0	(0.71)	0.0	(0.71)	0.0	(0.71)	0.0	(0.71)	2	(1.06)	0.0	(0.71)
Penicillium sp.	10	(3.16)	6	(2.32)	7	(1.86)	0.0	(0.71)	15	(3.83)	18	(4.79)	0.0	(0.71)
Stemphyllium sp.	1	(1.06)	1	(1.06)	3	(1.42)	8	(2.87)	0.0	(0.71)	0.0	(0.71)	0.0	(0.71)
Phoma sp.	3	(1.76)	0.0	(0.71)	0.0	(0.71)	3	(1.76)	0.0	(0.71)	0.0	(0.71)	0.0	(0.71)
Trichoderma sp.	1	(1.06)	0.0	(0.71)	0.0	(0.71)	0.0	(0.71)	3	(2.16)	0.0	(0.71)	4	(1.97)

[a] **Percentage data were transformed into arc sine angles before carrying out the analysis of variance to produce approximately constant variance**

LSD value = 1.355 at 0.05 **LSD value = 1.785 at 0.01**

Table (2): Analysis of variance of effects of fungi, Flax cultivars and their interaction on frequency of fungi isolated from Flax seeds.

Source of variation	Degrees of Freedom	Mean Square	F. Value	P>F
Replication	3	0.148	0.1564	
Cultivar(C)	6	0.774	0.8156	
Fungi (F)	14	14.121	14.8835	0.0000
CXF	84	3.839	4.0461	0.0000
Error	312	0.949		

Pathogenicity test:

Pathogenicity test of 14 *F.oxysporum* isolates Table (3), isolated from 7 flax cultivars indicate that there are a different levels of susceptibility to infection related to the tested 14 *F.oxysporum* isolates. For example the cv. Giza-8, isolate no.8 of *F.oxysporum* recorded (85.0%),whereas, isolate no.13 (73.33%) and the other six flax cultivars showed different levels of susceptibility to infection with different *F.oxysporum,* isolates. Data presented in Table (4), show that, the Anova analysis clarify that there are a significant differences in between the tested *F.oxysporum* isolats.

Table (3): Pathogenicity of *F. oxysporum* isolates, isolated from flax seeds on 7 flax cultivars under greenhouse conditions.

F.oxysporum isolates No.	Flax cultivars						
	Giza-8	Giza-7	Giza-6	Giza-5	Sakha-1	Sakha-2	Marline
1	41.66*	65.00	10.00	75.00	43.33	60.00	51.66
2	43.33	51.66	40.00	60.00	45.00	80.00	50.00
3	53.33	66.67	85.00	30.00	38.33	75.00	45.00
4	40.00	61.67	55.00	71.66	68.33	55.00	41.66
5	45.00	63.33	75.00	56.66	66.67	35.00	55.00
6	55.00	58.33	26.66	45.00	33.33	35.00	26.66
7	70.00	56.66	56.66	60.00	60.00	38.33	76.66
8	85.00	43.33	40.00	63.33	61.66	55.00	58.33
9	60.00	51.66	28.33	53.33	71.66	70.00	48.33
10	36.66	85.00	73.33	36.66	65.00	55.00	48.33
11	53.33	75.00	45.00	40.00	68.33	38.33	51.66
12	50.00	68.33	25.00	68.33	60.00	63.33	51.66
13	73.33	68.33	55.00	58.33	56.67	71.67	18.33
14	36.66	41.66	78.33	41.66	71.66	35.00	86.66
Control	3.33	3.33	3.33	1.66	3.33	5.00	1.66

***Data were recorded as mean (%) of 4 replicates**
LSD value = 1.662 at 0.05 LSD value = 8.785 at 0.01

Table (4): Analysis of variance of Pathogenicity of *F. oxysporum* isolates, isolated from flax seeds on 7 flax cultivars under greenhouse conditions.

Source of variation	Degrees of Freedom	Mean Square	F. Value	P<F
Replication	2	119.127	0.4636	
Cultivar (C)	6	642.011	2.4986	0.0235
Isolates(S)	14	4161.032	16.1941	0.0000
CXS	84	750.344	2.9202	0.0000
Error	208	256.947		

The shown phenogram in Fig.(1) are constructed based on cluster analysis of protein banding patterns obtained from the 14 *F.oxysporum*, isolates involved in flax seedling damping-off disease based on their virulence patterns on 7 flax cultivars. Two groups of *Fusarium oxysporum* isolates were classified by cluster analysis Fig. (1). The first group included isolates nos. 1, 2, 4, 6,9,12 and 13. The second group included isolate of *F. oxysporum* nos. 7, 8, 5,14,10,11 and 3, isolate no. 1, 12 are constructed in one group, they

recorded (41.66 & 50.00%, respectively). Also, isolates nos. 6, 13 are found in one group (55.0 & 73.33%, respectively) this results are in agreement with those reported by (Aly *et al.*, 2008 and Mansour *et al*.,2008),they mentioned that, there are a correlation between the pathogenicity and the protein banding patterns obtained from the isolated fungi .

In Fig.(2),the phenogram based on average linkage cluster analysis of electrophoretic protein patterns obtained by SDS-PAGE from 14 isolates of *F. oxysporium,* isolates from flax seeds divided the obtained isolates into two groups, the first at similarity 78.11% and the second at 55.29%, the two groups are constructed in similarity 42.03% .Also, Fig.(3). show the protein profiles obtained by SDS-PAGE from 14 isolates of *F. oxysporum* from flax seeds of different cultivar which illustrated this results.

Data shown in Table (5) showed the similarity coefficient matrix of *F.oxysporum* , isolates involved in flax seedling damping-off, indicate that there are a few significant correlations between the tested 14 isolates of *F. oxysporum.* For example isolates no.1 and no.12 .Another example is isolate no.6 and no.14 which clarify this results.

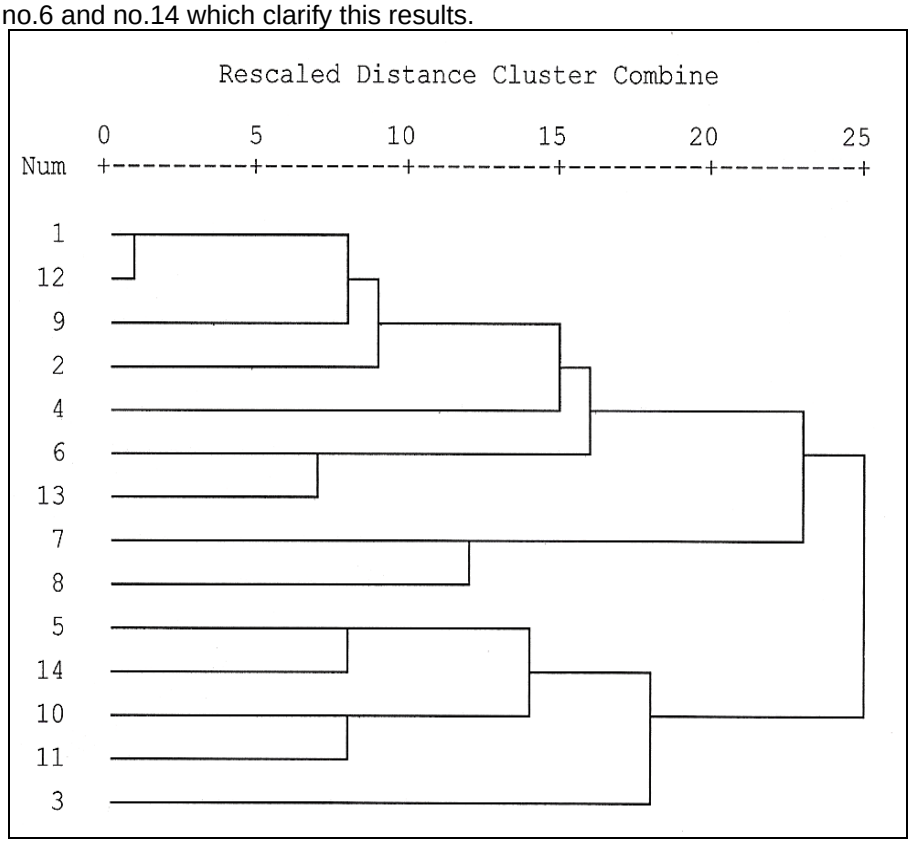

Fig.(1): Phenogram based on average linkage cluster analysis of 14 *F. oxysporum,* isolates involved in flax seedling damping- off based on their virulence patterns on 7 flax cultivars.

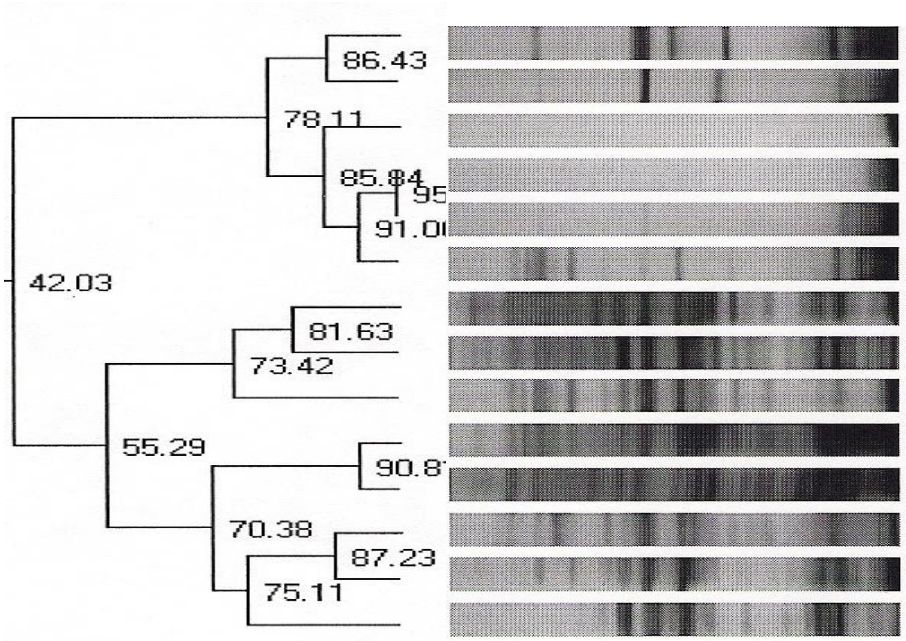

Fig. (2): Phenogram based on average linkage cluster analysis of protein patterns obtained by SDS-PAGE electrophoresis from 14 isolates of *F. oxysporum* from flax seeds.

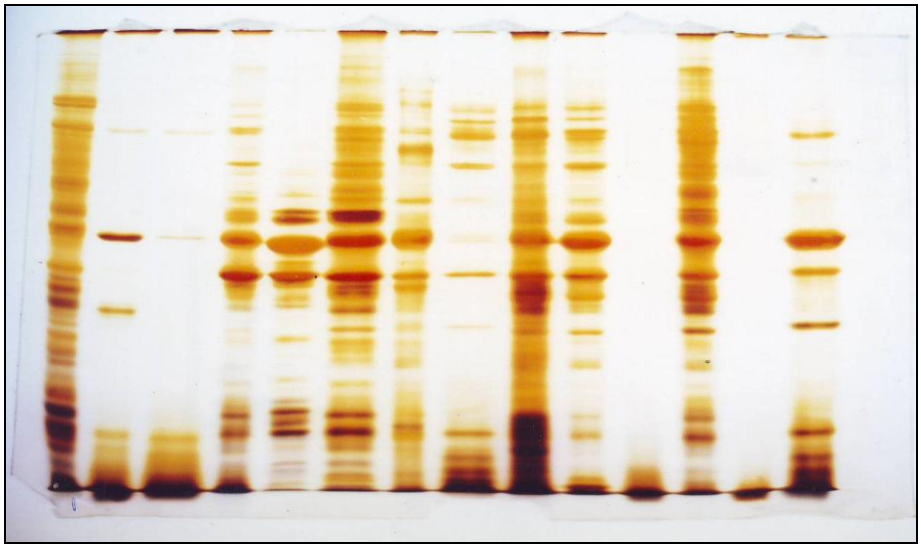

Fig. (3): Protein profiles obtained by SDS-PAGE from 14 isolates of *F. oxysporum* isolated from flax seeds of 7 cultivars.

Table (5): Similarity matrix of *F. oxysporum* isolates, involved in flax seedling damping-off based on their pathogenicity on 7 Flax cultivars.

Isolate No.	1	2	3	4	5	6	7	8	9	10	11	12	13
1													
2	0.626 [a]												
3	-0.523	0.085											
4	0.356	0.168	-0.280										
5	-0.474	-0.721	0.039	0.380									
6	0.456	-0.003	-0.122	0.073	0.222								
7	-0.131	-0.695	-0.518	-0.445	0.249	0.021							
8	0.174	-0.067	-0.567	-0.341	-0.535	0.337	0.420						
9	0.512	0.484	-0.402	0.175	-0.603	0.230	0.270	0.512					
10	-0.275	-0.191	0.573	0.275	0.580	-0.027	0.341	-0.814*	-0.269				
11	0.040	0.468	-0.116	0.143	0.416	0.404	0.251	-0.112	0.169	0.590			
12	0.947**	0.570	-0.520	0.445	-0.430	0.511	-0.210	0.202	0.701	-0.140	0.249		
13	0.091	0.246	0.306	0.263	-0.269	0.637	-0.613	0.156	0.343	0.111	0.054	0.231	
14	-0.555	-0.538	-0.035	-0.151	0.618	-0.776*	0.469	-0.337	-0.452	0.227	0.105	-0.575	-0.824*

[a] **Pearson correlation coefficient (r) is significant at P <0.01 (**) or P<0.05(*).**

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الفطريات المصاحبة لبذور الكتان والقدرة المرضية لعزلات الفيوزاريم أكسيسبورم والتفرقة بينهما بإستعمال التفريد الكهربى للبروتينات.

ضياء عبد الفتاح الوكيل*،معوض رجب عمر*،محمد أبو بكرمحمود*ورانيا زكرياأحمد ***
معهد بحوث أمراض النباتات، مركز البحوث الزراعية، الجيزة، مصر
** **كلية العلوم والدراسات الانسانية، جامعة الخرج، المملكة العربية السعودية**

أجرى حصر للفطريات المصاحبة لبذور ٧ أصناف من الكتان وهى جيزة ٨،جيزة ٧،جيزة ٦،جيزة٥،سخا١ ،سخا٢،مارلين. وأمكن عزل ١٥ نوعاً فطريا والتى تنتمى لعدد ١١جنس وكانت الفطريات المعزولة على التوالى أسبرجلس نيجر،أسبرجلس فلافس،أسبرجلس أوكراشيس، الترناريا الترناتا،ونوع من الكلادوسبوريم،ونوع من الكيتوميم ،والدريشيليرا،والابيكوكم،الفيوزاريم أكسيسبورم ،الفيوزاريم سولانى، الفيوزاريم مونيليفورم ،نوع من البنيسيليم ،ونوع من الاستمفيليم ،نوع من الفوما،نوع من التريكودرما ،على حين سجل الجنس أسبرجلس أعلى نسبة مئوية للاصابة مع الاصناف السبعة المختبرة للكتان تراوحت من(٢- ٢٠%) . أوضحت نتائج اختبار القدرة المرضية لعدد ١٤ عزلة من الفيوزاريم أكسيسبورم وذلك على السبعة أصناف من الكتان تحت ظروف الصوبة أن النسبة المئوية للاصابة هى المقياس لتقييم القدرة المرضية وأظهرت النتائج أن العزلة رقم ٨ سجلت ٨٥.٠%، ثم تلا ذلك العزلة رقم ١٣ والتى سجلت ٧٣.٣٣% على صنف الكتان جيزة ٨ .على حين كانت نتائج التحليل العنقودى طريقة مناسبة للتفرقة بين ١٤ عزلة تنتمى للجنس الفيوزاريم أكسيسبورم والمعزولة من بذور الكتان كما أظهرت نتائج التحليل العنقودى ان هناك ارتباط معنوى بين مجموعة من العزلات دون الاخرى .

الكلمات الدالة: الفطريات ، بذور الكتان ، عزلات الفيوزاريم اكسيسبورم ، التفريد الكهربى للبروتينات.

قام بتحكيم البحث

أ.د / محمد الششتاوى عبد ربه	**كلية الزراعة - جامعة المنصورة**
أ.د / محمد أحمد بيومى	**مركز البحوث الزراعية**