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Protein patterns in Relation to Virulence of *Sclerotium Cepivorum* Berk. The Incitant of White Rot of Garlic

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Abstract :

Six isolates of *Sclerotium cepivorum* Berk were isolated from naturally infected garlic plants collected from different localities of EL-Minya, Assiut and Sohag Governorates. Pathogenicty tests indicated that isolates No.2, 3 and 6 were highly pathogenic to garlic as compared with isolates No.1,4 and 5.

Protein of six isolates of *S. cepivorum* was compared by polyacrylamide gel electrophoresis (PAGE) and sodium dodecyel sulfate-polyacrylamide gel electophoresis (SDS-PAGE). Protein profiles separated by PAGE, isolate No. 1 showed the highest number of bands (20 bands), while isolate No. 4 showed the lowest number (15 bands). The number of bands of other isolates was 16 or 17 bands. Protein profiles separated by SDS-PAGE, isolate No. 5 showed the highest number of bands (19 bands) while isolate No. 3 showed the lowest number of bands (6 bands). The other isolates showed a number of bands ranged from 13 to 17 bands. On the basis of electrophoretic dissimilarities among protein banding paterns, isolates were grouped by claster analysis and the results were experessed as phenograms. Grouping the isolates based on PAGE analysis was associated with geographic of isolates,however, grouping the isolates based on SDS-PAGE was associated with virulence of isolates

Introduction :

White rot caused by the soil inhabiting fungus *Sclerotium cepivorum* Berk., is a very serious disease on garlic (*Allium sativum L.*) which causes tremendous losses to this crop in the field. It is widespread in many different countries all over the world and it was first observed in 1929 in Egypt (Nattrass 1931)In upper Egypt, in heavily infested soil infection

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in garlic fields reach 100%. therefore, growing garlic become of no economic .

Molecular biological approaches, i.e protein profile, isozyme analysis and PCR have been used to determine the variation within and between fungal species and isolates. Protein provide a direct measure of gene homology. Electrophotric protein analysis have been used to study the variations among different isolates and species of *Glomerella* , *cingulata* (Stipes and McCombs 1965), *Septoria* species, (Durbin,1966), *Phytophthora, cinnamoni,* (Gill and Zentmyer,1978), *Sclerotinia sclerotiorum, Sclerotinia trifoliorum* and *Sclerotinia minor* (Petersen *et al.,* 1982), *Botrytis* species (Backhouse *et al.*,1984), *Sclerotinia homoeocarpa, Sclerotium cepivorum* and *Lambertella subrenispora* (Novak and Kohn,1988) *Cephalosporium maydis* and *C. acrrmonium* (Abou-ELSeoud and Saeed 1990) *Sclerotium* species (Saeed and Abou-ELSeoud 1990), *Fusarium oxysporum* Schlect ex fr (Saeed, 1993) *Fusarium monilforme, F. proliferatum and F.subglutinana* (Vaguifalvi and Szecsi, 1994), *Fusarium oxysporum,* (Mandeel *et al.,* 1994) *Fusarium* spp. (Yilmattila *et al.,* 1996), *Fusarium culmorum* (Etebrian *et al.,* 1996) *Beauvera brongiartii* (Reineke and Zebitz, 1996) *Fusarium oxysporum* and *F. moniliforme* (EL-Zawahry,Hida *et al.,* 2000), *Fusarium specialis* (Moubasher and Baibridge, 2000) *Sclerotium cepivorum* Berk. (Mohamed,Nashwa 2004) and *Fusarium solani* and *Fusarium sambucunum* (Abo-El naga.Heidi and El – Aref,2005) .

In addition, molecular differences in protein patterns of pathogenic and non-pathogenic strains were used to determine the virulence related proteins (Wagih *et al.,* 1986 ; Abou-El Seoud and Saeed, 1990; Abo-El naga and El Araf, 2005).

The Present study is an attempt to understand the differences and the inter-relationships between 6 isolates of *Sclerotium cepivorum* in the protein patterns as well as its relation to virulence and geographic origin of isolates .

Material and Methods :

Isolation :

Natural diseased garlic plants showing white rot symptoms were collected from different locations of El-Minya; Assiut and Sohag, governorates of Egypt.

Infected plant parts were washed thoroughly with tap water then cut into small pieces (0.5 cm^2long) and surface sterilized by immersing them in 3% clorax (Sodium hypochloride)solution for three minutes, then washed by rinsing several times in steriled water.

Disinfested plant pieces were plated on Potato Dextrose Agar medium and inocubated at 20^oC for 7 days. The fungal isolates were purified by using hyphal tip isolation techniques as described by Brown (1924). The fungal

isolates were identified according to (Clements and Shear,1957)

Pathgenicity test:

Six isolated of *Sclerotium cepivorum* were tested for their pathogenicity on Chinese garlic cultivar as mentioned by (Abd-El-Rehim, 1984).This experiment was carried out under greenhouse conditions in 2005/2006 growing season. Data were recorded after 90 days from planting as a percentage of infection .

Extraction of fungal protein :

Protein extracts from *S. cepivorum* isolates were prepared according to (Guseva and Gromova, 1982), (Rataj – Guranowska *et al.*, 1984) and (Hussein, 1992) in the following way. Fungal isolates were grown for 22 days at 20^{o}C on liquid Czapek's medium the mycelium was harvested by filtration through cheesecloth, washed with distilled water several times and freezed –dried. The frozen mycelium was 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^{o}C. The protein content in supernatant was estimated according to (Bradfrod ,1976) by using bovine serum albumin as a standard protein, If protein concentration was low, protein would by precipitated from the clarified supernatant by adding ammonium sulfate at 70% of saturation (60 g / 100 mL) then kept in the refrigetor 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 hr. against the buffer and centrifugation at 11.000 rpm for 30 minutes. Protein was estimated in the obtained supernatant.

Electrophoresis of native protein (PAGE) :

Thawed protein-extract supernatant was mixed with equal volume of a solution containing 20% glycerol (v/v) and 0.1% bromophenol blue (v/v) in 0.15 M Tris-Hcl, pH 6.8. Twenty microliters of the resulting suspernsion (40 to 60 µg of protein) was subjected to electrophoresis in 2.5 mM Tris buffer containing 192 mM glycin at pH 8.3. Electrophoresis was conducted at room temperature (approximately 20 to 25^{o} C), for 9 hr. on an 15% poyacrylamide gel with a 6% stacking gel, at 20 and 10 mA, respectively, until the dye reached the bottom of the separating gel. Electrophoresis was performed in a vertical slab mold (16.5 × 14.5 × 0.1 cm). Gel was stained with silver metrate for the detection of protein bands (Sammons *et al.*, 1981).

Electrophoresis dissociated protein (SDS-PAGE) :

For electrophoresis of dissociated proteins, each supernatant

was mixed with an equal volume of a solution consisting of (by volume) 64% buffer (0.15 M Tis Hcl, pH 6.8); 20% glycerol; 6% Sodium dodecyl sulfate (SDS); 10% 2-6 mercaptoethanol and 0.1% bromophenol blue, before boiling in a water bath for 3 minutes .

Twenty-micro liter samples (40 µg of protein) were subjected to electrophoresis in 15% polyacrylamide prepared in 0.1% SDS (laemmli, 1970 and Latorre,*et al*,. 1995), The electrophoresis, staining, and distaining were conduced as described for native (undissociated) protein.

Gel analysis :

A gel decumeutation and analysis system (Uvitec cambridge, Uk was used to document the result of electrophoresis and to claster the electrophoretic patterns of proteins by the UPGMA.

Results :

Table (1) represent the pathogenic capabilities of the tested *Sclerotium cepivorum* isolates on Chinese garlic cultivar . Data indicate that the tested isolates proved to be pathogenic on the tested Chinese garlic cultivars causing white rot disease. Virulence of isolates on the tested garlic cultivar varied from highly virulent to weakly virulent. Isolates No. 2,3 and 6 were highly virulent isolates, however, isolates No. 1,4 and 5 were weakly virulent isolates

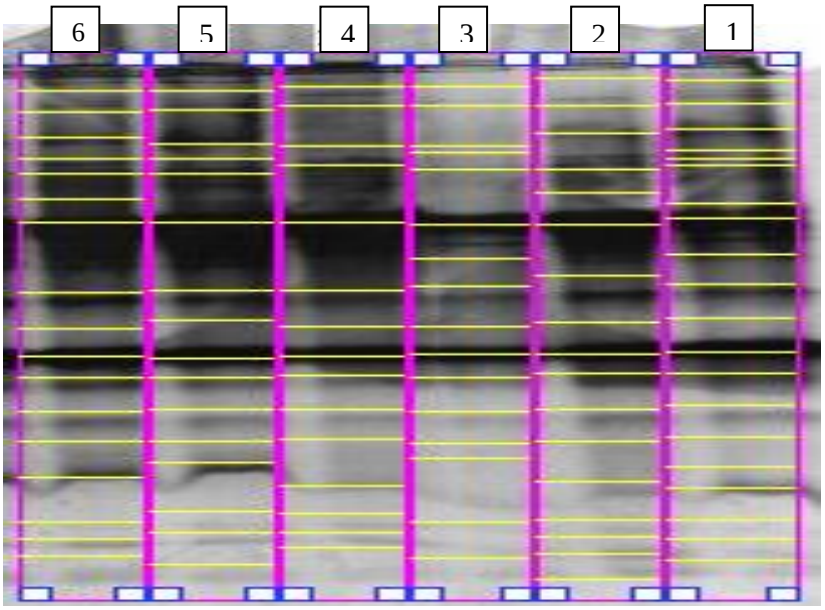
Table (1) : Pathogenic capabilities of six *S. cepivorum* isolates on Chinese garlic cultivar.

Isolate No.	Localities	Percentage of infected plants
1	El-Minya	21.42
2	El-Minya	85.71
3	Assiut	92.85
4	Assiut	14.28
5	Sohag	25.00
6	Sohag	89.28

LSD. At 5% 36.82

Data presented in Table (2) showed the protein profiles separated by PAGE. Isolate No. 1 showed the highest number of bands (20 bands), while isolate No. 4 showed the lowest number (15 bands). The number of bands of the other isolates was 16 bands for isolates No.3 and 5 and 17 bands for isolates No. 2and 6.

Fig(1) : Protein patterns obtained by polyacrylamide gel electropore sis (PAGE) from 6 isolates of *S. cepivorum*



Table(2) : Protein patterns obtained by polacrylamide gel electrophoresis (PAGE) from 6 isolates of *S.. cepivorum* .

	MW-RF					
1	0.063	0.063	0.055	0.055	0.039	0.043
2	0.102	0.098	0.091	0.091	0.091	0.087
3	0.150	0.161	0.165	0.165	0.142	0.134
4	0.189	0.189	0.201	0.177	0.209	0.173
5	0.217	0.217	0.307	0.209	0.252	0.189
6	0.264	0.307	0.433	0.311	0.311	0.201
7	0.307	0.433	0.500	0.374	0.406	0.272
8	0.437	0.488	0.555	0.425	0.492	0.299
9	0.504	0.559	0.591	0.500	0.551	0.366
10	0.555	0.594	0.654	0.551	0.587	0.421
11	0.594	0.654	0.709	0.594	0.654	0.484
12	0.657	0.713	0.795	0.657	0.713	0.547
13	0.713	0.752	0.846	0.717	0.787	0.587
14	0.780	0.843	0.882	0.744	0.858	0.646
15	0.862	0.882	0.909	0.862	0.890	0.705
16	0.894	0.941		0.929	0.921	0.760
17	0.925				0.969	0.799
18						0.858
19						0.894
20						0.933

Fig (2) showed a dendrogram based on claster analysis of the data showed in Table (2). The overall similarity level among the isolates was 10%. At this similarity level the isolates was divided into two remotely related groups, the first group included only isolate No. 1, while the second group included only isolate No. 1, while the second group included the other isolates, the latter group included two isolates from Assiut and two isolates from Sohag.

Similarly level (SL=35%) two isolates from Sohag and two isolates from Assiut (SL=25%). Isolate from El-Minya was placed in separate subclaster (SL=10%).

Fig(2): Phenogram based average linkage claster analysis of electro phoretic protein patterns obtained polacrfamide gel electro phoresis from 6 isolates of S. *cepivorum*.

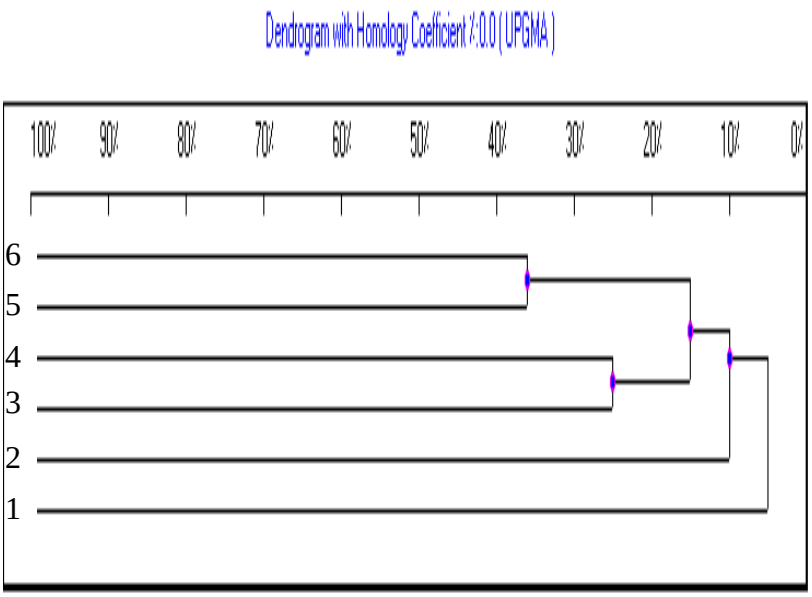
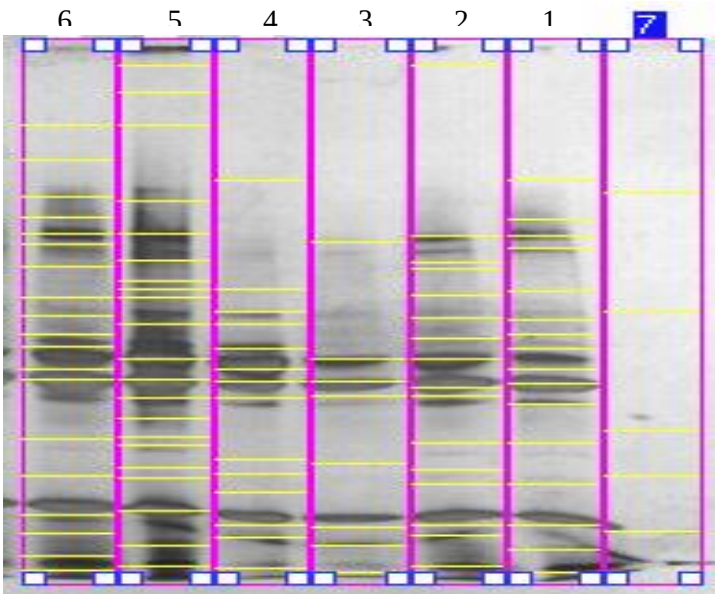


Table (3) showed the protein profiles separated by SDS-PAGE. Isolate No. 5 showed the highest number of bands (19 bands), while isolate No. 3 showed the lowest number of bands (6 bands). The other isolates showed a number of bands ranged from 13 to 17 bands.

Fig(3): Protein patterns obtained by sodium dodecyl sulfate-polacrylamide gel electroporesis (SDS-PAGE) from 6 isolates of *S. cepivorum.*



Table(3) : Protein patterns obtained by sodium dodyl sulfat- polya cylamide gel electrophoresis (SDS-PAGE) from 6 isolates of *S.cepivorum..*

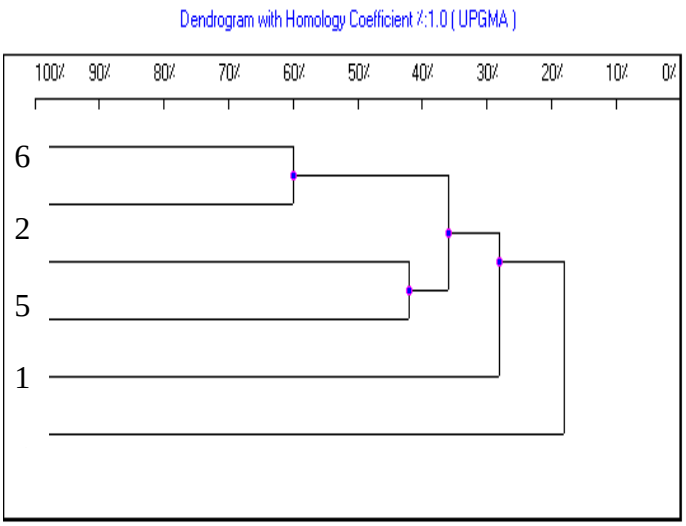
	MW-RF						
	L6	L5	L4	L3	L2	L1	L7
1	75.672	84.172	67.759	58.580	84.172	67.759	66.000
2	70.690	80.362	51.912	43.899	59.492	61.951	49.000
3	65.375	75.672	49.000	40.534	55.602	59.492	29.000
4	62.260	64.751	47.798	37.856	54.730	57.677	14.000
5	59.797	59.797	45.168	17.903	51.096	51.638	2.000
6	58.278	55.895	42.480	2.728	48.402	48.202	
7	55.020	53.022	40.534		46.320	46.755	
8	50.828	51.912	37.433		43.899	45.168	
9	48.602	50.828	19.291		39.445	42.480	
10	46.755	47.798	14.614		37.856	40.180	
11	45.406	43.899	9.671		24.961	36.098	
12	42.480	40.879	3.094		15.891	24.961	
13	40.879	37.433	0.667		11.713	11.713	
14	26.348	32.558			3.094	3.094	
15	14.000	27.028			1.111		
16	4.967	24.258					
17	1.556	16.550					
18		13.403					
19		8.738					

Fig(4) showed dendrogram by claster analysis of the data

shown in Table (3). The overall similarity level among the isolates was 15%.

At this level, the isolates were divided into two remotely related groups the first group included only isolate No. 3 while the second group included the other isolates. This group included two highly pathogenic isolates No.2and 6 (SL=60%), and two weakly pathogenic isolates No.1and 5 (SL=42%). Isolate No. 3 from Assiut was placed in a separate subclaster SL=25%.

Fig(4): Phenogram based average linkage claster analysis of electro phoretic protein pattern obtained by SDS-PAGE from 6 isolates of *S.cepivorum*..



Discussion :

The electrophoresis profiles of protein of 6 isolates of *S. cepivorum* showed differences in the number of bands and molecular weight of the proteins.

Groping the isolates based on PAGE analysis was associated with geographic origin of isolates.

Thus the two isolates from Sohag were included in one subclaster, while those from Assiut were placed in another subclaster. The low level of similarity among isolates from each governorates may indicate high level of genetic diversity within the population of each governorate.

The two isolates from El-Miya were anotable exception because they were rewately related from each other due to the presence of a heterogeneous population of isolator in El-Minya.

Grouping the isolates based on SDS- PAGE was associated with their virulence level regardless of their geographic origin.

The two highly pathogenic isolates No. 2 and 6 were included in one subclaster although isolate No. 6 came from Sohag, while isolate No. 2 came from El-Miya.

Similarity, the weakly pathogenic isolates No. 5 and I were placed in the same subclaster although isolates No, 5 came from Sohage, while isolate No. 1 came from El-Minya. Isolates No.3 and 4 came from Assiut however they were placed in remotely related subclasters because one of them No. 4 was weakly pathogenic, while the other isolate No. 3 was highly pathogenic. This result confirmed that SDS-PAGE grouping the isolates was associated with their pathogenicity and not their geographic origin. Such results are in agreement with those reported by (Wagih *et al.*,1986, Abu-El-Seoud and Saeed,1990, Saeed, 1993, El-Zawahry, Hida *et al.*, 2000, and Abo- Elnaga,.Heidi and Aref, 2005).

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النمط أو المحتوي البروتيني وعلاقته بالشدة المرضية للفطر *Sclerotium Cepivorum* المسبب لمرض العفن الأبيض في الثوم

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تم في هذه الدراسة عزل ستة عزلات من فطر *Sclerotium Cepivorm* من نباتات ثوم مصابة بمرض العفن الأبيض. جمعت من محافظات المنيا ، أسيوط وسوهاج. إجراء اختبار القدرة المرضية للعزلات علي نباتات الثوم. ووجد أن هناك تباين في القدرة المرضية للعزلات حيث تراوحت من عالية للعزلات2و3و6 إلي ضعيفة للعزلات5.4.1 .

كما أجريت دراسة مقارنة لأنواع البروتينات المستخلصة من ستة عزلات للفطر المختبر باستعمال تقنية التفريد الكهربي للبروتين الخام أو المفكك باستعمال مادة صوديوم دوديسيل سلفيت وقد أظهر التحليل الكهربي للبروتين الخام اختلاف العزلات فيما بينها من حيث عدد حزم البروتين في كل عزلة حيث احتوت العزلة رقم 1 علي 20 حزمة من البروتين الخام وأن العزلة رقم 4 احتوت علي 15 حزمة من البروتين وأن باقي العزلات تراوحت من 16 إلي 17 حزمة. وعند فصل البروتين بواسطة مادة صوديوم دودسيل سلفيد اختلفت العزلات من حيث عدد حزم البروتين حيث وجد أن العزلة رقم 5 احتوت علي 19 حزمة من البروتين وأن العزلة رقم 3 احتوت علي 6 حزم بروتين وأن باقي العزلات تراوحت من 13 إلي 17 حزمة .

استعمل التحليل العنقودي لتصنيف هذه العزلات بناء علي ما بينها من درجات تباين في أنماط البروتين وتم التعبير عن هذه النتائج في صورة فينوجرام . أظهرت الدراسة أن أنماط البروتين الخام كانت صالحة لتمييز عزلات الفطر من حيث موقعها الجغرافي أو مصدر العزل وأن أنماط البروتين المفكك يصلح لتمييز عزلات الفطر ذات القدرة المرضية العالية أو الضعيفة .