Journal of Sohag Agriscience (JSAS) 2023, 8(1):01-07

ISSN 2357-0725
https://jsasj.journals.ekb.eg
JSAS 2023; 8(1): 01-07

Received: 06-03-2023
Accepted: 12-03-2023

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Biochemical analysis of eight different *Fusarium oxysporum f.sp. lycopersici* isolates using SDS-PAGE marker

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Abstract

Tomato plants are affected by numerous environmental factors, especially biotic stresses such as fungi, bacteria, viruses, nematodes, and insect pests while growing that lead to yield damage. Globally, *Fusarium oxysporum f. sp. lycopersici* is considered a crucial pathogen that has been documented to cause damage to tomatoes by decreasing the yield in both open fields and the greenhouse. *Fusarium* wilt is a soil-borne fungal pathogen capable of surviving in the soil and crop debris for a decade and has been known to enter the plant through wounds on roots. SDS-PAGE analysis was done in eight isolates of *Fusarium oxysporum f.sp. lycopersici*. The results revealed that the studied isolates of *Fusarium* were clearly distinguished from each other at the studied biochemical level. The dendrogram obtained from the data showed that hierarchical clustering separated the isolates into three groups according to their similarity coefficients, confirming that some protein-based markers were associated. Therefore, biochemical markers associated with highly pathogenic *Fusarium* isolates offered a promising alternative to morphological markers to decide the appropriate selection of high pathogeneses for many biological applications.

Keywords:
Fungi, Fusarium, biotic stress, tomato, protein pattern.

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INTRODUCTION

The identification and taxonomy of fungal species and isolates are mainly based on their morphological and physiological characteristics and the hosts they infect. Biochemical changes associated with the induction of resistance are due to the response to inducing agents in plant pathogenesis (van Loon et al., 1998).

Recently, molecular techniques have been used to identify species and races of fungi and study their taxonomical relationships (Mazzola et al., 1996). Biochemical markers such as proteins and isozymes have offered promising morphological marker alternatives. These techniques have accurate and quick results and simplify. However, biochemical markers are sometimes affected by environmental factors, but their numbers are limited.

Sodium Dodecyl-Sulfat Polyacrylamid Gel Electrophoresis (SDS-PAGE) was used to determine any biochemical marker for the pathogenic ability. So, differences in protein patterns reflect genetic variation on the biochemical level and have been increasingly used to study the genetic variability of several microorganisms (Turner et al., 1999).

Therefore, this investigation aims to conduct electrophoretic studies through protein profiles among the studied *F. oxysporum f.sp. lycopersici* isolates.

MATERIALS AND METHODS

Isolation of fungal cultures

Fusarium isolates were isolated from wilt infested tomato plants. Infested samples were sterilized by dipping in 10% (w/v) sodium hypochlorite solution for (3–5) min and washed thrice with sterile water. Then, the infested samples were cut with a sterile blade and placed on the surface of potato dextrose agar media. PDA was amended with streptomycin antibiotic to minimize the chances of any bacterial growth. Plates were incubated at 28 ± 2 ^{o}C and observed periodically. The fungi were identified following sporulation and pure cultures were stored at 4 ^{o}C on PDA slants. All the previous procedures were done in the laboratory of plant Pathology, plant pathology dept., faculty of agric., Sohag University, Sohag, Egypt. The studied *Fusarium* isolates are (Fu-1, Fu-2, Fu-3, Fu-4, Fu-5, Fu-6, Fu-7 and Fu-8). The source of each isolates was infected tomato plants.

Protein extraction and estimation

Sample buffer

a) 7.88 gm. from tris HCL dissolves in 50 ml d-distal H_2O make solution 1M Tris HCL PH =6.8 to..
b) Mix with: (1 gm. SDS + 2.5 ml of β-mercaptoethanol + 2.5 ml of Tris HCL 1M pre-prepared) dissolve in 40 ml in…
c) Distal H_2O and complete total volume to 50 ml.

Electrode buffer

(6 gm. of tris base +28.84 gm. of glycine + 2gm. of SDS) and complete volume to 1000 ml d-distal H_2O to make 2X solution.

Loading dye

(0.005gm of bromophenol blue+ 12 ml glycerol) dissolve in 8 ml distal H_2O; the total volume 20ml solution.

Ammonium per sulphate (APS) 10%

0.5gm. of APS dissolves in 5ml of d-distal H_2O (Freshly prepared).

SDS 10%

0.5gm. of SDS dissolves in 5ml of d-distal H_2O (Freshly prepared).

Protein Extraction

The mycelial growth from the tested isolate of *F. oxysporum f.sp. lycopersici* and extracted by grinding 1g freeze-dried mycelium in pestle and mortar with liquid nitrogen according to the following steps: The samples are crushed; electrode buffer is placed on them, transferred to an eppindorf tube, boiled for 10 minutes, and centrifuged at 10,000 rpm. The supernatant is the isolated protein. The protein content in the supernatant was estimated according to the method of Bradford (1976) with bovine serum albumin as the standard protein. Protein content was adjusted to 2 mg/ml per sample.

Gel solution

A. **Stock 1:** (30 gm. of Acrylamide + 0.8 gm. of bis-acrylamide) and complete total volume to 100 ml with d-distal H_2O
B. **Stock 2:** (18.2 gm. of Tris-BASE or Tris-HCL) dissolve in 50ml H_2O; then complete total volume to 100ml with d-distal H_2O PH =8.8.
C. **Stock 3:** (6 gm. of Tris-HCL) dissolve in 50ml H_2O; then complete total volume to 100ml with d-distal H_2O PH =6.8.

Preparation of gel solution (large gel):

Chemicals	Separating gel			Stacking gel
CONC.	8%	12%	15%	5%
d-distal H2O	26.5 ml	20 ml	18 ml	11 ml
Stock 1	10 ml	24 ml	37.5 ml	2.6 ml
Stock 2	5621 ml	15 ml	19 ml	-
Stock 3	-	-	-	2 ml
10% SDS	0.5 ml	1 ml	0.75 ml	100 µL
10% APS	0.5 ml	1 ml	0.75 ml	100 µL
TEMED	04 µL	50 µL	100 µL	20 µL

Pouring the separating and staking gel

The separating gel was poured between glass sandwich using scientific instruments (San Francisco CA, USA, Model XPO77 Hoefer) and gently covered with 1 cm of water. Polymerization started within 25-30 min. after pouring. The stacking gel was then poured and allowed for polymerization after about 30 min.

Loading samples

Twenty micro-liters of the crude protein solution were applied to the wells of the stacking gel. The samples were covered with electrode buffer. Few drops of bromophenol blue (4 mg/100 ml deionized water) were added to the electrode (tracking dye).

Protein separation by SDS-PAGE

The samples were diluted in a ratio of (1: 1) with the sample buffer and heated to 95-100 ^oC for 5 min in hot water bath. Equivalent amounts of proteins (120 µl) from each sample were loaded into each well for each sample.

Gel running

Electrophoresis was performed in a vertical slab mold (Hoefer Scientific Instruments, San Francisco, CA, USA, model LKB 2001, measuring 16 x (18 x 0.15 cm). Electrophoresis was carried out at 30 milliamper (m.A.) at 10º C for 3 hours. The gel was run at 60V until the tracking dye approached the bottom of the cell. The protein bands were determined using protein markers of 66, 45, 36, 29, 24 and 20.1 kd (Sigma low molecular weight protein marker cat. No. 70 L Kit).

Staining buffer

(1gm. of Coomassic brilliant blue R + 180 ml of methanol +180 ml of d-distal H_2O + 40 ml Acetic acid glacial).

Destaining buffer

(100ml of Acetic acid glacial + 400 ml Methanol + 400 ml of d-distal H_2O).

Protein fixation and staining

The gels were fixed and stained overnight in a filtered staining solution containing 0.1% (w/v) Coomassie Brilliant Blue R dissolved in 30% (v/v) methanol, 10% (v/v) acetic acid and water.

Destaining of the gels

The gels were destained by successive immersion in the mixture of distaining solutions, until the background was clear. The distaining solution was 25% (v/v) methanol, 10% (v/v) acetic acid and distilled water. The staining and distaining procedures were performed at room temperature.

Photography and preservation

The gels were photographed, transferred to a preserving solution containing 10% (v/v) glycerol in a distaining solution for an hour and allowed to dry at room temperature when the surface of the gels appeared sticky, they were mounted between cellophane preserving sheets and left overnight for thorough drying at room temperature.

Data analysis and software

After electrophoresis, the protein profile was visualized with an UV transilluminator. Then, markers were scored from the gels as protein profile fragments present (1) or absent (0) in all the lanes. The bands were determined against a ladder of protein bands marker consisted of (11, 17, 20, 25, 35, 48, 63, 75, 100, 135, 180 and 245 kDa). Applying the Jaccard's coefficient to the binary matrix calculated the pairwise distance among the isolates, which were clustered by UPGMA

(Unweighted Pair Group method using arithmetic means).

RESULTS AND DISCUSSION

Protein analysis by (SDS-PAGE)

The total proteins of eight *Fusarium* isolates were extracted and analyzed using SDS-PAGE aiming to find protein markers associated with isolates (Fig1). Data in table (1) showed the presence of different protein bands in studied *Fusarium* isolates. Results that obtained on protein analysis were discussed according to numbers given in Table (2) which represents bands in Fig (2).

The obtained results revealed that bands (2,3,6,7,8) were a feature of *Fusarium* isolates(4,5,7) of highly pathogenic and Bands (1) for *Fusarium* isolates (5,6,7) which could be considered a distinguishable marker for these isolates. On the contrary, bands (3,9) were unique bands marked only in the *Fusarium* isolates(3,7) separately which could be low.

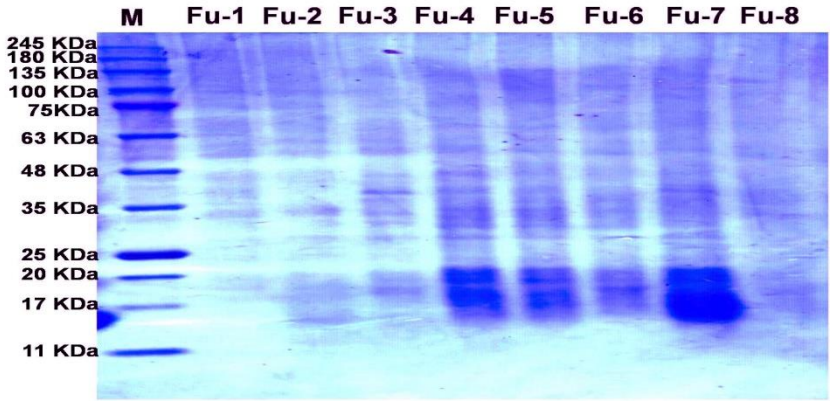

Fig. (1). Sodium Dodecyl Sulfate Polyacrylamid Gel Electrophoresis for protein extracted from Fu-1, Fu-2, Fu-3, Fu-4, Fu-5, Fu-6, Fu-7 and Fu-8 fusarium isolates.

Table. (1) Presence of protein bands in Fu-1, Fu-2, Fu-3, Fu-4, Fu-5, Fu-6, Fu-7 and Fu-8 fusarium isolates.

Bands No.	M (kDa)	Fu-1	Fu-2	Fu-3	Fu-4	Fu-5	Fu-6	Fu-7	Fu-8
Band 1	125	-	-	-	-	+	+	+	-
Band 2	41	-	-	+	+	+	-	+	-
Band 3	35	-	-	-	+	+	-	+	-
Band 4	33	-	-	-	+	+	-	-	-
Band 5	29	-	-	-	+	+	-	-	-
Band 6	21	-	-	-	+	+	+	+	-
Band 7	19	-	-	-	+	+	+	+	-
Band 8	17	-	-	-	+	+	-	+	-
Band 9	14	-	-	-	-	-	-	+	-

The correlation between all genotypes

Results in Table (2), and illustrated in dendrogram Fig. (2), showed obviously that, the relationship between the isolates Fu-4 and Fu-5 was (0.875). It means that the isolate Fu-4 was closely related to the isolate Fu-5 than other isolates. These two isolates Fu-4 and Fu-5 were distinguished with highly pathogenic in protein expression. On the other hand, the isolate Fu-5 was more related to the isolate Fu-6 than other isolates. These two isolates, Fu-5 and Fu-6 were associated with moderate pathogenic in protein expression. This trend might be due to the molecular affinity. However, no similarity was found between the most isolates.

Table (2) The similarity matrix between Fu-1, Fu-2, Fu-3, Fu-4, Fu-5, Fu-6, Fu-7 and Fu-8 *Fusarium* isolates, as revealed by (SDS-PAGE).

	Fu-1	Fu-2	Fu-3	Fu-4	Fu-5	Fu-6	Fu-7	Fu-8
Fu-1	1.000							
Fu-2	0.000	1.000						
Fu-3	0.000	0.000	1.000					
Fu-4	0.000	0.000	0.143	1.000				
Fu-5	0.000	0.000	0.125	0.875	1.000			
Fu-6	0.000	0.000	0.000	0.250	0.375	1.000		
Fu-7	0.000	0.000	0.143	0.556	0.667	0.429	1.000	
Fu-8	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000

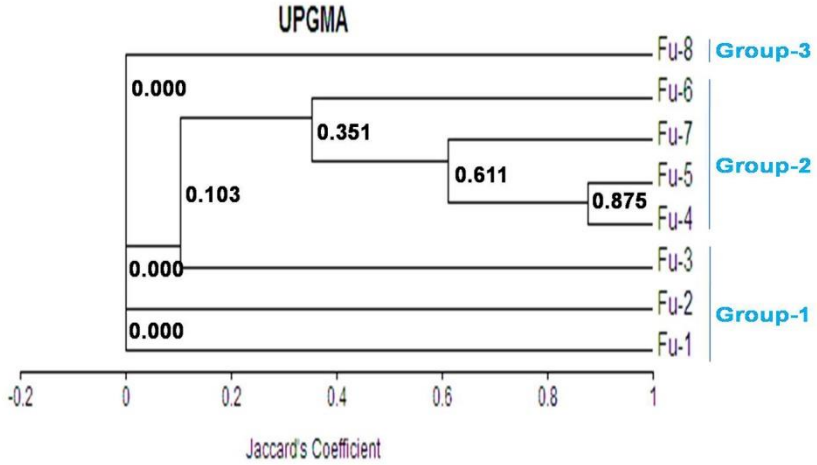

Fig. (2) Linkage dendrogram for the studied Fu-1, Fu-2, Fu-3, Fu-4, Fu-5, Fu-6, Fu-7 and Fu-8 *Fusarium* isolates based on protein polyacrylamid gel electrophoresis (SDS-PAGE).

DISCUSSION

The SDS-PAGE of protein pattern of eight *Fusarium* isolates showed different degrees of similarity due to differences in bands between the isolates. This finding indicated the genetic variability among the tested isolates. The obtained results agree with those obtained by Ibrahim et al. (2003) who revealed that variation could be considered as a reflection of the genetic variability in the tested isolates. These results are consistent with those by (Mohammadi et al., 2003). In addition, the geographical source of *Fusarium* did not affect on the variability among isolates indicating that the variability is not due to the environmental conditions, but may be due to some degrees of genetic variability. Moreover, protein analysis was used in several studies to provide information on the biochemical bases in *Fusarium* isolate (Li et al. 1983, Stein, 1983, Rier, et al. 1987 and Lin-chienyih et al. 1995). The non-reproducible bands interpreted according to presence of glycerol in the reaction buffer (Hai Lu and Negre.1993). In addition, the absence of these protein bands can be explained as a result of retarding the trigger of resistance gene transcription, which leads to pathogen-related proteins, as previously reported by Satheesh and Pari (2004) and El-Khallal (2007).

UPGMA is a simple agglomerative or hierarchical clustering method used in bioinformatics for the phylogenetic analysis. The results obtained in the present study are noteworthy and showed the similarity with the observations of Abd-Elsalam et al. (2003), Ingle and Rai (2011), Bonde et al. (2013) and Gupta et al. (2009).

CONCLUSION

SDS-PAGE analysis illustrated the existence of close relationship between the *Fusarium* isolates of high potential for pathogenic isolates (4, 5, and 7) at biochemical level. There were some protein-based markers associated with highly pathogenic. Therefore, biochemical markers associated with highly pathogenic of the studied *fusarium* isolates offered a promising alternative to morphological marker to decide the appropriate selection regarding highly pathogeneses to be used in many biological applications.

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التحليل البيوكيميائي لثمانية أنواع مختلفة من عزلات *Fusarium oxysporum f.sp. lycopersici* باستخدام محدد SDS-PAGE

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الملخص العربي

تتأثر نباتات الطماطم بالعديد من العوامل البيئية ، وخاصة الحيوية منها مثل الفطريات والبكتيريا والفيروسات والديدان الخيطية والآفات الحشرية أثناء النمو، مما يؤدي إلى تلف المحصول. وعلى الصعيد العالمي ، فإن فطرالـ *Fusarium oxysporum f. sp. Lycopersici* ، يعتبر أحد مسببات الأمراض الهامة التي تم توثيقها لإحداث ضرر للطماطم من خلال تقليل المحصول في كل من الحقول المستديمة والبيوت المحمية. مرض ذبول الفيوزاريوم هو أحد مسببات الأمراض الفطرية التي تنتقل عن طريق التربة ويمكنه البقاء على قيد الحياة في التربة وبقايا المحاصيل لمدة من الزمن ، ومن المعروف أنه يدخل النبات من خلال الجروح الموجودة على الجذور. تم إجراء تحليل SDS-PAGE في ثماني عزلات من *Fusarium oxysporum f.sp. lycopersici*. أظهرت النتائج أن عزلات الـ *Fusarium* التي تم دراستها تميزت بوضوح عن بعضها البعض على المستوى البيوكيميائي. كما أظهر مخطط الشجرة الذي تم الحصول عليه من البيانات أن التجميع الهرمي قسَّم العزلات إلى ثلاث مجموعات وفقًا لمعاملات التشابه فيما بينها ، مما يؤكد ارتباط بعض العلامات القائمة على البروتين. لذلك ، فإن العلامات البيوكيميائية المرتبطة بعزلات الـ *Fusarium* ذات القدرة المرضية العالية تقدم بديلاً واعدًا للعلامات المورفولوجية لتحديد الاختيار المناسب لأهم للمسببات المرضية للعديد من التطبيقات البيولوجية.