

**SEROLOGY AND PCR-BASED TECHNIQUES FOR DETECTION
 OF *FUSARIUM* WILT IN SESAME**

BY

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ABSTRACT

It is well known that serological methods are very useful for detecting some plant pathogens. In this study, the antisera of five *Fusarium* spp., i.e., *F. moniliforme*, *F. solani*, *F. oxysporum*, *F. roseum* and *F. semitectum* were used to investigate the serological relationships between them. Results illustrated that, there was a specificity as for *F. oxysporum* for infection with sesame cv. Giza-32 and sesame cv. Tushka-1. Polymerase chain reaction (PCR) detection of *F. oxysporum* isolates using F1 & F2 primers, showed a positive reactions with ten *F. oxysporum* isolates. DNA extracts of *F. solani*, *F. moniliforme* and *F. oxysporum* gave positive reactions compared with the positive control. PCR technique was able to detect very low amount of nucleic acid extracted from *F. oxysporum* in a dilutions ranged from 10^{-1} to 10^{-9} . In addition, results showed that the F1 & F2 primers were useful for detection of 12 *Fusarium* isolates collected from different locations. It could be concluded as PCR using the two primers would provide a powerful tool for detection of *F. oxysporum* isolates.

Key words: Sesame, Serological methods, *Fusarium*, PCR, Detection.

INTRODUCTION

Sesame (*Sesamum indicum* L.) plants are attacked by several soil and/or seed-borne fungi, which may cause seed decay, damping-off, root-rot, wilt disease and charcoal rot of sesame plants (Avila and Pinda, 1996). Charudattan and Devay (1972) demonstrated a common antigen relationship among *Fusarium* spp. and wilt-susceptible and wilt-tolerant varieties of cotton. When the common antigenic substance was isolated and purified, it was found to be a polysaccharide-protein complex. It was postulated that the common antigens may be involved in the establishment and survival of *Fusarium* isolates in host tissues. When the seed globulin of cotton cultivars susceptible to *Fusarium* wilt reacted with antiserum of *F. oxysporum* f.sp. *vasinfectum*, more precipitin lines were formed than the resistant cultivars. On the other hand, no obvious reaction was detected in case of antiserum of *F. moniliforme*, which was nonpathogenic on cotton (Abd El-Rehim *et al.*, 1988). Hussein *et al.* (1996) compared between *F. oxysporum*, *F. moniliforme*, and *F. solani*, isolated from diseased cotton seedlings infected with damping-off, by double

diffusion (DD) and immunoelectrophoresis (IE) techniques, where the species were grouped by the cluster analysis and the results were expressed as phenograms. The taxonomic relationships established based on DD matched those based on modern systems of morphological classification.

Diagnostic methods based on polymerase chain reaction (PCR) where the amplification of specific nucleic acid sequences have high analytical sensitivity to discriminate between different strains of *F. oxysporum* (Nazar *et al.*, 1991; Henson and French., 1993; Assigbelse *et al.*, 1994; Bentley *et al.*, 1994; Migheli *et al.*, 1997). Baayen *et al.* (2000) studied the development of specific PCR primers, which can reliably replace conventional isolation and pathogenicity test for the identification of *F. oxysporum* f.sp. *basilici*, specific races in *F. oxysporum* f. sp. *dianthi* & *gladioli*, and *F. oxysporum* f.sp. *albedinis*.

This study was designed to isolate and purify of *Fusarium* species or isolates from *Fusarium* wilt diseased-sesame seeds followed by raising antiserum sepecific to *Fusarium* spp. In addition, the role of DD and PCR techniques in detecting and differentiating the *Fusarium* spp. or isolates was studied.

MATERIALS AND METHODS

Isolation and identification of causal microorganism

Isolates of *F. oxysporum* were isolated and identified from samples of sesame cv. Giza 32 with *Fusarium* wilt lik-symptoms according to the method of Booth (1971) and Barnett and Hunter (1998).

Antisera raising

Protein(s) extract was prepared from the mycelia of *Fusarium* spp. grown for 22 days at 25-30°C on liquid (Cazpek's agar medium) according to the method of Rataj *et al.* (1984). The protein content in the supernatant was estimated by adding ammonium sulfate at 70% saturation as described by Bradford (1976) using bovin serum albumin (BSA) as a standard protein. Replicates of Newzealand rabbits, 3-4 kg weight, were immunized by antigens of the five *Fusarium* species to raise antisera as discribed by Hussein (1992).

DD technique

The technique was carried out according to Ouchterlony and Nilsson (1978). The central well was filled with antiserum of *Fusarium* sp. (5 species) and the peripheral wells were filled with antigens of *Fusarium* spp. Plates were kept in humid conditions at room temperature (18-24°C) in the dark for 48-72 hr. The developing precipitated lines were examined and recoded per each plate.

Extraction of proteins from different host plants

From seeds of sunflower cv. hybrid, peanut cv. Giza-5, sesame cv. Tushka-1, sesame cv. Giza-32, lupine cv. Local, soyabean cv. crawford, fabae bean cv. Giza 8 and chickpea cv. Balady; the protein extraction was prepared according to the method of Hussein (1992). The protein content in the supernatant was adjusted to a concentration of 3 to 4 mg/ml according to Bradford (1976) using BSA as a standard protein.

PCR detection

Fungal strains and culture medium

Twelve isolates of *F. oxysporum* f. sp. *sesami* were used in this study as shown in Table (1). Fungal isolates were grown on 75 ml potato dextrose broth in 500 ml Erlenmyer flasks at room temperature (Approx. 24±2°C). Cultures were filtered through a double layers of cheesecloth, and the mycelia were washed with sterile distilled water and freeze-dried (ISTA, 1995).

Table (1) : Isolates of *Fusarium* sp. used for PCR detection.

Isolates No.	Locations	Previous crops	Sampling date	Sesame host genotypes
1	Sharkia	Alfa-Alfa	May	Giza 32
2	Sharkia	Faba bean	May	Giza 32
3	Ismailia	Faba bean	June	Tushka 1
4	Ismailia	Alfa-Alfa	June	Shandawel
5	Giza	Alfa-Alfa	July	Shandawel
6	Giza	wheat	July	Giza 32
7	Giza	wheat	May	Giza 32
8	Benisuef	Faba bean	June	Giza 32
9	Benisuef	Alfa-Alfa	July	Tushka 1
10	Sohag	Alf-Alfa	July	Tushka 1
11	Sohag	Faba bean	June	Tushka 1
12	Sohag	Faba bean	May	Tushka 1

DNA extraction

In this experiment DNA was extracted from 5-7 days-old cultures of the fungal isolates according to the method described by (Sambrook *et al.*, 1989).

Primers used

Specific primers for the detection of *F. oxysporum* f. sp. *sesami* were kindly provided by Prof. Dr. Smith Parrey, Faculty of Agricultural Sciences, University of Alberta, T6G, Canada. The primers sequences and positions are described in Table (2).

Table (2): Primers name, sequences, and their orientation.

Primers name	Sequences (5'-----3')	Sizes (nts)	Orientation
H F1	ATCAAGAGCTAAAGGTCC	19	Forward
C F2	GGCATCTATCTTGGTCAA	19	Reverse

PCR mixture

PCR was performed in 50 µl total volume containing 100 ng of genomic DNA, 50 pmol of each primers (F1 and F2), 50X-PCR buffer, 3 mM MgCl₂, 200 mM each of dNTPs, 2.5 unit of *Taq* DNA polymerase (Promega, Madison, WI) and d.H₂O. The PCR cycle was denaturated at 94°C for 5 min, followed by 25

cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for one min. The final segment was extended to 5-7 min.

Agarose gel electrophoresis:

Aliquot (10 μ l) of PCR amplified DNA was added to 2 μ l of loading dye and directly analyzed by electrophoresis through horizontal slab gels contained 1% agarose and 1 X TAE buffer (Sambrook *et al.*, 1989). The gel was stained in ethidium bromide for 15 min (Sambrook *et al.*, 1989). Separated fragments were visualized using UVP Image Store 5000 (Gel Documentaion System, San Gabriel, CA).

For determination of the minimum amount of the fungal DNA that could be used as templates for PCR detection, variable dilutions of *F. oxysporum* f. sp. *sesami* genomic DNA ranging from 10^{-1} to 10^{-9} were used.

The presence of the expected PCR product was also detected in the DNA extracts of three *Fusarium* species (*F. oxysporum*; *F. solani* and *F. moniliforme*).

RESULTS

In this study, the characters of Genus *Fusarium* described by Commonwealth Mycological Institute (Booth, 1971) was followed for identification of the *Fusarium* isolates.

Serological studies

Results in Figure (1) investigated the similarity indicia that calculated by using all the resulting common antigens (specific and non specific ones) to differentiate between *Fusarium* spp. Data also indicate that there were noticeable differences between the different species of *Fusarium*, i.e. *F. oxysporum*, *F. moniliforme*, *F. solani*, *F. semitectum* and *F. roseum*.

Results in Figure (2) showed that DD reaction of the antiserum of *F. oxysporum* against antigens of 8 host plant, i.e., sunflower, peanut, sesame cv. Giza-32, sesame cv. Tushka-1, lupine local, soybean, chickpea and fabae bean, respectively, concluded that the tested antiserum of *F. oxysporum* were positive in reaction with the two sesame cultivars only cv. Giza-32 & Tushka1 and gave a positive reaction with the tested antiserum of *F. oxysporum*.

PCR detection of *Fusarium* sp.

The PCR technique provides a sensitive and specific technique for detection and identification of *F. oxysporum* f.sp. *sesami*, using a specific primer, and overcoming problems associated with other traditional detection methods. PCR has been utilized successfully to detect *F. oxysporum* f. sp. *sesami*. The DNA was amplified by PCR using F1 and F2 primers, the amplification products were analyzed by 1% agarose gel electrophoresis. DNA extracted from 10 isolates of *Fusarium oxysporum* f.sp. *sesami* consistently generated on amplification signal of the expected size using the primer set HF1 + CF2, while no amplification was obtained from DNA extracted from control lane 11 as shown in Figure (3).

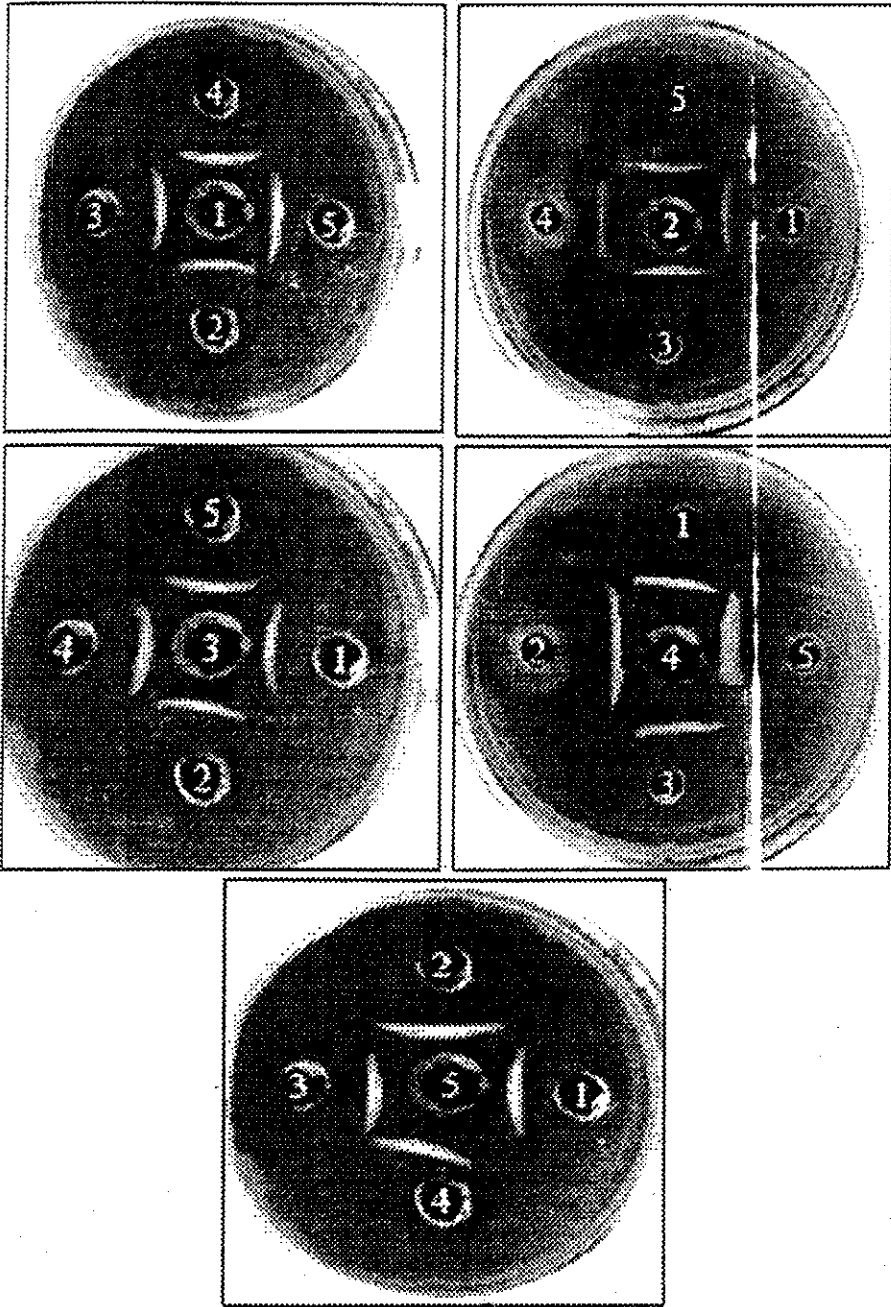


Figure (1): DD reactions of the antisera of *F. moniliforme* (1), *F. oxysporum* (2) *F. solani* (3) *F. roseum* (4) *F. semilectum* (5) against each others.

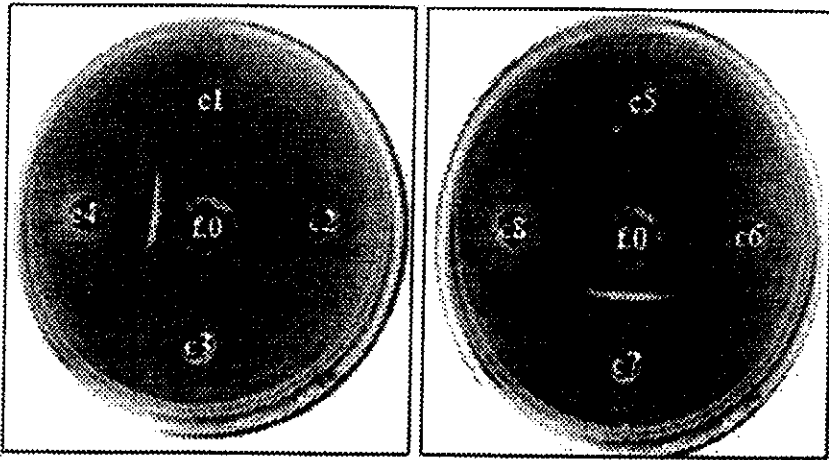


Figure (2): DD reaction of the antiserum of *F. oxysporum* in the center well (f0) against antigens of seeds of sunflower hybrid (c1), peanut cv. Giza 5 (c2), soyabean cv. Crawford (c3), sesame cv. Giza-32 (c4), lupine local (c5), chickpea cv. Balady (c6), sesame cv. Tushka 1 (c7), and fabae bean (c8).

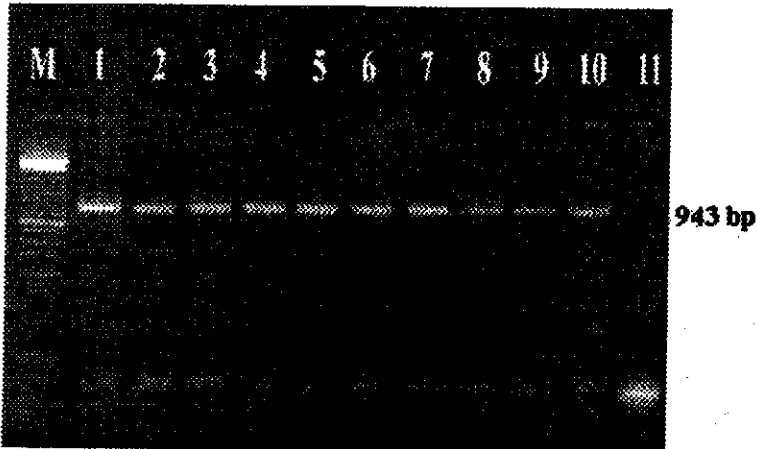


Figure (3): PCR detection of 10 isolates (lanes 1-10) *Fusarium oxysporum* f.sp. *sesami* isolated from sesame seeds. Lane 11: Negative control (PCR mixture with no DNA as a ten plates) and Lane M: molecular weight marker (100-1600 bp DNA ladder, Pharmacia).

DNA optimization for PCR detection

A range of dilutions of total genomic DNA from cultures of *Fusarium oxysporum* f.sp. *sesami* was tested to determine the sensitivity of PCR assay for DNA from *F. oxysporum* mycelium, 10^{-1} to 10^{-9} , the lowest detectable dilutions of fungal target DNA was 10^{-8} Figure (4).

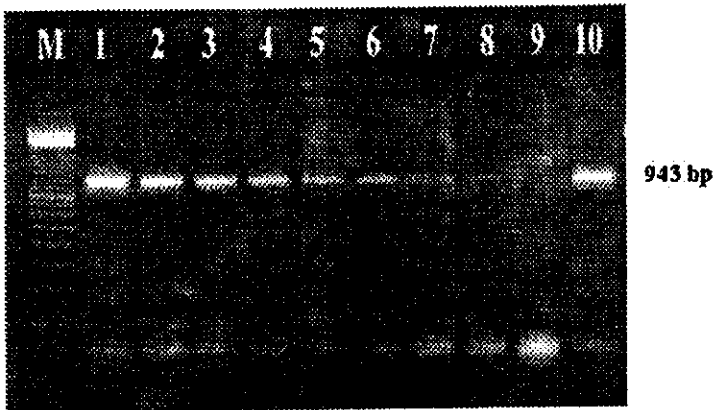


Figure (4): PCR detection of 9 DAN dilutions (10^{-1} - 10^{-9}) (Lanes 1-9) of *F. oxysporum*, f.sp. *sesami*; M: molecular weight marker (100-1600bp DNA ladder, Pharmacia).

Specificity of *F. oxysporum* isolates:

Specificity of F1 and F2 primers belonging to the *F. oxysporum* f.sp. *sesami* to the DNA genome of three *Fusarium* spp was tested as shown in Figure (5) in the presence of a positive control (Lane 5). Results showed that a single fragment of 943 bp specifically amplified from templates of *F. oxysporum* f.sp. *sesami* (Lane 3). No products were amplified from the DNA of *F. solani*, *F. moniliforme* (Lanes 1 and 2, respectively).

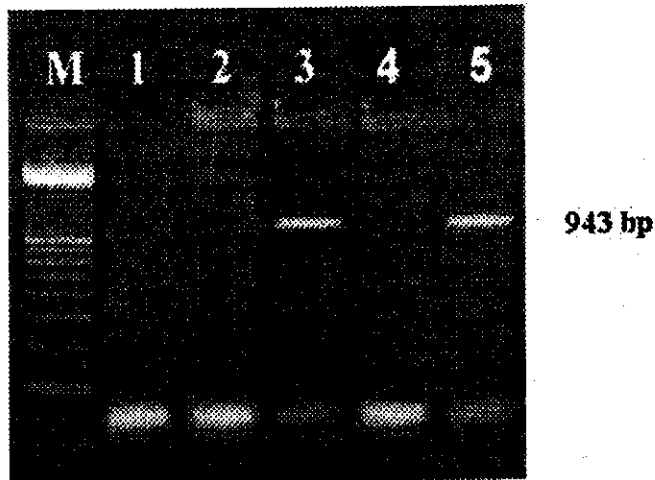


Figure (5): PCR detection using DNA extracts from three *Fusarium* spp. (Lane 3). *F. solani*, *F. moniliforme*, *F. oxysporum* f.sp.*sesami* (Lanes 1, 2 and 3, respectively). Lanes 4 and 5: Negatives and positive controls, respectively). Lane M: molecular weight marker (100-1600 bp DNA ladder, Pharmacia).

DISCUSSION

Due to the great importance of *Fusarium* spp., that affected sesame cultivars and caused a several diseases, the following study is directed to differentiate between the different *Fusarium* species.

As for serological studies, *Fusarium* species showed various homology percentages between them. On the base of general antigens, up to 90% homology percentage was expressed between *F. moniliforme* and *F. solani* and *F. roseum*. The obtained data suggested that two species of *Fusarium*; *F. moniliforme* and *F. solani* could put into one group different than the other species. These results are in agreement with those reported by Abd El- Satar *et al.* (2003) Bentley *et al.* (1994) and Chelkowski *et al.* (1999) they demonstrated that serological methods may be very useful for detecting some plant pathogens also, for the establishment of serological relationships between different fungal species or genera. Khalil (1981) reported the importance of using serological methods for studying the relations between infected and apparently healthy flax plants compared with control treatment.

Molecular diagnostics offers the potential for sensitive and specific detection of pathogens also, detection more than one pathogen in a single test by the use of primers (Molecular diagnostics). This method is also suitable for automation in short consuming time. However, the costs were higher than other conventional methods. Primers could be designed to be extremely specific and amplify a single pathogen from other very closely related species (Nazar *et al.*, 1991; Niessen *et al.*, 1997 and Thomas *et al.*, 1998) or more general to amplify a range of organisms (Wilits and Sherwood, 1999).

Two taxon-selective primers were designed for the internal transcribed spacer (ITS) region in the nuclear ribosomal repeat unit, these primers F1 and F2 were intended to be specific to *F. oxysporum* f.sp. *sesami* specificity of these primers were tested against three *Fusarium* species, the obtained results showed that, the primers described would provide a powerful tool for detection of *Fusarium oxysporum*. Also, this tool could be very valuable in defining and confirming the boundaries and relationships of classical sections and species. Whether these or similar techniques will replace microscopic examination of cultures and make identification easier for non specialists because, it is the favorable and easier for identifying fungal species (Baayen *et al.*, 2000 and Alves-Santos *et al.*, 2002).

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إستخدام الطرق السيرولوجية وتفاعل البلمرة المتسلسل للكشف عن فطر الذبول في السمسم.

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من المعروف أن الطرق السيرولوجية تعتبر أداة مفيدة جدا في الكشف عن بعض مسببات الأمراض النباتية وفي هذه الدراسة تم إعداد السيرم المضاد لخمسة أنواع من الفيوزاريوم شملت (فيوزاريوم أوكسيسبوريم و فيوزاريوم موتيليفورم و فيوزاريوم سولاني و فيوزاريوم روزيوم و فيوزاريوم سيمكتيم وذلك لدراسة العلاقات السيرولوجية بينهم.

أوضحت النتائج أنه يوجد تخصص بالنسبة للفطر فيوزاريوم أوكسيسبوريم في إصابته للسمسم صنفى جيزه ٣٢، وتوشكا ١، كما أجرى أيضا الكشف عن عزلات الفطر فيوزاريوم أوكسيسبوريم باستخدام تفاعل البلمرة المتسلسل PCR باستخدام نوعين من البانثات (F1 & F2) حيث أظهرت النتائج وجود تفاعلات موجبه مع عشرة عزلات من الفطر الفيوزاريوم أوكسيسبوريم. أما بالنسبة لمستخلصات الحامض النووى DNA للفيوزاريوم سولاني و فيوزاريوم موتيليفورم و الستيوزاريوم أوكسيسبوريم فإن جميعها قد أعطى تفاعلات موجبه مقارنة بالكنترول.