ISSN 1110-0419

Annals Of Agric. Sc., Moshtohor,
Vol. 45(2): 587-596, (2007).

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

BY

Mahdy, A.M.M.*; Nawal A. Eisa *; Faten, M. Abdel-Latif,*; El-Wakil, A. A. ** and El-Wakil, D. A. **

* Botany Dept., Fac. Agric., Moshtohor, Benha Univ., Egypt

** Plant Pathol. Res. Inst., Agric., Res.Center, Giza, Egypt

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 specificty as for *F. oxysporum* for infection with sesame cv. Giza-32 and sesame cv. Tushka-1. Ploymerase 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. oxysproum* 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. oxyspourm, 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 deveolpment of specific PCR primers, which can reliably replace conventional isolation and pathogenecity 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 purifiy 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 recroded 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 Agricul ural 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	ATTCAAGAGCTAAAGGTCC	19	Forward
C F2	GGCATCTATCTTGGTCAAA	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 $MgCl_2$, 200 mM each of dNTPs, 2.5 unit of *Taq* DNA polymerase (Promega, Madison, WI) and d.H_2O. 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 μl) of PCR amplified DNA was added to 2 μl of loading dye and directley analyzed by electrophoresis through horizontal slab gels contained 1% agarose and 1 X TAE buffer (Sambrook *et al.*, 1989). The gel was stained in ethedium bromide for 15 min (Sambrook *et al.*, 1989). Separated fragments were visulaized 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 singal of the expected size using the primer set HF1 + CF2, while no amplificaton was obtained from DNA extracted from control lane 11 as shown in Figure (3).

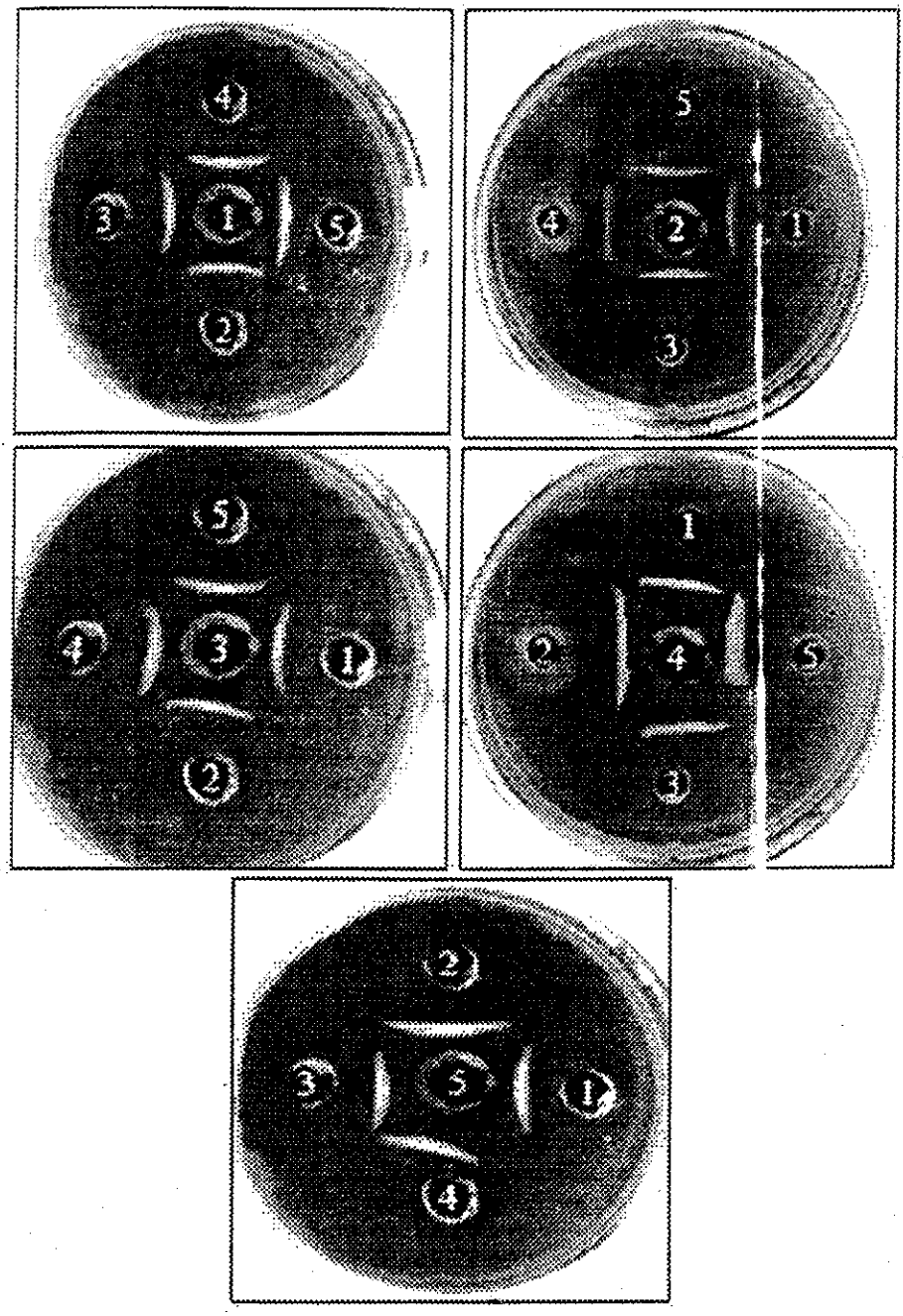

Figure (1): DD reactions of the antisera of *F. moniliforme* (1), *F. oxysporum* (2) *F. solani* (3) *F. roseum* (4) *F. semilectum* (5) against each others.

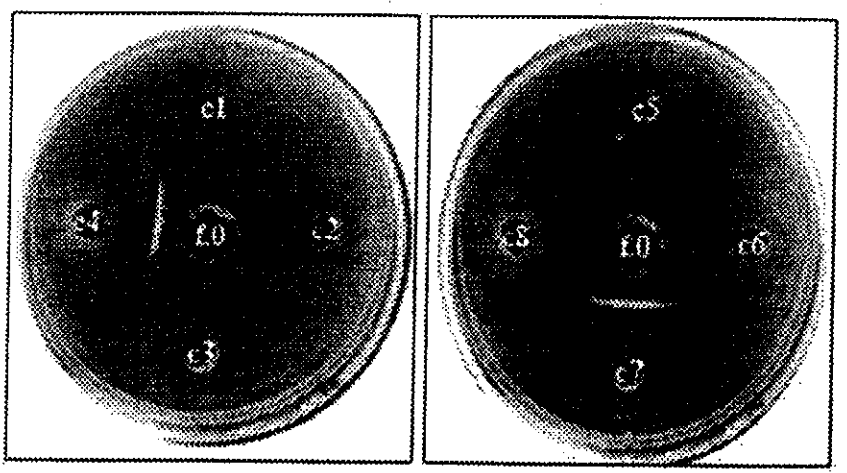

Figure (2): DD reaction of the antiserum of *F. oxysporum* in the center well (f0) against antigens of seeds of sunflower hyprid (c1), peanut cv. Giza 5 (c2), soyabean cv. crawford (c3), sesame cv. Giza-32 (c4), lupine local (c5), chikpea cv. Balady (c6), sesame cv. Tushka 1 (c7), and fabae bean (c8).

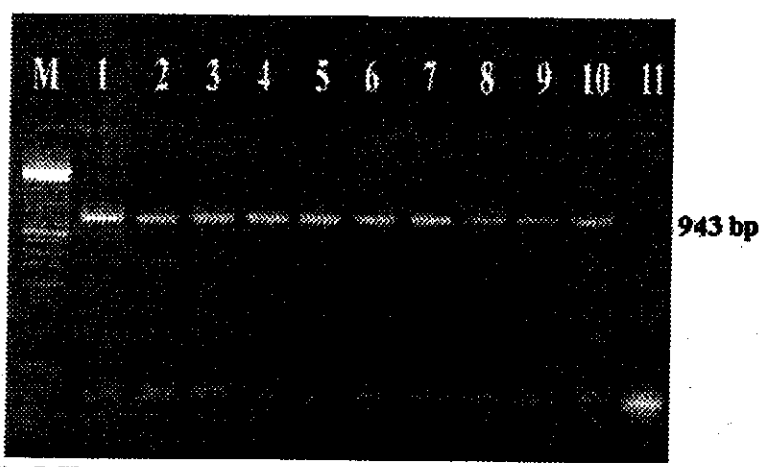

Figure (3): PCR detection of 10 isolates (lanes 1-10) *Fusarium oxysporum* f.sp. *sesami* isolated from sesame seeds. Lane 11: Negative control (PCR mixture with no DNA as a ten plates) and Lane M: molecular weight marker (100-1600 bp DNA ladder, Pharmacia).

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).

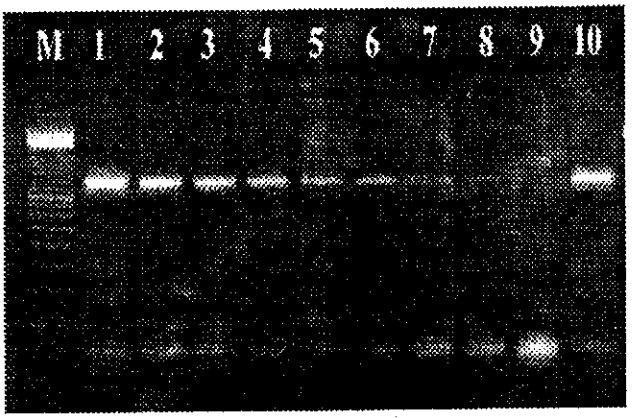

Figure (4): PCR detection of 9 DAN dilutions (10^{-1}-10^{-9}) (Lanes 1-9) of *F. oxysporum*, f.sp. *sesami*; M: molecular weight marker (100-1600bp DNA ladder, Pharmacia).

Specificty 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).

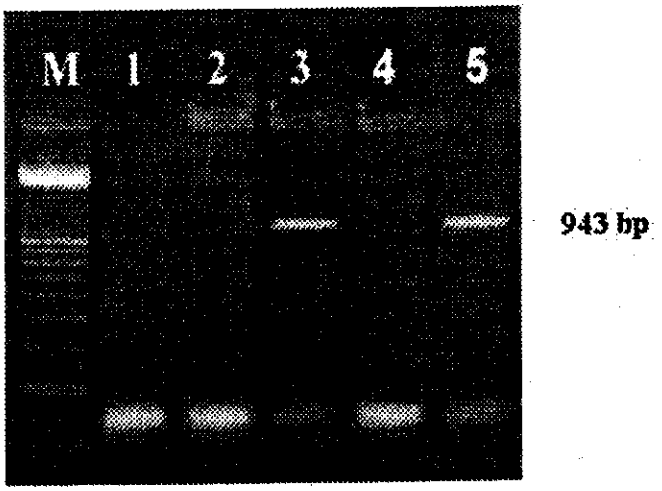

Figure (5): PCR detection using DNA extracts from three *Fusarium* spp. (Lane 3). *F. solani, F. moniliforme, F. oxysporum* f.sp.*sesami* (Lanes 1, 2 and 3, respectively). Lanes 4 and 5: Negatives and positive controls, respectively). Lane M: molecular weight marker (100-1600 bp DNA ladder, Pharmacia).

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 specifc 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 amplifiy 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 ribosmal 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 microsocpic 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).

REFERENCES

Abd El- Rehim, M.A.; Abou-Taleb, E.M. and Tohamy, A. (1988): Common antigen(s) in cotton to *Fusarium oxysporum* f.sp. *vasinfectum*. Phytopathology, 78: 217-233.

Abd El- Satar, M.A.; Khalil, M. S.; Mohamed, I.N.; Abd El-Salam, K.A. and Verret, J.A. (2003): Molecular phylogeny of *Fusarium* species by AFLP fingerprint. African J. of Biotechnology 2: 51-55.

Alves-Santos, F.M.; Ramos, B.; Garcia-Sanchez, M.A.; Eslava, A.P. and Diaz-Minguez, J. M. (2002): A DNA-based procedure for detection of *F. oxysporum* f. sp. *phaseoli*. Phytopathology. 92: 237-244.

Assigbelse, K.; Fernandez, B.D.; Boubions, M.P., and Geigor, J.P. (1994): Differentiation of *Fusarium oxysporum* f.sp. *vasinfectum* races on cotton by random amplified polymorphic DNA (RAPD) analysis. Phytopathology, 84: 622-623.

Avila, J.M., and Pinda, J.B. (1996): Behavior of ten sesame cultivars (*Sesamum indicum* L.) against *Macrophomina phaseolina* in Venezuela during three growing seaseons. Sesame and Safflower Newsletter, (11), 63-69.

Baayen, R.P.; Boogert, P.H.J.; Bonants, P.J.M.; Poli J.T.K.; Block,W. J.; and Walwijk, C. (2000): *Fusarium redolens* f. sp. *asparagi*, causal agent of asparagus root rot, crown rot and spear rot. European Journal of Plant Pathology 106: 907-912

Barnett, H.L. and Hunter, B.B. (1998): Illustrated Genera of Imperfect Fungi .Minesotta:Burgess Pub.Co., 241pp.

Bentley, S.; Pegg, K.G., and Dale, J.L. (1994): Optimization of RAPD-PCR finger priniting to analyze genetic variation within populations of *Fusarium oxysporum* f.sp. *cubenses*. Journal of Phytopathology, 142: 64-78.

Booth, C. (1971): The Genus *Fusarium*. Commonwealth Mycological Institute, Kew, Surrey, England.

Bradford, M.M. (1976): A rapid and sensitive method for the quantitation of microgram quantities protein utilizing the principle of protein-dye binding.Annals Biochemistrey, 62: 230-234.

Charudattan, R. and Devay, J.E. (1972): Common antigens among varieties of *Gossypium hirsutum* and isolates of *Fusarium* and *Verticillium* species. Phytopathology, 62: 230-234.

Chelkowski, J.; Bateman, G.L. and Mirocha, C.J. (1999): Identification of toxigenic *Fusarium* species using PCR assays. Journal of Phytopathology 147: 307-311.

Henson, J.M., and French, R. (1993): The polymerase chain reaction and plant disease diagnosis. Anal. Rev. Phytopathol. 31: 81-109.

Hussein, E.M. (1992): Biochemical and serological studies for determining susceptibility of cotton cultivars to *Fusarium oxysporum* f. sp. *vasinfectum* (In Russian). Ph.D. Thesis. All-Union Instiute of Plant Protection, Leningrad, USSR.

Hussein, E.M.; Aly, A.A.; Ashour, A.Z.A. and Nasr, S.M. (1996): Cluster analysis of serological protein patterns of three *Fusarium* species. J. Agric. Sci. Mansoura Univ., 21 : 3995-4012.

ISTA, (1985): International Rules for Seed Testing.Seed Science & Technology, 13: 12-329.

Khalil, M.S. (1981): Biochemical and serological studies on flax rust. Ph. D. Thesis, Fac. Agric., Cairo Univ., 143 pp.

Migheli, A.; Griatore, E.; Garibaldi, A.; Dshne, H. W.; Adam, G.; Diekmann, M.; Frahm, J.; Mauler-Machnik, A. and Halteren, P.V. (1997): Physiological race determination in *Fusarium oxysporum* f. sp. *dianthi* by pathogens. Proceedings 4th International symposium of the European Foundation for plant pathology. Bonn, Germany, 9-12 september. 203-206.

Nazar, R.N.; Hu., X.; Schmidt, J.; Culham., D. and Robb, J. (1991): Potential use of PCR-amplified ribosomal intergenic sequences in detection and differentiation of *Verticillium* wilt pathogens. Physiol. Mol. Plant 39: 1-11.

Niessen, M.L. and Vogel, R.F. (1997): Group specific PCR detecion of potential trichothecene producing *Fusarium* species in pure cultures and cereal samples system Appl. Microbiol . 21: 618-631.

Ouchterlony, O. and Nilsson, R. (1978): Immunodiffusion of experimental immunology (D.M. Weir, ed) 3rd ed. Blackwell Scientific Publications, Oxford pp. 218.

Rataj, G.; Wlatroszak, M.I. and Hornok, L. (1984): Serological comparison of two races of *Fusarium oxysporum* f.sp. *lupini* Phytopathology.10:221-225.

Sambrook, J.; Fritsch, E.F. and Maniatis, T. (1989): Molecular Cloning;A Laboratory Manual, 2nd ed.Cold Spring Harbor Laboratory Press, New York.

Thomas, J.E.; Reaves, J.C.; Taylor., E.J.A. and Kenyon., D.M. (1998): The development of new diagnostic techniques and their role in improving treatment strategies for seed-borne diseases. Brighton Crop Protection Confernce: Pests and diseases 3: 787-792.

Wilits, D.A. and Sherwood, J.E. (1999): Polymerase chain reaction detection of *Ustilago hordei* in leaves of susceptible and resistant barely varieties.Phytopathology, 89: 212-217.

إستخدام الطرق السيرولوجية وتفاعل البلمرة المتسلسل للكشف عن فطرالذبول فى السمسم.

عبده مهدى محمد مهدى*، نوال عبد المنعم عيسى*، فاتن محمود عبد اللطيف*، عبد الفتاح عبد الحميد الوكيل، ضياء عبد الفتاح الوكيل****

* قسم النبات الزراعى-كلية الزراعة بمشتهر- جامعة بنها.

** معهد بحوث أمراض النباتات- مركز البحوث الزراعية - مصر

من المعروف أن الطرق السيرولوجية تعتبر أداه مفيدة جدا فى الكشف عن بعض مسببات الأمراض النباتية وفى هذه الدراسة تم إعداد السيرم المضاد لخمس أنواع من الفيوزاريوم شملت (فيوزاريوم أوكسيسبوريم وفيوزاريوم موتيليفورم وفيوزاريوم سولانى وفيوزاريوم روزيوم وفيوزاريوم سيمكتيم وذلك لدراسة العلاقات السيرولوجية بينهم.

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