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DIVERSITY AMONG MYCELIAL COMPATIBILITY GROUPS OF *SCLEROTIUM ROLFSII*

*** Ahmed, Z. Huda, ** Harfoush I. Dorria**
**** Kamel, M.A., and * Shatout, M. Abeer**

** Mycology Research and Diseases Survey Department, Plant Pathol. Res. Inst., Agriculture Research Center (ARC). Giza, Egypt*
******* *Department of Plant Pathology, Fac. Agric., Ain shamis Univ., Cairo, Egypt.*

ABSTRACT

The fungus *Sclerotium rolfsii Sacc.* causes severe root rot to peanut crop (*Arachis hypogaea* L.). Molecular analysis preformed to assess the genetic diversity among 21 isolates of *S. rolfsii* collected from different hosts and geographically diverse locations in Egypt. The development of aversion reactions following mycelial parings of isolates in all possible combinations on PDA was used to differentiate 21 isolates of *S. rolfsii.* All total of 441 combinations only 76 one (17.2%) showed compatible reactions. Based on mycelial compatibility 7 group were identified among the different isolate. MCG 7 was a unique group conssist only one isolates isolated from guar plant. There was no clear relationship between host, geographic regions and MCGs. The extent of genetic diversity among MCG of *S. rolfsii* was studied using RAPD analysis. The genetic relationships among 12 isolates showed various reactions. High degree of genetic similarity was obtained between P2 and P6 isolates when using the two primers. These two isolates were also compatible and gave the same trend of disease incidence.

INTRODUCTION

Root and stem rot, caused by *S. rolfsii* is one of the most important diseases of peanut, worldwide. It can seriously reduce yield and pod quality (Cilliers *et al.* 2003 and Karthikeyan *et al.* 2006). Peanut crop sustain higher losses than any other agricultural crop (Aycock, 1966). *S. rolfsii* is an important soilborne pathogen that is distributed worldwide and causes various diseases to wide range of

plant species (Punja, 1988). Mycelial incompatibility is a phenomenon that occurs in a wide range of fungi, particularly in the homobasidomycetes (Rayner, 1991 and Worrall, 1997). When mycelia of different isolates belonging to the same species confront one another, either on agar media or suitable growth substrate, a distinct zone of demarcation (barrage or aversion zone) develops between the colonies. A recognition of non-self (all of recognition) from self is the underlying basis of the incompatible reaction (Worrall, 1997). It has been suggested that the incompatibility between vegetative hyphae serves to retain the genetic identity (individualism) of interacting strains by preventing neucular exchange (Rayner, 1991and Worrall, 1997). Isolates that exhibited a compatible reaction with one another could be placed in same group (MCG) (Rayner, 1991 and Worrall, 1997). In some fungi, agreater degree of genetic relatedness among isolates was correlated with weaker incompatibility reactions when paired (Worrall, 1997).

Mycelial incompatibility was previously demonstrated to occur between field isolates of *S. rolfsii* and was used to designate MCG (Punja and Grogan, 1983). It was assumed that isolates from same MCG shared greater genetic similarity compared with isolates from different MCG. For the genetic relationships among isolates of *S. rolfsii* from spercific geographic region, data showed that some members of a MCG were clonal in origin (Nalim *et al.* 1995 and Okabe *et al.* 2000) while other were not (Harlton *et al.* 1995 and Cilliers *et al.* 2000). In this study, isolates of *S. rolfsii* collected from different governorates in Egypt and from various hosts were assessed for genetic diversity. RAPD analysis was also used to determine the relationship between mycelial compatibility groups.

MATERIALS AND METHODS

Isolation of *sclerotium rolfsii*

Samples of different plants exhibiting typical symptoms of root rot were collected from different geographical localities with different kind of soil *i.e*. Sharqia, Behira, Qalubyia, Kafr El-Sheikh, and Bani-suwif governorates. In addition, also ten isolates were kindly obtained from Mycology Research & Diseases Survey Department, Plant Pathology Research Institute, (ARC). Four isolates from peanut (P2 from Giza, P3 from Ismailia and P6, P7 from Behira), one isolate from each of sesame Ss2 (Shariqia), sunflower Su1 (Giza), cotton Co

(Behira), bean be (Behira), guar Gu (Qalubyia) and cantaloupe C (Behira).

Diseased roots were thoroughly washed in tap water and surface sterilized with sodium hypochlorite (3.0%). Pieces of root tissues were rewashed twice in sterilized water, dried between sterilized filter papers, and cut into small pieces (1cm), then transferred into Petri dishes containing PDA medium. Plates were incubated at 25°C for seven days.The frequency of occurrence of *S. rolfsii* was recorded.

Variation in virulence among *S. rolfsii* isolates

Pathogenicity test of *S. rolfsii* isolates was carried out under greenhouse conditions in Plant Pathology Research Institute, (ARC). In this respect, pots (30 cm) were sterilized by immersing in 5% formalin solution for 15 minutes and left for one week until complete formalin evaporation. Pots were filled by sandy clay soil mixture (1:1w/w) which previously sterilized by autoclave.

Fungal inoculum was prepared by growing the fungus in glass bottles (500cc) containing sterilized sorghum grain medium for 15 days at 30°C. The soil was infested with different isolates, at the rate of 3% (w/w), and watered every two days for one week before planting. The same amount of autoclaved sorghum grain medium was added to the soil as control. Five seeds of peanut, Giza-6 cultivar, were swon in each pot and three replicate pots were used for each treatment.

Disease incidence was estimated after 15 and 30 days from planting. The fungus was reisolated from the diseased seedlings. Pathogenicity test was carried out according to Zayed *et al*. (1983).

$$\text{Disease incidence} = \sum (c - t)/c \times 100$$

C= control, t= treatment

Statistical analysis

Data were statistically analyzed according to the standard procedure in completely random design or split plot as mentioned by Snedecor & Cochran (1982).

Mycelial compatibility groupings

Mycelial compatibility grouping test was conducted for 21 isolates of *S. rolfsii,* according to Punja, *et al* (1983). Isolates were paired using mycelial discs in all possible combinations on 9.0 cm diameter petri dishes containing PDA medium.

Mycelial discs (5mm) were taken from the margin of actively growing colonies (7 days old) of each isolate were placed approximately 30 mm away from isolate and the other and incubated at 30°C. Three isolates were usually paired on one dish and the test was repeated twice. The pairings were examined after two weeks for the presence of an aversion or barrage reaction in the zone of mycelial contact. All isolates were self paired as controls. Isolates that formed an aversion reaction against all other isolates were assigned to a new MCG. MCG number designation was made arbitrarily without reference to host or geographical region of isolate.

Isolation of DNA:

DNA was isolated from 50 mg fresh mycelia of *S. rolfsii* using Qiagen Kit for DNA extraction. The extracted DNA was dissolved in 100 ul of elution buffer. The concentration and purity of the obtained DNA was determined by using "Gen qunta" system-pharmacia Bio-tech. The purity of the DNA for all samples was between 90-97% and the ratio between 1.7 – 1.8. Concentration was adjusted at 6 ng/ul for all samples using TE buffer PH 8.0.

Random amplified polymorphism DNA technique (RAPD):

Thirty ng from the extracted DNA were used for amplification reaction. The polymerase chain reaction (PCR) mixture contained PCR beads tablet (manufactured by Amessham pharmacia Bio-tech), which containing all of the necessary reagents except the primer and the DNA which add to the tablet.

The kits of Amessham pharmacia Bio-tech were also included the flowing primers. Five microliter of the primer 1 and 2 (10 mer) were added. The sequences of used
Primers are as shown:
Primer 1: 5- (GGTGCGGGAA)-3-
Primer 2: 5- (GTTTCGCTCC)-3-

The total volume was completed to 25 ul using sterile distilled water. The amplification protocol was carried out as follows using PCR unit II biometra.
Denaturation at 95 ˚C for 5 min.
45 cycles each consists of the following steps:
Denaturiation at 95 ˚C for 1 min.
Annealing at 36 ˚C for 1 min.
Extension at 72 ˚C for 2 min.
Final extension at 72 ˚C for 5 min.

Hold at 4 ˚C
7 ul of 6 X tracking buffer (manufactured by Qiagen Kit) were added to 25 ul of the amplification product.

Amplification product analysis:

The amplified DNA for all samples were electrophorased (15ul) using electrophoresis unit (WIDE mini-sub-cell GT Bio-RAD) on 1% agaose containing ethedium bromide (0.5 µg/ml). At 75 constant volt, and determine with UV transilluminator.

Gel analysis:

All kinds of gels (protein, isozyme, and DNA) were scanned for band Rf using gel documentation system (AAB Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6 C, Fullerton CA 92631). The different M.W. of bands were determined against PCR marker promega G 317A by unweighted pair-group method based on arithmetic mean (UPGMA)

RESULTS AND DISCUSSION

Results

Isolation of *S.rolfsii*:

A total of 21 isolates of *S. rolfsii* obtained from 10 different plant species from seven governorates in Egypt, were tested for their virulence on peanut (Giza6). Three isolates of S. *rolfsii* were isolated from each of peanut and sugerbeet, two isolate from pepper and one isolates from each of sesame, sunflower and soybean Table (1).

Variation in virulence among *S. rolfsii* isolates:

Pathogenicity test was carried out to investigate the virulence of 21 isolates of *S. rolfsii* on peanut plant (Giza 6). The fungus caused pre and post-emergence damping-off as well as root-rot and pod rot on mature plants (Fig1).

Data in Table (2) revealed that all *S. rolfsii* isolates were pathogenic to peanut seedling, in different levels. One isolate gave the highest percentage of pre-emergence damping off P3 85.4% followed by isolates Co and Sug3 (78.2%). The least virulent isolate was Su2 which gave 27.4 % pre-emergence damping off.

In case of post-emergence damping off, it was found that isolates Sug3 and Co gave the highest percentage of infection (66.6 %) followed by isolates Su1 and P2 (59%). Fig (1) illustrate diseases symptoms on peanut seedling by artificial inoculation

Mycelial compatibility among different 21 isolates of *S. rolfsii* was studied on PDA medium after 5 days. Compatible isolates were distinguished by the fusion of mycelia without aversion zone between isolates. Hyphae of incompatible isolates was followed by clearing zone developed in the region of interaction, (Fig 2). In some cases, sclerotia were produced at the contact edge of the mycelium. A total of 441 only 76 compatible reaction were resulted from different pairings. Seven mycelial compatibility groups (MCGs) were identified among the isolates (Table3). Isolates within the same mycelial compatibility group (MCG) did not show aversion zone. MCG7 was a unique group consisting of only one isolate (Gu) from guar. It was also noticed that different MCGs could be found within a specific geographic region. For example, MCG4 consisted of 2 isolates from Behira, one form soybean where the other from bean. The homogeneity in this group according to dendrogram was 86%. MCG5 consisted of 2 isolates from the same host (peanut) but they were isolated from different geographic regions (Ismailia and Behira).

RAPD – PCR

Molecular studies were performed to find the genetic differences between different isolates. Electrophortic patterns of RAPD - PCR products have been used as a powerful tool for the study of genetic variability of all isolates. RAPD patterns were obtained from the twelve isolates with two primers, these primers were chosen because they produced clear amplification patterns. With the two selected primers, amplification products demonstrated the extent of intra specific variation between 12 isolates of *S. rolfsii* from oil crops. The protocol described under the materials and methods was used successfully to isolate a high yield of genomic DNA was amplified by PCR.

Using primer 1, obtained data were documented and presented in Fig. (3). isolates P2 and P6, isolated from peanut from Giza and Behira governorates, showed several closely associated clusters, indicating a high degree of genetic similarity (83.38 %). Isolates P7 and P3, isolated from peanut from Behira and Ismailia governorates, showed a high degree of genetic similarity (95.7 %). Isolates P4and P5, isolated from peanut from Behira, showed several closely associated clusters, indicating a high degree of genetic similarity (95.49 %). Also, isolates Sb and P1 isolated from soybean and peanut

respectively from Behira governorate, showed several closely associated clusters, indicating a high degree of genetic similarity (95.8 %).

The obtained results of RAPD analysis using primer 2 were documented and presented in Fig. (4). Data revealed that, isolate P2 and P6, isolated from the same host(peanut) isolated from different governorate (Giza, Behira) had several closely associated clusters, indicating a high degree of genetic similarity (87.71 %). Meanwhile isolates P1 and Sb isolates from peanut and soybean respectively from Behira indicating (97%) genetic similarity

In comparison, isolates Su2 and P5 from different geographical origin Behira and Qalubyia respectively, and different hosts (sunflower and peanut) were in closely associated clusters (99.1%). Furthermore isolates Ss1 and P7 from sesame and peanut obtained from the same governorate showed (91.5%) similarity. The groupings were not correlated with host origin or geographic locations.

Table (1): Source of *S. rolfsii* isolates obtained from different hosts and different governorate in Egypt.

Isolate code	Host	Governorate
P1	Peanut	Behira
P2	Peanut	Giza
P3	Peanut	Ismailia
P4	Peanut	Behira
P5	Peanut	Behira
P6	Peanut	Behira
P7	Peanut	Behira
Ss1	Sesame	Behira
Ss2	Sesame	Sharkia
Su1	Sunflower	Giza
Su2	Sunflower	Qalubyia
Sb	Soybean	Behira
Co	Cotton	Behira
C	Cantaloupe	Behira
Pe1	Pepper	Qalubyia
Pe2	Pepper	Qalubyia
Sug1	Sugerbeet	Bani-suwif
Sug2	Sugerbeet	Kafr El-Sheikh
Sug3	Sugerbeet	Sharkia
Be	Bean	Behira
Gu	Guar	Qalubyia

peanut = P, sesame = Ss, sunflower = Su, soybean = Sb, cotton = Co, cantaloupe = C, pepper = Pe, sugerbeet = Sug, bean = Be, guar = Gu

Table (2): Pathogenicity of different *S. rolfsii* isolates on peanut (Giza 6) recorded after 15* and 30 days from sowing**

Isolates code	Pre-emergence damping off%*	Post-emergence damping off%**
P1	70.9^{abc}	50^{bc}
P2	56.4^{cde}	59.4^{ab}
P3	85.4^{a}	33.3^{def}
P4	56.5^{cde}	50^{bc}
P5	56.5^{cde}	25^{f}
P6	56.4^{cde}	22.2^{f}
P7	71^{abc}	33.3^{def}
Ss1	63.7^{bcd}	44.4^{cd}
Ss2	70.9^{abc}	50^{bc}
Su1	34.7^{fg}	59^{ab}
Su2	27.4^{g}	38.8^{cde}
Sb	41.9^{efg}	27.7^{ef}
Co	78.2^{ab}	66.6^{a}
C	49.2^{def}	21.5^{f}
Pe1	41.9^{efg}	33.3^{def}
Pe2	41.9^{efg}	38.8^{cde}
Sug1	70.9^{abc}	50^{bc}
Sug2	56.4^{cde}	44.4^{cd}
Sug3	78.2^{ab}	66.6^{a}
Be	41.9^{efg}	21.5^{f}
Gu	49.1^{def}	27.7^{ef}
Control	6.6^{h}	0^{g}
L.S.D(0.05)	18.75	13.55

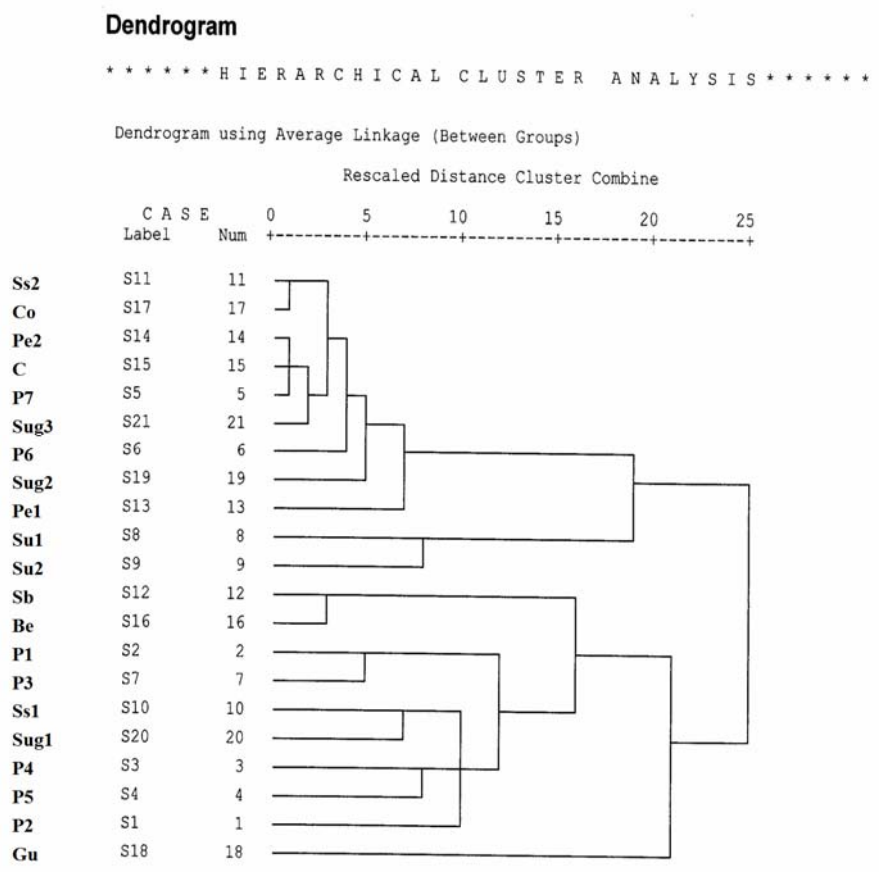
Fig (1): Symptom of peanut root rot caused by *S. rolfsii* (a.P3, b.P7 and c.Sug3).

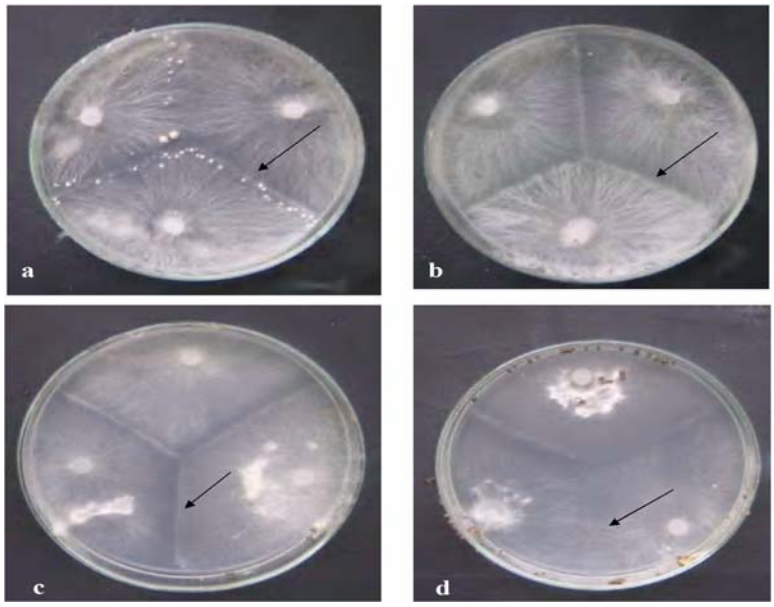
Note: white mycelium on crown area and pods (a and b), mycelium and sclerotia initially, were formed on crown area(c).

Mycelial compatibility groups:

Table (3): Mycelial compatibility groups of 21 *S.rolfsii* isolates according to dendrogram

MCG	Isolates
MCG1	Ss2 – CO
MCG2	Pe2 – C – P7 – P6 – Sug3 – Sug2-Pe1
MCG3	Su1 – Su2
MCG4	Sb – Be
MCG5	P1 – P3
MCG6	Ss1 – P4 – Sug1 – P5 – P2
MCG7	Gu





Fig(2): Types of reactions observed between mycelial compatibility groups of *S. rolfsii.* a,b and c Pairings of three isolates of *S. rolfsii* showing the development of incompatible reactions (clearing zones) at the contact edge of the mycelium. d. Pairing between isolates of *S.rolfsii* showing mycelial compatible. a.Note development of sclerotia on either side of the clearing zone.
Photograph was taken after 2 weeks of growth on PDA medium at 30°C.

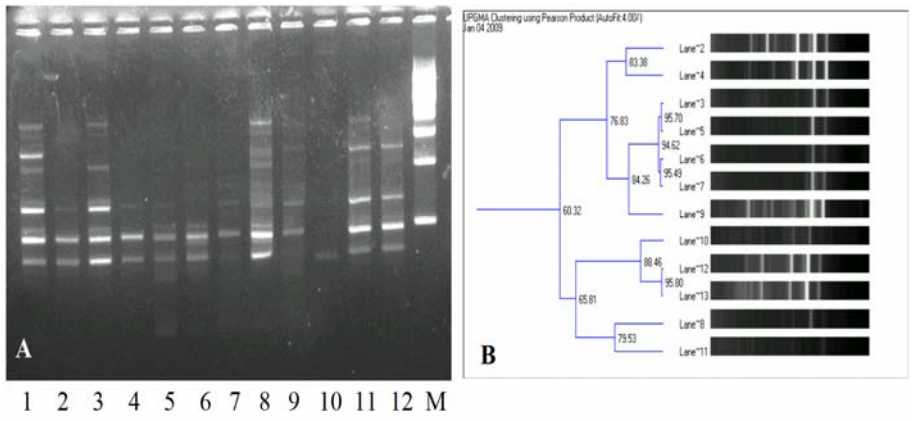


Fig (3): A. Electrophoretic analysis of RAPD banding patterns generated by primer1. M (DNA marker) for 12 isolates of *S. rolfsii*. B. Dendrogram constructed from analysis of DNA fragments amplified with primer 1 for 12 isolates of *S. rolfsii* using RAPD-PCR.

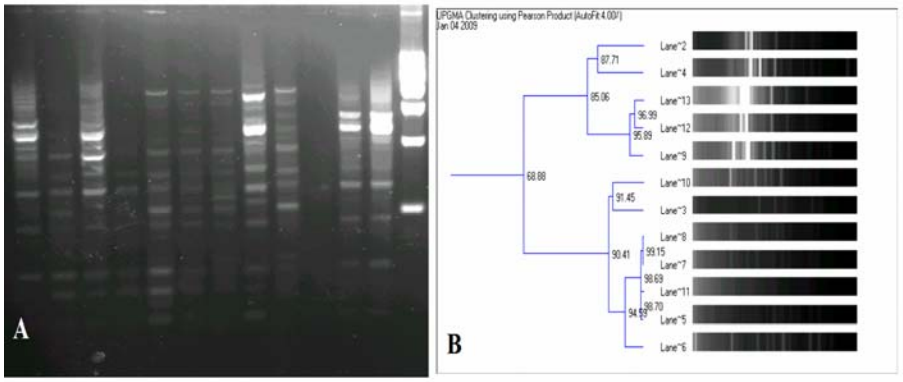


Fig (4):A. Electrophoretic analysis of RAPD banding patterns generated by primer2. M (DNA marker) for 12 isolates of *S. rolfsii*. B. Dendrogram constructed from analysis of DNA fragments amplified with primer 2 for 12 isolates of *S. rolfsii* using RAPD-PCR**.**

Discussion

The soil borne plant pathogen, *Sclerotium rolfsii*, *is* an important pathogen distributed worldwide and causes various diseases to wide range of plant species (Punja, 1985 and Ray and Mukherjee, 1997). This fungus causes a severe seed and soil born diseases to peanut crop in Egypt (Zayed *et al*. 1983). The pathogen attacks the roots, collar, stems, gynophores and pods of peanut plants (Punja, 1985). The fungus girdles the main stem and lateral branches, often killing the plant. The fungus can infect below ground, causes severely damaging pegs and pods (Franke *et al.* 1998).

In the present study, 21 isolates of *S. rolfsii were* obtained from different plant hosts from seven Egyptian governorates. All the isolates were pathogenic to peanut plants however with different degrees. Results are in agreement with results, of other authors (Harlton *et al*., 1995, Nalim *et al*., 1995, Okabe *et al*., 1998, Sharma *et al*., 2002 and Shukla and Pandey, 2007) where isolates of *S .rolfsii* have showed significant variations, not only in their morphology but also in their pathological behavior.

One method of indirectly measuring genetic variability among isolates of fungal plant pathogens is the determination of mycelial compatibility groups (MCGs) (Earnshaw and Boland, 1997). Characterization of MCG within a fungal species in particular of plant

pathogenic fungi, is a useful method to monitor distribution and spread of isolates over time (Anderson and Kohn 1995).

Also, mycelial compatibility had been used to examine genetic variability in numerous fungal plant pathogens such as *Cryphonectria parasitica* and *Ophiostoma ulmi* and *Fusarium oxysporum* as an effective means of identifying intraspecific variation within field population of a plant pathogen. The genetic consequences of mycelia incompatibility however remained unknown (Leslie, 1993).

In this study, testing 21 *S. rolfsii* isolates for mycelial compatibility, on PDA medium was carried out. Compatible isolates were distinguished by the fusion of mycelial without a version zone, where the incompatible reactions produced a barrage recognized by clearing zone in the region of interaction. Two out of 7 MCGs (MCG3 and MCG5) consisted of 2 isolates from same host but from different region, MCG4 consisted of 2 isolates from same region, but from different hosts. The spread of these isolates all over the country suggesting the movement of diseased plants, contaminated soil or agricultural equipment. The presence of several different MCG of *S.rolfsii* in a specific geographic localities or the same host species as shown in the present study has also been reported (Nalim *et al.* 1995, Okabe and Matsumoto, 2000 and Punja and Sun, 2001)

Interaction between different genotypes either prevent hyphae anastomosis or result in cell death following hyphal anastomosis (Leach and Yoder, 1983).

Cluster analysis dependent on data obtained by RAPD assay using two different primers, revealed that isolate P2 and P6, isolated from peanut from Giza and Behira governorates has the same disease severity 60% these two isolates were compatible and had closely associated clusters, that indicate a high degree of genetic similarity.

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التباين ما بين مجاميع التوافق الميسليوميه لفطر *Sclerotium rolfsii*

***هدى زكريا أحمد **درية ابراهيم حرفوش **مدحت كامل على *عبير محمد أحمد شلتوت**

*قسم تقسيم الفطريات وحصر الأمراض- معهد بحوث امراض النباتات مركز البحوث الزراعية
**قسم امراض النبات كلية الزراعة جامعة عين شمس

فطر *Sclerotium rolfsii* من الفطريات واسعة الانتشارعلي مستوي العالم. يصيب العديد من المحاصيل الحقليه والخضر والزيتية. وهو أحد فطريات التربه الذي لايكون طور جنسي في كثير من الاحيان، يعيش في التربه علي شكل ميسليوم واجسام حجرية.

خلال السنوات القليله الماضيه بدأ استخدام البصمه الوراثيه كآليه حديثه للتفرقه بين العزلات داخل النوع الواحد.

الهدف من إجراء هذا البحث هو محاوله التفرقه بين عزلات الفطر وتقسيمها الي مجاميع، معرفة العلاقه بين افراد المجموعه الواحده وأصل النبات المعزول من الفطر وكذا المنطقه.

وقد تم عزل 11 عزلة من 7 محافظات بجمهورية مصر العربية الشرقية – القليوبية – البحيره – كفر الشيخ – بني سويف.

وكذا عزل الفطر من عوائل مختلفة. فول سوداني – فول صويا– سمسم - دوار الشمس – فلفل – بنجر السكر . بالإضافه لما سبق تم الحصول علي 10 عزلات (معلومة المصدر المعزوله منه) من قسم تقسيم الفطريات وحصر الامراض بمركز البحوث الزراعية.

وعند اختبار القدرة المرضيه 21 عزله مختلفة علي نبات الفول السوداني، وجد ان جميع العزلات ممرضه مع اختلاف درجات الاصابه من عزله الي اخري. ولوحظ ايضا عدم وجود تخصص لهذا الفطر في الاصابه.

وبدراسة قدرة العزلات علي التوافق الميسليومي فيما بينهما. اظهرت النتائج وجود 76 توافق من 441 احتماليه بين العزلات بنسبة 17.02%.

كما امكن تقسيم العزلات الي سبع مجاميع مع ملاحظة وجود توافق بين افراد المجموعه الواحده واختلافها مع افراد المجاميع الأخري. وعدم وجود أي ارتباط بين افراد المجموعه والعائل الذي تم عزل الفطر منه و المنطقة المعزول منها.

وكمثال لذلك المجموعه رقم 3 تحتوي علي عزلتين من نفس العائل (دوار الشمس) من منطقتين مختلفتين (الجيزه والقليوبيه) وعلي العكس من ذلك المجموعه رقم 4 تحتوي علي عزلتين من نفس المنطقه (البحيره) تم عزلهما من نباتين مختلفين (فول السوداني- فول الصويا).

مما سبق يتضح عدم وجود اي ارتباط بين العائل او المنطقه وأفراد المجموعه الواحده.

اظهرت النتائج ايضاً ان كل من العزله P2 , P6 المعزولتان من الفول السوداني يوجد بينهما درجة عاليه من التماثل الجيني وكذا وجود توافق ميسليومي بينهما وقد اعطت نفس نتائج الاصابه علي نبات فول السوداني.