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Isolation and Molecular Characterization of *Rhizoctonia solani* the Causal Agent of Cotton Damping-Off Disease

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ABSTRACT

Cotton damping-off caused by *Rhizoctonia solani* is one of the most important and epidemic cotton diseases in cotton-growing regions worldwide, including Egypt. Isolates of *Rhizoctonia* were collected from infected cotton seedlings showing damping-off symptoms from the different cotton-growing area in Egypt. Twelve isolates of Rhizoctonia were obtained. Pathogenicity test of Twelve isolates of *R. solani* was evaluated on 5 cotton cultivars, Giza 80, Giza 87, Giza 90, Giza 92 and Giza 93, under greenhouse conditions. All tested isolates were able to infect cotton plants causing damping-off with different percentages of severities, isolate No. R8 and R9 showed significantly highest damping-off percentage, while the lowest percentage of damping-off percentage obtained from isolates No. R11 and R7. For identification of *R. solani* specific primers were used (ITS1 and GMRS-3) that confirmed that all the isolates were *R. solani*. The genetic diversity of studied isolates of the pathogen was determined by using random amplified polymorphic DNA (RAPD) markers. A total of 1583 fragments were detected when 18 RAPD markers were applied to 12 *R. solani* isolates. The phylogenic tree generated from neighboring joint analysis grouped *R. solani* isolates into groups. The results presented showed that the variability among the isolates of *R. solani* was high. Variation was found between the polymorphism showed by the isolates which caused the difference in pathogenicity of the isolates.

INTRODUCTION

Cotton (*Gossypium barbadense* L.) is one of the strategic farm crops, which is widely cultivated and traded across the world and one of the most important export crops of Egypt. Cotton seedling diseases are a worldwide problem; they are caused by a complex of soil-borne organisms. These organisms are found in all cotton-producing areas in Egypt, including *Rhizoctonia solani* and *Fusarium* spp. (Asran-Amal *et al*., 2005). *R. solani* Kühn. the anamorphic of *Thanatephorus cucumeris* (Frank.) Donk, causes seedling blight, pre-or post-emergence damping-off, sore shin and root rot of cotton seedlings. *R. solani* colonizes soft tissues and forms infection cushions. From these cushions, the fungus penetrates the epidermis and destroys plant cells (Watkins, 1981).

The pathogenicity of 39 isolates of *R. solani* AG-4 and one isolate belonging to AG-2-2 were evaluated under greenhouse conditions on cotton (Giza 75); most of the virulent isolates exhibited pre-emergence damping-off (El-Akkad-Salwa, 1997).

The pathogenicity of *R. solani* on Egyptian cotton is well documented in the literature. For example, Asran-Amal *et al.*, (2005) reported that *R. solani* was a major cause of cotton damping-off throughout much of the cotton-growing areas in Egypt.

Molecular techniques have become reliable and are highly suitable tools for identifying pathogen species and genetic variation within collections and populations (Sundravadana *et al.*, 2011).

Several studies have shown the importance of using genetic characterization in population studies of the fungus *Rhizoctonia*. Cluster analyses based on ribosomal DNA-internal transcribed spacer sequences of representatives of anastomosis groups and subgroups of *Rhizoctonia* spp. isolates have been studied (Carling *et al*., 2002 and Toda *et al*., 2004). Outbreaks of bordered sheath spots caused by *R. oryzae* have been reported in the rice-growing areas of Northern and Southern Japan as well as in Southeast Asia and the United States (Inagaki and Nakamoto,1982).

Different molecular markers have been used by researchers for genetic diversity and taxonomy of plant pathogens. Many molecular markers as randomly amplified polymorphic DNA (RAPD) (Dubey *et al*., 2012; Mehdi Nasr Esfahani 2020), inter-simple sequence repeats (ISSR) (Dubey *et al*., 2012 and Sharma *et al*., 2005), simple sequence repeats (SSR)(Dubey*et al*., 2012 and Mwang' Ombe *et al*., 2007) and internal transcribed spacer (ITS) (Godoy-Lutz *et al*., 2008 and Pannecoucque & Hofte 2008) have used for genetic diversity among *R. solani* isolates.

The objective of this study was to isolate and identify the causal agent of damping-off disease (*Rhizoctonia solani*) using molecular techniques and to determine the population structure of Rhizoctonia on cotton.

MATERIALS AND METHODS

Rhizoctonia solani Isolates:

Twelve isolates of *Rhizoctonia solani* were used in the current study, which were collected as showing in table 1.

Table 1: Code and number of collected *R. solani* isolates

S. Number	Isolates code	Collecting area
1	R1	Beni-Suef
2	R2	El-Behera
3	R4	El-Behera
4	R5	Dakhlia
5	R6	Dakhlia
6	R7	Gharbia
7	R8	Kafrelsheikh
8	R9	Menoufia
9	R11	Alexandria
10	R12	Alexandria
11	R13	Alexandria
12	R15	Alexandria

Pathogenicity Test:

Pathogenicity tests were carried out under greenhouse conditions. The fungal inocula were prepared using 500 ml conical flasks containing cornmeal–sand medium. Each flask contained clean sand (5 g), barley (100g) and enough tap water to cover the prepared mixture and autoclaved for 45 minutes at 121.5 ºC. The flasks were inoculated with each of the isolates and incubated at 27ºC for 7 days (White head, 1957).

Five grams of the fungus used to inoculate one kg soil on plastic pots (25cm in diameter) and covered by nylon sheet for 4 days, then sowing the infected soil by cotton seeds. The symptoms of damping-off were recorded according to Bheemaraya (2014).

Characterization and Identification of *R. solani* isolates:

1. DNA Extraction from *R. solani*

Mycelial cultures of 12 *R. solani* isolates were grown in potato dextrose broth media for 5 days at 28°C. Mycelia were harvested and DNA was extracted using CTAB method used by Murray and Thompson (1980).

Harvested mycelia were grounded in pre-chilled mortar and pestle with liquid nitrogen into a fine powder. Powdered mycelium was mixed with 800 µl pre-warmed (65°C) 2% Cetyl Trimethyl Ammonium Bromide (CTAB) DNA extraction buffer. The tubes were incubated in a water bath at 65°C for 1hr with gentle mixing by inverting the tubes at every 10 min intervals. After incubation 800 µl of chloroform: isoamyl alcohol (24:1) was added to each tube. The tubes were placed on a rotary shaker for 45 minutes. The mixture was centrifuged at 10,000 rpm for 15 min. The aqueous phase was transferred to a new tube and DNA was precipitated with 600 µl of ice-cold isopropanol and allowed to precipitate at -20°C overnight, followed by centrifuging at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was washed twice with 70 percent ethanol and air-dried for removal of ethanol. The DNA pellet was suspended in 100 µl TE buffer and stored at -20°C for further use.

2. Identification and Characterization of *R. solani* using Specific Primers:

Specific primers for *R.solani* (ITS1, GMRS-3)(Table 2) were tested for their specificity with purified genomic DNA extracted from the 12 isolates of the seedling damping-off pathogen. PCR was carried out in a thermal cycler with cycling conditions at 94°C for 5 min, followed by 35 cycles of 1min at 94°C, 1min at 65°C and 2min at 72°C, followed by final extension for 7min at 72°C.

Table 2: Specific primers for identification of *R. solani*

Primer	Sequence (5'-3')	Direction
ITS1	TCCGTAGGTGAACCTGCGG	Forward
GMRS-3	AGTGGAACCAAGCATAACACT	Reverse

Random Amplified Polymorphic DNA (RAPD-PCR) Analysis for the Variability of *R. solani* Isolates:

Eighteen RAPD primers (Macrogen Inc. Korea) (Table 3) were used to determine the polymorphism among 12 *R. solani* isolates. PCR reaction was performed in 20µl mixture. The PCR was performed with cycling conditions at 94^{o}C for 5min for initial denaturation, followed by 40 cycles of denaturation at 94^{o}C for 1 min, annealing for 1 min at 35^{o}C and extension at 72^{o}C for 1 min with final elongation at 72^{o}C for 7 min. The amplified products of PCR (10 µl) were subjected to electrophoresis using 1.5 % agarose gel prepared in 0.5X TE buffer. The gel was stained with red safe, visualized and photographed under UV light in gel documentation system.

DNA bands of PCR product were visualized on UV Transilluminator gel documentation system and photographed. The gel pictures were manipulated using Adobe Photoshop 8. The gels were scored for band presence or absence as (1) or (0), respectively. The total number of bands generated from each primer, as well as, the polymorphic bands number generated from each primer was calculated.

Fragments scored as present/absent. Fragment scoring and lane matching performed automatically on digital images of the gels, using Phoretix 1D advanced Version 4.00 (Phoretix International, Newcastle upon Tyne, UK). All but the faintest bands scored, where necessary scores and matches corrected manually. Clustering methods and similarity coefficients were tested using the procedures

SIMQUAL, SAHN, and TREE from the program NTSYSpc version 2.10 (Applied Biostatistics, Setauket, New York, USA). The clustering methods UPGMA, WPGMA, Complete-link, and Single-link were applied in all possible combinations with the similarity coefficients Dice, Jaccard and simple matching. (Rohlf, 2000) describes clustering methods and similarity coefficients

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Table 3: Code, names and sequence of RAPD primers

Code	Primer name	Sequence
01	OPA-08	GTGACGTAGG
02	OPA-11	CAATCGCCGT
03	OPA-13	CAGCACCCAC
04	OPB-17	AGGGAACGAG
05	OPC-01	TTCGAGCCAG
06	OPC-02	GTGAGGCGTC
07	OPC-05	GATGACCGCC
08	OPC-18	TGAGTGGGTG
09	OPE-20	AACGGTGACC
10	OPH-18	GAATCGGCCA
11	OPM-20	AGGTCTTGGG
12	OPN-09	TGCCGGCTTG
13	OPN-13	AGCGTCACTC
14	OPN-15	CAGCGACTGT
15	OPQ-01	GGGACGATGG
16	OPR-01	TGCGGGTCCT
17	OPZ-20	ACTTTGGCGG
18	P-14	CCACAGCACG

RESULTS AND DISCUSSION

Pathogenicity of *R. solani* on Cotton Cultivars:

Twelve isolates of *R. solani* from different geographical localities in Egypt and five cotton cultivars, namely, Giza 80, 87, 92, 90 and 93, were used throughout the present study**.** The frequency of pre-and post-emergence damping-off, caused by the tested *R. solani* isolates, was calculated (Table 4).

According to results (Table 4), the highest pre-emergence damping-off values were induced by isolate R8 (98.0%), followed by isolate R9 (94.0%), then isolate R5 (82.19%). On the other hand, the lowest pre-emergence damping-off values were obtained by R12and R11 isolates (16.62% and 17.71%, respectively), with significant differences between the isolate.

High significant variations were observed among the current cotton cultivars. The results indicated that Giza 80 was more tolerant, where the pre-emergence value was 18.74%, compared with that induced by the other susceptible cultivars, i.e., Giza 93 (61.52%), and the moderately tolerant cultivars Giza 87 (45.42%) and Giza 92 (48.11%)**.** These previous results were agreed with Monga and Raj (1994), Aqil and Batson (1999) and Asran-Amal (2001), who used twenty-eight isolates of *R. solani* obtained from cotton seedlings and twenty-three isolates from other hosts.

Results of post-emergence damping-off (Table 4) showed that, R2 isolate of *R. solani* recorded the highest value (10.96%)of post-emergence damping-off, followed by isolates R12, R6 and R13(9.71, 9.68 and 9.05%, respectively). However, the lowest values of post-emergence damping-off were obtained by R8 and R11 isolates (2.0 and 2.74%, respectively).On the other hand, the recorded data showed that cotton cultivar Giza 87 showed high susceptibility to the

fungal attack (6.84%), followed by Giza 92 and Giza 90 (6.67% and 6.55%, respectively, without significant differences). Giza 93 cv. showed the lowest post-emergence damping-off percentage (3.72%).

According to the results of the total damping-off values, presented in (Table 5) R8 isolate was the most virulent isolate (100%), whereas R11 isolate was the most avirulent isolate (20.45%). Moreover, Giza 80 cv. was the most tolerant (24.38%), while Giza 93 cv. was the most susceptible to *R. solani* Damping-off (64.94%). These results are in agreement with Moustafa *et al*. (1995), Asran-Amal *et al*. (2005) and Khan *et al*. (2017) they reported that *R. solani* was a major cause of cotton damping-off throughout much of the cotton-growing areas in Egypt.

Table 4: Pre- and post-emergence damping-off percentage caused by *R. solani* isolates on cotton cultivars

Isolates	Pre-emergence %*					Mean	Post-emergemce%**					Mean
	G90	G92	G93	G80	G87		G90	G92	G93	G80	G87	
***R. solani* (R1)**	88.89	86.00	92.50	20.33	77.67	**73.07 ^{d}**	11.11	3.33	0.00	10.00	11.11	**7.11^{c}**
***R. solani*(R2)**	47.80	56.30	44.67	3.33	16.67	**33.75^{f}**	11.11	16.67	3.70	6.67	16.67	**10.96^{a}**
***R. solani*(R4)**	92.23	90.00	92.57	3.33	88.87	**73.40^{d}**	7.40	0.00	3.70	16.67	0.00	**5.55^{de}**
***R. solani*(R5)**	92.60	86.63	96.20	46.67	88.87	**82.19^{c}**	7.40	3.33	0.00	6.67	0.00	**3.48^{fgh}**
***R. solani*(R6)**	60.16	40.00	51.67	0.00	55.67	**41.50^{e}**	7.40	13.33	11.00	0.00	16.67	**9.68^{ab}**
***R. solani*(R7)**	18.33	16.53	48.33	0.00	11.13	**18.86^{g}**	7.40	3.33	0.00	6.67	5.57	**4.59^{ef}**
***R. solani*(R8)**	100.0	90.00	100.0	100.0	100.0	**98.00^{a}**	0.00	10.00	0.00	0.00	0.00	**2.00^{h}**
***R. solani*(R9)**	100.0	100.0	100.0	70.0	100.0	**94.00^{b}**	0.00	0.00	0.00	20.00	0.00	**4.00^{fg}**
***R. solani*(R11)**	18.56	20.00	44.47	0.00	5.53	**17.71^{gh}**	3.70	10.00	0.00	0.00	0.00	**2.74^{gh}**
***R. solani*(R12)**	14.67	16.63	40.67	0.00	11.13	**16.62^{h}**	3.70	10.00	14.87	3.33	16.67	**9.71^{ab}**
***R. solani*(R13)**	18.83	23.33	37.03	0.00	11.13	**18.06^{gh}**	11.10	10.00	7.47	0.00	16.67	**9.05^{b}**
***R. solani*(R15)**	37.33	0.00	40.67	0.00	11.13	**17.82^{gh}**	14.83	6.67	3.70	3.33	5.57	**6.82^{cd}**
Control	10.27	0.00	11.00	0.00	12.67	**6.78^{i}**	0.00	0.00	0.00	0.00	0.00	**0.00^{i}**
MEAN	**53.82^{b}**	**48.11^{c}**	**61.52^{a}**	**18.74^{e}**	**45.42^{d}**		**6.55^{a}**	**6.67^{a}**	**3.72^{b}**	**5.64^{ab}**	**6.84^{a}**	

* Pre-emergence damping-off was measured as the average of 5 replicates after 14 days from cultivation.

** Post-emergence damping-off was measured as the average of 5 replicates after 21 days from cultivation.

LSD 0.05 for cultivar * isolate interaction (pre) = 4.06 **LSD 0.05 for cultivar * isolate interaction (post)= 3.41**

Table 5: Incidence of damping-off diseasecaused by *R. solani* isolates on cotton cultivars

Isolates	Total Infection %					MEAN
	G90	G92	G93	G80	G87	
***R. solani* (R1)**	100.0	89.33	92.50	30.33	88.77	**80.19^{d}**
***R. solani* (R2)**	58.91	72.97	48.37	10.00	33.33	**44.71^{f}**
***R. solani* (R4)**	99.63	90.00	96.27	20.00	88.87	**78.95^{d}**
***R. solani* (R5)**	100.0	89.97	96.20	53.33	88.87	**85.67^{c}**
***R. solani* (R6)**	67.57	53.33	62.67	0.00	72.33	**51.18^{e}**
***R. solani* (R7)**	25.73	19.87	48.33	6.67	16.70	**23.46^{i}**
***R. solani* (R8)**	100.0	100.0	100.0	100.0	100.0	**100.0^{a}**
***R. solani* (R9)**	100.0	100.0	100.0	90.00	100.0	**98.00^{b}**
***R. solani* (R11)**	22.27	30.00	44.47	0.00	5.53	**20.45^{j}**
***R. solani* (R12)**	18.37	26.63	55.53	3.33	27.80	**26.33^{gh}**
***R. solani* (R13)**	29.93	33.33	44.50	0.00	27.80	**27.11^{g}**
***R. solani* (R15)**	52.17	6.67	44.37	3.33	16.70	**24.65^{hi}**
Control	10.27	0.00	11.00	0.00	12.67	**6.77^{k}**
MEAN	**60.37^{b}**	**54.77^{c}**	**64.94^{a}**	**24.38^{e}**	**52.26^{d}**	

LSD0.05 for cultivar×isolate interaction= 4.39

Screening for *R. solani* Using Specific Markers:

The results in Figure 1 showed that all the tested isolates were *R. solani*, producing the same fragments at 500 bp. 12 isolates of *R. solani* were isolated from cotton seedlings showing damping-off symptoms, which also identified using specific primers using GMRS-3 and ITS1 primers, all the isolates were amplified with *R. solani* and gave a single band with 500 bp. This isolate gave amplification with *R. solani* specific primer GMRS-3 and ITS1 with 500bp single band.

With GMRS-3 and ITS1 primers, all the isolates hybridized with *R. solani* and gave a single band with 500 bp. This isolate gave amplification with *R. solani* specific primer GMRS-3 and ITS1 with 500 bp single band according to Inagaki (1998) and Kumari (2015).

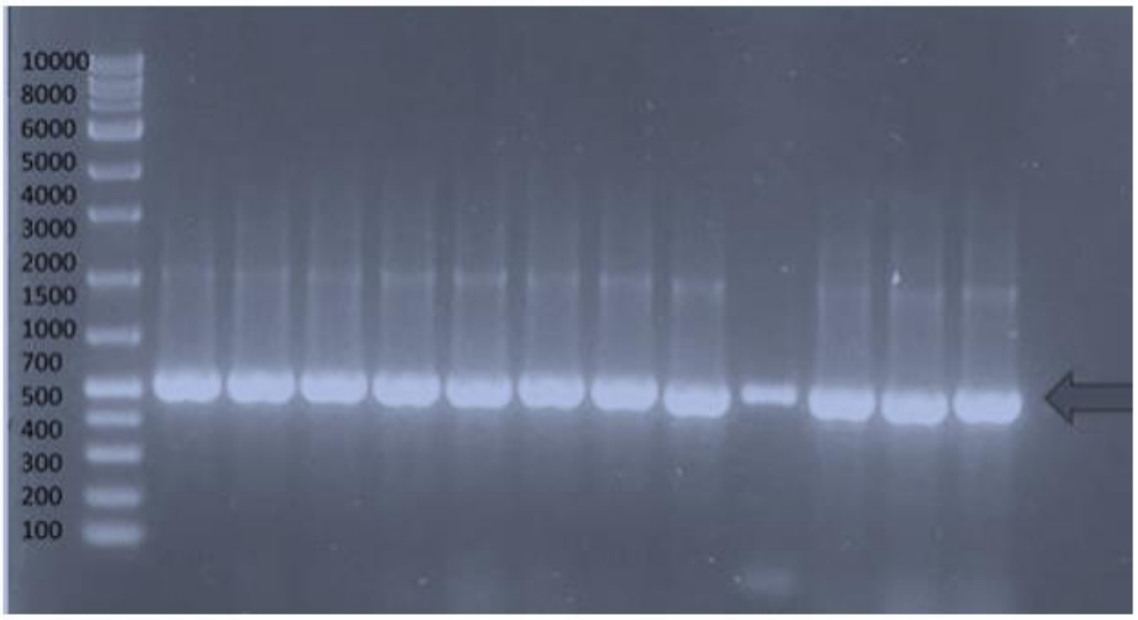

Fig. 1: DNA profile generated by *R. solani* specific primers (ITS1 and GMRS-3); M= 1 kb plus marker.

Characterization of *R. solani* isolates by Random Amplified Polymorphic DNA (RAPD-PCR):

Eighteen RAPD-PCR primers were used to differentiate between 12 *R. solani* isolates (Table 3). The total amplification fragments were detected for the 12 isolates in a total 1583 fragments and separates between isolates as follow: 122 (R1), 128 (R2), 133 (R4), 141 (R5), 139 (R6), 143 (R7), 140 (R8), 120 (R9), 124 (R11), 117 (R12), 131 (R13) and 145 (R15) (Fig.2).

The highest amplification fragments were recorded in isolate R15, while isolate number R12 showed the lowest fragments (Fig. 2). Among the investigated isolates, the obtained maximum numbers of fragments were ranged from 9 to 13 and (with an average number of 11.5 fragments). On the other hand, the minimum numbers of fragments ranged from 2 to 4 (3 fragments on average).

On the basis of data presented in Figure 2, it can be concluded that there were high genetic variations among the tested isolates and these data could be used to calculate the differentials among *R. solani* isolates, representing different localities in Egypt.

The genetic similarities among the tested 12 *R. solani* isolates were illustrated in (Figure 3), whereas genetic matrices were found**.** Clusters can be divided into two main groups by 27% genetic similarity, i.e., the first group includes *R. solani* isolate number R11, while the second group includes the rest of the tested isolates.

Within the second cluster divided by 30% genetic similarity into two sub-groups with 45% genetic similarity, i.e., the first one includes *R. solani* isolate R9, whereas the second one (47% genetic similarity) includes both R12 and R13 isolates (Fig. 3).

The second branch of the subgroup was divided into two sub-sub clusters with 33% genetic similarity, including *R. solani* isolatesR8 and R7 (75%) genetic similarity and R6 (62%) genetic similarity. The final cluster divided into two sub-sub clusters, i.e., the first branch includes *R. solani* isolate R15 only with genetic similarity 39%, whereas the second branch includes all of R1 isolates (genetic similarity-50%), R5 isolate (genetic similarity- 60%), R2 and R4 isolates (similarity- 88%)**.** These results were in the line with Mohammadi *et al*. (2003) and El-Zaidy *et al*. (2018), who studied the genetic variation among 20 isolates of *R. solani***.**

Using the same technique as Mohammadi *et al*., (2003) used random amplified polymorphic DNA (RAPD) markers to allow the identification of species or isolates, and the construction of dendrograms from the computed distances, their results genetic showed variation in Australian isolates of *R. solani* was analyzed by RAPD. All of the anastomosis groups (including subgroups) tested could be distinguished. For some groups, there was considerable variation in the fingerprint patterns between isolates. This variation was more marked between isolates from different geographic locations. Other groups showed very little variation between isolates. Also, Duncan *et al.* (1993) showed that RAPD analysis is a very useful alternative in anastomosis grouping for the identification of isolates of *R. solani.* Molecular markers are useful tools for detecting genetic variation in the population of *R. solani* (Monga *et al.*, 2004).

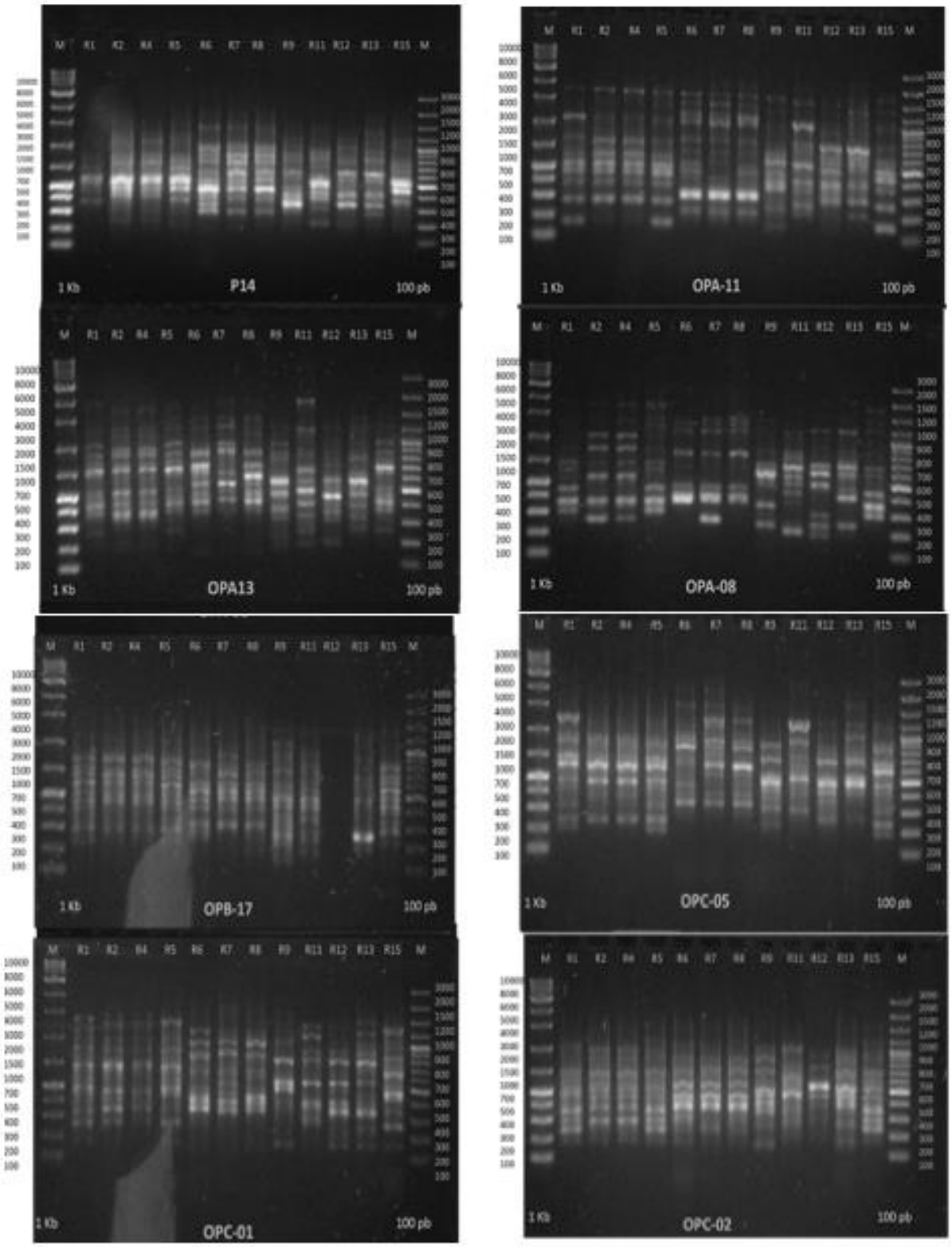

Fig. 2: Random amplified polymorphic DNA of 12 isolates *R. solani* using P-14, OPA-13, OPB-17, OPC-01, OPA-11, OPA-08, OPC-05 and OPC-02 primers, with 1 kb plus and 100 bp DNA ladder.

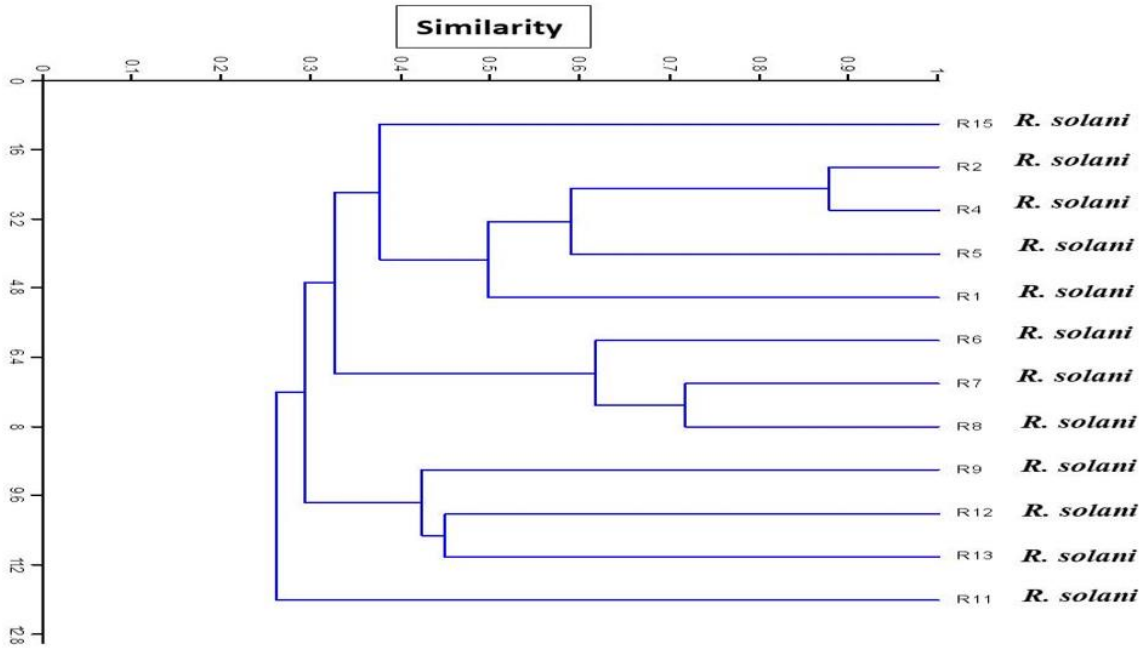

Fig. 3: Phylogenetic similarity of 12 *R. solani* isolates from different geographical locations in Egyptian governorates under circumstances based on RAPD-PCR primers.

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ARABIC SUMMARY

العزل والتعريف الجزيئى لفطر ريزوكتونيا سولانى المسبب لمرض موت بادرات القطن

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يعتبر مرض موت بادرات نبات القطن الناتج عن فطر الريزوكتونيا سولانى أحد اهم الامراض التى تصيب نبات القطن فى مناطق زراعتة على مستوى العالم وايضا مصر. تم جمع عزلات فطر الريزوكتونيا من بادرات القطن المصابة والمنزرعة فى بعض محافظات مصر. 12 عزلة لفطر الريزوكتونيا سولانى تم الحصول عليهم وتعريفهم. تم اجراء الشدة المرضية لجميع العزلات المتحصل عليها وذلك على 5 اصناف من القطن وهى جيزة 80, جيزة 87, جيزة 90, جيزة 92 وايضا جيزة 93 تحت ظروف الصوبة الزراعية. جميع العزلات المتحصل عليها اظهرت قدرة على احداث اصابة لنباتات القطن مع الاختلاف فى شدتها المرضية. أظهرت العزلة رقم R8والعزلة R9 اعلى شدة مرضية ونسبة اصابة باعراض موت البادرات مقارنة بباقى العزلات. بينما اقل شدة مرضية تم تسجيلها من العزلات رقم R11 و R7 . لتاكيد تعريف العزلات المتحصل عليها على المستوى الوراثى او الجزيئى تم استخدام بوادئ متخصصة لتعريف فطر الريزوكتونيا سولانى (ITS1 and GMRS-3) والتى أعطت نتيجة ايجابية وتأكيد أن العزلات جميعها تتبع فطر ريزوكتونيا سولانى. تم دراسة التباين الوراثى او الجزيئى للعزلات عن طريق استخدام المعلمات الوراثية RAPD. تم استخدام 18 بادئ وراثى والتى اعطت 1583 تتابع جزيئى مع 12 عزلة من الفطر. تم عمل شجرة وراثية من خلال النتائج المتحصل عليها من المعلمات الوراثية السابقة والتى اظهرت تباين كبير بين العزلات المختلفة من الفطر والتى ادت بالتبعية الى الاختلافات فى شدتها المرضية على النبات.