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RESEARCH ARTICLE

Genetic Relationship among Some Isolates of *Rhizoctonia Solani* Isolated from Some Infected Tomato Plants (*Solanum lycopersicum* L)

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Abstract

This study was carried out in the laboratory of Plant Virology at the Department of plants protection/ College of Agriculture/ University of Karbala to isolate and diagnose some isolates of fungi isolated from infected tomato plants in Najaf and Karbala provinces, Iraq. These isolates were diagnosed by using the polymerase chain reaction (PCR) technique and identifying the nucleotide sequence generated from PCR-amplified products using the universal primer pair ITS1 and ITS4. The analysis of nucleotide sequences, obtained from amplifying PCR Products, using the Basic Local Alignment Search Tool (BLAST) showed that all identified isolates belong to the fungus *R. solani*. From comparing the nucleotide sequences of the identified *R. solani* isolates, it was revealed a clear genetic variation among them as well as with other isolates of the same fungus previously registered in the National Center for Biotechnology Information (NCBI). The results also showed that two isolates among the other *R. solani* isolates were not previously registered in NCBI; their nucleotide identified sequences were deposited under the accession numbers: MH520074 and MH520071.

Keywords: *Rizoctonia solani*, Tomato, PCR, Nucleotide sequence, and ITS1-ITS4.

Introduction

Fungi are widespread in nature and have been discovered from remote, desert, polar-regions, tropical and subtropical regions in extreme environments worldwide [1, 2]. Many countries lack a precise identification of fungi and this reflects the international public opinion which says that 95% of fungi species are unknown and are still to be identified. Soil is the main source of microorganisms, including *Rhizoctonia solani* which has negative effects on different agricultural crops [3].

R. solani is dangerous because of its large host range and it can survive in extreme environmental conditions. *R. solani* isolates can be characterized by their genetic variation, morphological difference, and biochemical and molecular characteristics [4]. There is a crucial need to identify fungi precisely due to its importance to apply rapid and efficient methods in disease management. It was noted that depending on morphological characters in classifying fungi might give accurate results, but many researchers do not rely on these characteristics as they require sufficient experience in taxonomy, especially in fungal groups that are closely related such as *Fusarium* species, and they also need considerable time and effort [5].

Morphological identification is often inaccurate due to the morphological characteristics can be affected by environmental factors which in turn affect size, shape and colors of spores and fungal colonies [6]. Identification techniques that depend on molecular characteristics have participated in their accuracy and ability to study and reveal the genetic differences and eliminate the limitations of traditional methods in diagnosing many organisms [7,8].

PCR technique is one of the molecular techniques that depend on selecting and amplifying a specific region of the genome by depending on differences in DNA sequence of that regions and accordingly recognizing the genetic relationships in terms of similarity and difference between fungi species which will support the morphological diagnosis of the studied fungi [9].

This technique was used in diagnosing many microorganisms, including fungus such as *Fusarium* spp. And *R. solani*. In view of the importance of accurate fungus taxonomy, this study aimed to isolate and molecularly diagnose ten isolates of *R. solani* by using PCR and determining nucleotide sequences of the PCR-amplified products to know the genetic similarity and difference between those isolates and also with other isolates of *R. solani* previously diagnosed and registered in the National Center for Biotechnology Information (NCBI).

Materials and Methods

R. Solani Isolates

In order to isolate the pathogenic fungus *R. solani*, tomato plants with symptoms of wilt were collected from some farms in Najaf and Karbala provinces, Iraq. These plants were brought to the laboratory of Plant Virology at College of Agriculture/ University of Karbala to carry out for isolation of the causal agent. Plants roots were washed by tap water and cut into small pieces and then sterilized by Sodium hypochlorite (NaOCl) (1%) for two minutes. After that they were washed by sterilized distilled water to get rid of residues of sterilizer material and then dried by filtering paper.

They were cultured in Petri dishes containing Potato Dextrose Agar (PDA) and Chloramphenicol at a concentration of 200 mg/L.. All Petri dishes were incubation at a temperature of 25±2°C for four days.

Appeared *R. solani* isolates were purified on the same nutrient medium (PDA) and initially diagnosed using morphological characters mentioned by [10].

These fungal isolates were also diagnosed by using the polymerase chain reaction (PCR) technique and determining the nucleotide sequences of the generated PCR products amplified from the *R. solani* isolates as described later.

DNA Extraction, PCR amplification and DAN sequencing of rDNA-ITS region of *R. solani* Isolates

DNA for fungal isolates was extracted according to the method prescribed by the American Company ZYMO Research by using (Cat.No.D6005) provided by the same company. PCR was carried out by using (Maxime PCR PreMix (i-Taq), Cat. No.25026) provided by the Korean Company iNtRoN. PCR was carried out with a volume of 20 µl and containing 1 µl of each forward primer (ITS1: TCCGTA GGTGAACCTGCGG) and reverse primer (TS4: TTGATATGC TCCTCCGCTTA) and 1 µl of the extracted DNA.

All materials mentioned above were put in a test tube provided by the manufacturing company and the final volume was completed to 20 µl by adding nuclease-free water.PCR amplification was performed using the following conditions: Initial Denaturation for 5 minutes in a temperature of 98° C, followed by 35 cycles each consists of final denaturation for 40 sec at a temperature of 94° C, primer annealing for 40 sec at a temperature of 55° C. initial extension for 1 min, and final extension at 72°C for 5 min [11].

PCR-amplified products were electrophoretically separated on a 1% agarose gel for 140 min at 80 V and 400 mA and visualized with ethidium bromide under UV illumination, and images were captured using Vilber Lourmat, Taiwan, gel documentation system. PCR products amplified from *R. solani* isolates were sent along with forward and reverse primers used for PCR amplification, to the Company Microgen, South Korea, in order to determine the nucleotide sequences in both directions for each amplified PCR product.

All nucleotide sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) to be compared with data available at the National Center for Biotechnology Information (NCBI) [12].

Using the MEGA6 software, multiple alignments of the nucleotide sequences and construction of phylogenetic trees were performed using the neighbor joining method [13].

Results and Discussions

Isolating and Diagnosing *R. solani* Isolates

Ten isolates of *R. solani* were initially diagnosed and isolated from infected tomato plants depending on general characteristics described by [10] including fungal hypha is branched in a right angle, forming septa near branch initiation, hyphal branches striction, inability to form sexual and nonsexual spores, appearing a grade of brown color and forming different stone-fungal objects with a grading brown color (Fig. 1).

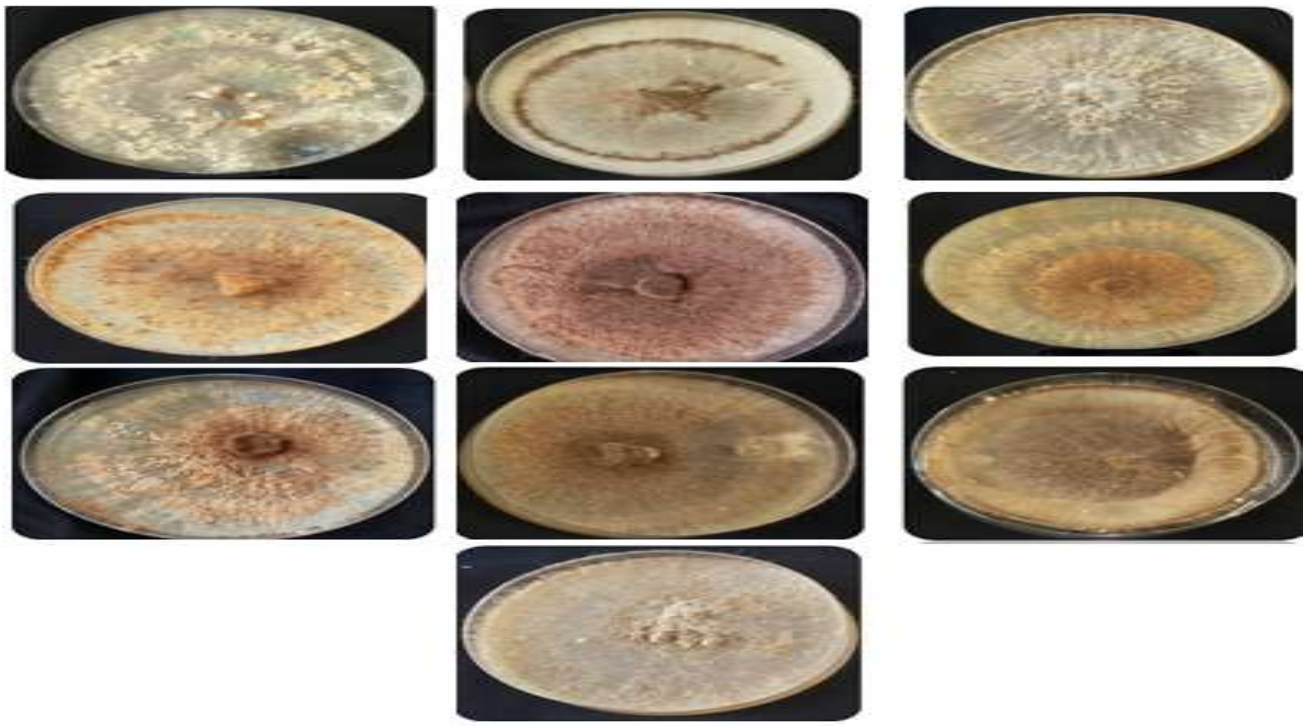

Figure 1: Colony morphology of the *R. solani* isolates as grown on PDA

Results of extracting DNA from these fungal isolates and exposing it to polymerase chain reaction (PCR) showed a possibility to amplify DNA products in different sizes ranged from 500bp and 750 bp using forward and reverse primers ITS1 and ITS4. (Fig. 2)

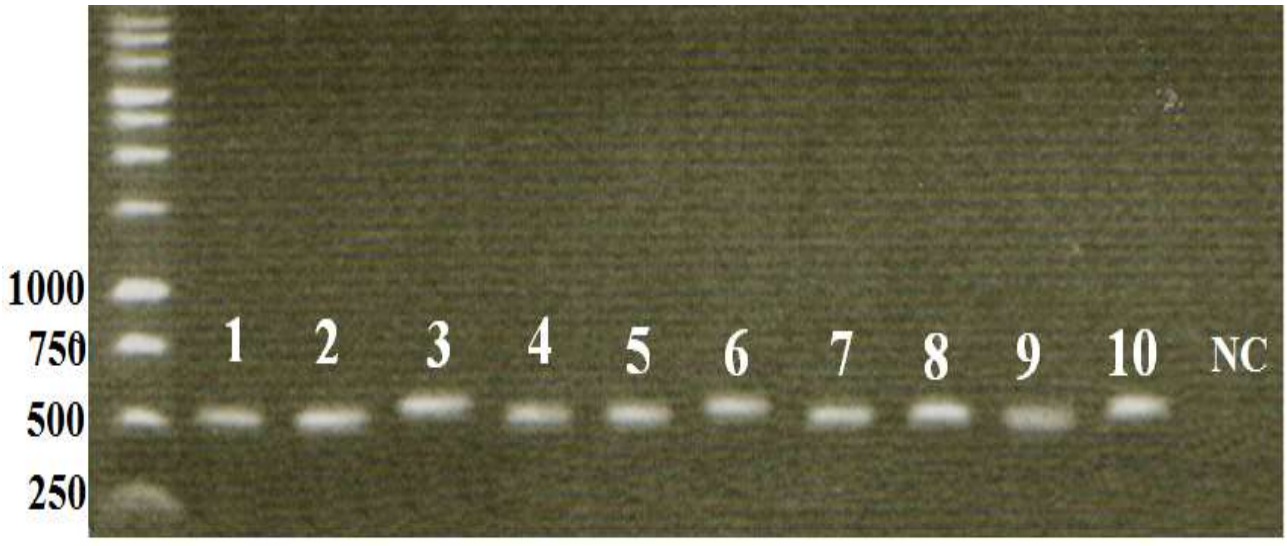

Figure 2: DNA products amplified by polymerase chain reaction (PCR) from *R. solani* isolates (1-10) isolated from infected tomato plants collected from in Najaf and Karbala provinces. M= 1Kbp DNA ladder marker. NC: Negative control (no template DNA added)

Results of nucleotide sequences analysis for PCR products amplified from the isolated fungal isolates by using BLAST showed that all fungal isolates belong to *R. solani. R. solani* isolates, isolated in this study, showed clear genetic variations among them in some positions of nucleotide sequences (Fig. 3) with a similarity percentage ranged from 91%-99% (Table 1 and Fig. 4).

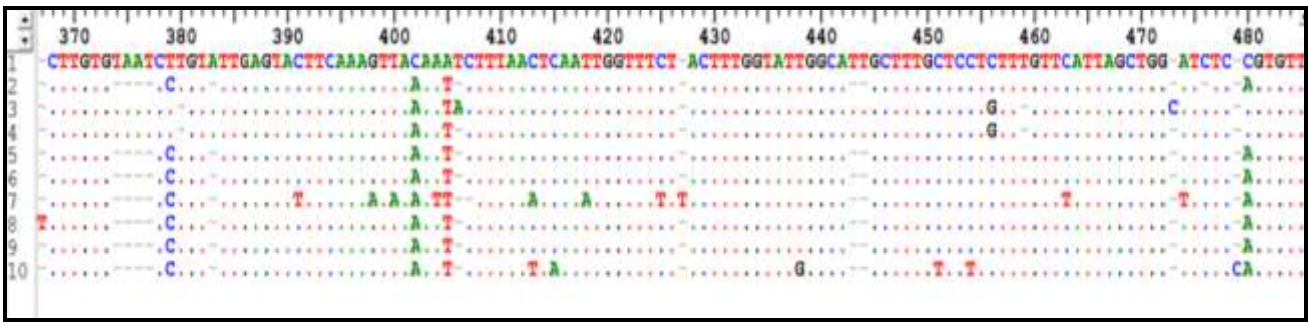

Figure 3: A graphical view of the similarity and difference in the ITS1, 5.8S rDNA and ITS4 sequences generated from the *R. solani* isolates obtained in this study. Identical nitrogenous bases are represented in dots. Numbers on the right side of the figure represent nucleotide sequences obtained from PCR products amplified from the different *R. solani* isolates

Table 1: Similarity rates among R. solani isolates obtained from infected tomato plants

										Isolate No.
									-	1
								-	98	2
							-	97	97	3
						-	99	97	98	4
					-	97	97	100	98	5
				-	100	97	97	100	98	6
			-	94	94	91	91	94	92	7
		-	93	99	99	97	96	99	97	8
	-	99	93	99	99	97	97	99	98	9
-	97	97	92	98	98	95	95	97	96	10
10	9	8	7	6	5	4	3	2	1	

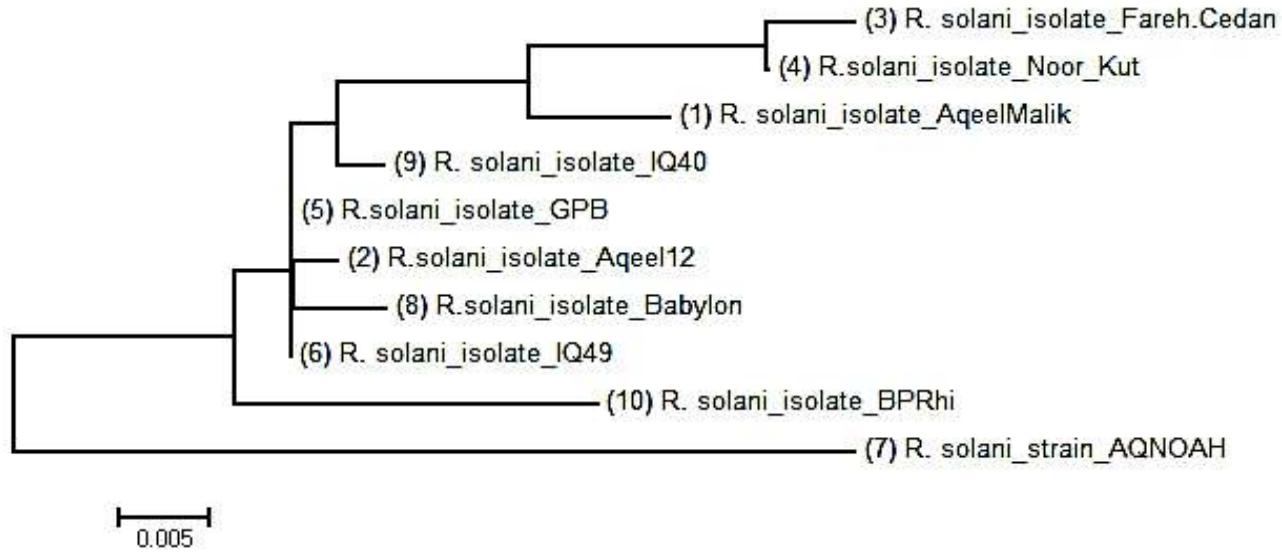

Figure 4: Phylogenetic tree generated using the neighbor-joining method based on a comparison of the ITS1, 5.8S rDNA and ITS4 sequences generated from *R. solani* isolated in this study

A BLAST search using the nucleotide sequences revealed that the *R. solani* isolates 3, 4, 5, 6, 7, 8, 9, and 10 showed a similarity percentage of 100% with some *R. solani* previously identified and registered in NCBI; whereas two isolates (1 and 2) gave a difference with the other isolate available in NCBI. From comparison of the sequence obtained from these *R. solani* (1 and 2) with the other *R. solani* isolates deposited in NCBI showed that the highest genetic similarity was 99% with the isolates previously registered in NCBI under the accession numbers: KX828173 and MK621284; therefore, these two isolates have registered in NCBI under the accession

numbers MH520074 and MH520071 respectively (Table 2).

Table 2: Summary of nucleotide sequences analysis of *R. solani* isolates obtained in this study

Isolate No.	Isolate name	Origin	Similarity (%)	Nearest relative *R. solani* isolate (Accession No.)	Sequenced base Similarity	Accession No. received from NCBI
1	AqeelMalik*	Iraq	99	KX828173	392/384	MH520074
2	Aqeel12*	Iraq	99	MK621284	619/623	MH520071
3	Fareh.Cedan*	Iraq	100	MH520073	413/413	-
4	Noor_Kut*	Iraq	100	MH520067	411/416	-
5	GPB*	India	100	MK621284	664/664	-
6	IQ49*	Iraq	100	KF372653	641/641	-
7	AQNOAH*	Iraq	100	KY055374	624/624	-
8	Babylon*	Iraq	100	KY283953	603/603	-
9	IQ40*	Iraq	100	KF372662	626/626	-
10	BPRhi*	India	100	KM434130	614/614	-

* R. solani isolates obtained in this study

It was also found from the neighbor-joining tree that *R. solani* isolate (AqeelMalik) appeared in a clade separated from most isolates previously registered in NCBI and it was closely similar to the Iraqi *R. solani* isolate (KX828173.1) by a similarity percentage of 99%.

It was clear through the same tree that *R. solani* fungal isolate (Aqeel12) was the most similar to the Iraqi *R. solani* isolate (MH520073.1) previously isolated from potato tubers. These two *R. solani* isolates obtained from this study had a similarity rate of 97-98%.

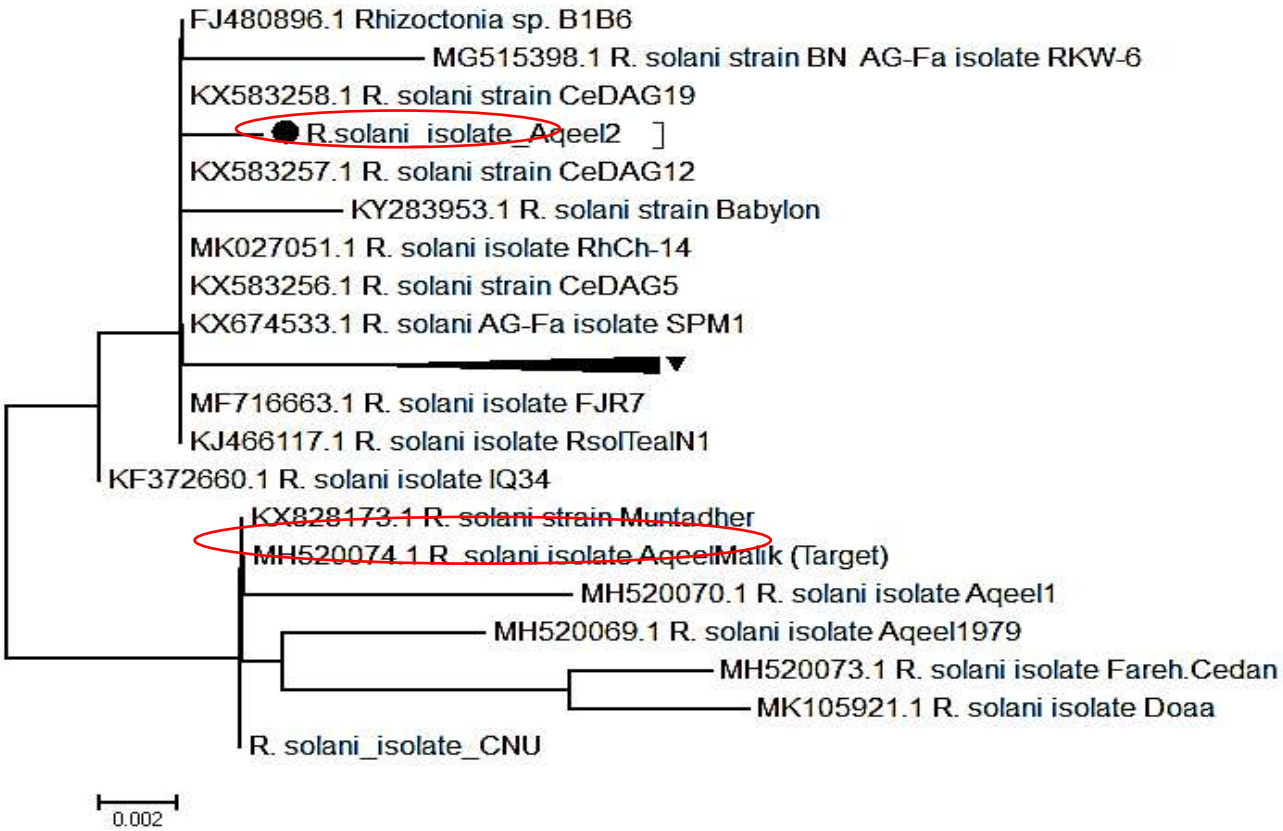

Figure 4: Neighboring-joining tree shows the genetic relationship for the novel *R. solani* isolates (MH520074) and (MH520071) isolated in this study from tomato plants

It was concluded from results obtained in this study that the most *R. solani* isolates obtained in this study have genetic differences among them as well as with other isolates previously registered in NCBI.

These results demonstrated that the two isolates (1 and 2) were not previously registered in NCBI; therefore, they were registered under the accession numbers MH520074 and MH520071, respectively.

In this study, PCR was used diagnose different isolates of *R. solani* because of its high accuracy in the identification of many organisms, including fungi, e.g. *Fusarium* spp., *R. solani*, and *Alternaria alternata* to

eliminate the problems of identification based on morphological characters [7, 14, 15].

Despite the usefulness of morphological characteristics in the identification of fungi and in placing the isolated fungi into smaller groups before using the other methods of identification. From previous studies, it was noticed that many problems can be occurred with the morphological identification of fungi such as the need of a high level of expertise, especially in the fungal species exceedingly related to each other, e.g. *Fusarium* spp., as well as the need for time and effort [1, 16]. Other factors can be affected the morphological characteristics, such as humidity, the type and nature of growth medium, and lighting which can affect the color, forms, and sizes of spores.

Molecular diagnosis technique depending on differences in DNA sequence of Internal transcribed spacer (ITS) has shown high efficiency in diagnosing many fungi such as *Fusarium* spp., *Cladosporium* spp. and *Fusarium verticillioides* [15, 17]. The accurate diagnosis for infecting fungus is considered one of the crucial needs to arrive effective methods in disease management and quarantine purposes to protect agricultural crops.

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