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Original Research Paper

Comparison of *Rhizoctonia solani* Isolated from Soil in Baghdad – Iraq Genetically with World Isolates

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R. solani **is a soil-borne Basidiomycete occurring worldwide. The objective of this study was to compare isolates of *Rhizoctonia solani* from some soil samples collected from different regions (Abu Ghraib, Yusifiyah, Al Rashidiya, Tarmiya, Doura and Quraiat) in Baghdad – Iraq. The degree of kinship for *R. solani* isolates was estimated by the PCR technology. A total of 24 isolates of *R. solani* were isolated from 114 samples of soil. Pathogenicity test using radish seedlings showed that 11 isolates were strong virulent, 5 isolates were moderately virulent, and 8 isolates showed low virulence. The colony morphology on PSA of isolates (IQ4, IQ6, IQ9, IQ14, IQ15, IQ16, IQ24, IQ25, IQ26, IQ28 and IQ50) showed typical features of *R. solani*. The sequences of 9 *R. solani* isolates from tomato plants deposited at NCBI – GenBank nucleotide database. The GenBank accession number for each isolate sequence was available since November 2013 as KF372663 – KF372673. The results of phylogenetic analysis showed that, identifying soil isolates were within AG4: two isolates, IQ4 and IQ6 were closely related to AG4-HGI and 9 isolates, IQ14, IQ15, IQ50, IQ9, IQ16, IQ24, IQ26, IQ25 and IQ28 were belonged to AG4–HG III (BS = 99%) and showed sequence similarity of 94, 97, 85, 96, 67, 69, 60, 74, 66, 66 and 65% respectively.**

Keywords: *Rhizoctonia solani*, soil, ITS, PCR.

INTRODUCTION

R. solani is the most widely known and most studied species of the genus *Rhizoctonia*. It was originally described by Julius Kühn who isolated it from potato in 1858. *R. solani* is a soil-borne Basidiomycete occurring worldwide, with complex biology (Carling and Sumner, 1992). Its highly destructive lifestyle as a non-obligate parasite causes necrosis and damping-off on numerous host plant species is interesting.

Because of the lack of conidia and the scarcity of the sexual spores, *R. solani* exists as a vegetative hyphae and sclerotia in nature. Sclerotia are an encapsulated, tight hyphal clump that protects and preserves the fungus over non-optimal environment. The fungus is dispersed mainly via sclerotia, contaminated plant material or soil, which were spread by wind, water or during agricultural practices such as tillage and seed transportation. The fungus can maintain in the soil as a saprophyte for long periods (Carling *et al*., 1999). Anastomosis groupings that are according to hyphal anastomosis exists among isolates having common biological affinities that greatly facilitated the identification of *R. solani* and other *Rhizoctonia* spp (Parmeter *et al*., 1967; Parmeter *et al*., 1969).

Anastomosis groups of the *Rhizoctonia* strains were determined from the sequence of the polymerase chain reaction (PCR) products of fungal ITS region amplified with universal primers ITS-1 and ITS-4 (White *et al*. 1990). Primer ITS-1 is located on the DNA sense strand (3' – 5') of the 18S rRNA gene and the ITS-4 on DNA antisense strand (5' – 3') of the 28S rRNA gene as explained by White *et al*. (1990). Gonzalez *et al*. (2001) used sequence analyses of the ITS region as well as part of the 28S rDNA to investigate

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whether Thanatephorus and Ceratobasidium represents distinct evolutionary lineages, and if anastomosis groups represent the most fundamental evolutionary units within *R. solani*. The objectives of this study was to compare *Rhizoctonia solani* isolated from soil in Baghdad – Iraq genetically with world isolates using the rDNA- internal transcribed spacer (ITS) region and sequence analysis.

MATERIAL AND METHODS

Isolates

114 soil samples were collected from different areas in Baghdad-Iraq (Abu Ghraib, Yusifiyah, Al Rashidiya, Tarmiya, Doura and Quraiat). *Rhizoctonia spp.* were isolated from the soil samples using the plant debris particle method (Boosalis and Scharen, 1959). Soil samples, 100 g were suspended in 2 liters of tap water in an Erlenmeyer flask by thoroughly shaking the suspension and letting the soil particles to settle down for several minutes. The floated plant debris particles on the surface were collected on a 60 mm mesh screen, washed with tap water and dry on filter paper. Single particles of plant debris were plated on Water Agar (WA) plates containing 250 µg/ml chloramphenicol.

The plates were incubated at 25± 1 C for 24 h. The hyphae growing from the particles were examined under microscope at low magnification for typical hyphal growth of *Rhizoctonia spp.* Hyphal tips were transferred to Potato Sucrose Agar (PSA) plates containing 250 µg/ml chloramphenicol. The plates were incubated at 25C for 2-3 days and pure cultures of *Rhizoctonia spp*. were transferred to slant PSA and kept at 4C until further investigation.

Pathogenicity tests

Pathogenicity test of *R. solani* isolates were evaluated by colonized agar disks (7-10 mm diam.) taken from the margins of 3 day-old cultures growing on PSA were transferred to the center of WA plates and incubated for 3 days at 25± 1 C in the dark. Six seeds of radish (*Raphanus sativus*) were placed on the margins of the *Rhizoctonia* colonies in separate plates. The pathogenicity of the isolates was evaluated after a further 6 days of incubation for radish at 25± 1 C.

Disease severity was assessed visually and scored using 1-5 disease severity index (DSI), where 1= <1 mm lesion; 2= 1- < 3 mm; 3=3- < 5 mm; 4=5- < 7 mm; 5= ≥ 7 mm or dead seedling. Isolates causing no symptoms or very mild symptoms (DSI = 0-0.3) were considered avirulent; isolates causing mild symptoms (DSI = 0.4-1.9) were considered low virulent; isolates causing moderate symptoms (DSI = 2-2.9) were considered moderately virulent; isolates causing severe symptoms (DSI = 3-3.9) were considered virulent and isolates causing very severe symptoms (4-5 DSI) were considered strongly virulent (Sneh *et al*., 2004).

Morphological characteristics

A 5 mm diameter mycelial disc from a 3-day- old PSA culture of *Rhizoctonia* spp isolates were placed in the middle of PSA Petri dishes and incubated at 25± 1 C for 21 days in the dark. The moniloid cells, sclerotia, right-angle branching, constriction as well as the formation of septa near the point of hyphal branching, dolipore septum and colony colors were observed three weeks after incubation (Misawa and Kuninaga, 2010). Number of nuclei accounted in young hyphae cells of *Rhizoctonia* spp. were examined by microscope using 400 x magnifications (Herr, 1979).

Molecular characteristics

Each *R. solani* isolate was grown in potato sucrose broth for 4-7days at 25± 1C. Mycelial mats were harvested by filtration, dried in room chamber and grounded into fine powder in liquid nitrogen and then stored in 20 °C until its use in DNA isolation. Genomic DNA was extracted from 100 mg of ground fungal tissue using the fungal DNA Kit (EZ-10 spin column fungal genomic DNA, Bioneer corporation, South Korea) and following the protocol recommended by the manufacturer Internal transcribed spacer region of ribosomal DNA (ITS) was amplified using ITS1 Forward primer (TCC GTA GGT GAA CCT GCG G) (length=19mer, molecular weight = 5844.7 g/mole, Bioneer Corporation) and ITS4 Reverse primer (TCC TCC GCT TAT TGA TAT GC) (length=20mer, molecular weight = 6033.8 g/mole, Bioneer Corporation) set of primers.

The PCR reaction was performed in 20 µL total volumes consisting of 5 µL of PCR PreMix [Top DNA polymerase 1 U + Each: dNTP (dATP, dCTP, dGTP, dTTP) 250µM +Tris-HCL (pH 9) 10 mM +KCL 30 mM + MgCl2 1.5 mM + stabilizer and tracking dye. Bioneer Corporation, South Korea], 5 µL of DNA template (concentration 40 – 50 ng), 3 µL of ITS1primer, 3 µL of ITS4 primer and 4µL of PCR deionized water. The amplification was performed in a PCR thermal cycler (My Genie 32 Thermal Block, Bioneer, South Korea). The cycle parameters were: An initial denaturation (95C, 2 min), 35 cycles of denaturation (94C, 30s), annealing (55C, 1 min) and extension (72C, 1 min).

Final extension was at 72C for 10 min. Following the PCR reaction, the amplified products (8 µL) were loaded in a 1.5% agarose gel stained with ethidium- bromide, together with 100 bp DNA marker (from 100 to 2000 base pairs, Bioneer Corporation, South Korea). Before loading, both samples and marker were dyed with Blue/Orange 6X Loading Dye (2 µL) used for tracking migration during electrophoresis. Electrophoresis was run at 80 V for 2 h. The DNA bands were visualized and photographed using special UV camera (Hsiang and Dean, 2001; Stojsin *et al*., 2007).

DNA sequencing and data analysis

After the amplification of the ITS region of the rDNA, each product was purified using the AccuPrep® PCR Purification Kit and protocol (Bioneer Corporation, Korea). Purified rDNA was sent to the DNA Sequencing Facility at Bioneer Corporation, Korea for sequencing. ITS sequence analysis was performed using on-line software CLUSTAL W2 and BLAST via http://www.ncbi.nlm.nih.gov, www.bioservers.org. All *R. solani* isolates sequences were aligned with software program CLUSTALW2. Sequence database from the National Center for Biotechnology Information (NCBI), which was downloaded from http://www.ncbi.nlm.nih.gov, was used for sequence information on selected *R. solani* isolates (Table 1).

Phylogenetic analysis was performed on all sequences obtained in this study and some available at GenBank were aligned with multiple aliment program ClustalW2. A tree showing the phylogenetic relatedness between isolates constructed on calculating pair – wise distances using the Jukes-Cantor model. A neighbor – joining tree was generated with bootstrap values based on 1000 replicates. By using the computer software package MEGA5.2. The tree was rooted with outgroup (Ohkura *et al*., 2009).

Table.1. Accession number of sequenced *Rhizoctonia solani* from different sources

AG & Subgroup	Source and geographic origin	GenBank Accession number
AG 1-IA	*Oryza sativa*, Japan	AB000017
AG 1-IB	*Beta vulgaris*, Japan	AB000038
AG 1-IC	*Beta vulgaris*, Japan	AB122142
AG 1-ID	Coffee	AB122128
AG 2-1	*Solanum tuberosum*, USA	AB000026
AG 2-2IIIB	*Beta vulgaris*, USA	AB054857
AG 2-2 IV	*Beta vulgaris*, USA	AB054859
AG 2-3	*Glycine max*, Japan	AB054870
AG-2-1-2t	Tulip, Netherlands	AB054850
AG-2-2 LP	Zoysia grass, Japan	AB054866
AG2-4	Maize, USA	AB054878
AG2-BI	Soil, Japan	AB054873
AG 3PT	*Solanum tuberosum*, USA	AB019013
AG 3TB	*Nicotiana tabacum*, USA	AB000001
AG 4HGI	*Beta vulgaris*, Japan	AB000028
AG 4HGII	*Beta vulgaris*, Japan	AB000033
AG4HGIII	*Vincetoxicum pycnostelma*,China	JQ669932
AG 5	*Beta vulgaris*, Japan	AF153777
AG 5	Sugar beet, Japan	AF354113
AG6-HGI	Soil, Japan	AB000019
AG 6GV1	Unknow n, Japan	AF153780
AG6-GV2	Unknow n, Brazil	AY154304
AG6-GV3	Wheat, South Africa	AF153788
AG6-GV4	Leaf litter, Australia	AF153785
AG 7	Soil, Japan	AB000003
AG 8	*Triticum aestivum*, Australia	AB000011
AG8ZGI-1	Soil, Australia	AF153795
AG8ZGI-2	Soil, Australia	AF153797
AG8ZGI-3	*Hordeum vulgare*, Australia	AF354068
AG8ZGI-4	*Hordeum vulgare*, Scotland	AF354066
AG9-TX	*Solanum tuberosum*, USA	AB000037
AG 9TP	*Solanum tuberosum*, USA	AB000046
AG 10	*Hordeum vulgare*, Australia	AF354071
AG 11	*Glycine max*, USA	AF354114
AG 12	*Pterostylis acuminata*, Australia	AF153803
AG13	Cotton, USA	AB275645
AG BI	Soil, Japan	AB000044

Table 2. Isolates code, geographic location, sources and disease severity index

***Rhizoctonia* spp. Isolate code**	**Regions Baghdad-Iraq**	**Disease severity index**
IQ- 1	Al Rashidiya	1.50
IQ- 2	Al Rashidiya	1.80
IQ- 3	Al Rashidiya	1.95
IQ- 4	Abu Ghraib	5.00
IQ- 5	Tarmiya	2.70
IQ- 6	Abu Ghraib	5.00
IQ- 7	Doura	2.80
IQ- 8	Doura	1.90
IQ- 9	Abu Ghraib	5.00
IQ-10	Tarmiya	1.90
IQ-12	Tarmiya	1.96
IQ-13	Doura	2.30
IQ-14	Abu Ghraib	5.00
IQ-15	Abu Ghraib	5.00
IQ-16	Abu Ghraib	4.30
IQ-19	Doura	2.40
IQ-20	Tarmiya	2.10
IQ-24	Yusifiyah	4.62
IQ-25	Yusifiyah	4.50
IQ-26	Yusifiyah	4.50
IQ-27	Doura	1.60
IQ-28	Yusifiyah	4.25
IQ-31	Doura	1.10
IQ-50	Abu Ghraib	4.37

* Scale: 0-0.3 DSI= Avirulent, 0.4-1.9 DSI= Low virulent, 2-2.9 DSI= Moderately virulent, 3-3.9DSI= Virulent and 4-5DSI= Strong virulent

Table.3. *Rhizoctonia solani* isolates sequences comparison with AG isolates from GenBank using Clustal W2

R. solani isolates	GenBank Accession Number	Sequence similarity (%)					
		AG1-IA AB000017	AG4-HGI AB000028	AG4-HGII AB000033	AG4-HGIII JQ669932	AG6-GV4 AF153785	AG7 AB000003
IQ 4	KF372663	76	94	89	83	76	73
IQ 6	KF372664	78	97	92	87	86	80
IQ 9	KF372665	67	67	68	69	68	67
IQ14	KF372666	67	74	73	85	69	67
IQ15	KF372667	75	82	76	96	78	75
IQ16	KF372668	57	57	57	60	59	58
IQ24	KF372669	70	68	68	74	73	70
IQ25	KF372670	65	62	62	66	65	65
IQ26	KF372671	64	64	64	66	65	63
IQ28	KF372672	62	62	62	65	64	63
IQ50	KF372673	64	60	65	67	66	66

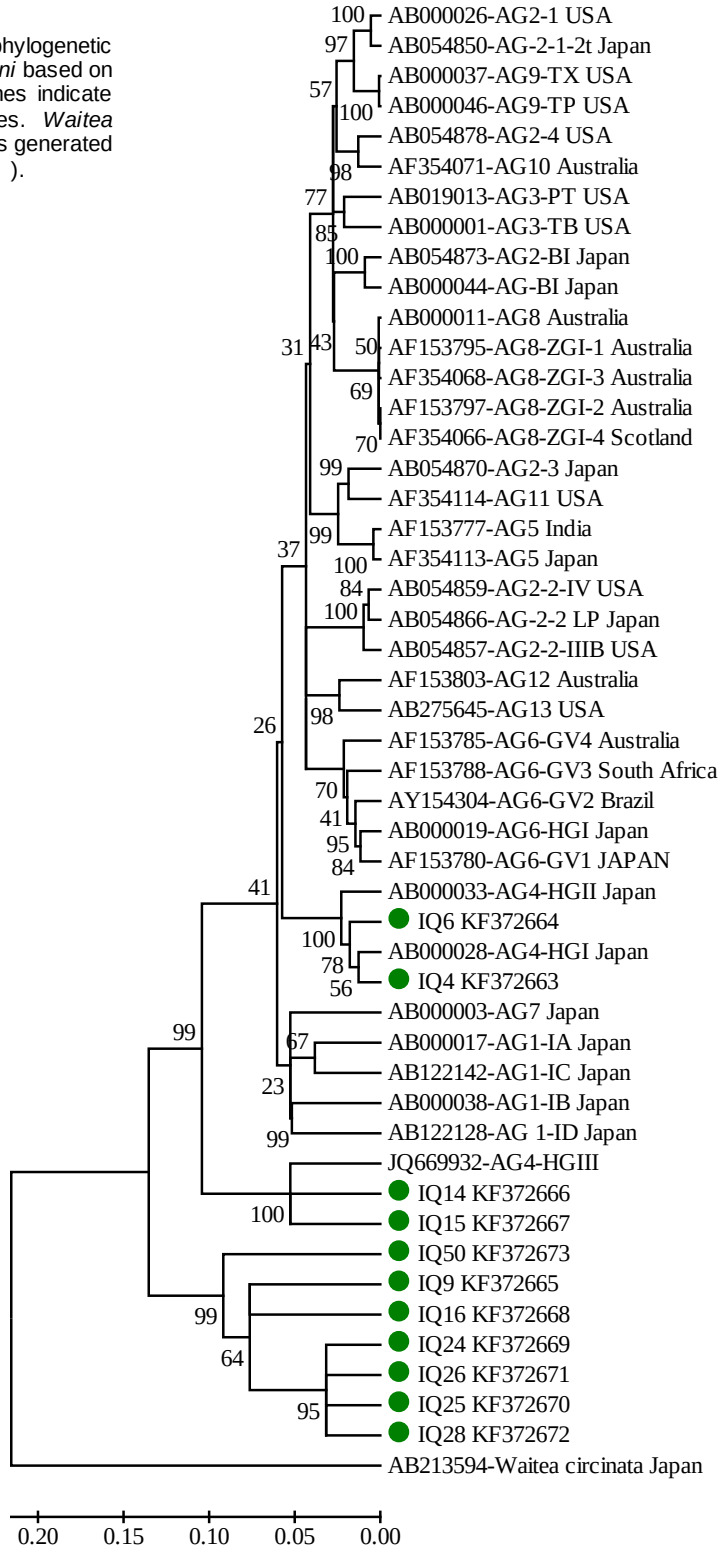

Fig.1. Neighbor-joining tree showing the phylogenetic relationship among the soil isolates of *R. solani* based on their ITS sequences. Numbers above branches indicate bootstrap percentages from 1000 replicates. *Waitea circinata* used as an outgroup. The sequences generated in present study were labelled with diamond ( ).

RESULTS AND DISCUSSION

Isolation of *Rhizoctonia spp.*

A total of 24 *Rhizoctonia spp.* isolates were obtained from 114 samples, from the soil of different six regions in Baghdad, Iraq, namely: Abu Ghraib, Yusifiyah, Al Rashidiya, Tarmiya, Doura and Quraiat (Table 2).

Pathogenicity tests

Results of pathogenicity test indicated *Rhizoctonia* isolates differed in virulence on radish seeds according to pathogenicity test method. Eleven isolates showed strong virulent on radish seedlings [Disease Severity Index (DSI) = 4 – 5] (IQ4, IQ6, IQ9, IQ14, IQ15, IQ16, IQ24, IQ25, IQ26, IQ28 and IQ50), Five isolates were moderately virulent (DSI= 2 – 2.9) (IQ5, IQ7, IQ13, IQ19 and IQ20) and Eight isolates were low virulent of Radish seedlings (DSI= 0.4 – 1.9) (IQ1, IQ2, IQ3, IQ8, IQ10, IQ12, IQ27 and IQ31) (Table 2).

The results of this study are consistent with results of previous study (Sneh *et al*., 2004) in which of the 206 *Rhizoctonia* spp isolates obtained from 135 soil samples collected from different fields in New Zealand, 55% were pathogenic on radish seedlings, 39 isolates were moderately virulent, 22 isolates were virulent and 53 isolates were strongly virulent. The host range of *R. solani* is very wide and it causes various diseases on important crop plants worldwide (Ogoshi, 1996). Because of high adaptability to environmental conditions and a wide host range, *R. solani* (teleomorph, *Thanatephorus cucumeris*) is distributed all over the world and is capable of causing severe crop losses on the Solanaceae (Ogoshi, 1987). Strong virulent of *Rhizoctonia* isolates have been selected for further studies.

Morphological characteristics

The isolates (IQ4, IQ6, IQ9, IQ14, IQ15, IQ16, IQ24, IQ25, IQ26, IQ28 and IQ50) showed typical features of *R. solani* complex including: (a) branching near the distal septum of cells in young, vegetative hyphae; (b) formation of a septum in the branch near the point of origin; (c) construction of the branch; (d) dolipore septum; (e) no clamp connection; (f) no conidium, except monilioid cells; (g) sclerotium not differentiated into rind and medulla; and (h) no rhizomorph. Has three or more nuclei per cell, larger hyphae (6-10 µm diameter) (Parmeter and Whitney, 1970; Ogoshi, 1975, 1987).

Sequencing of *Rhizoctonia* isolates

The sequences of the 11 *R. solani* isolates from soil (IQ4, IQ6, IQ9, IQ14, IQ15, IQ16, IQ24, IQ25, IQ26, IQ28 and IQ50) were recovered from Abu Ghraib, Yusifiyah, Al Rashidiya, Tarmiya, Doura and Quraiat - Baghdad, Iraq were deposited at NCBI – GenBank nucleotide database. The GenBank sent firstly submitted Bankit code number for isolates sequence as 1643201. The GenBank accession number for each isolate sequence was available since first of November 2013 as KF372663 – KF372673 (Table 3).

Molecular characteristics of isolates

The soil *R. Solani* isolates sequences were compared among themselves and 37 *R. solani* isolates were selected randomly chosen representative of *R. solani* AGs-1 to 13 and AG BI, whose sequences were downloaded from GenBank (Table 1). Phylogenetic analysis of *R. solani* isolates in this study was depended on two methods: First, in the neighbor – joining tree, collected *R. solani* isolates were inferred to belong to an AG, subgroup, or species if they formed a cluster including a reference sequence supported by a bootstrap value (BS) of 95% or higher, and were considered closely related to a group if supported by a BS lower than 95% (Ohkura *et al*., 2009).

Second, determining sequence similarity based on cross – comparison for detected anastomosis groups and subgroups of *R. solani* isolates were compared with randomly chosen representative from AGs 1 – 13 and AG BI from GenBank (www.ncbi.nlm.nih.gov). Analysis of ITS sequences was performed using on-line software Clustal W2 (www.ebi.ac.uk) (Stojsin *et al*., 2011).

The results of phylogenetic analysis identified soil *R. solani* isolates were as AG4: two isolates (IQ4 and IQ6) were closely related to AG4- HGI and 9 isolates (IQ14, IQ15, IQ50, IQ9, IQ16, IQ24, IQ26, IQ25 and IQ28) were belonged to AG4-HGIII (BS = 99%) (Fig.1). These soil isolates were showed sequence similarity 94, 97, 85, 96, 67, 69, 60, 74, 66, 66 and 65% respectively (Table 3).

The phylogenetic analysis clearly indicated that some of the Iraqi isolates of *R. solani* had variable ITS sequences, therefore, grouped separately in one major cluster, although they were from different places of origin. The present results also are in agreement with Kuninga *et al*. (1997) that observed 5.8s rDNA sequence to be completely conserved across all the AGs examined, whereas the ITS rDNA sequence was found to be highly variable among 45 isolates of *R. solani.*

The sequence homology in the ITS regions was above 96% for the isolates of the same subgroup. Ganeshamoorthi and Dubey (2013) found that rDNA-ITS sequence was variable of 50 chickpea isolates of *R. solani* grouped into seven AGs. The present study clearly highlights the genetic variation of the *R. solani* at the subgroup level, especially ITS region which was very much useful in intraspecific diversity of the *R. solani* and helps in development of AGs level diagnostic molecular markers. The present findings clearly indicated that soil population of *R. Salami* are variable with respect of AGs and genetic levels, but they are less variable in respect of ITS region.

The high degree of genetic similarity among the Baghdad population of *R. solani* may be evolved from a common ancestor. Some degree of variation was observed among the *R. solani* isolates which may be due to mutation, migration, mating compatibility, rarely sexual hybridization may provide an opportunity for developing genetic variability in a population even if the primary mode of reproduction is asexual. Climatic changes and global increase of temperature (Stojsin *et al*., 2011).

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