MOLECULAR CHARACTERIZATION OF *Rhizoctonia solani* ISOLATED FROM PEPPER PLANTS IN IRAQ BY USING PCR .

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ABSTRACT

Rhizoctonia solani is one of the most important vegetables plants pathogens which are distributed in soil worldwide. Three isolates are selected from eight *R. solani* isolates pathogenic for Pepper plants from different area in Baghdad – Iraq, according to the pathogenicity test on Radish seeds. The sequences of rDNA- ITS region of the Iraqi pepper isolates (IQ- 34, IQ-39 & IQ- 40) were showed variation in similarity. Phylogenetic tree based on rDNA-ITS regions indicated that the IQ-34 and IQ- 40 isolates from pepper belonged to AG5 and IQ- 39 isolate belonged to AG4-HGIII. The nucleotide sequence data were sent to International GenBank to check and registered. The GenBank send accession number for each sequence at Jul.2013 as: KF372660, KF372661 and KF372662 respectively.

Key words: *Rhizoctonia solani*, Pepper, pathogenicity, molecular characteristics, DNA sequencing, phylogenetic.

INTRODACTION

Genus Rhizoctonia is a highly heterogeneous group of filamentous fungi that share similarities in their anamorphic, sterile state. They do not produce asexual spores and sexual state occurs only rarely. The group contains several economically important and global plant pathogens like *Rhizoctonia solani* Kühn [telemorph *Thanatephorus cucumeris* (Frank) Donk] (Gonzáles García *et al*., 2006).

R. solani is the most widely known and most studied species of genus Rhizoctonia. It was originally described by Julius Kühn from potato in 1858. *R. solani* is soilborne Basidiomycete occurring world-wide, with complex biology. Its highly destructive lifestyle as a non-obligate parasite causes necrosis and damping-off on numerous host plant species. Because of the lack of conidia and the scarcity of the sexual spores, *R. solani* exists as vegetative hyphae and sclerotia in nature. The fungus is dispersed mainly via sclerotia, contaminated plant material or soil spread by wind, water or during agricultural practices such

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as tillage and seed transportation. The fungus can stay in the soil as a saprophyte for long periods (Ogoshi, 1087; Ogoshi *et al*., 1990).

Disease symptoms include leaf blights, leaf spots, damping-off, rots on roots, shoots and fruits, canker lesions on sprouts and stolon, and sclerotial diseases. However, some *R. solani* strains form symbiotic mycorrhizal relationships with orchid plants (Carling *et al*., 1999; Chang and Chou, 2007).

The host range of *R. solani* is wide and it causes various diseases on important crop plants of the world , including plant species in the *Solanaceae*, *Fabaceae*, *Asteraceae*, *Poaceae* and *Braccicaceae* as well as ornamental plants and forest trees (Ogoshi, 1996).

Hyphal fusion has been proved to be a reliable method for grouping *R. solani* strains into anastomosis groups (AGs) (Ogoshi, 1987). The concept has given rise to currently 14 AGs (Carling *et al*., 2002).

AGs 1, 2, 3 and 4 are the biggest pathogen groups, characterized individually in three different geographical locations, in Europe (Carling, 1996), in the North America (Parmeter et al., 1969), and in Asia (Watanabe and Matsuda, 1966).

The most informative DNA-based molecular technique for investigating diversity in Rhizoctonia isolates has been sequence analysis of ribosomal ribonucleic acid (rRNA) genes (28S) and the internal transcribed spacer (ITS) region (Gonzalez *et al*., 2001). Analyses of these genes have not only shown the genetic relatedness of Rhizoctonia isolates, but have also confirmed some of the anastomosis groupings.

In this study, detailed description of morphology characteristics, pathogenicity, and molecular characteristics of *R. solani* isolates from pepper plants root with a typical crown, which originate from different Baghdad regions- Iraq.

MATERIALS AND METHODS

Isolates

Isolates used in this study were collected during vegetative period in 2011 from five regions (Abu Ghraib, Yusifiyah, Al Rashidiya, Tarmiya and Doura) in Baghdad, Iraq. Diseased pepper plants (*Capsicum annuum* L.) showing symptoms of infection by *R.solani* was surface sterilized in 0.5% hypochlorite for 1 min and then plated on water agar (WA) with 250µg/ml chloramphenicol. After incubation at 25 ± 1 °C for 2-3 days, culture resembling *R.solani* were transferred to fresh plates to ensure purity. Isolations were transferred to slant PDA and kept on 4°C until further investigation.

Morphological characteristics

Tested isolates were examined for macroscopic characteristics typical of *R. solani*, such as development and change of mycelial color, sclerotia formation, mycelial appearance, branching of hyphae and existence of

multinucleate cells were also determined (Parmeter and Whitney, 1970; Herr, 1979).

Pathogenicity tests

Pathogenicity of tested *R. solani* isolates was evaluated by colonized agar disks (7-10 mm) taken from the margins of 3 day-old cultures growing on PDA were transferred to the center of water agar (WA) plates and incubated for 3 days.

Six pre-germinated 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 for radish at 25± 1 °C.

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

Molecular characteristics

Each isolate from *R.solani* was grown in potato dextrose broth for 4-7days at 25± 1 °C. Mycelial mats were harvested by filtration, dry in room chamber and ground to fine powder in liquid N and then stored in – 20 °C prior to DNA isolation.

Genomic DNA was extracted from 100 mg ground fungal tissue using the fungal DNA Kit (EZ-10 spin column fungal genomic DNA, Bioneer corporation , Korea) and following the protocol recommended by the manufacturer. Internal transcribed spacer region of ribosomal DNA was amplified using ITS1 F (TCC GTA GGT GAA CCT GCG G) and ITS4 R (TCC TCC GCT TAT TGA TAT GC) set of primers that were described by Hsiang and Dean (2001). The 20 µL reaction mixture for PCR amplification consisting of 5 µL of PCR PreMix (Bioneer Corporation, Korea), 5 µL of DNA template, 3 µL of ITS1, 3 µL of ITS4 and 4µL of PCR distilled water. The amplification was performed in PCR thermal cycler (My Genie 32 Thermal Block, Bioneer, Korea). The cycle parameters were: An initial denaturation (95°C, 2 min), 35 cycles of denaturation (94°C, 30s), annealing (55°C, 1 min) and extension (72°C, 1 min). Final extension was at 72°C for 10 min (Hsiang and Dean, 2001).

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 sending to sequenced in DNA Sequencing Facility at Bioneer Corporation, Korea. Analysis of ITS sequences

was performed using on-line software CLUSTALW. Sequence data base of National Centre for Biotechnology Information-NCBI) – GenBank, which was entered via web page www.ncbi.nlm.nih.gov, was used for information on *R. solani* isolates.

Phylogenetic analysis, all sequences obtained in this study and some available at GenBank were aligned with multiple aliment program ClustalW.
A tree showing the phylogenetic relatedness between isolates constructed from Maximum Composite Likelihood by the neighbor – joining method, using the computer software package MEGA5.2. the tree was rooted with an isolate of AG 6HGI (accession number : DQ301740) as out-group.

RESULTS AND DISCUSSION

Morphological characteristics

A total of 8 *Rhizoctonia spp.* isolates were obtained from 28 samples, from Pepper plants roots with typical symptoms of Rhizoctonia root rot was isolated (Table 1).

All isolates (IQ-18 , IQ-24 , IQ- 25 , IQ- 26 , IQ- 28 , IQ-34 , IQ- 39 & IQ-40) showed typical features of *R. solani* complex including brown pigmentation of hyphae, branching near distal septum, constriction of hyphae and formation of septum short distance from the place of branching, the presence of dolipore septa and multinuclear cells in young vegetative hyphae (Parmeter and Whitney, 1970).

Table 1. *Pathogenicity testing examined on Radish seeds to determine disease severity index of R. solani isolates used in this study.*

Rhizoctonia spp. Isolate code	Regions Baghdad-Iraq	Disease severity index
IQ- 18	Yusifiyah	2.6
IQ- 24	Yusifiyah	2.5
IQ- 25	Yusifiyah	2.3
IQ- 26	Yusifiyah	2.9
IQ- 28	Yusifiyah	2
IQ- 34	Yusifiyah	5
IQ- 39	Yusifiyah	5
IQ- 40	Yusifiyah	5

Pathogenicity tests

At the end of all pathogenicity experiments, re-isolations were done from all examined plant species with typical symptoms. *R. solani* was successfully re-isolated to confirm Koch's postulates. The results of the research showed three examined isolates proved to be strong virulent on radish seeds (IQ-34, IQ- 39 & IQ- 40). This isolates were selected for depth research in this study (table 1).

Also, isolates with the lowest pathogenicity (IQ- 18, IQ- 24, IQ- 25, IQ-26 & IQ- 28) on radish seeds test were taken for further research.

Molecular characteristics of isolates

The selected isolates sequences were compared between themselves and with 30 isolates (ClustalW), randomly chosen representative of *R. solani* AGs-1 to 12 and AG BI, whose sequences were downloaded from GenBank (Table 2). Similarity of these test sequences showed that homology ranged from 57 to 90%. ITS sequences of isolates IQ- 34, IQ- 39 & IQ-40 had the highest sequence similarity with Indiana isolate JF701784 that belong to AG 5 (81%), Australian isolate AF153795 that belong to AG8ZGI-1 (66%) and Indiana isolate JF701784 that belong to AG 5 (90%) respectively.

All sequencing results for *R.solani* isolates (IQ- 34, IQ- 39 & IQ- 40) were sent to International GenBank to check and registered. The GenBank send accession number for each sequence at Jul.2013 as: KF372660, KF372661 & KF372662 respectively (available now on www.ncbi.nlm.nih.gov).

This is the first detailed report representing the characteristic of *R. solani* on Pepper plants in Baghdad - Iraq with regard to its morphological, pathogenicity and phylogenetic analysis. Diseases caused by *R. solani* is worldwide of host plant species include Pepper plants (Sneh *et al*., 1991).

Integrated research of morphological, pathogenic and molecular characteristics serves for the determination of groups and subgroups in *R. solani.* The importance of correct determination of anastomosis groups within *R. solani* complex is very important because of different virulence levels present at different anastomosis groups (Carling *et al*., 2002).

Table 2. *R.solani sequences recovered from the GenBank (National Center for Biotechnology Information – NCBI) and sequence comparison with Iraqi Pepper isolates IQ-34, IQ-39 & IQ-40 using Clustal W.*

AG & Subgroup	Host and geographic origin	GenBank Accession number	Sequence similarity (%) (Pepper)		
			IQ-34	IQ-39	IQ-40
AG 1-IA	*Oryza sativa*, Japan	AB000017	70	62	71
AG 1-IB	*Beta vulgaris*, Japan	AB000038	62	63	72
AG 1-IC	*Beta vulgaris*, Japan	AB122142	72	62	80
AG 2-1	*Solanum tuberosum*, USA	AB000026	65	60	77
AG 2-2IIIB	*Beta vulgaris*, USA	AB054857	62	61	70
AG 2-2 IV	*Beta vulgaris*, USA	AB054859	64	61	70
AG 2-3	*Glycine max*, Japan	AB054870	62	64	75
AG 3	*Beta vulgaris*, USA	AB019006	64	61	73
AG 3PT	*Solanum tuberosum*, USA	AB019013	63	61	73
AG 3TB	*Nicotiana tabacum*, USA	AB000001	67	64	81
AG 4HGI	*Beta vulgaris*, Japan	AB000028	64	61	71
AG 4HGII	*Beta vulgaris*, Japan	AB000033	63	60	70
AG4HGIII	*Beta vulgaris*, USA	AF354075	65	60	77
AG 5	*Pea spp.*, India	JF701784	81	63	90
AG 6	*Pterostylis acuminata*, Australia	AF153784	71	65	82
AG 6GV	Soil, Japan	AF354101	74	64	80
AG 7	Soil, Japan	AB000003	70	61	78
AG 8	*Triticum aestivum*, Australia	AB000011	71	63	78
AG8ZGI-1	Soil, Australia	AF153795	70	66	82
AG8ZGI-2	Soil, Australia	AF153797	72	64	80
AG8ZGI-3	*Hordeum vulgare*, Australia	AF354068	67	63	79
AG8ZGI-4	*Hordeum vulgare*, Scotland	AF354066	69	60	76
AG 9	*Solanum tuberosum*, USA	AF354109	64	60	74
AG 9TX	*Solanum tuberosum*, USA	AB000037	64	60	78
AG 9TP	*Solanum tuberosum*, USA	AB000046	64	61	78
AG 10	*Hordeum vulgare*, Australia	AF354071	62	57	73
AG 11	*Glycine max*, USA	AF354114	69	63	76
AG 12	*Pterostylis acuminata*, Australia	AF153803	72	60	79
AG BI	Soil, Japan	AB000044	61	60	69

The *R. solani* diversity between Iraqi Pepper isolates and global isolates maybe back to this might occur due to climatic changes and global increase of temperature.

The Neighbor-joining phylogeny test based on sequences differences in the ITS – rDNA region (Fig.1) illustrates estimates of phylogenetic relationships among all AGs of *R.solani* , including Iraqi isolates (IQ- 34, IQ- 39 & IQ- 40).

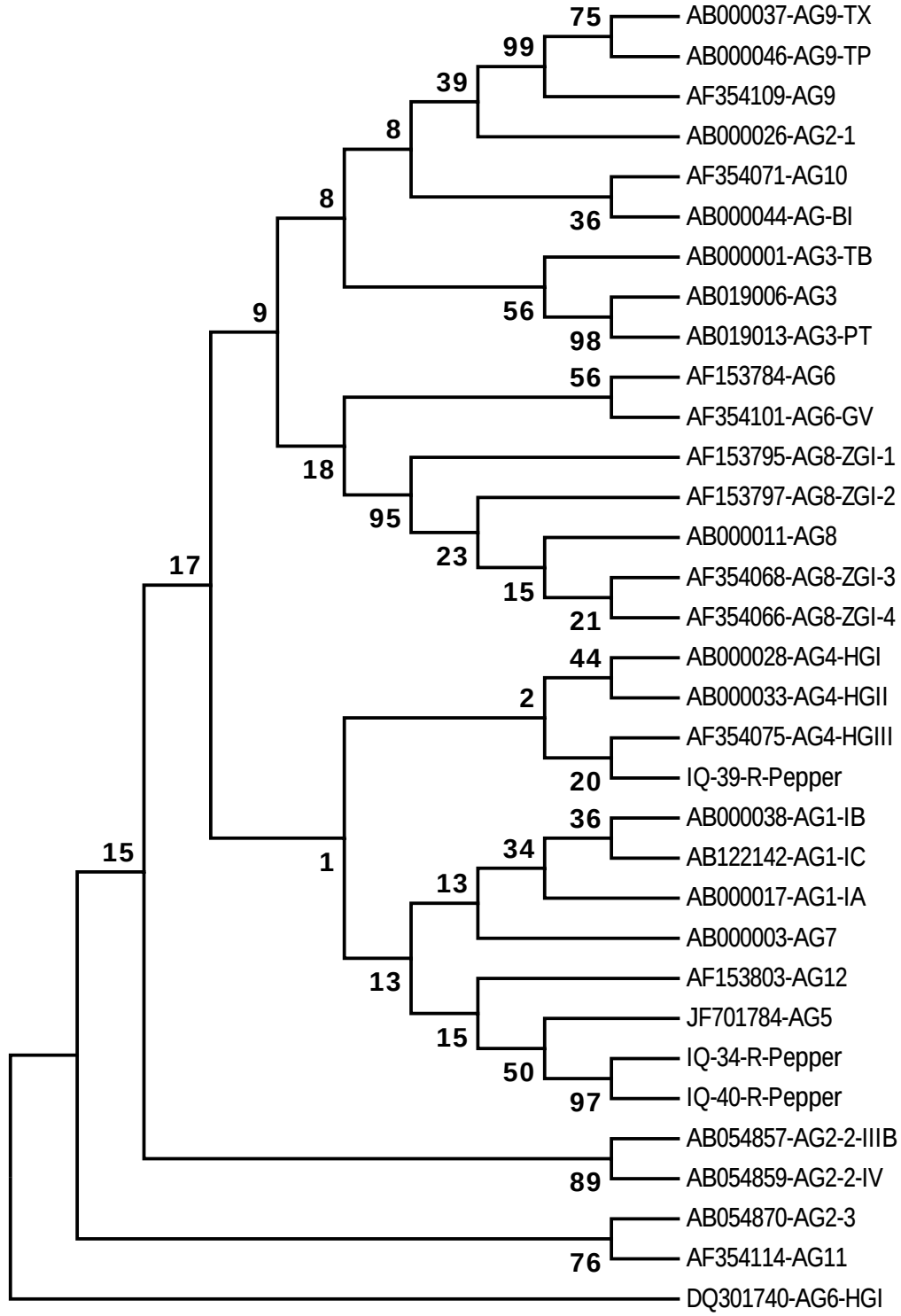

Fig 1. *Neighbor - joining tree illustrating relationships estimates of phylogenetic relationships of test isolates (IQ – 34, IQ -39 & IQ – 40) and all other AGs of R. solani. The number below each branch indicates the percentage of congruent clusters in 1000 bootstrap trials when values were greater than 50%.*

During the phylogenetic analysis in this study, the IQ- 39 isolate was found in a clustered with AG4- HGIII and IQ- 34, IQ- 40 isolates were clustered with AG5.

Sequencing and phylogenetic analysis of the ITS region has been confirmed to reliably divide isolates of *R. solani* into distinct groups and subgroups which correspond to the different anastomosis groups (carling *et al*. 2002).

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الخصائص الجزيئية لل *R. solani* المعزول من نباتات الفلفل في العراق باستخدام PCR.

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المستخلص

الفطر *R. solani* من الفطريات الممرضة المهمة على نباتات الخضر والذي ينتشر بشكل واسع في التربة حول العالم . تم اختيار ثلاث عزلات ممرضة من الفطر من اصل ثمانية عزلات جمعت من نباتات الفلفل من مناطق مختلفة في بغداد – العراق . وبعد اختبار امراضيتها على بذور الفجل. لعزلات الفلفل العراقية rDNA- ITS اظهر تسلسل القواعد النايتروجينية لمنطقة (IQ- 34, IQ-39 ، IQ- 40) بانها غيرمتشابة .

واشار اختبار شجرة الاصل التطوري اعتمادا على منطقة rDNA- ITS الى ان العزلات IQ- 40 و IQ-34 المعزولة من الفلفل تنتمي الى AG5 والعزلة IQ- 39 تنتمي الى AG4-HGIII . ارسلت بيانات تسلسل النيوكلوتيد الى GenBank الدولي للتدقيق والتسجيل . والذي ارسل ارقام تسجيل تسلسلات العزلات في تموز 2013 :KF372662، KF372661 و KF372660على التوالي .

الكلمات المفتاحية: *R. solani* ، الفلفل ، الامراضية ، الخصائص الجزيئية، تسلسل الدنا ، الأصل التطوري .