Biodiversity of *Rhizoctonia solani* AG3 and AG2-1 associated with potato diseases

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Abstract: Epidemiology of pathogenic variants of *Rhizoctonia solani* associated with potato crops in Egypt were analyzed. Twelve out of twenty isolates had comparable growth rates matching with that of the AG3 PT tester RS-114. Eleven isolates were multinucleate and anastomosed with each other and with RS114, confirming their membership of AG3. Rh21 was not AG3 but related to this group. The remaining 8 isolates did not anastomose with RS-114. Sequences of rDNA ITS of all isolates showed high similarities 98.8 and 98.7% with RS-114 and *R. solani* AG3 (JX050235), respectively; while Rh21 appeared to be distant 87.6% compared with RS-114 and 92.4% with AG2-1 *R. solani* (JQ676846). These results confirmed that all isolates except Rh21 belonged to AG3. A high frequency of SNPs was observed in 18S RNA and ITS1 rDNA. Genomic DNA of *R. solani* was also used for RAPD with 14 primers. The maximum number of bands was generated by primers OPX-13 and OPZ-11 (37 bands each) followed by OPV-17, OPS-19 and OPK-19. The 287 bands obtained with 14 primers were polymorphic showing high range of diversity with 100% polymorphism.
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1- Introduction

The soil-born plant pathogen *Rhizoctonia solani* Kuhn (*teleomorph: Thanatephorus cucumeris*) is a basidiomycetous fungus which produces sclerotia (Velvis and Jager, 1983) but does not generate any asexual form of propagation. The species is one of the most serious fungal plant diseases with a great diversity of host plants among which are several important crops (Woodhall *et al.,* 2007; Rashid *et al.,* 2013; Strausbaugh *et al.,* 2013). The fungus has a global distribution and causes a serious disease of potato cultivations in many regions (Lehtonen *et al.,* 2008a, b; Ritchie *et al.,* 2009).

Using hyphal fusion tests as criterion of identity, *R. solani* was divided into 14 fusion groups (AG-1 to AG-13 and AG-BI) (Carling *et al.,* 2002a; Lehtonen *et al.,* 2008b), with isolates found to infect potato plants predominantly belonging to sub-group AG-3 (Bandy *et al.,* 1988; Carling and Leiner 1990a). There are different 14 strains of *R. solani* according to genetically defined populations of anastomosis groups (AG) determined by anastomosis between hyphae of strain belong to the same AG (Carling *et al.,* 2002a).

Damage of potato plants by *R. solani* occurs primarily beneath the soil surface, where developing subterranean stems and stolons are particularly susceptible to attack (Banville et al. *et al.,* 1996). The presence of *R. solani* survival structures on the skin of progeny tubers results in misshapen tubers and seriously affects crop marketability (Carling and Leiner, 1986). Other sources of *R. solani* infection include seed tubers covered with sclerotia and mycelium, and the soil itself, which may contain different types, as well as variable levels of inoculum (Balali *et al.,* 1995).

The aim of this study is to determine the affiliation to mycelial compatibility groups (MCGs) of a set of isolates from Egyptian potato field soils, and to determine rates of genetic variation within and between MCGs. Sequencing of the partial ribosomal operon and RAPD analysis were used to describe the molecular diversity of *R. solani* isolates under study.

2- Materials and Methods

Collection of samples

Rhizoctonia solani was isolated from potato plants and potato field soil collected from potato fields, which distributed over Egypt governorates in 2012. Four other out-group control samples were collected from bean, eggplant, sugarbeet and cotton plants that show typical symptoms of *R. solani* infection (Table 1).

Isolation of *R. solani* from potato tubers and soil

R. solani was isolated from tuber-borne scleortia using the methods described by Balali *et al.* (1995). A minimum of four sclerotia were then removed from each tuber, placed on alkaline water agar medium and incubated at 25°C in the dark. Fungal colonies growing from the various samples were observed under a dissecting microscope every 24 h and hyphal tips from *R. solani* like colonies transferred to potato dextrose agar PDA (Difco) supplemented with

streptomycin 50 mgL^{-1} and incubated at 25°C in the dark.

Soil samples (1 kg) collected from potato fields were mixed thoroughly and sifted through 1 mm sieve. Two 100 g sub samples were used for fungal isolation as follows. Soil moisture was adjusted to 15% that resulted in -0.7 bar matric potential. Soil samples were placed on the surface of the water agar plate. Plates were incubated at 28°C and were examined after 18 h for the presence of typical *R. solani* mycelium using a dissecting microscope at X20 to X40 magnifications. Hyphal tips from *Rhizoctonia*-like colonies were transferred to potato dextrose agar (Difco) containing 1 ml/L lactic acid and incubated as described above.

Seventeen isolates were obtained from tubers-bearing sclerotia and four isolates from soil samples. Three other out-group control isolates were isolated from bean, eggplant and sugarbeet plants that show typical symptoms of *R. solani* infection (Table 1). *R. solani* AG-3PT reference strain (RS-114) was kindely provided by Prof. Dr. Marc Cubeta (North Carolina State University, USA). Pure cultures of the isolates were kept on PDA at 4°C as a stock culture and were renewed monthly.

Identification of *R. solani*

R. solani isolates identification and diversity were studied by examining the hyphal branching, growth rate determination, hyphal anstomosis test, somatic compatibility grouping, rDNA-ITS sequence analyses and randomly amplified polymorphic DNA (RAPD) analyses.

Radial growth rate determination

To determine the rate of radial growth of the *R. solani* isolates, a 5-mm-diameter mycelial disk from the edge of an actively growing colony was placed in the center of a 9-cm-diameter Petri plate containing PDA. Three replicate plates were used for each isolate. Petri plates were arranged in a randomized block design in the incubator. The colonies were measured after 24, 48, 72, and 96 h of incubation in the dark at 25°C.

Anastomosis group identification

R. solani isolates were tested for their ability to anastomose with a known AG-3PT tester isolate RS-114, using the clean slide technique of Kronland and Stanghellini (1988). A 5-mm-diameter disk from the edge of 3 day old colony of the unknown isolate was transferred to a glass slide cleaned by dipping in 95% ethanol, wiped dry then coated with thin layer of 2% water agar. A mycelia disc from a similarly grown AG-3PT tester culture was placed on the slide at 2 cm apart from the first disk. Two other slides were prepared at which the distances between the disks was 2.5 cm and 3 cm. Anastomosis was determined microscopically after the tester–unknown pairing were incubated for 48-72 h at room temperature (25±2°C). Hyphal interaction were assigned to one of the four categories in accordance with those described by Carling *et al.* (1988) and MacNish *et al.* (1997), i.e. C0= no recognition observed between hyphae, C1= hyphae contact, connection of wall but no membrane to membrane contact, C2= hyphae fusion resulting in the death of fused and adjacent cells, and C3= fusion of walls and membrane and no evidence of cell death (self-anastomosis). Each pair was repeated at least twice.

Determination of somatic compatibility grouping

To determine somatic compatibility, AG-3 PT was paired with the isolates on potato dextrose agar amended with 1% charcoal according to the procedure of MacNish *et al.* (1997). The isolates were grown for 4 days at 25°C and somatic interactions between isolates were determined macroscopically. Pairings were scored as somatically compatible when no reaction line was observed between paired isolates. Pairings were scored as somatically incompatible when a reaction line of raised hyphae was observed between paired isolates (MacNish *et al.*, 1997). Somatic compatibility experiments were conducted at least twice.

ITS region sequence analyses

DNA extraction and PCR: Genomic DNA was isolated from the hyphae of the 23 *R. solani* isolates grown for 3 days on PDA medium using GeneJET™ Genomic DNA Purification kit (Fermentas, Ontorio, Canada) according to the manufacture manual. Internal transcribed spacer (ITS region of the rRNA gene) was amplified using the universal ITS-1 TCCGTAGGTGAACCTGCGCAG and ITS-4 TCCTCCGCTTATTGATATGC primers that anneal to the flanking 18S and 28S rRNA gene (White et al. 1990). PCR amplification reaction mixture (50 µl) contained 25 µl Maxima® Hot Start PCR Master Mix (2X), 5 pmol of each primer, 5 µl of template DNA and 18 µl of nuclease free water. Amplification was performed in a thermal cycler DNA Engine Opticon 2 (Biorad, Hercules, CA) using the following program: initial denaturation 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. An aliquot (5 µl) of the reaction mixture was analysed on agarose gel. The PCR product was cleaned using the GeneJET™ PCR Purification Kit (Fermentas) according the manufactures manual. Direct sequencing of the purified PCR products were carried out in both directions using the universal ITS-1 and ITS-4 primers at the GATC Company, Germany using the ABI 3730xl sequencer.

Structural prediction of ITS2

The structural predictions are sensitive to possible errors in sequence annotation, e.g. possible

inclusion of nucleotides from the rRNA genes in the ITS sequences analyzed. Preliminary prediction of the secondary structure for ITS2 RNA using the developed homology-based algorithm, as implemented in the web server of the ITS2 database (Wolf *et al.*, 2005; Selig *et al.*, 2008) (http://its2.bioapps.biozentrum.uni-wuerzburg.de), suggested that, indeed, the ITS2 sequences of AG3 deposited to database might contain bases of the 28S rRNA gene. The ITS1, 5.8S rRNA and ITS2 sequences for AG3 were reported to contain 218, 155 and 270 bases, respectively, but the original information used to annotate difficult to trace. Annotation of ITS2 sequences was done according to the 5.8S-28S rDNA interaction (Côté and Peculis 2001; Keller *et al.*, 2009).

Randomly amplified polymorphic DNA (RAPD) Analysis

Fourteen primers developed by USA Operon Company were used in this study (Table 2). The total genomic DNA was extracted from the mycelia grown on potato dextrose agar (PDA). PCRs were performed in a final mixture of 20 µL containing 50 ng template DNA, 2 µL 10X reaction buffer (with 1.5 mmol/L Mg^{2+}), 0.2 mmol/L dNTP mixture, 0.2 µmol/L of primer, and 1 Unit of Taq DNA polymerase with the thermal profile: 45 cycles of 49ºC for 1 min, 36ºC for 1 min, and 72ºC for 2 min. PCR products were separated on 1.5% agarose gels.

3- Results

Twenty *Rhizoctonia solani* isolates were isolated from samples collected from diverse sources from geographically dispersed localities in Egypt. Isolates nos. Rh04, Rh05, Rh06, Rh07, Rh08, Rh09, Rh11, Rh12, Rh16, Rh17, Rh19 and Rh21 showed more or less similar mycelial growth rates ranging from 0.51-0.59 mmh^{-1} (Table 3), similar to that of the AG3 PT tester strain RS-114.

Rhizoctonia solani isolates Rh04-Rh09, Rh11, Rh12, Rh16, Rh17, Rh19 and Rh21 were multinucleate and anastomosed with each other and with the tester PT (RS114). Where the isolates Rh04-Rh09, Rh11, Rh12, Rh16, Rh17 and Rh19 showed hyphal wall and membrane fusion, no death in fused and adjacent cells was observed demonstrating perfect fusion with the PT tester strain (RS114). This confirmed that these isolates are belonging to Anastomosis group 3 (AG3). In contrast, isolate Rh21 had hyphal wall and membrane fusion, death in fused and adjacent cells with PT tester (RS114), which confirmed that this isolate is not AG3 but is related to this group (Table 4).

The data in Table 5 confirmed the data obtained from the anastomosis experiment, where the sequence obtained from the amplification of ITS region showed very high similarity of all *R. solani* isolates in between and with the sequence of PT tester (RS114) reached to 98.8% of similarity, while the isolate Rh21 showed less similarity with that obtained from other isolates with PT tester (RS114) and the other isolates reached to 87.6% of similarity. These results confirmed that all isolates belonging to AG3 except the isolate Rh21.

Similarities in ITS region between the isolated *R. solani* strains including the PT tester (RS114) were very high, with values up to 98.7% identity with an *R. solani* representative of AG3 deposited in GenBank under accession number JX050235. Isolate Rh21 showed 92.4% similarity with *R. solani* AG2-1 isolate GenBank JQ676846 (Table 6). These results are concordant with anastomosis experiments in that all *R. solani* isolates belong to AG3 except isolate Rh21 which is AG2-1.

Primers used amplified, Internal Transcribed Spacer (ITS) region including partial 18S and 28S in all isolates of AG3-PT anastomosis group, except Rh08 and Rh19 where 18S was not amplified. Comparing the sequences to those published in GeneBank (*R. solani* AG3 JX050235), a high degree of single nucleotide polymorphisms was noted in ITS1 with many deletions especially in the 18S gene. Where there was single nucleotide substitution (G/T) in different positions in isolates Rh11, Rh07, Rh11 and RS114 and Rh07. No insertions occurred in this region in all isolates. There were deletions in base (G) in different positions in isolates RS114, Rh04, Rh05, Rh06, Rh07, Rh08, Rh09, Rh12, Rh16 and R19. The 18S ribosomal RNA region in isolate Rh17 was identical to that in database (Table 7a).

The results in Table 7b revealed that in Internal Transcribed Spacer 1 (ITS1) region there were many substitutions of (C/G) in all isolates. (C/T) substitutions were observed at different positions in RS-114, RH04, Rh06, RH08, Rh09, Rh11, Rh12 and Rh19. (T/G) were observed at different positions in all isolates. (A/G) at position 13 in RS-114, Rh04, Rh06, Rh08, Rh09 and Rh11. (T/A) at position 14 in RS-114, Rh04, Rh06, Rh08 and Rh09. (C/A) at position 4 in Rh04 only. (T/C) were observed at different positions in Rh05, Rh06, Rh07, Rh08, Rh09, Rh11, Rh12, Rh16 and Rh19. (A/C) at position 13 in Rh05 only. (G/C) at position 12 in Rh11. Between bases number 16 and 17, many insertions were occurred, where (GT) in Rh05, Rh06, Rh09 and Rh12; (T) in Rh07, Rh08 and Rh16; and (G) in Rh19 only. Between bases number 44 and 45, there were insertion of (A) in RS114, Rh07 and Rh11. There were deletions of (T) at positions 9 and 10 in RS114, Rh04 and Rh17, where at position 6 in Rh12 only. Deletion of (C) was observed at position 8 in Rh17 only. These results when compared with *R. solani* JX050235 (AG3).

Table 1. The collected samples of *R. solani* isolates and their location

Code	Host plant	Origin	Location (Governorate)
RS114	**RS-114 (**AG3-PT tester)		
2	Potato	Soil	Gharbia
4	Potato	Tuber	Minoufiya 1 (Mnoof)
5	Potato	Tuber	Minoufiya 2 (Tamaly)
6	Potato	Tuber	Minoufiya 3 (Ashmoon)
7	Potato	Tuber	Beheira 1 (Al-Bostan)
8	Potato	Tuber	Beheira 2 (Esra and Meraj)
9	Potato	Tuber	Beheira 3 (AlTawheed)
11	Potato	Soil	Fayoum 1
12	Potato	Tuber	Fayoum 2
14	Potato	Tuber	Bani-Sewif (Sods Center)
15	Potato	Tuber	Bani-Sewif (Naser Center)
16	Potato	Tuber	Aswan
17	Potato	Tuber	Alexandria (Nubarya)
18	Potato	Tuber	Cairo (Maadi 1)
19	Potato	Tuber	Cairo (Maadi)
20	Potato	Soil	Kafr El-Sheikh
21	Potato	Tuber	Giza
22	Beans	-	-ve control
23	Egg plant	-	-ve control
24	Sugar beet	-	-ve control

Table 2. Sequence of RAPD-PCR primers used in this study

Primer	5' to 3'	Primer	5' to 3'
OPX-01	CTGGGCACGA	OPK-14	CCCGCTACAC
OPX-13	ACGGGAGCAA	OPK-19	CACAGGCGGA
OPW-05	GGCGGATAAG	OPK-20	GTGTCGCGAG
OPW-11	CTGATGCGTG	OPV-17	ACCGGCTTGT
OPW-13	CACAGCGACA	OPV-20	CAGCATGGTC
OPS-19	GAGTCAGCAG	OPM-03	GGGGGATGAG
OPZ-11	CTCAGTCGCA	OPD - 12	CACCGTATCC

Table 3. Radial mycelial growth rate of the isolated *Rhizoctonia solani* from different localities in Egypt

Code	Mycelia growth						Growth rate (mm/h)
	24 h	48 h	72 h	96 h	120 h	144 h	
RS-114	20.6	37.3	50.0	68.0	82.0	85.0	0.59
Rh2	26.3	33.0	36.5	38.0	41.0	46.3	0.32
Rh4	12.0	22.6	38.0	40.6	50.0	75.0	0.52
Rh5	9.0	16.0	21.3	38.6	64.0	85.0	0.59
Rh6	9.0	19.8	32.5	45.3	60.2	74.3	0.52
Rh7	9.6	21.3	33.0	46.3	63.6	75.6	0.51
Rh8	9.6	26.0	39.0	47.0	58.6	74.0	0.51
Rh9	8.6	16.0	21.0	38.3	58.0	74.0	0.51
Rh11	10.0	18.6	24.0	38.0	43.3	76.0	0.53
Rh12	9.3	24.6	32.6	43.3	55.6	75.0	0.52
Rh14	29.0	67.3	85.0	85.0	85.0	85.0	1.18
Rh15	30.3	85.0	85.0	85.0	85.0	85.0	1.77
Rh16	11.0	30.3	30.3	37.6	68.3	85.0	0.59
Rh17	13.3	22.6	29.6	46.3	59.0	75.0	0.52
Rh18	14.6	65.3	85.0	85.0	85.0	85.0	1.18
Rh19	11.3	25.0	41.6	55.6	78.0	85.0	0.59
Rh20	30.0	65.3	85.0	85.0	85.0	85.0	1.18
Rh21	8.6	15.6	30.6	44.3	65.3	80.5	0.56
Rh22	30.0	65.0	85.0	85.0	85.0	85.0	1.18
Rh23	19.3	47.0	85.0	85.0	85.0	85.0	1.18
Rh24	25.0	49.3	85.0	85.0	85.0	85.0	1.18

Table 4. Mycelial compatibility grouping of the isolated *Rhizoctonia solani*

Code	RS-114	Rh04	Rh05	Rh06	Rh07	Rh08	Rh09	Rh11	Rh12	Rh16	Rh17	Rh19	Rh21
RS-114	C3	C3	C3	C3	C3	C3	C3	C3	C3	C3	C3	C3	C2
Rh04		C3	C3	C3	C3	C3	C3	C3	C3	C3	C3	C3	C2
Rh05			C3	C3	C3	C3	C3	C3	C3	C3	C3	C3	C2
Rh06				C3	C3	C3	C3	C3	C3	C3	C3	C3	C2
Rh07					C3	C3	C3	C3	C3	C3	C3	C3	C2
Rh08						C3	C3	C3	C3	C3	C3	C3	C2
Rh09							C3	C3	C3	C3	C3	C3	C2
Rh11								C3	C3	C3	C3	C3	C2
Rh12									C3	C3	C3	C3	C2
Rh16										C3	C3	C3	C2
Rh17											C3	C3	C2
Rh19												C3	C2
Rh21													C3

C0, No interaction (not related)**; C1,** Hyphal wall contact**; C2,** Hyphal wall and membrane fusion, death in fused and adjacent cells (related)**; C3,** Hyphal wall and membrane fusion, no death in fused and adjacent cells (perfect fusion-closely related)**.**

Table 5. Relative similarity of sequence of ITS region of *R. solani* isolates with each other based on base sequences obtained

Code	Rh04	Rh05	Rh06	Rh07	Rh08	Rh09	Rh11	Rh12	Rh16	Rh17	Rh19	Rh21
RS-114 AB905378	97.9	96.6	98.4	96.8	97.5	97.8	96.9	97.2	97.5	97.8	97.6	86.7
Rh04 AB905379		95.6	96.9	95.4	96.6	95.9	95.2	96.6	96.5	96.5	96.9	85.6
Rh05 AB905380			96.6	98.5	96.9	97.2	98.1	97.3	97.4	96.9	97.5	84.8
Rh06 AB905381				97.5	97.6	98.2	96.2	98.1	98.8	97.8	98.1	86.6
Rh07 AB905382					96.5	97.7	97.9	96.5	97.5	96.9	97.8	84.5
Rh08 AB905383						97.5	96.6	97.5	98.9	97.5	98.1	87.6
Rh09 AB905384							96.9	97.8	98.4	97.5	97.8	85.8
Rh11 AB905385								96.0	97.2	97.1	97.3	84.9
Rh12 AB905386									97.8	96.8	97.6	86.2
Rh16 AB905387										97.9	98.7	87.4
Rh17 AB905388											97.2	86.7
Rh19 AB905389												86.3
Rh21 AB905390												

Table 6. Relative similarity of sequence of ITS region of *R. solani* isolates with *R. solani* species (AG3) sequences obtained from gene bank database

Code	RS-114	Rh04	Rh05	Rh06	Rh07	Rh08	Rh09	Rh11	Rh12	Rh16	Rh17	Rh19
JX050235	97.7	97.5	97.9	97.9	97.6	98.4	97.9	97.0	98.4	98.4	98.7	97.6
RS-114		97.9	96.6	98.4	96.8	97.5	97.8	96.9	97.2	97.5	97.8	97.6
Rh04			95.6	96.9	95.4	96.6	95.9	95.2	96.6	96.5	96.5	96.9
Rh05				96.6	98.5	96.9	97.2	98.1	97.3	97.4	96.9	97.5
Rh06					97.5	97.6	98.2	96.2	98.1	98.8	97.8	98.1
Rh07						97.5	97.7	97.9	97.5	97.5	96.9	97.8
Rh08							97.5	96.6	97.5	98.9	97.5	98.1
Rh09								96.9	97.8	98.4	97.5	97.8
Rh11									96.0	97.2	97.1	97.3
Rh12										97.8	96.8	97.6
Rh16											97.9	98.7
Rh17												97.2

Table 7a. Single nucleotide polymorphism (SNP) of *Rhizoctonia solani* isolates compared with the published sequences in gene bank database with accession number JX050235 for 18S ribosomal RNA region

Anastomosis group	Code	Substitution							Insertion			Deletion			
		G/T	A/G	T/G	A/C	G/A	T/A		140-141	193-194		G	A	T	C
AG3-PT	RS-114	3	-	-	-	-	-		-	-		1	-	-	-
	Rh04	-	-	-	-	-	-		-	-		1,2	-	-	-
	Rh05	-	-	-	-	-	-		-	-		1,2	-	-	-
	Rh06	-	-	-	-	-	-		-	-		1,2	-	-	-
	Rh07	2,3	-	-	-	-	-		-	-		1	-	-	-
	Rh08	-	-	-	-	-	-		-	-		1,2	-	-	-
	Rh09	-	-	-	-	-	-		-	-		1,2	-	-	-
	Rh11	1,2	-	-	-	-	-		-	-		-	-	-	-
	Rh12	-	-	-	-	-	-		-	-		1,2	-	-	-
	Rh16	-	-	-	-	-	-		-	-		1,2	-	-	-
	Rh17	-	-	-	-	-	-		-	-		-	-	-	-
	Rh19	-	-	-	-	-	-		-	-		1,2,3	-	-	-
AG2-1	Rh21	-	27,34, 35,40, 144	28,29, 139	30,21 7	140	146		T	CCCC		4,7,8,10, 16,18,19 ,22,23	6,11,12, 20,21,24 ,45,69,7 0,76	1,5,9, 15,25, 39,71, 75,77	2,3,13,1 4,17,26

5.8S is always highly conserved. The results of Table 7c showed that there were few substitutions in 5.8 ribosomal RNA region compared with ITS1 region, where (T/C) occurred at positions 92 and 93 in Rh04 and RS114, respectively; (T/A) at position 93 in Rh04 only; and (G/T) at position 150 in Rh09 only. There were no insertions or deletions in this region in all isolates when compared with AG3 in gene bank. There was single substitution of (T/C) at position 432 of IT2 region in Rh09 (Table 7c).

In Rh21 (Table 7a), there were many substitutions occurred in the 18S region, where (A/G) at positions 27, 34, 35, 40 and 144, (T/G) at positions 28, 29 and 139, (A/C) at positions 30 and 217, (G/A) at position 140, and finally (T/A) at position 146. There was insertion between bases numbers 140 and 141 (T) and bases numbers 193 and 194 (CCCC). Also, there were (G) deletions in positions 4, 7, 8, 10, 16, 18, 19, 22 and 23, (A) deletions at positions 6, 11, 12, 20, 21, 24, 45, 69, 70 and 76, (T) deletions at positions 1, 5, 9, 15, 25, 39, 71, 75 and 77, and (C) at positions 2, 3, 13, 14, 17 and 26 (Table 7a) when compared with that published in gene bank (JQ676846) (AG2-1). On the contrary there were no changes in bases occurred ITS1 region for Rh21 when compared with that in gene bank database (Table 7b).

Table 7b. Single nucleotide polymorphism of *Rhizoctonia solani* isolates compared with the published sequences in gene bank database for with accession number JX050235 for Internal Transcribed Spacer 1 (ITS1) region

Anastomosis group	Isolate Code	Substitution									Insertion		Deletion	
		C/G	C/T	T/G	A/G	T/A	C/A	T/C	A/C	G/C	16-17	44-45	T	C
AG3-PT	RS114	4,5	8	11,16	13	14	-	-	-	-	-	A	9,10	-
	Rh04	5	8	11,16	13	14	4	-	-	-	-	-	9, 10	-
	Rh05	4,5	-	16	-	-	-	7,9,10,11, 14	13	-	GT	-	-	-
	Rh06	5	4	7,11	13	14	-	15	-	-	GT	-	-	-
	Rh07	4	-	16	-	-	-	7,9,10,11,14	-	-	T	A	-	-
	Rh08	4,6	5	11	13	14	-	7	-	-	T	-	-	-
	Rh09	5,6	4	10,11,16	13	14	-	7,15	-	-	GT	-	-	-
	Rh11	4,8	5	15,16	13	-	-	7,9,11,14	-	12	-	A	-	-
	Rh12	5	4	11,16	-	-	-	7,10,14	-	-	GT	-	6	-
	Rh16	4,5,6	-	10,11	-	-	-	7,14	-	-	T	-	-	-
	Rh17	4,5	-	-	-	-	-	-	-	-	-	-	9,10	8
	Rh19	6	4,5	10,16	-	-	-	7,9,14	-	-	G	-	-	-
AG2-1	Rh21	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 7c. Single nucleotide polymorphism of *Rhizoctonia solani* isolates compared with the published sequences in gene bank database for 5.8S ribosomal RNA and Internal Transcribed Spacer 2 regions

Anastomosis group	Code	5.8S												ITS2
		Substitution							Insertion			Deletion		Substitution
		T/C	T/A	G/T	G/A	A/G	G/C		459-460	557-458		A		T/C
AG3-PT	RS-114	93	-	-	-	-	-		-	-		-		-
	Rh04	92	93	-	-	-	-		-	-		-		-
	Rh05	-	-	-	-	-	-		-	-		-		-
	Rh06	-	-	-	-	-	-		-	-		-		-
	Rh07	-	-	-	-	-	-		-	-		-		-
	Rh08	-	-	-	-	-	-		-	-		-		-
	Rh09	-	-	150	-	-	-		-	-		-		432
	Rh11	-	-	-	-	-	-		-	-		-		-
	Rh12	-	-	-	-	-	-		-	-		-		-
	Rh16	-	-	-	-	-	-		-	-		-		-
	Rh17	-	-	-	-	-	-		-	-		-		-
	Rh19	-	-	-	-	-	-		-	-		-		-
AG2-1	Rh21	-	-	-	435,708	707	703		T	A		699		-

5.8S ITS2 28S

JX050235 TTGGAGCATGCCTGTTTGAGTATCA ... TGATCTCAAATCAGGTAGGACTACC
RS-114 AG3PT TTGGAGCATGCCTGTTTGAGTATCA ... TGATCTCAAATCAGGTAGGACTACC
Rh04 TTGGAGCATGCCTGTTTGAGTATCA ... TGATCTCAAATCAGGTAGGACTACC
Rh05 TTGGAGCATGCCTGTTTGAGTATCA ... TGATCTCAAATCAGGTAGGACTACC
Rh06 TTGGAGCATGCCTGTTTGAGTATCA ... TGATCTCAAATCAGGTAGGACTACC
Rh07 TTGGAGCATGCCTGTTTGAGTATCA ... TGATCTCAAATCAGGTAGGACTACC
Rh08 TTGGAGCATGCCTGTTTGAGTATCA ... TGATCTCAAATCAGGTAGGACTACC
Rh09 TTGGAGCATGCCTGTTTGAGTATCA ... TGATCTCAAATCAGGTAGGACTACC
Rh11 TTGGAGCATGCCTGTTTGAGTATCA ... TGATCTCAAATCAGGTAGGACTACC
Rh12 TTGGAGCATGCCTGTTTGAGTATCA ... TGATCTCAAATCAGGTAGGACTACC
Rh16 TTGGAGCATGCCTGTTTGAGTATCA ... TGATCTCAAATCAGGTAGGACTACC
Rh17 TTGGAGCATGCCTGTTTGAGTATCA ... TGATCTCAAATCAGGTAGGACTACC
Rh19 TTGGAGCATGCCTGTTTGAGTATCA ... TGATCTCAAATCAGGTAGGACTACC

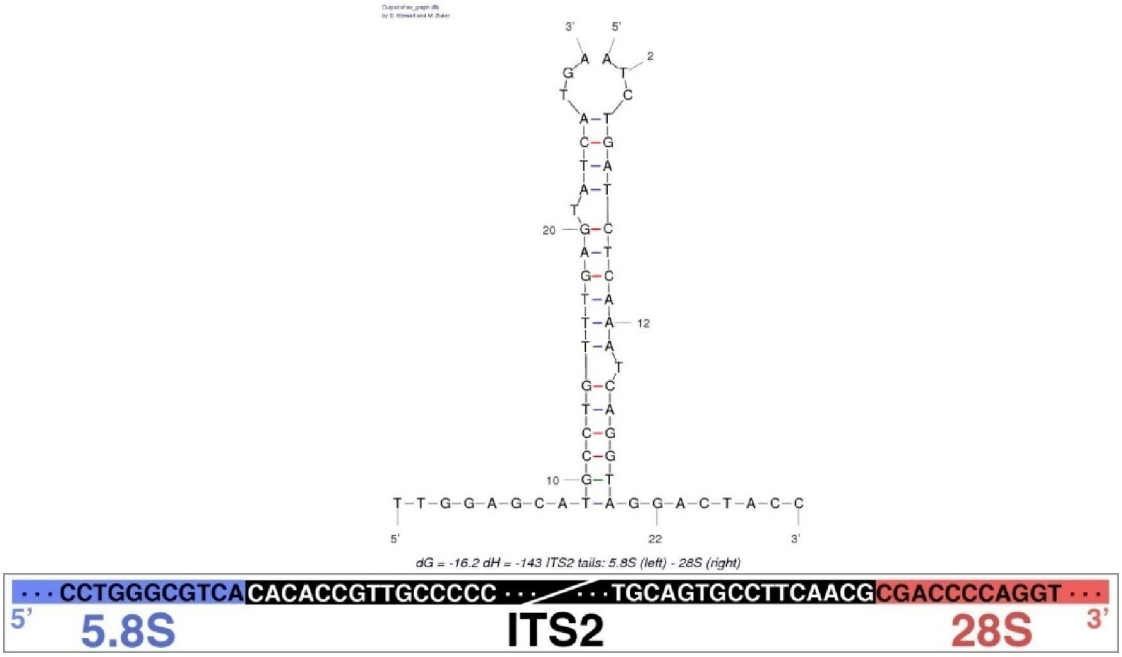

Figure 1. Annotation of the ITS2 sequence of *R. solani* (AG3). ITS2 sequences were annotated according to the flanking regions (5.8 and 28S rDNA), which interact during ribogenesis and location of ITS (shown below).

Table 8. Sequences of RAPD primers and product obtained from the amplification

Primer	Sequence (5′-3′)	Total of bands	Polymorphism (%)	Size range of amplicons
OPD-12	CACCGTATCC	10	100	0.4-1.0 kb
OPK-14	CCCGCTACAC	19	100	0.5-4.0 kb
OPK-19	CACAGGCGGA	25	100	0.3-2.0 kb
OPK-20	GTGTCGCGAG	15	100	0.3-1.0 kb
OPS-19	GAGTCAGCAG	26	100	0.3-1.5 kb
OPV-17	ACCGGCTTGT	29	100	0.3-3.5 kb
OPV-20	CAGCATGGTC	13	100	0.4-1.5 kb
OPW-05	GGCGGATAAG	10	100	0.2-0.8 kb
OPW-11	CTGATGCGTG	20	100	0.7-1.5 kb
OPW-13	CACAGCGACA	16	100	0.8-2.0 kb
OPX-01	CTGGGCACGA	22	100	0.3-1.5 kb
OPX-13	ACGGGAGCAA	37	100	0.3-1.0 kb
OPZ-11	CTCAGTCGCA	37	100	0.5-2.5 kb
OPM-03	GGGGGATGAG	8	100	0.3-0.8 kb
Total		287	100	

In isolate Rh21, there were many substitutions of (G/A) at positions 435 and 708, (A/G) at position 707, and (G/C) at position 703. Insertion of (T) was occurred at position between bases number 459 and 460, and insertion of (A) at position between bases number 557 and 458 when compared with that in database (AG2-1). Also, there was single deletion of (A) at position 699 (Table 7c).

The predicted secondary structure was used as criteria to refine the ITS2/28S junction in *R. solani* AG3, as shown in Figure 1. Consequently, the ITS2 sequences of AG3 strains determined in this study contained 235 nucleotides.

RAPD-PCR was performed on 12 *R. solani* together with control isolate (Rs114) using 14 universal RAPD primers. All 14 primers amplified genomic DNA of *R. solani,* with a maximum number of bands generated by primers OPX-13 and OPZ-11 (37 bands each) followed by OPV-17, OPS-19 and OPK-19 with 29, 26, and 25 bands, respectively. The lowest number of bands was obtained by primer OPM-03 (8 bands) (Table 8). Sizes of bands were between 0.2-4.0 kb. All 287 bands generated with 14 primers were polymorphic showing high range of variability with 100% polymorphism (Table 8).

4- Discussion

Rhizoctonia solani Kühn (teleomorph *Thanatephorus cucumeris* (Fr.) Donk) is an economically important soil borne plant pathogen infecting a wide range of agricultural and horticultural crops, causing several diseases worldwide (Ogoshi, 1987).

Twenty *R. solani* isolates were isolated and purified from the samples collected from different sources and different localities in Egypt. The majority of strains belonged to anastomosis group AG3 and had similar growth rates. In soil, AG 2-1 isolates grew significantly slower than AG-3 (Carling and Leiner, 1990a). Biological variability among the isolates of AG-3 is also expressed in severity of symptoms (Carling and Leiner 1986, 1990a; Bandy *et al.*, 1988; Anguiz and Martin 1989; Bains and Bisht 1995; Balali *et al.*, 1995).

Two isolates belonged to AGs other than AG-3, namely AG-2-1 (Carling and Leiner, 1986; Campion *et al.*, 2003) and AG-5 (Carling and Leiner 1986, 1990a; Bandy *et al.*, 1988; Anguiz and Martin 1989; Bains and Bisht 1995; Balali *et al.*, 1995) that can be pathogenic on potato. Woodhall *et al.* (2007) found that the majority (93%) of isolates of *R. solani* infecting potatoes in Great Britain belongs to AG-3, but a few isolates of AG-2-1 and AG-5 were also found.

Rhizoctonia solani consists of genetically defined populations that belong to different 'anastomosis group' (AG). The anastomosis-based system of grouping isolates of *R. solani*, though occasionally imprecise, is beneficial because it enables us to better understand this large and highly heterogeneous species. Use of hyphal anastomosis reactions has led to the characterization of 14 anastomosis groups in *R. solani*, AG-1 through AG-13 and AG-BI (Kronland and Stanghellini, 1988; Rashid *et al.*, 2013). Isolates originating from tubers with black scurf or different parts of the plants are generally associated with AG-3, whereas isolates from soil where potato were grown can include various other AGs (Truter and Wehner, 2004). According to the results of hyphal fusion test, *R.*

solani was divided into 14 fusion groups (AG-1 to AG-13 and AG-BI) (Ogoshi, 1987).

As species complex, *R. solani* is considered to be genetically diverse, showing variability in both pathogenicity as well as morphological and physiological characters (Ogoshi, 1987).

The status of AG-3 as the most prevalent AG in potato possibly this ubiquity reflects population dynamics of the pathogen due to global trading of tubers. Isolates of *R. solani* from potato most often belong to AG-3 (Bandy *et al.,* 1988; Bains *et al.,* 1995; Balali *et al.,* 1995; Banville *et al.,* 1996; Virgen-Calleros *et al.,* 2000; Cedeno *et al.,* 2001; Campion *et al.,* 2003; Justesen *et al.,* 2003; Truter and Wehner 2004; Woodhall *et al.,* 2007; Lehtonen *et al.,* 2008a). Lower frequencies of potato isolates belong to AG-2 (Cedeno *et al.,* 2001), AG-4 (Anguiz and Martin 1989; Virgen-Calleros *et al.,* 2000; Truter and Wehner 2004) and AG-5 (Bandy *et al.,* 1988; Bains *et al.,* 1995; Balali *et al.,* 1995; Virgen-Calleros *et al.,* 2000).

With the development of molecular diagnostic methods, assigning isolates to AGs became easier (Justesen *et al.,* 2003; Woodhall *et al.,* 2007; Lehtonen *et al.,* 2008a, b).

Ribosomal RNA genes (rDNA) are frequently used to study evolutionary relationships among organisms including fungi. In *R. solani*, subspecific groups, sometimes called intraspecific groups (ISGS) (Ogoshi 1987) are identified by restriction analysis of the rDNA ITS region (Liu *et al.,* 1993). In AG-3, Liu *et al.* (1993) reported that the pattern of rDNA variations revealed at least two ISGs (3A and 3B) within potato isolates.

The most significant advances in fungal taxonomy in general now are coming from approaches involving DNA sequence analysis. The comparison of rDNA-ITS nucleotide sequences has provided more informative data for evaluating the evolutionary relationships among of AG-4 isolates (Boysen *et al.,* 1996) and of other isolates in the *R. solani* complex (Kuninaga *et al.,* 1997).

The predicted secondary structure was used as criteria to refine the ITS2/28S junction in *R. solani* AG3. Consequently, the ITS2 sequences of AG3 strains determined in this study contained 235 nucleotides. The AG-3 isolates appeared to be genetically relatively homogenous as a group, based on the phylogenetic analysis of ITS sequences. However, while the differences in the ITS region were sufficiently large to reliably differentiate the AGs, the region is limited in size for detection of differences between isolates of the same AG. For detection of genetic diversity or drift and gene flow within AG-3, other methods will be required (Justesen *et al.,* 2003).

RAPD analysis, which is widely used because it is simple, inexpensive, and does not require complicated preparations, has been applied to differentiate strains belonging to the same species (Williams *et al.,* 1990). RAPD results are also easy to interpret, and prominent polymorphisms can be reliably reproduced (Zhou *et al.,* 2009). This technique has been used to reveal genetic diversity among isolates of *R. solani* (Sharma *et al.,* 2005).

Molecular markers are important tools for the characterization of genetic diversity in fungal pathogens where morphological features are either absent or not sufficient to allow intra-specific characterization (Sharma *et al.,* 2005). Three molecular markers, URPs, ISSR, and RAPD primers were reported to show good amplification of various plant pathogenic fungi (Aggarwal *et al.,* 2010) including *R. solani* (Sharma *et al.,* 2005; Zhou *et al.,* 2009). Sharma *et al.,* (2005) reported that the isolates of *R. solani* obtained from the same hosts and the same geographical regions showed similarity in DNA fingerprint profiles barring a few exceptions.

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