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Phenotypic diversity and molecular identification of the most prevalent anastomosis group of *Rhizoctonia solani* isolated from diseased faba bean plants

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Abstract: One hundred and thirty one isolates of *Rhizoctonia* spp. were isolated from faba bean plants showing root rot and stem canker collected from different fields in Delta region of Egypt. Forty six isolates were found to be polynucleate and the remaining isolates were binucleates. According to morphological features of isolates, they were classified into 12 groups. Polynucleate isolates were identified as *Rhizoctonia solani* (Kühn). The most aggressive isolate of *R. solani* was identified according to sequences of ITS1-5.8S rDNA-ITS4 and the sequence was compared with *Thanatephorus cucumeris* (teleomorphic phase) and other *R. solani* (NCBI GenBank). Sequence and comparison revealed that this isolate is *R. solani* AG4-HGI. Anastomosis test carried out between molecular identified isolate and 11 randomly chosen isolates resembles all groups of polynucleate *R. solani*. All tested isolates were completely fused between each other indicating that the prevalent AG of *R. solani* on faba bean is AG4-HG1.

Keywords: Anastmosis Group, Rhizoctonia Solani AG4-HGI, PCR, Grouping of isolates, Phylogenetic Analysis

1. Introduction

Rhizoctonia solani (Kühn) {telomorhp: *Thanatephorus cucumeris* (Frank) Donk} is one of the most important soil borne pathogens It infects many plants including faba bean (*Vicia faba* L.), causes very serious disease, ranging from damping-off to root rot and stem canker and may causes plant death (Salt, 1982; Lamari and Bernier, 1985; Omar, 1986; Elwakil *et al.,* 2009). Owing to the phenotypic diversity of *R. solani* isolates, it is considered that this fungus comprises "group" and led to a classification system based on anastmosis grouping **(AG).** This AG is comprised of isolates among which perfect hyphal fusion occurs. This classification was supported by molecular sequencing of ITS-5.8S rDNA-ITS4 (Kuninaga *et al*., 1997; Gonzales *et al*., 2001; Fenille *et al*., 2003).

This study was aimed to study morphological diversity among *R. solani* isolates and molecular identification of the most prevalent AG on faba bean in Egypt.

2. Materials and Methods

2.1. Isolation and Identification of Rhizoctonia spp.

Samples of diseased faba bean plants that showed root-rot and stem canker were obtained from different fields of Qualyobia and Giza Governorate. Procedure of isolation was carried out as mentioned earlier (Maha H. Mohamed *et al.,* 2014). Identification of isolated *Rhizoctonia* spp. was carried out according to the procedure adopted by Sneh *et al.* (1991).

2.2. Staining of Nuclei

All isolates of Rhizoctonia spp. were grown individually on glass slide covered by water agar. Growing hyphae were stained by trypan blue to visualize the nuclei content especially in the apical compartment of fungal hyphae (Burpee et al., 1978) Stained hyphae were examined under light microscopy (400X) in order to count the number of nuclei/apical compartment then photographed.

2.3. Grouping of Isolates

According to morphological features of *Rhizoctonia* spp. isolates *i.e* type of growth, density of aerial hyphae, mycelial color (melanization), density, distribution of sclerotia and number of nuclei/ apical compartments, all isolates were divided into groups. Every group contained approximately similar isolates.

2.4. Molecular Identification of Rhizoctonia solani

2.4.1. DNA Extraction

The most aggressive isolate on faba bean cotyledons (data not recorded) and identified as *Rhizoctonia solani* according to its morphological features (Singh *et al.,* 1991) was grown in 100 ml potato sucrose broth in 250 ml flasks inoculated with 0.5 cm mycelial plugs. Flasks were incubated at 25±1°C for five days in the dark. Fungal harvesting was carried out by filtering through Whatman No.1 filter paper. The mycelium was ground in liquid nitrogen and total DNA was extracted according to the protocol of **GeneJET** Plant genomic DNA purification Kit (Thermo) #K0791 (Kuramae-Izioka, 1997).

2.4.2. Polymerase Chain Reaction for Nucleotide Sequencing

Amplification of the nuclear rDNA ITS region including the 5.8 S rDNA was performed with primers ITS4 and ITS5 (White *et al.,* 1990). PCR reactions consisted of 25 µl volume containing 2 µl genomic DNA (5-10 µg/ µl), 2.5 µl 10X PCR buffer (QIAGEN), 5 µl Q-solution (QIAGEN), 0.5 µl dNTPs (10 mM, Fermentas GmbH), 1.75 µl of each primer (10 µM), 0.15 µl Taq polymerase (5 units /µl, Fermentas GmbH) and 11.35 µl sterile highly purified water. PCR was run in a Mastercycler Gradient (Eppendorf) with an initial denaturation step at 94ºC for 10 min followed by 35 cycles at these conditions: 1 min at 94ºC, 1 min at 55 C and 1 min at 72ºC, with a final extension step at 72ºC for 10 min by using Maxima Hot Start PCR Master Mix (Thermo)#K0221.

2.4.3. Sequencing and ITS Sequence Analysis

Sequencing of PCR product was carried out on GATC Company by use ABI 3730 xI DNA sequencer by using forward and reverse primers. All these procedures were carried out in Sigma **Scientific** Service Technical Support, Sigma Scientific **Services** Co.

2.4.4. **Phylogenetic** *Analyses*

Sequences obtained in the present study and some available at GenBank **were** aligned with the multiple alignment program Clustal X (Thompson *et al*., 1997). A phylogenetic tree was constructed from distance matrix values by the neighbor joining method (Saitou and Nei, 1987).

2.5. Anastomosis Test

Anastomosis test was carried out between the well identified isolate of *R. solani* and eleven isolates randomly chosen from all groups of polynucleate isolates. Test was carried out by culturing each pair of the isolates on glass slide covered by water agar. Types of fusion between every pair was examined using light microscopy (400X).

3. Results

A total of 131 isolates from faba bean plants showed typical symptoms of root rot were isolated. Source of isolates and plant age from which the fungus was isolated are presented in Table (1).

Table 1. *Origin of tester isolates of Rhizoctonia used in this study*

Source of isolates	Age of Plants	Number of samples with root rot-like symptoms	Number of isolates
El-Kaliobia	seedling	10	18
El-Kaliobia	adult	77	80
El-Sharkia	adult	40	17
El-Giza	adult	20	16
Total		147	131

3.1. Identification of Rhizoctonia

3.1.1. Morphological and Cultural Characteristics

Morphological studies of isolated *Rhizoctonia* spp. revealed that 46 isolates fitted well with *R. solani* characteristics according to criteria adopted by Sneh *et al.* (1991), which include number of nuclei, production of sclerotium and brown pigmentation, branching near distal septum, constriction of the branch base of hyphae and formation of septa within a short distance of the origin of branches. For determination of number of nuclei/apical compartment, colonies of *Rhizoctonia* spp. were stained with trypan blue. Forty six isolates were polynucleate containing 3 to 8 nuclei/apical compartment or moniloid hyphal cell (Fig. 1). Cultural characteristics such as colony diameter, morphology of mycelial growth and degree of pigmentation showed great diversity among the total of 131 isolates (Table 2) and Fig. (1). Isolates of *Rhizoctonia* are classified to twelve groups according to colonies' morphological characters. Seven of them belong to polynucleate *Rhizoctonia* and 5 groups belong to binucleate *Rhizoctonia*. Average of growth rate ± standard deviation was calculated. As presented in Table (2), polynucleate isolates have in general the higher rate of growth. Degree of pigmentation also varied between groups. Polynucleate *Rhizoctonia* are more pigmented than binucleate one.

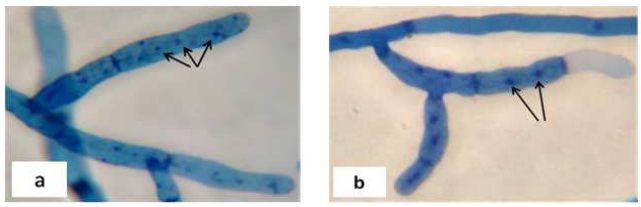


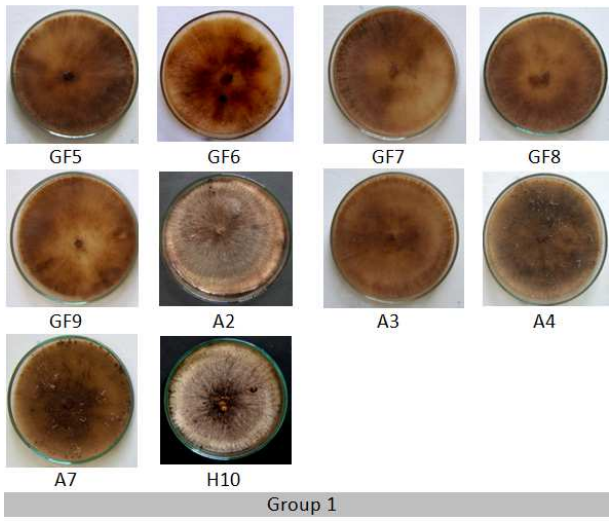
Fig. 1. *Hyphae of Rhizoctonia solani showing polynucleate (a) or/ binucleate (b) stained with trypan blue.Arrows indicate nuclei.*

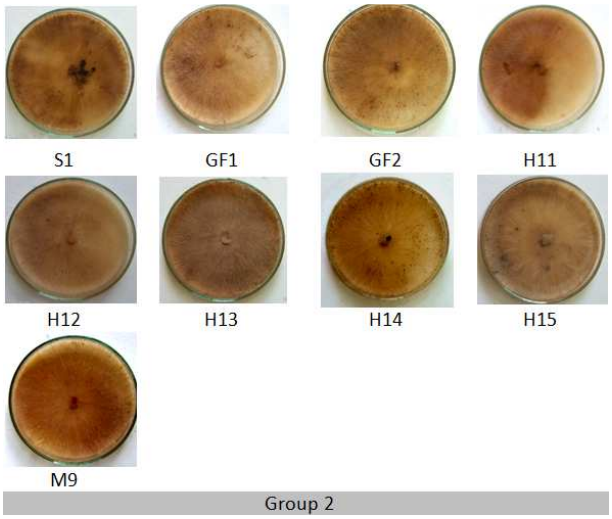
Table 2. *Radial colony growth, degree of melanin pigmentation production and number of nuclei of Rhizoctonia isolates growing on PSA medium; degree of pigmentation was determined according to Kim et al. (2001)*

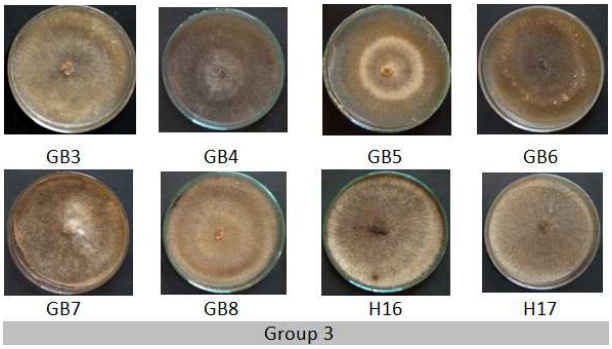
Isolates	Colony diameter (cm)	degree of melanin pigmentation	Source of isolate	Number of nuclei
Group 1				
Gf5	8.76	3	G	M
Gf6	8.65	3	G	M
Gf7	8.41	3	G	M
Gf8	8.50	3	G	M
Gf9	8.85	3	G	M
A2	7.16	3	K	M
A3	8.86	3	K	M
A4	7.75	3	K	M
A7	6.36	3	K	M
H10	7.25	3	S	M
SD	8.05 ± 0.87			
Group 2				
S1	7.05	2	K	M
Gf1	8.81	2	G	M
Gf2	9.00	2	G	M
H11	9.00	2	S	M
H12	9.00	2	S	M
H13	9.00	2	S	M
H14	8.80	2	S	M
H15	7.73	2	S	M
M9	9.00	2	K	M
SD	8.59 ± 0.71			
Group 3				
GB3	4.23	2	G	M
GB4	5.25	2	G	M
GB5	5.03	2	G	M
GB6	5.68	2	G	M
GB7	4.78	2	G	M
GB8	4.10	2	G	M
H16	8.03	2	S	M
H17	8.78	2	S	M
SD	1.73 ±5.73			
Group 4				
A1	8.06	2	K	M
A5	8.30	2	K	M
S2	8.23	2	K	M
S3	5.33	2	K	M
S4	4.30	2	K	M
GB1	3.76	2	G	M
B8	4.18	2	K	M
B10	3.23	2	K	M
B11	4.35	2	K	M
B12	3.83	2	K	M
SD	2.07±5.52			
Group 5				
Mo1	4.60	2	K	M
Mo2	5.46	2	K	M
Mo3	6.01	2	K	M
Mo4	5.43	2	K	M
Mo5	5.75	2	K	M
Gf3	5.96	2	G	M
Gf4	6.51	2	G	M
SD	0.59 ±5.67			
		Group 6		
Cu	7.20	2	K	M
		Group 7		
H7	5.75	0	S	M
		Group 8		
A15	2.51	0	K	B
A16	3.00	0	K	B
A17	3.25	0	K	B
A18	3.68	0	K	B
A20	3.06	0	K	B
A21	4.45	0	K	B
A22	3.11	0	K	B
A23	3.35	0	K	B
M1	3.21	0	K	B
M2	3.98	0	K	B
M3	4.86	0	K	B
M4	3.05	0	K	B
M5	3.31	0	K	B
M6	4.25	0	K	B
M7	3.43	0	K	B
M8	5.42	0	K	B
M11	3.75	0	K	B
M12	4.23	0	K	B
M13	3.13	0	K	B
H1	5.10	0	S	B
H2	5.98	0	S	B
H3	5.18	0	S	B
H4	5.90	0	S	B
H5	4.55	0	S	B
H6	5.16	0	S	B
H8	5.85	0	S	B
H9	5.33	0	S	B
S5	5.10	0	K	B
S6	5.35	0	K	B
S7	4.96	0	K	B
S8	2.90	0	K	B
S9	4.31	0	K	B
S10	3.40	0	K	B
S11	4.10	0	K	B
S12	5.26	0	K	B
S13	2.96	0	K	B
S14	4.91	0	K	B
S15	5.36	0	K	B
S16	3.73	0	K	B
S18	2.95	0	K	B
S19	4.00	0	K	B
B5	6.25	0	K	B
B6	5.76	0	K	B
B7	5.21	0	K	B
Mo6	6.46	0	K	B
Mo7	6.45	0	K	B
SD	1.11 ±4.38			
Group 9				
A6	6.86	2	K	B
A9	3.03	2	K	B
A10	9.00	2	K	B
A11	5.31	2	K	B
A12	6.56	2	K	B
A13	3.50	2	K	B
A14	3.26	2	K	B

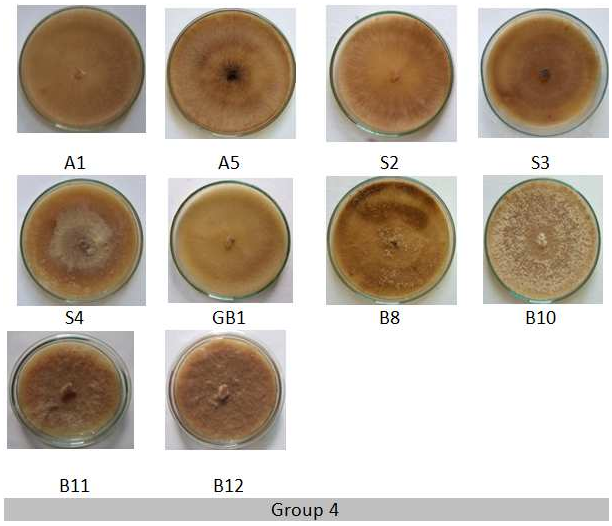
SD	2.24 ±5.36			
Group 10				
M14	7.20	2	K	B
M15	8.05	2	K	B
M16	6.95	2	K	B
M17	7.61	2	K	B
M18	7.30	2	K	B
M19	6.81	2	K	B
M20	6.11	2	K	B
M21	4.38	2	K	B
M22	6.96	2	K	B
M23	5.86	2	K	B
M24	6.88	2	K	B
M25	7.08	2	K	B
M26	7.65	2	K	B
M27	8.15	2	K	B
M28	6.95	2	K	B
M29	5.66	2	K	B
M30	6.51	2	K	B
M31	7.05	2	K	B
M32	7.10	2	K	B
M33	6.50	2	K	B
M34	6.93	2	K	B
M35	8.23	2	K	B
M36	6.50	2	K	B
M37	6.55	2	K	B
B2	6.01	2	K	B
A8	7.30	2	K	B
SD	6.85 ± 0.82			
		Group11		
M10	9.00	1	K	B
B1	7.56	1	K	B
SD	8.28 ± 1.01			
		Group12		
D	6.66	2	K	B

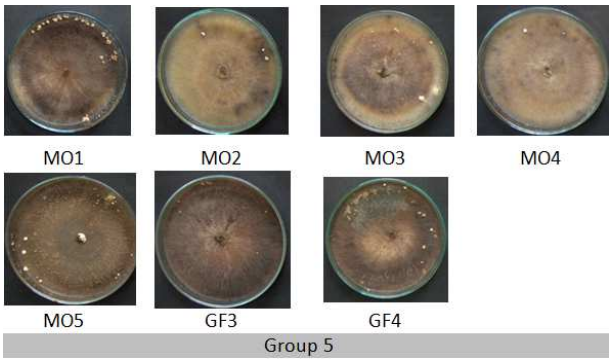
- Colony diameter (cm) between isolates average ± standard deviation (SD).
- Radial colony growth was recorded after 48 hours.
-melanin pigmentation production was a degree recorded after two weeks.
- G: El-Giza, S: El-Sharkia, K: El-Kaliobia.
- B: binuclear, M: multinuclear.









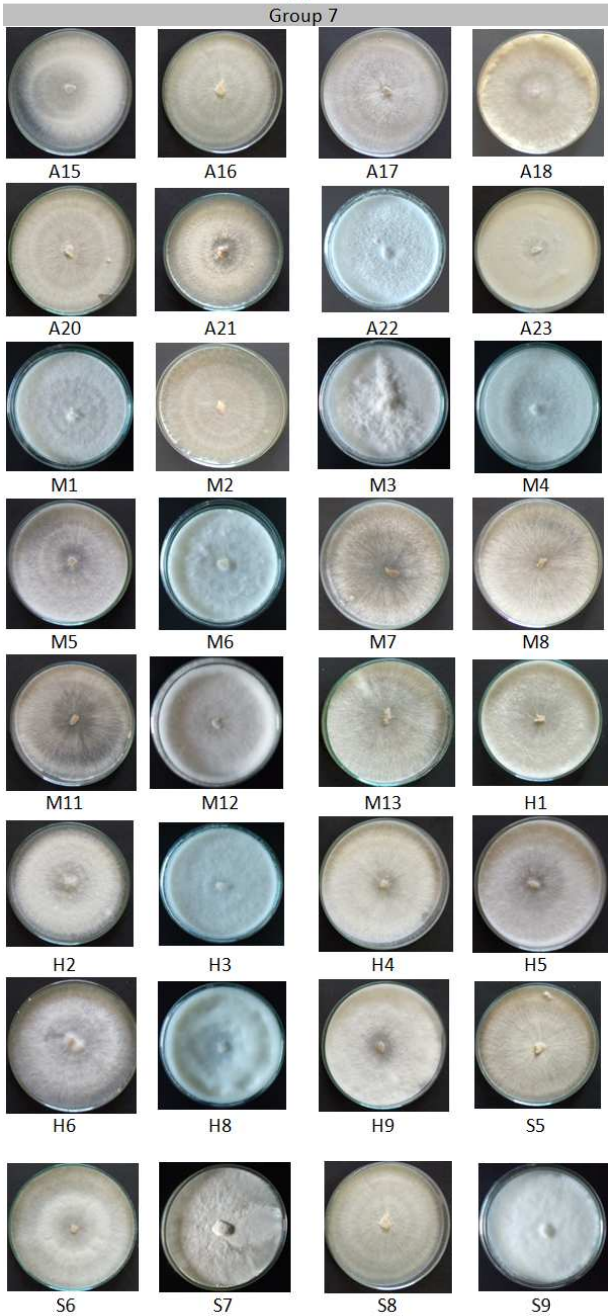


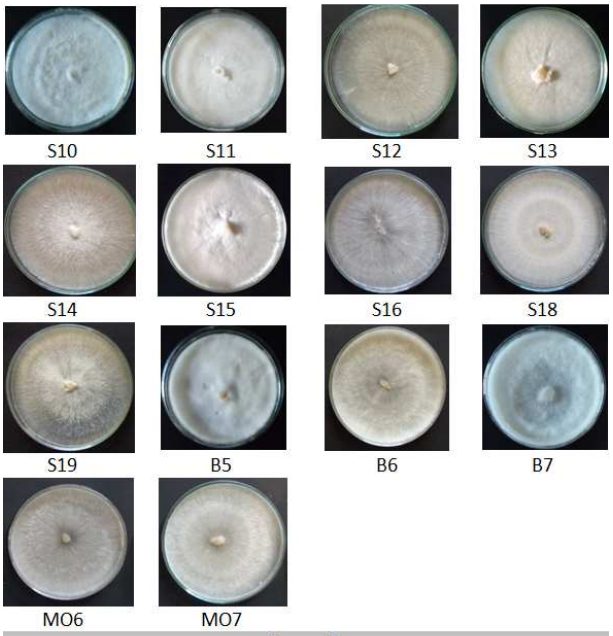
Cu

Group 6

H7

Group 7





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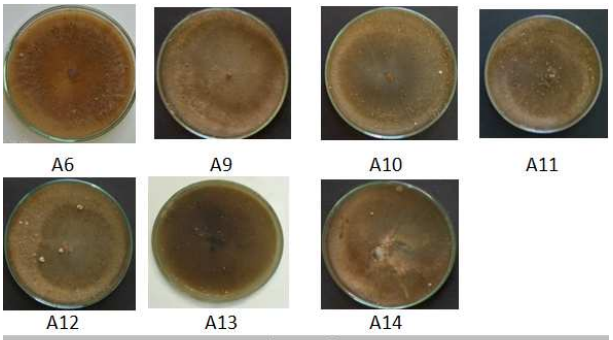
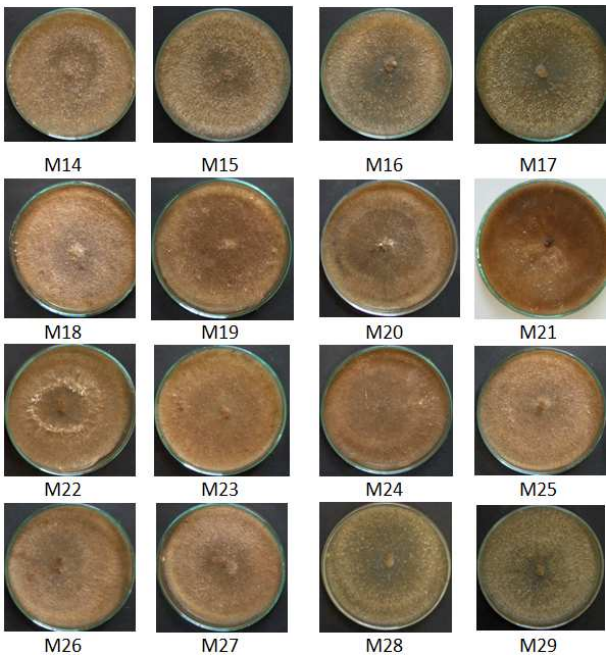


Group 9



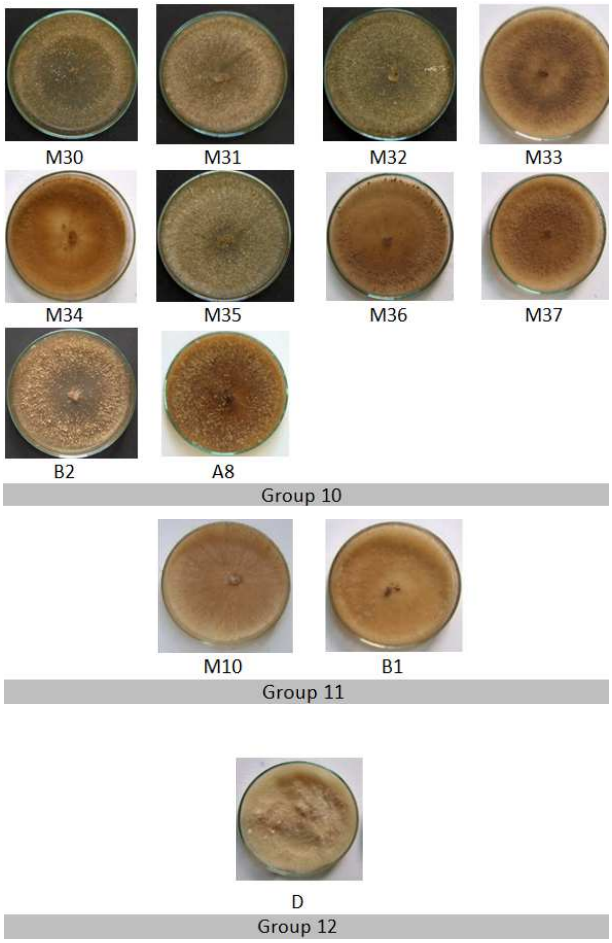


Fig. 2. *Grouping of R. solani isolates depend on cultural characteristics.*

3.1.2. Molecular Identification

DNA of the most aggressive faba bean isolate was amplified using universal primers ITS1 and ITS4 using PCR technique. Primers generated a fragment of approximately 700 bp long. The whole ITS region (ITS1, 5.8S rDNA and ITS4 of this isolate was sequenced in both directions with the same primers used for PCR amplifications. Sequences were aligned (Fig. 3).

Comparison of whole region (ITS1, 5.8S rDNA and ITS4) of isolate under study with that of *Thanatophorus cucumeris* (teleomorph) and other *R. solani* (anamorph) (NCBI GenBank) revealed that the similarity in nucleotide sequence between them reached 97% similarity (Table.3) and (Fig. 4). According to these comparisons, the isolate under study is identified as *R. solani* AG4 and the whole region (ITS1, 5.8S rDNA and ITS4) was registered in NCBI GeneBank with accession number KF907731.1.

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Fig. 3. *Sequences aligned with other known R. solani sequences from the NCBI GenBank.*

Table 3. *Comparison of whole region (ITS1, 5.8S rDNA and ITS4) of isolate under study with that of Thanatophorus cucumeris (teleomorph) and other R. solani (anamorph) (NCBI GenBank) revealed the similarity in nucleotide sequence between them.*

Description	Max score	Total score	Query cover	E value	Ident	Accession
Thanatophorus cucumeris isolate PT73 18S	1119	1119	93%	0.0	97%	JQ219361. 1
Rhizoctonia solani strain AG 4-HGI isolate LDDT01-1 18S	1110	1110	93%	0.0	97%	KF907731. 1
Rhizoctonia solani RT 14-2	1110	1110	93%	0.0	97%	FJ746939. 1
Rhizoctonia solani RT 26-4	1106	1106	93%	0.0	97%	FJ747974. 1
Thanatophorus cucumeris isolate Rh Faq	1106	1106	92%	0.0	98%	DQ021450. 1
Rhizoctonia solani isolate RS79	1101	1101	92%	0.0	97%	JQ616856. 1
Rhizoctonia solani isolate RT 26-1	1101	1101	93%	0.0	97%	FJ746973. 1
Thanitophorus cucumeris serain Rol 222	1101	1101	93%	0.0	97%	AY684924. 1
Rhizoctonia solani AG4 HGI isolate IBRS01	1097	1097	93%	0.0	97%	KF746162. 1
Thanitophorus cucumeris isolate CR-26	1097	1097	93%	0.0	97%	JF699277. 1
Thanitophorus cucumeris isolate KT63-1	1097	1097	93%	0.0	97%	EF203246. 1
Rhizoctonia solani strain 11D	1095	1095	93%	0.0	97%	JX294316. 1
Thanitophorus cucumeris	1094	1094	93%	0.0	97%	KJ715964. 1
Rhizoctonia solani isolate Remol	1094	1094	92%	0.0	98%	KC70958. 1

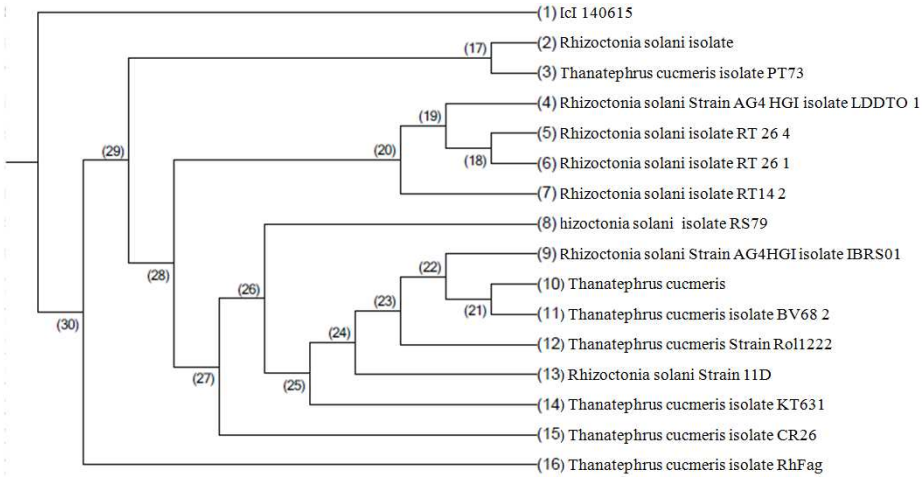
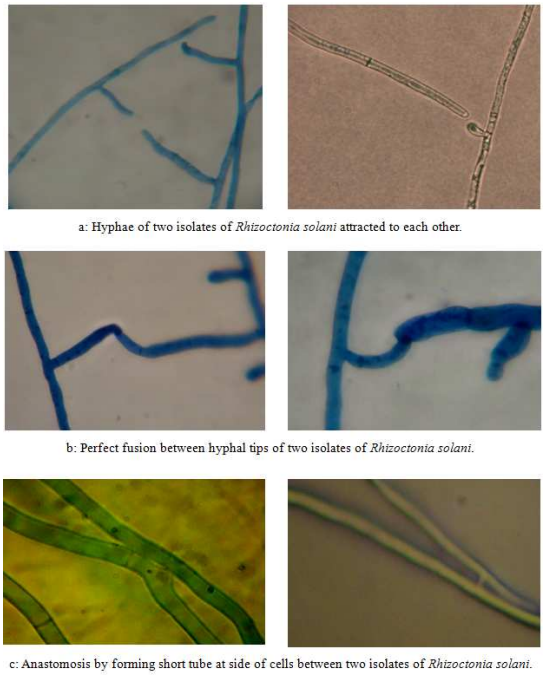


Fig. 4. *Comparison of whole region (ITS1, 5.8S rDNA and ITS4) of isolate under study with that of Thanatophorus cucumeris (teleomorph) and other R. solani (anamorph) (NCBI GenBank)*

Table 4. *Hyphal fusion among chosen polynucleate Rhizoctonia isolates.*

Isolates	A3	H10	GF6	S1	GF1	GF2	H14	M9	GB5	A5	MO4	Cu
A3	+	+	+	+	+	+	+	+	+	+	+	+
H10	+	+	+	+	+	+	+	+	+	+	+	
Gf6	+	+	+	+	+	+	+	+	+	+		
S1	+	+	+	+	+	+	+	+	+			
GF1	+	+	+	+	+	+	+	+				
GF2	+	+	+	+	+	+	+					
H14	+	+	+	+	+	+						
M9	+	+	+	+	+							
GB5	+	+	+	+								
A5	+	+	+									
MO4	+	+										
Cu	+											



a: Hyphae of two isolates of *Rhizoctonia solani* attracted to each other.

b: Perfect fusion between hyphal tips of two isolates of *Rhizoctonia solani*.

c: Anastomosis by forming short tube at side of cells between two isolates of *Rhizoctonia solani*.

Fig. 5. *Examples of fusion between two different isolates.*

3.2. Anastomosis Test

The accurate identified *R. solani* isolate was tested for fusion with another 11 isolates of polynucleate *R. solani* randomly chosen among polynucleate *R. solani* groups. As shown in Table (4). Data obtained indicated that all chosen tested isolates fused perfectly between them. Example of fusion are illustrated in Fig. 5 a, b, and c). This result indicates clearly that the randomly chosen *R. solani* isolates belong to the same Anastomosis group (AG4 HGI).

4. Discussion

The fungus *Rhizoctonia solani* (Kühn) **is** reported to cause economic losses in many economical plants including faba bean causing damping off, root and hypocotyl rots and stem canker. The tremendous diversity in morphology and pathogenecity of *R. solani* has led to classification system based on anastomosis grouping (AG). This AG is comprised of isolates among which hyphal fusion occurs and this classification system has been supported by molecular evidence (Kuninaga and Yokosawa, 1982 a&b, 1984, 1985; Vilgalys, 1988; Carling and Kuninaga, 1990; Kuninaga *et al.,*

1997; Gonzalez *et al.,* 2001; Fenille *et al*., 2003).

In this study a total of 131 isolates of *Rhizoctonia s*pp. were isolated from diseased faba bean plants collected from different provinces in Egypt. It is well established that genus *Rhizoctonia* classified according to the number of nuclei in the apical compartment into two groups: binucleate (in which apical compartments contains 2 nuclei) and polynucleate (in which apical compartments contain 3 to 11 nuclei). Data obtained indicated that binucleate *Rhizoctonia* comprise 64.88% from the total isolate, indicating that this group ~~is~~ dominates over polynucleate *Rhizoctonia* on faba bean plants. The behavior of binucleate *Rhizoctonia* and its effect on plants and its host range has not received adequate attention.

Isolated *Rhizoctonia* spp. was grouped into 12 groups according to their morphological characters, degree of melanization and number of nuclei. Polynucleate *Rhizoctonia* occupied 7 groups and binucleate ones occupied 5 groups. The diversities between polynucleate *Rhizoctonia* were very **strong** in comparison to binucleate groups and the divergence among polynucleate *Rhizoctonia* may be due to heterokaryosis and parasexual cycle within apical compartments resulting in these deviations in polynucleate *Rhizoctonia* spp. (Parmeter, *et al.,* 1963).

Twelve isolates resembling polynucleate *Rhizoctonia* groups were identified according to their morphological features according to Sneh *et al.* (1991). According to these features, isolates were identified as *Rhizoctonia solani* (Kühn). Anastomosis test was carried out between all isolates by culturing pairs of isolates facing each other on glass slide covered by water agar in sterilized Petri dish. It was observed that all isolates were perfectly fused between each other. Microscopic observation proved that hyphal apicals were attracted to each other and then fused (tip -by- tip). Another fusion was observed (apical -to- hyphal side; side -by- side), indicating that all tested isolates belong to the same AG.

One of these tested isolates which had high pathogenic effect on faba bean was chosen for further identification by rDNA - ITS sequences. Data obtained indicated that the similarities between tested isolate and *Thanatephorus cucumeris* (teleomorph of *R. solani*) reached 97% similarity and between tested isolate and *R. solani* isolates (anamorph) reached 97% similarities, indicating that the tested isolate is *R. solani* (Kühn) AG4-HG1.

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