Characterization of *Rhizoctonia* spp. Isolates Associated with Damping-Off Disease in Cotton and Tobacco Seedlings in Greece

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ABSTRACT

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Isolates of *Rhizoctonia* spp. were obtained during the spring of 2007 from diseased cotton and tobacco seedlings showing damping-off symptoms. The sampled fields were located in the main cotton- and tobacco-cultivating regions of central and northern Greece. Among the 79 isolates obtained from cotton plants, 17 were binucleate and 62 were multinucleate whereas, among the 89 isolates obtained from tobacco plants, 87 were multinucleate and only 2 were binucleate. Characterization of anastomosis groups (AGs) was performed with hyphal anastomosis reactions using tester isolates of known AG groups. Anastomosis reactions in cotton multinucleate isolates showed that 54 of them belonged in *Rhizoctonia solani* AG-4, 6 in AG-7, 1 in AG-2, and 1 in AG-3. In the 87 tobacco multinucleate isolates, anastomosis reactions showed that 70 of them belonged in *R. solani* AG-2, 16 in AG-4, and 1 in AG-5. In addition, molecular characterization was carried out using the specific ribosomal DNA internal transcribed spacer region, in a randomly selected number of isolates. In cotton, the most prevalent AG was AG-4, with 18 isolates to the subgroup HG-I, 1 isolate to the subgroup HG-II, and 7 isolates to the subgroup HG-III, followed by AG-7 (7 isolates), AG-2-1 (1 isolate), and AG-3 (1 isolate). In tobacco, the most prevalent group was AG-2-1 (70 isolates), followed by AG-4 (6 isolates to the subgroup HG-I and 5 isolates to the subgroup HG-III) and a single isolate belonging to AG-5. Phylogenetic analysis showed that the isolates were distinctly separated based on their AG types. Pathogenicity and aggressiveness of the isolates to several hosts was determined. AG-4 isolates from either cotton or tobacco were the most aggressive on the hosts tested, while AG-2-1 isolates were of moderate aggressiveness and were not pathogenic on barley.

Rhizoctonia spp. are soilborne pathogens attacking many of the agricultural and horticultural crops throughout the world and causing damping-off, root rot, foot rot, stem rot, fruit rot, and foliar blight in more than 150 plant species (33). In Greece, *Rhizoctonia* seedling blight is widespread throughout the country, causing significant losses due to pre- and post-emergence damping-off in field crops such as cotton, tobacco, canola, and sugar beet. Isolates within the genus *Rhizoctonia* vary greatly in their colony morphology, growth characteristics, and virulence on several hosts (15,33).

Although a preliminary discrimination can be achieved by classifying *Rhizoctonia* spp. into uninucleate, binucleate, or multinucleate groups based on the number of nuclei in vegetative hyphal cells, the most widely used method of classification of

Rhizoctonia spp. is based on grouping the isolates into anastomosis groups (AGs; 48). Hyphae of isolates will only anastomose each other if they are within the same AG. Classification in AGs is valid for both multinucleate (*Thanatephorus*) and binucleate (*Ceratobasidium*) taxa within the *Rhizoctonia* spp. complex. Fourteen AGs (AG-1 to AG-13) and one subgroup of bridging isolates AG2-BI have been described (8,9) in *Rhizoctonia solani* Kühn, the anamorph of *Thanatephorus cucumeris* (Frank) Donk. The latter isolates have the ability to anastomose with isolates of several AGs. Seven of the 14 AGs (AG-1, AG-2, AG-3, AG-4, AG-6, AG-8, and AG-9) have been further subdivided into subgroups based on several characteristics such as frequency of anastomoses, fatty acid and isozyme profiles, thiamine requirement, colony morphology, and host range of the isolates (33,37,48). Binucleate *Rhizoctonia* spp. are currently divided into 19 AGs, designated as AG-A through AG-S (38).

Rhizoctonia spp. are major threats in cotton and tobacco production worldwide, causing damping-off on young seedlings of both hosts. *R. solani* also causes target spot of tobacco, an important foliar disease

(22,47). Despite the significance of the cotton crop in several regions of the world and the high economic importance of *Rhizoctonia* spp. attacks on this crop, only a few reports have characterized *Rhizoctonia* spp. isolated from this host. According to these reports *R. solani* AG-4 is the predominant AG (6,26,28). In addition, isolates of *R. solani* AG-7 (2), AG-13 (8), and a binucleate *Rhizoctonia* sp. (26) have been recovered from diseased cotton seedlings. Similarly, in tobacco, the predominant AG associated with damping-off is the AG-4 (16,32), while isolates of AG-1 to AG-5 have also been recovered from tobacco (27,32). Isolates of AG-1, AG-2, AG-4, and AG-5 are associated with damping-off symptoms while those of AG-3 are associated with foliar symptoms (target spot) (10,17,23,27).

Characterization of *Rhizoctonia* populations within a cropping region can help in the development of environmentally friendly and sustainable crop management systems. Factors affecting the dynamics, existence, and distribution of AGs in the soil include crop rotation, tillage (13,36), climate, and soil characteristics (14,54). Although the occurrence of *Rhizoctonia* spp. is well documented in Greece, there are no studies related to the occurrence of AGs and subgroups of *R. solani* or binucleate *Rhizoctonia* spp. Therefore, this study was initiated to characterize the AGs of isolates of *Rhizoctonia* spp. obtained from cotton and tobacco seedlings grown on cultivated fields in central and northern Greece. We examined those isolates based on (i) nuclear staining, (ii) observation of hyphal anastomosis with tester isolates, (iii) sequence analysis of internal transcribed spacer (ITS) region on ribosomal (r)DNA, (iv) mycelial growth in different temperatures, and (v) pathogenicity tests on five crops, including tobacco and cotton.

MATERIALS AND METHODS

Pathogen isolation. A survey was conducted in the main cotton- and tobacco-cultivating regions of Greece in spring 2007 to obtain isolates of *Rhizoctonia* spp. (Fig. 1). In each region, 3 to 15 fields were inspected for seedlings showing symptoms of damping-off. Diseased cotton and tobacco seedlings were arbitrarily selected at several sites in each field and transported

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to the laboratory in individual polyethylene bags to prevent cross contamination.

The surface of symptomatic roots and hypocotyls was disinfected with a 10% sodium hypochlorite solution for 1 min and washed three times with sterilized distilled water. Pieces from the diseased roots and hypocotyls were placed on 1.5% water agar, acidified with lactic acid (10%, pH 4.5) and incubated at 25°C in the dark (Libeler incubators). Two petri dishes, each containing three to five segments, were prepared from each plant. After 2 to 3 days, hyphae grown from segments were observed microscopically for morphological characteristics of *Rhizoctonia* spp. and hyphal tips from *Rhizoctonia* spp.-like colonies were transferred on potato dextrose agar (PDA; Ovoid, Unipart Ltd., Basingstoke, England). From each petri dish, only one isolate was kept in pure culture. The cultures were incubated at 25°C in the dark for 3 days and then were stored at 10°C until use. In total, 79 isolates of *Rhizoctonia* spp. were obtained from 34 cotton fields (COT isolates) and 89 isolates from 23 tobacco fields (TOB isolates) (Table 1).

Nuclear staining. Nuclei of each isolate were stained for confirmation of binucleate or multinucleate status based on the methodology described by Bandon (4). Single 5-mm-diameter disks of 2-day-old cultures

grown on PDA were transferred on clean, sterilized glass slides covered by 2% water agar. The slide cultures were incubated in a moist chamber at 25°C in the dark. After 2 to 3 days, the slides were removed from the moist chamber and one drop of safari O and one drop of 3% KOH were added. The number of nuclei per cell was counted from 20 cells in each isolate under ×400 magnification. Measurements were taken two times.

Hyphal anastomosis. The determination of the AGs was performed using tester isolates of known AG provided by Dr. R. Grouch (Institute of Vegetable and Ornamental Crops, Gerbera, Germany) and Dr. J. H. M. Schneider (Institute of Sugar Beet Research, Bergen op Zoom, Netherlands) (Table 2).

Anastomosis reactions were observed using the glass-slide technique, following the method described by Carling (7). A 5-mm mycelial plug of an actively growing culture of each isolate and a 5-mm mycelial plug of each tester isolate was placed 1 to 2 cm apart on water-agar-coated, sterile glass slides. The glass slides were placed in a moist chamber and incubated at 25°C in the dark until the growing hyphae came into contact (2 to 3 days). The region of the overlapping hyphae was stained with one drop of safari O and one drop of 3% KOH and examined microscopically at

×400 and ×1,000 to determine anastomosis reactions. The categories of hyphal anastomosis reactions were scored as follows (11): C0, no interaction; C1, hyphal contact; C2, hyphal fusion with killing reaction; and C3, fusion with no cell death. Isolates were placed into AGs based on the presence of at least five C2 reactions with each of the tester isolates per microscopic field. The anastomosis determination procedure with the tester isolates was conducted two times.

DNA extraction. To determine sequence similarity of the rDNA-ITS region, 36 COT isolates and 34 TOB isolates were used. The isolates were selected to represent all the groups identified in the anastomosis reaction tests. Each isolate was grown in potato dextrose broth (Sigma-Aldrich, St. Louis), filtrated, dried, lyophilized, ground to a fine powder using micro pestles (Eppendorf International, Weaseling, Germany), and stored at −20°C until use. DNA was extracted using Quay Pure gene Core Kit A (Siegen GmbH, Hilden, Germany) according to the manufacturer's protocol. The concentration of the extracted DNA was measured by UV spectrophotometer (Gene Quant2; Pharmacia Biotech, Cambridge, UK).

DNA amplification and sequencing. ITS1 and ITS2 regions, including the ribosomal 5.8S RNA gene, were amplified using the universal ITS-1 (5-TCCGTA GGTGAACCTGCGG-3) and ITS-4 (5-TCCTCCGCTTATTGATATGC-3) primers that anneal to the flanking 18S and 28S rRNA genes (53). The PCR amplification reactions were performed in a 50-μl mixture containing 5 μl of polymerase chain reaction (PCR) buffer (1×) (Dynasty II Hot Start Reaction Buffer, Fanzines, Espoo, Finland), 2 μl of dNTPs (200 μM), 0.5 μl of each primer (0.5 μM) (Lark Technologies Inc., Essex, UK), 0.5 μl (1 U per 50 μl) of *Taq* DNA polymerase (Dynasty II Hot Start; Fanzines), 5 μl of template DNA (50 ng), and 36.5 μl of sterile highly puri-

Fig. 1. Map of Greece showing tobacco and cotton production areas where seedlings were collected for isolation of *Rhizoctonia* spp. 1 = Larissa, 2 = Pieria, 3 = Imathia, 4 = Pella, 5 = Kilkis, 6 = Thessaloniki, 7 = Serres, 8 = Drama, 9 = Xanthi, and 10 = Rodopi.

Table 1. Geographic origin and number of *Rhizoctonia* spp. isolates obtained from cotton and tobacco fields of central and northern Greece

Region, crop	No. of fields	No. of Isolates
Cotton isolates		
Serres	6	12
Drama	2	2
Thessaloniki	1	1
Pella	5	14
Imathia	12	23
Larissa	8	27
Total	34	79
Tobacco isolates		
Pieria	1	2
Xanthi	2	13
Kilkis	1	5
Serres	15	44
Rodopi	4	25
Total	23	89

fied H₂O. In all PCR sets, a negative control without DNA was included.

PCR mixtures were covered with a drop of mineral oil to prevent evaporation and incubated in a PTC 200 thermal cycler (MJ Research, Waltham, MA) using the following conditions: DNA denaturation for 5 min at 94°C and 30 cycles of DNA denaturation for 30 s at 94°C, primer annealing for 30 s at 57°C, and extension of DNA for 1 min at 72°C. DNA amplification was terminated by primer extension for 5 min at 72°C and final incubation at 4°C. After the end of each reaction, set aliquots of the reaction mixture (5 µl) were analyzed on 2% agarose gel in Tris-borate EDTA buffer, supplemented with ethidium bromide (0.1 µg ml⁻¹) by electrophoresis, and viewed under UV light.

PCR products of each isolate were purified using the Qiaquick PCR Purification Kit (Siegen GmbH). The purified products were subjected to sequencing in both directions using the universal primers ITS-1

and ITS-4. The sequencing was carried out by Lark Technologies Inc.

Sequence data and phylogenetic analysis. Sequences were aligned using the computer software package Clustal W 2.0.9 (25,51) and BioEdit 7.0.9.0 (18). The alignment of both directions in each isolate was checked visually. The obtained sequences of all isolates were compared with sequences in the National Center for Biotechnology Information (NCBI) data base using BlastN 2.2.18 (55). Sequence similarity between the ITS1-5.8S-ITS2 sequences of isolates obtained in this study and those of isolates already deposited in GenBank was checked using Clustal W 2.0.9. Phylogenetic analyses were performed using neighbor-joining method (40) based on the alignment using ITS1-5.8S-ITS2 sequences of the isolates used in this study and isolates of known AG. The computational analysis to generate the phylogenetic tree was performed using Mega 4.0 (50). The distances in the ITS-

5.8S rDNA region were determined by Kimura's two-parameter model (21), omitting all sites with gaps. A bootstrap analysis of 1,000 replications was carried out. Sequences of *R. cerealis* (GenBank accession no. AJ302009) and binucleate *Rhizoctonia* AG-F (accession nos. AB219144 and DQ102433) were used as outgroups for rooting the phylogenetic trees constructed from isolates obtained from cotton and tobacco, respectively.

Hyphal growth response at different temperatures. Hyphal growth rate of 86 isolates was measured at six different temperatures (10, 15, 20, 25, 30, and 35°C) on PDA. Isolates were selected to include all the AGs and subgroups revealed in the study. Mycelial plugs (5 mm in diameter) from 3-day-old cultures were transferred to the center of 9-cm petri dishes. The cultures (two replicate cultures per isolate and temperature) were incubated in the dark. The experiments for all the isolate-temperature combinations were carried simultaneously in different growth chambers. Two perpendicular colony diameters were measured on the bottom of each plate at 24-h intervals until the colony had reached the edge of the petri dish. Agar plug diameters were subtracted from every measurement. The average daily hyphal growth rate per isolate was calculated per temperature. The experiment was performed two times.

Pathogenicity and aggressiveness. In all, 21 COT isolates and 20 TOB isolates were selected to represent the AGs and subgroups revealed in the study. The pathogenicity and aggressiveness of the selected isolates was tested on cotton (cv. Flora), tobacco (cv. Xanthi), barley (cv. Mutsu), radish (cv. Saxa 2), and sugar beet (cv. Creta) using a method modified from Carling et al. (9). Each isolate used as inoculum was incubated on PDA plate for 2 to 3 days. A 5-mm agar plug with mycelium actively grown on PDA was transferred on the center of 9-cm petri dish

Table 2. Tester isolates of *Rhizoctonia solani* used to determine anastomosis groups (AGs) of isolates of the pathogen collected from cotton and tobacco grown in Greece

AG	Isolate code	Host	Country	Source
1-1A	CS-KA	Rice	Japan	Schneider
1-1A	PRG-97-1	Rice	Japan	Grosch
1-1B	B-19	Sugar beet	Japan	Schneider
1-1C	BV-17	Sugar beet	Japan	Schneider
2-1	Ps-4	Bean	Japan	Schneider
2-1	SH-3	Soil	Japan	Grosch
2-2IIIB	C-96	Bent grass	Japan	Schneider
2-2IV	RI-64	Sugar beet	Japan	Schneider
3	ST-11-6	Potato	Japan	Schneider
3	B-15	...	Israel	Grosch
4-HGI	AH-1	Peanut	Japan	Schneider
5	GM-10	Soybean	Japan	Schneider
5	SH-4	...	Japan	Grosch
6	6R-1	...	Israel	Grosch
7	1-529	...	Japan	Grosch
8	08-01	Wheat	United States	Grosch
9	09-01	...	...	Grosch
10	10-03	Alfalfa	Australia	Grosch
12	12-01	Plant residues	Australia	Grosch
13	13-01	Cotton	United States	Grosch

Table 3. Nuclear status of *Rhizoctonia* spp. isolates and anastomosis grouping (AG) of *R. solani* obtained from diseased cotton and tobacco seedling plants from northern and central regions of Greece

Region, crop	Number of isolates ^y			Anastomosis group (AG) ^z				
	Total	Binucleate	<i>Rhizoctonia solani</i>	AG-2	AG-3	AG-4	AG-5	AG-7
Cotton isolates								
Serres	12	1 (8.3)	11 (91.7)	0	1 (9.0)	10 (91.0)	0	0
Drama	2	0	2 (100.0)	0	0	2 (100.0)	0	0
Thessaloniki	1	0	1 (100.0)	0	0	1 (100.0)	0	0
Pella	14	4 (28.5)	10 (71.5)	0	0	6 (60.0)	0	4 (40.0)
Imathia	23	9 (39.1)	14 (60.9)	0	0	13 (92.8)	0	1 (7.2)
Larissa	27	3 (11.1)	24 (88.9)	1 (4.2)	0	22 (91.6)	0	1 (4.2)
Total	79	17 (21.5)	62 (78.5)	1 (1.6)	1 (1.6)	54 (87.1)	0	6 (9.7)
Tobacco isolates								
Pieria	2	1 (50)	1 (50)	0	0	0	1 (100.0)	0
Xanthi	13	0	13 (100.0)	13 (100.0)	0	0	0	0
Kilkis	5	0	5 (100.0)	5 (100.0)	0	0	0	0
Serres	44	1 (2.2)	43 (97.8)	36 (83.7)	0	7 (16.3)	0	0
Rodopi	25	0	25 (100.0)	16 (64.0)	0	9 (36.0)	0	0
Total	89	2 (2.2)	87 (97.8)	70 (80.5)	0	16 (18.4)	1 (1.1)	0

^y Number in parenthesis is the percentage of the total number of isolates.

^z Number in parenthesis is the percentage of the of the total number of isolates per AG.

containing 2% water agar. Sterile PDA plugs were used as controls. After 2 days of incubation, 10 seeds of each host were placed at equal distances in a circular pattern along the outer edges of the petri dishes. Prior to use, seed were surface sterilized in 10% NaOCl for 1 to 2 min. Petri dishes were incubated at 21°C in the dark. Three days after the placement of the seed into the water agar medium, damage was assessed on the developing cotton, barley, radish, and sugar beet seedlings, where 0 = no damage; 1 = minor discoloration of the hypocotyl; 2 = discoloration plus small (<1-mm-diameter) necrotic lesions on the stem, hypocotyl, or root; 3 = discoloration plus large (>1-mm-diameter) necrotic lesions on the stem, hypocotyl, or root; and 4 = seedling dead (9). The disease index scale was applied to all hosts except tobacco. Due to the small size of tobacco seedlings, pathogenicity on this host was determined by counting the number of dead 3-day-old seedlings. For each isolate, three replicate petri dishes were used and the experiment was carried out two times.

Data analysis. Data of isolates' mycelial growth rate and aggressiveness on several hosts were subjected to analysis. Due to uneven number of isolates within groups and subgroups, nonparametric analysis was performed. Overall differences in mycelial growth rates at the temperatures tested and the aggressiveness data of each AG or subgroup were tested with the Kruskal-Wallis test and, in case of a significant result, pairwise comparisons were performed by means of a series of Mann-Whitney tests. Due to the relatively high number of pairwise comparisons, the significance level per comparison was adjusted according to Bonferroni's method ($P = 0.002$). All the statistical analyses were performed using SPSS (SPSS Inc., Chicago).

RESULTS

Isolation and identification. In total, 168 isolates of *Rhizoctonia* spp. were obtained from diseased cotton and tobacco seedling plants. The isolates were collected from 57 fields distributed across the 10 most important cotton- and tobacco-cultivating regions of Greece (Table 1). Nuclear staining using safari O revealed that multinucleate isolates were dominant among isolates obtained from cotton (79.5%) and tobacco (97.8%) (Table 3). The remaining isolates were binucleate.

Anastomosis grouping. All multinucleate isolates were paired with tester isolates of *R. solani* AGs AG-1 to AG-13. Positive anastomosis reactions were observed with the tester isolates of AG-2, -3, -4, -5, and -7 (Table 3). Among 62 COT isolates, 54 isolates gave a C2 reaction with the AG-4 tester isolate, 6 isolates with AG-7, and one isolate each with AG-2 and AG-3. Similarly, among the 87 multinucleate

Table 4. Hyphal anastomosis reactions and analysis of the ribosomal (r)DNA internal transcribed spacer region of *Rhizoctonia solani* and two binucleate *Rhizoctonia* spp. isolated from cotton and tobacco seedlings in Greece

Isolate name	Anastomosis grouping ^y	rDNA sequencing	Accession no. ^z
Cotton isolates			
P20B	AG-7	AG-7	FJ480891
P2B3	AG-4	AG-4-HG-I	FJ480867
P4B3	AG-7	AG-7	FJ480892
P7B2	AG-7	AG-7	FJ480893
AMP4B	AG-4	AG-4-HG-III	FJ480881
L2B7	AG-4	AG-4-HG-I	FJ480874
L3B1	AG-7	AG-7	FJ480894
L3B10	AG-4	AG-4-HG-I	FJ480865
L4B2	AG-4	AG-4-HG-I	FJ480868
L4B9	AG-4	AG-4-HG-I	FJ480864
L6B8	AG-2	AG-2-1	FJ480890
L7B1	Binucleate	AG-F	FJ480897
L7B5	AG-4	AG-4-HG-III	FJ480882
L7B10	AG-4	AG-4-HG-I	FJ480866
L8B2	AG-4	AG-4-HG-I	FJ480875
L10B7	AG-4	AG-4-HG-I	FJ480873
SB2B	AG-4	AG-4-HG-III	FJ480883
SG1B	AG-4	AG-4-HG-I	FJ480876
SD2B	AG-4	AG-4-HG-I	FJ480869
SZ6B	AG-3	AG-3	FJ480889
SH3B	AG-4	AG-4-HG-I	FJ480863
D2B21	AG-4	AG-4-HG-III	FJ480884
D3B28	AG-4	AG-4-HG-II	FJ480880
TH1	AG-4	AG-4-HG-III	FJ480886
A8B	AG-4	AG-4-HG-III	FJ480887
G9B	AG-4	AG-4-HG-I	FJ480871
D1B	AG-4	AG-4-HG-I	FJ480870
D3B	AG-4	AG-4-HG-I	FJ480862
H3B	AG-4	AG-4-HG-I	FJ480872
T4B	AG-4	AG-4-HG-III	FJ480885
I9B	AG-4	AG-4-HG-I	FJ480879
K1B	AG-4	AG-4-HG-I	FJ480878
K9B	AG-7	AG-7	FJ480895
M9B	AG-4	AG-4-HG-I	FJ480877
B1B6	Binucleate	AG-F	FJ480896
H20B	AG-4	AG-4-HG-III	FJ480888
Tobacco isolates			
SAK2	AG-4	AG-4 HG-III	FJ480930
SAK3	AG-4	AG-4 HG-III	FJ480927
SAK5	AG-2	AG 2-1	FJ480920
SAK7	AG-2	AG 2-1	FJ480902
SBK7	AG-2	AG 2-1	FJ480903
SCK1	AG-2	AG 2-1	FJ480921
SCK2	AG-2	AG 2-1	FJ480904
SCK7	AG-4	AG-4 HG-I	FJ480922
SDK3	AG-4	AG-4 HG-III	FJ480928
SDK4	AG-2	AG 2-1	FJ480901
SEK1	AG-2	AG 2-1	FJ480900
SZK3	AG-2	AG 2-1	FJ480905
SZK5	AG-2	AG 2-1	FJ480906
SZK8	AG-4	AG-4 HG-III	FJ480929
SHK4	AG-2	AG 2-1	FJ480907
SHK8	AG-4	AG-4 HG-I	FJ480923
SIK1	AG-2	AG 2-1	FJ480908
SKK2	AG-2	AG 2-1	FJ480899
SKK3	AG-2	AG 2-1	FJ480898
SMK1	AG-2	AG 2-1	FJ480909
SOK1	AG-2	AG 2-1	FJ480910
KA5K	AG-2	AG 2-1	FJ480911
KB9K	AG-2	AG 2-1	FJ480913
KB10K	AG-2	AG 2-1	FJ480912
KC7K	AG-4	AG-4 HG-I	FJ480925
KC10K	AG-4	AG-4 HG-I	FJ480924
KD5K	AG-4	AG-4 HG-I	FJ480926
KD7K	AG-2	AG 2-1	FJ480914
XK2	AG-2	AG 2-1	FJ480915
XK9	AG-2	AG 2-1	FJ480916
KK4	AG-2	AG 2-1	FJ480917
DOI4K	AG-2	AG 2-1	FJ480918
DOI7K	AG-2	AG 2-1	FJ480919
LIT3K	AG-5	AG-5	FJ480931

^y Anastomosis groups (AGs) based on hyphal anastomosis reactions with tester isolates.

^z Accession number of sequences obtained from the National Center for Biotechnology Information.

TOB isolates, 70 isolates gave a C2 reaction with the AG-2 tester isolate, 16 isolates with AG-4, and one isolate with AG-5 (Table 3).

ITS and phylogenetic analysis. Most of the isolates tested yielded a 700-bp product, whereas the binucleate isolates and the isolates belonging in AG-7 yielded

a 680-bp product. Sequence analysis of the PCR products and comparison with already existing sequences in NCBI showed that, among the 36 COT isolates tested, 18 belonged to AG-4-HG-I, 1 to AG-4-HG-II, 8 to AG-4-HG-III, 1 belonged to AG-2-1, 1 to AG-3, 5 to AG-7, and 2 binucleate isolates to AG-F (Table 4). Similarly, among the 34 TOB isolates tested, 24 belonged to AG-2-1, 5 to AG-4-HG-I, 4 to AG-4-HG-III, and 1 to AG-5 (Table 4). For both hosts, molecular data were in accordance with the classification of the isolates based on the results of the hyphal anastomosis.

The alignment of the obtained sequences showed that the 5.8S rDNA gene sequence was completely conserved in all the isolates tested independently of AG classification (*data not shown*; sequences are available upon request). The phylogenetic tree of COT isolates was rooted using a *R. cerealis* sequence (GenBank accession no. AJ302009) while the phylogenetic tree of the TOB isolates was rooted using two binucleate *Rhizoctonia* spp. isolates (accession nos. AB219144 and DQ102433). The phylogenetic trees showed that the isolates tested separated distinctly based on their AG and that each AG clustered together. The phylogenetic tree for the cotton isolates was mainly separated into two clades (Fig. 2). One clade was composed of isolates AG-2-1 and AG-3, and another contained AG-4, AG-7, and binucleate AG-F. Each HG in AG-4 was separated in the clade composed of AG-4 isolates. Similarly, the phylogenetic tree for the tobacco isolates was also separated into two clades (Fig. 3). One clade was composed only of AG-2-1 isolates and another was composed of AG-4 and AG-5 isolates. Both phylogenetic trees also revealed that isolates obtained from different regions could not be separated into distinct clades on the basis of their geographic origin.

Hyphal growth response at different temperatures. As a group, binucleate isolates had an optimum growth temperature of 30°C. They showed only a slight growth at 10°C but grew well at 35°C (Table 5). On average, the AG-2-1 isolates grew more slowly than isolates of other AGs. The AG-3 isolate had an optimum growth temperature of 25°C but showed minimal growth at 10 and 35°C. The AG-4 and AG-7 isolates had an optimum growth at 30°C, grew well at 35°C, and showed minimal growth at 10°C. The AG-4 subgroups did not vary significantly in growth rate, with the exception of the AG-4-HG-II isolate, which had a reduced growth rate at 30°C and grew only slightly at 35°C. The AG-5 isolate had the ability to grow at both 10 and 35°C and had an optimum temperature of 25 to 30°C (Table 5).

Pathogenicity and aggressiveness. On cotton plants, the isolate AG-2-1 and the binucleate isolates obtained from cotton were less aggressive, with disease index

Fig. 2. Phylogenetic tree constructed by the neighbor-joining method based on the sequences of the internal transcribed spacer (ITS)1-5.8S-ITS2 ribosomal DNA regions of *Rhizoctonia* spp. isolated from cotton seedlings. Distances were determined by Kimura's two-parameter model, omitting all sites with gaps. A *Rhizoctonia cereale* sequence (AJ302009) was used as an outgroup. Numbers next to the branches represent the percentage of congruent clusters in 1,000 bootstrap trials when the values were more than 70%. Numbers in parenthesis are the accession numbers of sequences obtained from the National Center for Biotechnology Information.

values of 1.0 and 1.4, respectively, compared with other COT isolates belonging to several AGs (Table 6). Even lower was the disease index value of 0.3 caused on cotton by the AG-5 isolate obtained from tobacco. The most aggressive isolates on cotton were the AG-4 isolates, independently of their host of origin, and the AG-7 isolates obtained from cotton.

Similarly, on radish and sugar beet, the AG-2-1 and the binucleate isolates were the least aggressive, with disease index values of 0.8 to 2.8 (Table 6). The remaining isolates tested were equally aggressive on both hosts.

Barley was the least susceptible host. Binucleate, AG-2-1, AG-3, and AG-5 isolates caused no symptoms on barley plants.

AG-7 and AG-4 isolates caused damage on barley seedlings with disease index values ranging from 0.0 to 1.4 (Table 6).

On tobacco, the most aggressive isolates were AG-4-HG-I, AG-4-HG-III, and AG-7 causing symptoms on 46 to 76% of the seedlings (Table 6). Interestingly, the AG-4-HG-II isolate from cotton caused no symptoms on tobacco seedlings. The binu-

Fig. 3. Phylogenetic tree constructed by the neighbor-joining method based on the sequences of the internal transcribed spacer (ITS)1-5.8S-ITS2 ribosomal DNA regions of *Rhizoctonia solani* isolated from tobacco seedlings. Distances were determined by Kimura's two-parameter model, omitting all sites with gaps. Two binucleate *Rhizoctonia* sequences were used to root the tree. Numbers alongside branches represent the percentage of congruent clusters in 1,000 bootstrap trials when the values were more than 70%. Numbers in parenthesis are the accession numbers of sequences obtained from the National Center for Biotechnology Information.

cleate and AG-3 isolates from cotton were less aggressive on tobacco, causing symptoms on 3 and 20% of the seedlings, respectively. The AG-2-1 isolates, independently of their host of origin, were moderately aggressive, causing symptoms on 35 to 40% of tobacco seedlings (Table 6).

DISCUSSION

The results of the study indicated that damping-off disease of cotton and tobacco seedlings in Greece caused by *Rhizoctonia* spp. is mainly due to *R. solani*. Variability in the number and frequency of different AGs was observed on both hosts.

The relatively high proportion of binucleate *Rhizoctonia* spp. (21.5% of isolates) isolated from cotton in this study was also found previously in Spain (26). In reports from other hosts such as sugar beet (20), soybean (12), or cauliflower (35), the proportion of binucleate isolates was low. Molecular data presented in the current report showed that binucleate *Rhizoctonia* isolates from cotton were of AG-F. To the best of our knowledge, this is the first report of AG-F isolates attacking cotton worldwide.

AG-4 predominated among the cotton isolates of *R. solani*, followed by AG-7, which agrees with previous reports from other countries (2,3,6,26,28). Isolates of AG-4 were obtained from all the sampled cotton fields in all the sampling regions. In this study, samples were taken from latitudes ranging from 39 to 41°N and distribution of AG-4 isolates varied with latitude, as previously observed in the United States (19). Isolation of AG-2 and AG-3 *R. solani* from cotton is reported, to the best of our knowledge, for first time.

AG-2-1 was the predominant AG isolated from tobacco, followed by AG-4. Previous reports suggested that damping-off of tobacco seedlings is most closely associated with AG-4 (16,32). The effect of the temperature and differences in the soil texture, along with possible effects of the differences in agronomic practices among different countries, may explain the observed prevalence of AG 2-1 isolates in tobacco seedlings in Greece in contrast to reports of AG-4 isolate prevalence in other countries. In addition, one AG-5 isolate was recovered. To the best of our knowledge, infection of tobacco seedlings by

AG-5 *R. solani* has been previously reported only in Italy (31).

The levels of variability in ITS1 and ITS2 for the isolates examined were similar to those observed in previous studies (5,23,24,41,45). Construction of phylogenetic trees showed that the three different AGs (AG-2, AG-4, and AG-5) recovered from tobacco isolates were located in three different clades on the phylogenetic tree, suggesting low genetic relatedness among them. Phylogenetic analysis of the cotton isolates showed that the AG-2 and AG-3 isolates were located on the same clade, suggesting high genetic similarity, as previously reported (45). This may explain the morphological similarity of these isolates. Similarly, the binucleate isolates of AG-7 and the *R. solani* subgroups of AG-4 and the AG-7 isolates were located on the same clade. This genetic relatedness of binucleate and AG-4 isolates has been observed in another recent study (44). Such results are informative for evaluating the evolutionary relationships among the several AGs of *R. solani* and contribute to the better understanding of the biology the species complex.

Table 5. Radial growth rate (mm/24 h) on potato dextrose agar of *Rhizoctonia* fungi by anastomosis group (AG) and subgroup that were isolated from cotton and tobacco seedlings in northern and central regions of Greece

AG	No. of isolates	Temperature (°C) ^z					
		10	15	20	25	30	35
Cotton isolates							
Binucleate	16	3.4 a	10.9 a	16.4 a	21.4 a	28.8 a	21.0 ab
AG-2-1	1	2.3 a	5.2 a	19.5 ab	22.0 ab	19.0 a	3.7 a
AG-3	1	4.3 a	12.6 ab	11.6 a	18.3 a	15.8 a	8.0 a
AG-4-HG-I	18	4.6 a	17.0 b	26.0 b	31.0 b	33.3 a	19.2 a
AG-4-HG-II	1	8.7 a	16.0 ab	23.7 ab	24.0 ab	24.2 a	7.3 a
AG-4-HG-III	8	6.4 a	17.1 b	24.1 ab	28.1 b	31.0 a	23.9 b
AG-7	5	4.9 a	15.9 ab	23.3 ab	24.9 ab	33.8 a	26.3 c
Tobacco isolates							
Binucleate	2	1.8 a	9.4 a	19.0 a	22.5 ab	28.2 ab	24.2 b
AG-2-1	24	5.3 a	11.3 a	18.5 a	19.0 a	16.2 a	4.3 a
AG-4-HG-I	5	3.2 a	16.6 a	25.1 a	30.2 b	33.4 b	16.9 b
AG-4-HG-III	4	3.6 a	16.9 a	24.6 a	28.6 ab	31.0 b	23.7 b
AG-5	1	7.8 a	14.7 a	25.0 a	25.5 ab	26.5 ab	14.1 b

^z Least square mean values per temperature followed by the same letter are significantly different at $P = 0.002$ according to the nonparametric tests of Kruskal-Wallis and Mann-Whitney.

Table 6. Disease severity on cotton, radish, sugar beet, and barley seedling plants and disease incidence on tobacco seedling plants caused by binucleate *Rhizoctonia* spp. and *Rhizoctonia solani* isolates belonging to different anastomosis groups (AGs) and subgroups^z

AG	No. of isolates	Cotton	Radish	Sugar beet	Barley	Tobacco
Cotton isolates						
Binucleate	2	1.4 a	2.5 b	2.2 a	0.0 a	3.8 a
AG-2-1	1	1.0 a	0.8 a	2.8 a	0.0 a	37.5 b
AG-3	1	3.3 c	3.6 cd	3.4 b	0.0 a	20.0 ab
AG-4-HG-I	8	3.3 c	3.5 cd	3.8 c	0.8 c	76.3 c
AG-4-HG-II	1	2.7 b	3.7 cd	4.0 c	1.1 cd	2.5 a
AG-4-HG-III	5	2.4 b	3.5 c	3.8 c	1.4 d	46.0 b
AG-7	3	3.5 c	3.8 d	3.9 c	0.3 b	53.3 b
Tobacco isolates						
Binucleate	2	1.4 a	2.4 ab	2.8 ab	0.0 a	35.0 a
AG-2-1	10	1.3 a	1.6 a	2.6 a	0.0 a	31.3 a
AG-4-HG-I	4	2.4 a	3.5 ab	4.0 b	0.7 ab	68.1 b
AG-4-HG-III	2	3.0 a	3.9 b	4.0 ab	1.2 b	52.5 ab
AG-5	1	0.3 a	3.2 ab	4.0 ab	0.0 a	30.0 a

^z Disease severity was measured using a disease index scale ranging from 0 (no damage) to 4 (seedling dead), and disease incidence was measured as percentage of dead seedling plants. Least square means followed by different letter within the columns are not significantly different according to the nonparametric tests of Kruskal-Wallis and Mann-Whitney at $P = 0.002$.

As reported in previous studies (39,46), AG-4 and AG-7 isolates had higher growth rates than the remaining AGs and subgroups at temperatures of 10 to 30°C. The AG-7 isolates showed higher growth rates at 30 and 35°C compared with other isolates, as previously reported (2). The similarity of our results to those from countries with diverse climates suggests that most isolates and subgroups of *R. solani* do not exhibit temperature adaptation across geographic areas (19). However, the lower mycelial growth rate of the AG-2-1 isolates compared with that of AG-4, and the lower temperature optima for these isolates, agree with previous reports (34,43,46). AG-2-1 isolates are known to prevail in colder conditions than AG-4 isolates (48,49). In the current study, the AG-2-1 isolates were obtained mainly from tobacco fields located in cool, semi-mountain regions where tobacco is cultivated in Greece.

Results of pathogenicity studies were similar to previous reports (1,2). AG-2-1 isolates were less aggressive than AG-4 and AG-7 isolates and did not cause any symptoms on barley. *R. solani* AG-2-1 is a heterogeneous subgroup in terms of both genetic variability and virulence on several hosts (9,35,42,49,54). Although AG-5 isolates are considered to be weak pathogens (30), in the current study the AG-5 isolate was highly aggressive against radish and sugar beet and was moderately aggressive on tobacco. Nicoletti and Lahoz (31) also found that AG-5 was not aggressive on tobacco seedlings. Although pathogenicity to different hosts has been used in the past to classify AGs and subgroups of *R. solani* (9), it seems that pathogenicity is not a reliable characteristic of these groups (29,33,52).

In Northern Greece, tobacco and cotton are rotated with susceptible hosts such as sugar beet, tomato, and maize, suggesting that crop rotation may be insufficient for the successful management of diseases caused by *R. solani* and binucleate *Rhizoctonia* spp. Small grains that are less susceptible to the AGs recovered in the study could be included in the rotation schemes. Wheat and barley are the two main small-grain species cultivated in northern Greece. The predominant AGs found in this study should be tested for pathogenicity on the main wheat and barley cultivars cultivated in the region to determine their suitability as rotation crops. Furthermore, based on the findings of the current report, the efficacy of fungicides applied either as seed treatments in cotton or as soil-drench in tobacco should be investigated.

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