Characterization of AG-13, a Newly Reported Anastomosis Group of *Rhizoctonia solani*

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ABSTRACT

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Rhizoctonia solani anastomosis group (AG)-13 was collected from diseased roots of field grown cotton plants in Georgia in the United States. Isolates of AG-13 did not anastomose with tester isolates of AG-1 through AG-12. Mycelium of all isolates of AG-13 were light brown but darkened as cultures aged. All isolates produced aerial mycelium. Concentric rings were visible after 3 to 4 days of growth but disappeared as cultures aged and darkened. Individual sclerotia were up to 1.5 mm in

diameter, similar in color to the mycelium, and generally embedded in the agar. Clumps of sclerotia up to 5 mm in diameter were produced on the agar surface. All attempts to induce basidiospore production were unsuccessful. The 5.8S region of the rDNA from isolates of AG-13 was identical in length and sequence to isolates of all other AGs of *R. solani*. Length and sequence of the internal transcribed spacer (ITS) regions of rDNA from isolates of AG-13 were unique among AGs of *R. solani*. Similarity between AG-13 and other AGs of *R. solani* ranged from 68 to 85% for ITS region 1 and 85 to 95% for ITS region 2. Selected isolates of AG-13 caused minor or no damage to barley, cauliflower, cotton, lettuce, potato, and radish in laboratory or greenhouse studies.

Rhizoctonia solani Kühn, (teleomorph *Thanatephorus cucumeris* (A. B. Frank) Donk) is a plant-pathogenic fungus that causes disease on a broad array of host plant species (30). It often is associated with subterranean portions of plants, although leaf spots and web blights also are well known (30). *R. solani* is called a species complex because it contains many related but genetically isolated subspecific groups (1,3,25). Major subspecific groups of *R. solani* traditionally have been identified on the basis of hyphal anastomosis reactions and are called anastomosis groups (AGs) (6,11). Currently there are 13 AGs described in the literature (AG-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -12, and -BI) although, in a recent paper (8), a proposal was made to define AG-BI as a subset of AG-2. In addition to AG-2, AG-1, -3, -4, -6, -8, and -9 also have been divided into subsets (8,25). The four AGs described initially, (AG-1, -2, -3, and -4) (27) cause important diseases on plants worldwide, whereas AGs described later are less destructive pathogens and generally have more restricted geographic distributions. AG-12 is unique among AGs of *R. solani* because many of its isolates are mycorrhizal in the field with the Australian orchid *Pterostylis acuminata* R.Br (11).

One of the many plant species that *R. solani* can attack is cotton (*Gossypium hirsutum* L.), on which it causes a root and hypocotyl rot. In random sampling and during a systematic season-long sampling of Georgia cotton fields for incidence of *R. solani* and other root pathogens, most isolates of *R. solani* recovered were members of AG-4, a root rotting *R. solani* commonly associated with cotton. However, 15 isolates that would not anastomose with testers representing the known AGs also were recovered. The objective of this work was to characterize AG-13 by describing: (i) its unique anastomosis reaction among AGs of *R. solani*; (ii) its cultural characteristics on potato dextrose agar (PDA); (iii) its

hyphal diameter, nuclei per cell, and radial growth rate; (iv) its virulence on selected hosts; (v) its cellular fatty acid methyl esterase profile; and (vi) its rDNA-internal transcribed spacer (ITS) region length and sequence. These evaluative procedures include many of the methods typically used when characterizing newly identified AGs, or subsets of AGs, of *R. solani*. A preliminary report of some of these findings has been published in which we proposed designating the new group AG-13 (7).

MATERIALS AND METHODS

Isolate collection. Fifteen isolates of AG-13 were recovered from cotton plants collected from fields in three different counties in Georgia. Isolate 177 was collected on the University Research Farm in Burke County, GA in 1996. This field of Dothan loamy sand had been in continuous cotton for more than 3 years. Isolates Hud 2-1 and Hud 2-8 were collected in Dooley County, GA in 1996. The soil in this field also was a Dothan loamy sand and, in the 2 years prior to sampling, was planted to cotton and snap bean (*Phaseolus vulgaris* L.). The 12 remaining isolates of *R. solani* AG-13, including RB-45, -251, -301, -303, -329, -331, -337, -357, -384, -386, -395, and -429, were collected from a field on the Coastal Plain Experiment Station, Tift County, GA in 1998. That Tifton loamy sand field had been planted to cotton for the previous 2 years. The 15 isolates were recovered from diseased root tissue of 15 different cotton plants that ranged in age from 7 to 28 days. Segments (1 cm) of root tissue were excised from each plant, placed in 0.5% NaOCl for 3 min, rinsed in sterile water, then placed onto PDA (Difco Laboratories, Detroit) in petri dishes. As they appeared, individual fungal colonies possessing the general characteristics of *R. solani* were transferred to PDA.

Isolate characterization. AG determinations were made according to a modified method of Parmeter et al. (5,27). Anastomosis reactions were grouped into categories where category 0 (C0) is no reaction and C3, the clonal anastomosis reaction, has an

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appearance equivalent to self anastomosis (6). To assess similarity within AG-13, 14 of the 15 isolates of AG-13 were paired with each other in all possible combinations.

Attempts were made to induce isolates of AG-13 to sporulate on 1.5% water agar, 2% V8 juice agar (18 g of agar plus 20 ml of V8 juice per liter), and with the soil overlay technique (24). For each of the three methods, two or more petri dishes were prepared and incubated at 20 to 23°C for 21 days. Cultures were examined every second or third day for the presence of hymenia and basidiospores. All 15 isolates were evaluated on the three media.

Cultural characteristics of AG-13 were observed by growing each isolate on PDA at 20 to 23°C in the dark. Three petri dishes of each isolate were grown in each of two runs. Each time, observations of cultural characteristics were made over a 21-day period.

Hyphal diameter and the number of nuclei per cell were determined on cultures grown at 20 to 23°C on cellophane dipped in dilute (13 g of PDA per liter) PDA overlaying 1.5% water agar in petri dishes. Mycelium was stained with 3% KOH and safranin O (5) and examined with bright field optics at $\times 400$. Three petri dishes of each isolate were grown for each of two runs. Hyphal diameter was determined on isolates 177, Hud 2-1, Hud 2-8, RB-337, RB-386, RB-395, and RB-429 by measuring 20 cells per isolate per dish at right angles to the longitudinal cell wall. Using the same seven isolates, nuclei were counted in 20 cells per isolate

per dish in cells selected from a location in the stain gradient where nuclei and septa could be clearly observed.

Radial growth at 25°C of six randomly selected isolates of AG-13 (177, Hud 2-1, Hud 2-8, RB-357, RB-395, and RB-429) plus one isolate each of AG-1 (M43), -2-1 (F56L), -3 PT (SCL24), and -4 (AH-1) was determined by growing them on PDA in the dark. Mycelial disks (4 mm in diameter) cut from the advancing edge of growing colonies of each tested isolate were placed in the center of 100-by-15-mm petri dishes containing PDA. Radial growth was measured at 24 and 72 h after incubation began. Each treatment was replicated three times and the experiment was repeated once.

Virulence determination. Isolates of AG-13 were recovered from field-grown cotton plants; therefore, virulence of all 15 isolates was determined on cotton in a greenhouse study. Inoculum was produced by seeding flasks containing a sterilized cornmeal/sand (CMS) mixture (3 g of cornmeal, 100 g of sand, and 20 ml of distilled water) with each isolate followed by a 14-day incubation at 20 to 21°C. Six seeds of the cotton cv. Paymaster 1220 were planted at a depth of 2.5 cm in 20-by-200-cm pots containing 15 ml of CMS inoculum and 2.5 liters of Tifton loamy sand. Inoculum of the appropriate fungal isolate was mixed with the soil in each pot prior to planting. The number of healthy, diseased, and dead plants was counted after 17 and 28 days. Fungi were isolated from dead plants of each treatment to confirm Koch's postulates.

TABLE 1. Tester isolates representing the 12 established anastomosis groups (AGs) of *Rhizoctonia solani* used to determine the anastomosis group identity of AG-13^a

AG	Subgroup ^b	Isolate	Origin	Host	Collector or supplier
1	IA	CS-Ka	Japan	<i>Oryza sativa</i>	S. Kuninaga
1	IB	SFBV-1	Japan	<i>Beta vulgaris</i>	S. Kuninaga
1	IC	M43	Quebec	<i>Pinus resinosa</i>	N. A. Anderson
2	I	F56L	Alaska	<i>Solanum tuberosum</i>	D. E. Carling
2	I	457	Japan	Unknown	E. E. Butler
2	BI	AI1-4	Japan	Soil	S. Kuninaga
2	BI	TE2-4	Japan	Soil	S. Kuninaga
2	2IIIB	C330	Japan	<i>O. sativa</i>	A. Ogoshi
2	2IV	RI-64	Japan	<i>B. vulgaris</i>	A. Ogoshi
3	PT	ST-11-6	Japan	<i>S. tuberosum</i>	A. Ogoshi
3	PT	SCL24	Alaska	<i>S. tuberosum</i>	D. E. Carling
3	TB	263	Georgia	<i>Nicotiana tabacum</i>	A. Csinos
4	HG-I	AH-1	Japan	<i>Arachis hypogaea</i>	S. Kuninaga
4	HG-II	RR5-2	Japan	<i>B. vulgaris</i>	S. Kuninaga
5	...	ST-6-1	Japan	<i>S. tuberosum</i>	A. Ogoshi
5	...	Rh184	Japan	<i>B. vulgaris</i>	S. Naito
6	HG-I	HAM1-1	Japan	Soil	S. Kuninaga
6	GV	NKN2-1	Japan	Soil	S. Kuninaga
6	?	SPb	NSW, Australia	Leaf litter	E. J. Pope
7	...	1535	Japan	Soil	S. Kuninaga
7	...	1556	Japan	Soil	S. Kuninaga
7	...	92PT123-1A	Arkansas	Soil	C. S. Rothrock
7	...	91ST8057-2A	Arkansas	Soil	C. S. Rothrock
7	...	91ST8112-9A	Arkansas	Soil	C. S. Rothrock
8	ZG1-1	89570	Western Australia	<i>Hordeum vulgare</i>	G. C. MacNish
8	ZG1-1	91784	Western Australia	<i>Lupinus angustifolius</i>	G. C. MacNish
8	ZG1-2	90812	Western Australia	<i>L. angustifolius</i>	G. C. MacNish
8	ZG1-2	SA50	Southern Australia	<i>Avena sativa</i>	A. Dubé
8	ZG1-3	SA1512	Southern Australia	<i>H. vulgare</i>	S. M. Neate
8	ZG1-4	88351	Western Australia	<i>H. vulgare</i>	G. C. MacNish
8	ZG1-4	91006	Western Australia	<i>H. vulgare</i>	G. C. MacNish
8	ZG1-5	91623	Western Australia	<i>H. vulgare</i>	G. C. MacNish
8	ZG1-5	92547	Western Australia	<i>H. vulgare</i>	G. C. MacNish
9	TP	V12M	Alaska	<i>S. tuberosum</i>	D. E. Carling
9	TX	S9R1	Alaska	Soil	D. E. Carling
10	...	W395	Washington	<i>Triticum aestivum</i>	A. Ogoshi
10	...	W3751	Washington	<i>T. aestivum</i>	A. Ogoshi
11	...	R1352	W. Australia	<i>L. angustifolius</i>	M. W. Sweetingham
11	...	ROTH26	Arkansas	<i>O. sativa</i>	C. S. Rothrock
12	...	H-1	NSW, Australia	<i>Pterostylis acuminata</i>	A. Perkins
12	...	W-1	NSW, Australia	<i>P. acuminata</i>	E. Pope
12	...	SWd	NSW, Australia	Leaf litter	E. Pope

^a Each isolate in this list was paired with *R. solani* AG-13 isolates Hud 2-1, Hud 2-8, and 177. All pairings gave the C0 anastomosis reaction.

^b ... indicates subgroups either do not exist or have not been formally designated; ? indicates subgroup identity not known.

Five replicates of each of the 16 treatments were arranged in a randomized complete block design and the experiment was repeated once. Data were analyzed by analysis of variance and means were separated with Duncan's multiple range test.

Virulence of isolates 177, Hud 2-1, and Hud 2-8 and one isolate each from AG-1 (M43), -2-1 (KAB1), -2-2 (RI-64), -3 PT (SCL24), and -4 (AH-1) was determined on germinating seeds of radish (*Raphanus sativus* L.), cauliflower (*Brassica oleracea* L., var. *botrytis* L.), head lettuce (*Lactuca sativa* L.), and barley (*Hordeum vulgare* L.) developing on 2% water agar in 100-by-15-mm petri dishes. These hosts were chosen for use in this study because they have been used in other recent characterizations of AGs and subsets of AGs of *R. solani* (8,11). Each petri dish was seeded in the center with a 7-mm-diameter disk cut from the growing edge of the appropriate fungal isolate growing on PDA. Sterile PDA disks were used in the control treatments. Two days after seeding of the petri dishes with the various fungus treatments, 18 surface-sterilized (0.5% NaOCl for 1 min) seeds (six seeds per dish) were evenly spaced on the agar surface in a circle half way between the center and the edge of the dish. The growing fungal colonies reached the seeds just as they began to germinate. Stacks of petri dishes were placed in plastic boxes in a growth chamber in the dark and incubated at 21°C. Three and eight days later, assessments of disease on seedlings were made on a 0-to-4 scale in which 0 = no disease; 1 = minor discoloration of hypocotyl; 2 = discoloration plus small (<1 mm in diameter) necrotic lesions on stem, hypocotyl, or root; 3 = discoloration plus large (>1 mm in diameter) necrotic lesions on stem, hypocotyl, or root; and 4 = seedling dead. Experiments to determine virulence on radish, cauliflower, lettuce, and barley were arranged in a randomized complete block design and were repeated once.

Virulence also was determined on potato (9) for isolates 177, Hud 2-1, and Hud 2-8 plus one isolate of AG-3 (SCL24). Surface-disinfested potato seed pieces were placed 15 cm deep in pasteurized soil inoculated with the appropriate fungal isolates. Damage was assessed according to a rating scale described by Carling and Leiner (9) after 5 weeks at 10°C in a dark growth chamber. The five treatments were arranged in a randomized complete block design and the study was repeated once. Data were analyzed by analysis of variance and means were separated with Duncan's multiple range test.

Cellular fatty acid methyl ester analysis. Of the 15 isolates of AG-13, 14 (except 177) were grown at 27°C on 100-by-15-mm petri dishes containing PDA. Four 3-mm-diameter mycelial pieces were inoculated into flasks containing 500 ml of potato dextrose broth (PDB) and incubated at 27°C for 4 days. The mycelium was removed from the flasks and 2 g was weighed, washed with sterile distilled water for 1 min, then placed into a 13-by-100-mm screw-top test tube for fatty acid extraction using previously described methods (32,33). Fatty acid methyl ester (FAME) compositions were determined for each isolate using gas chromatography procedures from the Hewlett-Packard application note 228-41 (Hewlett-Packard, Pittsburgh, PA) (4) as originally adapted for *Rhizoctonia* spp. (31). A composite profile for *R. solani* AG-13 isolates was generated based on the fatty acid composition of each of the five replicates of each isolate. All data was stored as a library reference to make it possible for future comparisons with other samples of *Rhizoctonia*.

DNA isolation. Six isolates of AG-13 (177, Hud 2-1, Hud 2-8, RB-251, RB-357, and RB-429) were incubated at 25°C in PDB for 4 days. Mycelial mats were harvested by filtration, blotted dry, lyophilized, ground to a fine powder, then stored at -20°C prior to DNA isolation. Genomic DNA was extracted according to a modification of a previously described procedure (19). Mycelial powder was suspended in NTE buffer (100 mM NaCl, 30 mM Tris-HCl, 10 mM EDTA, 10 mM 2-mercaptoethanol, pH 8.0) containing 0.5% (vol/vol) Nonidet P-40 and centrifuged at 10,000 rpm for 1 min. The pellet was resuspended in NSE (100 mM NaCl, 200 mM

sucrose, 10 mM EDTA, pH 4.4) before adding TES buffer (10 mM Tris-HCl, 250 mM EDTA, pH 9.2, 2.5% sodium dodecyl sulfate). The homogenate was incubated at 55°C for 30 min. The solution was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (50:48:2) and centrifuged at 10,000 rpm for 20 min. The upper layer was mixed with 0.1 volume of 3 M sodium acetate, then with 0.54 volume of isopropanol. The DNA pellet was collected, rinsed with 70% ethanol, then dried under vacuum.

rDNA-ITS sequence analysis. The region of the rDNA repeat from the 3' end of the 18S gene to the 5' end of the 28S gene were amplified using six polymerase chain reaction (PCR) primers: ITS 2, 3, 4, 5, NS 7 (36), and LR3 (29). Each primer was linked to the Rhodamine-labeled primers RV-M and M4 (TaKaRa, Ltd., Shiga Prefecture, Japan) for the cycle sequencing reactions. The PCR amplification reactions were performed in a 50-μl mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM MgCl₂, 200 μl of each of the four deoxynucleoside triphosphates, 5 pmol of each primer, 1.0 μl of template, and 2.5 units of *Taq* polymerase. The amplification was performed with a thermocycler MP (TaKaRa, Ltd.). The cycle parameters included an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. A single-stranded DNA template was prepared using a previously described procedure (35). One of the PCR primers was biotinylated; the biotinylated PCR products were immobilized on streptavidin-coated paramagnetic beads (Dynabeads M-280 streptavidin, DYNAL, Oslo, Norway), and single-stranded DNA was prepared according to the manufacturer's instructions. Both strands then were sequenced directly by cycle sequencing using a Thermo Sequenase premixed cycle sequencing kit (Amersham Life Science, Buckinghamshire, UK) and the Rhodamine-labeled primers described above. The sequencing products were separated in 6% polyacrylamide gel, and the fragments were monitored using a HITACHI DNA sequencer SQ5500E (Hitachi Ltd., Tokyo).

Sequence analysis. The sequence data were checked between complementary strands and the resulting sequences were aligned using the computer software package CLUSTAL V (13). The alignment of all sequences was checked visually. DNA sequence similarity based on the proportion of different nucleotide sites, including insertion-deletion sites, was calculated from the data using the Lipman-Pearson algorithm (20) in the DNASIS software package (Hitachi Ltd., Tokyo). Estimates of phylogenetic relationships were determined by the maximum parsimony method from the

	# 177	Hud 2-1	Hud 2-8	RB 45	RB 251	RB 301	RB 329	RB 331	RB 337	RB 357	RB 384	RB 386	RB 395	RB 429
#177	C3	C2	C2	C2	C2	C2	C2	C2	C2	C2	C2	C2	C2	C2
Hud 2-1		C3	C2	C2	C2	C2	C2	C2	C2	C2	C2	C2	C2	C2
Hud 2-8			C3	C2	C2	C2	C2	C2	C2	C2	C2	C2	C2	C2
RB 45				C3	C2	C2	C2	C2	C2	C2	C2	C2	C2	C2
RB 251					C3	C2	C2	C2	C2	C2	C2	C2	C2	C2
RB 301						C3	C2	C2	C2	C2	C2	C2	C2	C2
RB 329							C3	C2	C2	C2	C3	C3	C2	C2
RB 331								C3	C2	C2	C2	C2	C2	C2
RB 337									C2 = "STRONG"	C3	C3	C2	C2	C3
RB 357										C3	C2	C2	C2	C3
RB 384											C3 = "CLONAL"	C3	C2	C2
RB 386												C3	C2	C2
RB 395													C3	C2
RB 429														C3

Fig. 1. Anastomosis reactions among 14 isolates of *Rhizoctonia solani* anastomosis group (AG)-13 paired in all combinations.

Phylogenetic Analysis Using Parsimony (PAUP) program 3.1.1 (D. L. Swofford, 1993, Illinois Natural History Survey, Champaign). Maximum parsimony trees were identified using the heuristic search option in PAUP using only informative characters. The strength of the internal branches from the resulting trees was assessed by bootstrap analysis using 1,000 replications. *Thanatephorus orchidicola* (ATCC 24752) and *T. pennatus* (ATCC 64537) were included as outliers.

RESULTS

Anastomosis reactions. Isolates 177, Hud 2-1, and Hud 2-8 were paired with two or more tester isolates representing each of the 12 AGs of *R. solani* (Table 1). AGs were represented by two or more testers each and, in addition, many AG subsets were represented by one or more testers. Thus, a total of 42 tester isolates were included. All three isolates of AG-13 gave C0 reactions with each of the 42 testers.

Of the 15 isolates of AG-13, 14 were paired in all combinations (Fig. 1). Three multimember vegetatively compatible populations (VCPs) (21) were identified by the anastomosis reactions of these pairings: (i) isolates RB-45 and RB-251, (ii) isolates RB-329, RB-384, and RB-386, and (iii) isolates RB-337, RB-357, and RB-429. Isolates in the same VCP give the C3 anastomosis reaction with one another. The remaining six isolates represented single-member VCPs because each gave the C2 anastomosis reaction with all other AG-13 isolates.

Isolate characterization. Mycelia of all tested isolates of AG-13 were light brown during early growth and all produced large amounts of aerial hyphae throughout the growth cycle. As cultures aged, their color darkened and most were very dark brown after 21 days. Concentric rings formed on all cultures by day three but rings tended to disappear as cultures matured and darkened. By day six, sclerotia formed near the edge of the petri dishes. However, after 21 days of growth, sclerotia were scattered randomly about the agar surface as well as in the agar. Individual sclerotia were tan when young, generally dark brown when mature, and up to 1.5 mm in diameter with clumps up to

5 mm in diameter. Some sclerotial clumps were found on the agar surface but most clumps and individual sclerotia were embedded in the agar.

The average hyphal diameter of seven isolates was 8.30 μ m and ranged from 8.15 to 8.48 μ m. The average number of nuclei per cell for the same seven isolates was 3.9 and ranged from 3.6 to 4.3. Despite repeated attempts, none of the isolates of AG-13 produced hymenia or basidiospores on 1.5% water agar, 2% V8 juice agar, or on soil overlay dishes.

After 24 and 72 h, the mean radial growth at 25°C of the six isolates of AG-13 was 32.7 mm (range 29.6 to 38.3) and 85 mm (no range because all six isolates had grown to the edge of the plates by 72 h), respectively. The radial growth of the four control isolates was 27.3 (range 22.3 to 37.5) after 24 h and 78.9 (range 70.3 to 85.0) mm after 72 h.

Virulence determination. Damage to shoot and root tissues of potato seedlings by isolates of AG-13 was limited to a few small (<2 mm in diameter) lesions. This was in contrast to the large girdling lesions and heavy damage caused on both shoot and root tissue by the isolate of AG-3 PT. Damage induced by isolates of AG-13 on cotton seedlings was limited to tissue discoloration and small lesions on root and hypocotyl. As with potato, it was possible to recover AG-13 isolates (14 of 15 in the cotton study) from these lesions, but the amount of damage to the cotton seedlings infected with AG-13 was not significantly different from the non-inoculated control. Isolates of AG-13 caused tissue discoloration and small necrotic lesions on or near the hypocotyl of cauliflower and radish by 8 days after inoculation (Table 2). Isolates of AG-1, -2, and -4 killed all cauliflower seedlings and all but one radish seedling by 8 days after inoculation, whereas AG-3 and the non-inoculated treatment caused no symptoms on either host. Only 2 of 18 lettuce seedlings displayed symptoms in response to inoculation with AG-13, and these symptoms were limited to a slight tissue discoloration. The AG-4 isolate killed 72% of the lettuce seedlings but the isolates of AG-1, -2, -3, and the noninoculated treatment caused no damage to lettuce. One lettuce seed inoculated with AG-2 was not viable. Isolates of AG-13 caused no damage to barley.

TABLE 2. Damage to germinating seedlings of radish, cauliflower, head lettuce, and barley caused by isolates *Rhizoctonia solani* anastomosis group (AG)-1, -2-1, -3, -4, -13, and a noninoculated control^a

Host	AG	Subgroup	No. of isolates	3 days after inoculation ^b			8 days after inoculation ^b		
				None	Medium	Dead	None	Medium	Dead
Radish	...	...	...	18	0	0	18	0	0
Radish	1	IC	1	0	16	0	0	0	16
Radish	2	1	1	0	14	0	0	0	14
Radish	3	PT	1	5	13	0	0	18	0
Radish	4	HGI	1	0	9	9	0	1	17
Radish	13	...	3	4	13	0	0	18	0
Cauliflower	...	...	...	17	0	0	17	0	0
Cauliflower	1	IC	1	0	18	0	0	0	18
Cauliflower	2	1	1	0	16	0	0	0	16
Cauliflower	3	PT	1	15	3	0	0	18	0
Cauliflower	4	HGI	1	0	2	16	0	0	18
Cauliflower	13	...	3	13	4	0	0	18	0
Lettuce	...	...	...	17	0	0	18	0	0
Lettuce	1	IC	1	17	0	0	17	0	0
Lettuce	2	1	1	17	1	0	17	0	1
Lettuce	3	PT	1	18	0	0	18	0	0
Lettuce	4	HGI	1	1	5	12	0	5	13
Lettuce	13	...	3	17	1	0	16	2	0
Barley	...	...	...	16	0	0	16	0	0
Barley	1	IC	1	18	0	0	2	16	0
Barley	2	1	1	17	0	0	0	17	0
Barley	3	PT	1	15	0	0	9	6	0
Barley	4	HGI	1	10	7	0	3	14	0
Barley	13	...	3	16	0	0	16	0	0

^a Experiment was run twice with similar results. Data shown are from the second run.

^b Medium = medium damage; tissue discoloration and necrosis starting at hypocotyl and sometimes extending down the root or up the stem. Number indicates the number of seedlings per plate exhibiting each symptom.

Cellular FAMES. Five common FAMES ranging from C15 to C18 were present in one or more of *R. solani* AG-13 isolates evaluated in this study. Approximately 95% of the FAMES present were the straight-chain fatty acids palmitic (16:0), oleic (18:1 cis 9), and stearic (18:0) acids. Also, pentadecanoic acid (15:0) was identified in two-thirds of the AG-13 isolates and 9-heptadecanoic acid (17:1 cis 9) in isolate RB-303. Myristic acid (14:0) was detected in a third of all isolates, but levels were low and thus were excluded from the summary list of FAMES provided by the MIDI program.

rDNA-ITS sequence analysis. The 5.8s region of the rDNA of the six tested isolates of AG-13 was identical in length and sequence to that of isolates representing all other AGs of *R. solani* (17). The ITS 1 region began 63 positions from the 5' end of primer ITS 5 and was 210 bp long. ITS 2 began 127 positions from the 5' end of primer ITS 3 and was 282 bp long. The ITS 1 regions of the six isolates of AG-13 evaluated were identical (Fig. 2), whereas similarity in the ITS 2 region ranged from 99 to 100% (0 to 3 nucleotide differences [n.d.]). When compared with all other AGs, isolates of AG-13 showed similarity levels of 68 to 85% (31 to 67 n.d.) in ITS 1 and 85 to 95% (15 to 42 n.d.) in ITS 2 (Fig. 2). A parsimony tree based on sequence differences in the ITS-5.8S rDNA region (Fig. 3) illustrates estimates of phylogenetic relationships among all AGs (AG-2 is presented as subsets -2-1, -2 BI, -2-2 IIIB, -2-2 IV, -2-2 LP, -2-3, and -2-4) of *R. solani*, including AG-13. In this tree, AG-13 is found in a cluster that includes all AGs of *R. solani* except AG-4.

DISCUSSION

Fifteen isolates of AG-13 were recovered from diseased hypocotyl and root tissue taken from cotton plants growing in the field. These 15 isolates represent less than 10% of the *R. solani* (most were AG-4) recovered from cotton over a 2-year period. When the AG-13 isolates were inoculated onto cotton plants in the greenhouse, damage was limited to discoloration on roots and hypocotyl. Similarly, isolates of AG-13 caused incipient tissue discoloration and necrotic lesions on potato stems. On germinating seedlings of radish and cauliflower, isolates of AG-13 caused tissue discoloration and necrotic lesions. Virulence of isolates of AG-13 in each of these controlled studies was lower than anticipated and reasons for this are uncertain at this time. One possible

explanation is that field symptoms originally attributed to AG-13 were the result of combined infections with other pathogens, including *R. solani* AG-4, in the field environment. Further study in the field is needed to ascertain the real potential of AG-13 to damage cotton and other crops.

Selected tester isolates of AG-13 did not anastomose (C0) with tester isolates representing all known AGs of *R. solani*. The absence of even the weak bridging (C1) anastomosis reaction illustrates the completeness of separation between AG-13 and all other AGs in the *R. solani* complex. All possible pairings were made among isolates of AG-13 and, in each pairing, either the C2 or the C3 anastomosis reactions was observed. That the preponderance of intra-AG-13 pairings yielded C2 rather than C3 anastomosis reactions illustrates a degree of variation within the AG. This is in contrast to the lack of variation, indicated by a high percentages of C3 (clonal) anastomosis reactions, observed among isolates within groups such as AG-3 TB (16). Among 14 isolates of AG-13, nine VCPs (21,26) were detected, six with a single member each and three with multiple members. Specific comments regarding the incidence of C3 reactions within AG-13 and its relationship to the magnitude of spatial separation cannot be made because physical distances between sampled plants were not recorded at the time of isolate collection. It is known, however, that members of the same VCP always came from the same field but that each isolates of AG-13 was collected from a different plant. These observations suggest that the incidence of clonality, as indicated by the C3 anastomosis reaction, may decrease as the distance between collection points increases (11,26). However, additional field collections, including collections of multiple isolates from the same plant or plant organs (26), are needed to verify this suggestion as well as to estimate the extent of clonality in AG-13.

Measurement of FAME content is another simple and useful technique for identifying groups within *R. solani*, one that effectively separates isolates of AG-13 from other AGs of *R. solani*. In other studies, linoleic, stearic, and palmitic acids were the three most common FAMES detected in isolates of *R. solani* AG-1 IA (31), AG-2-2 (32), AG-3 (34), and AG-4 (33), accounting for more than 90% of total FAMES in those isolates, although linoleic acid was not found in the tested isolates of AG-13. The absence of linoleic acid in AG-13 potentially could be used as an additional tool for separating it from other AGs; however, in another study

Fig. 2. Sequence similarity of rDNA-internal transcribed spacer (ITS) regions among isolates of anastomosis group (AG)-13 and all other AGs of *Rhizoctonia solani*. Other AGs and AGs subsets included in this figure are AG-1, -2-1, -2 BI, -2-2 IIIB, -2-2 IV, -2-3, -2-4, -3, -4, -5, -6, -7, -8, -9, -10, -11, and -12.

(4), linoleic acid also was absent in certain isolates of AG-4 and AG-7. Evaluation of additional isolates is needed to confirm the usefulness of this technique for specifically identifying isolates of AG-13.

Physical parameters including color, general colony morphology, number of nuclei per cell, and hyphal diameter, as well as growth rate at 25°C, fall within the range generally accepted for *R. solani* (10,24), but all efforts to induce hymenium and basidio-

spore production were unsuccessful, leaving us without this key piece of classical taxonomic information. However, sporulation by AG-12 also has not been observed either in the field or in the laboratory (11) and many isolates of most AGs of *R. solani* have been neither observed to sporulate in the field nor induced to sporulate in the laboratory (2). All other data, most notably that from rDNA-ITS sequence analysis (17), supports the placement of AG-13 within the *R. solani* complex.

AG-13 is the most recent of many AGs and subsets of AGs of *R. solani* that have been discovered and characterized in the past 10 years. Newly characterized AGs include AG-11 (12), AG-12 (11), and now AG-13, whereas newly characterized subsets of AGs include AG-1 ID (28), Nt isolates (18,23), AG-2-2 LP (14), AG-2-3 (22), AG-2-4 (8), and AG-3 TB (16,34). Although all are groups within the *R. solani* complex, it is important to remember that AGs and subsets of AGs are defined based on different criteria. Each AG, including AG-13, is a unique group within *R. solani* and can be separated from all other AGs based on hyphal anastomosis reaction alone. Subsets of AGs, on the other hand, cannot be differentiated from one another based on anastomosis reaction (8; D. E. Carling, unpublished data). For example, AG-1 ID (28) cannot be distinguished from the other three subsets of AG-1 based on anastomosis reaction but instead requires use of criteria such as host range, virulence, or certain molecular tests for identification. AG-2-1, -2-2 IIIB, -2-2 IV, -2-2 LP, -2-3, and -2-4 all are subsets of AG-2 (8), but they cannot be differentiated from one another based on anastomosis reactions (8). Also, isolates of AG-3PT and AG-3TB cannot be differentiated based on anastomosis reaction but easily can be separated based on host range or rDNA-ITS sequence (16,34). AG-13, and all other AGs of *R. solani*, can be differentiated from one another based on anastomosis reaction alone.

The relatively low level of virulence, compared with AG-1, -2, -3, and -4, observed in the isolates of AG-13 evaluated herein suggests that AG-13 will generate little interest among plant pathologists. However, as with AG-11 and -12, AG-13 provokes questions regarding when it appeared and how it survives. Although a recent discovery may suggest recent evolution of AG-11 (12,15), AG-12 (11), AG-13, and newly reported subsets of different AGs (8,14,16,22,28,34), there is no evidence to support this view. Rather, low soil populations, low virulence, limited host range and narrow or remote geographic distribution, or combinations of these criteria, have allowed these groups, until now, to escape detection. It is reasonable to believe that these newly described AGs and subsets of AGs have been a component of their environments for as long as the earliest described AGs of *R. solani* and that they are being detected only now due to more thorough examinations of all isolates of *R. solani* being collected. If detailed evaluation of isolates of *R. solani* continues, it is also reasonable to expect that additional AGs and subsets of AGs will be discovered in the future.

Isolates 177, Hud 2-1, and Hud 2-8 of AG-13 of *R. solani* are on file as MYA-986, MYA-984, and MYA-985, respectively, in the American Type Culture Collection.

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Fig. 3. A single most parsimonious tree based on sequences of the internal transcribed spacer (ITS) regions of rDNA illustrating estimates of phylogenetic relationships of anastomosis group (AG)-13 and all other AGs of *Rhizoctonia solani* (AG-2 is presented as subsets -2-1, -2 BI, -2-2 IIIB, -2-2 IV, -2-2 LP, -2-3, and -2-4). The numbers above each branch denote an estimated number of substitutions and the number below each branch indicates the percentage of congruent clusters in 1,000 bootstrap trials when values were greater than 50%. Alignment appeared ambiguous in some areas due to numerous deletions and insertions and these areas were excluded from analysis. The length of the tree is 339 steps with a consistency index (CI) of 0.512 and a retention index (RI) of 0.578. *Thanatephorus orchidicola* and *T. pennatus* were used as outgroups.

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