Characterization of the Crater Disease Strain of *Rhizoctonia solani*

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ABSTRACT

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Crater disease (CD) of wheat is caused by a *Rhizoctonia solani* strain of ambiguous phylogeny. Anastomosis reactions confirmed placement of CD-causing *R. solani* in anastomosis group (AG) 6, with results indicating a closer affinity to AG-6 GV than to AG-6 HG. Cultures of CD isolates were initially white to cream, turning a yellowish light brown after 10 days. Concentric rings of dark and light mycelium were evident from an early stage. Mycelium generally was appressed to the agar surface, with sparse aerial growth. A few light-colored, irregularly shaped sclerotia could be discerned after 2 weeks. The mean hyphal diameter of CD-causing *R. solani* was 7.46 μm (ranging from 5.0 to 10.0 μm), and

cells contained a mean number of four (ranging from two to eight) nuclei, compared to a mean hyphal diameter of 8.58 and 8.42 μm and a mean nuclear number of six and four for AG-6 HG and AG-6 GV, respectively. The CD isolates had a slower growth rate (15.3 mm/day) than AG-6 HG (29.1 mm/day) and AG-6 GV (22.6 mm/day) but, like AG-6, were thiamine prototrophic. Conspicuous nodulose swellings were produced by CD-causing *R. solani* on roots of wheat, and infection resulted in retarded shoot growth. Smaller nodules were evident on bean and soybean roots. Fingerprint patterns generated for the various isolates with four enzymes, *HpaII*, *Sau3AI*, *TaqI*, and *CfoI*, showed the presence of a unique 610-bp fragment in the pathogen. It is proposed that CD-causing *R. solani* isolates represent a distinct intersterility group within AG-6 that is more related to subgroup GV than to subgroup HG.

Rhizoctonia patch disease (RP), which affects cereal production in many parts of the world (1,14,24,34), is attributed to root infection by *Rhizoctonia solani* Kühn (21). A disease resembling RP occurs in wheat fields on the Springbok Flats of South Africa (5), where it is referred to as crater disease (CD). Like RP, CD is caused by *R. solani*, but infection results in production of nodulose swellings and sclerotial sheaths on seminal roots (5,27) rather than girdling and rotting of roots, as is found with RP (11,17, 24,34). Bare patch is ascribed to root infection by anastomosis group (AG) 8 (15), whereas recent evidence (3) assigned the CD-causing *R. solani* to AG-6. However, unlike other isolates of AG-6 (or any other AG of *R. solani*), the CD AG-6 also produces bridging reactions with *R. solani* AG-1 IA (3). Furthermore, AG-6 previously has been found only in nonpathogenic association with orchid roots (30), turfgrass (22,37) as an inhabitant of uncultivated soil (9), and more recently as a root pathogen of apple (12), whereas the CD-causing *R. solani* causes a destructive disease of wheat.

Considering the polymorphic and phylogenetically diverse nature of AG-6 (7), it seems reasonable to assume that the CD-causing *R. solani* could constitute a separate subgroup within this AG. Vilgalys and Gonzalez (33) indicated a clear differentiation between various *R. solani* AGs based on restriction fragment length polymorphisms (RFLPs) of rDNA, whereas Vilgalys (31) showed that isolates within particular intraspecific subgroups (ISGs) share a high degree of DNA sequence complementarity among their genomes. The current report characterizes the CD pathogen and specifically compares it with existing tester isolates of AG-6 by means of standard techniques as well as genetic typing by RFLP.

MATERIALS AND METHODS

***R. solani* isolates.** Twenty isolates of CD-causing *R. solani* were acquired from soil and wheat roots on the Springbok Flats of South Africa. Eleven isolates (CD-95, CD1005, CD1010, CD1081, CD1224, CD1243, CD1102, CD1113, CD1033, CD1058, and CD1065) were from wheat roots, collected at five sites on the southern Springbok Flats, two isolates (CD1168 and CD1242) were from a location on the middle Springbok Flats, and four isolates (CD1036, CD1044, CD1171, and CD1194) were from a location on the northern Springbok Flats. Two isolates (CD1028 and CD1238) were obtained from roots of wheat planted in soil artificially infested with isolate CD16-6 on the middle Springbok Flats, and one isolate (CD-G) was from artificially infested soil (CD16-6) in a greenhouse. Isolate CD16-6 was isolated from wheat roots collected at a site on the southern Springbok Flats. An isolate (CD1260) from wheat roots from Hanang wheat farms in Tanzania and an isolate from roots of *Acacia tortilis* (Forsk.) Hayne subsp. *heteracantha* (Burch.) Brenan (umbrella-thorn tree) (CD1261), recently shown to anastomose with CD-causing *R. solani* (13), also were included. All of the above isolates possess the same anastomosis affiliation and, therefore, hereafter are referred to as CD-causing *R. solani* or CD isolates. Tester isolates representing 11 AGs of *R. solani* were used (Table 1).

AG determination. AG affinities with the various *R. solani* AG tester strains were established for six CD isolates by a modification (2) of the method described by Parmeter et al. (18). Reactions observed were assigned to one of four categories: C0 = no anastomosis reaction; C1 = hyphal contact and apparent joining of walls but no membrane-to-membrane contact; C2 = wall contact and pore plus death of anastomosing and adjacent cells; and C3 = fusion of walls and membranes (2). At least five fusion sites were observed for each positive anastomosis reaction.

Characteristics of isolates of CD-causing *R. solani*. All isolates were grown on potato dextrose agar (PDA) in the dark at 25°C. Observations regarding colony morphology were made over

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a 3-week period. Mature hyphae from the periphery of actively growing 7-day-old PDA cultures of 12 CD isolates and AG-6 GV and AG-6 HG tester strains were mounted on slides and stained with 0.05% trypan blue in lactophenol. Hyphal diameter was determined under bright-field illumination at 400× magnification, and the mean for each isolate was calculated from 20 observations. Mature, actively growing hyphae from the above CD, AG-6 GV, and AG-6 HG isolates on PDA were mounted on microscope slides, stained with acridine orange (35), and observed at 400× magnification under bright-field illumination. The median number of nuclei per cell was calculated from 30 observations per isolate.

Radial growth was determined by inoculating PDA plates in triplicate with all tester isolates of AG-6 GV and AG-6 HG and all 20 isolates of CD-causing *R. solani*. Cultures were maintained at 25°C in the dark, and radial growth was recorded every second day until the colony reached the edge of the plate. Growth rate was expressed as the increase in colony diameter per day.

Erlenmeyer flasks containing Czapek-Dox broth with or without 10⁻⁵ M thiamine hydrochloride (21) were inoculated in triplicate with three 4-mm-diameter mycelial disks of an isolate representing AG-3 (prototrophic), AG-5 (auxotrophic), and AG-6 HG and AG-6 GV. These were compared with three CD isolates, as well as one from Tanzania and one from *A. tortilis* subsp. *heteracantha*. Mycelial dry mass was determined by filtering, drying at 70°C, and weighing the biomass after 2 weeks of shake-incubation at 25°C in the dark. Induction of sporulation of CD-causing *R. solani* was attempted on 1.5% water agar, V8 juice agar (18 g of agar plus 20 ml of V8 juice per liter), and by the soil overlay technique (16).

Pathogenicity determination. Pathogenicity of four CD isolates and AG-6 HG and AG-6 GV was determined on a selection of reported host plants of *R. solani*: bean (*Phaseolus vulgaris* L.), carrot (*Daucus carota* L.), cotton (*Gossypium hirsutum* L.), lettuce (*Lactuca sativa* L.), potato (*Solanum tuberosum* L.), radish (*Raphanus sativus* L.), soybean (*Glycine max* (L.) Merr.), tomato (*Lycopersicon esculentum* Mill.), and wheat (*Triticum aestivum* L.). Screening involved inoculation of *R. solani* isolates (CD95, CD1005, CD1260, and CD1261) into 500-ml Erlenmeyer flasks containing 200 ml of malt-extract broth. After 21 days of stationary growth at 25°C, mycelium and sclerotia were retrieved by filtration through Whatman No. 1 filter paper (Whatman, Maidstone, England), rinsed in sterile distilled water (SDW), and homogenized in an Ultra-turrax blender (Janke and Kunkel GmbH & Co., Staufen, Germany). Inoculum was added to sterile sand/vermiculite (3:1, vol/vol) to a final concentration of 0.1 % (wt/vol). The inoculum-containing mixture was dispensed into 250-ml polystyrene cups, with control cups receiving only a sterile sand/vermiculite mixture. Cups were planted to the various crops (three pregerminated seeds per cup or, with potato, three excised sprouts per cup), with five replicate cups per isolate per crop. Cups were maintained in a greenhouse at 21 to 27°C and watered regularly with tap water. Seedlings were removed from cups 3 weeks after planting, and their roots were washed free of soil and examined for necrosis and nodulous swellings. Roots were cut into segments ≈10 mm long, surface-disinfested for 1 min in 3% NaOCl, rinsed in SDW, and plated on PDA augmented with 50 mg of rifampicin per liter. The remaining roots were cleared and stained with trypan blue as described by Phillips and Hayman (19) and examined microscopically for

TABLE 1. Anastomosis reactions between crater disease (CD) and tester isolates of *Rhizoctonia solani* representing 11 anastomosis and intraspecific groups (AG and ISG, respectively) of *R. solani*

Tester isolate			Anastomosis reaction ^a					
AG	ISG ^y	Origin	CD-95	CD1010	CD1243	CD-G	CD1260	CD1261
1	IA	ATCC 66159 ^w	C0	C0	C0	C0	C0	C0
1	IA	Carling Cs-Ka ^x	C0	C1	C1 (2) ^y	C0	C0	C1
1	IB	Carling SFBV-1	C0	C0	C0	C0	C0	C0
1	IB	ATCC 66151	C0	C0	C0	C0	C0	C0
1	IC	Carling 43	C0	C0	C0	C0	C0	C0
1	IC	ATCC 44661	C0	C0	C0	C0	C0	C0
2	1	Carling F56L	C0	C0	C0	C0	C0	C0
2	1	ATCC 62805	C0	C0	C0	C0	C0	C0
2	1	ATCC 44658	C0	C0	C0	C0	C0	C0
2	2	ATCC 44659	C0	C0	C0	C0	C0	C0
2	2	Ogoshi RI-64 ^z	C0	C0	C0	C0	C0	C0
3		Carling W14L	C0	C0	C0	C0	C0	C0
3		ATCC 14006	C0	C0	C0	C0	C0	C0
3		ATCC 62803	C0	C0	C0	C0	C0	C0
4		ATCC 48802	C1 (3)	C1 (2)	C0	C0	C1	C0
4	HGI	ATCC 44662	C1	C0	C0	C0	C1	C0
4	HGII	ATCC 42127	C0	C0	C0	C0	C0	C0
5		Ogoshi ST-6-1	C0	C0	C0	C0	C0	C0
5		ATCC 46138	C0	C0	C0	C0	C0	C0
6		Ogoshi Fri 1-41	C1 (2)	C1	C2 (3)	C2	C2 (1)	C1
6	HG	ATCC 76129	C1	C2 (1)	C2 (2)	C1	C1	C2 (2)
6	HG	Carling HAM1-1	C1	C1	C2	C2 (2)	C1	C2 (3)
6	GV	ATCC 76130	C2 (3)	C1	C2 (4)	C2 (2)	C2	C2 (2)
7		Carling 1529	C0	C0	C0	C0	C0	C0
8		Carling C1WA	C0	C0	C0	C0	C0	C0
9		ATCC 62804	C0	C0	C0	C0	C0	C0
10		ATCC 76107	C0	C0	C0	C0	C0	C0
BI		Carling AI 1-4	C0	C0	C0	C0	C0	C0

^a Anastomosis reaction rating: C0 = no anastomosis reaction; C1 = hyphal contact and apparent connection of walls but no membrane-to-membrane contact; C2 = wall connection and pore plus death of anastomosing and adjacent cells.

^y Where not presented, ISGs either do not exist (AG-3, -5, -7, -8, -9, -10, and -BI) or are unknown (AG-4 and -6).

^w American Type Culture Collection, Rockville, MD.

^x D. E. Carling, Agricultural and Forestry Experimental Station, University of Alaska, Fairbanks, Palmer.

^y Numbers in parentheses indicate the number of C1 or C2 reactions recorded. If no number in parentheses is listed, five or more reactions were observed.

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fungus structures. Shoots were dried for 48 h at 70°C and weighed. The entire experiment was repeated once.

DNA extraction. Twenty-eight tester isolates of *R. solani*, representing twenty AGs/ISGs, and ten CD isolates (CD-95, CD1243, CD1113, CD1168, CD1242, CD1036, CD1194, CD1238, CD1260, and CD1261) were grown on PDA. Four 5-mm-diameter plugs from the periphery of an actively growing culture of each isolate were inoculated into 200 ml of malt-extract broth in a 250-ml Erlenmeyer flask. Stationary flasks were incubated for 8 to 14 days at 25°C, after which the mycelium was harvested by filtration, washed with SDW, blot-dried between filter paper, immersed in liquid nitrogen, and lyophilized (20). The dry material was ground with a mortar and pestle and kept at 4°C until used. Genomic DNA was extracted from the various isolates according to a procedure described by Cubeta et al. (4). Each sample was diluted with SDW to a working concentration of 20 ng of DNA per µl and verified spectrophotometrically (23).

Primers. Two oligonucleotides, LR7 (5'-TACTACCACCAAGATCT-3') and LROR (5'-ACCGCTGAACCTTAAGC-3'), were synthesized by the Department of Biochemistry, University of Cape Town, South Africa, for amplification of ribosomal DNA homologous to positions 17 to 1488 within the 25S rRNA encoding gene. These primers previously successfully amplified rDNA in various other fungi, including ascomycetes and basidiomycetes (4).

Polymerase chain reaction (PCR) conditions. PCR was performed in 100-µl volumes containing 20 ng of template DNA, 0.6 µM LR7 and 0.6 µM LROR, 10 µl of recommended 10× buffer, 2 mM MgCl₂, 100 µM each dCTP, dGTP, dATP, and dTTP, and 0.5 units of *Taq* DNA polymerase (Promega, Whitehead Scientific, Cape Town, South Africa). Forty PCR cycles were performed on an automated thermocycler device (Hybaid Omnigene, South African Scientific Industries, Johannesburg), with the following parameters: 94°C denaturation step (1 min); 50°C annealing step (2 min); and primer extension at 72°C (90 s). PCR prod-

ucts were analyzed by electrophoresis of a sample (5 µl) in horizontal agarose gels, and the size of products was verified by comparison with a molecular weight marker (marker VI, Boehringer Mannheim, Johannesburg, South Africa).

Restriction digestion of PCR products. Amplified PCR products were purified by phenol-chloroform-isoamyl alcohol extraction. For restriction analysis, each PCR product was digested with *Cfo*I, *Hpa*II, *Sau*3AI, and *Taq*I following the manufacturer's (Boehringer) directions. These enzymes previously have been effective for demonstrating RFLPs in binucleate *Rhizoctonia* rDNA (4). After digestion, samples were subjected to electrophoresis in a 2.5% agarose gel for 15 h at 2 V cm⁻¹. Gels were stained with ethidium bromide and photographed before final analysis. All restriction analyses were performed at least twice.

RESULTS

Anastomosis grouping. When paired with itself, complete fusion of walls and membranes (C3 reaction) occurred in each of the six CD-causing *R. solani* isolates. Pairings between different CD isolates consistently yielded C2 reactions (wall contact and pore plus death of anastomosing and adjacent cells). None of the CD isolates anastomosed with tester isolates representing AG-1 IB, -1 IC, -2, -3, -4 HGII, -5, -7, -8, -9, -10, or -BI, indicating the absence of anastomosis relationships between these AGs and CD-causing *R. solani* (Table 1). Tester isolate Carling Cs-Ka (from the collection of D. E. Carling, Agricultural and Forestry Experiment Station, University of Alaska, Fairbanks, Palmer) (but not ATCC 66159 [from the American Type Culture Collection, Rockville, MD]) of AG-1 IA produced C1 reactions with three CD isolates. C1 reactions also were evident in pairings between AG-4 and AG-4 HGI and two CD isolates, whereas one CD isolate produced a C1 reaction with only AG-4. The most distinct anastomosis reaction of CD isolates occurred with AG-6. Five of the six CD isolates produced at least two C2 reactions with AG-6 GV, whereas four isolates produced a C2 reaction with AG-6 HG, albeit not consistently with both isolates of the latter.

Characteristics of isolates of CD-causing *R. solani*. Cultures of CD isolates were initially white to cream, turning yellowish light brown after 10 days. Concentric rings of dark and light mycelial growth were evident from an early stage. Mycelium generally was appressed to the agar surface, with sparse aerial development. A limited number of light-colored, irregularly shaped sclerotia could be discerned after 2 weeks. These turned dark brown with age, producing a brown exudate. Cultures of AG-6 were darker than those of CD isolates. Mycelium of AG-6 GV isolates appeared dark brown, aerial, and cottony. No concentric rings were evident, and sclerotia were small and few in number. Mycelium of AG-6 HG isolates was dark brown, appressed to the agar surface, and did not have concentric rings. Sclerotia were produced mainly in the center of colonies and occasionally at the margin. The sclerotia often aggregated into large compound clusters that were a darker brown than the mycelium. The mean

TABLE 2. Thiamine requirement of crater disease (CD) isolates of *Rhizoctonia solani* and of isolates representing anastomosis groups (AG) 3, 5, 6 GV, and 6 HG

Isolate	Mycelial dry mass after 2 wk (mg) ^y		
	Czapek (A)	Czapek + T ^z (B)	B/A
AG-3 (ATCC 62803)	127 a	137 a	1.07
AG-5 (ATCC 46138)	237 b	460 a	1.94
AG-6 GV (ATCC 76130)	297 a	313 a	1.05
AG-6 HG (ATCC 76129)	252 a	249 a	0.99
CD-95	213 a	203 a	0.95
CD1005	143 a	146 a	1.02
CD1065	93 a	95 a	1.02
CD1260	105 a	98 a	0.93
CD1261	137 a	140 a	1.02

^y Mycelial dry mass values are the mean of three replicates; values in rows followed by the same letter do not differ significantly according to *F* test (*P* ≤ 0.05).

^z Czapek-Dox medium augmented with 10⁻⁵ M thiamine hydrochloride (T).

TABLE 3. Pathogenicity of crater disease (CD) isolates of *Rhizoctonia solani* and tester isolates of anastomosis group (AG) 6 GV (ATCC 76130) and 6 HG (ATCC 76129) to various plant species

Isolate	Dry shoot mass of 10 plants after 3 wk (g) ^z									
	Bean	Beetroot	Carrot	Cotton	Lettuce	Potato	Radish	Soybean	Tomato	Wheat
CD-95	0.82 ab	0.31 a	0.36 a	0.75 a	0.64 a	1.90 a	1.13 a	0.48 a	0.74 a	0.29 b
CD1005	0.78 ab	0.36 a	0.40 a	0.60 a	0.64 a	2.04 a	0.98 a	0.51 a	0.76 a	0.38 b
CD1260	0.96 a	0.33 a	0.34 a	0.70 a	0.62 a	1.82 a	0.90 a	0.50 a	0.66 a	0.31 b
CD1261	0.89 a	0.29 a	0.43 a	0.65 a	0.74 a	2.30 a	1.43 a	0.54 a	0.64 a	0.30 b
AG-6 GV	0.64 b	0.38 a	0.41 a	0.78 a	0.77 a	2.10 a	1.21 a	0.54 a	0.74 a	0.61 a
AG-6 HG	0.70 b	0.35 a	0.38 a	0.82 a	0.68 a	2.16 a	1.31 a	0.55 a	0.67 a	0.55 a
Control	0.90 a	0.34 a	0.40 a	0.80 a	0.72 a	2.23 a	1.22 a	0.64 a	0.70 a	0.57 a

^z Mean of five replicates; values in columns followed by the same letter do not differ significantly according to Duncan's multiple range test (*P* = 0.05). A repetition of the experiment showed the same tendencies.

hyphal diameter of the CD isolates was 7.46 μm (ranging from 5.0 to 10.0 μm), and the mean hyphal diameter of AG-6 GV and AG-6 HG was 8.42 μm (ranging from 6.25 to 10.0 μm) and 8.58 μm (ranging from 7.5 to 10.0 μm), respectively. Cells of the CD isolates contained a mean of four (ranging from two to eight) nuclei per hyphal cell. The mean number of nuclei in cells of AG-6 GV was also four (ranging from three to six), whereas AG-6 HG contained a mean of six nuclei (ranging from four to eight) per cell.

The mean radial growth rate of the 20 CD isolates at 25°C was 15.3 mm/day, compared to 22.6 and 29.1 mm/day for AG-6 GV and AG-6 HG, respectively. The growth rate of thiamine prototrophic *R. solani* (AG-3 and AG-6) isolates was the same in the presence or absence of thiamine, whereas auxotrophic AG-5 developed significantly more slowly in medium that did not contain the vitamin (Table 2). CD isolates grew at the same rate with or without thiamine, indicating their thiamine prototrophy. None of the CD isolates produced a teleomorph with any of the methods used.

Pathogenicity determination. CD isolates of *R. solani* suppressed shoot growth of only wheat (Table 3) and produced nodulose swellings on roots from which *R. solani* was reisolated readily. Small root nodules yielding *R. solani* were present on roots of bean and soybean in cups infested with CD-causing *R. solani*, but plant growth was not affected significantly. Chlorosis of leaves and necrotic lesions on roots was evident in radish. Crowns of beetroot plants showed a slight discoloration. No nodules, necrotic lesions, or other symptoms indicative of infection were discernible in carrot, cotton, lettuce, potato, or tomato. Clearing and staining of roots revealed superficial colonization of beetroot, carrot, lettuce, radish, and tomato roots by CD-causing *R. solani*, but the fungus could not be isolated from them or from the roots of any of the other crops, except wheat, bean, and soybean. AG-6 GV and AG-6 HG isolates reduced the shoot mass of bean and produced necrotic lesions on roots from which *R. solani* was reisolated. No pathogenicity to any of the other crop species was apparent, and the two AG-6 ISGs could not be reisolated from roots or crowns of the crops.

Amplification of rDNA. Two sizes of rDNA amplicons were obtained after amplification with LR7 and LROR. All CD isolates and those of AG-1 IA, -1 IB, -3, -5, -6, -6 GV, -8, -9, and -BI produced a single 1.8-kb fragment, whereas AG-1 IC, -4, -4 HGI,

-4 HGII, -6 HG, -7, and -10 produced a 1.4-kb fragment. AG-2, AG-2-1, and AG-2-2 tester isolates yielded fragments of both sizes.

Restriction analysis of amplified rDNA. The number of fragments obtained from individual isolates after restriction enzyme digestion ranged from two to five (Table 4). Apart from those listed, all isolates produced a common 250-bp fragment after digestion with *TaqI*, 100- and 240-bp fragments after digestion with *CfoI*, and a 280-bp fragment after digestion with *HpaII*. Fingerprint patterns of the 10 CD isolates were similar. The unique 610-bp fragment obtained when rDNA from these isolates was digested with *HpaII* (Table 4) is of particular importance. The pattern of five bands, 610, 500, 280, 240, and 140 bp, furthermore, could be distinguished clearly from the patterns exhibited by all other *R. solani* isolates.

Corresponding patterns were evident for AG-1 IA and AG-1 IB, but the two AG-1 IC isolates differed entirely from the patterns with regard to RFLP patterns of all four enzymes. The single isolates representing AG-4, AG-4 HGI, and AG-4 HGII were identical with respect to all four RFLPs. Isolate AG-6 (Ogoshi Fri-141 [from the collection of A. Ogoshi, Faculty of Agriculture, Hokkaido University, Kita-ku, Japan]) showed the same banding pattern as AG-6 GV and, thus, is considered representative of the latter ISG.

DISCUSSION

Interisolate anastomosis and RFLPs showed that the various CD-causing *R. solani* isolates are similar regardless of geographic origin, i.e., the Springbok Flats of South Africa or Hanang wheat farms in Tanzania, or host, i.e., wheat or *A. tortilis* subsp. *heteracantha*. Isolates varied somewhat with respect to anastomosis reaction with different AG tester strains, but the only pairing of CD-causing *R. solani* that resulted in a C2 anastomosis response was with the two ISGs of AG-6. In accordance with Carling et al. (3), bridging (C1 reaction) occurred with AG-1 IA, albeit with only one of the tester strains and only between this strain and South African CD isolates, whether from wheat or *A. tortilis* subsp. *heteracantha*. In contrast, Carling et al. (3) reported that the wheat isolates from South Africa and Tanzania, but not the one from *A. tortilis* subsp. *heteracantha*, bridged with AG-1 IA. The current investigation also showed bridging between CD isolates and AG-4/AG-4 HGI, a phenomenon that has not been observed

TABLE 4. Restriction fragment sizes obtained from anastomosis group (AG) and crater disease (CD) isolates of *Rhizoctonia solani* after digestion with *CfoI*, *HpaII*, *Sau3AI*, and *TaqI*

AG	No. of isolates	Restriction fragment sizes (bp)			
		<i>CfoI</i> ^a	<i>HpaII</i> ^y	<i>Sau3AI</i>	<i>TaqI</i> ^z
1 IA	2	860, 550, 160	890, 500, 140	1,040, 780, 140	1,020, 640
1 IB	2	860, 550, 160	890, 500, 140	1,040, 780, 140	1,020, 640
1 IC	2	550, 380, 160	430	790, 560, 140	550, 410
2	1	890, 710, 380	740, 460, 430	760, 550, 110	1,070, 640, 550
2-1	3	860, 710, 380	890, 740, 430	1,040, 790, 140	1,020, 640, 550
2-2	2	890, 710, 380	920, 740, 460	1,040, 790, 140	960, 700
3	3	860, 710	890, 740	760, 650, 370, 110	700, 640, 350
4	1	710, 380	740, 430	560, 500, 280, 130	640, 550
4 HGI	1	710, 380	740, 430	560, 500, 280, 130	640, 550
4 HGII	1	710, 380	740, 430	560, 500, 280, 130	640, 550
5	2	860, 710	890, 740	1,040, 790, 140	1,020, 640
6	1	860, 710	530, 500, 360, 240	1,010, 510, 250, 110	1,020, 640
6 GV	1	860, 710	530, 500, 360, 240	1,010, 510, 250, 110	1,020, 640
6 HG	2	680, 380	500, 410, 240	560, 510, 250, 110	640, 550
7	1	710, 380	500, 430, 240	790, 560, 140	640, 550
8	1	860, 710	890, 740	760, 520, 250, 190, 110	1,020, 640
9	1	830, 710	890, 740	1,040, 790, 140	980, 640, 110
10	1	710, 380	740, 430	790, 560, 140	640, 550
BI	1	860, 710	920, 740	1,010, 760, 110	1,020, 640
CD	10	860, 710	610, 500, 240, 140	1,010, 760, 110	1,020, 640

^a All isolates produced common 100- and 240-bp fragments after digestion with *CfoI* in addition to those listed.

^y All isolates produced a common 280-bp fragment after digestion with *HpaII* in addition to those listed.

^z All isolates produced a common 250-bp fragment after digestion with *TaqI* in addition to those listed.

before. Although bridging indicates a real but distinct relationship between *R. solani* isolates (32), it is subservient to C2 anastomosis reactions for demonstrating authentic associations. Placement of CD-causing *R. solani* within AG-6 (3), therefore, is endorsed. The fact that AG-6 bridges with AG-8 and AG-BI (29), whereas CD isolates do so with AG-1 IA and AG-4, nevertheless points to differences between the two groups. Fruiting of CD-causing *R. solani* could not be induced, and despite various attempts (5,26), including the current one, its teleomorph remains unknown.

The two ISGs of AG-6 have not yet been characterized separately, and previous descriptions of AG-6 by Kunita et al. (8, 9) presumably were based on the cultural characteristics of both. According to these reports, AG-6 produces yellowish- or dark-brown cultures with loose-textured sclerotia, has a mean hyphal diameter of 8.6 µm, contains 3 to 15 nuclei per cell, and is thiamine auxotrophic. The current investigation showed the main difference between the two ISGs to be the cottony appearance of AG-6 GV colonies as opposed to the appressed growth of AG-6 HG and the inclination of the latter to produce sclerotia in clusters. Cultures of CD-causing *R. solani* differed from these by having a lighter color, producing pale sclerotia, and exhibiting concentric rings of light and dark mycelial growth. The CD pathogen grew more slowly and had thinner hyphae than both AG-6 ISGs. Its nuclear number was the same as that of AG-6 GV (four per cell) but less than that of AG-6 HG (six per cell). Although hyphal width and growth rate are not valid criteria for separating *R. solani* AGs or ISGs (22,36), little variation exists in colony pigmentation and sclerotial characteristics within AGs (25,37). The conspicuously lighter color of CD-causing *R. solani* cultures indicate that the strain differs from existing ISGs of AG-6.

Apart from its peculiar mode of pathogenicity, the CD-causing *R. solani* further differs from AG-6 in that it is virulent as opposed to the generally accepted nonpathogenic nature of the latter (9,22, 30,37). The CD pathogen previously was believed to attack only species in the Poaceae, e.g., wheat, barley (*Hordeum vulgare*), oat (*Avena sativa*), rye (*Secale cereale*), maize (*Zea mays*) (5), millet (*Panicum miliaceum*) (26), and graminaceous weeds such as *Brachiaria eruciformis* and *Pogonarthria squarrosa* (28). However, except for wheat, millet and oat, authentic field symptoms have not been observed in these crops. This investigation confirmed the virulence of CD-causing *R. solani* to wheat and further showed that the pathogen is capable of infecting leguminous crops such as bean and soybean. Infection of these plants, resulting in the formation of the characteristic root nodules, did not, however, lead to significant impediment of shoot growth, though given more time for disease to develop it is possible that growth retardation would have occurred. The infection of legumes by CD-causing *R. solani* also lends credibility to its association with *A. tortilis* subsp. *heteracantha*.

In this investigation, the major groups that could be recognized according to the size of amplified 25S rDNA products (1.4 and 1.8 kb) corresponded to the different size classes of rDNA PCR amplicons reported by Cubeta et al. (4) for binucleate *Rhizoctonia* and are thought to represent different length-variants of the rRNA gene. Although not in accord with the anastomosis affinities of *R. solani* proposed by Sneh et al. (29), the differentiation was considered sufficiently significant to separate isolates yielding a 1.4-kb amplicon from those producing a 1.8-kb amplicon. This apparent unrelatedness of the two groups was substantiated by differences in the restriction fragment sizes observed between them after enzymatic digestion particularly with *HpaII* and *Sau3AI*, as well as separate clustering of the different size groups by Cubeta et al. (4). Because the study primarily concerned molecular characterization of CD-causing *R. solani*, not an analysis of variation between different AGs, isolates yielding 1.4-kb amplicons, were excluded from subsequent comparisons.

The 10 CD isolates all yielded a single size PCR amplicon that exhibited similar patterns with each of the four enzymes used.

They shared among them a unique and exclusive 610-bp fragment after digestion with *HpaII*, a characteristic that could facilitate accurate identification of isolates as CD-causing *R. solani* in future studies. In *Rhizoctonia*, RFLP fingerprints generally are consistent with the anastomosis status of a particular isolate (4,6,33). Liu et al. (10) for instance, showed AG-10 to be genetically distinct from other groups in the *R. solani* species complex through analysis of the 18S rDNA region of *R. solani* by means of a PCR-based restriction mapping procedure.

R. solani AG-6 is a genetically polymorphic group composed of strains with partial phylogenetic relatedness (7). Genomic DNA hybridization divided AG-6 into two genetically different ISGs: with AG-6 HG is a homogeneous group, and AG-6 GV consists of genotypically variable isolates (7). The molecular genetic typing done here supports this separation and also links the CD-causing *R. solani* closer to AG-6 GV than to AG-6 HG. It also showed that the CD pathogen differs molecularly from all other *R. solani* AGs associated with bare patch disease of cereals, i.e., AG-8, -3, -4, -5, -9, and -10 (17).

Anastomosis grouping remains the accepted criterion for classification of *R. solani* (16,32), and Carling et al. (3) accordingly placed the CD pathogen in AG-6. Considering its anastomosis and bridging reactions, morphological characteristics, unique ability to produce nodulose swellings on roots of infected plants, and significant differences in RFLP banding patterns, it is proposed that the pathogen represents a distinct ISG within AG-6.

LITERATURE CITED

1. Benedict, W. G., and Mountain, W. B. 1956. Studies on the etiology of a root rot of winter wheat in southwest Ontario. Can. J. Bot. 34:159-174.
2. Carling, D. E., Leiner, R. H., and Kebler, K. M. 1987. Characterization of a new anastomosis group (AG-9) of *Rhizoctonia solani*. Phytopathology 77:1609-1612.
3. Carling, D. E., Meyer, L., and Brainard, K. A. 1996. Crater disease of wheat caused by *Rhizoctonia solani* AG-6. Plant Dis. 80:1429.
4. Cubeta, M. A., Echandi, E., Abernethy, T., and Vilgalys, R. 1991. Characterization of anastomosis groups of binucleate *Rhizoctonia* species using restriction analysis of an amplified ribosomal RNA gene. Phytopathology 81:1395-1400.
5. Deacon, J. W., and Scott, D. B. 1985. *Rhizoctonia solani* associated with crater disease (stunting) of wheat in South Africa. Trans. Br. Mycol. Soc. 85:319-327.
6. Duncan, S., Barton, J. E., and O'Brien, P. A. 1993. Analysis of variation in isolates of *Rhizoctonia solani* by random amplified polymorphic DNA assay. Mycol. Res. 97:1075-1082.
7. Kunita, S., and Yokosawa, A. 1984. DNA base sequence homology in *Rhizoctonia solani* Kühn. IV. Genetic relatedness within AG-6. Ann. Phytopathol. Soc. Jpn. 50:346-352.
8. Kunita, S., Yokosawa, R., and Ogoshi, A. 1978. Anastomosis grouping of *Rhizoctonia solani* Kühn isolated from non-cultivated soils. Ann. Phytopathol. Soc. Jpn. 44:591-598.
9. Kunita, S., Yokosawa, R., and Ogoshi, A. 1979. Some properties of anastomosis groups 6 and BI in *Rhizoctonia solani* Kühn. Ann. Phytopathol. Soc. Jpn. 45:207-214.
10. Liu, Z. L., Domier, L. L., and Sinclair, J. B. 1995. Polymorphism of genes coding for nuclear 18S rRNA indicates genetic distinctiveness of anastomosis group 10 from other groups in the *Rhizoctonia solani* species complex. Appl. Environ. Microbiol. 61:2659-2664.
11. MacNish, G. C. 1984. *Rhizoctonia* patch in the Western Australian grain belt. Plant Pathol. 33:355-359.
12. Mazzola, M. 1997. Identification and pathogenicity of *Rhizoctonia* spp. isolated from apple roots and orchard soils. Phytopathology 87: 582-587.
13. Meyer, L., Wehner, F. C., Kuwite, C. A., and Piening, L. 1996. Crater disease and patchy stunting of wheat caused by the same strain of *Rhizoctonia solani*. Plant Dis. 80:1079.
14. Murray, D. I. L., and Nicholson, T. H. 1979. Barley stunt disorder in Scotland. Plant Pathol. 28:200-201.
15. Neate, S. M., and Warcup, J. H. 1985. Anastomosis grouping of some isolates of *Thanatephorus cucumeris* from agricultural soils in South Australia. Trans. Br. Mycol. Soc. 85:615-620.
16. Ogoshi, A. 1987. Ecology and pathogenicity of anastomosis and intra-specific groups of *Rhizoctonia solani* Kühn. Annu. Rev. Phytopathol. 25: 125-143.

17. Ogoshi, A., Cook, R. J., and Bassett, E. N. 1990. *Rhizoctonia* species and anastomosis groups causing root rot of wheat and barley in the Pacific Northwest. *Phytopathology* 80:784-788.
18. Parmeter, J. R., Jr., Sherwood, R. T., and Platt, W. D. 1969. Anastomosis grouping among isolates of *Thanatephorus cucumeris*. *Phytopathology* 59:1270-1278.
19. Phillips, J. M., and Hayman, D. S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.* 55:158-161.
20. Raeder, U., and Broda, P. 1985. Rapid preparation of DNA from filamentous fungi. *Let. Appl. Mycol.* 1:17-20.
21. Rovira, A. D., Ogoshi, A., and McDonald, H. J. 1986. Characterization of isolates of *Rhizoctonia solani* from cereal roots in South Australia and New South Wales. *Phytopathology* 76:1245-1248.
22. Rowley, L. V. 1991. Applied epidemiology of *Rhizoctonia* spp. on turfgrass. M.S. thesis. University of Massachusetts, Amherst.
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
24. Samuel, G., and Garrett, S. D. 1932. *Rhizoctonia solani* on cereals in South Australia. *Phytopathology* 22:827-836.
25. Sherwood, R. T. 1969. Morphology and physiology in four anastomosis groups of *Thanatephorus cucumeris*. *Phytopathology* 59:1924-1929.
26. Smith, E. M. 1988. Etiology and control of crater disease of wheat. D.Sc. (Agriculture) thesis. University of Pretoria, Pretoria, South Africa.
27. Smith, E. M., and Wehner, F. C. 1986. Induction of crater disease of wheat in the field by artificial infestation of the soil with *Rhizoctonia solani*. *Phytophylactica* 18:127-130.
28. Smith, E. M., and Wehner, F. C. 1989. *In vivo* and *in vitro* effects of alternative crops on *Rhizoctonia solani* associated with crater disease of wheat. *Phytophylactica* 21:61-64.
29. Sneh, B., Burpee, L., and Ogoshi, A. 1991. Identification of *Rhizoctonia* Species. The American Phytopathological Society, St. Paul, MN.
30. Uetake, Y., Obayashi, K., Ogoshi, A., and Tsutsui, K. 1988. Identification of *Rhizoctonia* species isolated from orchids. *Ann. Phytopathol. Soc. Jpn.* 54:114.
31. Vilgalys, R. 1988. Genetic relatedness among anastomosis groups in *Rhizoctonia* as measured by DNA/DNA hybridization. *Phytopathology* 78:698-702.
32. Vilgalys, R., and Cubeta, M. A. 1994. Molecular systematics and population biology of *Rhizoctonia*. *Annu. Rev. Phytopathol.* 32:135-155.
33. Vilgalys, R., and Gonzalez, D. 1990. Ribosomal DNA restriction fragment length polymorphisms in *Rhizoctonia solani*. *Phytopathology* 80:151-158.
34. Weller, D. M., Cook, R. J., MacNish, G., Bassett, E. N., Powelson, R. L., and Petersen, R. R. 1986. *Rhizoctonia* root rot of small grains favored by reduced tillage in the Pacific Northwest. *Plant Dis.* 70:70-73.
35. Yamamoto, D. T., and Uchida, J. Y. 1982. Rapid nuclear staining of *Rhizoctonia solani* and related fungi with acridine orange and with safranin O. *Mycologia* 74:145-149.
36. Zhang, M. 1994. *Rhizoctonia solani* Kühn anastomosis groups and fungi associated with turfgrasses affected by brown patch. M.S. thesis. Department of Agronomy, University of Maryland, College Park.
37. Zhang, M., and Dernoeden, P. H. 1995. Facilitating anastomosis grouping of *Rhizoctonia solani* isolates from coolseason turfgrasses. *HortScience* 30:1260-1262.