Characterization of *Rhizoctonia* spp. Recovered from Crop Plants Used in Rotational Cropping Systems in the Western Cape Province of South Africa

Y. T. Tewoldemedhin, University of Stellenbosch, Department of Plant Pathology, Matieland 7602, Stellenbosch, South Africa; **S. C. Lamprecht,** University of Stellenbosch, Department of Plant Pathology and Plant Protection Research Institute (ARC), Private Bag X5017, Matieland 7599, Stellenbosch, South Africa; **A. McLeod,** University of Stellenbosch, Department of Plant Pathology; and **M. Mazzola,** United States Department of Agriculture–Agricultural Research Service, Tree Fruit Research Laboratory, Wenatchee, WA 98801

ABSTRACT

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Isolates of *Rhizoctonia* spp. associated with barley, canola, clover, lucerne, lupin, annual *Medicago* spp. (medic), and wheat were recovered during the conduct of a 4-year (2000 to 2003) crop rotation trial in the Western Cape province of South Africa. These isolates were characterized by determining their anastomosis group (AG), in vitro optimum growth temperature, and pathogenicity toward emerging and 14-day-old seedlings of all the aforementioned crops. During the 4-year rotational trial, 428 *Rhizoctonia* isolates, in all, were obtained. The most abundant multinucleate AG was AG-4 HG-II (69%), followed by AG-2-1 (19%), AG-3 (8%), AG-2-2 (2%), and AG-11 (2%). The population of binucleate *Rhizoctonia* spp. comprised AG-K (53%), AG-A (10%), AG-I (5%), and unidentified AGs (32%). The optimal time for isolating *Rhizoctonia* spp. was found to be at the flowering or seedpod stage (20 to 22 weeks after planting). Temperature studies showed that isolates belonging to AG-2-2, AG-4 HG-II, and AG-K had significantly higher optimum growth temperatures than those from other AGs. In pathogenicity assays conducted on emerging as well as 14-day-old seedlings, isolates of AG-2-2 and AG-4 HG-II were the most virulent on all crops. *Rhizoctonia solani* AG-2-1 was highly virulent on canola, moderately virulent on medic and lupin, weakly virulent on lucerne and barley, and nonpathogenic on wheat. AG-11 isolates were moderate to weakly virulent on all crops, with the exception of barley and wheat. AG-3 was weakly virulent on canola, lupin, and medic. AG-K was the only binucleate *Rhizoctonia* sp. capable of inciting disease in our assays. This is the first comprehensive study to elucidate the identity and potential importance of *Rhizoctonia* spp. as a yield limiting factor in crop production systems in the Western Cape province of South Africa.

Additional keywords: sequence analysis

Plant-pathogenic *Rhizoctonia* spp. occur worldwide and are economically important pathogens on vegetable and field crops, turf grasses, ornamentals, and fruit and forest trees (1,2,43). Among *Rhizoctonia* spp., the multinucleate species complex *Rhizoctonia solani* has been studied most extensively, having a host range of more than 250 plant species and being capable of inciting disease under diverse environmental conditions. Isolates of *R. solani* are separated by hyphal incompatibility into anastomosis groups (AGs), with the level of host specificity varying widely among AGs (2). Currently, *R. solani* can be divided into 14 anastomosis groups: AG-1 to AG-10, AG-BI (42), AG-11 (8), AG-12 (7), and AG-13 (6). Several AGs have been subdivided further into subgroups that differ for one or more biochemical, genetic, or pathogenic characteristics (13,26). *R. solani* can adversely impact the growth of both annual and perennial crops and may do so in both agricultural and forest ecosystems (28), with disease being incited at virtually any period of plant development. Disease symptoms range from damping-off to hypocotyl and coleoptile rot of young seedlings to root and crown rot of established plants.

Binucleate *Rhizoctonia* spp. may function as plant pathogens; however, the majority exhibit a saprophytic habit or, less commonly, develop a symbiotic association with plants (43). Although many species of binucleate *Rhizoctonia* have been described (9), the most commonly used system for classifying binucleate *Rhizoctonia* isolates is the one proposed by Sneh et al. (42), which classified binucleate *Rhizoctonia* isolates into AG-A through S. Although binucleate *Rhizoctonia* spp. occur worldwide, distribution of the various groups is poorly documented, with many groups appearing to be saprophytic, including AG-C, -H, -K, -L, -N, and -O (9). Diseases incited by pathogenic isolates include sharp eyespot of cereals and yellow patch of turf (*Agrotis palustris* Huds.), as well as damping-off and root rot of strawberry (*Fragaria ananassa* Duch.), sugar beet (*Beta vulgaris* L.), vegetables, and many other hosts (9).

In South Africa, *Rhizoctonia* spp. often have been isolated from barley (*Hordeum vulgare* L.), canola (*Brassica napus* L. var. *oleifera* DC), clover (*Trifolium* spp.), lucerne (*Medicago sativa* L.), lupin (*Lupinus* spp.), medic (annual *Medicago* spp.), and wheat (*Triticum aestivum* L.) (S. C. Lamprecht, E. E. Auret, and J. C. Janse van Rensburg, *unpublished data*). *Rhizoctonia* isolates have not been well characterized in South Africa, with only *R. solani* AG-6 and *R. cerealis* Van der Hoeven, which cause crater disease of wheat in summer rainfall regions of South Africa, being characterized to the species or AG level (36,39). *Rhizoctonia* spp. are known to have a significant impact on stand establishment and yield of these crops in other countries (4,14,17,19,25,33,35,47,48), suggesting that they have the potential to limit crop production within the rotation systems employed in South Africa. Therefore, characterization of *Rhizoctonia* spp. associated with barley, canola, clover, lucerne, lupin, medic, and wheat need further investigation.

The objectives of this study were to (i) determine the optimal developmental stage for isolating *Rhizoctonia* spp. associated with the seven rotation crops barley, canola, clover, lucerne, lupin, medic, and wheat; (ii) determine the distribution of *Rhizoctonia* spp. isolated from these crops among the known AGs; and (iii) assess the nature of the relationship between representatives of the various AGs and the different crop species.

MATERIALS AND METHODS

Field trial. *Rhizoctonia* spp. were isolated from a crop rotation trial over a 4-year period (2000 to 2003) at the Tygerhoek experimental farm, Riviersonderend. The land used for the rotation trial previously was planted to lucerne for 4 years. The following crops were included in the trial: canola (cv. Varola 50), barley (cv. Clipper), alfalfa or lucerne (cv. SA Stan-

Corresponding author: S. C. Lamprecht
E-mail: lamprechts@arc.agric.za

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dard), lupin (*Lupinus angustifolius* cv. Wonga), wheat (cv. SST57), and a mixture of medic (*M. truncatula* cvs. Mogul, Parabinga, and Sephi), and clover (*Trifolium michelianum* cv. Patrick). The trial site contained four different rotation systems, each consisting of four replicates. The four rotation systems included system one (barley and wheat), system two (barley, medic-clover mixture, and wheat), system three (barley, canola, lupin, and wheat), and system four (lucerne monoculture). Systems one, two, and three included 10 crop sequences over the 4 years (Table 1). Planting dates for all crops in the 4 years were either mid-April or early May, except for lucerne which, being a perennial crop, was planted only in 2000. The medic-clover mixture also re-established itself in 2002 and 2003.

Table 1. Rotation systems (four) and within-rotation system crop sequences (n = 10) used in this study

Rotation system	Crop sequence (2000-2001-2002-2003)[z]
1	W-B-W-B
	B-W-B-W
2	M-W-M-B
	W-M-B-M
	B-M-W-M
	M-B-M-W
3	L-W-C-B
	W-C-B-L
	C-B-L-W
	B-L-W-C
4	Luc-Luc-Luc-Luc

[z] W = wheat B = barley, M = medic-clover mix, L = lupin, C = canola, and Luc = lucerne.

Table 2. *Rhizoctonia* isolates used for conventional hyphal fusion reactions, in vitro temperature growth studies, and pathogenicity testing

Nuclear status, AG[y]	Accession number[z]	Origin
Multinucleate		
AG-2-1	PPRI 7426	Lupin
	PPRI 7427	Canola
	PPRI 7428	Medic
AG-2-2	PPRI 7429	Medic
	PPRI 7430	Medic
AG-3	PPRI 7431	Lupin
	PPRI 7432	Canola
	PPRI 7433	Wheat
AG-4 HG-II	PPRI 7434	Barley
	PPRI 7435	Wheat
	PPRI 7436	Medic
	PPRI 7437	Barley
	PPRI 7438	Wheat
	PPRI 7439	Medic
AG-11	PPRI 7440	Lupin
	PPRI 7441	Lupin
Binucleate		
AG-A	PPRI 7414	Lucerne
	PPRI 7415	Barley
	PPRI 7416	Medic
AG-I	PPRI 7420	Barley
	PPRI 7421	Lupin
	PPRI 7422	Wheat
AG-K	PPRI 7423	Lucerne
	PPRI 7424	Lupin
	PPRI 7425	Canola
Un. species	PPRI 7417	Wheat
	PPRI 7418	Wheat
	PPRI 7419	Wheat

[y] AG = anastomosis group and Un. species = unidentified *Rhizoctonia* spp.

[z] Cultures deposited in the National Collection of Fungi at the ARC-Plant Protection Research Institute in Pretoria, South Africa.

Sampling and isolation. Plants were collected at three sampling times in 2000, 2001, and 2003 (i.e., at the seedling [4 to 6 weeks after planting], midseason [12 to 14 weeks after planting], and flowering or seedpod [20 to 22 weeks after planting] stages). In 2002, plants were sampled only at the flowering or seedpod stages. In all, 60 plants were collected from 10 sampling locations (6 plants/location) along a zigzag ("W") pattern through each experimental plot at each sampling time. This study was an attempt to survey the diversity of *Rhizoctonia* spp. associated with this cropping system; therefore, plant symptoms were not used as a factor in selecting samples. Plants were washed under running tap water to remove adhering soil and rinsed twice in sterile distilled water. Small pieces of root and hypocotyl, coleoptile, or crown tissue with lesions were excised and plated onto the following growth media: water agar (WA) (Agar Bacteriological, Biolab Diagnostics, Midrand, South Africa), WA amended with novostreptomycin at 200 μg ml^{-1}, and potato dextrose agar (PDA) (Biolab Diagnostics) amended with novostreptomycin at 200 μg ml^{-1}. In all, 18 hypocotyl, coleoptile, or crown and 18 root pieces were plated for each experimental plot at each sampling time for each crop. The total number of pieces plated for each crop at each sampling time was 864, 288, 144, 288, 516, and 864 for barley, canola, lucerne, lupin, medic-clover mixture, and wheat, respectively. The total number of pieces plated per crop depended upon the number of plots containing the specific crops within the rotation trial.

The rotation system experiment was a randomized complete block design structure with 20 main plots (treatments) randomly replicated within four blocks. The 20 main plots (rotation systems one, two, and three) consisted of a 10-by-2 factorial (10 crop sequences × two tillage practices) with three rotation systems. Three sampling times per year were used as a subplot factor, which can be referred to as a random split-plot in time (24). A further sub-subplot was the *Rhizoctonia* groups (binucleate and multinucleate) isolated from the plant samples.

Nuclear staining. The nuclear status of multiple cells of more than one hyphae of all *Rhizoctonia* spp. isolates was determined by staining using a fluorocrome, acridine orange stain followed by fluorescence microscope imaging (51). The nuclei of 20 cells per isolate were counted in order to determine the nuclear status of each isolate.

AG typing through sequence analyses of the rDNA internal transcribed spacer (ITS) regions. Mycelia of *Rhizoctonia* cultures that were grown on PDA plates at 25°C with a 12-h photoperiod for 5 days were used for DNA extractions. DNA was extracted from mycelia according to the protocol of Lee and Taylor (22).

Polymerase chain reaction (PCR) amplification of *Rhizoctonia* DNA was conducted using the primer set internal transcribed spacer (ITS)4 (5′-TCCTCC GCTTATTGATATGC-3′)/ITS5 (5′-GGA AGTAAAAGTCGTAACAAGG-3′) (49). Reactions were carried out in a volume of 50 μl that included 1 μl of diluted DNA (5 to 10 ng), 2.5 units of Amplitaq Gold DNA polymerase, 1× manufacturer's reaction buffer, 3 μl of a 25-mM $MgCl_2$ solution, and 200 μM each dNTP. Amplification reactions were conducted in a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA) using reaction conditions of initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 60 s, 50°C for 45 s, and 72°C for 60 s, with a final extension step at 72°C for 7 min.

The resulting amplification products were cloned into the vector pCR 4-TOPO (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cloning reaction (2 μl) was used to transform chemically competent *Escherichia coli* TOP10 cells according to the manufacturer's instructions (Invitrogen). Plasmid DNA was isolated from transformed colonies using the S.N.A.P. miniprep plasmid purification kit (Invitrogen). Plasmid DNA containing the appropriate-sized amplification product insert was identified through *Eco*RI restriction digestion and subsequently utilized in DNA sequence reactions.

Sequencing reactions were conducted using the CEQ DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA). Reactions were performed in a total volume of 20 μl containing 2 μl of plasmid DNA (10 to 20 ng), 2 μl of M13 reverse primer (1.78 pMol μl^{-1}), 4.0 μl of manufactured Master Mix, and 12 μl of dH_2O, with an initial denaturation at 96°C for 60 s followed by 30 cycles of 96°C for 20 s, 50°C for 20 s, and 60°C for 20 s. Upon completion of the sequencing reaction, stop solution (2 μl of 3 M NaOAc, pH 5.2, and 2 μl of 100 mM EDTA, pH 8.0) and 1 μl of glycogen was added prior to ethanol precipitation of DNA. Samples were resuspended in 40 μl of sample loading solution and analyzed using a CEQ 8000 Genetic Analysis system. For each resulting sequence, a BLAST search was performed on GenBank to identify the most closely related species.

AG typing using conventional hyphal fusion reactions. The AG of 28 representative isolates was confirmed by conventional hyphal anastomosis testing (Table 2). These isolates, which also were charac-

terized through sequence analyses of the rDNA-ITS region, were paired with known tester strains (Table 3) on 2% WA in petri dishes. The cultures were incubated under cool white light with a 12-h photoperiod at 25°C. Analysis of hyphal fusion was conducted by examining the zone of hyphal interaction at ×40 magnification using a light microscope.

Temperature growth studies. The effect of temperature on in vitro radial growth of 28 *Rhizoctonia* isolates, representing nine AGs (Table 2), was studied at seven different temperatures (5, 10, 15, 20, 25, 30, and 35°C). Mycelial plugs (0.6 cm in diameter) were transferred from the edge of 5-day-old actively growing colonies to PDA. Cultures were incubated in the dark and colony radius was measured in four directions for each PDA plate after 48 h. Each isolate was replicated three times at each temperature.

Pathogenicity trials. Three independent trials were conducted. The first trial determined the optimal inoculum concentration based on seedling emergence. The second trial tested pathogenicity and relative virulence of the nine different AGs based on seedling emergence of the seven rotation crops. The third trial tested pathogenicity and relative virulence of all AGs based on rot development on 14-day-old seedlings of the seven rotation crops.

Pathogenicity trials were conducted using plastic pots 13 cm in diameter with a holding capacity of 700 g of planting medium. The planting medium was made up of equal quantities of soil, perlite, and sand, which was pasteurized (30 min at 83°C) for 3 days before being mixed with inoculum (inoculum concentration and emerging seedling trial) or sown with seed (14-day-old seedling trial). All trials were conducted in a growth chamber employing a 15°C night and 25°C day temperature regime, 10-h photoperiod, and relative humidity of 60 to 70%. Pots were watered every third day to field capacity.

To establish an appropriate inoculum concentration for use in subsequent trials, canola and wheat were inoculated with AG-2-1 (PPRI 7426), AG-4 HG-II (PPRI 7434), and AG-K (PPRI 7423) at five inoculum concentrations (0.005, 0.0158, 0.05, 0.158, and 0.5% mass inoculum/mass planting media [wt/wt]) 1 day prior to planting seed. Inoculum was prepared on millet seed as previously described by Strauss and Labuschagne (44). Controls consisted of noninoculated millet seed at the five different concentrations. Pathogenicity and relative virulence of each isolate at different inoculum concentrations were determined by calculating the percent damping-off and percent reduction in dry mass of seedlings 14 days after planting. Percent damping-off caused by each AG on a specific crop was determined by using the following formula: ([number of emerged seedlings in control crop A – number of emerged seedlings in AG-inoculated crop A]/number of emerged seedlings in control crop A) × 100. The percent dry mass reduction was determined using a similar formula, except that dry mass was used instead of number of emerged seedlings. The experiment used a randomized complete block design structure. The treatment design for the trial was a 3–by-2–by-5 factorial (three AGs, two crops, and five concentrations). An experimental unit consisted of a pot with 50 seed. The trial was conducted twice, with three replicates per treatment.

The capacity of 28 *Rhizoctonia* spp. isolates representing nine AGs (Table 2) to reduce plant emergence and biomass of seven rotation crops, including canola (cv. Varola 50), clover (cv. Patrick), lucerne (cv. SA Standard), lupin (cv. Wonga), medic (cv. Sephi), wheat (cv. SST57), and barley (cv. Clipper), was determined. Inoculation of planting media was conducted as in the concentration trial, except that one inoculum concentration (0.05%, wt/wt) was used. Pathogenicity and relative virulence were determined as described above. The experimental design was a randomized block design. The treatment design was a 29-by-7 factorial (28 isolates representing nine AGs and a control, and seven crops) with two replications per treatment. An experimental unit consisted of a pot with 50 seed for all the crops except lupin, where 20 seed were planted in each pot. The trial was conducted twice.

The 28 *Rhizoctonia* spp. isolates (Table 2) also were evaluated for their ability to cause rot (root, hypocotyl, or coleoptile) of 14-day-old seedlings. Pots were filled with pasteurized planting medium and an empty test tube (17 by 150 mm) was inserted in the center of each pot. Five planting holes were made around each test tube at 1 cm from the test tube. The same seven crop cultivars used in the emerging seedling pathogenicity trial also were used in the 14-day-old pathogenicity trial. At 14 days after planting, the test tube was removed from each pot and planting medium amended with 0.5% (wt/wt) inoculum was placed in the holes left by the test tube. Root rot was rated according to MacNish et al. (27) on a 0-to-4 scale, where 0 = no obvious symptoms, 1 = slight discoloration or small superficial lesions, 2 = moderate discoloration or extensive but nongirdling lesions, 3 = extensive discoloration of tissue or girdling lesions, and 4 = plant dead. Coleoptile (barley and wheat) and hypocotyl (canola, clover, lupin, lucerne, and medic) rot also were rated on a 0-to-4 scale as described for root rot. The experimental and treatment design for the trial was the same as for the emerging seedling trial (a 29–by-7 factorial with two replications [pots] per treatment). An experimental unit consisted of a pot with 25 seed for each crop except for lupin, in which 15 seed were planted in each pot. The trial was conducted twice and utilized a randomized block design.

Statistical analysis. For the rotation system, analysis each year was done separately because crop sequences differed from one year to the next. Although isolations were done separately from hypocotyls, coleoptiles, crowns, and roots, these data were combined for the analysis of variance. Analysis of variance was performed for each separate year using SAS (version 8; SAS Institute, Inc., SAS Campus Drive, Cary, NC) and the student's *t* test and least significant difference (LSD) were calculated to compare means of significant effects at the 5% significance level.

For the temperature growth studies, second-order polynomials were fitted on the radial length over temperature for each isolate by AG combination and replicates (41). The optimum growth temperature and area under each curve were calculated. These measurements were subjected to analysis of variance and the means were compared using student's *t* test and LSD at the 5% significance level.

Table 3. *Rhizoctonia* isolates used as reference isolates for conventional hyphal fusion studies[z]

***Rhizoctonia* group, AG**	**Isolate**	**Collector or supplier**
Multinucleate		
AG-2-1	H-24	Mazzola, M.
AG-2-2	455-11	Mazzola, M.
AG-3	PPRI 2152 (W14L)	PPR/Ogoshi 1988
AG-4	6-3-6	Mazzola, M.
	PPRI 2153 (F 10)	PPRI/Ogoshi 1988
AG-4 HG-II	RH 165	Mazzola, M.
AG-11	WAC9938	Barbetti, M.
	WAC10000	Barbetti, M.
	WAC10001	Barbetti, M.
Binucleate		
AG-A	PPRI 2130 (C-517)	PPRI/Ogoshi 1993
AG-D	PPRI 2135 (OR706)	PPRI/Ogoshi 1993
	C-610	Mazzola, M.
AG-I	AV-2	Mazzola, M.
AG-K	PPRI 2141 (AC-1)	PPRI/Ogoshi 1993

[z] AG = anastomosis group and PPRI = Plant Protection Research Institute (Agricultural Research Council).

Statistical analyses of data were conducted similarly for all three pathogenicity trials. Levene's variance ratio test (23) was performed to test for homogeneity of trial variances between the trial repeats. In these analyses, data of the two independent trials were considered block treatments and the replications within each trial were used as subsamples, providing that Levene's variance ratio test showed homogeneity in trial variance. Data were subjected to analysis of variance using SAS (version 8; SAS Institute, Inc.), and the Shapiro-Wilk test was performed to test for normality (37). In cases where deviations from normality were due to kurtosis and not skewness, the data were accepted as reliable and the results were interpreted without transformation (10). Pearson correlations between variables also were calculated.

RESULTS

Sampling and isolation. In all, 428 *Rhizoctonia* spp. isolates were obtained during the 4 years of the crop rotation trial. Nuclear staining of the isolates revealed the presence of 104 (24%) multinucleate (*R. solani*) and 324 (76%) binucleate *Rhizoctonia* isolates. The highest number of multinucleate isolates were isolated from medic-clover (37 isolates), followed by canola and wheat (20 isolates each), barley (17 isolates), lupin (10 isolates), and lucerne (0 isolates). The highest number of binucleate isolates were isolated from medic-clover (132 isolates), followed by canola (53 isolates), wheat (50 isolates), barley and lucerne (31 isolates each), and lupin (27 isolates). Significantly ($P \leq 0.05$) higher recovery of binucleate compared with multinucleate isolates was obtained for all 4 years (Table 4). In all 4 years, the recovery frequency of the two *Rhizoctonia* groups differed significantly ($P = 0.0105$ [2000], $P = 0.0464$ [2001], $P = 0.0060$ [2002], and $P = 0.0062$ [2003]). Due to the relatively low number of isolates obtained in each year within the four different rotation systems, statistical analyses could not be conducted to evaluate whether different rotation systems affected the incidence of *Rhizoctonia* spp. on the different crops.

Sampling time significantly ($P \leq 0.05$) affected the frequency of isolation of *Rhizoctonia* groups in 2000 ($P = 0.0003$) and in 2001 ($P = 0.0037$), but not in 2003 ($P = 0.1104$). In 2000, 2001, and 2003, recovery of *Rhizoctonia* spp. was higher during the flowering or seedpod stage than during the seedling or midseason stages (Table 5).

AG typing through sequence analyses of the rDNA ITS regions. Based upon sequence analysis, the population (324 isolates) of multinucleate *R. solani* contained isolates belonging to AG-4 HG-II (69%), AG-2-1 (19%), AG-3 (8%), AG-2-2 (2%), and AG-11 (2%). The population (104 isolates) of binucleate *Rhizoctonia* spp. included isolates belonging to AG-K (53%), AG-A (10%), AG-I (5%), and unidentified groups (32%). A large number of binucleate isolates (unidentified group) did not exhibit a clear genetic affinity with any binucleate *Rhizoctonia* spp. according to the ITS sequences contained in the GenBank library. Among the multinucleate *Rhizoctonia* groups, AG-2-1 was isolated from canola, lupin, medic, and wheat; AG-3 from canola, lupin, and wheat; and AG-4 HG-II from barley, canola, lupin, medic, and wheat. However, AG-2-2 and AG-11 were isolated only from medic and lupin, respectively. Binucleate *Rhizoctonia* AG-A was isolated from all the crops in the study, except from lupin; AG-I was isolated from barley, canola, lupin, and wheat and AG-K and the unidentified binucleate group were isolated from all the crops in the study.

AG typing using conventional hyphal fusion reactions. All *R. solani* isolates were anastomosed successfully with the respective tester isolates and all exhibited a C2 reaction as described by Carling (5). The multinucleate *Rhizoctonia* isolates that were identified in this manner included AG-2-1 (three isolates), AG-2-2 (two isolates), AG-3 (three isolates), AG-4 HG-II (six isolates), and AG-11 (two isolates). AG-I (three isolates) and AG-K (three isolates) were the only binucleate AGs that were anastomosed successfully with the tester isolates. The unidentified binucleate *Rhizoctonia* group failed to anastomose with tester isolates of AG-A, AG-I, AG-D, and AG-K. The AG group of isolates as determined through conventional anastomosis typing corresponded with the identification determined through sequence analyses of the ITS region. The only exception was three AG-A isolates that did not anastomose with the tester AG-A isolate PPRI 2130 (C-517).

Temperature growth studies. Significant differences in the temperature for optimum growth as well as growth rate at different temperatures (area under the curve) was observed among and within the nine AGs (Table 6). *Rhizoctonia* AG-2-2, AG-4 HG-II, and AG-K had significantly ($P \leq 0.05$) higher optimum growth temperatures than the other AGs (Table 6).

Pathogenicity trials. In all the pathogenicity tests, variance for the data from the two independent trials was comparable based on Levene's variance ratio test (23). Consequently, data from repeat trials were combined in all the analyses. In both the inoculum concentration and emerging seedling pathogenicity trials, correlation analyses showed that there was a very high correlation ($P < 0.0001$) between percent damping-off and percent dry mass reduction. Therefore, only percent damping-off subsequently will be discussed as a measure of pathogenicity and virulence.

In the inoculum concentration trial, the multinucleate *R. solani* isolates (AG-2-1 and AG-4 HG-II) were more virulent on canola than on wheat. Therefore, at high inoculum concentrations (0.158 and 0.5%, wt/wt), significant differences in the extent of damping-off of canola caused by AG-2-1 and AG-4 HG-II could not be distinguished (Fig. 1). In contrast, on wheat, significant ($P \leq 0.05$) differences in percent damping-off between the multinucleate isolates could be discerned at all inoculum concentrations, except at the lowest inoculum concentration (0.005%; Fig. 1). Binucleate *Rhizoctonia* AG-K caused low percentages of damping-off of canola as

Table 4. Mean percent recovery of bi- and multinucleate *Rhizoctonia* groups from crop rotations over a 4-year period

	Recovery (%)[y]			
***Rhizoctonia* group**	**2000**	**2001**	**2002**	**2003**
Binucleate	1.19 a	0.80 a	1.42 a	0.91 a
Multinucleate	0.42 b	0.38 b	0.35 b	0.23 b
P value	0.0105	0.0464	0.0060	0.0062
LSD ($P = 0.05$)[z]	0.544	0.408	0.490	0.435

[y] Means within a year (in a column) followed by the same letter do not differ significantly ($P = 0.05$).
[z] LSD = least significant difference.

Table 5. Effect of sampling time on the mean percent recovery of *Rhizoctonia* isolates from crop rotations over a 4-year period

	Recovery (%)[y]			
Sampling time[z]	**2000**	**2001**	**2002**	**2003**
1	0.42 b	0.05 b	…	0.35 a
2	0.17 b	0.40 b	…	0.42 a
3	1.81 a	1.32 a	0.89	0.96 a
P value	0.0003	0.0037	…	0.1104
LSD ($P = 0.05$)	0.482	0.559	…	0.638

[y] Means within a year (in a column) followed by the same letter do not differ significantly ($P = 0.05$); … = plants sampled only at third sampling time in 2002.
[z] Sampling time: 1 = seedling stage (4 to 6 weeks after planting), 2 = midseason (12 to 14 weeks after planting), and 3 = flowering stage (20 to 22 weeks after planting); LSD = least significant difference.

well as wheat at all inoculum concentrations (Fig. 1).

In the emerging seedling trial, significant crop–AG interactions were recorded for both damping-off ($P < 0.0001$) and dry mass reduction ($P < 0.0001$). There were no significant differences in percent damping-off ($P > 0.05$) or percent dry mass reduction ($P > 0.05$) caused by different isolates within seven of the nine AGs. However, a significant difference was present in percent damping-off ($P < 0.0001$) and percent dry mass reduction ($P < 0.0001$) caused by isolates within AG-2-1 and AG-2-2.

Pathogenicity and relative virulence of the *R. solani* AGs varied toward seedlings of the seven test crops. *R. solani* AG-2-1 caused significant damping-off of canola, lucerne, lupin, and medic, with canola and lupin being the most susceptible. Barley, clover, and wheat were not susceptible to AG-2-1 (Table 7). *R. solani* AG-2-2 caused significant damping-off (between 22 and 88%) on all seven crops (Table 7). Inoculation of the seven crops with AG-2-2 resulted in significantly more damping-off in canola, lupin, and medic (more than 87%) than the other crops. Clover and lucerne had the least amount (<25%) of damping-off when inoculated with AG-2-2 (Table 7). Similar to AG-2-2, *R. solani* AG-4-HG-II also was capable of causing significant damping-off on all seven crops. *R. solani* AG-4-HG-II was significantly less virulent on barley and wheat than on the other five rotation crops. *R. solani* AG-3 was least virulent of all the multinucleate AGs on all seven crops, causing significant damping-off only on canola, lupin, and medic (Table 7). *R. solani* AG-11 was moderately virulent on canola, clover, lucerne, lupin, and medic, not causing more than 50% damping-off (Table 7).

The binucleate *Rhizoctonia* isolates did not cause significant damping-off on most of the crops (Table 7). *Rhizoctonia* AG-I and AG-A caused significant damping-off only on lupin (7.92%) and canola (8.29%), respectively. *Rhizoctonia* AG-K caused a significant amount of damping-off (between 12 and 22%) on canola, lupin, clover, and medic (Table 7).

The number of *Rhizoctonia* AGs capable of inciting disease on any specific crop plant varied among those evaluated in this study (Table 7). Barley and wheat were least susceptible, suffering significant damping-off only when inoculated with two AGs (AG-2-2 and AG-4 HG-II). Clover and lucerne were susceptible to four AGs (AG-2-2, AG-4 HG-II, AG-11, and AG-K), showing between 8 and 84% damping-off. Medic was susceptible to six AGs, with more than 22% damping-off caused by all AGs. Lupin and canola were susceptible to six of the AGs (Table 7).

In the 14-day-old seedlings trial, significant crop–AG interactions were recorded for root ($P < 0.0001$), coleoptile ($P = 0.0228$), and hypocotyl ($P < 0.0001$) rot. Significant differences in root rot severity caused by isolates within AG-4 HG-II ($P = 0.0010$) and AG-K ($P = 0.0125$) and for coleoptile rot caused by isolates within AG-4 HG-II ($P = 0.0398$) were observed.

In the 14-day-old seedling inoculation experiments, pathogenicity and relative virulence of *R. solani* AGs differed among the seven rotation crops examined. *R. solani* AG-2-1 caused the highest amount of root rot on 14-day-old canola and lupin seedlings, whereas medic and canola seedlings had the highest hypocotyl rot rating when inoculated with this AG. *R. solani* AG-2-1 was only weakly virulent or not pathogenic to barley, clover, lucerne, and wheat (Table 7). *R. solani* AG-2-2 caused significant rot (root, hypocotyl, or coleoptile) on 14-day-old inoculated seedlings of all test crops except clover (Table 7). Lupin and medic were most susceptible to AG-2-2, having significantly more root and hypocotyl or coleoptile rot than lucerne, barley, and wheat (Table 7). *R. solani* AG-3 caused significant root rot on canola and lupin, as well as slight but significant hypocotyl rot on 14-day-old inoculated medic seedlings (Table 7). *R. solani* AG-4 HG-II caused significant disease (either root, hypocotyl, or coleoptile rot) on 14-day-old seedlings of all the rotation crops, although it was only weakly virulent on barley and wheat (Table 7). *R. solani* AG-11 was mildly virulent and caused significant root rot on all crops except barley and wheat (Table 7).

Isolates of binucleate *Rhizoctonia* spp. did not cause significant aboveground disease symptoms (coleoptile or hypocotyl rot) on 14-day-inoculated seedlings of any of the crops except for AG-K, which caused significant hypocotyl rot on medic (Table 7). *Rhizoctonia* AG-K also caused significant root rot on 14-day-old canola, lucerne, lupin, and medic seedlings (Table 7). None of the other binucleate *Rhizoctonia* spp. isolates caused

Table 6. Comparison of temperatures at optimum radial growth and total growth area under the curve (growth rate) for the nine anastomosis groups (AGs) of *Rhizoctonia* following a temperature growth study at seven different temperatures[x]

AG[y]	Optimum temperature[z]	Area under the curve
AG-2-1	21.65 d	492.18 e
AG-2-2	25.90 a	549.88 c
AG-3	21.72 d	521.08 d
AG-4 HG-II	22.43 c	637.95 a
AG-11	21.63 d	572.87 b
AG-A	20.97 e	419.61 g
AG-I	20.70 e	296.91 i
AG-K	23.21 b	464.38 f
UNBR	21.04 e	318.37 h
P value	<0.0001	<0.0001
LSD ($P = 0.05$)	0.350	10.286

[x] Values followed by the same letter in a column do not differ significantly ($P = 0.05$).
[y] UNBR = unidentified binucleate *Rhizoctonia* spp. and LSD = least significant difference.
[z] Temperature at optimum growth.

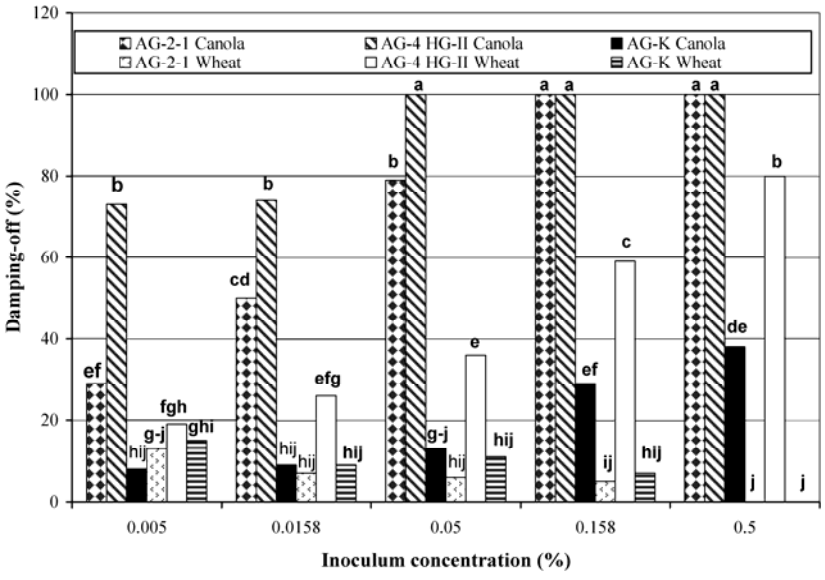

Fig. 1. Influence of inoculum concentration on percent damping-off of emerging canola and wheat seedlings caused by different anastomosis groups (AGs) of *Rhizoctonia* spp. Values are the mean percent damping-off of two replications and data are pooled over two experiments.

significant root rot on any of the test crops (Table 7).

DISCUSSION

This study characterized *Rhizoctonia* populations associated with crop species (barley, canola, lucerne, lupin, medic-clover, and wheat) that were grown within a 4-year crop rotational trial in the Western Cape province of South Africa. A significantly higher frequency of binucleate *Rhizoctonia* isolates, which are generally less virulent than multinucleate isolates (42), were present within the crop rotation trial. Similarly, in Australia, binucleate isolates were isolated at a higher frequency from lupin and cereal seedlings (25,45). Contrarily, a number of researchers isolated higher numbers of multinucleate than binucleate *Rhizoctonia* spp. from barley, canola, and wheat in various regions of the world (14,31,52). The higher numbers of binucleate *Rhizoctonia* spp. recovered in this study could be due to the lack of surface sterilization of plant materials prior to conducting isolations, thus favoring recovery of bi- versus multinucleate isolates. However, this seems unlikely because various investigations following the same procedure obtained a greater number of multi- than binucleate isolates (25,31,45). A more likely explanation for the high incidence of binucleate isolates would be the continuous lucerne cropping history of the study site, which had been established since 1996. Various studies have documented that prolonged crop monoculture can induce *Rhizoctonia* spp.-suppressive soils (29,43). Future research should be aimed at determining whether a higher incidence of nonpathogenic binucleate *Rhizoctonia* spp. is associated with disease suppression.

Rhizoctonia spp. were more frequently isolated from plants at the flowering or seedpod stage than at the seedling stage. A factor contributing to this trend could have been the more favorable temperatures (20 to 25°C) for growth of the *Rhizoctonia* AGs during September and October (flowering or seedpod stage) compared with less favorable temperatures (below 20°C) in July and August (midseason). Although isolates from all AGs tested exhibited in vitro growth at a wide range of temperatures (ranging from 15 to 30°C), virulence was clearly evident at temperatures ranging from 20 to 25°C in plant assays. In Western Australia, an increase in recovery of *Rhizoctonia* isolates also was obtained from July to August (25).

The identification of *Rhizoctonia* AGs through sequence analyses allowed the typing of a large number of isolates (428 isolates). Sequence analyses identified five multinucleate *Rhizoctonia* AGs (AG-2-1, AG-2-2, AG-3, AG-4 HG-II, and AG-11) and three binucleate *Rhizoctonia* AGs (AG-A, AG-I, and AG-K). One limitation to sequence identification of *Rhizoctonia* AGs is that relatively little sequence data are available for isolates from the binucle-

Table 7. Mean percent damping-off of emerging seedlings, and root, coleoptile, and hypocotyl rot index of 14-day-old seedlings caused by different *Rhizoctonia* anastomosis groups (AGs) in artificially inoculated steam pasteurized soil[v]

AG type	Barley	Canola	Clover	Lucerne	Lupin	Medic	Wheat
	Damping-off (%)[w]						
AG-2-1	1.15 rs	96.16 a	5.28 o–s	10.46 m–p	58.75 f	44.82 g	3.69 p–s
AG-2-2	45.69 g	87.60 cd	22.23 ijk	25.00 ij	88.13 bcd	91.55 a–d	45.95 g
AG-3	0.99 rs	27.11 i	4.53 o–s	4.08 p–s	7.92 m–r	13.96 lmn	2.02 rs
AG-4-HG-II	19.26 jkl	95.58 ab	74.99 e	84.40 d	92.92 abc	93.92 abc	22.15 ijk
AG-11	7.55 m–s	18.96 jkl	9.75 m–q	15.16 klm	50.00 h	34.80 h	4.57 o–s
AG-A	2.28 qrs	8.29 m–r	3.10 p–s	5.85 o–s	5.00 o–s	5.63 o–s	1.52 rs
AG-I	3.06 p–s	4.40 p–s	4.65 o–s	5.68 o–s	7.92 m–r	6.76 n–s	2.02 rs
AG-K	4.01 p–s	12.10 l–o	8.02 m–r	6.03 o–s	10.42 m–p	22.30 ijk	2.18 qrs
UNBR[x]	3.69 p–s	6.78 n–s	4.26 p–s	3.37 p–s	6.67 n–s	2.70 qrs	3.69 p–s
Control	0.00 s	0.00 s	0.00 s	0.00 s	0.00 s	0.00 s	0.00 s
	Root rot[y]						
AG-2-1	0.06 s	1.85 cde	0.26 p–s	0.64 l–p	1.75 def	0.97 h–m	0.07 rs
AG-2-2	0.18 p–s	1.66 def	0.24 p–s	0.74 k–o	3.00 a	1.80 def	0.28 o–s
AG-3	0.20 p–s	0.62 l–q	0.16 qrs	0.40 o–s	0.97 i–m	0.38 o–s	0.02 s
AG-4-HG-II	0.20 p–s	1.63 d–g	2.28 bc	1.36 f–j	2.91 a	2.38 b	0.18 p–s
AG-11	0.16 qrs	1.61 d–g	1.93 bcd	1.19 g–k	1.04 h–l	1.56 d–h	0.13 rs
AG-A	0.08 rs	0.36 o–s	0.12 rs	0.03 s	0.15 qrs	0.26 p–s	0.01 s
AG-I	0.08 rs	0.32 o–s	0.03 s	0.20 p–s	0.00 s	0.36 o–s	0.05 s
AG-K	0.02 s	1.40 e–i	0.47 n–s	0.93 j–n	1.53 d–h	1.14 g–k	0.04 s
UNBR	0.03 s	0.53 m–r	0.26 p–s	0.15 qrs	0.08 rs	0.27 p–s	0.09 rs
Control	0.00 s	0.00 s	0.00 s	0.00 s	0.00 s	0.00 s	0.00 s
	Coleoptile or hypocotyl rot[z]						
AG-2-1	0.67 cd	2.45 bc	0.47 ijk	1.28 fg	0.77 hi	2.19 cd	0.28 de
AG-2-2IV	1.66 ab	1.13 gh	0.41 ijk	1.48 efg	3.03 a	2.19 cd	0.66 cd
AG-3	0.20 de	0.37 ijk	0.00 k	0.17 jk	0.26 jk	0.76 hi	0.00 e
AG-4-HG-II	1.93 a	0.42 ijk	2.14 cd	1.32 efg	2.76 ab	2.96 a	1.15 bc
AG-11	1.34 b	0.38 ijk	1.73 def	1.48 efg	0.63 ij	2.78 ab	0.24 de
AG-A	0.12 de	0.00 k	0.05 k	0.13 jk	0.11 k	0.37 ijk	0.10 e
AG-I	0.14 de	0.00 k	0.00 k	0.13 jk	0.03 k	0.35 ijk	0.00 e
AG-K	0.39 de	0.34 ijk	0.18 jk	0.18 jk	0.23 jk	1.79 de	0.18 de
UNBR	0.16 de	0.06 k	0.10 k	0.14 jk	0.16 jk	0.15 jk	0.04 e
Control	0.00 e	0.00 k	0.00 k	0.00 k	0.00 k	0.00 k	0.00 e
LSD ($P = 0.05$)	0.551	0.495	0.495	0.495	0.495	0.495	0.551

[v] Values are the mean percent damping-off, root, coleoptile, and hypocotyl rot (lesion) of two replications. Data are pooled over two experiments. Values followed by the same letter do not differ significantly ($P = 0.05$).

[w] P value < 0.0001 and least significant difference (LSD; $P = 0.05$) = 7.638.

[x] UNBR = unidentified binucleate *Rhizoctonia* spp.

[y] The value index for rot was a 0-to-4 scale, where 0 = no obvious symptoms, 1 = slight discoloration or small superficial lesions, 2 = moderate discoloration or extensive but nongirdling lesions, 3 = extensive discoloration of tissue or girdling lesions, and 4 = plant dead. P value < 0.0001 and LSD ($P = 0.05$) = 0.468.

[z] Coleoptile rot was assessed for barley and wheat, and hypocotyl rot for canola, clover, lupin, lucerne, and medic, on a 0-to-4 scale as described above. P value = 0.0228 (coleoptile) and <0.0001 (hypocotyl).

ate groups. As a result, a subset (32%) of the binucleate isolates from this study remained unidentified. The accuracy of our sequence identification was assessed through conventional hyphal anastomosis reaction studies on a subset of isolates. These analyses confirmed most identifications based upon ITS sequence data, with the exception of the AG-A isolates that did not exhibit anastomosis with the reference isolate. However, this result is inconclusive because only one reference isolate of AG-A was available. Further investigations using additional AG-A reference isolates are required to clarify this finding.

In general, binucleate *Rhizoctonia* isolates were either nonpathogenic or weakly virulent, even though they were associated with most crops. This suggests that binucleate isolates were not important pathogens within the crop rotation trial. However, it is noteworthy that, in this study as well as others (18,20,25), binucleate isolates sometimes can be pathogenic, especially those within AG-K. In our study, isolates of AG-K caused significant damping-off or rot on 14-day-old canola, lucerne, lupin, and medic seedlings. Similarly, AG-K was reported as moderately or weakly virulent toward canola (18). The binucleate AG-A and AG-I were able to cause only minor, though statistically significant, rates of damping-off of emerging canola and lupin seedlings. This significant damping-off caused by these AGs is difficult to interpret. It can be hypothesized that these isolates will most likely not be pathogenic in natural soil ecosystems where inoculum concentrations may be lower than that evaluated in this study. This is supported by the inoculum concentration trial data that showed a significant increase in percent damping-off caused by a binucleate isolate (AG-K) with increasing inoculum concentration on a more susceptible crop such as canola. Another factor that will influence the pathogenicity of AG-I and AG-A in natural soil ecosystems is interaction with a competitive soil microflora that can significantly reduce the capacity of *Rhizoctonia* isolates to incite disease as well as suppressing population levels (30,38).

The *R. solani* AGs that are most likely to play an important role in yield reduction of rotations crops used in the Western Cape province include AG-2-1, AG-2-2, and AG-4-HG-II, because they were most virulent on specific rotation crops. *R. solani* AG-2-1 was highly virulent on canola seedlings, similar to other parts of the world where it is limiting canola production (11,12,14,18). This AG also was pathogenic on lupin and medic but has not been reported pathogenic on these crops elsewhere, except for one report of its pathogenicity toward lupin (45). *R. solani* AG-2-2 was pathogenic on all the rotation crops, even though previous studies have reported it as being pathogenic only toward wheat and clover (34,50). *R. solani* AG-4 HG-II was highly virulent toward lupin, medic, clover, and canola. *R. solani* AG-4, of which the subgroup has not been specified, is a well-known pathogen of canola (11,12,14,16,17,46,53) and lucerne (16,47), and also previously has been found pathogenic to lupin (26), medic (13), and clover (32).

Some of the *R. solani* AGs (e.g., AG-2-2 and AG-4-HG-II) were shown to be pathogenic to some of the test crops, but were never isolated from these crops in the field trial. This could be indicative of some biotic or abiotic factors within the rotation trial that are suppressing infection or populations of these AGs. Prolonged monoculture of lucerne within the trial site could have induced this disease suppression (29). Another factor contributing to the absence of these pathogenic multinucleate isolates could have been due to lucerne residue that can in itself suppress pathogenic *Rhizoctonia* spp. (43).

R. solani AG-3 and AG-11 were only weakly to moderately virulent on some of the crop plants tested. AG-3 was weakly virulent on canola, lupin, and medic seedlings, and this is the first report of AG-3 functioning as a pathogen in association with these crops. A previous report had indicated that AG-3 is not pathogenic towards lupin (21).

R. solani AG-11 has been reported as a pathogen of lupin (45) and, in our studies, was most virulent on this plant host. Isolates of this AG were weakly to moderately virulent on all other test crops, with the exception of barley and wheat. This appears to be the first report of AG-11 as a pathogen of canola, clover, medic, and lucerne worldwide.

Knowledge of the appropriate inoculum concentration is of utmost importance to differentiate pathogenicity and relative virulence among *Rhizoctonia* isolates. Inoculum concentrations that are too high can result in an inability to distinguish between highly virulent isolates, whereas a too-low inoculum concentration can result in an inability to distinguish among weakly virulent isolates. In these trials, 0.05% (wt/wt) inoculum concentration was found most appropriate for determining pathogenicity and relative virulence of *Rhizoctonia* AGs on emerging seedlings of wheat and canola.

Plant assays clearly demonstrated that the relative virulence of *Rhizoctonia* isolates is influenced by inoculum concentration utilized. Consequently, differences in inoculum concentration used in various other studies (3,25,31,45,52) could explain discrepancies in the pathogenicity of some AGs on certain crops as determined in this study, relative to previous reports. Additional factors influencing apparent virulence include host cultivar and environmental conditions (40). Therefore, future studies should evaluate more cultivars of the currently evaluated rotation crops. Furthermore, it is important that future studies should establish inoculum concentrations of specific AGs in natural soil ecosystems using techniques such as quantitative real-time PCR.

Determining *Rhizoctonia* AGs associated with rotation crops is essential for development of effective integrated management strategies, including crop rotation and seed treatments. This is especially important because the identification and confirmation of pathogenicity of the various AGs toward the seven rotation crops evaluated in this study suggest that *Rhizoctonia* spp. are contributing to difficulties in seedling establishment of these crops observed in the Western Cape region of South Africa (S. C. Lamprecht, E. E. Auret, and J. C. Janse van Rensburg, *unpublished data*). The identification of specific pathogenic AGs will allow for the evaluation of different fungicide seed treatments for control of these AGs (16), because *Rhizoctonia* spp. of different AGs can differ in sensitivity to the same fungicide (15). Knowledge of the host range of each AG also will allow for the development of appropriate crop sequences to be used in rotation systems.

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