Sequence-Tagged Site Markers to Identify *Rhizoctonia solani* AG 4 or 8 Infecting Wheat in South Australia

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ABSTRACT

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Polymerase chain reaction with random amplified polymorphic DNA primers was used to generate polymorphisms from *Rhizoctonia solani* isolates of anastomosis groups (AG) 4 and 8. Products specific to AG 4 and 8 were selected, cloned, sequenced, and used in conjunction with a published AG 8 sequence to obtain four sequence-tagged site (STS) markers that produced different sized products from AG 4 and 8 isolates. A positive control primer (for ribosomal DNA [rDNA]) was mixed with the STS markers, but to obtain products from both the rDNA and the *R. solani* DNA, the magnesium concentration had to be increased. At the higher magnesium concentration, the specificity of one AG 8 primer changed to encompass all *R. solani* tested. Wheat root DNA reduced the sensitivity of the STS primers. Wheat plants were inoculated with *R. solani* AG 4 or 8 isolates, and DNA extracted from tissue samples was tested with mixtures of the ribosomal and AG-specific STS primers. The results yielded both ribosomal and AG-specific markers, illustrating that this technique can be used to identify *R. solani* within wheat roots.

Additional keywords: binucleate *Rhizoctonia*, *Triticum aestivum*.

Rhizoctonia solani Kühn is the cause of "Rhizoctonia bare patch," a root disease of cereal seedlings in southern Australia (15,16,20, 21), South Africa (6), and the United States (18). In southern Australia, *R. solani* anastomosis group 8 (AG 8) is the primary pathogen associated with bare patch, but AG 4 and 2 also are found on cereal roots (12,15,16). Although *R. solani* can be isolated from diseased wheat roots, determination of the AG is difficult. A simple method to determine the AG would assist in understanding the ecology of the pathogen and the epidemiology of the disease (17).

Current methods for determining the intraspecific groups of *R. solani* involve pathogenicity testing (17,20), isoenzyme analysis (15,16), determination of the AG (17,18,21), and DNA analysis (5, 8,11,14,23) of pure cultures. Restriction fragment length polymorphism (RFLP) analysis of *R. solani* requires large amounts of fungal DNA, and the probes are based on ribosomal DNA (rDNA) (23) and genomic *R. solani* DNA (11). Polymerase chain reaction (PCR) is a rapid method that requires very little DNA (10) to detect specific DNA sequences and has been used with primers based on rDNA to characterize pure cultures of binucleate *Rhizoctonia* (5).

In this study, we set out to develop a method to identify the dominant *R. solani* groups (AG 4 and 8) in South Australian wheat fields. Random amplified polymorphic DNA (RAPD) (24) primers were used to find DNA fragments specific to these two AG. Specific sequence-tagged site (STS) primers were designed from these fragments by cloning and sequencing. These primers were used to differentiate AG 4 and 8 from other fungi. Wheat infected with *R. solani* AG 4 or 8 was tested by PCR using the STS primers, and the appropriate STS markers were produced.

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MATERIALS AND METHODS

DNA extraction. The *R. solani* isolates used in this study are described in Table 1. The other fungi used in this study (Table 2) are described in Brisbane et al. (2). All fungi were initially grown on half-strength potato-dextrose agar (Oxoid, Basingstoke, Hampshire, England). Plugs (9 mm diameter) taken from the edge of actively growing colonies were used to inoculate 300 ml of one-sixth–strength Czapek-Dox broth (1) supplemented with 0.8 g of yeast extract per liter. After 7 to 10 days, the mycelium was harvested, washed with water, blotted with a paper towel, freeze-dried, and stored at –20°C until required. The dried mycelium was ground to a powder with sand and liquid nitrogen in a pestle and mortar or with steel balls in a 2-ml tube on a vortex mixer (4). Fifty milligrams was extracted with buffer (1 ml) containing 200 mM Tris-HCl (pH 8.5), 250 mM NaCl, 25 mM EDTA, and 0.5% (wt/vol) sodium dodecyl sulfate. To 1 volume of suspension was added 0.7 volumes of phenol and 0.3 volumes of chloroform. The suspension was mixed by inverting the tube several times and centrifuging for 30 min. RNase A (25 µl at 20 mg/ml) was added to the supernatant, which was extracted with chloroform/isoamyl alcohol (24:1, vol/vol), and the DNA was precipitated with isopropanol (19).

PCR. The RAPD 10-mer primers (Operon Technologies, Alameda, CA) were designated OPD-01 to -20. A 20 µl volume of typical PCR reaction contained 25 ng of DNA in 1× *Taq* buffer (Promega, Madison, WI), 1 unit of *Taq* DNA polymerase, 2 mM $MgCl_2$, 20 pmol of primer, and 0.2 mM dNTPi. Reactions were performed on a programmable thermal controller (MJ Research, Watertown, MA). The program for the RAPD primers was (step 1) 4 min at 95°C; (step 2) 1 min at 94°C; (step 3) 1 min at 36°C; (step 4) 1 min at 72°C; and 45 cycles from steps 2 to 4 followed by a final step of 4 min at 72°C. Products from the RAPD reactions were separated on 4% Nu Sieve GTG agarose (FMC Corporation, Rockland, ME) at 6 V/cm and visualized with ethidium bromide.

TABLE 1. *Rhizoctonia solani* isolates used in this study

Culture number[a]	Anastomosis group	Zymogram group	Origin	Location[b]	Original number[a]	Source
21	AG 8	ZG1	Wheat	Avon, S.A.		H. McDonald
759	AG 4	ZG8	Soil	Moonta, S.A.	F87	J. F. De Beer
1325	AG 3	nd[c]	Potato		F86	J. F. De Beer
1328	AG 8	ZG1	Wheat	Moonta, S.A.	F95	J. F. De Beer
1342	AG 4	ZG8	Wheat	Moonta, S.A.	L54	J. F. De Beer
1343	AG 4	ZG8	Wheat	Moonta, S.A.	L24	J. F. De Beer
1344	AG2-1	nd	Wheat	Bow Hill, S.A.		S. Neate
1346	AG 8	ZG1-5	Wheat	Cook Plains, S.A.	JW92	J. H. Warcup
1347	AG 8	ZG1-1	Wheat	Wirulla, S.A.	JW126	J. H. Warcup
1348	AG 8	ZG1	Barley	Spalding, S.A.		H. McDonald
1418	AG 8	ZG1-2	Barley	Woodchester, S.A.		S. Neate
1427	nd	CZG5[d]	Soil	Kapunda, S.A.		S. Neate
1471	nd	CZG3	Wheat	Wanilla, S.A.		S. Neate
1499	nd	CZG5	Medic	Alford, S.A.		S. Neate
1510	AG 8	ZG1-2	Barley	Perponda, S.A.		S. Neate
1541	AG 8	ZG1-3	Barley	Rudall, S.A.		S. Neate
1661	AG 4	nd	Cotton	Narrabri, N.S.W.		H. Ogle
1662	AG 4	nd	Cotton	Narrabri, N.S.W.		H. Ogle
1746	AG 4	ZG 8	Cucumber	S.A.		G. Masuhara
1761	AG 4	nd	Medic	Michigan, USA	F125	N. T. Flentje
1783	AG1-1B	nd		Japan	B19	A. Ogshi

[a] Culture collection of S. M. Neate.
[b] S.A. = South Australia; N.S.W. = New South Wales, Australia.
[c] nd = not determined.
[d] CZ = binucleate *Rhizoctonia*.

TABLE 2. Fungi other than *Rhizoctonia solani* used in this study

Culture[a]	Fungus	Origin	Source
FAC-2028	*Curvularia inaequalis*	Barley	J. R. Harris
FAR-2061	*Embellisia chlamydospora*	Barley	J. R. Harris
F 1771	*Fusarium avenaceum*		L. W. Burgess
F. 5618	*F. culmorum*		L. W. Burgess
FQB-305	*F. culmorum*	Wheat	J. R. Harris
FMH-183	*F. graminearum*	Barley	J. R. Harris
F. 4616	*F. oxysporum*		L. W. Burgess
FAR-2153	*Phoma chrysanthemicola*	Oats	J. R. Harris
FYR-2226	*P. macrospinosa*	Barley	J. R. Harris
FYH-2224	*Trichoderma koningii*	Barley	J. R. Harris
FNC-1666	*T. viride*	Barley	J. R. Harris

[a] Culture collection of J. R. Harris (2).

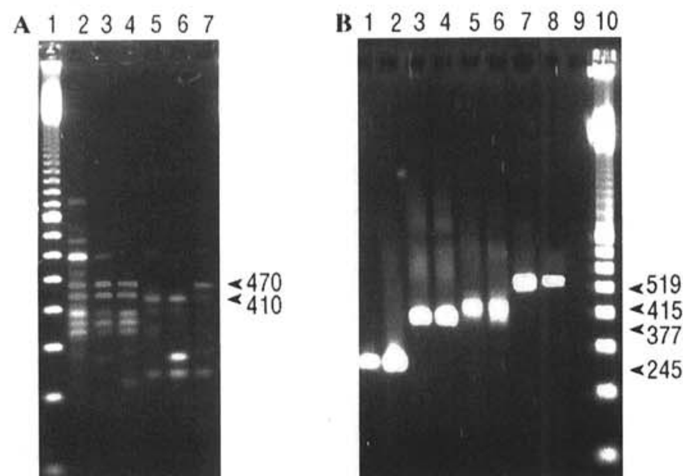

Fig. 1. Ethidium bromide-stained polymerase chain reaction products after electrophoresis in 4% 3:1 Nu Sieve gel. **A,** Products from random amplified polymorphic DNA primer OPD-05 with *Rhizoctonia solani* anastomosis groups (AG) 4 and 8 DNA showing different banding patterns for each AG. Lane 1, 100-bp ladder; lane 2, AG 4 #759; lane 3, AG 4 #1342; lane 4, AG 4 #1343; lane 5, AG 8 #1328; lane 6, AG 8 #1348; lane 7, AG 8 #1418. **B,** Products from sequence-tagged site primers for two AG of *R. solani*. Lane 1, RA-245 and AG 8 #21; lane 2, RA-245 and AG 8 #1328; lane 3, R8-377 and AG 8 #1346; lane 4, R8-377 and AG 8 #1510; lane 5, R4-415 and AG 4 #759; lane 6, R4-415 and AG 4 #1343; lane 7, R4-519 and AG 4 #759; lane 8, R4-519 and AG 4 #1342; lane 9, blank; lane 10, 100-bp ladder.

The PCR program for the STS primers was similar to that described above, except the annealing temperature (step 3) was 60°C, and the number of cycles was 30. Generally, 25 ng of fungal DNA was used in each reaction, and the $MgCl_2$ concentration was 2 mM for tests on the specificity of single primer sets. The concentration of $MgCl_2$ was increased to 4 mM when mixed sets of primers were used. Products were separated in 1.5% agarose MP (Boehringer GmbH, Mannheim, Germany) in pH 8 Tris-acetate buffer (13) at 6 V/cm and visualized with ethidium bromide.

STS primers. To obtain clones from the RAPD bands specific to the two AG, the bands were cut from the 4% Nu Sieve gel and extracted with QIAEX (QIAGEN Ltd., Chatsworth, CA). The DNA was ligated into the T tailed vector pBluescript SK± (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The ligation mixture was added to competent Sure (Stratagene) *Escherichia coli* cells, and the transformants were grown on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates following the manufacturer's instructions. Plasmids were extracted by the alkaline lysis procedure (13) and either sequenced manually with a version 2 Sequenase DNA sequencing kit (United States Biochemical Corporation, Cleveland) or with an automated ABI 373A DNA sequencer apparatus. Primers designed from these sequences had an annealing temperature of 60°C in 50 mM salt.

Detection of *R. solani* on wheat roots. Wheat seeds (cultivar Spear) were surface-sterilized with sodium hypochlorite (6% active chlorine), washed with sterile water, sown in glass tubes containing sterile sand (3), and inoculated with 5-mm agar plugs of *R. solani* AG 4 #759, #1343, #1746, or AG 8 #1347. The glass tubes were placed in a glasshouse, and the *R. solani* infected the wheat roots and shoots; diseased tissues were excised, and the DNA was extracted using the method of Doyle and Doyle (7). Fifty nanograms of this DNA was used in the PCR with STS primers.

RESULTS

Development of STS primers. RAPD primers OPD-01 to -20 were used in PCR analysis with 25 ng of DNA from *R. solani* AG 8 #21. Primers that gave no bands were not considered further. The remaining primers were used with DNA from AG 4 and 8 isolates to detect polymorphisms. Some RAPD primers, e.g., OPD-05, gave different patterns for *R. solani* AG 4 and 8 (Fig. 1A), and

bands shared by AG 4 or 8 were detected. One of the PCR products from AG 8 culture #1328 using OPD-05 was a 410-bp band. Since this band appeared to be specific for AG 8, it was excised from a gel, cloned, and sequenced. From this sequence, two 20-mer primers with an annealing temperature of 60°C were selected and designated RA-245 a and b (Table 3); they produced a 245-bp product. In a similar fashion, a PCR product from AG 4 culture #1342 and OPD-05 was specific for AG 4. This 470-bp band (Fig. 1A) was sequenced, and a primer set for a 415-bp product was constructed (R4-415 a and b; Table 3). The AG 4 culture #1342 and OPD-04 gave a 559-bp band not seen in the products from AG 8 isolates; this band also was cloned and sequenced, and a primer set was designed to give a product of 519 bp (R4-519 a and b; Table 3). A second primer set for AG 8 was designed from an *R. solani* AG 8 repetitive DNA sequence (14). This 25- and 20-mer primer set (designated R8-377 a and b; Table 3) produced a 377-bp product.

In addition to the STS primers for AG 4 and 8, we constructed a primer set (RIB-727 a and b; Table 3) based on the sequence of a 26S ribosome from *Saccharomyces cerevisiae* (9). This was used as a positive internal control of the PCR and produced a 727-bp product.

PCR of STS primers with *R. solani* and other fungi. STS primer sets RA-245 and R8-377 (AG 8) and R4-415 and R4-519 (AG 4) were used in PCR reactions with the appropriate *R. solani* AG to generate products (Fig. 1B). The specificity of these PCR primers was tested with 19 *R. solani* isolates (Table 4). The AG 8 cultures gave the correct size marker with primer sets RA-245 and R8-377, and no product was obtained with primer set R4-519. Only one AG 8 isolate (#1347) gave a 415-bp product with the R4-415 primers. The AG 4 isolates all gave the correct size products with both AG 4 primers and no product from R8-377. Only AG 4 isolate #1661 gave a 245-bp product with RA-245 (Table 4). The other AG did not form products with the AG 8 primers, but two isolates, #1783 (AG1-1B) and #1471 (CZG5), gave a 519-bp product with the R4-519 primers. In every case when an unexpected product was obtained, the other primer for that AG did not give a product.

The results of PCR with the STS primers for other fungi that were tested are shown in Table 5. The primers RA-245 and R4-415 gave no products with the DNA of these fungi. However, the AG 8 primer R8-377 gave a 377-bp product with *Fusarium avenaceum*, *Trichoderma koningii*, and *T. viride* and a trace of product with three other *Fusarium* species. The AG 4 primer set R4-519 gave products smaller than 519 bp with several non-*Rhizoctonia* fungi. A 519-bp product was obtained with primer RA-519 and *Phoma macrospinosa* #FYR-2226 (Table 5), and there was no product from primer R4-415 with the non-*Rhizoctonia* fungi. The STS primer set RA-245 also was tested (in 2 mM $MgCl_2$) with DNA from 15 fungi (other than those shown in Table 2), including species of *Gaeumannomyces*, *Alternaria*, and *Aspergillus*, and no product was obtained (data not shown).

The desired product was not always obtained from the tests with these primers. On some occasions, this was due to impure (or too little) DNA, and on other occasions, there was a failure of the *Taq* polymerase, so a positive internal control was needed. For this control, we chose a primer, RIB-727, based on rDNA (9).

PCR with mixed primer sets. Frequently, when the control primers were mixed with other STS primers, there was a failure to produce one of the products. In 16 reactions with RIB-727 and various other STS primers, AG 8 isolate #1347 gave the 727-bp product on only 8 occasions. However, when the magnesium concentration was increased to 4 mM, all tests of #1347 with RIB-727 and other primers gave the 727-bp product. In general, primer RIB-727 mixed with other STS primers in 2 mM $MgCl_2$ gave the 727-bp product in 50% of the reactions (74 performed), but with 4 mM $MgCl_2$, the product was obtained in 90% of the reactions. A negative aspect of increasing the magnesium concentration was decreased specificity of the RA-245 primers. The mixed primer sets RIB-727 and RA-245 gave the 727- and 245-bp products with all *R. solani* isolates, and some, such as AG 2-1, produced additional bands (Fig. 2A). Mixtures of RIB-727 and R4-519 gave a 519-bp product with AG 4 isolates and no product with AG 8 isolates (Fig. 2B). There was no change in specificity of STS primers R4-415 and R8-377 at 4 mM magnesium.

TABLE 3. Polymerase chain reaction primer designations

Number	Anastomosis group[a]	Culture number	Length (bp)	Primer length	Sequence of specific primers
R4-415 a	AG 4	1342	415	18-mer	gcgcttcacatggggcaa
R4-415 b				22-mer	gactttcctgggttagccacac
R4-519 a	AG 4	1342	519	24-mer	cattgtaaaaagagtgcggtgggt
R4-519 b				21-mer	ttagtaacttgggagccggtt
RA-245 a	AG 8	1328	245	20-mer	gagcactatcggcgagcgag
RA-245 b				20-mer	gtttcctcgcccctgtctgc
R8-377 a	AG 8		377	25-mer	cccgatcgtcacccctgaaggtgaa
R8-377 b				20-mer	tactaacggtgttcggtgcc
RIB-727 a	*S. cerevisiae*[b]		727	18-mer	cggcgagtgaagcggcaa
RIB-727 b				21-mer	ttcaccatctttcgggtccca

[a] *Rhizoctonia solani* anastomosis groups.
[b] *Saccharomyces cerevisiae.*

TABLE 4. Specificity of the sequence-tagged site primers and *Rhizoctonia solani* cultures at 2 mM $MgCl_2$

Culture number[a]	Anastomosis group	Zymogram group	AG 4 primers[b-d]		AG 8 primers[b,d]	
			R4-415	R4-519	RA-245	R8-377
21	AG 8	ZG1	–	–	+	+
1328	AG 8	ZG1	–	–	+	+
1346	AG 8	ZG1-5	–	–	+	+
1347	AG 8	ZG1-1	+	–	+	+
1418	AG 8	ZG1-2	–	–	+	+
1510	AG 8	ZG1-2	–	–	+	+
1541	AG 8	ZG1-3	nd	–	+	+
759	AG 4	ZG8	+	+	–	–
1342	AG 4	ZG8	+	+	–	–
1343	AG 4	ZG8	+	+	–	–
1661	AG 4	nd	+	+	+	–
1662	AG 4	nd	+	+	–	–
1761	AG 4	nd	+	+	–	–
1325	AG 3	nd	–	nd	nd	–
1344	AG2-1	nd	–	–	–	–
1783	AG1-1B	nd	–	+	–	–
1427	nd	CZG5	–	–	–	–
1471	nd	CZG3	–	+	–	nd
1499	nd	CZG5	–	–	–	nd

[a] Culture collection of S. M. Neate.
[b] – = no product.
[c] + = product of correct size.
[d] nd = not determined.

TABLE 5. Specificity of the sequence-tagged site primers and fungi other than *Rhizoctonia solani* at 2 mM $MgCl_2$

Culture number[a]	Culture name	AG 4 primers[b-d]		AG 8 primers[b,d,e]	
		R4-415	R4-519	RA-245	R8-377
FAC-2028	*Curvularia inaequalis*	–	–	–	–
FAR-2061	*Embellisia chlamydospora*	–	–	–	–
F 1771	*Fusarium avenaceum*	–	s	–	+
F. 5618	*F. culmorum*	–	–	–	tr
FQB-2305	*F. culmorum*	–	s	–	tr
FMH-2183	*F. graminearum*	–	–	–	tr
F. 4616	*F. oxysporum*	–	s	–	tr
FAR-2153	*Phoma chrysanthemicola*	–	–	–	–
FYR-2226	*P. macrospinosa*	–	+	–	–
FYH-2224	*Trichoderma koningii*	–	–	–	+
FNC-1666	*T. viride*	–	–	–	+

[a] Culture collection of J. R. Harris (2).
[b] – = no product.
[c] s = short product.
[d] + = correct size product.
[e] tr = trace of product.

Detection of *R. solani* DNA in plant tissues. The sensitivity of the RA-245 primer set was tested in the presence and absence of DNA extracted from wheat roots. In the absence of DNA from wheat roots, the RA-245 primers gave a decreasing amount of product with 50, 5, and 0.5 ng of DNA from AG 8 isolate #21 (Fig. 3A). Thus, the RA-245 primers could detect less than 5 ng of AG 8 DNA. However, in the presence of 50 ng of wheat root DNA, only 50 ng of AG 8 DNA gave a band, indicating that the presence of wheat root DNA decreased the sensitivity of STS primers. *Rhizoctonia*-inoculated root tissue that had many lesions and was dark in color gave less DNA than infected tissue that showed little color change. The results of PCR with mixed primers RIB-727 and RA-245 and DNA extracted from wheat roots infected with various *R. solani* isolates are shown in Figure 3B. In noninfected roots, only the 727-bp product was produced, indicating a successful PCR and the presence of rDNA but no *R. solani* DNA. In infected tissue, both the 727- and 245-bp products were present (Fig. 3B).

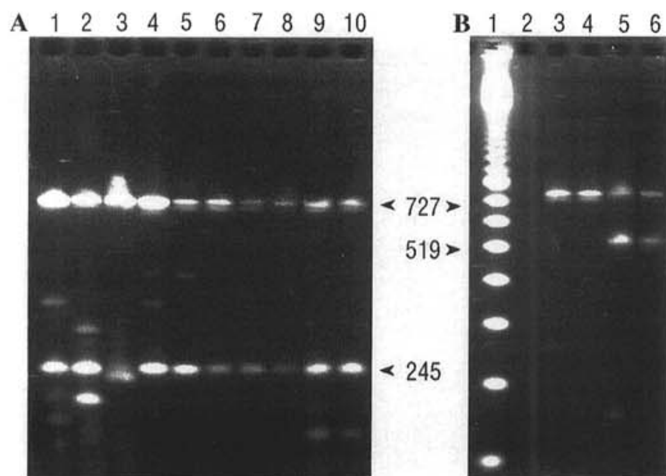

Fig. 2. Polymerase chain reaction products after electrophoresis in 4% 3:1 Nu Sieve gel. **A,** Products from mixed primer sets RIB-727 and RA-245 at 4 mM Mg^{2+}. Lane 1, *Rhizoctonia solani* anastomosis group (AG)1-1B #1783; lane 2, AG2-1 #1344; lane 3, binucleate *Rhizoctonia* #1471; lane 4, AG 3 #1325; lane 5, AG5 #1616; lane 6, AG 8 #1347; lane 7, AG 8 #1328; lane 8, AG 8 #1418; lane 9, AG 4 #759; lane 10, AG 4 #1343. **B,** Mixed primer sets RIB-727 and R4-519 at 4 mM Mg^{2+}. Lane 1, 100-bp ladder; lane 2, blank; lane 3, AG 8 #1328; lane 4, AG 8 #1418; lane 5, AG 4 #759; lane 6, AG 4 #1342.

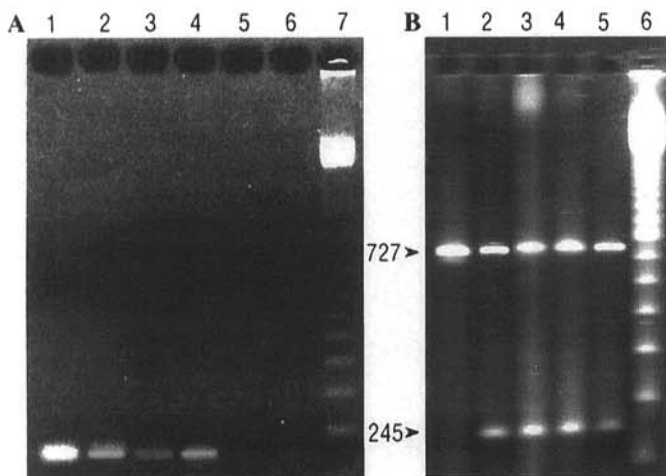

Fig. 3. Polymerase chain reaction (PCR) with DNA from wheat roots. **A,** Sensitivity of RA-245 primer set to different amounts of DNA from *Rhizoctonia solani* anastomosis group (AG) 8 culture #21 at 2 mM Mg^{2+}, with and without added wheat root DNA (WDNA). Electrophoresis in 1.5% agarose. Lane 1, 50 ng of #21; lane 2, 5 ng of #21; lane 3, 0.5 ng of #21; lane 4, 50 ng of WDNA + 50 ng of #21; lane 5, 50 ng of WDNA + 5 ng of #21; lane 6, 50 ng of WDNA + 0.5 ng of #21; lane 7, 100-bp ladder. **B,** PCR in 4 mM Mg^{2+} with mixed primers RIB-727 and RA-245 and DNA extracted from wheat roots infected with various *R. solani* isolates. Electrophoresis in 4% 3:1 Nu Sieve gel. Lane 1, uninfected roots; lane 2, AG 4 #759; lane 3, AG 4 #1343; lane 4, AG 4 #1746; lane 5, AG 8 #1347; lane 6, 100-bp ladder.

DISCUSSION

The ability to detect and identify phytopathogens using PCR technology has great potential for enhancing diagnostic capabilities. This technology is particularly suited to identifying *R. solani* AG because the standard method of searching for hyphal anastomoses on an agar plate is more time-consuming than a PCR test. In a previous taxonomic study using PCR for binucleate *Rhizoctonia* species (5), the primers were based on rDNA sequences. Thus, we knew that portions of the rDNA of *Rhizoctonia* were suitable for PCR, and we based our control primer on a conserved rDNA sequence fairly close to the sequence used by Cubeta et al. (5). Because this sequence is present in fungi and plants, it gives a product with DNA extracted from roots. This internal control overcame a common problem with PCR, the occurrence of false negative results (10). Thus, when a PCR produced the control product (727 bp), it showed that rDNA was present and also that the *Taq* DNA polymerase had not been inhibited by materials extracted with the DNA.

STS markers can be based on microsatellite DNA, which has been successful in other systems (22). Southern blots showed that microsatellite sequences $(GACA)_4$, $(GT)_{10}$, and $(GA)_{10}$ are present throughout the *R. solani* genome (data not shown). However, we chose to use RAPD products rather than microsatellites to develop the STS markers, because RAPD products can be rapidly obtained and visualized to determine whether they are specific for the required AG. The specific product is cut from the gel and cloned, whereas with microsatellites clones are first made and then screened for specificity. Once the sequence of the RAPD product is known, the length of the STS product (which must be shorter than the sequence) is determined. It was assumed that the probability of finding the selected AG-specific sequence in other fungi was low. However, since this is uncertain, two STS markers were developed for each AG so two independent tests could be made.

The STS primer sets produced the expected products with DNA from the appropriate AG (Fig 1B; Table 4), but occasionally there was an unexpected product. If Australian isolates have a narrow genetic base, this problem could be worse when this method is used in other areas of the world. However, Duncan et al. (8) used PCR to examine many Australian isolates and state, "Our studies reveal there is a high level of heterogeneity in some of the groups." Similarly Neate et al. (15) who sampled South Australian soils state "Seven different zymogram groups were detected…these zymogram groups were found in all localities sampled." Thus, the evidence suggests Australian *Rhizoctonia* spp. are heterogeneous, so we expect this work can be applied in other countries without much difficulty. The STS primer sets are good but not perfect for identifying AG 4 and 8 isolates. If identification is made with one primer set, it then can be confirmed with the other primer set.

We assumed that by mixing various primers together more information would be obtained from a single PCR. However, the addition of the control primer to the STS primers did not always lead to the expected two products. Frequently only one product was obtained; this was usually, but not invariably, the shorter product. We discovered that increasing the $MgCl_2$ concentration gave both products on most occasions. It was not surprising that this increase in $MgCl_2$ concentration affected the specificity of RA-245, because increased magnesium concentration reduced the stringency with which the primers bind to DNA (10). Because RA-245 at 4 mM $MgCl_2$ gave a product with every *Rhizoctonia* isolate tested, this provided a presumptive test for the presence of *Rhizoctonia*.

It is now possible to determine whether wheat is infected with *R. solani* by extracting DNA from wheat tissue and doing a PCR with both RA-245 and RIB-727 primers. A 727-bp product indicates that rDNA is present and that the PCR has been successful, and a 245-bp band indicates that DNA from *R. solani* is present. Subsequent tests with STS primers at 2 mM $MgCl_2$ will determine if the *R. solani* is AG 4 or 8. These primers were designed for southern Australian wheat fields, where the dominant pathogenic *Rhizoctonia* is AG 8. Its applicability to other areas will depend on the AG present, and new primers will need to be devised for other AG. For multiplex PCR, the problems associated with mixed primer sets will need to be investigated further, and DNA sequences that vary in length with each AG may have to be found.

This paper extends previous work using molecular approaches for the identification of intraspecific *Rhizoctonia* groups. RFLP and RAPD methods (5,14,23) require that the DNA be extracted from a pure culture of the fungus, whereas STS primers can identify *R. solani* DNA extracted from infected plant tissue. The STS primers are probably the method of choice for future development of rapid diagnostic methods for *Rhizoctonia* in wheat plants.

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