

Genetic Diversity of Double-Stranded RNA from *Rhizoctonia solani*

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Paper 1427 of the Journal Series of the Maine Agricultural Experiment Station.

We thank Dr. R. F. Bozarth for the dsRNA molecular weight standards, Dr. Y. Koltin for the dsRNA preparation from isolate I 13, and Dr. D. E. Carling for sharing with us unpublished information about vegetative compatibility in AG 5. This work was supported by USDA Competitive Grant 84-CRCR-1-1430 and the Maine Agricultural Experiment Station.

Accepted for publication 5 January 1990.

ABSTRACT

Bharathan, N., and Tavantzis, S. M. 1990. Genetic diversity of double-stranded RNA from *Rhizoctonia solani*. *Phytopathology* 80:631-635.

Genetic relationships among double-stranded RNA (dsRNA) components from 42 isolates of the basidiomycete *Rhizoctonia solani* were examined by Northern blot hybridization. Isolates from five anastomosis groups (AG) representing a wide range of virulence were included in the study. There exists a relatively high degree of genetic heterogeneity among dsRNAs from a single isolate or isolates from the same AG. Results from several RNA-RNA hybridization experiments revealed a lack of genetic relatedness among dsRNA populations from different AGs. Cross-hybridization did occur, however, among dsRNA segments from three

hypovirulent isolates belonging to AG 2, 3, and 5. Evidence presented here confirms findings of earlier reports as to the ubiquitous presence of dsRNA in *R. solani*, irrespective of degree of pathogenicity. Given the genetic diversity of dsRNAs occurring in this plant pathogen, our data provide no support for broad generalizations made in the past as to the association of dsRNA with virulence or hypovirulence in *R. solani*. In contrast, there appears to be a direct or indirect correlation between specific dsRNA species and hypovirulence.

The soilborne basidiomycete *Rhizoctonia solani* Kühn is an important plant pathogen with a wide host range that includes several crop species. A phenomenon similar to the transmissible cytoplasmic hypovirulence of *Endothia parasitica* Murrill (1,14) has been described in *R. solani* (11-13). Only three out of 13 isolates were found to possess dsRNA and were hypovirulent, whereas virulent isolates had no detectable dsRNA. A diseased isolate (189a), a member of anastomosis group (AG) I, contained three dsRNAs of cytoplasmic origin but no detectable amounts of viruslike particles (13). More recently, Zanzinger et al (33) reported that dsRNA was ubiquitous in natural populations of *R. solani* and found no apparent correlation between the presence of dsRNA and degree of pathogenicity. Hyakumachi et al (19) also observed that dsRNA was quite prevalent among Japanese isolates of the fungus which were both hypovirulent and highly virulent. In contrast, Finkler et al (16) suggested that dsRNA was associated with virulence in *R. solani*. Some of the dsRNAs present in this basidiomycete were found to be of viral nature (16,31,33).

The conflicting reports on the potential role(s) of dsRNA in *R. solani* could be attributed to one or a combination of the following factors: the complex genetics of *R. solani*, a species consisting of at least 10 genetically isolated AGs (26); the lack of a bioassay for the introduction of purified mycovirus or dsRNA into suitable genotypes; and the genetic diversity of dsRNA populations occurring in this fungus. We report here data concerning the genetic relationships among dsRNA segments from 42 isolates of *R. solani* belonging to five AGs and of varying degrees of pathogenicity (virulence). Preliminary findings have been presented (5-7).

MATERIALS AND METHODS

Fungal isolates and dsRNA extraction. A list of the isolates of *R. solani* used in this study is shown in Table 1. Cultures were grown at room temperature in 1-L flasks containing 150 ml of malt extract broth (30 g/L) for 18-21 days. Mycelium was

washed several times with sterile deionized water, and dsRNA was extracted from 20-g samples as described by Morris and Dodds (24). DsRNA eluted from the CF-11 column was freed from DNA or ssRNA contaminants by nuclease treatment following the method of Hoch et al (18), except that each DNase and RNase digestion was followed by treatment with proteinase K for 30 min at 37 C.

Gel electrophoresis of dsRNA. Fractionation and estimation of molecular weights of dsRNA components were carried out as described previously (9) by SDS polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous buffer system (21). DsRNA was separated on slab gels (0.1 × 14 × 17 cm) with a 2.5% stacking gel (2 cm) and 7.5% resolving gel (15 cm). Samples containing approximately 5 µg of dsRNA were electrophoresed at 8 V/cm for 18 hr. Following electrophoresis, gels were stained with 0.002% (w/v) ethidium bromide for 20 min at room temperature. DsRNA bands were visualized under 300 nm of ultraviolet light and photographed. Molecular weight markers included dsRNAs from virus-infected *Helminthosporium maydis* Nisik (8.4 kb), *Penicillium chrysogenum* Thom (average 2.9 kb and 0.7 kb), and *P. stoloniferum* Thom (average 1.4 kb), which were kindly donated by R. F. Bozarth.

Electrophoretic transfer of dsRNA to nylon membrane. DsRNA was denatured by soaking the gels in 0.05 M NaOH and 0.01 M NaCl for 30 min at room temperature with constant shaking. The gels were then neutralized in 0.1 M Tris-HCl, pH 7.5 for 60 min. Prior to electro-blotting, the gels were equilibrated in electro-blot buffer (0.01 M Tris, pH 7.8, 5 mM sodium acetate, and 0.5 mM EDTA) for 20 min. Electrophoretic transfer onto the Hybond nylon membranes (Amersham Corp., Arlington Heights, IL) was performed as recommended by the manufacturer with a Trans Blot Cell (Bio-Rad Laboratories, Richmond, CA) at 100 V, 1.23 A for 3-4 hr at 4 C. The dsRNA was immobilized on the nylon membrane by crosslinking with UV light for 10-15 min or by baking (80 C, 2 hr).

Hybridization studies. Individual dsRNA segments were selected as probes on the basis of frequency of occurrence, AG, size, and degree of virulence of the fungal isolates from which they were derived. Individual dsRNA bands were eluted from low-melting-point agarose (SeaPlaque) (FMC Corp., Rockland,

TABLE 1. List of isolates of *R. solani* indicating anastomosis grouping (AG) and degree of pathogenicity

Isolate	AG	Hosts tested ^a	Pathogenicity ^b
Rhs 23, Rhs 36, 189HT3	1	Soybean, sugarbeet	V
Rhs 51B, Rhs 52	1	Soybean, sugarbeet	MV
Rhs 8, 189a	1	Soybean, sugarbeet	H
Rhs 11, Rhs 16a, Rhs 56	2	Cabbage, radish	V
Rhs 47, 27ACT, Rhs 717, S284	2	Cabbage, radish	HV
Rhs 12, Rhs 27, Rhs 43, Rhs 45, Rhs 2	3	Potato ^c	V
Rhs 42, Rhs 44	3	Potato ^c	MV
Rhs 1A1, Rhs 50	3	Potato ^c	HV
Rhs 3, Rhs 24, Rhs 112, Rhs 113	4	Bean, tomato	V
I 13	4	Bean, tomato, carrot, cotton, onion, radish	V
Rhs 32	4	Bean, tomato	MV
Rhs 15, Rhs 33, Rhs 35, 223 SEE, JMW 4, F 19	4	Bean, tomato	HV
Rhs 9, Rhs 10, Rhs 19, Rhs 22, Rhs 53, Rhs 59	5	Lupine, potato, tomato	P
Rhs 1	5	Lupine, potato, tomato	NP

^aThirty to 50 surface-sterilized seeds were planted in sterilized soil mixed with a specified amount of inoculum from each isolate. Virulence was determined as percent damping off relative to the number of emerged, uninoculated control plants.

^bIsolates of *R. solani* from AGs 1, 2, 3, and 4 were designated as virulent (V), moderately virulent (MV), or hypovirulent (HV), whereas AG 5 isolates were grouped into pathogenic (P) or nonpathogenic (NP).

^cVirulence ratings were based on the percentage of tuber sprout tissue decayed. A total of at least 10 single-eye seed pieces were cut from tubers of cultivar Katahdin and suberized in a moist chamber at room temperature for 2 wk before planting (inoculation).

ME) as described by Maniatis et al (23) or by using strips of DEAE membrane following the method of Dretzen et al (15). Individual dsRNA segments (1–5 µg) were partially hydrolyzed by boiling in 100 µl of deionized formamide (nucleic acid grade, Bethesda Research Laboratories, Inc., Gaithersburg, MD) for 20 min (25) and 5'-end labeled with T₄ polynucleotide kinase essentially as described by Jordan and Dodds (20). Specific activities ranged from 5 × 10⁶ to 1 × 10⁸ cpm/µg RNA.

RNA-RNA blot hybridization. Membranes were prehybridized for 3–4 hr at 42 C in polythene heat-sealable bags with 0.25 ml/cm² prehybridization solution containing 50% (v/v) formamide, 5× Denhardt's reagent (1× = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 5× SSPE (1× = 0.018 M NaCl, 10 mM NaPO₄, pH 7.7, 1 mM EDTA), denatured salmon sperm DNA (200 µg/ml), and 0.1% (w/v) SDS. Hybridization was at 42 C for 24 hr in 0.1 ml/cm² of hybridization solution (50% formamide, 5× Denhardt's solution, 4× SSPE, 0.1% SDS, 200 mg/ml of salmon sperm DNA, and approximately 1 × 10⁸ cpm of 5' end-labeled RNA probe). The hybridized blots were washed with 0.1× SSC, 0.1% SDS unless otherwise indicated. Washing was at 68 C twice for 20 min each time and finally once at 70 C for 10 min. Damp membrane blots wrapped in Saran Wrap were autoradiographed using Kodak X-OMAT AR film and intensifying screens. Exposures were at –80 C for 2–8 days. Each membrane was hybridized to the next probe of choice after the labeled RNA probes were removed from the hybridized blots (22). Membranes were incubated with X-ray films for at least 6–8 days to verify that the original probe had been completely removed before reprobing.

RESULTS

DsRNA was detected consistently in all of the isolates of *R. solani* included in this study, and the banding patterns in SDS-PAGE gels were consistent from experiment to experiment. The 42 fungal isolates contained a total of 163 dsRNA components that were classified into three size groups, e.g., large (L) 4.6 kb or larger, medium (M) 1.1 to 4.5 kb, and small (S) 0.5 to 1.0 kb (Fig. 1). Two AGs (AG 1 and AG 4) contained no dsRNAs of the S group, whereas the proportion of the L fragments ranged from 32% in AG 2 to 60% in AG 5. The frequencies of the different dsRNA groups (L, M, or S) in hypovirulent isolates were similar to those found in virulent isolates (data not shown). Experiments with nuclease digestion (pancreatic DNase I or pancreatic RNase A in the presence of low or high salt) as well as alkali hydrolysis

confirmed that the largest size bands (>9 kb) were dsRNA. Detailed characterization of some of these molecules will be reported in the near future.

One of the objectives of this study was to examine whether dsRNAs of the same (or very similar) electrophoretic mobility and relatively high frequency within an AG or across AGs were genetically related. Individual dsRNA segments were purified and used as probes to determine whether sequence homology exists among dsRNAs found in a single isolate. The data suggested the existence of considerable sequence heterogeneity among dsRNAs from a single isolate. For example, the high molecular weight (>8.4 kb) and the 0.66-kb dsRNA fragments from isolates 189a (AG 1) and 27ACT (AG 2) hybridized to the homologous fragment but not to the other dsRNAs from the respective isolates (Fig. 2). Similar results were obtained with dsRNAs from other isolates tested in AG 3, AG 4, and AG 5 (results not shown).

Several Northern blot hybridization experiments were carried out to evaluate potential genetic relationships in dsRNA populations within an AG. Results of hybridization experiments dealing with isolates of AG 2 are depicted in Figure 3. The 2.3-kb dsRNA fragment of Rhs 11 hybridized to all of the dsRNA

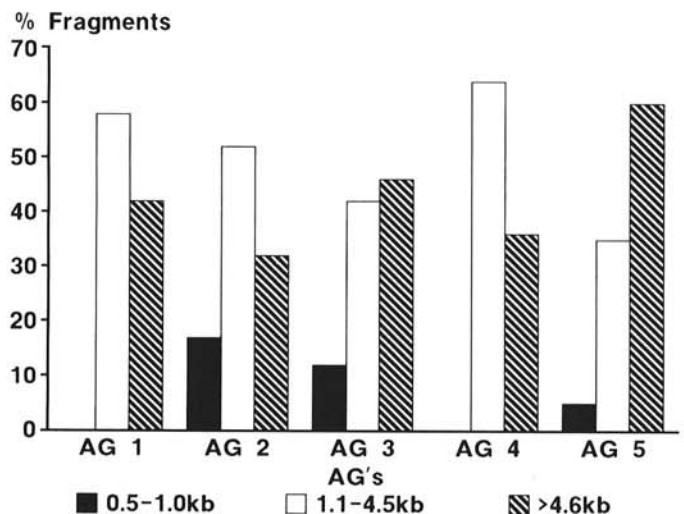


Fig. 1. Distribution of dsRNA segments from 5 AGs of *Rhizoctonia solani* according to size: small (0.5–1.0 kb), medium (1.1–4.5 kb), or large (>4.6 kb).

segments from the same isolate (Rhs 11) as well as those of S 284 (Fig. 3C, lanes 3 and 7). No detectable hybridization was observed with the remaining dsRNA components from the other isolates included in the experiment. DsRNA fragments from three different isolates were used as probes to study sequence similarity among dsRNAs from different isolates in AG 4. None of the dsRNA probes hybridized to dsRNA components from the other isolates that were included in the blot (Fig. 4B-D). The largest dsRNA component from isolate Rhs 19 was one of the probes used to examine potential relatedness among dsRNAs from AG 5 isolates, and it was found to be related to dsRNAs from all of the isolates tested with the exception of Rhs 59 (Fig. 5). Not all dsRNA bands on the blot hybridized to the Rhs 19 probe, however.

The dsRNA species that were used as probes to study sequence similarity among dsRNAs within an AG also were used to study possible relationships among dsRNAs from different AGs. From a total of 45 hybridization experiments, only three hypovirulent isolates, Rhs 47 (AG 2), K1AI (AG 3), and Rhs 1 (AG 5), cross-hybridized when the 6.4-kb fragment of K1AI was used as a probe (Fig. 6). In each of the three strains, cross hybridization was specific for certain dsRNA components, and not all of the segments present were able to hybridize. Similar results were observed when cross-hybridizing dsRNAs from isolates Rhs 47 and Rhs 1 were used as probes against the same strains.

DISCUSSION

We have found that dsRNA in *R. solani* is ubiquitous, and that virulent, hypovirulent, and avirulent (e.g., nonpathogenic) isolates of this basidiomycete all possess dsRNA. Thus, any potential relationships between dsRNA and virulence in *R. solani* would be part of a complex genetic phenomenon. These results are in direct disagreement with earlier reports about the exclusive association of dsRNA with hypovirulence (13) or virulence (16)

and confirm previous findings suggesting the ubiquity of dsRNA and the lack of an apparent correlation between the mere presence of dsRNA and virulence in *R. solani* (19,33). The virulence (degree of pathogenicity) of the isolates used in this study was assessed on more than one host or the major host (e.g., AG 3 vs. potato) several times over a period of 6 yr. Conflicting results about the role of viruslike particles (VLPs) or dsRNA in the virulence of other fungal plant pathogens (18,28,29,30) are common in the literature. Stanway and Buck (30) surveyed more than 100 isolates of *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier, incitant of the take-all disease of wheat, and found no correlation between virulence of the fungus and the presence or number of dsRNA segments. They observed, however, that hypovirulence did correlate with the presence of specific dsRNAs. Frick and Lister (17) examined 20 isolates of *G. graminis* and found considerable serotype variation, not only among isolates of different geographical origins, but also among isolates taken from the same field in the same year. They suggested that "biotype variation between VLPs of different isolates, may be considered as an explanation for the apparent inconsistencies and complexities for their putative association with fungal virulence." To date, no serological data are available to draw a consensus on the serotype diversity of mycoviruses in *R. solani*. Serological diversity would show differences in viral epitopes representing only a fraction of the viral genome, whereas this study provides dsRNA hybridization data that reflect genetic relationships more accurately and includes dsRNAs not associated with viruslike particles.

The genetic diversity among dsRNA segments found in a single isolate of *R. solani* (Fig. 2) was not unexpected. Fungal viruses are known to have multipartite genomes (8). Furthermore, mixed viral infections with two or more unrelated viruses have been reported to occur in other fungi (4,10). Interestingly, none of the dsRNA probes from AG 4 hybridized to dsRNA segments from isolates of AG 4 other than the one from which the probe originated. Vilgalys (32) also reported low genomic DNA hybridization values (30-62%) for isolates of AG 4 compared to the overall intragroup DNA hybridization which was >57% in other AGs. The lack of genetic relatedness among dsRNAs from different isolates of AG 4 suggests that horizontal transmission of mycoviruses or other cytoplasmic genetic determinants among AG 4 isolates might be quite limited under natural conditions. In contrast, the high degree of vegetative compatibility among AG 5 isolates (D. E. Carling, *personal communication*) appears to result in a greater genetic homogeneity in the dsRNA population of AG 5 (Fig. 5). DsRNA segments from isolates of the same

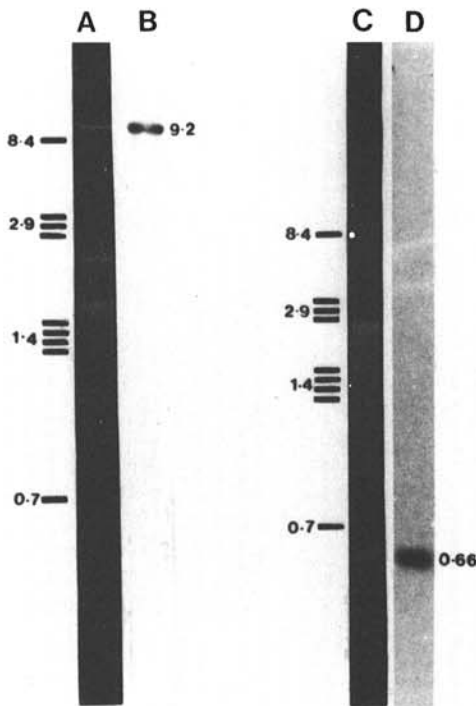


Fig. 2. Fractionation of dsRNA from isolates of *Rhizoctonia solani* 189a (AG 1) (A) 27ACT (AG 2) (C) on 7.5% SDS-polyacrylamide gels stained with ethidium bromide. Lanes B and D show hybridization to the single dsRNA components 9.2 kb and 0.66 kb, respectively. Sizes of dsRNA molecular weight standards are shown to the left of lanes A and C.

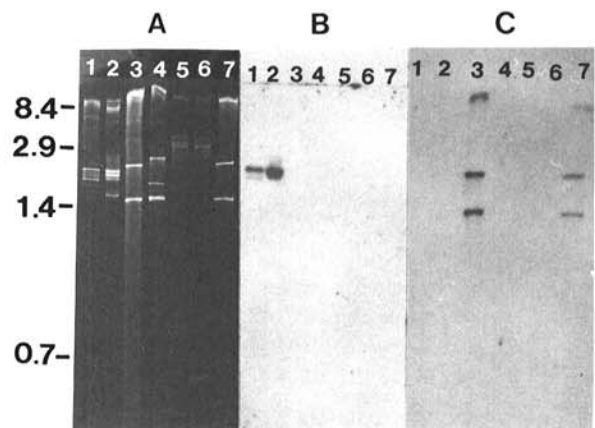


Fig. 3. Electrophoretogram of dsRNA segments from AG 2 isolates of *Rhizoctonia solani* in a 7.5% SDS-polyacrylamide gel stained with ethidium bromide (A). DsRNA was electrophoretically transferred to a nylon membrane and hybridized to the 2.4-kb dsRNA from isolate 27 ACT (B), or to the 1.8-kb dsRNA from Rhs 11 (C). Lanes 1 to 7 correspond to fractionated dsRNA from AG 2 isolates 27 ACT, Rhs 717, Rhs 11, Rhs 47, Rhs 56, Rhs 16, and S 284, respectively. The numbers on the left indicate the size (kb) of the dsRNA standards.

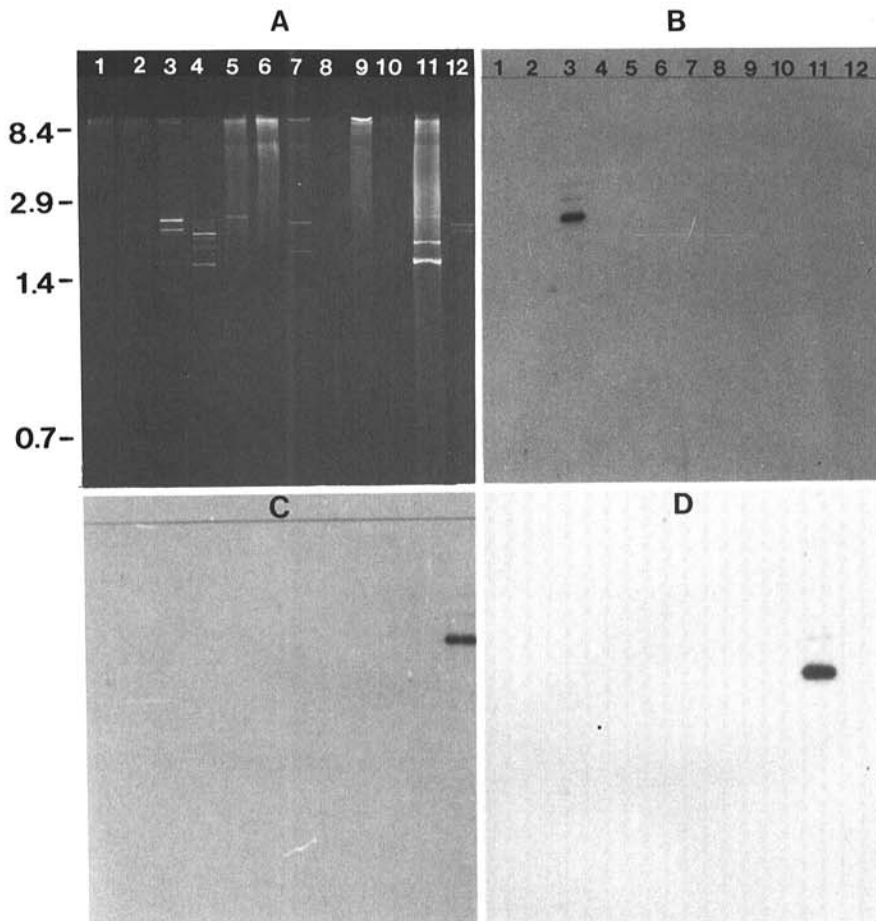


Fig. 4. Ethidium bromide-stained dsRNAs from 12 AG 4 isolates of *Rhizoctonia solani* electrophoresed in a 7.5% SDS-polyacrylamide gel (A). Several weakly stained dsRNA bands are not visible on panel A. Following electrophoretic transfer of the dsRNAs to a nylon membrane, the blot was hybridized to the 2.5-kb dsRNA from isolate Rhs 15 (B), the 2.4-kb dsRNA from Rhs 32 (C), or the 2.2-kb dsRNA from isolate I 13 (D). Lanes 1 to 12 correspond to fractionated dsRNA from isolates Rhs 3, Rhs 33, Rhs 15, Rhs 35, Rhs 24, Rhs 112, Rhs 113, JMW4, F19, 223 SEE, I 13, and Rhs 32, respectively. The numbers on the left indicate the sizes (kb) of the dsRNA standards.

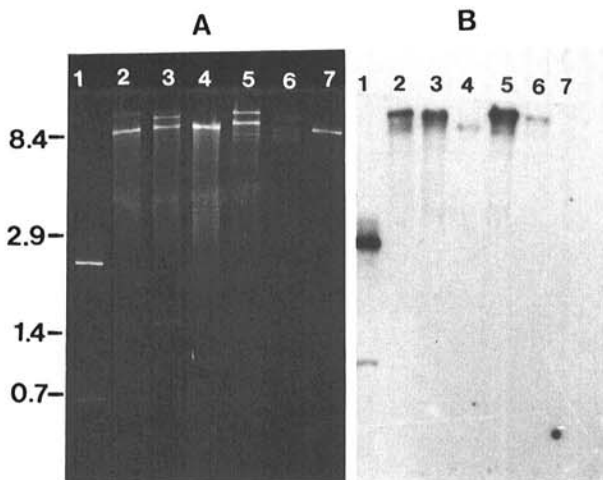


Fig. 5. Electrophoretogram of dsRNAs from AG 5 isolates of *Rhizoctonia solani* in a 7.5% SDS-polyacrylamide gel stained with ethidium bromide (A). DsRNA was transferred electrophoretically to a nylon membrane, and the blot was hybridized to largest dsRNA segment from isolate Rhs 19 (B). Lanes 1-7 correspond to fractionated dsRNAs from isolates Rhs 1, Rhs 22, Rhs 10, Rhs 53, Rhs 19, Rhs 9, and Rhs 59, respectively. The sizes (kb) of the dsRNA molecular weight markers are shown on the left.

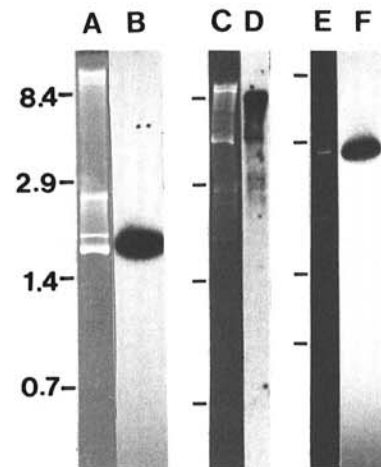


Fig. 6. Cross hybridization among dsRNAs from three hypovirulent isolates of *Rhizoctonia solani* belonging to different AGs. Lanes A, C, and E show fractionated dsRNA from isolates Rhs 47 (AG 2), Rhs 1A1 (AG 3), and Rhs 1 (AG 5), respectively. Electrophoretically transferred dsRNAs were hybridized to the 6.4-kb dsRNA from Rhs 1A1 (lanes B, D, and F, respectively). Bars to the left of lanes A, C, and E indicate the positions of the dsRNA molecular weight standards.

AG that had the same electrophoretic mobility did not necessarily cross-hybridize (e.g., the 1.8-kb dsRNA segments from isolates Rhs 47 and S 284 [Fig. 3], or the 2.5-kb segments from isolates Rhs 15 and Rhs 32 [Fig. 4]). These results, as well as data discussed below, support the view that conclusions about the presence or absence of genetic relatedness between dsRNA bands cannot be based on electrophoretic mobility. When the fastest (1.8 kb) dsRNA of Rhs 11 (AG 2) was used as a probe, it hybridized to all three discrete bands from the same isolate as well as three dsRNAs from isolate S 284 (AG 2) (Fig. 3). Whereas the two faster dsRNAs from these two isolates appear to be of the same size (1.8 and 2.6 kb, respectively), the slow dsRNA band of Rhs 11 is significantly larger than the respective band of isolate S 284 (Fig. 3A and C). Interestingly, Rhs 11 is virulent, while the S 284 is hypovirulent. One might speculate that the slow dsRNA band of S 284 suffered a deletion which resulted in the conversion of an originally virulent isolate to the hypovirulent S 284. A similar yet more obvious case of a potential dsRNA deletion leading to hypovirulence was observed in AG 5. When the slowest dsRNA band of Rhs 19 was used as a probe, it hybridized to the high molecular weight dsRNAs (>8.4 kb) from all of the virulent isolates with the exception of Rhs 59. The same probe hybridized to the significantly smaller dsRNA bands (2.3 kb and 0.45 kb, respectively) of the hypovirulent isolate Rhs 1 (Fig. 5), which contains no detectable amounts of a high molecular weight dsRNA. It is noteworthy that isolate Rhs 1 is nonpathogenic on three different hosts, e.g., potato, tomato (3; Bandy and Tavantzis, unpublished), and lupine (Leach and Tavantzis, unpublished), whereas all of the other five "hybridizing" isolates (i.e., Rhs 9, 10, 19, 22, and 53) are pathogenic on all three hosts.

Several hybridization experiments were carried out under high stringency conditions to determine whether dsRNA populations from different AGs are genetically related. The lack of cross hybridization among dsRNAs from isolate members of different AG was expected, considering that the various AGs of *R. solani* are genetically isolated (2,27). In spite of their genetic isolation, however, cross hybridization did occur among three dsRNA segments of varying sizes (i.e., 1.8, 6.4, and 2.3 kb) from isolates Rhs 47 (AG 2), Rhs 1A1 (AG 3), and Rhs 1 (AG 5), respectively (Fig. 6). The fact that all three isolates are hypovirulent suggests that the common sequence might be responsible for their hypovirulence condition and may have been present in an ancestral genotype from which the corresponding three AGs (AG 2, AG 3 and AG 5) evolved in a divergent manner. Work is under way to unveil the nature of the above sequence and to determine whether it can be used as a hypovirulence-inducing agent in *R. solani* or other fungal plant pathogenic species.

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