Genetics

Spontaneous Appearance of Genetically Distinct Double-Stranded RNA Elements in *Rhizoctonia solani*

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ABSTRACT

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A tuberborne sclerotium of the plant-pathogenic basidiomycete *Rhizoctonia solani* gave rise to a culture (Rhs 1AP) that exhibited a sector (Rhs 1A1) with reduced pigmentation and growth rate. Rhs 1AP is virulent on potato, which is the only known host plant for Rhs 1AP. In contrast, Rhs 1A1 is hypovirulent or nonpathogenic on potato. The virulent isolate Rhs 1AP contains two double-stranded RNAs (dsRNAs) of 23 and 6.5 kb, whereas the hypovirulent Rhs 1A1 possesses three dsRNAs in addition to the two found in Rhs 1AP. The apparent sizes of the three novel dsRNAs are 25, 3.7, and 1.2 kb. We constructed complementary DNA libraries of these dsRNAs and conducted Northern blot hybridization analysis, which showed that the dsRNAs with corresponding sizes (23 and 6.5 kb) occurring in both cultures are genetically similar, if not identical. The five dsRNAs, however, are not related to one another. For over 12 yr, both the phenotype and the dsRNA content of Rhs 1AP and Rhs 1A1 remained stable until 2 yr ago, when Rhs 1AP gave two more sectors (Rhs 1A2 and Rhs 1A3) exhibiting a slow growth habit. Interestingly, both of the slow-growing subcultures lacked the original Rhs 1AP dsRNAs (23 and 6.5 kb). Rhs 1A2 and Rhs 1A3 have had stable phenotypes and dsRNA patterns since the time of their emergence. All of the cultures (Rhs 1A1, Rhs 1A2, and Rhs 1A3) derived from Rhs 1AP contained the 1.2-kb dsRNA. Polymerase chain reaction analysis of total DNA showed that sequence(s) genetically related to the 3.7-kb dsRNA element are found in genomic DNA from Rhs 1AP and Rhs 1A1.

Additional keywords: cloning, genomic DNA integration.

Rhizoctonia solani Kühn (teleomorph *Thanatephorus cucumeris* (A. B. Frank) Donk) is a collective species consisting of over 10 anastomosis groups (AG) (33). Each AG is an evolutionary unit in the sense that it is a genetically isolated, noninterbreeding population with a distinct host range (2). A cytoplasmically controlled degenerative disease of *R. solani* was reported in 1978 (11,13). The disease condition was characterized by a loss of mycelial pigmentation, reduced growth rate and sclerotial production, the presence of double-stranded RNA (dsRNA), and hypovirulence similar to that reported in *Cryphonectria parasitica* (1,15). Three of 13 *R. solani* strains had dsRNA and were hypovirulent, whereas 10 virulent isolates contained no detectable dsRNA (13). In contrast, Finkler and coworkers (18) reported that dsRNA was present only in virulent strains of the fungus. Our research group initiated a study the results of which were in disagreement with both of the above reports. Our initial work suggested that 1) dsRNA is ubiquitous in natural populations of *R. solani* and 2) there is no apparent correlation between the mere presence of dsRNA and virulence (43). Isolates from five AGs (AG-1–AG-5) and several host species per isolate were used (42). Moreover, an extensive survey conducted in Japan (22) and data from two recent independent surveys conducted in Florida (41) and Louisiana (24) confirmed our findings. Hypovirulent isolates of *R. solani* are capable of providing biocontrol against virulent isolates of this pathogen (4,12). Therefore, it is important to understand the mechanism(s) that bring about the hypovirulence phenomenon and whether or not certain dsRNA elements are involved in the expression of virulence in *R. solani*.

We showed that the conflicting reports on the role of dsRNA in the expression of virulence in *R. solani* might be attributed, at least in part, to the high degree of genetic diversity among dsRNA elements occurring in natural populations of the fungus

(8,9). Our data provided several lines of indirect evidence suggesting that specific dsRNA elements might be involved in regulating virulence in *R. solani* (8,9). Furthermore, curing dsRNA through hyphal-tip subculture of the AG-3 isolate Rhs 41 increased its virulence fourfold (35).

Here we report that spontaneous changes are taking place in the dsRNA pattern of a virulent isolate of *R. solani*. These changes coincide with variations in the phenotype of the resulting cultures.

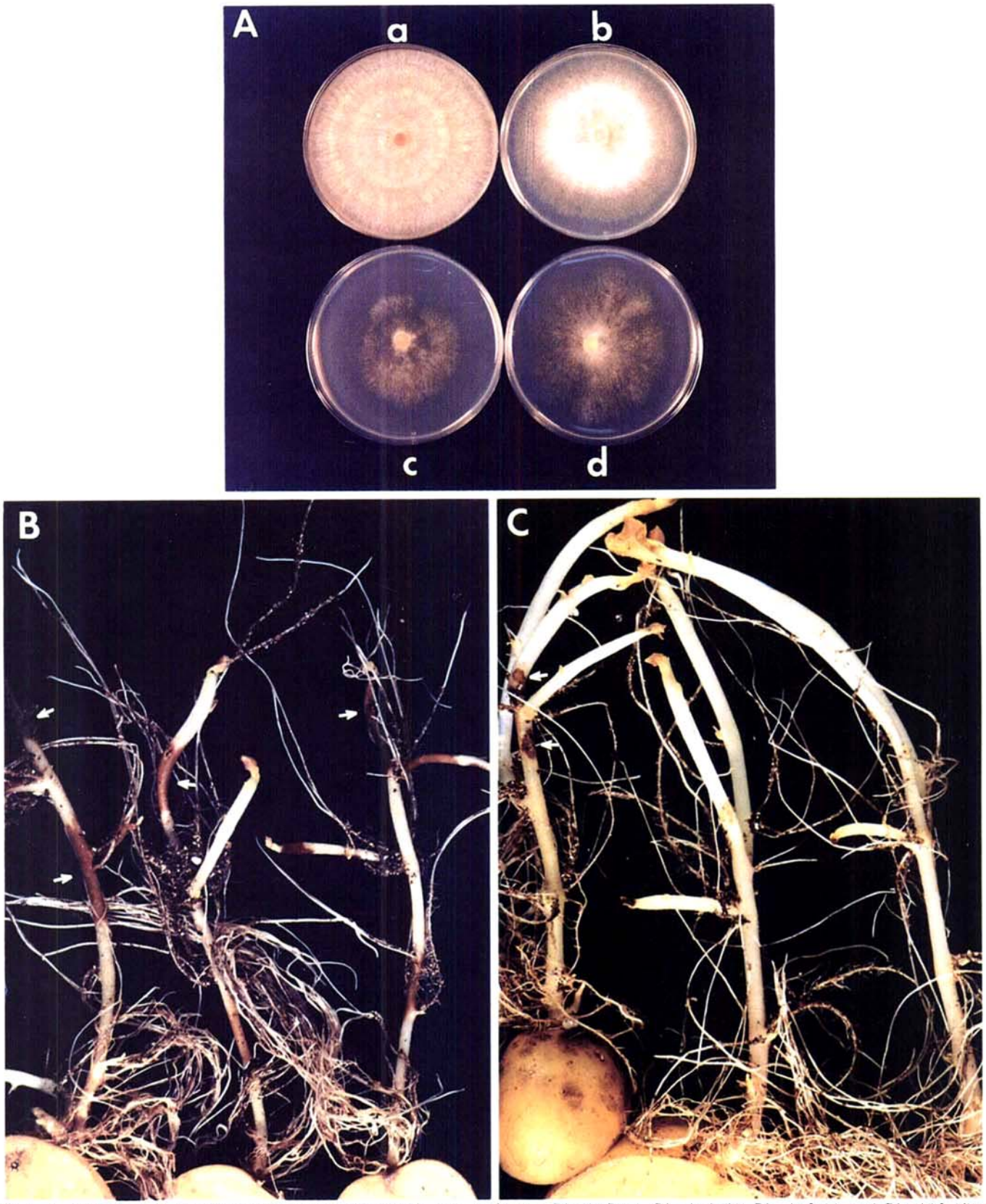

Fig. 1. A, Seven-day-old mycelial cultures of the anastomosis group 3 isolates Rhs 1AP (a), Rhs 1A1 (b), Rhs 1A2 (c), and Rhs 1A3 (d) on potato-dextrose-yeast agar media; **B,** sprouts of potato cultivar Katahdin infected with *Rhizoctonia solani* isolate Rhs 1AP; and **C,** potato sprouts infected with *R. solani* isolate Rhs 1A1. Arrows indicate lesions caused by the respective fungal isolates. Although two superficial lesions caused by Rhs 1A1 are shown here, Rhs 1A1 typically causes no lesions on most sprouts inoculated with it. No visible lesions were observed in mock-inoculated sprouts (not shown).

In addition, the novel dsRNAs that appear in the sectors (subcultures) are genetically unrelated to the dsRNAs occurring in the parental culture. To our knowledge, this is the first report on cloning of dsRNA from *R. solani.* Parts of this work have been reported previously (25,26).

MATERIALS AND METHODS

Fungal isolates. Isolate Rhs 1AP (Fig. 1A,a) originated from a tuberborne sclerotium and was shown to be a member of AG-3 possessing a relatively high degree of virulence (Fig. 1B). Isolate Rhs 1A1 (Fig. 1A,b) originated as an Rhs 1AP sector characterized by reduced pigmentation and growth rate as compared with Rhs 1AP. More importantly, Rhs 1A1 was found to be hypovirulent (often nonpathogenic) (Fig. 1C) and significantly reduced the amount of rhizoctonia disease caused by virulent AG-3 isolates in potato both in the field and in the greenhouse (4). Pathogenicity tests with potato cultivar Katahdin sprouts were conducted as described by Bandy and Tavantzis (3).

The Rhs 1A1 sector appeared in the sclerotial culture of Rhs 1AP (in 1982) and has retained the same phenotype as that of the original sector. More recently (1991), two more sectors, Rhs 1A2 and Rhs 1A3 cultures (Fig. 1A,c and d, respectively), have been isolated from Rhs 1AP. They grow on solid media at a rate significantly slower than that of Rhs 1AP and Rhs 1A1 (Fig. 1A).

Mycelium was grown in 1-L flasks containing 150 ml of malt extract broth (30 g/L) for 2 wk at room temperature unless otherwise indicated. Mycelium was harvested by filtration, washed with sterile deionized water, frozen in liquid nitrogen, and stored at −80 C.

Extraction and purification of dsRNA. The procedure for extraction of dsRNA from fungal mycelium was as described by Morris and Dodds (28) with minor modifications. dsRNA preparations were freed from traces of single-stranded RNA and DNA by using pancreatic ribonuclease A in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and pancreatic DNase I, respectively, as described by Hoch et al (21). Electrophoretic fractionation of dsRNA preparations was carried out as described previously (8). DNA size standards included λ phage DNA digested with *Hin*dIII and the 1-kb DNA ladder (Bethesda Research Laboratories, Gaithersburg, MD).

Cloning of dsRNA. Complementary DNA (cDNA) libraries of individual, gel-eluted dsRNA bands were prepared by using the SuperScript cDNA synthesis kit (Bethesda Research Laboratories) and following the directions of the supplier, except that dsRNA templates were denatured with methylmercuric hydroxide (20 mM) and first-strand synthesis was performed at 22 C (5 min), 45 C (30 min), and 50 C (30 min). Double-stranded cDNAs were ligated to *Eco*RI-*Sal*I-*Not*I adapter before they were ligated to the pBluescript SK +/− phagemid (Stratagene, La Jolla, CA). Recombinant plasmids were purified, and the sizes of the cDNA clones were determined by digestion with *Not*I and fractionation in 1% agarose gels.

Northern blot analysis of dsRNA. Purified dsRNA samples were fractionated in 1% agarose gels in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0) at 25 V for 15 h at room temperature and denatured by soaking the gels in 50 mM NaOH for 30 min at room temperature unless otherwise indicated. Gels were neutralized with 1.5 M Tris-HCl, pH 7.5, containing 0.5 M NaCl for 40 min (two 20-min changes) and blotted in a standard capillary blotting apparatus as described previously (37). Blots (Hybond-N, Amersham Corp., Arlington Heights, IL) were baked at 80 C for 2 h, prehybridized (27) at 65 C for 2 h, hybridized with oligomer-primed ^{32}P-labeled cDNA fragments (17) at 68 C overnight, and washed (8). Hybridized blots were dried and used to expose X-ray films (Eastman Kodak, Rochester, NY); intensifying screens were used. Denaturing formamide-formaldehyde RNA gels and Northern blots were performed according to Maniatis et al (27) with minor modifications.

In experiments involving L_1- or L_2-specific radiolabeled probes, several nonoverlapping cDNA clones were used as a mixture to ensure that most of the sequence of these large dsRNAs was represented in the respective probes.

PCR analysis of sequences specific to the L_1 and M_1 dsRNAs. Oligomeric primers (20–24 bases) were designed from sequence data of 3.7- and 25-kb dsRNA-specific cDNA clones (D. K. Lakshman and S. M. Tavantzis, *unpublished data*). Primers P4 (TCATGGCTGTAATCGGGTGT) and P13 (TTAAGTGCGGAATGAAGCAA) were specific to the 3.7-kb (M_1) dsRNA and were expected to generate a 585-bp polymerase chain reaction (PCR) DNA fragment. Primers P19 (CCAACAACAACCTGTCTTAAGC) and P20 (GGAACCTTGATACCTCATATGTC) were specific to the 25-kb (L_1) dsRNA and were expected to generate an 164-bp PCR DNA fragment. Reverse transcriptase PCR was performed with the GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, CT) according to the supplier's specifications. Total dsRNA (1 μg) from Rhs 1A1 or Rhs 1AP was denatured by boiling for 2 min, and cDNA was synthesized at 42 C for 1 h by using avian myeloblastosis virus reverse transcriptase and a primer (P4 or P19). PCR was performed in a 100-μl reaction (10 mM Tris, pH 8.3, 2 mM $MgCl_2$, 200 μM of each of the four dNTPs, 0.5 μM of each of the two primers [P4 and P13 or P19 and P20], and 2.5 U of *Taq* DNA polymerase [added at 82 C]) in a model 480 DNA thermal cycler (Perkin-Elmer Cetus) programmed for 35 cycles at 95 C for 1 min, 64 C for 1 min, and 72 C for 1 min. At the end of the last cycle, reactions were extended for 7 min at 72 C and stored at 4 C until used for gel electrophoresis.

For genomic PCR, total DNA was extracted from the Rhs 1A1 or Rhs 1AP isolate as described by Raeder and Broda (30). Contaminating RNAs were removed by incubation with DNase-

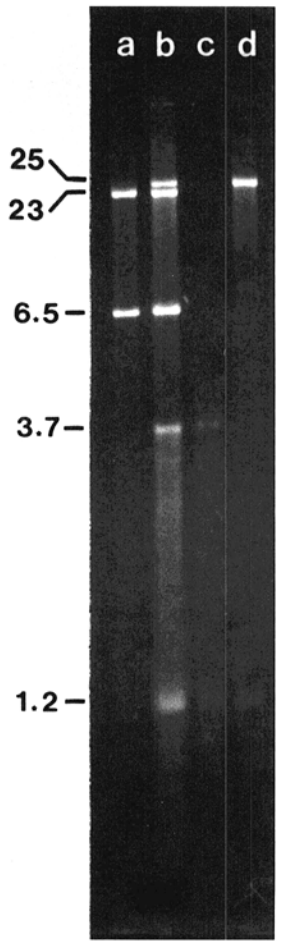

Fig. 2. Electrophoretogram of double-stranded RNA (dsRNA) of Rhs 1AP (lane a), Rhs 1A1 (lane b), Rhs 1A2 (lane c), and Rhs 1A3 (lane d) stained with ethidium bromide following electrophoresis on a 1% agarose gel. The numbers on the left indicate the approximate sizes (kb) of the dsRNA bands.

free RNase Plus (5 Prime →3 Prime, Inc. Boulder, CO). One microgram of genomic DNA was used in a 100-μl PCR reaction.

DNA PCR fragments were fractionated in 1.5 or 2% agarose gels in 0.5× TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.3), visualized by staining with ethidium bromide, blotted onto Hybond-N nylon membranes, and hybridized to the respective (L_1- or M_1-specific) cDNA probe as described previously (27).

RESULTS

dsRNA patterns in the four cultures of *R. solani*. Mycelium was pulverized to fine powder with a mortar and pestle in the presence of liquid nitrogen. This method enabled us to obtain large-sized dsRNA bands without smearing and allowed the consistent detection of dsRNA elements occurring in relatively small amounts. The parental virulent isolate Rhs 1AP exhibited two dsRNAs of 23 (L_2) and 6.5 kb (L_3) (Fig. 2, lane a). The hypovirulent derivative Rhs 1A1 possessed three dsRNAs in addition to the two dsRNAs found in the parental Rhs 1AP. The apparent sizes of the novel dsRNAs were 25 (L_1), 3.7 (M_1), and 1.2 (S_1) kb (Fig. 2, lane b). It is important to note here that the dsRNA patterns of Rhs 1AP and Rhs 1A1 have remained stable for 12 yr. Of the two slow-growing subcultures, Rhs 1A2 has the 3.7- and 1.2-kb dsRNAs (Fig. 2, lane c), whereas Rhs 1A3 possesses the 25- and 1.2-kb dsRNAs (Fig. 2, lane d). The dsRNA sizes are approximations, since DNA size standards were used in this study. Our previous estimates for the Rhs 1A1 dsRNAs were based on dsRNA size standards (7,8) and were very similar to those reported here.

Cloning of dsRNA. The pBluescript SK +/− phagemids were used as vectors in the cDNA cloning experiments. The size range of double-stranded cDNA varied with the dsRNA template. Electrophoretic analysis of cDNA before cloning indicated that near full-length cDNA was synthesized in most cases. cDNAs were cloned into the *Eco*RI site of the pBluescript multiple cloning site through the addition of *Eco*RI-*Sal*I-*Not*I adapters to the insert's blunt ends. Clones 3.0–4.0 kb in size comprised a significant portion of the cDNA libraries for the larger dsRNAs (3.7–25 kb).

Northern blot analysis of dsRNA. Before the different dsRNAs were cloned, a Northern blot containing purified dsRNA from the four cultures was hybridized with 5′-end, ^{32}P-labeled, denatured, 25-kb dsRNA (L_1) prepared as described previously (8). The RNA probe hybridized to the corresponding L_1 band but did not cross-hybridize with any of the other dsRNAs (data not shown). Similar results were obtained when a single-stranded, ^{32}P-labeled cDNA of L_1 (data not shown) or a mixture of nonoverlapping L_1 cDNA clones (Fig. 3A) was used as a probe. Only Rhs 1A1 (Fig. 3A, lane b) and the slow-growing Rhs 1A3 (Fig. 3A, lane d) contained the 25-kb dsRNA. The high-stringency conditions for hybridization and washing described in Materials and Methods were used in all experiments involving DNA probes.

When a mixture of several cDNA clones representing different regions of L_2 (23-kb dsRNA) was used as a probe, it hybridized only to the respective L_2 band (Fig. 3B). Autoradiograms of this and other blots exposed for much longer periods failed to detect any cross-hybridization between the L_2 probe and the rest of the dsRNA species. Moreover, neither of the slow-growing cultures, Rhs 1A2 and Rhs 1A3, contained L_2 (Fig. 3B, lanes c and d).

A ^{32}P-labeled 4.0-kb cDNA clone of L_3 (6.5-kb dsRNA) hybridized only to the homologous band in isolates Rhs 1AP and

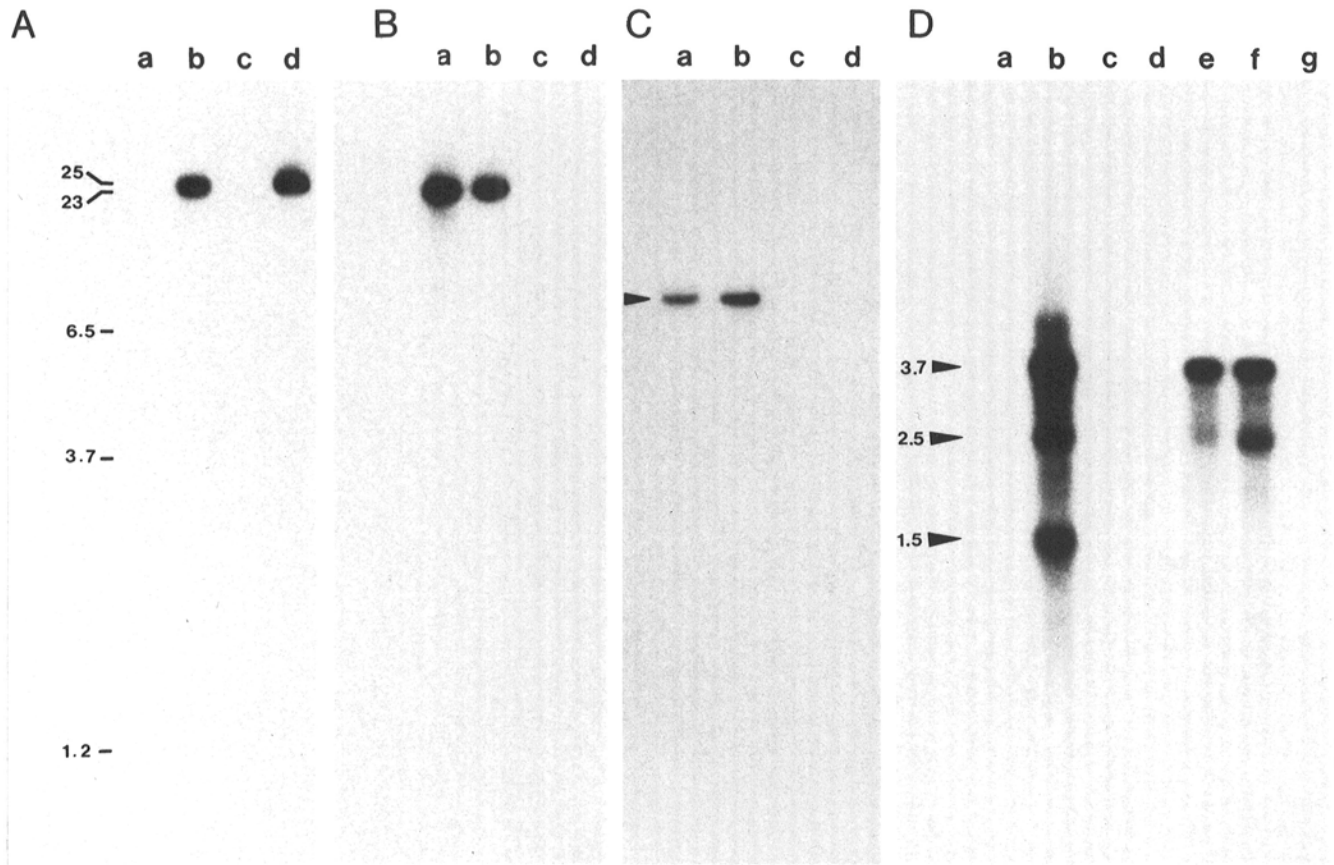

Fig. 3. A–C, Autoradiograms of Northern blots of double-stranded RNA (dsRNA) preparations eluted with cellulose CF-11 obtained from Rhs 1AP (lane a), Rhs 1A1 (lane b), Rhs 1A2 (lane c), and Rhs 1A3 (lane d). Blots were hybridized with a mixture of radiolabeled, nonoverlapping cDNA clones of the 25-kb dsRNA (L_1 **[A]**), the 23-kb dsRNA (L_2 **[B]**), or a 4.0-kb clone of the 6.5-kb dsRNA (L_3 **[C]**). **D,** Autoradiogram of a Northern blot of RNA eluted with cellulose CF-11 from Rhs 1AP (lane a) and Rhs 1A1 (lane b) and RNase-treated (in 0.3 M NaCl) RNA from Rhs 1A3 (lane d), Rhs 1A2 (lane e), Rhs 1A1 (lane f), and Rhs 1AP (lane g) hybridized with a radiolabeled, 3.4-kb cDNA clone of the 3.7-kb dsRNA (M_1) from Rhs 1A1. No sample was loaded in lane c. Numbers and arrows on the left of the panels indicate size (kb) and position of the five dsRNAs.

Rhs 1A1 (Fig. 3C, lanes a and b, respectively). Again, neither of the two slow-growing cultures, Rhs 1A2 and Rhs 1A3, possessed L_3.

When a 3.4-kb cDNA clone (clone 31) of M_1 (3.7-kb dsRNA) was used as a probe, it hybridized to the M_1 band and to the 2.5-kb band ($M_{1,1}$) that often accompanies the M_1 dsRNA species in purified RNase-untreated dsRNA preparations of Rhs 1A1 (Fig. 3D, lane b). This probe (clone 31) also hybridized to a 1.5-kb band ($M_{1,2}$) and to a streak located right above the 3.7-kb band (Fig. 3D, lane b). More importantly, the parental virulent culture Rhs 1AP did not contain the M_1 dsRNA species (Fig. 3D, lanes a and g). In addition, the slow-growing culture Rhs 1A3 did not have M_1 (Fig. 3D, lane d). The $M_{1,2}$ RNA species was sensitive to RNase treatment in 0.3 M NaCl and was therefore probably single-stranded RNA (Fig. 3D, lanes e and f).

In a subsequent experiment, the M_1, $M_{1,1}$, $M_{1,2}$, and 1.2-kb (S_1) bands and the streak above M_1 were eluted from low-melting-temperature agarose (27), electrophoresed on a 1% formamide-formaldehyde gel, blotted, and hybridized with the M_1-specific cDNA clone (clone 31) . Interestingly, upon denaturation, the $M_{1,2}$ RNA species shifted to the 3.7-kb single-stranded position (Fig. 4, lane b), whereas the $M_{1,1}$ (2.5-kb) and M_1 (3.7-kb) bands remained at their corresponding single-stranded positions (Fig. 4, lanes c and d, respectively). Furthermore, a relatively strong signal corresponding to a dimer of M_1 (7.5 kb) and a weaker signal representing larger molecules were associated with the M_1 sample (Fig. 4, lane d). Finally, the sample containing the streak that migrates right above M_1 gave a band comigrating with M_1 and a weak signal comigrating with that of the previous sample (Fig. 4, lane e).

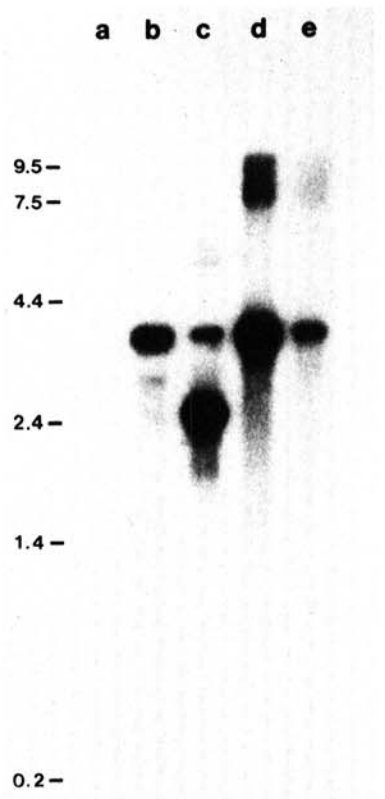

Fig. 4. Northern blot of a formamide-formaldehyde denaturing agarose gel hybridized with a radiolabeled 3.4-kb cDNA clone of the M_1 (3.7-kb) double-stranded RNA (dsRNA) element from the Rhs 1A1 culture. The RNA species analyzed on this gel were extracted from Rhs 1A1 and eluted as single bands from native agarose gels. Lane a, the 1.2-kb dsRNA (S_1); lane b, the 1.5-kb RNA related to M_1; lane c, the 2.5-kb dsRNA related to M_1; lane d, the 3.7-kb dsRNA (M_1); and lane e, the RNA streak migrating right above M_1. Numbers on the left show the position and size (kb) of RNA size standards (Bethesda Research Laboratories).

When total dsRNA preparations were used as templates, only reverse transcriptase PCR reactions containing Rhs 1A1 dsRNA gave the 585-bp DNA fragment (Fig. 5A, lanes c and c′), but dsRNA templates from both isolates gave the 164-bp DNA fragment (Fig. 5B, lanes a, b, a′, and b′). Southern blot hybridization analyses showed that these PCR fragments are genetically related to the 3.7- and 25-kb dsRNA, respectively.

In genomic DNA PCR, reactions containing total DNA from Rhs 1A1 and Rhs 1AP gave a 585-bp DNA PCR product genetically related to the 3.7-kb dsRNA element (Fig. 5A, lanes a, b, a′, and b′). In contrast, neither of the two total DNA templates gave the 164-bp DNA fragment (Fig. 5B, lanes c, d, c′, and d′). Moreover, the PCR-Southern blot hybridization data suggested that the 25-kb (L_1) dsRNA occurs in Rhs 1AP at a very low titer (Fig. 5B, lanes b and b′). It should be noted that L_1 was not detectable in Rhs 1AP by Northern blot hybridization analysis (Fig. 3A, lane a) even after prolonged exposures of the X-ray film to the respective hybridized blot (data not shown).

DISCUSSION

This is the first study showing the emergence of three novel dsRNAs in a fungal culture. The three dsRNAs are genetically different from one another, but they are also unrelated to dsRNAs found in the parental culture. The conversion of the parental virulent *R. solani* isolate Rhs 1AP to the hypovirulent Rhs 1A1 coincided with the appearance of the three novel dsRNAs (L_1, M_1, and S_1) in subculture Rhs 1A1. By comparison, vigor reduction coincided with the loss of the two original Rhs 1AP dsRNAs (L_2 and L_3) in subcultures Rhs 1A2 and Rhs 1A3. We are currently working on the development of a transformation system for the Rhs 1AP isolate that will allow introduction of full-length cDNAs or selected open reading frames (ORFs) of particular dsRNAs to provide direct evidence of whether or not they are responsible for the hypovirulence (Rhs 1A1) or the reduced vigor (Rhs 1A2 and Rhs 1A3) phenotype.

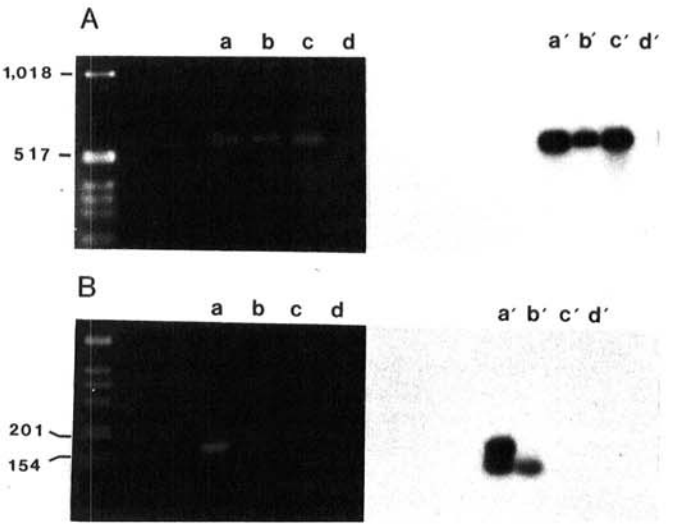

Fig. 5. A, Analysis of polymerase chain reaction (PCR) products of total DNA from Rhs 1A1 (lanes a and a′) or Rhs 1AP (lanes b and b′) or total double-stranded RNA (dsRNA) from Rhs 1A1 (lanes c and c′) or Rhs 1AP (lanes d and d′). PCR was performed with primers (P4 and P13) specific to M_1 dsRNA, and products were analyzed on 1.5% agarose gels, blotted, and radioactively probed with cDNA clones of the M_1 dsRNA (lanes a′–d′). **B,** Analysis of PCR products of total dsRNA from Rhs 1A1 (lanes a and a′) or Rhs 1AP (lanes b and b′) or total DNA from Rhs 1A1 (lanes c and c′) or Rhs 1AP (lanes d and d′) with primers (P19 and P20) specific to L_1. PCR products were analyzed on 2% agarose gels, blotted, and radioactively probed with cDNA clones of the L_1 dsRNA (lanes a′–d′).

The phenotype and dsRNA content of the four cultures (Rhs 1AP, Rhs 1A1, Rhs 1A2, and Rhs 1A3) have been stable since the time of their respective initial isolations. Over the course of 12 yr involving numerous subculturings, the original sclerotium-derived Rhs 1AP gave only the three sectors described here. A similar phenomenon of changing dsRNA profiles has been observed in certain subcultures of the basidiomycete *Agaricus bisporus* exhibiting a diseased phenotype (29). It was not reported, however, whether the "new" dsRNAs were genetically related to those occurring in the parental culture. Moreover, in *A. bisporus,* the new dsRNAs were smaller than some of the original dsRNAs. In our model, one of the new dsRNAs (L_1) is the largest of the five elements. Interestingly, Tartaglia et al (34) reported dsRNA pattern variability attributed to the presence of several deletion mutant dsRNAs in the causal agent of chestnut blight, *C. parasitica.*

Large dsRNAs, such as the 25- (L_1) or 23-kb (L_2) dsRNA, have been found in several fungi and plants (6,10,19,29,39). Some of these dsRNAs are found in mitochondria. Preliminary work in our laboratory suggests that L_1, L_2, and M_1 dsRNAs are located in the mitochondria and that L_3 and S_1 are associated with the 100,000-*g* pellet of the cytoplasmic fraction (7). A substantial portion of the L_2 and M_1 elements were found in this pellet, but it is not clear whether this can be attributed to ruptured mitochondria or to movement of the elements from one cellular location to another. Shapira et al (32) reported that a 12.7-kb dsRNA, shown to cause hypovirulence in *C. parasitica* (14), has a strand that possesses a 3′ poly(A) terminus and two contiguous large ORFs. The 12.7-kb dsRNA has five distinct domains with significant sequence similarity to conserved domains (e.g., RNA polymerase, helicase, and proteases) found within polyproteins of the plant potyvirus group that consists of positive-sense RNA virus members (23). Sequence analysis of the 3.7-kb dsRNA (M_1) from Rhs 1A1 and partial sequencing of the 23-kb dsRNA (L_2) from Rhs 1AP (23) show that they carry genes of a viral nature (D. K. Lakshman and S. M. Tavantzis, *unpublished*).

The 3.4-kb cDNA clone of M_1 (clone 31) was the only radiolabeled probe that hybridized to a band other than that corresponding to the cloned dsRNA (Fig. 3D, lane b). Two bands migrating at the positions of 2.5 ($M_{1,1}$) and 1.5 kb ($M_{1,2}$) cross-hybridized with this clone. Upon denaturation, the 3.7- and 2.5-kb elements migrated to their corresponding single-stranded positions, thus suggesting that they are typical dsRNA helices (Fig. 4). These results are in accordance with the RNase treatment data, which showed the two bands from Rhs 1A1 to be resistant to RNase digestion at 0.3 M NaCl (Fig. 3D, lane f). In contrast, the third cross-hybridizing band ($M_{1,2}$) shifted position from an apparent size of 1.5 kb in the native gel to that of 3.7 kb in the formamide-formaldehyde gel (Fig. 4, lane b). Most importantly, denatured $M_{1,2}$ comigrated with the denatured M_1 dsRNA element (Fig. 4, lanes b and d), so it appears that $M_{1,2}$ is a full-length transcript of the M_1 dsRNA element possessing extensive intramolecular base pairing that enables it to bind the CF-11 cellulose and elute with the dsRNA fraction. Furthermore, $M_{1,1}$ should be a subgenomic replicative form of M_1. Ongoing work in our laboratory is addressing these hypotheses.

Some of the dsRNAs found in *R. solani* are components of encapsidated mycoviruses (18,36) possessing RNA-dependent RNA polymerase activity and protein-encoding dsRNA genome segments (36). Castanho et al (13) were unable to detect viruslike particles in dsRNA-containing *R. solani* cultures. Other basidiomycetes have been reported to possess unencapsidated dsRNA (5,16), whereas dsRNA associated with hypovirulence in *C. parasitica* is packaged in membranous vesicles (20). The five dsRNAs in this study are associated with complexes that sediment upon ultracentrifugation (*unpublished data*), but it is unknown whether these complexes consist of typical virus capsids, vesicles, or other entities. Work is underway to determine the nature of the structures associated with the dsRNA elements described in this paper.

One of the most important questions concerns the origin of the novel dsRNAs (L_1, M_1, and S_1) that appear in the Rhs 1A1 subculture. The PCR and Southern blot analyses showed that M_1-related sequence(s) (3.7 kb) are found on the genomic DNA of both Rhs 1AP and Rhs 1A1 (Fig. 5A). Also, L_2-related sequences (23 kb) are found on genomic DNA of both of these isolates (N. Bharathan, D. K. Lakshman, and S. M. Tavantzis, *unpublished*). In contrast, these experiments suggest that L_1 (25 kb) does not exist in DNA form in either of the two isolates (Fig. 5B). L_1, however, is found in total dsRNA preparations from Rhs 1AP in very low amounts (Fig. 5B). Characteristically, Northern blot hybridization analyses failed to detect the 25-kb dsRNA in Rhs 1AP (Fig. 3A). The PCR data also showed that L_1 occurs in Rhs 1A1 as more than one variant and in titers greater than that found in Rhs 1AP (Fig. 5B). We are currently examining whether the M_1- and L_2-related sequences exist on the nuclear or the mitochondrial DNA of the fungus. Although other explanations exist, some dsRNA elements might use a retroviral or retroposon mode of replication that involves a chromosomal or a mitochondrial copy of their respective sequences. Schuster and Brennicke (31) described an ORF with high homology to reverse transcriptase in the mitochondrial DNA of *Oenothera.* This ORF is located between a sequence fragment from mitochondrial DNA and a fragment of nuclear origin. They proposed that an interorganellar transfer of genetic information might occur through RNA in conjunction with a local reverse transcription and genomic integration. Similarly, sequence homology between large dsRNAs and nuclear DNA has been described in a number of plants (38–40).

LITERATURE CITED

1. Anagnostakis, S. L., and Jaynes, R. A. 1973. Chestnut blight control: Use of hypovirulent cultures. Plant Dis. Rep. 27:225-226.
2. Anderson, N. A. 1982. The genetics and pathology of *Rhizoctonia solani.* Annu. Rev. Phytopathol. 20:329-347.
3. Bandy, B. P., and Tavantzis, S. M. 1990. Effect of hypovirulent *Rhizoctonia solani* on Rhizoctonia disease, growth, and development of potato. Am. Potato J. 67:189-199.
4. Bandy, B. P., Zanzinger, D. H., and Tavantzis, S. M. 1984. Isolation of anastomosis group 5 of *Rhizoctonia solani* from potato field soils in Maine. Phytopathology 74:1220-1224.
5. Barroso, G., and Labarer, E. J. 1990. Evidence for viral and naked double-stranded RNAs in the basidiomycete *Agrocybe aegerita.* Curr. Genet. 18:231-237.
6. Beilharz, M. W., Cobon, G. S., and Nagley, P. 1982. A novel species of double-stranded RNA in mitochondria of *Saccharomyces cerevisiae.* Nucleic Acids Res. 10:1051-1069.
7. Bharathan, N. 1989. Cytoplasmic hypovirulence in *Rhizoctonia solani.* Ph.D. diss. University of Maine, Orono.
8. Bharathan, N., and Tavantzis, S. M. 1990. Genetic diversity of double-stranded RNA in *Rhizoctonia solani.* Phytopathology 80:631-635.
9. Bharathan, N., and Tavantzis, S. M. 1991. Assessment of genetic relatedness among double-stranded RNAs from isolates of *Rhizoctonia solani* from diverse geographic origins. Phytopathology 81:411-415.
10. Brown, G. G., and Finnegan, P. M. 1989. RNA plasmids. Int. Rev. Cytol. 117:1-56.
11. Castanho, B., and Butler, E. E. 1978. Rhizoctonia decline: A degenerative disease of *Rhizoctonia solani.* Phytopathology 68:1505-1510.
12. Castanho, B., and Butler, E. E. 1978. Rhizoctonia decline: Studies on hypovirulence and potential use in biological control. Phytopathology 68:1511-1514.
13. Castanho, B., Butler, E. E., and Shepherd, R. J. 1978. The association of double-stranded RNA with Rhizoctonia decline. Phytopathology 68:1515-1519.
14. Choi, G. H., and Nuss, D. L. 1992. Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. Science 257:800-803.
15. Day, P. R., Dodds, J. A., Elliston, J. E., Jaynes, R. A., and Anagnostakis, S. L. 1977. Double-stranded RNA in *Endothia parasitica.* Phytopathology 67:1393-1396.
16. Dickinson, M. J., and Pryor, A. J. 1989. Encapsidated and unencapsidated double-stranded RNAs in flux rust, *Melampsora lini.* Can. J. Bot. 67:1137-1142.
17. Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.
18. Finkler, A., Koltin, Y., Barash, I., Sneh, B., and Pozinak, B. 1985. Isolation of a virus from virulent strains of *Rhizoctonia solani.* J.

Gen. Virol. 66:1221-1232.
19. Fukuhara, T., Moriyama, H., Pak, J. Y., Hyakutake, H., and Nitta, T. 1993. Enigmatic double-stranded RNA in Japonica rice. Plant Mol. Biol. 121:1121-1130.
20. Hansen, D. R., Van Alfen, N. K., Gillies, K., and Powell, W. A. 1985. Naked dsRNA associated with hypovirulence of *Endothia parasitica* is packaged in fungal vesicles. J. Gen. Virol. 66:2605-2614.
21. Hoch, J. G., Tavantzis, S. M., Campana, R. J., and Anagnostakis, S. L. 1985. Evaluation of the presence of double-stranded RNA in *Ceratocystis ulmi.* Can. J. Bot. 63:297-300.
22. Hyakumachi, M., Sumino, A., Ueda, I., and Shikada, E. 1985. Relationship between the presence of dsRNA in *Rhizoctonia solani* and pathogenicity. Ann. Phytopathol. Soc. Jpn. 51:372-373.
23. Koonin, E. V., Choi, G. H., Nuss, D. L., Shapira, R., and Carrington, J. C. 1991. Evidence for common ancestry of a chestnut blight hypovirulence-associated double-stranded RNA and a group of positive-strand RNA plant viruses. Proc. Natl. Acad. Sci. USA 88:10647-10651.
24. Kousik, C. S., Snow, J. P., and Valverde, R. A. 1994. Comparison of double-stranded RNA components and virulence among isolates of *Rhizoctonia solani* AG-1 IA and AG-1 IB. Phytopathology 84:44-49.
25. Lakshman, D. K., and Tavantzis, S. M. 1993. Cloning of double-stranded RNA (dsRNA) elements associated with hypovirulence in *Rhizoctonia solani.* (Abstr.) Phytopathology 83:245.
26. Lakshman, D. K., and Tavantzis, S. M. 1993. Viral genes carried on two double-stranded RNAs (dsRNA) from *Rhizoctonia solani.* (Abstr.) Phytopathology 83:1424.
27. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
28. Morris, T. J., and Dodds, J. A. 1979. Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. Phytopathology 69:854-858.
29. Morten, K. J., and Hicks, R. G. T. 1992. Changes in double-stranded RNA profiles in *Agaricus bisporus* during subculture. FEMS Microbiol. Lett. 91:159-164.
30. Raeder, U., and Broda, P. 1985. Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. 1:17-20.
31. Schuster, W., and Brennicke, A. 1987. Plastid, nuclear and reverse transcriptase sequences in the mitochondrial genome of *Oenothera:* Is genetic information transferred between organelles via RNA? EMBO J. 6:2857-2863.
32. Shapira, R., Choi, G. H., and Nuss, D. L. 1991. Virus-like genetic organization and expression strategy for a double-stranded RNA genetic element associated with biological control of chestnut blight. EMBO J. 10:731-739.
33. Sneh, B., Burpee, L., and Ogoshi, A. 1991. Identification of *Rhizoctonia* Species. American Phytopathological Society, St. Paul, MN.
34. Tartaglia, J., Paul, C. P., Fulbright, D. W., and Nuss, D. L. 1986. Structural properties of double-stranded RNAs associated with biological control of chestnut blight fungus. Proc. Natl. Acad. Sci. USA 83:9109-9113.
35. Tavantzis, S. M. 1988. Biological control of Rhizoctonia disease and increased growth response of potato effected by hypovirulent *R. solani.* Pages 44-46 in: Innovations in Pest Management. S. Engelstad, W. M. Coli, and J. L. Carlson, eds. University of Massachusetts Press, Amherst.
36. Tavantzis, S. M., and Bandy, B. P. 1988. Properties of a mycovirus from *Rhizoctonia solani* and its virion-associated RNA polymerase. J. Gen. Virol. 69:1465-1477.
37. Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose papers. Proc. Natl. Acad. Sci. USA 77:5201-5205.
38. Turpen, T., Garger, S. J., and Grill, L. K. 1988. On the mechanism of cytoplasmic male sterility in the 447 line of *Vicia faba.* Plant Mol. Biol. 10:489-497.
39. Waharchuk, D. A., and Hamilton, R. I. 1985. Cellular double-stranded RNA in *Phaseolus vulgaris.* Plant Mol. Biol. 5:55-63.
40. Wang, B., Li, Y. N., Zhang, X. W., and Wang, J. Z. 1990. Double-stranded RNA and male sterility in rice. Theor. Appl. Genet. 79:556-560.
41. Washington, J. R., and Martin, F. N. 1991. Characterization of double-stranded RNA, and DNA plasmids occurring in Florida isolates of *Rhizoctonia solani* anastomosis group 4. (Abstr.) Phytopathology 81:1162.
42. Zanzinger, D. H. 1985. Double-stranded RNA in soil-borne isolates of *Rhizoctonia solani* obtained from potato fields in Maine. Ph.D. diss. University of Maine, Orono.
43. Zanzinger, D. H., Bandy, B. P., and Tavantzis, S. M. 1984. High frequency of finding double-stranded RNA in naturally occurring isolates of *Rhizoctonia solani.* J. Gen. Virol. 65:1601-1605.