The Association of Double-Stranded RNA with Rhizoctonia Decline

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ABSTRACT

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The cause of Rhizoctonia decline, a degenerative disease of *Rhizoctonia solani* was explored. All attempts to isolate and purify a mycovirus or detect viruslike particles (VLPs) in crude or semipurified preparations failed. Nucleic acids were extracted from frozen mycelium and double-stranded ribonucleic acid (dsRNA) was selectively purified. Diseased isolate 189a of *R. solani* contained three segments of dsRNA with molecular weights approximately equal to 2.2, 1.5, and 1.1 × 10^6. Healthy isolate 189HT5 usually contained no detectable dsRNA, but occasionally preparations contained traces of the same-sized segments of dsRNA as in 189a. Elution properties from cellulose columns, insensitivity to DNase, insensitivity to RNase in high ionic strength buffer (2× SSC), but sensitivity in low ionic strength buffer (0.1× SSC), and staining characteristics with ethidium bromide and toluidine blue O, verified that the three segments were dsRNA. The association of dsRNA with disease was shown by converting healthy 189HT5, which contained no detectable dsRNA, to the diseased-type by anastomosis with diseased 189a; the resulting converted 189HT5 mycelium contained the same three segments of dsRNA as in 189a. Twelve additional isolates of *R. solani* were examined for the presence of dsRNA. Two that were diseased contained unique segments of dsRNA, different from those in isolate 189a. The other ten isolates were healthy and contained no detectable dsRNA.

Additional key words: *Thanatephorus cucumeris*, fungal virus, mycovirus, cytoplasmic inheritance.

Rhizoctonia decline is a degenerative disease of *Rhizoctonia solani* Kuehn [=*Thanatephorus cucumeris* (Frank) Donk] (4, 5) which alters the morphology and physiology of affected isolates (4) as well as reduces their virulence (5). Diseases of other plant pathogenic fungi have been reported: *Endothia parasitica* (7, 9, 27); *Helminthosporium victoriae* (17); *H. oryzae* (18); *H. maydis* (19); *Gaeumannomyces graminis* (13, 26); and *Colletotrichum lindemuthianum* (25). However, etiological determinations of the diseases affecting these fungi remain unresolved.

Mycoviruses or viruslike particles (VLPs) have been reported in over 30 species of plant pathogenic fungi (14). In most instances, infection with mycoviruses has been suggested because of the presence of VLPs, but no symptoms were attributed to their presence. To date there are only three examples in which mycoviruses or dsRNA have been associated with altered fungal phenotypes; those are the killer systems in *Saccharomyces cerevisiae* (1) and *Ustilago maydis* (29) and the 'La France' disease of *Agaricus bisporus* (21). As a result, most mycoviruses are considered latent (16, 28) and of little biological consequence to the host fungus.

Nonetheless, diseases of fungi, particularly plant pathogens, due to mycoviruses are receiving considerable attention because of their potential for biological control of plant disease. For example, Van Alfen et al. (27) suggested the use of hypovirulent strains of *Endothia parasitica* for the control of chestnut blight. Recently Day et al. (7) have associated this hypovirulence with the presence of dsRNA. The results presented in this paper indicate that Rhizoctonia decline is caused by a cytoplasmic, dsRNA mycovirus. A preliminary report has been published (6).

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MATERIALS AND METHODS

Isolates and cultures.—Most of the work reported here was done with isolate 189 (ATCC 13248), anastomosis group 1, of *R. solani*. Strain 189a is severely diseased and isolate 189HT5 is a healthy, hyphal-tip strain (4). Twelve other field isolates of *R. solani* from anastomosis groups 1, 2, 3, and 4 also were used; some were healthy and others were diseased. Mycovirus-containing cultures of *Penicillium chrysogenum* Thom (ATCC 9480) and *P. stoloniferum* Thom (ATCC 14586) were used for the extraction of VLPs and dsRNA. All isolates were maintained on potato-dextrose agar (PDA) at room temperature (approximately 25 C).

For mass mycelial propagation, isolates were grown in still culture on Blue Ribbon malt extract broth [3% Blue Ribbon malt extract (Premier Malt Prod., Inc., Milwaukee, WI 53201) in distilled water, pH 4.5-5.0]. One-liter Erlenmeyer flasks or 800-ml Roux culture bottles containing 250 ml of malt broth were inoculated with 1 ml of a culture homogenate of each isolate (previously grown 7-21 days on PDA and blended with 100 ml of sterile distilled water). After 3 to 4 wk of incubation the mycelial mats were harvested by vacuum

filtration. The mycelium was washed three times with distilled water, squeezed dry, weighed into 10 g samples and frozen at −20 C. Some of the frozen samples were lyophilized and ground in a Wiley mill (A. H. Thomas Co., Philadelphia, PA 19106) using 250-μm (mesh) screen.

Extraction and purification of viruslike particles.—Either frozen, lyophilized, or freshly harvested mycelium of isolates 189a or 189HT5 was used for extraction and purification of VLPs using methods described by Wood et al. (30), Bozarth (2), and Moffitt and Lister (22). Several buffer systems with different ionic strengths and pH values were tested for each method: ie, 0.01-0.1 M potassium phosphate buffer, pH 6.0, 7.2, and 8.0; 0.01 - 0.1 M sodium acetate buffer, pH 5.0; and 0.01 M sodium borate buffer, pH 9.0. Following differential centrifugation of virus extracts, the final high speed pellets were suspended in the appropriate buffer and analyzed by rate-zonal centrifugation in density gradients. The latter consisted of using 100-400 mg/ml sucrose density gradients which were centrifuged for either 1.5-2.0 hr in a Beckman SW41 rotor (Beckman Instruments, Palo Alto, CA 94304) at 39,000 rpm and 5 C, or 2.5-3.0 hr in a Beckman SW25.1 rotor at 23,000 rpm at 5 C. The gradients were photometrically scanned at 254 nm and fractionated with an ISCO density gradient fractionator (ISCO Instrumentation Specialists, Co., Lincoln, NE 68505). Carbon-coated grids with a collodion backing were floated on density gradient fractions, the grids were drained, and stained with 2% potassium phosphotungstate, pH 7.2. All specimens were examined on an RCA EMU-3H electron microscope.

Penicillium chrysogenum mycovirus (PcV) and *P. stoloniferum* mycovirus (PsV) were extracted and purified using either the method described by Wood et al. (30) or Bozarth (2). The VLPs in these preparations were compared with the contents of preparations from *R. solani*.

Extraction and purification of DsRNA.—Isolation and purification of dsRNA directly from mycelium of *R. solani*, *P. chrysogenum*, or *P. stoloniferum* was accomplished using a modification of the cellulose, chromatography method (8) as described by Morris (23). For each gram of frozen mycelium, 0.5 ml extraction buffer (0.2 M glycine, 0.1 M Na_2HPO_4, 0.6 M NaCl, and 1.0% sodium dodecyl sulfate, adjusted to pH 9.5 with 5 M NaOH), one drop of mercaptoethanol, 2 ml of water-saturated phenol containing 0.1% 8-hydroxyquinoline, and 2 ml of chloroform-pentanol (25:1, v/v) was homogenized at high speed in a Waring Blendor for 5 min at 4 C. Conveniently, 50 g of frozen mycelium was extracted. The homogenate was centrifuged at 10,000 rpm in a Sorvall SS-34 rotor (Norwalk, CT 06470) for 15 min at 5 C. The upper aqueous phase was removed with a wide-mouth pipette and to it was added 95% ethanol to make the final concentration of ethanol 15%. For each 10 ml of the 15% ethanol solution, 0.5 g cellulose powder was added (Biorad Cellex N-1, Biorad Laboratories, Richmond, CA 94804). This cellulose-15% ethanol mixture was stirred for 5 min and then centrifuged at 7,000 *g*. The supernatant was discarded and the cellulose was washed with a solution of 15% ethanol-STE buffer (STE buffer: 0.5 M Tris, 0.01 M Na_2EDTA, 1.0 M NaCl, pH 6.9) and then recentrifuged. This wash procedure was repeated three times. Following the last wash the cellulose-15% ethanol-STE mixture was poured into a glass chromatography column, 1.0 cm I.D. × 30 cm. The cellulose was washed continually with 15% ethanol-STE until the eluate was clear. This procedure removes all species of nucleic acids (ribosomal RNA, transfer RNA, and DNA) except dsRNA and viroid material. The dsRNA was then eluted from the cellulose with 100% STE and precipitated from solution by adding 2.5 parts of cold 95% ethanol and 0.1 parts 3 M sodium acetate, pH 5.0. This solution was placed at −20 C overnight. The precipitate that formed was collected by low-speed centrifugation, and was dissolved in a suitable buffer for electrophoretic analysis. Ultraviolet absorption spectra of all dsRNA preparations were made using a Beckman DK-2A spectrophotometer. All preps had an A_{260nm}/A_{280nm} ratio greater than 2.0 with maximum absorption at 256 nm and minimum absorption at 228 nm. For determining concentrations of dsRNA, an extinction coefficient of 20 was used.

Polyacrylamide gel electrophoresis (PAGE).—Analysis by PAGE was done with 2.4% gels according to the methods of Loening and Ingle (20). A stock acrylamide solution of 15% polyacrylamide and 0.75% bisacrylamide was prepared. A mixture of 5 ml stock acrylamide solution, 19.75 ml glass-distilled water, 6.25 ml of 5× running buffer (running buffer: 36 mM Tris, 30 mM sodium dihydrogen phosphate, 1 mM EDTA, pH 7.6), 0.25 ml of 10% ammonium persulfate (w/v), and 0.025 ml N, N, N′, N′-tetramethylethylenediamine (TEMED) was degassed under vacuum for about 1 min, pipetted into glass tubes (0.6 cm I.D. × 10 cm) and overlayered with running buffer. After polymerization, the tubes were placed in electrophoresis chambers, running buffer was added, and the gels were prerun for 30 min at 5 mA/tube. Samples containing 10-100 μg of dsRNA in 10% sucrose solution were pipetted onto gel surfaces and current was applied for 4-5 hr at 5 mA/tube and 20 C. The gels were removed from the glass tubes, rinsed in glass-distilled water for 30-60 min, and scanned at 260 nm using a Gilford spectrophotometer with linear transport (Gilford, Oberlin, OH 44074). The gels were stained overnight in an aqueous solution of 0.01% toluidine blue O, destained with water, and rescanned at 590 nm.

Nuclease treatments.—Sensitivity of dsRNA preparations to ribonuclease (RNase) and deoxyribonuclease (DNase) was tested by incubating nucleic acid samples with 1.0 μg RNase (bovine pancreatic ribonuclease A; Sigma Chemical Co., St. Louis, MO 63118) per ml in either 0.1× SSC or 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0). Other samples were treated with 10 μg DNase (RNase-free deoxyribonuclease; Worthington Biochemical Corp., Freehold, NJ 07728) per ml in 0.01 M TKM buffer (0.01 Tris, 0.01 M KC1, 0.01 M $MgCl_2$, pH 7.4). During nuclease treatments, samples were incubated at 37 C for 30 min after which they were chilled, precipitated with ethanol, centrifuged at low speed, and the pellet dissolved in buffer and placed on gels. Some dsRNA samples were first fractionated by PAGE and then the gels were soaked for 8 hr at 24 C using the same RNase and DNase treatments as described here. The gels were scanned at A_{260nm} before and after the nuclease treatments.

Molecular weight determinations.—Molecular weight

estimates for the three segments of dsRNA from 189a were calculated from standard curves plotting electrophoretic mobility versus the log of the molecular weights using the dsRNA from PcV and PsV as standards. A mixture of PcV-dsRNA and PsV-dsRNA were co-electrophoresed with the dsRNA of 189a using 2.4% gels run for 5 hr at 5 mA/gel and 20 C. Molecular weight values used for the three segments of PcV-dsRNA were 2.18, 1.99, and 1.89×10^6 and for the five segments of PsV-dsRNA 1.11, 0.99, 0.94, 0.89, and 0.46×10^6 were used (15). Molecular weight estimates were based on measurements from 12 gels.

RESULTS

Attempts to detect viruslike particles.—We were unable to isolate a mycovirus from diseased 189a or healthy 189HT5 mycelium regardless of the extraction or purification method used. Exhaustive examination of crude and semipurified mycelial extracts as well as the sucrose gradient fractions for recognizable VLPs with the electron microscope proved negative. However, despite the lack of evidence for the presence of a mycovirus for Rhizoctonia decline, it is possible that a mycovirus exists in a low titer or is not purifiable or

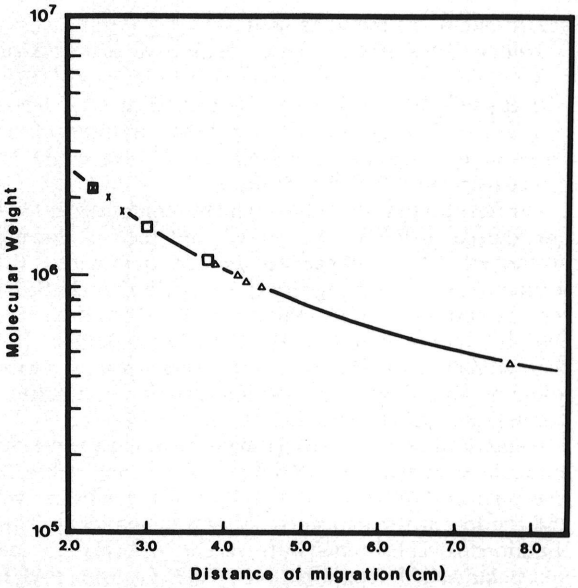

Fig. 2. Semilogarithmic relationship between electrophoretic mobility and molecular weight for dsRNA from PcV (X), PsV (Δ), and diseased isolate 189a of *Rhizoctonia solani* (□). Gel electrophoresis was done with 2.4% gels for 5 hr at 5 mA/tube and 20 C.

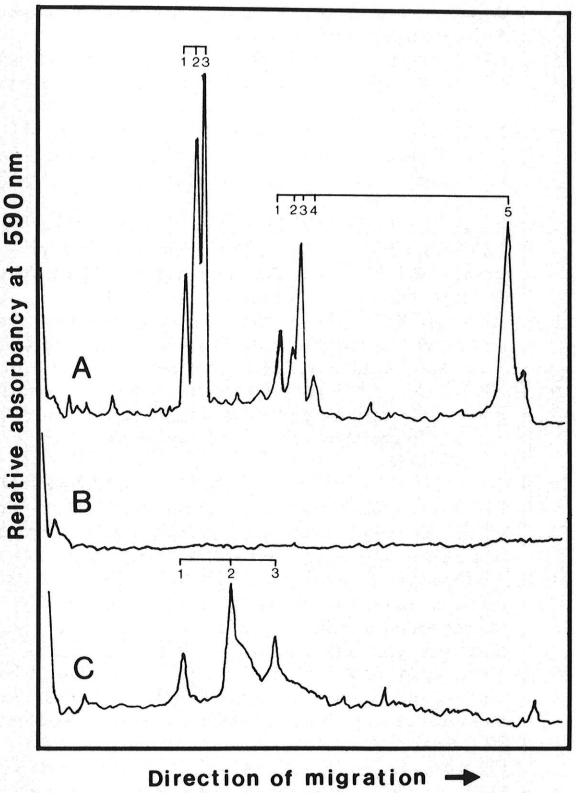

Fig. 1-(A to C). Electrophoretograms of polyacrylamide gels of dsRNA samples from mycelium of **A)** mycovirus-containing isolates of *Penicillium chrysogenum* and *P. stoloniferum*, **B)** healthy isolate 189HT5 of *Rhizoctonia solani*, and **C)** diseased isolate 189a of *R. solani*. Gels (2.4%) were run 5 hr at 5 mA/tube and 20 C, stained with toluidine blue O, and scanned at 590 nm.

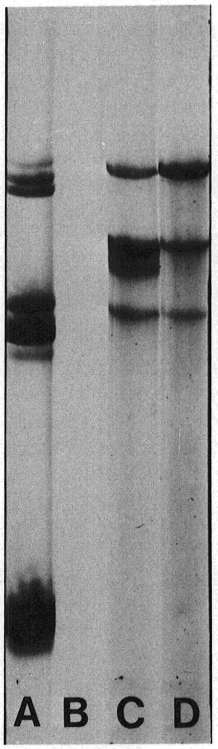

Fig. 3-(A to D). Polyacrylamide gel electrophoresis of dsRNA fractions isolated from **A)** PcV and PsV, **B)** healthy isolate 189HT5 of *Rhizoctonia solani*, **C)** diseased isolate 189a of *R. solani*, and **D)** 189HT5 converted to the disease-type by anastomosis with 189a. Gels (2.4%) were run for 5 hr at 5 mA/tube and 20 C, and stained with toluidine blue O.

detectable by the methods used.

Analysis of dsRNA.—Three distinctive segments of dsRNA were consistently isolated from diseased 189a, whereas healthy 189HT5 usually contained no dsRNA (Fig. 1, Fig. 3,A-C). Occasionally, however, traces of the same sized segments of dsRNA as in 189a could be detected in the 189HT5 preparations.

Verification that the three segments contained in 189a are indeed dsRNA was based on their following properties: (i) resistance to DNase treatments, (ii) resistance to RNase in high ionic strength buffer (2× SSC) but susceptibility at low ionic strengths (0.1× SSC), (iii) elution from cellulose with 100% STE buffer, (iv) characteristic pink-band color when stained with toluidine blue O (11), and (v) intercalation of ethidium bromide into the band material.

A standard curve used to estimate the molecular weight of the three segments of dsRNA in 189a is shown in Fig. 2. The relationship between migration distance in the gel and the log of the molecular weights is nonlinear. This situation is consistent with recent information on molecular weight determinations of dsRNA using PAGE (3, 10, 12). By interpolation, average molecular weight values of 2.2, 1.5, and 1.1×10^6 were estimated for the three segments of dsRNA in 189a.

Transmission of dsRNA.—To determine whether the three segments of dsRNA in 189a were associated with Rhizoctonia decline, a healthy isolate of 189HT5 was converted to the disease type by anastomosis with 189a (4). The converted 189HT5 mycelium was extracted for dsRNA and comparisons were made with similar extracts from the original 189HT5 and 189a cultures. The original 189HT5 contained no detectable dsRNA, but both the original 189a and the converted 189HT5 contained the three segments of dsRNA associated with disease in isolate 189a (Fig. 3).

Content of dsRNA in other isolates of Rhizoctonia solani.—Twelve other isolates of *R. solani* representing each of the four anastomosis groups were analyzed for dsRNA by PAGE. Segments of dsRNA were found in isolates S-239 and C-229 (24) both of which exhibited disease symptoms similar to 189a. The other 10 isolates appeared healthy and contained no detectable dsRNA. The segments in S-239 and C-229 were electrophoretically different from each other and from the three segments in isolated 189a, even though molecular weight estimates were not determined. Thus, the respective sizes of dsRNA were isolate specific.

DISCUSSION

The presence of dsRNA in diseased isolates and the strong correlation between transmission of dsRNA from 189a to 189HT5 by anastomosis resulting in disease of the 189HT5, indicates that dsRNA is involved in Rhizoctonia decline. Whether the dsRNA itself is the disease agent or Rhizoctonia decline is due to a dsRNA mycovirus is unknown. Inasmuch as most mycoviruses contain segmented dsRNA genomes, it would be premature to conclude that a mycovirus is not involved in Rhizoctonia decline on the basis of our inability to isolate or detect recognizable VLPs.

Previously, we reported that although 189HT5 is symptomless, it must contain the disease agent of Rhizoctonia decline based on observations that some single basidiospore cultures degenerate similarly to 189a single basidiospore cultures (4). Since nucleic acid preparations of healthy 189HT5 can contain traces of similar-sized segments of dsRNA as in 189a, it seems 189HT5 contains either a mild symptomless strain of the Rhizoctonia decline disease agent or there exists a quantitative relationship between the presence of dsRNA and the degree of symptom expression.

In short, Rhizoctonia decline is apparently due to a transmissible, cytoplasmic determinant and the association of dsRNA with disease suggests the disease agent is either dsRNA per se or a mycovirus containing dsRNA.

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