Assessment of Genetic Relatedness Among Double-Stranded RNAs from Isolates of *Rhizoctonia solani* from Diverse Geographic Origins

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ABSTRACT

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The degree of genetic relatedness among double-stranded RNA (dsRNA) components from 51 isolates of *Rhizoctonia solani* of diverse geographic origins was analyzed by RNA-RNA hybridization. The cultures included in this study were classified into five anastomosis groups (AGs 1–5), and originated from different geographic locations in the United States, Japan, and Israel. Radioisotope-labeled total dsRNA from eight Japanese cultures (AGs 1, 2, 4, and 5) did not hybridize to northern blots of total dsRNA from American cultures of the corresponding AGs. By contrast, total dsRNA from a Japanese AG 3 isolate hybridized to dsRNA from two of the nine North American cultures tested. Isolates of the same AG and obtained from the same field contained dsRNAs, some of which had the same size; only a few of these dsRNAs cross-hybridized. Quantitative analysis of hybridizing dot-spots showed that sequence homology among cross-hybridizing dsRNAs from isolates of the same AG but different geographic locations ranged from 32 to 80%.

The basidiomycete *Rhizoctonia solani* Kühn (teleomorph = *Thanatephorus cucumeris* (Frank) Donk) is an important pathogen of potato and several other crop species worldwide. The phenomenon of cytoplasmically transmitted hypovirulence in certain isolates of *R. solani* is correlated to the presence of double-stranded RNA (dsRNA) (4–6). The similarities between the cytoplasmically transmitted hypovirulence of *R. solani* and that of *Endothia parasitica* (1,7) and the potential of exploiting this phenomenon to biologically control the ubiquitous soilborne plant

pathogen motivated further studies of the system of *R. solani*. Although Finkler and co-workers (8) reported that dsRNA is associated with virulence in *R. solani*, extensive studies conducted by two independent groups in the United States (17) and Japan (11) showed that dsRNA occurs in nonpathogenic, hypovirulent, and highly virulent isolates. Furthermore, Bharathan and Tavantzis (3) demonstrated that a high degree of genetic diversity exists among dsRNA components from a single culture of *R. solani* or from cultures that belong to the same AG but represent a wide range of virulence on the same set of hosts. The same study provided correlative evidence suggesting that specific dsRNA segments may be associated with virulence, whereas other dsRNAs may confer hypovirulence.

The objective of this work was to address the question of genetic relatedness among dsRNAs that originated from different continents. The data presented here might provide explanations about the contradictory results reported by different research groups working on the biological role of dsRNA in *R. solani*.

MATERIALS AND METHODS

Fungal isolates. The 51 isolates of *R. solani* that were used in this study are members of five different anastomosis groups (AG) (Table 1). Thirty-one of these isolates were collected from potato fields in Maine. Additional isolates from the continental United States were obtained from E. E. Butler (University of California, Davis); E. G. Ruppel (USDA-ARS, Fort Collins, CO); and D. R. Sumner and D. K. Bell (Coastal Plain Experiment Station, Tifton, GA). The nine Japanese isolates were obtained from A. Ogoshi (Hokkaido University). Purified dsRNA of isolate I 13 (AG 4) from Israel was provided by Y. Koltin. All fungal cultures were maintained on potato-dextrose yeast agar (2). Virulence of these isolates was determined as described previously (2,3).

Isolation and analysis of dsRNA. Mycelial cultures were grown and processed as described previously (3), and dsRNA was extracted as described by Morris and Dodds (13). After elution of the dsRNA from the CF-11 column, potential DNA and ssRNA contaminants were removed by nuclease treatment, following the methods of Hoch et al (10) with a proteinase-K (50 μg/ml) incubation (30 min at 37 C) after each RNase or DNase treatment. dsRNA samples were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (3). Molecular weight markers were dsRNAs from virus-infected *Helminthosporium maydis* (8.4 kb), *Penicillium chrysogenum* (average 2.9 and 0.7 kb), and *P. stoloniferum* (average 1.4 kb), which were kindly donated by R. F. Bozarth (Indiana State University).

Electrophoretic transfer of dsRNA to nylon membranes was done as described previously (3). dsRNA was denatured in 2.2 M formaldehyde and 50% formamide for 15 min at 65 C before spotting onto nylon membranes that had been equilibrated with 2× SSC (1× SSC = 0.15 M sodium chloride, 0.15 mM sodium citrate, pH 7.0). The denatured dsRNA was applied as 10-μl duplicate spots that were allowed to dry at room temperature and was immobilized on the membranes as described previously (3).

Total or single dsRNA bands were used as probes on the basis of geographic origin, size, frequency of occurrence, AG, and degree of virulence of the fungal isolate from which they were derived. Individual dsRNA bands were eluted from low-melting-temperature agarose and radioisotope-labeled as described previously (3). Specific activities of probes ranged from 5×10^6 to 1×10^8 cpm/μg of RNA. RNA-RNA hybridization was done according to Bharathan and Tavantzis (3).

The extent of sequence homology between cross-hybridizing dsRNA segments was estimated by the dot-spot hybridization procedure. Total or single bands of dsRNA samples (1-μg) were diluted serially with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) and applied as 10-μl duplicate spots onto nylon membranes. Prehybridization and hybridization of these membranes to the appropriate dsRNA probe were as described above. After autoradiography, filter segments containing each spot were excised, solubilized in an NCS solubilizer (Amersham Corp., Arlington Heights, IL) at 50 C for 2 hr, and mixed with 5 ml of an organic-counting scintillant (OCS, Amersham) (9,15). Genetic relatedness between two individual dsRNA components was expressed as the ratio (percentage of hybridization) of the radioactivity (cpm) attributed to the cross-hybridization reaction over the counts corresponding to the hybridization of the radioisotope-labeled probe with the same amount of its homologous (self) dsRNA species.

RESULTS

All of the isolates of *R. solani* used in this study contained one or more dsRNA components (Table 1). Several northern blots of dsRNA preparations representing all of the isolates listed in Table 1 were hybridized with a number of selected radioisotope-labeled probes from all five AGs. A compilation of the reactions indicating genetic relatedness (including self-hybridizations) is presented in Table 1. It should be noted that in all but one case, cross hybridization involved dsRNAs from cultures of the same AG.

The dot-spot hybridization technique was used to determine the relationship among dsRNAs of American and Japanese isolates of *R. solani*. Denatured dsRNA from isolates of different AGs were spotted (approximately 2–3 μg) on nylon membranes and probed with denatured total dsRNA derived from Japanese isolates. Results indicate no cross hybridization among dsRNAs isolates in AGs 1, 2, 4, and 5 (Fig. 1A, B, D, and E) from the United States and Japan. In AG 3, however, dsRNA from the Japanese isolate STII 6 hybridized strongly to dsRNA from the Maine cultures Rhs 1A1 and 12 (Fig. 1C).

A 2.5-kb dsRNA species was found in five AG 4 cultures (Rhs 15, 24, 32, 33, and 35) originating from northern Maine. Some of these cultures were obtained from the same field. Two of the 2.5-kb dsRNAs (Rhs 15 and 32) were used as probes in separate experiments. The probes hybridized to their homologous bands, but there was no cross hybridization with any of the remaining 2.5-kb fragments either in low- or high-washing strigency conditions. In fact, there was no cross hybridization among dsRNAs within AG 4 with any of the probes used, including total dsRNA from isolate I 13 from Israel.

Estimates of sequence homology among cross-hybridizing dsRNAs, as determined by dot-spot hybridization, were the means of duplicate spots at three different amounts dsRNA applied to the filter (1.0, 0.5, and 0.25 μg). The ratios (see Materials and Methods) were within one or two percentage points at the above three levels and were consistent from experiment to experiment, but dropped gradually as the amount of cross-hybridizing dsRNA available became limiting. The relatedness between cross-hybridizing dsRNAs from American cultures of *R. solani* varied from 32 to 80% for dsRNAs belonging to isolates of the same AG; whereas, in the only case of relatedness among dsRNAs from isolates belonging to different AGs (Rhs 1, 1A1, and 47), sequence homology varied from 43 to 54%. Total dsRNA from the Japanese AG 3 isolate STII 6 showed homologies of 31 and 50% with cross-hybridizing total dsRNA from the Maine AG 3 isolates Rhs 12 and 1A1, respectively.

DISCUSSION

Like the North American cultures (3), nine Japanese isolates belonging to five different AGs contained dsRNAs the majority of which were in the medium-size category (Table 1). Radioisotope-labeled total dsRNA from nine Japanese isolates of *R. solani*, representing five AGs, were used as probes to study their potential relationship with dsRNA from 41 American cultures and one isolate from Israel. Not a single cross hybridization was observed in four out of five AGs. Interestingly, dsRNA from one of the three Japanese AG 3 cultures cross-hybridized with dsRNA from two of the nine Maine AG 3 isolates tested. This might suggest that propagules of AG 3 *R. solani* of the

TABLE 1. Geographic origin, dsRNA content, and genetic relatedness among dsRNA components from isolates of *Rhizoctonia solani* belonging to five anastomosis groups (AG)

AG/isolate	Virulence[a]	Geographic origin[b]	Size of dsRNA components (kb)	Hybridization of dsRNA components to probes[c]			
AG 1				189a/L	189a/2.3	Rhs 52/L	IC BV7/T
Rhs 23	VR	NM	L, 1.8, 1.6	···	···	L,1.8	···
Rhs 36	VR	NM	L, 2.3, 2.2, 2.1, 1.8	···	···	···	···
189HT3	VR	CA	L, 2.3, 1.8	···	2.3, 1.8	···	···
Rhs 51	MV	CM	L	···	···	L	···
Rhs 52	MV	CM	L, 2.9	···	···	L	···
189a	HV	CA	L, 2.3, 1.8	L	2.3, 1.8	···	···
Rhs 8	HV	SM	L	···	···	L	···
CS21A	PA	JP	L, 7.8, 5.4	···	···	···	···
IBShiba2	PA	JP	L, 7.8	···	···	···	···
ICBV7	PA	JP	2.3, 1.8	···	···	···	T
AG 2				27ACT/2.4	27ACT/0.6	Rhs 11/1.8	C1165/T
Rhs 11	VR	NM	L, 2.6, 1.8	···	···	L, 2.6, 1.8	···
Rhs 16	VR	NM	L, 4.7, 0.9, 0.7	···	···	···	···
Rhs 56	VR	CM	L, 4.3, 0.8	···	···	···	···
Rhs 47	HV	GA	L, 2.7, 2.2, 1.8	···	···	···	···
27ACT	HV	GA	L, 2.4, 2.2, 0.6	2.4, 2.2	0.6	···	···
Rhs 717	HV	GA	L, 2.4, 2.3, 1.9	2.4, 2.3	···	···	···
S284	HV	CO	L, 2.7, 1.7	···	···	L, 2.7, 1.7	···
FC25	PA	JP	L, 7.8, 2.2	···	···	···	···
IVRI645	PA	JP	L, 7.8, 4.3, 2.2	···	···	···	···
C1165	PA	JP	L, 7.6	···	···	···	T
AG 3				Rhs 1A1/6.4	Rhs 50/L	Rhs 27/2.4	St116/T
Rhs 2	VR	SM	L_1, L_2, 8.3, 2.4, 2.0	L	···	···	···
Rhs 12	VR	NM	L, 2.8, 2.5, 0.9	L	L, 2.8, 2.5	···	T
Rhs 27	VR	NM	L, 2.9, 2.4, 1.7	···	···	2.9, 2.4, 1.7	···
Rhs 43	VR	NM	L, 8.3, 6.1, 2.4, 1.8	L	···	···	···
Rhs 45	VR	NM	L, 2.6, 1.8	···	···	···	···
Rhs 42	MV	NM	L, 8.3, 6.4, 2.8, 2.4, 1.7	···	···	2.4, 1.7	···
Rhs 44	MV	NM	L, 8.3, 7.4, 6.1, 2.4, 1.7	···	···	1.7	···
Rhs 1A1	HV	NM	L, 6.4, 4.0, 3.7, 3.1, 2.5, 0.9, 0.6	L, 6.4, 3.7, 3.1	···	···	T
Rhs 50	HV	CM	L, 0.9	L	L	···	···
ST116	PA	JP	L, 7.8, 4.3, 2.0, 1.8, 1.6, 0.6	···	···	···	T
AG 4				Rhs 15/2.5	Rhs 32/2.5	I 13/T	HGR101
Rhs 3	VR	NM	2.5, 2.3	···	···	···	···
Rhs 24	VR	NM	2.5, 2.2	···	···	···	···
Rhs 32	MV	NM	2.5, 2.2	···	2.5, 2.2	···	···
Rhs 15	HV	NM	2.5, 2.2, 1.8, 1.4	2.5	···	···	···
Rhs 33	HV	NM	2.5, 1.4	···	···	···	···
Rhs 35	HV	NM	7.0, 2.3, 2.2, 2.0, 1.8, 1.7	···	···	···	···
Rhs 112	VR	GA	8.3	···	···	···	···
Rhs 113	VR	GA	2.3, 2.0	···	···	···	···
223SEE	HV	GA	8.3	···	···	···	···
JMW4	HV	CT	5.0	···	···	···	···
Frank19	HV	WT	L	···	···	···	···
I 13	PA	IS	2.5, 2.2, 2.0	···	···	T	···
HGR101	PA	JP	1.8, 1.6	···	···	···	T
AG 5				Rhs 19/L	Rhs 53/L	GM10/T	
Rhs 9	PA	NM	L, 2.1, 1.5	L	L	···	
Rhs 10	PA	NM	L, 3.5, 3.3, 1.4, 0.8	L	···	···	
Rhs 19	PA	NM	L, 8.4, 7.4, 6.6, 6.0	L	L	···	
Rhs 22	PA	NM	L, 3.7, 3.5, 1.6	L	···	···	
Rhs 53	PA	CM	L, 3.7, 3.6	L	L	···	
Rhs 59	PA	CM	L, 7.4, 5.0, 3.9	···	L	···	
Rhs 1	NP	SM	2.3, 0.45	2.3, 0.45	2.3	···	
GM10	PA	JP	L	···	···	T	

[a] VR = virulent, MV = moderately virulent, HV = hypovirulent, PA = pathogenic, NP = nonpathogenic.

[b] NM = northern Maine, CM = central Maine, SM = southern Maine, CA = California, JP = Japan, GA = Georgia, CO = Colorado, CT = central Texas, WT = west Texas, IS = Israel.

[c] Probes are arranged according to AGs. Each is identified according to a code in the form xx/yy, in which xx represents the fungal isolate and yy represents the size of the restriction fragment in kb. L = large (>8.4 kb); T = total dsRNA; and ··· = no detectable hybridization. Other values are the sizes of the hybridizing fragments in kb.

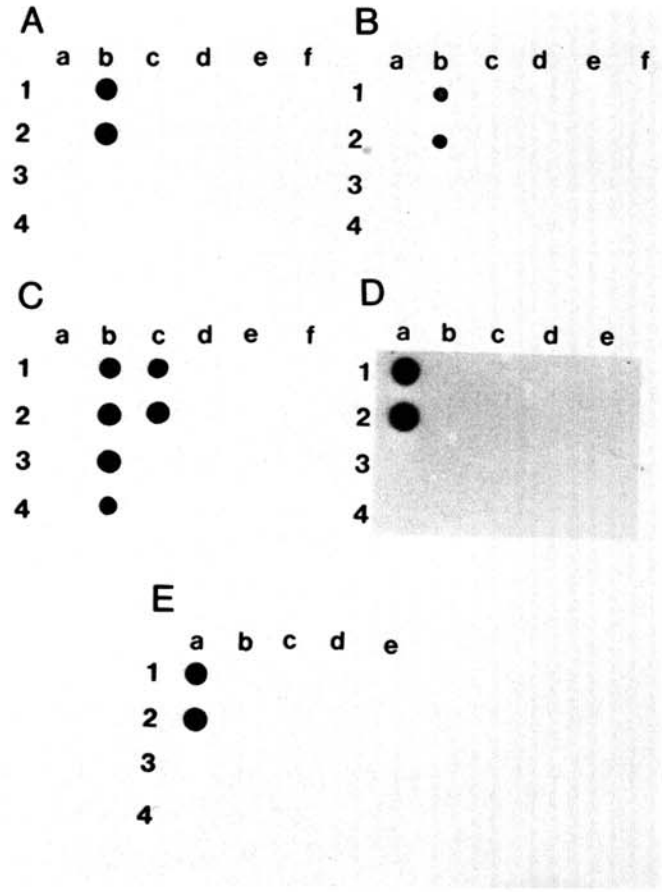

Fig. 1. Dot-spot hybridization of total dsRNA from 41 American cultures of *R. solani*, nine Japanese cultures, and one Israeli culture. The designated dsRNA preparations (10-μl containing 2–3 μg) were spotted in duplicate onto a nylon membrane and probed with radioisotope-labeled total dsRNA from selected cultures of Japanese origin. Panels A–E show membranes containing dsRNA from members of AGs 1–5 that were probed with dsRNA from Japanese isolates ICBV7, C1165, ST116, HGR101 and GM 10, respectively. Numbers 1–4 on the left of each panel and letters A–F show the positions of the designated dsRNA samples. A mixture of dsRNA standards (see Materials and Methods) was used as the control. **A,** 189a (A1–2), ICBV7 (B1–2), IBShiba2 (C1–2), CS 2 (D1–2), Rhs 51 (E1–2), Rhs 36 (F1–2), 189HT3 (A3–4), and control dsRNA (B3–4). **B,** 27ACT (A1–2), C1165 (B1–2), FC 25 (C1–2), RI645 (D1–2), Rhs 16 (E1–2), S284 (F1–2), Rhs 717 (A3–4), Rhs 11 (B3–4), Rhs 47 (C3–4), Rhs 56 (D3–4), and control dsRNA (E3–4). **C,** Rhs 2 (A1–2), ST116 (B1–2), Rhs 12 (C1–2), Rhs 42 (D1–2), Rhs 43 (E1–2), Rhs 45 (F1–2), Rhs 44 (A3–4), Rhs 1A1 (B3–4), Rhs 27 (C3–4), Rhs 50 (D3–4), and control dsRNA (E3–4). **D,** HGR101 (A1–2), Rhs 15 (B1–2), Rhs 24 (C1–2), Rhs 113 (D1–2), I 13 (E1–2), Rhs 3 (A3–4), Rhs 33 (B3–4), Rhs 35 (C3–4), Rhs 112 (D3–4), F 19 (E3–4). **E,** GM 10 (A1–2), Rhs 59 (B1–2), Rhs 10 (C1–2), Rhs 9 (D1–2), Rhs 1 (E1–2), Rhs 53 (A3–4), Rhs 22 (B3–4), Rhs 19 (C3–4), and control dsRNA (D3–4).

same origin (e.g., Central America) were introduced to the two countries directly or indirectly through potato germ plasm.

An important finding of this work is that the origin of dsRNA in *R. solani* is quite complex. dsRNA species of the same size and derived from cultures collected within a single field or county might or might not share common nucleotide sequences (Table 1). By contrast, cross hybridization did occur between dsRNAs from cultures collected in locations as distant as Maine and Colorado or Maine and Japan (Table 1). Similarly, dsRNA from a Michigan isolate (MCI) of *E. parasitica* did not cross-hybridize with any dsRNAs from seven cultures originating from that state (14). In contrast, all three dsRNAs found in the soilborne isolate Rhs 11 (northern Maine) cross-hybridized to three dsRNAs of similar size from the corn isolate S 284 (Colorado) (Table 1). In fact, the degree of relatedness (approximately 80% homology) between two pairs (similar size) of these dsRNAs was the highest among cross-hybridizing fragments examined in this study.

Although there is a considerable genetic diversity of dsRNA species within a natural population of *R. solani* occurring in the same location (field, county, or state), genetic relatedness among dsRNAs from isolates of common geographic origin is higher than that observed among isolates of distant geographic origin. For example, most of the L dsRNAs from AG 5 found in Maine are cross-hybridizing but none of them appeared to be related with the L dsRNAs from the Japanese isolate GM 19 (Table 1). These results are in accordance with findings of an earlier report, which showed that dsRNA from a Michigan isolate (GH 2) of *E. parasitica* cross-hybridized with dsRNA from most of the Michigan isolates tested but with none of the other state or European isolates that were included in the study (14). Similarly, L'Hostis and co-workers (12) reported that there was genetic relatedness among dsRNAs from American strains of *E. parasitica* or among dsRNAs from strains of European origin, but there was little or no cross hybridization between dsRNA from strains originating from different continents.

The fact that there was no cross hybridization between the L bands of the AG 1 cultures 189a and 189HT3 was surprising because the latter is a hyphal-tip isolate of the former. Both of these cultures were kindly provided to us by E. E. Butler (4–6). The dsRNA patterns of the two cultures were quite similar (Table 1). The two medium-size bands (2.3 and 1.8 kb) cross-hybridized and had the same migration rate and intensity. In contrast, the L band of 189a was significantly more prominent than that of 189HT3 but because of their slow migration rate it was not clear as to whether or not they comigrated. This lack of cross hybridization between the L bands of isolates 189a and 189HT3 was observed in a number of independent experiments. Thus, it appears that hyphal-tipping removed an L dsRNA species from 189a that might be involved in the suppression of virulence in this isolate. Similarly, hyphal-tipping of the moderately virulent AG 3 isolate Rhs 41 (16) resulted in the elimination of three out of four dsRNA bands and a dramatic increase in virulence (16; D. H. Zanzinger and S. M. Tavantzis, *unpublished*).

LITERATURE CITED

1. Anagnostakis, S. L., and Day, P. R. 1979. Hypovirulence conversion in *Endothia parasitica*. Phytopathology 69:1226-1229.
2. 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.
3. Bharathan, N., and Tavantzis, S. M. 1990. Genetic diversity of double-stranded RNA in *Rhizoctonia solani*. Phytopathology 80:631-635.
4. Castanho, B., and Butler, E. E. 1978. Rhizoctonia decline: A degenerative disease of *Rhizoctonia solani*. Phytopathology 68:1505-1510.
5. Castanho, B., and Butler, E. E. 1978. Rhizoctonia decline: Studies on hypovirulence and potential use in biological control. Phytopathology 68:1511-1514.
6. Castanho, B., Butler, E. E., and Shepherd, R. J. 1978. The association of double-stranded RNA with Rhizoctonia decline. Phytopathology 68:1515-1519.
7. 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.
8. Finkler, A., Koltin, Y., Barash, I., Sneh, B., and Pozniak, D. 1985. Isolation of a virus from virulent strains of *Rhizoctonia solani*. J. Gen. Virol. 66:1221-1232.
9. Gallitelli, D., Hull, R., and Koenig, R. 1985. Relationships among viruses in the tombus virus group: Nucleic acid hybridization studies. J. Gen. Virol. 66:1523-1531.
10. 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.
11. Hyakumachi, M., Sumino, A., Ueda, I., and Shikata, E. 1985. Relationship between the presence of dsRNA in *Rhizoctonia solani* and the pathogenicity. Ann. Phytopathol. Soc. Jpn. 51:372-373.
12. L'Hostis, B., Hiremath, S. T., Rhoads, R. E., and Ghabrial, S. A. 1985. Lack of homology between double-stranded RNA from European and American hypovirulent strains of *Endothia parasitica*. J. Gen. Virol. 66:351-355.
13. 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.
14. Paul, C. P., and Fulbright, D. W. 1988. Double-stranded RNA molecules from Michigan hypovirulent isolates of *Endothia parasitica* vary in size and sequence homology. Phytopathology 78:751-755.
15. Thomas, P. S. 1983. Hybridization of denatured RNA transferred or dotted to nitrocellulose papers. Methods Enzymol. 100:255-266.
16. Zanzinger, D. H. 1985. Classification of and detection of double-stranded RNA (dsRNA) in soilborne isolates of *Rhizoctonia solani* Kühn obtained from potato fields in Maine. Ph.D. thesis. University of Maine, Orono. 120 pp.
17. 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.