Etiology

Association of Double-Stranded RNA with Low Virulence in an Isolate of *Leucostoma persoonii*

Sue Hammar, D. W. Fulbright, and G. C. Adams

Graduate research assistant, associate professor, and assistant professor, respectively, Department of Botany and Plant Pathology, Michigan State University, East Lansing 48824.

Journal Article 12922 of the Michigan Agriculture Experiment Station.

We are grateful to E. Endert-Kirkpatrick and D. F. Ritchie for isolate 14.4A, to T. Proffer for the teleomorph, and to A. L. Jones for the donation of mature peach trees.

This research was supported by USDA grant 85-CRSR-2-2551 and 88-34152-3380.

Accepted for publication 4 January 1989 (submitted for electronic processing).

ABSTRACT

Hammar, S., Fulbright, D. W., and Adams, G. C. 1989. Association of double-stranded RNA with low virulence in an isolate of *Leucostoma persoonii*. Phytopathology 79:568-572.

Nine segments of double-stranded RNA (dsRNA) were identified in an isolate of *Leucostoma persoonii* (14.4A) demonstrating low virulence, abnormal culture morphology, and an inability to produce pycnidia when grown on culture media. Treatments of isolate 14.4A that altered the number of dsRNA segments also changed the virulence and morphology of the resulting cultures. When all dsRNA segments in isolate 14.4A were eliminated, the resulting cultures had increased virulence, sporulated, and grew similar to typical isolates of *L. persoonii*. Four segments of dsRNA were transferred by hyphal anastomosis from isolate 14.4A to a genetically marked strain. Based on dsRNA segment elimination patterns and dsRNA tranfer data, isolate 14.4A may be infected with at least two distinct dsRNA viruses.

Additional keywords: *Cytospora*, hypovirulence, mycovirus, *Valsa*, viruslike particle.

Cytospora canker of peach is caused by *Leucostoma cincta* (Pers.:Fr.) Hoehn. (anamorph = *Leucocytospora cincta* (Sacc.) Hoehn) and *Leucostoma persoonii* (Nits.) Hoehn. (anamorph = *Leucocytospora leucostoma* (Pers.) Hoehn.). Cytospora canker is characterized by tissue necrosis and perennial cankers, which are usually accompanied by copious gum exudation. Infected branches wilt and die back from girdling and plugging of the xylem vessels (4,16,18). Fungicides have not been effective in controlling the disease.

Biological control as a means of reducing the impact of Cytospora canker is appealing. Reduced virulence or hypovirulence in some pathogenic fungi is associated with the presence of double-stranded RNA (dsRNA) and has been associated with biological control of plant diseases (7,8,26). Hypovirulence has best been described in the plant pathogen *Cryphonectria parasitica* (= *Endothia parasitica*) (1,26), which causes a canker disease on chestnut (*Castanea* spp.) similar to the perennial Cytospora canker on stone fruits.

The purpose of this study was to select and characterize hypovirulent isolates of *Leucostoma* spp. based on virulence assays and the presence and transmission of dsRNA molecules.

MATERIALS AND METHODS

Isolates. Isolates used in virulence tests were recovered from cankers on peach trees grown in Michigan, California, Pennsylvania, West Virginia, and North Carolina (Table 1). Cultures were maintained on Leonian's malt agar (LMA) (21) and stored on peach bark agar (22) at 4 C with annual subculturing.

Isolates were identified as *L. persoonii* and as *L. cincta* based on colony color, size of pycnidia, lobate or uniformly radial colony margin (27), presence or absence of growth at 37 C (19), and by comparison of cultural morphology with single ascospore isolates from an ascocarp identified as *L. persoonii*.

Virulence tests. The virulence of isolates identified as

© 1989 The American Phytopathological Society

Leucostoma spp. was evaluated on 8-yr-old peach trees (*Prunus persica* (L.) Batsch 'Garnet Beauty'), and on apple fruit (*Malus domestica* L. 'Golden Delicious'). Peach trees were inoculated in October 1985 following the method of Scorza and Pusey (23). Two-year-old peach branches measuring 17 mm in diameter were wounded to the xylem with an empty stapling gun. The wound area was sprayed for 5 sec with a commercial aerosol freezing product (100% dichlorofluoromethane, Chemtronics, Inc., Hauppauge, NY) and inoculated with a 5-mm mycelial plug taken from the margin of a 5-day-old culture on LMA. The inoculations were wrapped with Parafilm to prevent dessication. Canker lengths were measured the following May after the bark was stripped off to reveal the length of the necrotic area distal to the inoculation point.

Golden Delicious apple fruit were inoculated by removing a 9-mm-diameter × 4-mm-deep plug of tissue with a sterile cork borer (13). Mycelial plugs were placed mycelium side down in contact with the wounded apple tissue. The site of inoculation was covered with tape, and the apples were placed in open plastic bags. The width of each lesion was measured after 10 days.

The presence of *Leucostoma* spp. in inoculated tissues was verified by reisolation from the lesions. Peach twigs with bark were surface sterilized by soaking in a 0.5% sodium hypochlorite solution for 2–3 min and blotted dry with sterile paper towels. Apple tissue was surface sterilized by swabbing the lesion margin with 95% ethanol. Tissue was excised from the margin of the lesions and embedded in either LMA or PDA and incubated at room temperature.

Isolation of dsRNA. DsRNA was extracted using modifications (14) of the procedures by Day et al (8) and Dodds (9). Nucleic acid samples were layered on 5% polyacrylamide slab gels (16 × 18 cm) and electrophoresed at 40 mA for 12 hr. Molecular weight estimates of dsRNA were calculated from standard curves plotting electrophoretic mobility versus the log of the molecular weights using coelectrophoresed dsRNA standards from reovirus serotype 3 (24), *C. parasitica* (GH2) (14), and *Bipolaris maydis* (ATCC 32450). Segments on gels were identified as dsRNA based on their resistance to RNase at high ionic strength (0.3 M NaCl), sensitivity to RNase at low ionic strength, and resistance to DNase treatments (14).

TABLE 1. Virulence of isolates of *Leucostoma* spp. on 10 trees of Garnet Beauty peach ranked in decreasing order by canker length and the association of double-stranded RNA with each isolate

State of origin[a]	Isolate	Mean canker length (cm)[b]	dsRNA[c]
MI	MR-1	11.00 a[d]	−
NC	8.2	9.98 ab	−
CA	F-46	9.83 abc	−
CA	CL-5	8.40 bcd	−
MI	C10.8	7.78 bcde	−
MI	H9.5	7.64 bcdef	−
WV	C-J-1	7.50 cdef	−
WV	C-S-20	7.24 defg	−
MI	G-3	7.08 defgh	−
MI	F-4	6.59 defghi	−
CA	Ma-4	6.49 defghij	−
WV	C-MI-5	6.48 defghij	−
PA	P-1	6.06 defghijk	−
WV	C-jm-18	5.94 efghijk	−
MI	10.9	5.46 efghijk	−
NC	9.2	5.34 fghijk	+
NC	14.1	5.11 ghijk	−
CA	F-45	4.92 ghijk	−
NC	14.4A[e]	4.81 hijk	+
MI	H7.13	4.79 hijk	−
MI	H6.15	4.75 hijk	−
MI	H9.11	4.71 ijk	−
MI	11.11	4.18 jk	−
CA	I-80	4.02 k	−
	Control[f]	0.5 l	−

[a]State where isolates were recovered.

[b]Inoculations were made by placing mycelium into wounds made with an empty stapling gun followed by freeze injury. The sites of inoculation were wrapped in Parafilm. Seven months later the bark was removed and canker lengths (distal from the point of inoculation) were measured. Means are based on 10 replications (10 trees, 1 branch/tree).

[c]−, dsRNA not detectable; +, dsRNA detectable by gel electrophoresis.

[d]Means not followed by same letters are significantly different by the least significant difference test (LSD), $P = 0.05$.

[e]Higher ranking due to the contamination of three of the 10 replications. The remaining seven replications had a mean cankers width of 1.98 cm.

[f]Controls were inoculated with a sterile plug of LMA medium rather than with mycelium.

Curing experiments. Five procedures were used to obtain subcultures of isolate 14.4A that exhibited colony morphology considered normal for isolates of *L. persoonii*. First, mycelial plugs of 14.4A were transferred to media amended with 0.25–50 μg of cycloheximide (Sigma Chemical Co., St. Louis, MO) per milliliter (13) or 25–50 μg of ribavirin (Sigma) per milliliter. After 2 wk, agar plugs were transferred from the margins of the colonies to fresh PDA. Second, hyphal tips were excised and subcultured from 2-day-old cultures of 14.4A grown on LMA. Initially a total of 100 hyphal tips were taken from four colonies and each again grown for 2 days. Then, a hyphal tip was taken from each of these resulting colonies and grown for 2 days. This was repeated 10 consecutive times. The resulting 100 colonies were grown for 4 wk. Third, the mycelium from the margin of an expanding lesion in apple fruit inoculated with isolate 14.4A was recovered as described in virulence tests. Fourth, isolate 14.4A was grown at various temperatures. Fifteen 4-day-old cultures were transferred to LMA, and the plates were incubated in the dark at 33, 36, or 38 C for 2 wk. Mycelial transfers were made to LMA, and the plates were incubated at room temperature with ambient laboratory lighting. Fifth, protoplasts were isolated and regenerated.

To obtain protoplasts, mycelia were grown 36 hr in 500-ml flasks containing 100 ml of a complete broth medium without glucose (8). Mycelia were collected with a Büchner funnel and incubated 3 hr at 33 C in a solution containing 1 ml of 1.0 M sodium phosphate buffer at pH 5.8 and 9 ml of 1.0 M mannitol amended with a 1-ml filter-sterilized solution containing 10 mg of Novozyme 234 (Novo Laboratory, Inc., Wilton, CT) and 1 mg of chitinase from *Serratia marcescens* (Sigma). The protoplasts were separated from large hyphal fragments by filtering the solution through four layers of sterile cheesecloth, then through a 15-μm mesh nylon filter (Tetko, Inc., Elmsford, NY) and collected in a centrifuge tube. Protoplasts were centrifuged at 2,000 rpm for 5 min, resuspended in 1.0 M mannitol, recentrifuged, resuspended in 2 ml of 1.0 M mannitol and layered onto 4 ml of 1.0 M sucrose. The solution was centrifuged at 1,000 rpm for 5 min. The protoplasts, located in an opaque interface between the two solutions (17), were removed and added to 2 ml of 1.0 M mannitol. The layering procedure was repeated until a highly purified protoplast preparation was obtained. The final protoplast pellet was diluted in 1.0 M mannitol to 10^2 protoplasts per milliliter and pipetted onto complete agar medium (8) supplemented with 1.0 M mannitol. Within 72 hr after plating, 50–100 regenerated protoplasts showing hyphal initiation were individually transferred to LMA.

Benomyl-resistant mutants. Benomyl-resistant (Ben^R) mutants were isolated by collecting pycnidia from 4-wk-old colonies and grinding them in sterile water with a mortar and pestle. The slurry was passed twice through a 5-μm filter so that only the conidia in water remained. One milliliter of a solution of 10^6 conidia per milliliter was pipetted onto LMA amended with 4 μg of benomyl per milliliter. The conidia were irradiated for 1.5 min with a 254 nm of ultraviolet light (uv) from a UVSL-58 Mineralight lamp with an output of 1.25×10^4 ergs $cm^{-2}sec^{-1}$. This exposure killed approximately 90% of the conidia. Irradiated plates were stored in the dark until conidia germinated and formed colonies. These colonies were subcultured onto LMA and LMA amended with benomyl, and growth was compared among the presumptive Ben^R mutants and the nonmutated parent isolate.

Transfer of dsRNA. To transfer dsRNA from isolate 14.4A to Ben^R mutants, mycelial plugs of 14.4A and a Ben^R mutant were placed side by side in a petri dish containing LMA or Difco oatmeal agar. Plates were allowed to incubate until the hyphae of

the two isolates made contact. Mycelial plugs were removed from various areas on the plate and subcultured on LMA amended with 4 μg of benomyl per milliliter. Isolates that grew on the benomyl-amended media were assayed for the presence of dsRNA.

RESULTS

Isolates. The absence of the sexual states, in particular *L. cincta*, on peach cankers necessitated the identification of isolates T-5, C10.8, C11.7, R-1, 8.2, RD-1, M-5, and 14.4A as *L. persoonii* and isolates 14.1 and 9.2 as *L. cincta* based on cultural characteristics (19,27). Thus, our species designations are tentative. The identification of 14.4A as *L. persoonii* is based on the cultural characteristics of the dsRNA-free subcultures (isolates 103, 105, and 107) derived from 14.4A. Isolate 14.4A had an abnormal culture morphology including lysing hyphal tips and did not produce pycnidia or conidia in culture or on inoculated stems. Ultrastructural examination has revealed the presence of viruslike particles (VLPs) at high concentrations in the cytoplasm (25). Other isolates tested were found to have the normal or expected morphology of *Leucostoma* spp. in culture (27) and produced pycnidia and spores in culture and on peach tree cankers.

Virulence tests. Virulence assays performed with apple fruit indicated that isolate 14.4A was the least virulent of the isolates tested (Table 2). In orchard tests with other isolates, 14.4A was one of the least virulent (Table 1). Isolate 14.4A would possibly have had the lowest ranking in Table 1, but contamination of three inoculation sites with wild-type strains is thought to have increased the lesion size. The mean for the seven remaining uncontaminated inoculated sites was 1.98 cm. While most of the isolates tested ranked differently in virulence from one test to another (Tables 1 and 2, i.e., isolate 14.1 and 8.2), isolate 14.4A was consistently ranked low in all virulence assays performed (unpublished data).

Screening isolates for dsRNA. The only field isolates in which dsRNA was detected were 14.4A and 9.2 (Fig. 1). Nine segments of dsRNA were visible in nucleic acid extracts from 14.4A subjected to polyacrylamide gel electrophoresis. Segments B, C, and D were consistently associated with 14.4A. Segments A and F were usually present but stained fainter with ethidium bromide than segments B, C, and D after polyacrylamide gel electrophoresis. Segments E, G, H, and I appeared inconsistently. Segments B, C, and D ranged in size from a molecular weight of approximately $2\text{–}3 \times 10^6$. The estimate of 3×10^6 is probably low since mobilities of the standards used did not bracket the segment being compared. The segments found in 14.4A were identified as dsRNA based on their resistance to RNase at high ionic strength (0.3 M NaCl), sensitivity to RNase at low ionic strength (in water), and resistance to DNase treatments.

TABLE 2. Assay of virulence of isolates of *Leucostoma persoonii* on apple fruit

State of origin[a]	Isolate	Mean lesion width (cm)[b]	dsRNA[c]
NC	14.1	8.33 a[d]	−
MI	T-5	7.77 ab	−
NC	9.2	6.80 bc	+
MI	C10.8	6.67 bc	−
MI	C11.7	6.58 bc	−
MI	R-1	6.53 bc	−
NC	8.2	6.48 c	−
MI	RD-1	6.08 c	−
MI	M-5	5.72 c	−
NC	14.4A	1.52 d	+
	Control[e]	0.5 e	−

[a]State where isolates were recovered.
[b]Inoculations made by placing mycelial plugs into wounds made by removing apple tissue with a cork borer. The site of inoculation was covered with tape and the apples were placed at room temperature in open plastic bags. Lesions were measured after 10 days. Each value is the mean of 10 replications.
[c]−, dsRNA not detectable; +, dsRNA detectable by gel electrophoresis.
[d]Means not followed by same letters are significantly different by the least significant difference test (LSD), $P = 0.05$.
[e]Controls were inoculated with a sterile plug of LMA medium rather than with mycelium.

Isolate 9.2 consistently yielded three dsRNA segments distinct from those found in isolate 14.4A (Fig. 1).

Curing dsRNA from isolate 14.4A. The various curing procedures yielded isolates varying in culture morphology from isolate 14.4A. Preliminary analysis of these variants indicated that certain dsRNA segments were not detectable after electrophoresis in polyacrylamide gels stained with ethidium bromide. Therefore, these variants were considered partially cured of dsRNA. Partially cured subcultures of 14.4A were obtained by subculturing hyphal tips, reisolating 14.4A from apple fruit, growing isolates on ribavirin, or by protoplast regeneration (Table 3). Unlike the other field isolates of *Leucostoma* spp. tested, isolate 14.4A did not grow on PDA containing 0.25–5 of μg of cycloheximide per milliliter. Isolate 14.4A grew only at 33 C, and none of these cultures were cured of dsRNA. Partially cured isolates were also obtained from older cultures that had been frequently subcultured onto laboratory media.

The morphologies of all partially cured isolates were indistinguishable from one another regardless of the curing method by which they were derived. The partially cured isolates were darker than isolate 14.4A, the mycelium was more compact in

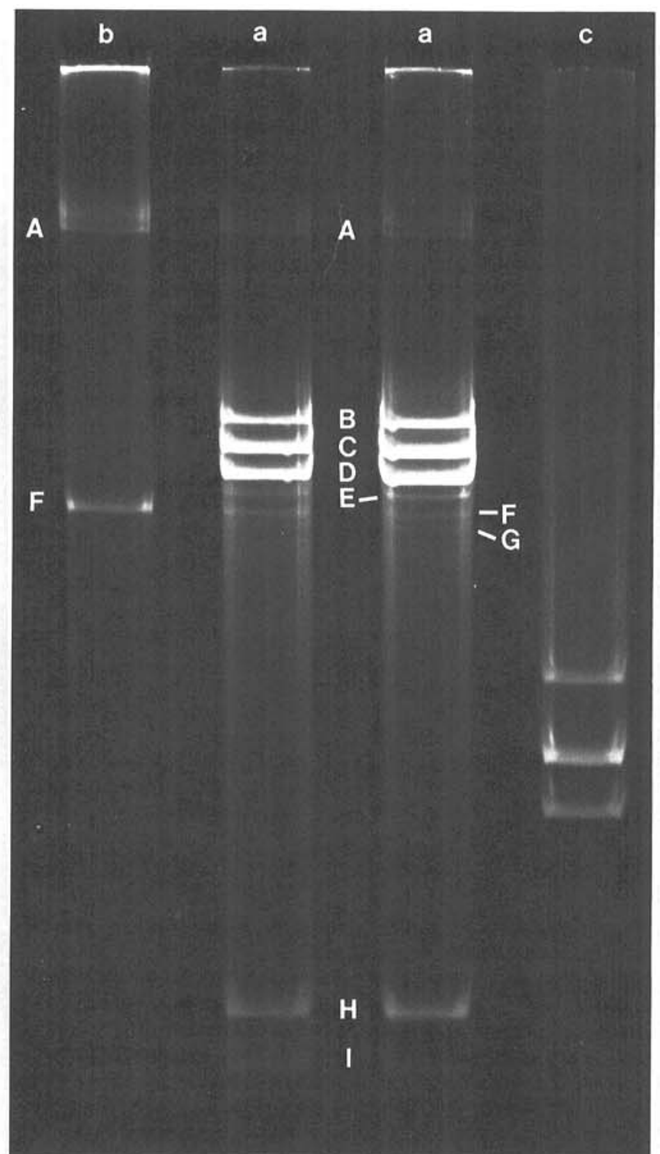

Fig. 1. An ethidium bromide stained 5% polyacrylamide gel with specified segments of dsRNA extracted from *Leucostoma* spp. **A,** isolate 14.4A; **B,** partially cured isolate of 14.4A; and **C,** isolate 9.2.

texture, hyphae did not lyse at the tips, and the cultures formed fruiting bodies that lacked conidia (Fig. 2). All (> 100) partially cured isolates tested retained dsRNA segments A and F (Fig. 1), but segments B, C, D, E, G, H, and I were not detected in these isolates. The partially cured isolates were consistently more virulent than 14.4A in apple fruit assays; however, virulence still could not be considered normal since virulence in peach tree assays was not significantly greater than isolate 14.4A (Table 4).

The majority of the colonies regenerated from protoplasts of 14.4A exhibited the cultural phenotype and banding pattern of partially cured strains. However, three protoplast colonies (103, 105, and 107) were blackish grey in pigmentation with a more lobate colony margin than isolate 14.4A or the partially cured isolates (Fig. 2). Isolates 103, 105, and 107 formed pycnidia and produced conidia in culture, resembled field isolates in colony morphology, and exhibited virulence equivalent to field isolates in both apple and peach tree virulence assays (Table 4). DsRNA was not detected in isolates 103, 105, and 107 after 15 assays, and these isolates were therefore considered dsRNA-cured isolates of 14.4A.

Transmission experiments. Benomyl-resistant mutants were derived from the dsRNA-cured isolates 105 and 107. Because 105 and 107 originated from protoplasts of 14.4A, it was expected that hyphal fusion should occur readily among isolates 105, 107, and 14.4A. Mutants 105 Ben^R and 107 Ben^R had no detectable levels of dsRNA when assayed by gel electrophoresis and were indistinguishable in virulence from cured isolates 105 and 107 before exposure to uv light (Table 4).

TABLE 3. Ratio and percentage of recovery of isolates of *Leucostoma persoonii* with the partially dsRNA-cured morphology[a] after treatment of isolate 14.4A with various curing procedures

Treatment[b]	Proportion of 14.4A morphology to partially cured morphology	Percent partially cured
Hyphal-tip	20:980	98
Apple fruit	12:38	76
Ribavirin	25:2	7.4
Cycloheximide	NG[c]	...
Temperature (33 C)	15:0	0
Protoplasts	4:96	96

[a] Isolate lacking specific dsRNA segments and having darker pigmentation, more compact mycelium with no lysing hyphal tips, and producing pycnidia with no conidia.
[b] Various treatments include: hyphal-tip, removing hyphal tips from actively growing cultures; apple fruit, reisolation of mycelium from inoculated apple fruit lesions; ribavirin, growth on media containing 25–50 μg of ribavirin per milliliter; cycloheximide, growth on media containing 0.25–50 μg of cycloheximide per milliliter; temperature, growth of cultures at 33 C; and protoplasts, regeneration of protoplasts.
[c] NG, no growth of isolate 14.4A on cycloheximide-amended media.

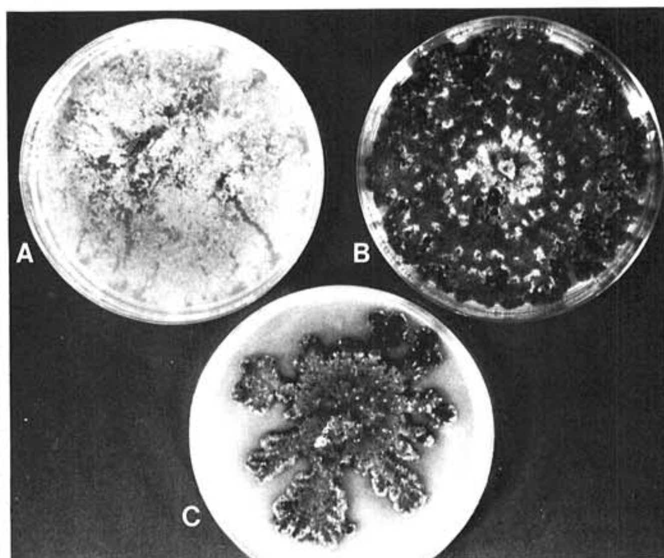

Fig. 2. Characteristic cultural morphology of dsRNA infected and cured strains of *Leucostoma persoonii.* **A,** Strain 14.4A with light pigmentation, less compact mycelium, and no pycnidia; **B,** partially cured strain with darker pigmentation, compact mycelium, and pycnidial initials; and **C,** cured strain with sporulating pycnidia, and lobate colony margins characteristic of most isolates of *L. persoonii* from cankers on peach.

TABLE 4. Comparison of virulence of wild-type isolates of *Leucostoma persoonii* 14.4A, and subcultures of 14.4A, on peach tree branches (17 mm diameter)

Isolate	Mean canker length (cm)[a]	dsRNA[b]
105 (cured 14.4 A)	4.67 a[c]	−
107 (cured 14.4 A)	4.48 a	−
T-5 (wild-type)	4.11 a	−
R-1 (wild-type)	3.65 a	−
105 Ben^R	3.80 a	−
Partially cured 14.4A	1.13 b	+
14.4A	0.72 b	+

[a] Inoculations were made by placing mycelium embedded in Leonian's malt agar into wounds made by an empty stapling gun followed by freeze injury. The sites of inoculation were wrapped in Parafilm. Seven months later the bark was removed and canker lengths were measured. Means are based on 10 replications (10 trees, 1 branch/tree).
[b] −, dsRNA not detectable; +, dsRNA detactable by gel electrophoresis.
[c] Means followed by same letters are not significantly different by the least significant difference test (LSD), $P = 0.05$.

When placed together on the surface of LMA or oatmeal agar, the hyphae of the two inoculum plugs (14.4A and 105 Ben^R) grew together if the fusion plates were preinoculated with 14.4A 2 days before inoculation with 105 Ben^R. If the fusion plate was not preinoculated with 14.4A, the mutant overgrew the plate and restricted the growth of isolate 14.4A. No sectors with altered morphology were observed at the interface between the two isolates (2). Subcultures resistant to benomyl and with dsRNA were obtained after transferring a portion of mycelium from the zone of interaction between the paired strains to a plate of LMA with 4 μg of benomyl per milliliter. To verify that the sector was not a spontaneous benomyl-resistant mutant of 14.4A, 80 plates of LMA amended with 4 μg of benomyl per milliliter were each inoculated with four mycelial plugs of 14.4A. No colony growth or hyphal sectors were produced after 2 mo of incubation. The dsRNA-infected 105 Ben^R was morphologically distinct from the dsRNA-free 105 Ben^R and isolate 14.4A. The mycelium was less compact and had a brown pigmentation instead of the blackish grey color of 105 Ben^R. The strain retained the ability to form pycnidia, however. In a virulence test conducted in apple fruit the infected mutant was significantly less virulent ($P = 0.05$) than the dsRNA-free mutant (data not shown). The efficiency of transfer of dsRNA from isolate 14.4A to 105 Ben^R was very low. DsRNA segments A, B, C, and E were found in the converted mutant after gel electrophoresis, but staining was faint, indicating a low titer.

DISCUSSION

Most mycoviruses are asymptomatic or latent in their hosts (15). While this may be the case with isolate 9.2 of *L. persoonii*, it does not appear to be the case with isolate 14.4A. We have found a correlation between the diseased state of 14.4A and the presence of dsRNA. This is illustrated by changes in growth, sporulation, and increased virulence when dsRNA is systematically eliminated from 14.4A.

Although it is difficult to completely cure 14.4A of dsRNA, some segments of dsRNA can be readily eliminated. When the partially cured phenotype of 14.4A was obtained, dsRNA segments B, C, and D were not detected. These three segments were always lost in concert. This suggests an interdependence of all three segments, or dependence of one or more segments on another. In comparison to the easily eliminated B, C, and D segments of dsRNA found in 14.4A, segments A and F of the partially cured strains were difficult to eliminate. The phenotypic changes that accompany the loss of the B, C, and D segments are characteristic

and constant, including the absence of lysing hyphal tips, more compact mycelium, increased virulence, and formation of sterile pycnidial initials. These apparent fruit bodies reveal only masses of intertwined hyphae and no fertile conidiogenous layer in electron microscopy (25). The phenotypic changes correlated with loss of all dsRNA segments, including A and F, are also constant and include an even greater increase in virulence, formation of conidia, increased hyphal pigmentation, and a more lobate (more normal) colony on PDA. These results indicate that 14.4A might have a mixed infection of at least two different dsRNA viruses.

Mixed viral infection is common in fungi and has been reported in *C. parasitica* (10,11) and *Gaeumannomyces graminis* (6). DsRNA segments A and F appear to be quite stable in isolate 14.4A of *L. persoonii*, which is still reduced in virulence and conidiation when these segments are present. These partially cured isolates are morphologically less debilitated than the noncured isolate 14.4A. The VLPs of 14.4A have not been observed in partially cured strains (Snyder, Adams, and Fulbright, *unpublished*). Currently, we are attempting to infect protoplasts of isolates of *L. persoonii* with the purified VLPs of isolate 14.4A.

Viruslike particles have not been associated with transmissible cytoplasmic factors that diminish vigor in cultural growth and host pathogenicity in plant pathogenic fungi (20). In *C. parasitica* the dsRNA is either free in the cytoplasm or associated with membranous vesicles (12). No VLPs have been isolated or detected in the dsRNA infected isolate of *R. solani*, which has been reported involved in Rhizoctonia decline (7). VLPs have been found in *G. graminis* var. *tritici*, but they were not considered a cause for the reduction in pathogenicity (5).

In *C. parasitica* where dsRNA is cytoplasmically borne, the transmission of the dsRNA by hyphal anastomosis from hypovirulent to vegetatively compatible virulent isolates occurs readily (3). Additionally, transmission of dsRNA sometimes occurs between isolates that are not vegetatively compatible (1,26). It is surprising that detecting dsRNA transfer between 14.4A and a cured 14.4A Ben^R mutant appears to be difficult since here anastomosis is a selfing response rather than fusion between two different isolates. One explanation for this difficulty is that the lysing hyphal tips of 14.4A might hinder hyphal anastomosis. It is interesting to note that the dsRNA segment pattern found after transmission of dsRNA from 14.4A to 105 Ben^R included only segments A, B, C, and E. This sequence of segments was different than that of 14.4A or of any of the partially cured isolates.

Isolate 9.2 was the only other isolate in these studies found to contain dsRNA. The dsRNA did not affect the virulence or morphology of isolate 9.2. The mere presence of dsRNA in *L. persoonii* does not necessarily indicate that an isolate will be hypovirulent. Isolate 9.2 was found to carry dsRNA molecules representing different sizes than those dsRNA molecules in 14.4A, and this implies that isolate 9.2 is infected with a different "virus." Also, this indicates that specific molecules may confer specific phenotypic changes in the host fungus.

The discovery of isolate 14.4A is an indication that hypovirulent strains exists in natural *Leucostoma* populations. The partially cured strains may be better for potential biological control of Cytospora canker. These strains still have very low virulence but lack the debilitation of isolate 14.4A. These strains do not sporulate, however, and natural spread in the orchard may prove difficult.

LITERATURE CITED

1. Anagnostakis, S. L. 1978. The American chestnut: New hope for a fallen giant. Conn. Agric. Exp. Stn. Bull. 777, New Haven, CT. 9 pp.
2. Anagnostakis, S. L., and Day, P. 1979. Hypovirulence conversion in *Endothia parasitica*. Phytopathology 69:1226-1229.
3. Anagnostakis, S. L., and Waggoner, P. E. 1981. Hypovirulence, vegetative incompatibility, and growth of cankers of chestnut blight. Phytopathology 71:1198-1202.
4. Banko, T. J., and Helton, A. W. 1974. Cytospora induced changes in stems of *Prunus persica*. Phytopathology 64:899-901.
5. Buck, K. W. 1986. Viruses of the wheat take-all fungus, *Gaeumannomyces graminis* var. *tritici*. Pages 221-236 in: Fungal Virology—an Overview in Fungal Virology. K. W. Buck, ed. CRC Press, Boca Raton, FL.
6. Buck, K. W., Almond, M. R., McFadden, J. J. P., Romanos, M., and Rawlinson, C. J. 1981. Properties of thirteen viruses and virus variants obtained from eight isolates of the wheat take-all fungus, *Gaeumannomyces graminis* var. *tritici*. J. Gen. Virol. 53:235-245.
7. Castanho, B., Butler, E. E., and Shepherd, R. J. 1978. The association of double-stranded RNA with Rhizoctonia decline. Phytopathology 68:1515-1519.
8. 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.
9. Dodds, J. A. 1970. Revised estimates of the molecular weights of dsRNA segments in hypovirulent strains of *Endothia parasitica*. Phytopathology 70:1217-1220.
10. Elliston, J. E. 1985. Preliminary evidence for two debilitating cytoplasmic agents in a strain of *Endothia parasitica* from western Michigan. Phytopathology 75:170-173.
11. Elliston, J. E. 1985. Further evidence for two cytoplasmic hypovirulence agents from western Michigan. Phytopathology 75:1405-1413.
12. Ellzey, J. T., Hammons, T. L., and Cooper, M. O. 1986. An ultrastructural study of membrane-bounded particles within hypovirulent strains of *Endothia* (*Cryphonectria*) *parasitica*. Mycologia 78:313-315.
13. Fulbright, D. W. 1984. Effect of eliminating dsRNA in hypovirulent *Endothia parasitica*. Phytopathology 74:722-724.
14. Fulbright, D. W., Weidlich, W. H., Haufler, K. Z., Thomas, C. S., and Paul, C. P. 1983. Chestnut blight and recovering American chestnut trees in Michigan. Can. J. Bot. 61:3164-3171.
15. Ghabrial, S. A. 1980. Effects of fungal viruses on their hosts. Annu. Rev. Phytopathol. 18:441-461.
16. Hampson, M. C., and Sinclair, W. A. 1973. Leucostoma canker of peach: Symptoms in relation to water supply. Plant Dis. Rep. 45:591-597.
17. Hashiba, T., and Yamada, M. 1982. Formation and purification of protoplasts from *Rhizoctonia solani*. Phytopathology 72:849-853.
18. Helton, A. W., and Randall, H. 1975. Cambial gummosis in *Prunus domestica* infected with *Cytospora cincta*. Plant Dis. Rep. 59:340-344.
19. Hildebrand, E. M. 1947. Perennial peach canker and the canker complex in New York, with methods of control. Cornell Exp. Stn. Mem. 276. 566 pp.
20. Hollings, M. 1978. Mycoviruses-viruses that infect fungi. Adv. Virus Res. 22:2-53.
21. Leonian, L. H. 1923. The physiology of perithecial and pycnidial formation in *Valsa leucostoma*. Phytopathology 13:257-272.
22. Royse, D. J., and Ries, S. M. 1978. The influence of fungi isolated from peach twigs on pathogenicity of *Cytospora cincta*. Phytopathology 68:603-607.
23. Scorza, R., and Pusey, P. L. 1984. A wound-freezing inoculation technique for evaluating resistance to *Cytospora leucostoma* in young peach trees. Phytopathology 74:569-572.
24. Shatkin, A. J., Sipe, J. D., and Loh, P. 1968. Separation of ten reovirus genome segments by polyacrylamide gel electrophoresis. J. Virol. 2:986-991.
25. Snyder, B. A., Adams, G. C., and Fulbright, D. W. 1989. Association of a virus-like particle with a diseased isolate of *Leucostoma persoonii*. Mycologia 81(In press).
26. Van Alfen, N. K. 1982. Biology and potential for disease control of hypovirulence of *Endothia parasitica*. Annu. Rev. Phytopathol. 20:349-362.
27. Willison, R. S. 1936. Peach canker investigations II. Infection studies. Can. J. Res. 14:27-44.