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Sue Ann Hammar

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Gerald C Adams

Major professor

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By

Sue Ann Hammar

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ABSTRACT

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By

Sue Ann Hammar

An isolate of Leucostoma persoonii with low virulence, abnormal morphology, and lacking conidia was found to contain double-stranded RNA (dsRNA) as determined by polyacrylamide gel electrophoresis. Specific dsRNA segments were lost in partially cured subcultures resulting in increased but not normal virulence or sporulation. Protoplast derived dsRNA-free subcultures had normal virulence and sporulation. A mixed viral infection was indicated. The optimal experimental design for evaluating isolate virulence in the orchard, based on sampling variances, was to inoculate one branch per tree in 6-9 trees for each isolate. Criteria for differentiating L. cincta from L. persoonii by cultural characteristics was evaluated. Vegetative compatibility (vc) groupings were determined for two orchards by pairing isolates derived from cankers. Isolates within an orchard were in numerous vc groups. Isolates from several cankers within one tree and among closely spaced trees usually differed in vc grouping. The epidemiology and sexuality of L. persoonii is discussed.

To my parents

For teaching me the value of an education

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GENERAL INTRODUCTION

Cytospora canker of peach is of considerable economic importance and is the limiting production factor for this crop in most northern climate peach growing areas (9,14,25,29). This disease is also known as peach gummosis, valsa canker, perennial canker, die-back of peach, and peach canker (30,46). The same organisms that cause Cytospora canker on peach also cause an important disease on apple, cherry, apricot, prune, and plum (19,20). The disease is often destructive in young orchards where it causes premature death of the trees. Older infected trees gradually lose productivity and longevity is decreased (36,44).

Symptoms of Cytospora canker on peach include dieback of twigs and branches, girdling and wilting of branches and major limbs, perennial cankers on the trunk, scaffold limbs, and branches, and premature leaf senescence and defoliation (9,18,40). Cankers are first apparent in the spring at nodes and fruit pedicels (14,36). Typical canker symptoms are collapsed cortical tissue, bark necrosis, gumming, pycnidia, and a zonate margin (6).

The causal agents of the disease are Leucostoma cincta (Pers.: Fr.) Hoehn. [anamorph= Leucocytospora cincta (Sacc.) Hoehn.] and Leucostoma persoonii (Nits.) Hoehn. [anamorph= Leucocytospora leucostoma (Pers.) Hoehn.]. The fungi causing Cytospora canker on peach were originally placed in the genus Valsa in 1849 based on the oblique position and circinate pattern of emerging perithecia in the determinant stroma. As early as 1917 Hoehnel separated species of Valsa sensu lato into Valsa sensu stricto and Leucostoma (3,26). Leucostoma is differentiated from

Valsa sensu stricto by the presence of a distinctive dark conceptacle of compact fungal tissue deliminating the stromata around the fruiting bodies. The anamorphs that developed with a similar conceptacle were segregated from Cytospora and placed in Leucocytospora (3,26,37). The anamorph and teliomorph of the species that cause Cytospora canker of peach form the distinctive conceptacle.

The genus Leucostoma is in the Diaporthese. The pycnidia, and when present, perithecia, develop in a stroma. Perithecia are oblique with few to numerous beaks converging and erumpent through the disc (3,29). Stromatic tissues are prosenchymatous or pseudoparenchymatous, forming determinent white or grayish brown ectostromatic discs. The stromata are delimited by a dark conceptacle. The conidia and ascospores are hyaline, cylindroid to allantoid, and one-celled. Asci are short-stalked and ellipsoid and have an apical ring mostly visible as two strongly refractive bodies. The asci often become free from their attachment and may exit the perithecia in a slime matrix. The conidia are produced in a simple, irregularly chambered pycnidial cavity which is formed either in the center of a perithecial stroma or alone in a stroma. The pycnidium usually opens by one central pore through which conidia exude in long threads or cirri. Kern (29) recognized two species associated with Cytospora canker of peach based on differences in color and shape of the stromatic disc and ascospore size. In L. persoonii the stromatic disc is whitish or white and lenticular in shape with ascospores 8-14 x 1.5-3 u. In L. cincta the disc is grayish brown to dark brown and in shape with ascospores 8-20 x 2-4 u. The thickness and shape of the conceptacle and ascospore size range all serve to delimit species in the genus Leucostoma (3,29). Unfortunately L. persoonii and L. cincta are not

readily distinguishable by cultural characteristics or anamorph morphology and the teleomorph is scarce or ephemeral. For these reasons plant pathologists are seldom able to positively identify the pathogens and thus the pathology literature becomes difficult to interpret. Lukezic et al. (32) have suggested that the two species are synonymous, both being derivable from ascospore progeny of a single perithecium. However, other researchers recognize them as distinct, occuring in the same geographic locale or on the same tree (15,and L. Spielman, personal communication).

The first mention of the parasitic nature of Leucostoma spp. on peach in the American literature was made in 1900 by Stewart et al. (38). Aderhold's reference in 1903 to attack of frost-weakened trees was one of the first recognitions of the importance of predispositional factors in host susceptibility to the disease (1). In 1916, Walton and Babcock (43) found that infections could be initiated by inoculating Leucostoma spp. on tree trunks, larger branches, and twigs of peach trees. In 1918, McCubbin (33) discovered that V. leucostoma was able to invade healthy tissues and form cankers when inoculated onto dead or dying tissue. Defago (1935) conducted an extensive investigation of Valseae parasitic on declining stone fruit trees (13). He concluded that the degree of infection was governed by both virulence of the isolate and host vigor. Willison (46,47) obtained maximum necrosis with inoculations made at leaf fall. Summer inoculations were comparatively inocuous under certain conditions. Willison also reported on the characteristics and roles played by the two species of Valsa responsible for peach canker.

Leucostoma spp. are wound-parasites (20,46,48) and are most likely to

cause infection when the trees are dormant (7,14). The fungus enters wounds caused by low temperature winter injury to peach flower buds, twigs, and bark (14,21,22,40), pruning (25,47), sunscald (10), and fruit moth and borer injuries (10,42). When the fungus enters the wound it colonizes the bark and cambial tissues and xylem tissue in advance of visible canker margins (9,47). The development of the disease is associated with numerous stress factors including moisture stress (6), nutrient stress (eg. potassium stress), and high clay content in the soil (5,6). Once in the xylem the fungus may plug vessels or locally destroy the cambium and impede water flow causing wilting and defoliation (2,9,18). Canker expansion is greatest just prior to bud break while host activity is slight and temperatures are increasing and approaching optimal for fungal growth (25,28,46). These pathogens are able to inhibit or overcome host- mediated nonspecific responses such as callus formation, periderm differentiation, and lignification in the bark and the xylem (8).

Attempts to control Cytospora canker of peach using fungicides has been ineffective due to the deep penetration of the fungus in the host (23,35) and the complexity of predisposing and interacting factors (41). Pruning and other cultural practices are often inadequate for satisfactory control (44) and are often time consuming, labor intensive, and costly (41). Cytospora canker might be controlled biologically. Work on induced resistance suggests the virulence of the pathogen can be reduced if the peach trees are inoculated previously with avirulent pathogens (10,24,27). The disease might also be controlled using hypovirulence. Hypovirulence is cytoplasmically transmissible and results in attenuation of virulence of the pathogen (42).

The most thorough studies on hypovirulence are those on the plant pathogen Cryphonectria parasitica [Murr.] Barr. (=Endothia parasitica (Murr.)), the causal agent of chestnut blight. Hypovirulence of C. parasitica has been correlated with the presence of double-stranded RNA (dsRNA) molecules (12,42). Hypovirulent strains of C. parasitica were first discovered in Europe where they were reducing the impact of virulent strains of the pathogen (17). Grente and Sauret (17) demonstated that these hypovirulent strains were dominant when mixed in a tree with normal virulent isolates. This dominance was later found to be due to transmission of dsRNA molecules from hypovirulent to virulent isolates (42). Biological control of chestnut blight has occured naturally in Italy (34) and in Michigan (16) and the disease is being controlled in France by artificial spread of similar strains (17). In all of these cases, the presence of hypovirulent strains of C. parasitica is correlated with the survival of the chestnut trees in spite of infection (16,17).

The purpose of this research was to explore the possibilities of using hypovirulence as a means of biologically controlling Cytospora canker of peach. To detect hypovirulent isolates of Leucostoma, a reliable virulence test was developed to screen for isolates with reduced virulence. Secondly, dsRNA was found associated with the loss of virulence in an isolate of L. persoonii . Thirdly, the vegetative compatibility system for L. persoonii was examined to determine if the spread of hypovirulence would be possible within the fungal population. These tests also gave us insight into some aspects of the biology of the fungus and epidemiology of disease spread.

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SECTION I

The Association of Double-Stranded RNA with Low Virulence and Loss of Sporulation in an Isolate of *Leucostoma persoonii* from Peach

The Association of Double-stranded RNA with Low Virulence and Loss of Sporulation in an Isolate of *Leucostoma persoonii* from Peach

ABSTRACT

An isolate of *Leucostoma persoonii* (14.4A) with low virulence, abnormal culture morphology, and an inability to produce pycnidia when grown on culture media was found to contain nine segments of double stranded RNA (dsRNA) when nucleic acid extracts from mycelium were examined by polyacrylamide gel electrophoresis. Some dsRNA segments were eliminated when hyphal tips of 14.4A were removed and subcultured. The formation and regeneration of protoplasts and the reisolation of 14.4A from susceptible plant tissue also led to the loss of dsRNA. Specific dsRNA segments were more easily eliminated than others. Partially cured strains were more virulent than 14.4A in apple fruit virulence assays but comparable in virulence to the uncured 14.4A in peach tree assays. Cured strains, in which dsRNA was not detectable, were obtained through protoplast regeneration and selection for conidia production. The dsRNA-free cultures grew similar to isolates considered normal and demonstrated increased virulence on peach trees when compared with fully virulent isolates. Four detectable segments of dsRNA were transferred by hyphal anastomosis from 14.4A to a genetically marked strain. These results indicate that 14.4A may be infected with two distinct dsRNA viruses.

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.] Symptoms of cytospora canker include cambial tissue necrosis and perennial cankers which are occasionally accompanied by copious gum exudation. Infected branches wilt and die back from girdling and plugging of the xylem vessels (4,18,20). Fungicides have not been effective in controlling the disease.

The isolate of L. persoonii used in this study is dramatically reduced in virulence, has an abnormal culture morphology and contains double-stranded RNA (dsRNA) and virus-like particles. Although the vast majority of mycoviruses apparently have no deleterious effect on their host (17), some mycoviruses adversely affect the virulence of their host. This suggests that some plant pathogenic fungi might be controlled with viral infections (23).

Biological control of Cytospora canker is an appealing concept. 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 (8,9,29). Hypovirulence has best been described in the plant pathogen Cryphonectria parasitica [Murr.] Barr. (=Endothia parasitica (Murr.)) (2,29), which causes a perennial canker disease similar to Cytospora canker.

The purpose of this study was to determine if the presence of dsRNA could be directly correlated with the loss of virulence and other abnormal characteristics of L. persoonii. This was accomplished by eliminating the dsRNA from the fungus and determining whether or not virulence was regained. In addition, we attempted transmission of the

dsRNA to non-infected strains.

MATERIALS AND METHODS

Isolates

Isolates used in virulence tests were recovered from cankers on peach trees grown in Michigan, West Virginia, California, Pennsylvania, and North Carolina (Tables 1-2). Cultures were maintained on Leonian's Malt Agar (LMA) (24) and stored on peach bark agar (25) at 4 C with annual subculturing.

Isolates were identified as L. persoonii or L. cincta based on colony color, size of pycnidia, whether or not the colony margin was lobate or uniformally radial (31) and by the presence or absence of growth at 37 C (21). The morphology of each isolate was also compared to the morphology of single ascospore isolates of L. persoonii.

Virulence Tests

The virulence of isolates of 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 of 1985 following the method of Scorza and Pusey (26). Two-yr-old peach branches measuring 17 mm in diameter were wounded to the xylem with an empty, hand-held 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. Inoculations were wrapped with parafilm to prevent dessication. Canker lengths were measured the following May after stripping off the bark to reveal the length of the necrotic area distal to the inoculation point.

The virulence test using apple fruit was performed by removing a 9 mm diameter x 4 mm deep plug of tissue with a sterile cork borer. Mycelial plugs were placed mycelium side down in contact with the wounded apple tissue (15). The site of inoculation was covered with tape and the apples were placed at room temperature 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. Wood pieces were surface sterilized by soaking in a 10% solution of a commercial laundry bleach (5.25% NaOCl) 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 potato dextrose agar (Difco, PDA) and incubated at room temperature.

Isolation of dsRNA

DsRNA was extracted using modifications (16) of the procedures of Day et al. (9) and of Dodds (10). Nucleic acid samples were layered on 5% polyacrylamide slab gels and electrophoresed at 40 mA for 12 hr. Molecular weight estimates of dsRNA were calculated from standard curves plotting electrophoretic mobility verses the log of the molecular weights using coelectrophoresed standards of reovirus serotype 3 (27), C. parasitica (GH2) (16), and Bipolaris maydis (ATCC# 32450).

Curing experiments

Five procedures were used to obtain subcultures of isolate 14.4A with colony morphology considered typical for L. persoonii isolates. First, mycelial plugs of 14.4A were transferred to LMA media ammended with

either 0.25 to 50 ug of cyclohexamide (Sigma Chemical Co., St. Louis, Mo) per milliliter (15) or 25 to 50 ug ribavirin (Sigma Chemical Co., St. Louis, Mo) per milliliter. After 2 wk, agar plugs were transferred from the margins of the colonies to fresh PDA. Second, 100 hyphal tips were taken from four 2-day-old colonies of 14.4A grown on LMA. After 2 days growth, a hyphal tip was transferred from each resulting colony and the process was continued ten consecutive times at 2-day intervals. The resulting 100 colonies were grown for 4 wk (the length of time for typical cultures to sporulate) before evaluation. Third, reisolations were made from the margin of an expanding lesion in apple fruit inoculated with 14.4A. Fourth, 15, 4-day old cultures of 14.4A were transferred to LMA, and the plates were incubated in the dark at 33, 36, and 38 C for 2 wk. Mycelial transfers of any living colonies 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 (9). Mycelia were collected with a buchner funnel and incubated 3 h at 33 C in a solution containing 1 ml of 1 M sodium phosphate buffer at pH 5.8 and 9 ml of 1 M mannitol amended with a filter sterilized 1 ml solution containing 10 mg of Novozyme 234 (Novo Laboratory, Inc., Wilton, CN) and 1 mg 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 um mesh nylon filter (Tetko, Inc., Elmsford, NY) and collecting the filtrate in a centrifuge tube. Protoplasts were centrifuged at 2000 rpm for 5 min, resuspended in 1 M mannitol, recentrifuged, resuspended in 2

ml of 1 M mannitol and layered onto 4 ml of 1 M sucrose. The solution was centrifuged at 1000 rpm for 5 min. The protoplasts, located in an opaque interface between the two solutions (19), were removed and added to 2 ml of 1 M mannitol. The layering procedure was repeated until a highly purified protoplast preparation was obtained. The final protoplast pellet was diluted in 1 M mannitol to 10^2 protoplasts per milliliter; 1 ml was pipetted onto 1 M mannitol in complete medium agar (9). In 48-72 h after plating, regenerated protoplasts were individually transferred to LMA. In each of 20 experiments, 50-100 protoplasts showing hyphal initiation were individually transferred.

With each of the five procedures, subcultures exhibiting a more normal cultural morphology or showing sporulation were tested for dsRNA by polyacrylamide gel electrophoresis.

Benomyl-resistant mutants

Benomyl-resistant mutants were isolated by collecting pycnidia from 4-wk-old colonies of dsRNA-free 14.4A subcultures and grinding them in sterile water with a mortar and pestle. The slurry was passed twice through a 5 um filter so that only the conidia in water remained. One ml of a solution of 10^6 conidia per milliliter was pipetted onto LMA amended with 4 ug benomyl per milliliter. The conidia were irradiated for 1.5 min with a 254 nm 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. Six benomyl-resistant mutants were tested to verify lack of dsRNA prior to transmission experiments.

Transfer of DsRNA

To transfer dsRNA from isolate 14.4A to benomyl-resistant (Ben^R) isolates, mycelial plugs of 14.4A and a Ben^R isolate 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. Plugs were removed from various areas on the plate and subcultured on LMA amended with 4 ug benomyl per milliliter. Isolates that contained dsRNA and grew on the benomyl amended media were assumed to have been converted by isolate 14.4A.

RESULTS

Isolates

Isolate 14.4A, designated as L. persoonii, had an abnormal culture morphology including lysing hyphal tips and did not produce pycnidia or conidia in culture or inoculated stems. All other isolates tested had a normal or expected morphology of Leucostoma spp. (31) and produced spores in culture.

Virulence tests

Inoculations performed on apple fruit indicated that 14.4A was the least virulent of the isolates tested (Table 2). In orchard tests, 14.4A was one of the least virulent as evidenced by the small cankers it caused in relation to most other isolates tested (Table 1). It's ranking in Table 1 is higher than expected due to the contamination of 14.4A inoculation sites with wild type strains. While most of the isolates tested had variable virulence from one test to another (Table 1 and 2; isolate 14.1 and 8.2), isolate 14.4A was consistantly very low in all virulence assays (unpublished data).

Screening isolates for dsRNA

The only field isolates in which dsRNA was detected by electrophoresis were 14.4A and 9.2 (Table 1 and 2). Nine segments of dsRNA were visible in nucleic acid extracts from 14.4A subjected to polyacrylamide gel electrophoresis (Fig 1). Segments B, C, and D were consistantly associated with 14.4A. Segments A and F were usually present but stained much fainter with ethidium bromide than segments B, C and D after polyacrylamide gel electrophoresis. Segments E, G, H, and I appeared inconsistantly. Segments B, C, and D ranged in size from a molecular weight of 2.90×10^6 to 2.05×10^6. 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 (H_2O), and resistance to DNase treatments.

Isolate 9.2 consistantly yielded three distinct dsRNA segments in every gel observed (Fig 1).

Curing of isolate 14.4A

All partially cured isolates were indistinguishable from one another regardless of the curing method from which they were derived. The partially cured isolates were darker in pigment than 14.4A, the hyphae were more compact in texture and no longer lysed at the tips, and formed fruiting bodies which lacked conidia (Fig 2). Of the one-hundred partially cured strains tested, all retained segments A and F (Fig 1) but lost segments B, C, D, E, G, H, and I. The partially cured strains were consistantly more virulent than 14.4A in apple fruit assays (Table 2), however, virulence still could not be considered normal since virulence in peach tree assays was not significantly greater than isolate 14.4A (Table 3).

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[c]	Isolate	Canker length (cm)[a]		DsRNA[b]
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	0.5	L	

a/Inoculations were made by placing mycelium embedded in LMA into wounds made by an empty hand-held 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 ten replications (10 trees, 1 branch/tree).

b/-,no dsRNA present; +,dsRNA present as determined by gel electrophoresis

c/Initials of state where isolates were recovered

d/Means followed by the same letters are not significantly different (P=.05)

e/Higher ranking due to the contamination of three of the ten replications. The mean for the seven uncontaminated reps = 1.98.

Table 2. Assay of virulence of isolates of Leucostoma persoonii on apple fruit

State of origin[c]	Isolate	Lesion width (cm)[a]		DsRNA[b]
NC	14.1	8.33	A[d]	-
MI	T-5	7.77	AB	-
NC	9.2	6.80	BC	+
MI	10.8	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	0.5	E	

a/ Inoculations were 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 ten replications.

b/-,no dsRNA present; +,dsRNA present as determined by gel electrophoresis

c/Initials of state where isolates were recovered

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

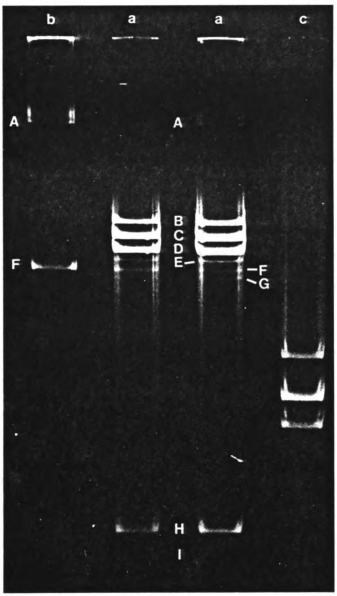

Figure 1. Photograph of an ethidium bromide stained 5% polyacrylamide gel showing specified segments of dsRNA extracted from Leucostoma spp. a) isolate 14.4A, b) partially cured 14.4A, and c) isolate 9.2.

Partially cured subcultures of 14.4A were readily obtained by subculturing hyphal tips, reisolation of 14.4A from apple fruit or protoplast regeneration. The percentage of subcultures with the partially cured phenotype (method, 14.4A phenotype: partially cured phenotype) were: hyphal tipping, 980:20= 98%; apple fruit, 38:12= 76%; protoplast regenerates, 96:4= 96%; treatments of 25 ug/ml Ribavirin, 2:25= 7.4%; and cyclohexamide treatment, 0:0= 0%. Higher concentrations of Ribavirin did not yield a greater percentage of partially cured strains. Unlike the other field isolates tested, 14.4A did not grow on PDA containing concentrations of 0.25 to 5.0 ug/ml cyclohexamide. Isolates with the partially cured morphology were also readily obtained from older cultures that had been frequently subcultured onto laboratory media. Isolate 14.4A grew only at 33 C and none of the subcultures were cured of dsRNA.

The majority of the colonies regenerated from protoplasts of 14.4A exhibited the banding pattern and cultural phenotype of partially cured strains: however, three protoplast colonies (103, 105, and 107) were blackish grey in pigmentation with a more lobate colony margin than 14.4A or the partially cured strains (Fig 2). The protoplast regenerated cultures formed pycnidia and conidia in culture, resembled field isolates in colony morphology, and exhibited virulence equivalent to field isolates in peach tree virulence assays (Table 3). No dsRNA was detected in these isolates in 15 attempts by electrophoresis.

Transmission experiments

The Ben^R mutants were derived from the dsRNA cured isolates 105 and 107. Since 105 and 107 originated from protoplasts of 14.4A, hyphal

Table 3. Comparison of virulence of Leucostoma persoonii wild-type isolates, 14.4A, and subcultures of 14.4A on branches (17 mm diam.) on peach trees

Isolate	Mean canker length (cm)[a]		DsRNA[b]
Cured 14.4A-105	4.67	A[c]	-
Cured 14.4A-107	4.48	AB	-
Cured 14.4A-103	4.33	AB	-
Wild-type MT5	4.11	AB	-
Wild-type MR-1	3.65	AB	-
Benomyl resistant cured 14.4A	3.80	AB	-
Partially cured 14.4A	1.13	C	+
14.4A	0.72	C	+

a/Inoculations were made by placing mycelium embedded in LMA into wounds made by an empty hand-held 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 ten replications (10 trees, 1 branch/tree).

b/-,no dsRNA present; +,dsRNA present as determined by gel electrophoresis

c/Means followed by same letters are not significantly different by the least significant difference test (LSD), P=0.05.

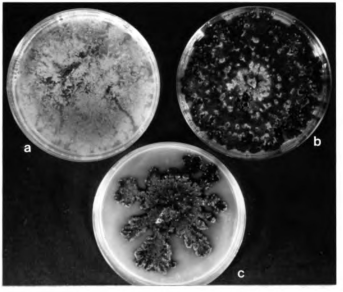

Figure 2. Characteristic cultural morphology of 30-day-old dsRNA infected and cured strains of Leucostoma persoonii on LMA a) strain 14.4A with light pigmentation, less compact mycelium, and no pycnidia, b) "partially cured" strain with darker pigmentation, compact mycelium, and pycnidial initials, and c) "cured" strain with sporulating pycnidia, and lobate colony margins characteristic of most isolates of L. persoonii from cankers on peach.

fusion should occur readily between a Ben^R marked mutant and 14.4A. The Ben^R mutants had no detectable levels of dsRNA when evaluated by gel electrophoresis and were indistinguishable in morphology and virulence from 105 and 107 prior to exposure to uv light.

The hyphae of the two inoculum plugs appeared to intermingle freely when the fusion plates were preinoculated with 14.4A two days prior to inoculation with the mutant. If the fusion plate was not preinoculated with 14.4A, the mutant overgrew the plate and restricted 14.4A growth. No sectors with altered morphology were observed at the interface between the two isolates (1). Transmission by hyphal anastomosis of dsRNA from 14.4A to a Ben^R mutant was successful, although the efficiency of transmission was very low. DsRNA segments A, B, C, and E were found in the converted mutant after gel electrophoresis but they stained faint indicating a low titer. The converted mutant was obtained after transferring a portion of mycelium from the zone of interaction between the paired strains to a plate of LMA with 4 ug benomyl/ml. After incubation for 3 wk the culture produced a hyphal sector. Subcultures from this sector contained dsRNA and grew on LMA ammended with benomyl. To verify that the sector was not a spontaneous mutant of 14.4A, 80 plates of LMA ammended with 4 ug/ml benomyl were each inoculated with four mycelial plugs of 14.4A. No colony growth or hyphal sectors were produced after 2 months incubation. The infected mutant was morphologically distinct from the original mutant and from 14.4A. The colony characteristics of the dsRNA containing benomyl resistant mutant have been altered from that of the dsRNA-free mutant. The mycelium was less dense and had a brown pigmentation instead of the blackish grey color of the original mutant. The strain retained the ability to form

pycnidia. In preliminary virulence tests in apple fruit the infected mutant is less virulent than the dsRNA-free mutant ($P = 0.05$).

DISCUSSION

Most mycoviruses are asymptomatic or latent in their hosts (17). This does not appear to be the case with L. persoonii isolate 14.4A. We have found a correlation between the diseased state of 14.4A and the presence of dsRNA. This is illustrated by change in growth, sporulation, and increased virulence when dsRNA is systematically eliminated from 14.4A.

Although it is difficult to completely cure 14.4A, some segments of dsRNA can be readily eliminated. When the partially cured phenotype of 14.4A is obtained, dsRNA segments B, C, and D are lost. 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, 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, D segments are very characteristic and constant, including the absence of lysing tips, more compact hyphal growth, 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 (28). The phenotypic changes correlated with loss of segments 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. This suggests 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 (11,12) and G. graminis (6). DsRNA segments A and F appear to be quite stable in the fungus which is still reduced in virulence and conidiation when these segments are present. These partially cured strains are less debilitated to hyphal growth than the combined infection seen in 14.4A. Isolate 14.4A contains the dsRNA of the less debilitating infection seen in the partially cured strains as well as many additional dsRNA segments B, C, D, E, G, H, and I. The virus-like particles (VLP's) of 14.4A are not present in the partially cured strains (Snyder et al., unpublished). The additional dsRNA segments may be the genome of the VLP's. The partially cured strains may contain a virus that has lost the capacity to encapsidate the dsRNA. The unencapsidated virus apparently replicates its genome effectively in the rapidly dividing hyphal tip cells making it difficult to eliminate. In contrast, the virus that forms VLP's may not assemble the particles fast enough to keep pace with the growth of hyphal tip cells. Further studies would be required to determine the degree of relatedness, if any, of the two debilitating agents.

So far, virus-like particles have not been associated with transmissible cytoplasmic factors that diminish vigor in cultural growth and host pathogenicity in plant pathogenic fungi (22). In C. parasitica the dsRNA is either free in the cytoplasm or associated with membranous vesicles (14). No VLP's have been isolated or detected in the dsRNA infected isolate of R. solani, which has been reported involved in Rhizoctonia decline (8). VLP's have been found in G. graminis var. tritici, but they were not generally a cause for the reduction in pathogenicity (7).

The concentration of VLP's in the cytoplasm of 14.4A is unusually high in several thin sections observed with transmission electron microscopy (28) The particles appear side-by-side, filling the cytoplasm between organelles. The presence of the VLP's is associated with lysis of hyphal tips. It is therefore not surprising that infected hyphae are less capable of causing disease. Assessing the role of the VLP's in reducing virulence is difficult. No VLP's can be detected in the partially cured strains, and the strains do not appear as debilitated as 14.4A. In laboratory tests using apple fruit, the isolates with no detectable VLP's are consistantly more virulent. In peach trees, however, both 14.4A and partially cured isolates were very low in virulence. Thus, elimination of detectable particles and associated dsRNA segments still results in low virulence and no sporulation. The dsRNA segments remaining in the partially cured strains apparently are more important than the VLP's in determining virulence on the host.

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 (2,29). It is surprising that detecting dsRNA transfer between 14.4A and a cured 14.4A Ben^R mutant appears to be rare because this is considered a selfing response and not fusion between two different isolates. One explanation is that the lysing hyphal tips of 14.4A might hinder hyphal anastomosis with another isolate. Hyphal tip lysis may make isolate 14.4A too debilitated to use for transmission of dsRNA.

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 lysis of hyphal tips. Thus, hyphal anastomosis and cytoplasmic transfer of the dsRNA may occur more readily with the partially cured strains. These strains do not sporulate, however, and natural spread in the orchard may prove difficult.

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. This indicates that specific molecules may confer specific phenotypic changes in the host fungus. This may be due to the lack of specific genes on the dsRNA that interfere with fungal host metabolism, the tolerance of the fungal host to the virus infection, or attenuation of the virus. Even in the C. parasitica system, a few strains have been found where dsRNA does not correlate with hypovirulence in the infected strains. These strains are nearly as pathogenic as the virulent isolate (13,30).

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SECTION II

Vegetative Compatibility in *Leucostoma persoonii*

Vegetative Compatibility in *Leucostoma persoonii*

ABSTRACT

Isolates of *Leucostoma persoonii* were paired on various media to determine a suitable medium for differentiating vegetative compatibility (vc) groupings. Vegetative incompatibility was evident only on oatmeal agar as dark lines and pycnidia forming along the line of mycelial contact between expanding colonies. Ascospore colonies derived from a single perithecium segregated into several vc groups indicating that the fungus outcrosses and is most likely heterothallic with several alleles controlling vegetative compatibility. Isolates of *L. persoonii* from cankers on peach trees within a single orchard clustered into numerous vc groups. Isolates from several cankers within one tree and among closely spaced trees usually differed in vc grouping. Spatial clustering of vc groups in the orchard were not aggregated. Conidia from a single pycnidium were in one vc group. The frequency and spatial arrangement of vc groups in an orchard indicates that ascospores (or air-blown conidia) could play a major role in dissemination and in initiation of new infections of peach.

Cytospora canker of peach is an important disease affecting the longevity and productivity of peach trees [*Prunus persica* (L.) Batsch] in most peach growing regions of the United States. Peach trees are susceptible to two fungal species, *Leucostoma persoonii* (Nits.) Hoehn.

[anamorph= Leucocytospora leucostoma (Pers.) Hoehn.] and Leucostoma cincta (Pers.:Fr.) Hoehn. [anamorph= Leucocytospora cincta]. Symptoms of the disease may include perennial cankers on the laterals, scaffold branches, and the trunk of the tree, and twig and branch dieback (17). The corresponding reduction in tree vigor may result in tree death (22). Currently, there are no effective chemical or cultural controls for peach canker.

A potential biological control for Cytospora canker is through the use of hypovirulence. Hypovirulent strains of fungi contain cytoplasmically transmissible determinants which reduce the pathogenicity of virulent wild-type strains (23). Double-standed RNA (dsRNA) has been assumed to be responsible for the hypovirulence and natural recovery phenomena of chestnut blight caused by Cryphonectria parasitica (Murr.) Barr (11).

Low virulence has been associated with the presence of dsRNA in an isolate of L. persoonii (13). The potential for biological control of Cytospora canker of peach using hypovirulence may be dependent on transmission of the dsRNA to virulent Leucostoma isolates through hyphal anastomosis. Separating isolates of L. persoonii into vegetatively compatible (vc) groups would aid studies of transmission of dsRNA between two isolates. Identifying the frequency of different vc groups in nature would assist in ascertaining the potential spread of the dsRNA within the population of the pathogen.

The study of the frequency of vc groups in an ascocarp, on a tree, on nearby trees, or throughout an orchard or geographical area might provide valuable information on the sexuality of the pathogen and on the epidemiology of the disease. For example, one vc group in an ascocarp

would indicate a homothallic (homomictic) sexuality whereas several groups would be indicative of outcrossing or heterothallism (dimixis). If dimictic, the frequency of vc groups in a tree and nearby trees might provide evidence indicating whether disease is spreading primarily through infection by conidia or ascospores. The frequency of vc groups within an orchard or a geographical area might provide an estimation of the frequency of sexual recombination in that region.

The objective of this research was to develop methods to first examine vegetative compatibility in L. persoonii and then to look at populations of L. persoonii within certain peach orchards. The sexuality of the fungus is revealed and the implications of the frequency of vc groups is discussed in reference to both the transmission of dsRNA mediated hypovirulence and the epidemiology of Cytospora canker of peach.

MATERIALS AND METHODS

Isolates

The isolates used in this study were obtained from cankers on 8 to 9-yr-old peach trees in two orchards in Clarksville, Michigan. Twenty four trees from two rows were sampled in one orchard (orchard A) and 42 trees from five rows were sampled in a second orchard (orchard B). One canker was sampled from each tree and multiple cankers were sampled from eight trees. Excised pieces (2 x 2 cm) of bark and wood from the margins of cankers were sterilized by soaking in a solution of 0.5% sodium hypochlorite for 3 min and then blotting dry between sterile paper towels. The excised pieces were embedded in petri dishes containing Leonian's malt agar (LMA)(16) and incubated at room

temperature (20-24 C) for 4-6 days. Isolations were maintained and stored on LMA. Species determinations of the Clarksville isolates were made based on colony color, morphology, pycnidial size, and maximum temperature for growth (13).

For ascospore isolations, peach branch segments containing sexual fruiting bodies were collected by Tyre Proffer in a Michigan State University orchard and by Allan Biggs in orchards in Ontario, Canada. Perithecia were dissected from stroma on surface disinfested stems with a sterile scalpel. Perithecia were pushed and rolled over solidified water agar to remove conidia from the surface of the fruiting bodies. After slicing open the perithecia, ascospores were dispersed in 1 ml of sterile distilled water with a hand-operated tissue homogenator. The slurry was filtered through a double layer of 15 um nylon mesh filter (Tetko, Inc., Elmsford, NY) and then diluted to 10^2 ascospores per milliliter. One-tenth ml of the ascospore solution was streaked across the medium surface on each petri plate containing LMA. Plates were incubated 24-36 hr at room temperature. Single germinated ascospores were isolated with the aid of a dissecting microscope and sterile, drawn glass needles, and were transferred to slants containing LMA. Single conidia were isolated from a pycnidium in the same manner.

Determination of vegetative compatibility

Cultures were grown for 4 days on LMA under fluorescent lights (General Electric 20W cool-white lamps) at 20-24 C. Plugs (4 mm diameter) from colony margins of the cultures were paired on various media to determine the best medium for detecting the mycelial interaction zones (barrages) characteristic of vegetative incompatibility reactions (2,21). Plugs were placed 1 cm apart from each

other in 100 x 15 mm petri plates such that 21 plugs were on each plate (19). Each isolate was paired with itself and with each of the other isolates. The pairing plates were incubated in the dark at room temperature for 10-20 days and then, based on the absence or presence of barrage zones (21) between isolates, rated as compatible or incompatible respectively. The media tested were Difco potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI), acidified PDA, PDA plus 1% activated charcoal (neutralized activated charcoal, Sigma Chemical Co., St. Louis), 2% water agar, Endothia complete agar (9), Leonian's malt agar (LMA), Difco corn meal agar, Difco oatmeal agar, oatmeal agar (10), Clarified oatmeal agar, CV agar, V-8 juice agar (18), 1.25% malt extract agar, and Neurospora synthetic crossing medium (25). Clarified oatmeal agar was made as follows: 75 g of oatmeal in 1 L of water was autoclaved for 5 min., blended in a waring blender for 5 min, and poured through two layers of cheese cloth. The resulting liquid was centrifuged at 11,000 rpm for 30 min and 200 ml of the supernatant was diluted to 1 L with water, 1 ml of a vitamin stock solution (22) and 20 g agar were added and the medium was autoclaved for 30 min. CV agar consisted of 10 g maltose, 1.2 g KH_2PO_4, 0.6 g $MgSO_4 \cdot 7H_2O$, 1 ml of vitamin stock solution (1), 1 ml trace element stock solution (1), and 20 g agar per liter. Each compatibility test was conducted at least twice and each treatment plate in a test was replicated twice. All subsequent studies were conducted as above on clarified oatmeal agar.

RESULTS

When pairing L. persoonii isolates on media the most frequent problem encountered was the formation of an irregularly lobate colony margin and

avoidance between colonies. Avoidance was manifested in several ways: the hyphae from paired isolates would grow away from each other in opposite directions, the hyphae of the two isolates would meet and grow around each other with no mergence, or the colonies would cease growth before hyphae made contact. L. persoonii grew sporadically or not at all on 2% water agar which was used as the preconditioning step for vc pairing in Leucostoma kunzei (19). Preconditioning was not necessary with L. persoonii.

Isolates grew with uniformly radial margins, merged, and exhibited no immediate antagonism only on the media containing oatmeal. In 7-16 days some adjacent colonies formed dark brown or black lines (barrage zones) at points of contact (Fig. 1). In 30-35 days pycnidia formed along the barrage line. Such pairings were considered vegetatively incompatible. Pairings were considered vegetatively compatible when merging isolates formed no barrage zone.

Growth and interactions on clarified oatmeal agar were comparable to oatmeal agar. The clarified medium was preferred for testing compatibility because the clarity allowed easier recognition of the barrage reaction (Fig. 1). Oatmeal agar and clarified oatmeal agar were prepared fresh because Difco oatmeal agar gave inconsistant results. Results on oatmeal containing media were variable if media were poured too deeply (>45 ml in 100 x 15 petri plates) or when plates were not incubated in the dark. Light caused the hyphae of L. persoonii to pigment, and mask barrage zones.

Sexuality of L. persoonii

All conidial isolates derived from a given pycnidium were vegetatively compatible and did not form barrage zones when paired.

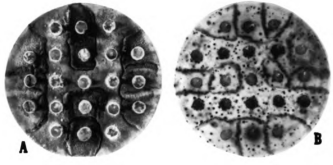

Figure 1. Comparison of the clarity of reaction lines (barrage zones) formed at the line of contact between 15-day-old incompatible colonies of Leucostoma persoonii. Pairings made on (A) oatmeal agar and (B) clarified oatmeal agar.

Whereas, single ascospore isolates from a given perithecium often exhibited barrage zones. Numerous (greater than six) vc groups were present in a perithecium. This indicates that alleles at several loci control vegetative compatibility and that outbreeding has occurred. Thus, L. persoonii is most likely a heterothallic (dimictic) pyrenomycete. Production of the perithecium is necessary to confirm sexuality. This, however, has not yet been achieved.

Distribution of vc groups

In orchard A, 13 vc groups were identified among 24 isolates sampled from 24 trees in two rows. Groups 1 and 2 contained four isolates each, and groups 3 and 4 contained three isolates each. In some instances adjacent trees were infected with strains from the same vc group but more frequent were adjacent trees with cankers caused by isolates in different vc groups (Fig. 2).

In orchard B, 23 vc groups were identified among 65 isolates from cankers on 42 trees in five rows. Several vc groups occurred with relative frequency in the orchard; Vc groups 9, 12, 23, 10, and 11 were recovered from 10, 9, 6, 5, and 4 trees respectively. Other vc groups were represented by a single isolate (groups 1, 3, 5-8, 14, 15, 17-22). Spatial distribution of vc groups in orchard B did not appear aggregated or clustered around foci (Fig. 3., eg., note vc groups 9, 11, and 12).

In orchard B, isolates taken from two or more cankers within a tree generally differed in vc grouping (Fig. 3). In one tree (Fig. 3., row 1 tree 12) five isolates, each from a different canker, belonged to different vc groups, whereas, in a second tree (row 5 tree 6), isolates from six cankers all belonged to the same vc group. Isolates from four of five cankers in each of two trees (row 4 tree 4 and row 5 tree 14)

were different vc grouping. The remaining four trees that were sampled contained two to three cankers each, and all cankers within a tree were found to be caused by isolates from different vc groups.

Orchard A and orchard B were adjacent, separated by a one lane road. Three isolates in orchard A were vegetatively compatible with four isolates in orchard B, however, no other vc group was present in both orchards.

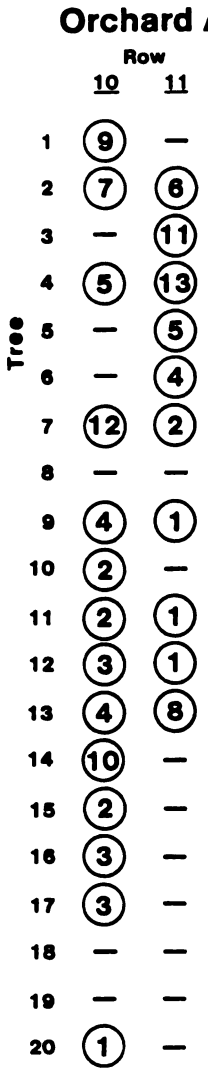

Figure 2. Two rows of peach trees in orchard A. Numbers identify the vegetative compatibility groups of isolates of Leucostoma persoonii isolated from a canker in each tree.

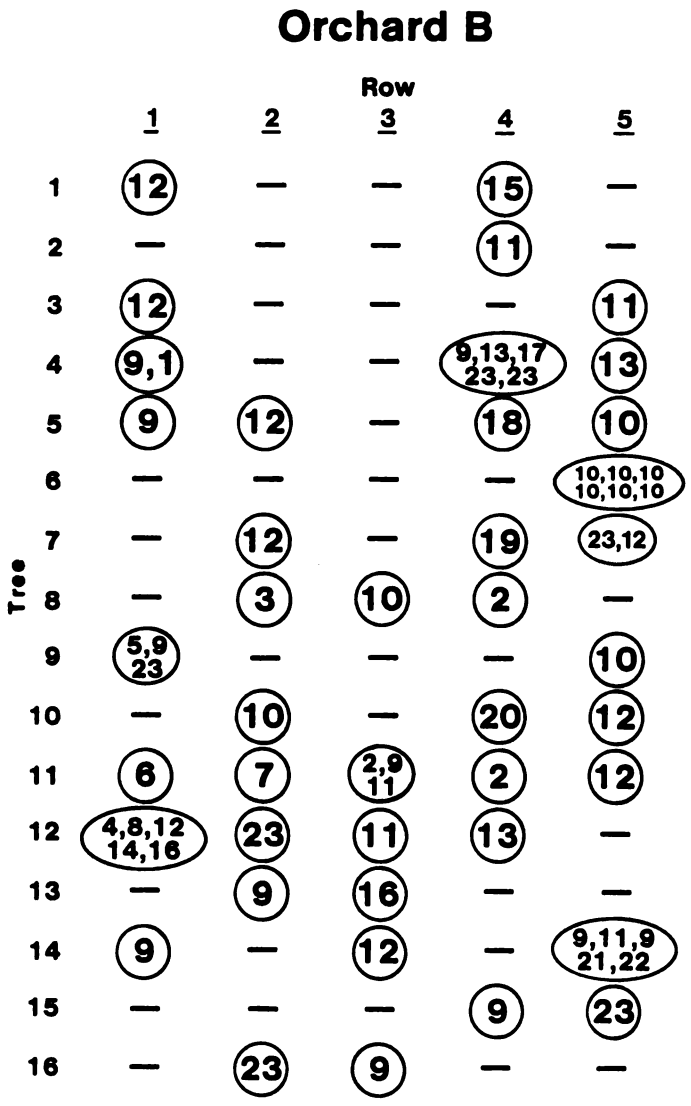

Figure 3. Spatial arrangement of trees and vegetative compatibility groups of 65 isolates of Leucostoma persoonii isolated from 42 trees in orchard B.

DISCUSSION

The difficulties encountered when pairing isolates on media other than oatmeal agar were avoidance, irregular radial growth, and growth inhibition. We initially hypothesized that these growth patterns might be due to vitamin deficiencies in the media. However, pairings on media containing all common vitamins (including choline, myo-inositol, thiamine, and biotin which are reported to be growth requirements of some isolates of L. persoonii (17)) did not significantly alter the growth characteristics. We also hypothesized that avoidance and growth inhibition might be due to excretion of toxic metabolites in the media during growth. However, the addition of activated charcoal to the media to absorb waste products did not improve growth. Further experiments will be required to achieve an understanding of the unique characteristics of oatmeal media in inducing mycelial reactions.

Based on the number of vc groups found in this study, there appears to be high diversity in the L. persoonii population, even within an orchard. Isolates within one tree and between adjacent trees characteristically differ in vc grouping. The results indicate that a reappraisal of the prevailing view that rain spread conidia are the effective infective propagules is warranted (7,26). The great number of vc groups identifiable in close proximity to one another in the orchard suggest a hypothesis that the primary propagules of infection might be ascospores. However, if conidia are spread long distances by wind, a preponderance of vc groups similarly might result. Conidia are generally believed to spread infection because masses of conidia are present exuding from very numerous pycnidia on most cankers. Also, the conidia are infective when trees are wound inoculated with pure suspensions of

conidia in water (13,20). The alternative infective propagule, the ascospore, is rare or at least the ascocarp is generally rarely seen by investigators and, when found, occurs sparsely in comparison to pycnidia. It is our interpretation that the preponderance of vc groups in an orchard is most likely due to ascospore dissemination of L. persoonii but further research is needed on propagule dissemination to clarify the roles of conidia and ascospores.

The lack of occurrence of common vc groups in isolates from adjacent trees does not support prevailing views on the primary importance of infected nursery seedlings serving as foci of infection in newly established orchards (24). Conidia forming on an infected seedling adjacent to disease-free plants should spread the disease to eventually produce an aggregation of trees with cankers caused by isolates of a common vc group. Such a clustered pattern of vc groups within an orchard was not seen in our studies. Therefore, the initial infective propagules might more likely be air blown ascospores or conidia disseminated from other orchards. The orchard in study, however, had been established for 9 years. We have planted new orchards to monitor spread of infection from nursery seedlings.

Bertrand and English (7) suggested conidia would be the major or only inoculum in orchards where standard practices of pruning out diseased branches are followed because perithecia are formed on branches 2 years after branch death. In Michigan we found the perfect state on living scaffold branches. Both the frequency and site of canker formation in Michigan thus precludes effective pruning. Thus, ascospores might be important propagules even in well pruned orchards.

The collections of the sexual spores from perithecia on cankers and

the pairing of the spores from a perithecium on oatmeal agar uncovered the many vc groups segregating during recombination. The results confirm the outcrossing behavior of L. persoonii and reveal that multiple loci control vc compatibility. Apparently, L. persoonii is heterothallic but an alternative possibility is that, like C. parasitica, it may be a homothallic fungus that has the capacity to out breed (2), and readily does so. Leonian (16) mentioned the formation of a sexual state in culture by an isolate of L. persoonii thus indicating the species might be homothallic. However, fructification in culture has not been confirmed by subsequent researchers and has not been repeatable in our laboratory.

It is hypothesized that a natural method of virus transmission in fungi is through hyphal anastomosis and heterokaryon formation (14). Vegetative compatibility or the ability of fungal mycelia to fuse and establish cytoplasmic contact is important in determining the spread of cytoplasmic virus-like infections in a fungal population (8). Hyphal anastomosis is controlled by vegetative compatibility (vc) genes (2,4,8). It is suggested that incompatibility is a cellular defense against genetic infection and might serve to protect mycelia from invasion of viruses and other suppressive cytoplasmic determinants following hyphal anastomosis (8). Vegetative incompatibility may reduce the effectiveness of viral transfer and biological control of diseases in hypovirulent systems (2,4,6). For instance, biological control of chestnut blight in Europe has been successful (11) , however, there is no evidence that hypovirulence is spreading measurably in the United States (6). Isolates of C. parasitica (a homothallic Pyrenomycete) from France are in a single vc group and in Europe only a few groups exist

(12). In the United States, 35 vc groups have been reported in Connecticut alone (5). The proliferation of vc groups is the probable factor limiting establishment of biological control in Connecticut, in contrast to Europe where there is little diversity (6). Incompatibility is a disadvantage for the spread of dsRNA associated hypovirulence in a homothallic fungus. It is encouraging, however, that some vegetatively incompatible pairings of C. parasitica allow the transfer of hypovirulence determinants (3). This phenomenon termed hypovirulence conversion compatibility is less stringent than vegetative compatibility (2,6).

The influence of vc groups on the spread of dsRNA and associated virus particles can only be hypothesized at this stage of our understanding of hypovirulence in L. persoonii. However, it is probable that the multiplicity of vc groups will slow the natural spread of hypovirulence (3). L. persoonii may differ from C. parasitica in being heterothallic. Heterothallism might favor transmission if dsRNA or virions can be transmitted during mating . The virulence reducing factors in our studies of L. persoonii (13) are dsRNA and virus-like particles (VLP's). The spread of VLP's, evidently constructed with a capsid in order to survive and infect outside the cytoplasm, might have a mechanism to overcome the barrier to hyphal anastomosis and plasmogamy imposed by the vc or sexual compatibility systems. L. persoonii and its virulence reducing dsRNA and VLP's might afford a good model system for research exploring the factors controlling transmission of hypovirulence, dsRNA, and virions in a frequently outcrossing (probably heterothallic) ascomycete with multiple loci controlling vc compatibility.

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SECTION III

Evaluating Virulence in *Leucostoma* for Screening Peach Germplasm for Resistance to Cytospora Canker

Evaluating Virulence in *Leucostoma* for Screening Peach Germplasm for Resistance to Cytospora Canker

ABSTRACT

A wide range in virulence was found among 24 isolates of *Leucostoma persoonii* and *Leucostoma cincta* after inoculation of each isolate on one branch per tree in ten peach trees (*Prunus persica* (L.) Batsch cv. 'Garnet Beauty'). The most reliable method for consistantly obtaining successful infection of peach with *Leucostoma* spp. was the combination of wounding to xylem depth, injuring to surrounding tissues with freezing, inoculating, and wrapping the wound in parafilm. An optimal experimental design for detecting differences in virulence among isolates of *Leucostoma* spp., and differences in tolerance of peach to Cytospora canker, was developed based on measurement of variance in susceptibility of inoculated trees and branches. Inoculating more than one branch per tree did not contribute to reducing experimental error but increasing the number of trees inoculated (one branch/tree) to greater than four trees significantly reduced the statistical variance. Inoculating six to nine trees, one branch/tree, gave high precision in the detection of differences in virulence or tolerance while utilizing few trees and reducing labor. Criteria for differentiating *L. cincta* and *L. persoonii* in culture was examined and a system based on the colony margin, size of pycnidia, maximum temperature for growth, and colony color was found to be suitable.

Cytospora canker of peach caused by Leucostoma cincta (Fr.) Hoehn. [Imperfect state, Leucocytospora cincta (Sacc.) Hoehn.] and Leucostoma persoonii (Nits.) Hoehn. [imperfect state, Leucocytospora leucostoma (Pers.) Hoehn.] is a destructive disease of peach in Michigan (19). The disease is often the limiting factor in peach production as well as a deterrent to replanting and establishment of new orchards (24). Cytospora canker is also an important disease of prune, apricot, cherry, and plum (8,17). The disease is characterized by premature leaf senescence, twig and branch dieback, and extensive perennial cankers on the trunk, branches, and scaffold limbs (16,20,35). Cytospora canker is difficult to control. Once a tree is infected, the disease can not be controlled using fungicides (18) and pruning out the infection is not always practicle. All of the currently grown peach cultivars are susceptible to Cytospora canker (6,10).

The most advantageous means of controlling Cytospora canker of peach would be the introduction of horticulturally acceptable disease resistant cultivars. Breeding programs have been initiated to develop canker resistant varieties (24,36) but no germplasm with suitable disease tolerance has been identified (10,24,28). Recently, an effort has been initiated in the Department of Horticulture at Michigan State University to identify genetic resistance to Cytospora canker in a brood-based population of Prunus persica (L.) Batsch. Essential needs of the breeding program include the means for consistant measurement of genotypic variation in host response to inoculation with Leucostoma and measurement of the relative virulence of the isolates used in screening the germplasm for resistance to Cytospora canker.

To effectively determine genotype variation in susceptibility it is

desirable to know approximately how many branches in a tree or how many trees of one genotype need to be inoculated (experimental size) to determine real differential responses. Additionally, such information is needed to determine the relative virulence of the fungal isolates used in the screening. Others have reported differences in peach varietal resistance to cankers and differences in isolate virulence (3,17,36,38). However, variation due to sampling error (canker size on inoculated branches of a tree) and replication error (canker size on different inoculated trees of one genotype) was not reported. Generally, four or fewer trees of one cultivar were inoculated at numerous loci or branches to determine cultivar or isolate responses (3,17,29,36,38). One concern in inoculating several loci on a few trees is that it has been demonstrated that differences in host vigor (7) and in environmental stresses on hosts (3,26) affect susceptibility of individual trees to Cytospora canker. For example, variations in the clay content of soils within one orchard can account for 88% of the variation in susceptibility to Cytospora canker among prune trees (1). Also, trees in an orchard are likely to be individually subjected to different severities of postharvest moisture stresses, scale injury, and nematode infections which influence relative susceptibility to Cytospora canker (2,13,29). Thus, variation among trees of one genotype might mask differences between cultivars or fungal isolates if inadequate replications are tested. Another concern is that branches of different diameter or wood age might similarily increase variation and hinder accurate determination of cultivar response or isolate virulence.

A reliable method for testing differences in virulence among isolates of Leucostoma might provide information on disease resistance by

influencing the experimental designs for peach breeding programs. It could also be important for clarifying the relative importance of host and pathogen variation. Additionally, it is important to determine the relative virulence of isolates of L. persoonii and L. cincta since they have been reported to differ greatly. In one study (38) two of ten isolates of L. persoonii on peach were avirulent and in a separate study (17) one of four isolates of L. cincta were avirulent. When seventy-six isolates (mostly L. persoonii) were inoculated on french prune, two isolates produced cankers greater than twice the size formed by 70% Of the remaining isolates (3). L. persoonii has been reported to be of low pathogenicity compared to L. cincta on peach (19,37), however, in other research L. persoonii was more virulent in warm weather (mean temperature greater than 16 C) and L. cincta more virulent in cool weather (1,19,36).

The objective of this study was to determine the relative virulence of a number of isolates of L. persoonii and L. cincta on a uniform source of germplasm. The contributions to variation in the experimental results are calculated for the error in sampling and the error in replications. From this data we develop recommendations for approximating the optimal experiment size and design for detecting a desired percentage difference in virulence among isolates of the pathogen while minimizing expenditure of plants and labor. From this information we extrapolate the experimental size and design required to detect a desired amount of cultivar resistance (expressed as percentage reduction in canker size compared to a susceptible cultivar) with a selected level of assurance that the detected resistance is genuine. The evaluations of peach cultivars for resistance to Cytospora canker

reported in the literature are discussed in relation to experimental design. Criteria for characterizing isolates of Leucostoma spp. from peach are discussed and methods of inoculating the trees are examined.

MATERIALS AND METHODS

Twenty-four isolates were obtained from Michigan, North Carolina, and West Virginia. Cultures were grown on Leonian's malt agar (LMA)(25) for 5 days at 25 C prior to use in inoculation experiments.

Designation of isolates as L. cincta or L. persoonii was based on a comparison of their cultural characteristics after 30 days growth at 25 C on LMA to descriptions by Hildebrand (19) and Willison (37). Characteristics that were examined included (i) whether the colony margin was lobate with restricted growth, or uniformily radial growing to fill the culture dish, (ii) whether pycnidia were small (<1 mm) or large (1-3 mm), (iii) the colony color, and (iv) the ability to grow at 37 C. In addition, the cultural characteristics of the 24 isolates were compared to the cultural characteristics of single ascospore isolates of L. persoonii derived from perithecia.

Variations in the inoculation method were tested to determine the optimum procedure for obtaining consistant infection. In this test the trunk of 62 2-year-old trees were mechanically wounded with a hand held stapling gun followed either with or without the aerosol freeze treatment. The wounds were then wrapped in either parafilm or cloth tape (which fell off after 2 weeks.) The freeze spray and wrap treatments were tested in all combinations and replicated three times.

The wound freeze inoculation method of Scorza and Pusey (32) was used in the isolate virulence study. The inoculation site was first cleaned

with gauze soaked in 95% ETOH. The trees were wounded down to xylem depth using an empty hand held stapling gun. The resulting 12 x 2 mm wound was frozen by spraying with a commercial aerosol cryogen (100% Dichlorofluoromethane, Chemtronics, Inc., Hauppauge, NY) for five seconds at a distance of approximately 15 cm. Inoculum consisted of a 5 mm diameter mycelial plug. The inoculum was placed on the wounded bark and wrapped in parafilm to prevent dessication. Control trees were wounded in an identical manner except a plug of LMA was substituted for the mycelial plug. Ten vigorous 8-yr-old peach trees (P. persica cv. 'Garnet Beauty') were each inoculated with nine isolates of L. cincta and 15 isolates of L. persoonii in October 1985 (isolate virulence plot). Each of the 24 isolates and a control were placed on each tree and there were ten replications. Each inoculation was made on a separate 2-yr-old branch at a point on the branch measuring 17 mm in diameter. Branches were removed and canker length was measured (length of necrotic area distal to inoculation point) at the time of leaf elongation during the spring following inoculation. Statistical analysis of data included analysis of variance (ANOV) of randomized complete block design with trees as blocks (replicates). Isolates were ranked according to average canker length using the least significant difference (LSD) at the P= 0.05 level.

An isolate was inoculated into a single tree on 20 separate branches to test for within tree variation (branch variance plot). Branches were measured with a caliper and a portion of the branch with 2-yr-old wood and measuring 17 mm in diameter was inoculated. Isolate NC 9.2 contained double-stranded RNA (dsRNA) and was included to evaluate whether wounded and inoculated branches were successfully colonized by the inoculum or

invaded by wild strains.

Cankered branches that had been inoculated the previous fall with isolate NC 9.2 were removed from the ten trees and brought into the laboratory. Pieces of tissue were removed along the side of the necrotic margin of the canker and soaked in a 0.525% hypochlorite solution for 3 min. The tissue was blotted dry with sterile paper towels and transferred to LMA. The ten resulting cultures were tested for the presence of dsRNA using a modified double cellulose column extraction procedure and electrophoresing on a 5% polyacrylamide slab gel (11,14).

The following methods were used to develop an experimental design providing the most efficient determination of the relative virulence of a pathogen or the relative resistance of a peach cultivar. The experimental design requiring the least labor and providing the greatest reduction in sampling and experimental errors was determined using a method from Sokal and Rohlf (33). The sample variance (MSS) from the ANOV of data of the branch variance plot and the mean square error (MSE) from the ANOV of data of the virulence plot were used to determine the minimal experiment size and optimal design for reducing component variance. This was achieved by the construction of two tables, one of the total number of observations or inoculations required for various experimental designs (Table 3A), and a second table (Table 3B) of the sampling variance expected for each of the experimental designs in Table 3A. The values in Table 3B are calculated using the following formulae (33).

Sampling error = MSS

$$\textbf{Replication error} = \frac{\text{MSE} - \text{MSS}}{\text{(no. of branches inoculated per tree)}}$$

Sampling variance=

$$\frac{\text{sampling error}}{\text{(no. of trees inoculated)} \times \text{(no. of branches inoculated/tree)}} + \frac{\text{replication error}}{\text{(no. of trees inoculated)}}$$

The experimental design is chosen from Table 3 A & B with the number of trees to be inoculated. When limiting the size of the experiment to a certain number of trees an estimation can be made of the ability of the experiment to detect differences in isolate virulence or cultivar resistance.

Differences in isolate virulence or cultivar resistance (expressed as a percent difference in canker length following inoculation with a fungal isolate) are detectable at a chosen magnitude with a chosen assurance that the detected differences are true. Using MSE as an estimate of the variance, choosing the assurance level desired at 90% (0.90) and the level of significance to be used in the actual experiment at P= 0.05, the following formula and a table of t values are used to estimate detectable differences (34):

$$\text{Percent detectable difference in isolate virulence or cultivar resistance (canker length)} = \frac{2(\text{MSE})\ (t_0 - t1)^2}{\text{number of trees inoculated per isolate or per cultivar}}$$

t_0 = t table (two tailed) value for P= 0.05 using degrees of freedom associated with MSE.

t_1 = t table (two tailed) value for P = 2(1 - 0.90) = 0.20. [0.90 is the assurance level chosen] using degrees of freedom associated with MSE.

RESULTS

Among the 24 isolates tested, 15 were identified as L. persoonii and nine as L. cincta. All isolates of L. persoonii had a lobate, restricted colony margin, an olivaceous or darker colony color after 30 days growth on LMA, and small (<1 mm) pycnidia (Fig 1). Comparisons with cultures derived from single ascospores from an ascus identified as L. persoonii aided our identification of L. persoonii by cultural characteristics but the sexual state of L. cincta was not available. The eight L. cincta isolates were more variable (Table 1). Six of the isolates had characteristic, uniformally radial margins, large pycnidia (1-3 mm) often having a cottony appearance, and an olivaceous to buff colony color (Fig 1). The other two isolates had uniformally radial margins but lacked the characteristic pycnidia. The Fenn isolate (Table 1) had small (<1 mm) pycnidia and isolate F-46 had no observable pycnidia after 30 days. These two isolates were identified as L. cincta based on colony color, margin, and inability to grow at 37 C. Isolates designated as L. cincta failed to grow at 37 C whereas those designated as L. persoonii

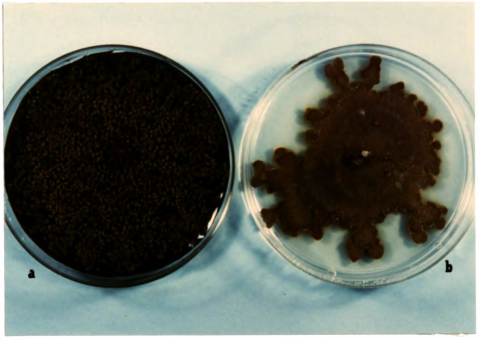

Figure 1. Colony characteristics of a) L. cincta with a uniformally radial colony margin and large (1-3 mm) pycnidia and b) L. persoonii with a lobate colony margin and small (<1 mm) pycnidia.

TABLE 1. Isolates of Leucostoma spp.: Origin, colony characteristics, author designated species, and source or reference

Isolate	Origin[a]	Colony color[b]	Colony[c] Margin	Pycnidium[d] Size	Growth at 37 C	Designated[e] Species	Source or Reference
NC8.2	NC	Olivaceous buff	F	L	-	c	(12)
NC9.2	NC	Olivaceous	F	L	-	c	(12)
NC14.2	NC	Olivaceous	L	S	+	p	(12)
NC14.1	NC	Greenish olivaceous	F	L	-	c	(12)
C-j-1	WV	Greenish olivaceous	L	S	+	p	ATCC 58386[f]
C-MI-5	WV	Olivaceous	L	S	+	p	C.L. Wilson
C-jm_18	WV	Olivaceous	L	S	+	p	C.L. Wilson
C-S-20	WV	Isabelline	L	S	+	p	C.L. Wilson
H10.9	MI	Greenish olivaceous	L	S	+	p	S.A. Hammar
H9.11	MI	Iron grey	L	S	+	p	S.A. Hammar
H9.5	MI	Greenish olivaceous	L	S	+	p	S.A. Hammar
H8,15	MI	Olivaceous	L	S	+	p	S.A. Hammar
H7.13	MI	Fuscous black	L	S	+	p	S.A. Hammar
G-3	MI	Dk. Mouse grey	F	L	+	c	S.A. Hammar
MR-1	MI	Olivaceous	L	S	+	p	S.A. Hammar
F-4	MI	Buff	F	S	-	c	S.A. Hammar
P-1	PA	Grey olivaceous	F	L	+	p	B.A. Snyder
11.11	MI	Greenish olivaceous	L	S	+	p	S.A. Hammar
10.8	MI	Olivaceous	L	S	+	p	S.A. Hammar
C1-5	CA	Greenish olivaceous	F	L	-	c	J.M. Ogawa
Ma-4	CA	Olivaceous	L	S	+	p	J.M. Ogawa
I-80	CA	Olivaceous	F	L	ND	c	J.M. Ogawa
F-45	CA	Olivaceous	F	L	-	c	oc1 (3)
F-46	MI	Honey	F	ND	-	c	F-40 (3)

a/ Initials of state where isolate originated. All except F-45 and F-46 were from peach

b/ Color key used: Rayner's Colour Chart (31)

c/ F= full uniformally radial margin, L= lobate margin

d/ S= small (<1 mm diameter) pycnidia, L= large (1-3 mm diameter) pycnidia

e/ p= L. persoonii, c= L. cincta

f/ American Type Culture Collection

grew at 37 C or greater temperatures.

The best inoculation method for the virulence screen was determined in a preliminary test. The largest and most consistant cankers were obtained using Scorza's method (32). The most effective treatment consisted of the freeze spray in combination with the parafilm wrap (Table 2). Infection was not as consistant when the freeze spray was eliminated or when the wound was wrapped with tape instead of parafilm. Therefore, we used the freeze spray in combination with the parafilm wrap for all subsequent inoculation experiments.

In the virulence screen all inoculations resulted in canker formation except the controls. The results show a wide range in virulence of the isolates. The virulence of isolates [based on mean canker length of ten replications (trees)] are listed in order of decreasing virulence (Table 3A). Isolate I-80 from California is the least virulent with a mean canker length of 4.02 cm, while Riley, a Michigan isolate, is the most virulent with a mean canker length of 11.0 cm. The geographic origin of an isolate was not a factor in its virulence. For instance, the four most virulent isolates were isolated from Michigan, North Carolina, and California, while the four least virulent isolates were isolated in Michigan and California. The relative virulence of L. cincta compared to L. persoonii was not appreciably different and L. cincta isolates were dispersed evenly among the L. persoonii isolates in the virulence ranking (Table 3A). Individual trees of a common cultivar (scion germplasm) varied significantly in their responses to the pathogen. When the variation between trees was removed by the two-way ANOV the different Leucostoma isolates were revealed to have significant differences in virulence (Table 3B). Branches within a

Table 2. Analysis of variance table for optimum inoculation procedure[a] of Leucostoma isolates in peach

	Degrees of freedom	Mean square	F value
Isolate[b]	2	25.154	12.58**
Freeze spray alone	1	.302	.15 ns[c]
Isolate x freeze spray	2	.863	.43 ns
parafilm wrap alone	1	.667	.33 ns
Isolate x parafilm wrap	2	.174	.09 ns
freeze spray x parafilm wrap	1	5.523	2.76**
Isolate x freeze spray x parafilm wrap	2	9.730	4.87**
Error	24	2.000	

a/2-yr-old trees were mechanically wounded with a stapling gun followed either with or without an aerosol freeze treatment. The wounds were wrapped in tape (which fell off and exposed the wound within 2 wk) or parafilm.
b/Two isolates were inoculated three times for each treatment. The two isolates were significantly different in their virulence.
c/ns = not significant

Table 3A. Virulence of isolates of Leucostoma spp. on 10 trees of Garnet Beauty peach ranked in order of decreasing canker length

Origin	Isolate	Species designation	Average canker length (cm)[b]	
MI[a]	R-1	L. persoonii	11.00	A[c]
NC	8.2	L. cincta	9.98	AB
CA	F-46	L. cincta	9.83	ABC
CA	CL-5	L. cincta	8.40	BCD
MI	10.8	L. persoonii	7.78	BCDE
MI	H9.5	L. persoonii	7.64	BCDEF
WV	C-j-1	L. persoonii	7.50	CDEF
WV	C-S-20	L. persoonii	7.24	DEFG
MI	G-3	L. cincta	7.08	DEFGH
MI	F-4	L. cincta	6.95	DEFGHI
CA	Ma-4	L. persoonii	6.49	DEFGHIJ
WV	C-MI-5	L. persoonii	6.48	DEFGHIJ
PA	P-1	L. persoonii	6.06	DEFGHIJK
WV	C-jm-18	L. persoonii	5.94	EFGHIJK
MI	H10,9	L. persoonii	5.46	EFGHIJK
NC	9.2	L. cincta	5.34	FGHIJK
NC	14.1	L. cincta	5.11	GHIJK
CA	F-45	L. cincta	4.92	GHIJK
NC	14.4A	L. persoonii	4.81	HIJK
MI	H7.13	L. persoonii	4.79	HIJK
MI	H6,15	L. persoonii	4.75	HIJK
MI	H9.11	L. persoonii	4.71	IJK
MI	11,11	L. persoonii	4.18	JK
CA	I-80	L. cincta	4.02	K
	Control		0.5	L

a/Initials of state where strains originated
b/Length of canker distal to the inoculation point
c/Means followed by the same letters are not significantly different by the least significant difference test (LSD) (P=0.05)

B. Randomized Complete Block Design

	Degrees of freedom	Mean square	F significant at P less than:
Total	228		
Peach tree (reps)	9	53.015	.000
Isolates	23	36.033	.000
Error	196	10.116	

single tree did not vary significantly in susceptibility. The standard deviation (S) for branches within a single tree was .453 whereas the standard deviation between trees was 3.18. This is strong indication that there is considerably more variation between trees than within a tree.

Cultures reisolated from branches on ten trees inoculated with strain NC 9.2 exhibited the typical dsRNA banding pattern of NC 9.2 in all instances. This provided assurance that cankers were caused by the strains used in the tests.

The ANOV of the data from the virulence plot and the branch variance plot permit the calculations in Table 4A & B. Examination of Table 4A & B reveal that increasing the number of branches inoculated per tree has an insignificant effect on reducing the sample variance. Increasing the number of trees inoculated (one branch per tree) greatly reduces the sample variance. However, increasing the number of trees inoculated above nine gives minor reductions in variance that perhaps are not worth the increased labor or plant material. From our data, the experimental design of choice for the most efficient determination of the relative virulence of an isolate of the pathogen or the relative resistance of a peach cultivar would be to inoculate one branch per tree and use between six and nine trees to screen a cultivar or a fungal isolate.

Replicating inoculations on ten trees detects differences as low as a 4.6% in virulence (canker length) at $P = 0.05$ and with an assurance of 90% that the detectable difference is true. This can be interpreted as an ability to detect a 4.6% difference in resistance to canker in peach cultivars when ten trees per cultivar are inoculated, one branch per tree.

A. Total observations (inoculations)

Number of branches inoculated per tree	Number of trees inoculated					
	1	3	6	9	12	15
1	1	3	6	9	12	15
2	2	6	12	18	24	30
3	3	9	18	27	36	45
4	4	12	24	36	48	60
5	5	15	30	45	60	75
10	10	30	60	90	120	150
20	20	60	120	180	240	300

B. Sampling variances

Number of branches inoculated per tree	Number of trees inoculated					
	1	3	6	9	12	15
1	10.12	3.37	1.69	1.12	0.84	0.67
2	9.68	3.23	1.61	1.08	0.81	0.65
3	9.54	3.18	1.59	1.07	0.80	0.64
4	9.49	3.15	1.58	1.06	0.79	0.63
5	9.42	3.14	1.57	1.05	0.79	0.63
10	9.3	3.11	1.55	1.04	0.78	0.62
20	9.3	3.10	1.55	1.03	0.77	0.62

Table 4. Choice of experimental design for the most efficient determination of the relative virulence of the pathogen or resistance of a cultivar requiring the least labor and cost (observations of inoculated branches and trees) and providing the most accurate experimental results (greatest reduction in sample variance). A) Total number of observations (or inoculations) required with different inoculation models (experimental designs). B) Sampling variances expected for the different designs in A.

DISCUSSION

The analysis of the plot data reveal the relative importance of sampling error and replication error. Calculations based on the data are used to estimate the optimal experimental size and design for screening isolates of Leucostoma spp. to determine their relative virulence. This experimental design should minimize the sources of experimental variance that would interfere with the precision of screening peach germplasm for resistance to Cytospora canker. Variance due to inoculation of different branches in a tree is essentially of no consequence. Thus, inoculating more than one branch per tree gives no increased precision in differentiating isolate virulence or varietal resistance. Variance due to inoculation of several trees (one branch per tree) of the same germplasm is the major source of experimental error. Increasing the number of plants inoculated greatly increased the precision of the screening tests. The inoculation of more than four trees is necessary to detect the true response of the trees of one germplasm to the virulence of a tested isolate of the pathogen. Under our experimental conditions, the experimental size and design for optimal precision using the least labor and amount of trees of one germplasm will require inoculation of one branch per tree for six to nine trees (Table 4A and B). We believe that the sources of experimental error under these conditions should be similar in other locales. In our plots there are no recognizable environmental factors that might account for the variance seen among individual trees of the same germplasm. Apparently, there is no significant variation between branches of uniform diameter and age on a peach tree in regards to response to pathogenesis.

Assuming that the identified sources of experimental error in our

tests are similar to those of past tests, it becomes difficult to interpret the results of experiments where numerous branches or loci were inoculated on four or fewer trees in screening tests. It is noteworthy that two studies evaluating resistance to Cytospora canker in peach trees (27,32) utilizing numerous replications found only weak or insignificant disease resistance. This is clear evidence that the existing level of Leucostoma resistance in North American germplasm is quite low.

The morphological and pathological characteristics of isolates used in a breeding program need to be thoroughly described prior to screening of germplasm so that species identification will be universally interpretable. Identification of the pathogen to species is not easily achieved with Cytospora canker. Cytospora canker is caused by two species that are considered to be distinct in the north east and north central states, although Lukezic et al. (29) have questioned the validity of separating the two species. The important characteristics for identification are those of the sexual fruiting bodies (teleomorphs) which do not form under laboratory conditions. Because of the relative scarcity of the teleomorph of the two species in nature, L. cincta and L. persoonii are commonly identified by cultural characteristics (19,36). Researchers generally have utilized the cultural characteristics described by Willison (37) as criteria for differentiating L. cincta from L. persoonii. In this study we found the culture photographs and optimum growth temperature studies by Hildebrand (19) to also be useful for distinguishing between the two species. Colony color was the most variable characteristic and was the least important for characterization. Hildebrand found that the maximum growth

temperature for L. cincta was 30 C whereas for L. persoonii it was 39 C. Only isolates designated as L. persoonii (based on lobate colony margin, small pycnidia, and colony color on LMA) were able to grow at 37 C in our studies. Thus, differences in cultural characteristics were further corroborated with differences in maximum temperature allowing growth. Growth at 37 C is apparently a relatively reliable character for distinguishing L. cincta from L. persoonii. Differentiating species by cultural characteristics, however, was dependant on growth on LMA at standard temperature and light conditions because differences in isolates designated as L. cincta and L. persoonii were often indistinct when grown on other media. Kern (23) suggested that the typical specimens of L. cincta and L. persoonii might represent extremes and that intermediate forms were possible. Many of the isolates that we have examined were intermediate in pycnidial size, colony color, colony margin, or temperature maximum. No characteristic was singly reliable in distinguishing cultures of the two species, however, the multiple characteristics used in this study are suggested as useable until isozyme patterns or more precise criteria become available.

Our data show significant variation in virulence among isolates of L. cincta and L. persoonii. We have not found significant numbers of avirulent isolates as reported in other studies (17,38). However, the evident differences in virulence emphasize the importance of obtaining a measurement of the relative virulence of an isolate prior to utilizing it for screening germplasm. We did not observe the virulence differences between L. cincta and L. persoonii as reported in the literature (19,37). Hildebrand (19) noted that L. cincta always caused larger cankers than L. persoonii when trees were inoculated during the cooler

months of the year. Our tests showed an even distribution in virulence between the two species. The cooler temperatures of Michigan winter and early spring did not favor the growth of L. cincta over L. persoonii. Similarily, Dhanvantari (9) found that in trees artificially inoculated in autumn the two species of Leucostoma were equally virulent. The geographic origin of the isolates tested was not correlated with the level of virulence.

Peach trees in the orchard are most susceptible to infection in the early spring following dormancy (4,5,22). Thus, trees should be inoculated in late fall or winter. Differences in methods of inoculating peach trees with Leucostoma spp. can affect the reliability of resistance evaluations. Others have reported that the combination of injury to xylem depth, freeze injury to surrounding tissues, and wrapping the wound in parafilm is most reliable in inducing infection beyond the injured area (32). The importance of freeze injury and the superiority of parafilm wrap to cloth tape for providing a more optimum environment for infection is confirmed in our tests.

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APPENDIX

Isolation of DsRNA

Cultures were grown in complete broth medium without glucose (12) for 10-14 days. After harvesting the mycelial pad was pressed between layers of absorbent paper to remove excess liquid. The fungal tissue (0.5-6.0 g) was frozen in liquid nitrogen and ground in a chilled mortar and pestle with glass beads (490 um). The resulting fine powder was added to centrifuge tubes and combined with 10 ml STE buffer (0.05 M Tris, 0.1 M NaCl, and 0.001 M EDTA, pH 7.0) containing 0.1% bentonite, 1.5 ml 10% sodium dodecyl sulfate, and 15 ml of redistilled phenol saturated with STE buffer. Following centrifugation at 8,000 rpm for 15 minutes, the upper aqueous phase was removed and added to 95% ethanol for a final concentration of 15% ethanol. This was passed through cellulose columns (Whatman CF-11) and washed with 15% ethanol-STE to remove all the species of nucleic acids (ribosomal RNA, transfer RNA, and DNA) except dsRNA and viroid material. The dsRNA was eluted from the cellulose with STE and collected. Ethanol was added to make a final concentration of 15% ethanol-STE. The column procedure was repeated and the solution was stored overnight at -20C. The precipitate that formed was collected by centrifugation and then resuspended in electrophoresis buffer (0.04 M Tris, 0.02 M sodium acetate, 0.001 M EDTA, pH 7.8). The samples were layered on 5% polyacrylamide slab gels (17.5 cm wide x 16 cm long x 1.5 mm thick) and electrophoresed at 40 mA for 12 hr. The gels were placed in a 500 ml ethidium bromide solution (0.05 ug/ml) for 30 minutes followed by destaining for 15 min in H_2O. Banding patterns were recorded

by placing gels on an ultraviolet transilluminator and photographing through a No. 4 yellow filter with Polaroid type 55 film. Some gels were treated with deoxyribonuclease (DNase) and with ribonuclease (RNase) to remove DNA or ssRNA. Gels were placed in a solution of RNase A (Sigma) at a concentration of 50 ug/ml in a 0.3 M NaCl solution for 30 minutes. The gel was stained with ethidium bromide, observed, and then placed in a RNase and water solution to remove all RNA's. If any bands remained after the RNase in water treatment, they were treated with 10 ug/ml DNase I (Sigma) in 5 mM magnesium chloride and stained again with ethidium bromide to determine if the bands were DNA. Bands digested by ribonuclease in water but not by RNase in NaCl were assumed to be dsRNA (13)