Vegetative Compatibility in *Leucostoma persoonii*

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ABSTRACT

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Isolates of *Leucostoma persoonii* were paired on various media to determine a suitable medium for differentiating vegetative compatibility (VC) groupings. Barrage reactions were evident only on oatmeal agar as dark lines with pycnidia forming along the zone of mycelial contact between expanding colonies (vegetatively incompatible reaction). Conidia from a single perithecium were in one VC group (VCG). Ascospore colonies derived from individual pycnidia in several stroma on different branch cankers always segregated into several VCGs per perithecium, 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 an orchard were of numerous VCGs. Isolates from several cankers within one tree and among closely spaced trees usually differed in VC grouping. Spatial clustering of VCGs in an orchard did not appear aggregated. A reappraisal of ascospores as important infective propagules is warranted by the high frequency and dispersed spatial arrangement of VCGs in Michigan peach orchards.

Additional keywords: Cytospora canker, perennial canker, *Prunus persica*, sexuality.

Cytospora canker of peach is an important disease affecting the longevity and productivity of peach trees (*Prunus persica* (L.) Batsch) in northern peach-growing regions of the United States. Peach trees are susceptible to both *Leucostoma persoonii* Höhn. (anamorph = *Leucocytospora leucostoma* (Pers.) Höhn) and *Leucostoma cincta* (Fr.:Fr.) Höhn (anamorph = *Leucocytospora cincta* (Sacc.) Höhn), causal agents of Cytospora canker. Symptoms of the disease include perennial cankers on lateral and scaffold branches and the trunk of the tree, and twig and branch dieback (25). The corresponding reduction in tree vigor

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may result in rapid tree death under conducive environmental conditions (23). 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 that reduce the pathogenicity of virulent wild-type strains (24). Double-stranded RNA (dsRNA) has been assumed to be responsible for the hypovirulence and natural recovery phenomena of chestnut blight caused by *Cryphonectria parasitica* (Murr.) Barr (13).

Low virulence has been associated with the presence of dsRNA in an isolate of *L. persoonii* (14). The potential for biological control of Cytospora canker of peach using hypovirulence may be dependent on transmission of the dsRNA to virulent isolates of *Leucostoma* through hyphal anastomosis. Separating isolates of *L. persoonii* into vegetatively compatible groups (VCGs) would aid studies of transmission of dsRNA between two isolates. Identifying the frequency of different VCGs in nature would assist in ascertaining the potential spread of the dsRNA within the population of the pathogen.

The study of the frequency of VCGs in an ascocarp, on a tree, on nearby trees, or throughout an orchard or geographical area might provide valuable information on the sexual condition of the pathogen and on the epidemiology of the disease. For example, one VCG in an ascocarp would indicate homothallism (homomixis), whereas several VCGs would be indicative of outcrossing or heterothallism (dimixis). If the pathogen is dimictic, the frequency of VCGs in a tree and nearby trees might provide evidence indicating whether disease is spreading primarily through infection by conidia or ascospores. The frequency of VCGs 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 determine if a system of vegetative compatibility occurs in *L. persoonii* and, if so, to employ the methods to estimate the frequency of the VCGs within certain orchards. The implications of the frequency of VCGs are discussed in reference to the sexuality of the fungus, the epidemiology of Cytospora canker of peach, and the transmission of dsRNA-mediated hypovirulence.

MATERIALS AND METHODS

Isolates. The isolates used in the orchard studies were obtained from cankers on 8- to 9-yr-old peach trees in two orchards in Clarksville, MI. 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). Trees were spaced 4 m within and 8.5 m between rows. One canker was sampled from each tree, and multiple cankers were sampled from eight trees. Excised pieces (2 × 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 pieces were embedded in petri dishes containing Leonian's malt agar (LMA) (17) and incubated at room temperature (20–24 C) for 4–6 days. Isolations were maintained and stored on LMA. Species determinations of the canker isolates were made based on colony color, morphology, pycnidial size, maximum temperature for growth, and similarity to ascospore-derived cultures (1).

For ascospore isolations, three peach branch segments containing sexual fruiting bodies were collected in peach orchards: one by Tyre Proffer at the Michigan State University (MSU) Botany Farm (BF), one by Sue Hammar at the MSU horticulture station in Clarksville, MI (HS), and one by Alan Biggs in Vineland, Ont. (VO), Canada. The teleomorphs were determined to be *L. persoonii* based on morphology (16). Stroma on surface-disinfested cankers were dissected horizontally with a sterile scalpel until the perithecia were well exposed. A sterile needle was used to lift an individual perithecium out of a stroma. Several perithecia were removed from each stroma, and several stroma were sampled per canker. The inner matrix of excised perithecia was squash mounted and examined in sterile water on a sterile slide for mature ascospores. Ascospores were washed onto a petri plate containing LMA and spread across the medium surface. 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 cirrus in the same manner. Single ascospore colonies were designated as originating from a specific branch canker, stroma, and perithecium: for example, BF-S1P2-1. The colony BF-S1P2-1 is single ascospore 1 from perithecium 2 (P2) in stroma 1 (S1) on the Botany Farm branch canker (BF). Ten viable ascospores were isolated from each of 9 perithecia (3 stroma) from canker BF, 10 perithecia (3 stroma) from canker HS, and 1 perithecium from VO. Ascospores from a perithecium were paired in all possible combinations. Sixty-seven ascospore colonies from 7 perithecia (3 stroma) from canker HS were paired to determine the total number of VCGs. Ten conidia from each of three pycnidia from canker VO were paired in all possible combinations. The number of VCGs was determined as described below.

Determination of vegetative compatibility. Cultures were grown for 4 days on LMA under fluorescent lights (General Electric 20-W 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 (3,22). Plugs were placed 1 cm apart from each other in 100 × 15 mm petri plates such that 21 plugs were on each plate (20). Each isolate was paired with itself and with each of the other isolates. The pairing plates were incubated in the dark at room temperature (22–26 C) for 10–20 days and then, based on the absence or presence of barrage zones (22) between isolates, rated as compatible or incompatible, respectively. The 14 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, MO), 2% water agar, *Endothia* complete agar (10), LMA, Difco cornmeal agar, Difco oatmeal agar, oatmeal agar (11), clarified oatmeal agar, Czapek's agar amended with 1 ml of a vitamin stock (2), V-8 juice agar (19), 1.25% malt extract agar, and *Neurospora* synthetic crossing medium (26). 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 cheesecloth. The resulting liquid was centrifuged at 11,000 rpm for 30 min, and 250 ml of the supernatant was diluted to 1 L with water. One milliliter of a vitamin stock solution (2) and 20 g of agar were added, and the medium was autoclaved for 30 min. Subsequent studies were conducted as above on clarified oatmeal agar. All compatibility tests were conducted four times, and each treatment plate in a test was replicated twice.

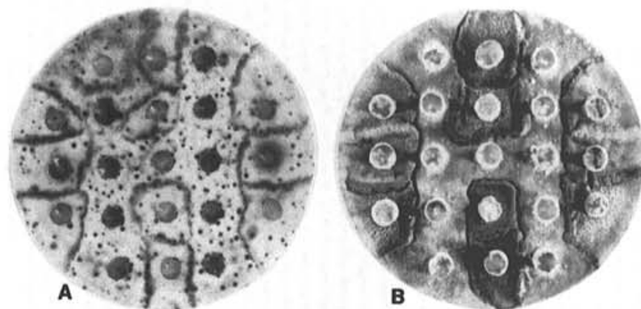

Fig. 1. Comparison of the clarity of reaction lines (barrage zones) formed at the line of contact between vegetatively incompatible pairings of colonies of *Leucostoma persooni* made on: **A,** oatmeal agar, and **B,** clarified oatmeal agar.

RESULTS

When pairing isolates of *L. persoonii* on media, the most frequent problem encountered was the formation of an irregularly lobate colony margin and repulsion between colonies. 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). Pycnidia formed along the barrage line if plates were incubated 30–35 days. Such pairings were considered to be vegetatively incompatible, and when merging isolates formed no barrage zone, they were considered to be vegetatively compatible.

Growth and interactions on clarified oatmeal agar were comparable to those on 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 inconsistent results. Incubation in the dark was necessary because light caused the hyphae of *L. persoonii* to pigment and mask barrage zones.

L. persoonii grew sporadically or not at all on 2% water agar which was used as the preconditioning step for VC pairing in *L. kunzei* (Fr.:Fr.) Munk (20). Preconditioning was not necessary with *L. persoonii*.

Sexuality of *L. persoonii*. All conidial isolates derived from a given pycnidium did not form barrage zones when paired and were vegetatively compatible, whereas single ascospore isolates from a given perithecium often exhibited barrage zones. Ten ascospores from BF-S2P1 paired in all possible combinations yielded 10 VCGs, as did 10 spores from each of the following perithecia: BF-S2P3, BF-S3P2, BF-S3P3, HS-S3P4, and HS-S3P5. Fewer than 10 VCGs/10 spores were present among the other perithecia as follows: BF-S2P7 and BF-S4P2 with 8 VCGs, BF-S2P5 and VO-S1P1 with 7 VCGs, HS-S3P8 and HS-S2P2 with 5 VCGs, HS-S3P1 with 4 VCGs, HS-S2P1 with 3 VCGs, and HS-S1P1 and HS-S3P7 with 2 VCGs. HS-S3P2 yielded 5 VCGs/9 spores, and HS-S1P3 yielded 3 VCGs/8 spores. Sixty-seven single ascospore colonies from 3 stroma and 7 perithecia of the HS canker yielded 12 VCGs in the ratios 30:21:5:2:2:1:1:1:1:1:1:1. In contrast to *L. kunzei*, no multimerge isolates were evident (20).

Distribution of VCGs. In orchard A, 13 VCGs 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 VCG, but more frequently cankers on adjacent trees were caused by isolates in different VCGs (Fig. 2).

Orchard A

	Row 10	Row 11
1	9	—
2	7	6
3	—	11
4	5	13
5	—	5
6	—	4
7	12	2
8	—	—
9	4	1
10	2	—
11	2	1
12	3	1
13	4	8
14	10	—
15	2	—
16	3	—
17	3	—
18	—	—
19	—	—
20	1	—

Fig. 2. Two rows of peach trees in orchard A (24 trees from two rows). Numbers identify the vegetative compatibility groups of isolates of *Leucostoma persoonii* isolated from a canker in each tree. Trees were spaced 4 m within and 8.5 m between rows.

Orchard B

	Row 1	Row 2	Row 3	Row 4	Row 5
1	12	—	—	15	—
2	—	—	—	11	—
3	12	—	—	—	11
4	9,1	—	—	9,13,17,23,23	13
5	9	12	—	18	10
6	—	—	—	—	10,10,10,10,10,10
7	—	12	—	19	23,12
8	—	3	10	2	—
9	5,9,23	—	—	—	10
10	—	10	—	20	12
11	6	7	2,9,11	2	12
12	4,8,12,14,16	23	11	13	—
13	—	9	16	—	—
14	9	—	12	—	9,11,9,21,22
15	—	—	—	9	23
16	—	23	9	—	—

Fig. 3. Spatial arrangement of trees and vegetative compatibility groups of 65 isolates of *Leucostoma persoonii* isolated from 42 trees in orchard B. Trees were spaced 4 m within and 8.5 m between rows.

In orchard B, 23 VCGs were identified among 65 isolates from cankers on 42 trees in five rows. Several VCGs occurred repeatedly in the orchard; VCG 9, 12, 23, 10, and 11 were recovered from 10, nine, six, five, and four trees, respectively. Other VCGs were represented by a single isolate (groups 1, 3, 5–8, 14, 15, 17–22). Spatial distribution of VCGs in orchard B did not appear aggregated or clustered around foci (Fig. 3), for example; note VCG 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 VCGs, whereas, in a second tree (row 5, tree 6), isolates from six cankers all belonged to the same VCG. Isolates from four of five cankers in each of two trees (row 4, tree 4, and row 5, tree 14) were in different VCGs. 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 VCGs.

Orchard A and orchard B were adjacent, separated by a one-lane road. Four isolates in orchard A (VCG 2, Fig. 2) were vegetatively compatible with three isolates in orchard B (VCG 2, Fig. 3). However, no multimerge groups were evident, and no other VCG was present in both orchards.

DISCUSSION

The difficulties encountered when pairing isolates on media other than oatmeal agar were repulsion, irregular radial growth, growth inhibition, and lack of barrage formation. Pairings on media containing all vitamins reported to be growth requirements of some isolates of *L. persoonii* (18) did not significantly alter the growth characteristics. The addition of activated charcoal to the media to absorb waste products did not improve growth. Further experiments will be required to understand the unique characteristics of oatmeal media in inducing mycelial reactions. Oatmeal agar also induces mycelial reactions in *Monilinia fructicola* (G. Wint.) Honey (22).

Based on the number of VCGs found in this study, there appears to be a high degree of VCG diversity in the population of *L. persoonii*, even within an orchard. Isolates within one tree and between adjacent trees usually differed in VC grouping. The results indicate that a reappraisal of the prevailing view that rain-spread conidia are the effective infective propagules is warranted (8,27). The large number of VCGs identifiable in close proximity to one another in the orchard suggests a hypothesis that the primary propagules of infection might be ascospores. Conidia generally are 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 (1,21). 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 VCGs 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.

In a newly established orchard, 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 VCG. Such a clustered pattern of VCGs within an orchard was not seen in our studies. However, the orchard in our study had been established for 9 yr. Perhaps, nursery seedlings possess latent infections caused by many VCGs of *L. persoonii* that then serve as foci of conidial infection in newly established orchards (25). We have planted new orchards to monitor spread of infection from nursery seedlings.

Bertrand and English (8) suggested that conidia would be the major or only inoculum in orchards in California where standard practices of pruning out diseased branches are followed because perithecia are formed on branches 2 yr 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. Ascospores might be important propagules even in well-pruned orchards in Michigan.

The collections of the sexual spores from perithecia on cankers from three locations and the pairing of the spores on oatmeal agar revealed that many VCGs occurred in a perithecium. Frequently, 10 ascospores segregated into 10 different VCGs, indicating that more than 10 VCGs probably occur per perithecium following sexual recombination.

The simplest scenario is that one fertilization event occurs on a canker which initiates the many stroma and perithecia. Then ascospores in each perithecium would segregate into the same VC groupings. However, we do not know whether conidia can spermatize ascogonia in individual stromata or in individual perithecia of *L. persoonii*. If the latter scenario occurs, then hundreds of VCGs might appear among ascospores formed on a single canker. Our results discussed below are suggestive of the simpler scenario.

The 67 ascospores from canker HS (3 stroma and 7 perithecia) segregated into 12 VCGs; 10 of these sometimes were evident in 10 ascospores of 1 perithecium. If we assume that vegetative compatibility is governed by loci having 2 alleles and that absolute dominance occurs, then a minimum of 3 VC loci segregated during sexual recombination. However, the ratios of segregating VCGs (30:21:5:2:2:1:1:1:1:1:1:1) did not suggest 3 loci. Chi-squared analysis of these data was not possible because many of the 12 classes were observed only once. Two thousand four hundred additional pairings would be needed to obtain at least two observations of each of the classes, and most likely other unique classes would appear as single observations.

The results confirm the outcrossing behavior of *L. persoonii* and reveal that multiple loci control vegetative compatibility. Apparently *L. persoonii* is heterothallic. Less likely it is a homothallic fungus that has the capacity to outcross like *C. parasitica* (3) but frequently does so. Leonian (17) mentioned the formation of a sexual state in culture by an isolate of *L. persoonii*, thus indicating that the species might be homothallic. However, fructification in culture has not been confirmed by subsequent researchers.

It has been hypothesized that a natural method of virus and dsRNA transmission in fungi is through hyphal anastomosis and heterokaryon formation (15). Hyphal anastomosis in ascomycetes is controlled by vegetative compatibility genes (3,7,9). Vegetative incompatibility reduces the effectiveness of dsRNA transfer and thus biological control of diseases in hypovirulent systems (3,6,7). The proliferation of VCGs is the probable factor limiting establishment of biological control of *C. parasitica* in North America, in contrast to Europe where biological control is successful and there is little diversity (4,6,12,13). The virulence-reducing factors in our studies of *L. persoonii* (14) are dsRNA and viruslike particles (VLPs). The influence of VCGs on the spread of dsRNA and associated VLPs can only be hypothesized at this stage of our understanding of hypovirulence in *L. persoonii*. However, it is probable that the multiplicity of VCGs will limit the natural spread of hypovirulence (5). *L. persoonii* may differ from *C. parasitica* in being heterothallic, and heterothallism might favor transmission if dsRNA or virions can be transmitted during mating.

LITERATURE CITED

1. Adams, G. C., Hammar, S. A., and Iezzoni, A. 1989. Optimum sample size for detecting virulence differences in *Leucostoma* isolates from peach. Plant Dis. 75:754-759.
2. Adams, G., Johnson, N., Leslie, J. F., and Hart, P. 1987. Heterokaryons of *Gibberella zeae* formed following hyphal anastomosis or protoplast fusions. Exp. Mycol. 11:339-353.
3. Anagnostakis, S. L. 1977. Vegetative incompatibility in *Endothia parasitica*. Exp. Mycol. 1:306-316.
4. Anagnostakis, S. L. 1983. Conversion to curative morphology in *Endothia parasitica* and its restriction by vegetative compatibility. Mycologia 75(5):777-780.
5. Anagnostakis, S. L., and Day, P. R. 1979. Hypovirulence conversion

in *Endothia parasitica*. Phytopathology 69:1226-1229.
6. Anagnostakis, S. L., and Kranz, J. 1986. Diversity of vegetative compatibility groups of *Cryphonectria parasitica* in Connecticut and Europe. Plant Dis. 70:536-538.
7. Anagnostakis, S. L., and Waggoner, P. E. 1981. Hypovirulence, vegetative incompatiblity, and growth of cankers of chestnut blight. Phytopathology 71:1198-1202.
8. Bertrand, P. F., and English, H. 1976. Release and dispersal of conidia and ascospores of *Valsa leucostoma*. Phytopathology 66:987-991.
9. Caten, C. E. 1972. Vegetative incompatibility and cytoplasmic infection in fungi. J. Gen. Microbiol. 72:221-229.
10. 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.
11. Gooding, G. V., and Lucas, G. B. 1959. Factors influencing sporangial formation and zoospore activity in *Phytophthora parasitica* var. *nicotianae*. Phytopathology 49:277-281.
12. Grente, J. 1981. Les variants hypovirulents de l'*Endothia parasitica* et la lutte biologique contra le chancre du châtaignier. Ph.D. thesis. Université de Bretagne Occidentale, Brest, France. 195 pp.
13. Grente, J., and Berthely-Sauret, S. 1978. Research carried out in France into diseases of the chestnut tree. Pages 88-92 in: Proc. Am. Chestnut Symp. West Virginia University Books, Morgantown.
14. 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.
15. Hollings, M. 1978. Mycoviruses—Viruses that infect fungi. Adv. Virus Res. 22:2-53.
16. Kern, H. 1955. Taxonomic studies in the genus *Leucostoma*. Mich. Acad. Sci. Arts Lett. 40:9-22.
17. Leonian, L. H. 1923. The physiology of perithecial and pycnidial formation in *Valsa leucostoma*. Phytopathology 13:257-272.
18. Lukezic, F. L., DeVay, J. E., and English, H. 1965. Comparative physiology and pathogenicity of *Leucostoma persoonii* and *Rhodosticta quercina*. Phytopathology 55:511-518.
19. Mircetich, S. M., and Matheron, M. E. 1976. Phytophthora root and crown rot of cherry trees. Phytopathology 66:549-558.
20. Proffer, T. J., and Hart, J. H. 1988. Vegetative compatibility groups in *Leucocytospora kunzei*. Phytopathology 78:256-260.
21. 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.
22. Sonoda, R. M., Ogawa, J. M., Essar, T. E., and Manji, B. T. 1982. Mycelial interaction zones among single ascospore isolates of *Monilinia fructicola*. Mycologia 74:681-683.
23. Tekauz, A., and Patrick, Z. A. 1974. The role of twig infections on the incidence of perennial canker of peach. Phytopathology 64:683-688.
24. Van Alfen, N. K., Jaynes, R. A., Anagnostakis, S. L., and Day, P. R. 1975. Chestnut blight: Biological control by transmissible hypovirulence in *Endothia parasitica*. Science 189:890-891.
25. Wensley, R. N. 1964. Occurrence and pathogenicity of *Valsa* (*Cytospora*) species and other fungi associated with peach canker in Southern Ontario. Can. J. Bot. 42:841-857.
26. Westergaard, M., and Mitchell, H. K. 1949. *Neurospora* B. A synthetic medium favoring sexual reproduction. Am. J. Bot. 34:573-577.
27. Willison, R. S. 1936. Peach canker investigations. II. Infection studies. Can. J. Res. 14:27-44.