A Wound-Freezing Inoculation Technique for Evaluating Resistance to *Cytospora leucostoma* in Young Peach Trees

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ABSTRACT

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A reliable method of inoculating young peach (*Prunus persica*) trees (0.95–1.6 cm in diameter) with *Cytospora leucostoma* is reported. Trees were wounded to xylem depth with an empty handheld stapling gun. The wound area was frozen with a commercial aerosol tissue-freezing product and inoculated with a suspension containing 10^7 pycniospores of *C. leucostoma* per milliliter. Cultivar susceptibility was rated by measuring the area and extension of xylem necrosis. The selection of the least susceptible and most susceptible cultivars as determined by these ratings was consistent for trials conducted for 3 yr, and agreed with previously reported field ratings. Although significant differences in susceptibility to *C. leucostoma* were found, the resistance reaction was weak. Significant year and year × cultivar effects indicated that nongenetic influences were a major component of variability.

Additional key words: canker, disease screening.

Cytospora cincta Sacc. and *C. leucostoma* (Pers.) Sacc. (perfect states *Valsa cincta* Fr. and *V. leucostoma* Fr.) are important pathogens of peach and other stone fruit trees and limit fruit production in some areas (9,10,12). Symptoms include cankering of the trunk and branches, branch dieback, and progressive weakening of the tree. A number of inoculation techniques have been used in studies of infections by *Cytospora* and host resistance. These include the "bark-flap," "bored hole," and "impact-wound" methods (8), the "crush-wound" method (14), "bark grafting" (17), surgical removal of various tissue layers, and the application of cold and hot awls (19). Natural infections have also been evaluated (11,13,15). No method has proven completely reliable as an indicator of innate resistance to *Cytospora*, and in some tests quite opposite results have been obtained when comparing artificial inoculations and field resistance (13,19). Impact and crush wounds are difficult to apply to small (<1.6 cm diameter) plants. The bark-flap and bored-hole methods are time consuming and as such would not be suited to evaluation of large numbers of seedlings. The development of efficient, reliable inoculation methods for seedling trees would allow for early selection of resistant genotypes and subsequent development of resistant cultivars.

This paper reports the development of a reliable technique for inoculating young budded peach trees with *C. leucostoma* and an evaluation of resistance in peach.

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MATERIALS AND METHODS

Five peach cultivars (Dixired, Elberta, Loring, Redhaven, and Sunhigh), chosen on the basis of their use in previous inoculation studies with *Cytospora* (14,18,19), were tested as dormant trees in 1980. All were obtained in February from a single nursery in Tennessee and had been propagated on Lovell peach seedling rootstock. They were sized at 90–125 cm in height (1.25–1.6 cm in diameter), except for those Redhaven which were 60–90 cm in height (0.95 cm in diameter). Plants had been stored 80 days at 4–8 C prior to inoculation.

A virulent isolate of *C. leucostoma* was isolated locally from an infected peach tree and used as the inoculum source. It was identified in culture as *C. leucostoma* according to the description by Willison (20). Cultures were grown on a peach-bark-extract agar medium (16). The inoculation site was cleaned with a cloth soaked in 70% EtOH. Tissue in the inoculation area was injured by mechanical wounding, killed by artificial freezing, or subjected to a combination of both treatments. A handheld stapling gun, without staples, was used for mechanical wounding; the head of the gun was held firmly against the bark as the trigger was pulled. This technique produced a wound ~12 mm long × 2 mm wide × 2 mm deep. A frozen area ~25 × 15 mm was produced by spraying with a commercial aerosol tissue freezing product (Cryokwik, Damon/IEC Division, Needham Hts., MA 02194) for 5 sec from a distance of 8 cm. Low temperature measured with a 0.102-mm-diameter (36-gauge) copper-constantan thermocouple reached −33 C at the stem surface. Inoculum consisted of 5-mm-diameter disks of mycelium on agar medium or 6-mm-diameter disks of filter paper soaked in a suspension containing 10^7 spores per milliliter. Inoculum was placed on the injured bark. Deionized, distilled H_2O was used for the controls. Treated areas were wrapped in plastic budding tape, and the trees were placed under mist for 48 hr. Five trees with two wounds per tree were used for each treatment. They were placed in a warm greenhouse (25–30 C day, 20–23 C night) and the roots were covered with composted sawdust. Inoculation of dormant trees and subsequent exposure to warm temperatures were chosen to simulate conditions during the period of greatest susceptibility in the field (1,2,10,17). After 50 days, all trees had leafed out and were evaluated for length and width of necrotic xylem and amount of gumming and pycnidium formation. Area of necrosis was calculated from length × width at inoculation site; this is not a true measure of area but serves as a simple, relatively rapid approximation that should reflect the same relative differences among cultivars as true area. Length or area of necrosis due to infection by *Cytospora* was determined by subtracting mean length or area of control wounds from mean length or area of inoculated wounds.

Young dormant trees for 1981 and 1982 inoculations were obtained in February or March from commercial nurseries in Michigan, Oregon, Pennsylvania, and Tennessee and had been budded onto Halford or Lovell seedling rootstocks. A wide range of plant sources was chosen to test the ability of the wound-freeze (WF) inoculation technique to give consistent infection success among trees grown in widely different pretreatment environments. Scion diameter ranged from 1.0 to 1.6 cm. Trees were stored at 4–8 C for 3–8 wk prior to inoculation. They were then mechanically wounded, frozen, and inoculated with a spore suspension of the same isolate of *C. leucostoma* used in 1980. Twenty microliters of a suspension of 10^7 spores per milliliter was pipetted into each wound. Wounds were wrapped in Parafilm, a paraffin-base laboratory film. Pre- and postinoculation procedures followed those for 1980 except that inoculated plants were not held under mist before being placed in a warm greenhouse, since in separate tests this was not found to be necessary for establishment of the pathogen. In 1981, 10 trees each (one wound per tree) of the following cultivars were inoculated per treatment: Canadian Harmony, Dixired, Elberta, Harbelle, Harken, Loring, Redhaven, Reliance, and Sunhigh. In 1982, 15 inoculated trees and five control trees (one wound per tree) of the same cultivars were tested.

Statistical analyses included analyses of variance (ANOVA) split plot design for 1980 data with cultivars and inoculation methods as variable factors (Table 1) and ANOVA split plot design for combined 1981 and 1982 data (Tables 2 and 3), using cultivars and years as factors. The relationship between length and area of necrotic xylem was tested by using linear regression analysis. All tests of significance were performed at either the $P = 0.01$ or 0.05 level.

RESULTS

Infections that developed from mechanical wounding prior to inoculation produced gum but generally did not expand. Infections of freeze-injured trees produced gum and typically supported pycnidial development; however, except in cultivar Loring they did not significantly advance beyond the area killed by freezing. Only the combination of mechanical wounding and freeze injury resulted in a significant amount of infection beyond the injured area in all cultivars (Table 1). Gum exudation and pycnidial development was also observed in all cultivars subjected to the WF treatment. Uninoculated mechanical and freeze-induced wounds occasionally produced small amounts of gum in cultivars Elberta, Loring, Redhaven, and Sunhigh.

There was a significant correlation between length of necrotic xylem and area as measured in this study. The coefficient of

TABLE 1. Comparison of inoculation methods for use in the screening of young budded peach cultivars for resistance to *Cytospora leucostoma*

	Inoculation method[w]				
Cultivar	Spore inoc.[x]	Wound + spore inoc.	Wound + mycelium inoc.	Freeze + spore inoc.	Wound + freeze + spore inoc.
Mean area of cankers[y]					
Loring	15.3 a C[z]	40.4 a C	171.1 a C	1,157.6 a B	3,261.7 a A
Elberta	9.4 a B	141.2 a B	99.9 a B	106.4 b B	2,202.5 b A
Redhaven	0.0 a B	644.6 a B	0.0 a B	188.6 b B	1,116.1 c A
Sunhigh	0.0 a A	7.9 a A	18.3 a A	0.0 b A	351.4 d A
Dixired	1.2 a A	64.3 a A	36.2 a A	66.4 b A	190.5 d A
Mean length of cankers[y]					
Loring	1.7 a C	2.3 a BC[z]	12.6 a B	24.8 a B	71.8 a A
Elberta	2.0 a B	5.8 a B	3.4 a B	2.2 b B	52.2 b A
Redhaven	0.0 a B	10.0 a B	0.1 a B	7.3 b B	30.6 c A
Sunhigh	0.0 a A	1.5 a A	1.6 a A	1.4 b A	15.7 cd A
Dixired	0.6 a A	5.0 a A	2.0 a A	0.6 b A	12.8 d A

[w] Wound = mechanical injury caused by an unloaded handheld stapling gun; conidial inoculation = 6-mm-diameter filter paper disks saturated with a suspension containing 10^7 conidia per milliliter; mycelial inoculation = 5-mm-diameter agar disk with mycelium of *C. leucostoma*; and freeze = freeze injury caused by spraying a commercial aerosol freezing product for 5 sec from a distance of 15 cm.

[x] Inoculation site unwounded and unfrozen.

[y] Area or linear extension of xylem necrosis expressed as area or length of inoculated wounds minus area or length control wounds in millimeters.

[z] Means within a column sharing a lower case letter in common are not significantly different, $P = 0.05$, according to Duncan's multiple range test. Means between columns sharing an upper case letter in common are not significantly different, $P = 0.05$, according to Duncan's multiple range test.

TABLE 2. Average area and linear extension (mm) of xylem necrosis of young budded peach trees following artificial wound-freeze (WF) inoculations with *Cytospora leucostoma* in 1981 and 1982 and comparison of the WF technique with published susceptibility ratings of mature field-grown trees

Cultivar	WF		Luepschen et al[w]	Luepschen[x]	Dhanvantari & Dirks[y]
	Area[v]	Length	1975	1981	1983
Elberta	2,987 a[z]	102 a	126 ab	···	106.8
Sunhigh	1,544 b	53 bc	···	···	68.8
Loring	1,502 b	73 b	143 a	···	···
Redhaven	1,488 b	58 bc	107 bc	···	···
Canadian Harmony	1,408 b	55 bc	···	166 ab	···
Dixired	1,255 b	55 bc	···	···	···
Harken	537 c	38 cd	98 bc	···	···
Reliance	460 c	22 d	···	···	···
Harbelle	416 c	34 cd	···	104 b	77.7

[v] Area and length of xylem necrosis expressed as area or length of inoculated wounds minus that of control wounds (mm).
[w] Linear extension (mm) of canker on limbs of 5- to 7-yr-old trees; values represent 3-yr means. *C. leucostoma* (14).
[x] Canker area (cm^2) on 3- to 4-cm-diameter limbs of 8-yr-old trees. *C. leucostoma* (13).
[y] Linear extension of xylem necrosis (mm) on 1-yr-old twigs of 3- to 5-yr-old trees; values represent 3-yr means. LSD = 28.3 mm. *C. cincta* (6).
[z] Means within a column sharing a letter in common are not significantly different, $P = 0.05$, according to Duncan's multiple range test.

correlation each year was greater than 0.93. Mean separations of area measurements were slightly more distinct than those of lengths (Table 2).

In 1980, four discrete classes of infection based on area of xylem necrosis were identified by WF. Cultivars Dixired and Sunhigh exhibited the smallest areas of infection, followed by Redhaven and Elberta. Infections on cultivar Loring had the largest areas (Table 1). Cultivar × inoculation method interactions were significant ($P = 0.01$) based upon area and length measurements (Table 1). The WF technique was significantly different from other techniques when used on the more susceptible cultivars tested in 1980 (Loring, Elberta, and Redhaven). It was not significantly different from the other techniques when used on the less susceptible cultivars Sunhigh and Dixired.

The combined data for 1981 and 1982 inoculations separated cultivars into three distinct infection classes based on area of necrotic xylem (Table 2). Cultivars Harbelle, Reliance, and Harken exhibited the smallest average areas of necrosis, while cultivar Elberta exhibited the greatest. Cultivars Dixired, Canadian Harmony, Redhaven, Loring, and Sunhigh were intermediate. While statistically significant differences in reaction to *Cytospora* are apparent in our data (Table 2), differences in infection between years and cultivar × year interactions were also significant (Table 3).

DISCUSSION

Injury to xylem depth is necessary for reliable infection by *Cytospora* (8,19). The destruction of relatively large areas of tissue has been shown to promote infection, possibly due to the release of cell substances which provide substrate for fungal growth (5,19). The artificial freezing process provides relatively large areas of cell destruction, thus enhancing colonization by *Cytospora*.

Our results indicate that only the combination of mechanical injury to xylem depth and freeze injury to a relatively large area was effective in separating young trees of cultivars into classes of susceptibility. The relative susceptibility of these cultivars inoculated by the WF technique is in agreement with previously reported ratings of mature field-grown trees except in the case of cultivar Sunhigh (Table 2).

Measurement of necrotic xylem area generally resulted in more distinct mean separation (Tables 1 and 2). This increase in precision must be weighed against the relative ease of making length measurements. In a breeding program where only the most resistant material will be saved, the slight loss in accuracy may be more than offset by the time saved through simple measurements of length.

TABLE 3. Analysis of yearly variability on expression of infection by *Cytospora* in young budded peach trees[a]

Infection	Source	df	MS	F	P > F
		Cultivars inoculated in 1981 and 1982			
Area	Cultivar	8	13,482,712	11.52	0.0001
	Year	1	98,674,849	84.29	0.0001
	Cultivar × year	8	9,538,440	8.15	0.0001
	Error	164	1,170,618		
Length	Cultivar	8	11,522	8.69	0.0001
	Year	1	250,507	188.97	0.0001
	Cultivar × year	8	7,307	5.51	0.0001
	Error	164	1,326		

[a] ANOVA split plot design, factors = cultivars and years.

A significant cultivar × year interaction for infection of peach by *Cytospora* was reported by Dhanvantari and Dirks (6). Although the interaction between cultivar and environment alters the yearly ranking of some cultivars in our study, the most- and least-susceptible cultivars based upon area or linear extension of xylem necrosis were generally consistent. In at least two of the three years, cultivars Elberta, Loring, and Sunhigh were among the most susceptible and cultivars Reliance, Harbelle, and Harken were the least susceptible. Sunhigh was the only cultivar that gave a markedly different rating in one year. In 1980, it was rated as one of the least susceptible cultivars, but in 1981 and 1982 it was rated among the most susceptible. The reason for this is unclear. The yearly variability suggests that resistance is weak. A lack of field resistance to *Cytospora* in peach has been previously noted (13). In the field, where trees are subject to greater environmental stress, the levels of resistance in existing cultivars as detected through artificial inoculation are not high enough to prevent infection or advance of the disease, although these processes may take place more slowly in cultivars rated as less susceptible through artificial inoculation.

When the ancestry of the peaches inoculated in this study is considered, it becomes clear that the genetic base is narrow. Most cultivars are derived from repeated matings of cultivars J.H. Hale and South Haven progeny (3,4,7). Cultivar J.H. Hale was among the most susceptible of the cultivars tested by Luepschen et al (14). Cultivar Elberta, the female parent of cultivar J.H. Hale, has been rated among the most susceptible cultivars (6,13) as it was through WF inoculations. It should not be expected that great differences in resistance will be found in such a limited gene pool, especially since most peach cultivars are selected for fruit qualities and not for resistance to *Cytospora*. Through transgressive segregation and selection pressure, small gains in resistance can be made as is apparently the case with cultivars Harbelle and Harken. Selected in Canada, a region of intense disease pressure from *Cytospora*, cultivars Harbelle and Harken are consistently the least susceptible cultivars, although their ancestry is based almost exclusively on cultivars J. H. Hale and South Haven.

High levels of resistance can be achieved only through the search for new germ plasm exhibiting such resistance. The WF technique provides a simple, reproducible, and relatively rapid method of inoculating young trees and evaluating resistance to *Cytospora*. Resistance ratings obtained by this method follow those generally obtained through artificial inoculation of mature trees. Thus, it appears that this method would be useful in selecting for resistance to *Cytospora*. Resistant phenotypes can be pruned below the infected area. Lateral buds reestablish growth. Selection of resistant parental germ plasm and strong selection pressure against susceptible progenies would facilitate the development of more resistant cultivars.

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