Effect of Exposure to Freezing Temperatures on Necrosis in Sweet Cherry Shoots Inoculated with *Pseudomonas syringae* pv. *syringae* or *P. s. morsprunorum*

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ABSTRACT

Sobiczewski, P., and Jones, A. L. 1992. Effect of exposure to freezing temperatures on necrosis in sweet cherry shoots inoculated with *Pseudomonas syringae* pv. *syringae* or *P. s. morsprunorum*. Plant Dis. 76:447-451.

Dormant 1-yr-old shoots of two sweet cherry (*Prunus avium*) cultivars were wound-inoculated with *Pseudomonas syringae* pv. *syringae* or with *P. s. morsprunorum* and then incubated sequentially at 15, −10, and 15 C for 7, 1.5, and 10 days, respectively. Dark-stained necrotic tissue extended downward from the point of inoculation at the tip of the shoots. Inoculations with *P. s. syringae* resulted in significantly greater necrosis than inoculations with *P. s. morsprunorum*. The population and the distribution of bacteria in shoots just before exposure to freezing temperatures were greater for *P. s. syringae* than for *P. s. morsprunorum*. Ten days after exposure to freezing temperatures, populations of both pathovars were much higher in shoots subjected to −10 C for 1.5 days than in shoots maintained at 15 C through the experimental period. The extent of necrosis in wound-inoculated shoots of the cultivar Hedelfingen decreased, whereas necrosis in shoots of the cultivar Gold increased with the period of dormancy. When inoculations preceded exposure to freezing temperatures by 7 days, the extent of necrosis in dormant shoots of cultivars inoculated with *P. s. syringae* was as follows (in decreasing order): Napoleon, Emperor Francis, Gold, Nelson, Ulster, Sam, Vega, Windsor, Schmidt, Hedelfingen, Valera, Vic, and Viva. Cultivars Ulster, Vega, and Napoleon developed the most necrosis when inoculated with *P. s. morsprunorum*.

Additional keywords: bacterial canker

The influence of freezing temperatures on the severity of bacterial canker of flower buds and leaves of sour cherry has been well documented (13,21,22), but little is known about the influence of freezing temperatures on the severity of canker of woody parts of cherry trees. However, on apricot branches and peach shoots inoculated with *Pseudomonas syringae* pv. *syringae* van Hall (6,7,18) and on peach shoots inoculated with *P. s. persicae* (Prunier et al) Young et al (15), it was demonstrated that the severity of bacterial canker increased when the branches or shoots were exposed to freezing temperatures a few days after inoculation. Garrett and Flecher (4) developed a screening method for evaluating the resistance of cultivars of sweet cherry to bacterial canker based on the inoculation of 2-yr-old dormant shoot growth with *P. s. morsprunorum* (Wormald) Young et al followed 7 days later by exposure of the shoots to −5 C.

The objectives of this research were to determine the effect of exposing dormant sweet cherry (*Prunus avium* L.) shoots inoculated with *P. s. syringae* or *P. s. morsprunorum* to freezing temperatures on the development of necrotic tissue in the shoots. We also determined if this method could be used to evaluate the susceptibility of cultivars of sweet cherry to bacterial canker.

This research was supported in part by USDA Agreement 88-34152-3380.

Accepted for publication 3 December 1991 (submitted for electronic processing).

MATERIALS AND METHODS

Bacteria. Strain 315-2-1 of *P. s. syringae*, isolated in Michigan in 1990 from sweet cherry fruit, was ice-nucleation positive. Strain 212-02 of *P. s. morsprunorum*, isolated in Michigan in 1989 as an epiphyte from blossoms of sweet cherry and characterized in an earlier study (11), was ice-nucleation negative. Both strains were cytochrome oxidase negative (8) and produced fluorescent colonies when cultured on King's medium B (KB) (5) for 3 days at 22 C. Strain 315-2-1 produced large, black, necrotic lesions, and strain 212-02 produced small, brown lesions when inoculated into cherry fruitlets. Identity of the strains was reconfirmed before they were used in this study by subjecting them to the GATTa determinative tests (9,14).

Inoculum. The strains were grown on KB for 2 days at 22 C. Cells were suspended in 0.01 M potassium phosphate buffer (pH 7.2), and the suspension was adjusted to 0.04 absorbance at 625 nm on a Spectronic 20 colorimeter (Bausch and Lomb, Rochester, NY) to give 1×10^8 cfu/ml. The concentration of viable bacteria in the suspension was confirmed by dilution plating on KB.

Effect of inoculation and subfreezing temperatures. Terminal and lateral shoots were collected during December–March from 13-yr-old sweet cherry trees of the cultivars Gold and Hedelfingen in an orchard near East Lansing, MI. A total of 240 shoots, 25–30 cm long, were collected at random from four to six trees of each cultivar on each sampling date. The shoots were placed in 0.02% sodium hypochlorite for 5 sec, rinsed in sterile distilled water, placed in 96% ethanol for 5 sec, and rinsed again in sterile distilled water. After the shoots had dried, the basal end of each was dipped about 2 cm into melted paraffin. Then the tip of each shoot was removed 2–3 cm below the apical bud and discarded. Immediately, a 20-μl drop of bacterial suspension was placed on the cut end of the shoot. Each drop contained 1.5×10^6 viable bacteria based on dilution plate analysis. Only buffer was applied to cuts on control shoots. After the liquid had been absorbed, the cut surface was wrapped with Parafilm and the shoots were transferred to autoclaved glass jars, one treatment per jar, with cotton saturated with water in the bottom. Each jar was covered with plastic to maintain a moist atmosphere, and all of the jars were brought to 15 C in a controlled temperature chamber. After 7 days, half of the shoots, both inoculated and noninoculated, were transferred from the 15-C chamber to a freezer at −10 C. After 1.5 days, the shoots were returned to 15 C for 10 days. This temperature sequence was previously used to study the effect of exposure to cold on the development of necrosis in peach shoots inoculated with *P. s. syringae* (18).

In an second experiment on 14 December 1990, terminal and lateral shoots were collected at random from four to six trees in the East Lansing orchard, inoculated as described in the first experiment, and then placed in a chamber maintained at 10 C. The temperature regime for freezing the shoots was as previously described for branches of apricot (7). After 10 days, two-thirds of the shoots, both inoculated and noninoculated, were removed from the 10-C chamber, and half of the shoots were placed at −5 C and half at −10 C. After

3 days, all shoots were returned to the 10-C chamber for 12 days.

The bark was cut away from the xylem of each shoot with a razor blade at the end of each test, and the length of brown discoloration (necrosis) was measured. Measurements were made of necrosis in the bark (phloem) as well as that in the shoot (cambium and xylem).

Reisolation of bacteria. Tissue at the margin between diseased and apparently healthy tissue of 10 shoots per treatment was tested for the presence of *P. s. syringae* and *P. s. morsprunorum* as previously described (12). Fifty fluorescent bacterial colonies per treatment with common colony type on KB were tested for cytochrome oxidase (8) and ice nucleation activity at −5 C (droplet freezing procedure of Lindow et al [10]). Ten 10-μl drops per isolate were spotted onto paraffin-coated aluminum foil boats floating on ethylene glycol/water (1:1) in a refrigerated bath (Lauda RM20, Brinkmann Instruments, Westbury, NY). If the drops froze within 2 min, the isolate was considered to be *P. s. syringae*. If the drops did not freeze within 2 min, the isolate was considered to be *P. s. morsprunorum*.

Distribution of bacteria in inoculated shoots. The presence of bacteria within shoots was assayed 7 and 18.5 days after inoculation. Isolations were made in February and again in March from five shoots per treatment. The surface of the shoots was disinfested with cotton saturated with 95% ethanol. The top 5 cm of each shoot was cut into 1-cm sections, each section was diced into smaller pieces, the pieces from each section were placed into preweighed test tubes with 10 ml of potassium phosphate buffer, and the tubes were reweighed. After 0.5–1 hr, aliquots were plated in a dilution series on KB. Fluorescent colonies were counted after 3–4 days at 22 C, and bacterial identifications were made as described above.

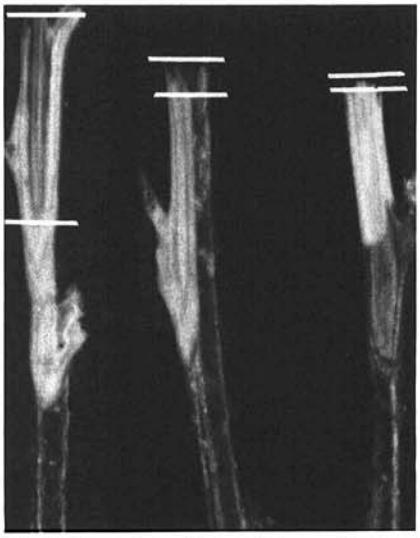

Fig. 1. Necrosis in the cambium and xylem of excised shoots of Hedelfingen sweet cherry subjected to 15, −10, and 15 C for 7, 1.5, and 10 days, respectively. Shoots were inoculated with *Pseudomonas syringae* pv. *syringae* (left), *P. s. morsprunorum* (center), or noninoculated (right). Area between white lines on each shoot is the length of necrosis measured at the end of each experiment.

Cultivar susceptibility. A total of 180 dormant shoots were collected on 16 January 1991 from two trees of each of 13 cultivars in a 12-yr-old sweet cherry orchard at the Northwest Michigan Horticultural Experiment Station, Traverse City. The techniques for inoculating, freezing, and evaluating the shoots were as described for the first experiment.

Statistical methods. Factorial analyses of variance for a completely random design available through MSTAT-C (MSTAT, Michigan State University, East Lansing, MI) were conducted to evaluate the effects of inoculation, freezing, and time of sampling on the length of necrosis in the cambium and xylem of shoots of each cultivar. Data for each sampling date (first experiment) and for each cultivar (second experiment) were subjected to one-way analyses of variance and means were separated by Duncan's multiple range test ($P = 0.05$). Means for cultivars were differentiated by one-way analyses of variance followed by Duncan's multiple range test.

RESULTS

Dormant shoots of sweet cherry inoculated with *P. s. syringae* or *P. s. morsprunorum* developed a necrosis in the cambium, xylem, and phloem similar to that observed in nature (Fig. 1). Generally, the extent of necrosis was greater in the cambium and xylem than in the phloem; therefore, only data for the cambium and xylem are presented here.

The length of necrosis was affected by whether the shoots were inoculated with *P. s. syringae*, *P. s. morsprunorum*, or noninoculated; whether or not they were subjected to freezing temperatures; and when in winter the samples were collected from the orchard (Table 1). There also was a significant ($P = 0.001$) amount of interaction among each of the factors (Table 1).

Negligible necrosis was observed in the cambium and xylem of noninoculated shoots of both cultivars maintained at 15 C and in shoots inoculated with *P. s. morsprunorum* and maintained at 15 C (Table 2). Significantly ($P = 0.05$) more necrosis was found in inoculated shoots subjected to −10 C for 1.5 days than in inoculated shoots held at 15 C throughout the experiment. Inoculations with *P. s. syringae* resulted in significantly greater necrosis than inoculations with *P. s. morsprunorum*, except for cultivar Gold in December. The length of necrosis for shoots of Hedelfingen inoculated with *P. s. syringae* and exposed to −10 C for 1.5 days tended to decrease, whereas that for shoots of Gold tended to increase with the period of dormancy (Table 2).

The length of necrosis in the cambium and xylem of shoots inoculated with *P. s. syringae* or *P. s. morsprunorum* and exposed to −5 C for 3 days did not differ significantly from the length of necrosis in shoots inoculated with the respective pathovars and incubated continuously at 10 C (Table 3). The greatest length of necrosis was observed in shoots inoculated with *P. s. syringae* or *P. s. morsprunorum* and exposed to −10 C for 3 days starting 12 days after inoculation.

Detection of bacteria in inoculated shoots. *P. s. syringae* and *P. s. morsprunorum* were consistently reisolated from the border of healthy and necrotic tissues of excised shoots, both frozen and nonfrozen, inoculated in December. No fluorescent, oxidase-negative bacteria were isolated from noninoculated shoots.

Distribution of bacteria in shoots. For shoots removed from the orchard in February, populations of bacteria 7 days after inoculation and just before exposure to freezing temperatures were greater for *P. s. syringae* than for *P. s. morsprunorum* (Fig. 2). Most of the bacteria were recovered from the first 1-cm section below the inoculation site. Ten days after the cold treatment, populations of both pathovars were higher in shoots subjected to −10 C for 1.5 days than in

Table 1. Summary of analysis of variance for length of necrosis in the cambium and xylem of excised sweet cherry shoots sampled four times during the winter of 1990–1991 and inoculated with two pathovars of *Pseudomonas syringae* before they were subjected to 15, −10, and 15 C for 7, 1.5, and 10 days, respectively, or maintained at 15 C for 18.5 days

Source	df	Mean square	
		Hedelfingen	Gold
Inoculum	2	1,532.3***[z]	1,621.4***
Treatment	1	2,335.4***	1,678.3***
Inoculum × treatment	2	547.3***	711.1***
Sample date	3	175.6***	47.2***
Inoculum × sample date	6	130.7***	31.7***
Treatment × sample date	3	127.2***	47.4***
Inoculum × treatment × sample date	6	118.3***	30.6***
Error	72	2.6	3.2

[z] F value significant at $P < 0.001$.

Table 2. The length of necrosis in excised sweet cherry shoots as affected by collection date, exposure to cold temperature, and inoculation with two pathovars of *Pseudomonas syringae* before they were subjected to 15, −10, and 15 C for 7, 1.5, and 10 days, respectively, or nonfrozen and maintained at 15 C for 18.5 days.

Cultivar	Collection date (1990–1991)	Necrosis in the cambium and xylem (mm)					
		P. s. syringae		*P. s. morsprunorum*		Noninoculated	
		Frozen	Nonfrozen	Frozen	Nonfrozen	Frozen	Nonfrozen
Hedelfingen	13 December	33.0 a[z]	6.2 b	7.5 b	2.5 bc	3.1 bc	0.2 c
	10 January	27.2 a	4.2 c	5.7 b	2.9 d	2.8 d	0.5 e
	14 February	22.0 a	6.0 c	8.2 b	2.5 d	2.1 de	0.6 e
	15 March	16.2 a	5.4 b	5.5 b	2.8 c	1.4 d	0.4 d
Gold	13 December	18.0 b	7.2 c	25.0 a	2.2 d	1.5 d	0.2 d
	10 January	20.7 a	3.8 bc	6.3 b	2.0 c	1.7 c	0.5 c
	14 February	39.1 a	7.4 c	15.0 b	1.7 d	1.4 d	0.3 d
	15 March	16.3 a	5.0 b	5.0 b	2.5 c	1.3 cd	0.3 d

[z] Each value is the mean of four replications of 10 shoots each. Means in a row followed by different letters are significantly different according to Duncan's multiple range test ($P = 0.05$).

Table 3. The length of necrosis in excised dormant sweet cherry shoots as affected by exposure to cold temperatures and inoculation with *Pseudomonas syringae* pv. *syringae* (Pss) or *P. s. morsprunorum* (Psm) before exposure to 10, −10 or −5, and 10 C for 10, 3, and 12 days, respectively, or nonfrozen and maintained at 10 C for 25 days

Cultivar	Temperature (C)	Necrosis in cambium and xylem (mm)		
		Pss	Psm	Noninoculated
Hedelfingen	−10	26.2 a[z]	6.8 bc	4.0 cd
	−5	6.7 bc	2.6 d	1.2 d
	10	8.3 b	2.2 d	1.0 d
Gold	−10	24.2 a	7.6 b	1.9 d
	−5	3.9 c	2.1 d	0.9 e
	10	4.4 c	1.6 de	1.1 de

[z] Each value is the mean of four replications of 10 shoots each. Mean values for each cultivar followed by different letters are significantly different according to Duncan's multiple range test ($P = 0.05$).

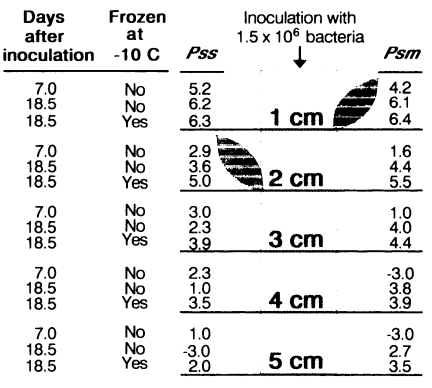

Fig. 2. Effect of exposure to −10 C for 1.5 days on the distribution of *Pseudomonas syringae* pv. *syringae* (*Pss*) and of *P. s. morsprunorum* (*Psm*) 7 and 18.5 days after inoculation. Data expressed as log (number of bacteria per milligram of tissue). Mean of five replications.

shoots maintained at 15 C throughout the experimental period (Fig. 2). Similar results were obtained when the study was repeated in March.

Cultivar susceptibility. There were significant differences in the extent of necrosis among shoots from the 13 cultivars (Table 4). The cultivars with the greatest necrosis after inoculation with *P. s. syringae* and exposure to freezing temperatures were Napoleon, Emperor Francis, Gold, Nelson, and Ulster (in decreasing order); whereas the cultivars with the greatest necrosis after inoculation with *P. s. morsprunorum* and exposure to freezing temperatures were Ulster, Vega, and Napoleon. As in the two previous experiments, the extent of necrosis in the cambium and xylem of nonfrozen shoots was shorter than in frozen shoots. There were discrepancies between frozen and nonfrozen shoots in the ranking of cultivar susceptibility within each of the pathovars.

DISCUSSION

It was possible to induce dark-staining necrotic tissue extending downward from the point of inoculation at the tip of dormant sweet cherry shoots by exposing the shoots to freezing temperatures in the laboratory 7–10 days after inoculation. This necrosis is a frequent expression of bacterial canker (6,7,15, 18). The extent of the necrosis was increased when sweet cherry shoots were exposed to freezing temperatures after inoculation with *P. s. syringae* or *P. s. morsprunorum*. Previously, bacterial canker was increased when apricot branches were frozen after inoculation with *P. s. syringae* (6,7) and when peach shoots were frozen after inoculation with *P. s. syringae* (18) or *P. s. persicae* (15). Our results and results reported from the United Kingdom (4) extend the concept of synergism between bacterial canker and freezing to sweet cherry and to *P. s. morsprunorum*. However, the extent of necrosis with *P. s. morsprunorum* was less than with *P. s. syringae*.

In our studies, necrosis after inoculation with both pathovars developed in all tissues, but it was less severe in the phloem than in cambium and xylem, especially the outer xylem, of both frozen and nonfrozen shoots. The extent of necrosis in the phloem, cambium, and xylem was similarly dependent on temperature treatment. However, in experiments on apricot and peach (6,18), necrosis did not develop in the phloem of nonfrozen branches and shoots, but it did develop in the xylem independently of temperature treatment. On peach, the length of necrosis was similar on frozen and nonfrozen shoots (18). Necrosis in the xylem was more extensive than in the phloem on frozen apricot branches and peach shoots (6,18). In later experiments conducted in the same sequence of temperatures that we used, necrosis of the xylem on apricot branches was less severe than in the phloem and it occurred on shoots independently of the temperature treatment (7). Thus, our findings indicate that the effect of freezing temperatures on bacterial canker development in sweet cherry shoots was different than that reported for apricot and peach because the amount of necrosis in the xylem was temperature dependent. But its effect was similar in that necrosis was generally much less severe in the phloem than the xylem.

Our finding that the exposure of inoculated shoots of sweet cherry to −5 C did not increase the development of bacterial canker is contrary to previous studies with apricot branches in Hungary (7) and with peach shoots in France (15). It is possible that sweet cherries are more resistant to freezing than peaches and apricots. Also, shoots collected in Michigan are likely exposed to lower winter temperatures than those collected in Hungary or France. Dormant shoots collected in regions with mild winters are probably not as well acclimated to freezing temperatures as dormant shoots collected in regions with severe winters. Therefore, the selection of −5 or −10 C

Table 4. Length of necrosis in the cambium and xylem (mm) of 1-yr-old excised shoots of sweet cherry cultivars exposed to freezing temperatures after inoculation with pathovars of *Pseudomonas syringae*

Cultivar	*P. s. syringae*		*P. s. morsprunorum*		Noninoculated	
	Frozen	Nonfrozen	Frozen	Nonfrozen	Frozen	Nonfrozen
Napoleon	35.3 a[z]	20.7 a	5.7 a–c	4.2 a	0.9 cd	0.8 d
Emperor Francis	26.0 b	12.5 b	4.8 b–d	2.2 c	1.0 c	0.4 cd
Gold	24.7 bc	4.0 f	5.5 bc	2.1 c	1.2 bc	0.6 bc
Nelson	23.0 bc	7.2 de	4.5 b–e	2.8 c	0.8 cd	0.3 c–e
Ulster	23.1 bc	6.2 ef	7.1 a	4.0 ab	1.7 b	0.4 cd
Sam	22.3 cd	5.7 ef	4.3 c–e	2.8 c	0.9 cd	0.3 c–e
Vega	22.0 cd	9.8 cd	6.0 ab	3.0 bc	1.3 bc	0.0 e
Windsor	21.6 cd	10.6 bc	...	...	1.1 bc	...
Schmidt	18.9 de	10.7 bc	3.7 de	2.7 c	0.8 cd	0.5 cd
Hedelfingen	17.4 ef	7.9 de	3.8 de	1.9 c	0.2 d	0.3 c–e
Valera	16.3 e–g	8.2 c–e	4.7 b–d	2.2 c	2.4 a	0.2 de
Vic	15.2 fg	12.6 b	4.7 b–d	4.2 a	0.9 cd	0.2 de
Viva	13.7 g	3.9 f	3.0 e	2.5 c	1.3 bc	1.2 a

[z] Each value is the mean of four replications with seven to eight shoots per replicate. Means in a column followed by different letters are significantly different according to Duncan's multiple range test ($P = 0.05$).

as the temperature for conducting this assay probably depends on the level of acclimation of the shoots.

The susceptibility of trees to bacterial canker and to injury from freezing has been reported to change over the course of the winter. Weaver (18) found that shoots of peach had greater susceptibility during deep dormancy than during the dehardening period, whereas Davis and English (3) reported that the susceptibility of peach tissue varied directly with the degree of dormancy. Also, Chandler and Daniell (2) demonstrated that the susceptibility of peach trees to bacterial canker was higher at the beginning of and during full dormancy than at other times. Our results and those of Weaver (18) suggest that this relationship was cultivar dependent. On cultivar Hedelfingen, susceptibility tended to decrease as the degree of dormancy increased in time, but on the cultivar Gold, the opposite trend occurred. It is possible that the beginning of dormancy and its course was different for both cultivars. Differences in the susceptibility of shoots in various months may be related to systemic movement of bacteria in the tissue, toxin production, or the utilization of nutritional compounds that alter the shoot's physiology (7). Weaver (19) suggested that the ice nucleation activity of *P. s. syringae* may increase its ability to cause bacterial canker, but Vigouroux (16,17) proposed that increased water soaking associated with freezing and thawing of the tissues favored the ingress and spread of *P. s. persicae* in peach and apricot stems.

In our study and in that of Klement et al (7), more bacteria were recovered from shoots exposed to −10 C than from shoots not exposed to freezing temperatures. Although populations of both pathovars increased after exposure of shoots to −10 C, *P. s. morsprunorum* caused significantly less necrosis than *P. s. syringae*. This result suggests that the number of bacteria was not the limiting factor related to the amount of necrosis. Although the shoots were subjected to freezing temperatures, the cambium and xylem probably were not injured at −10 C because the necrosis was not uniformly distributed across the length of the shoot. Also, temperatures of −14.5 to −25.5 C were required to injure the cambium and xylem in 50% of the shoots of sweet cherry cultivar Napoleon collected in the same months that these shoots were collected (P. Sobiczewski, *unpublished data*). Monitoring the temperature of the tissue to detect the exotherm-inducing temperature, as done recently for pear blossoms (20), should be done in future experiments to establish if the difference in the amount of necrosis inducted by *P. s. syringae* and *P. s. morsprunorum* is associated with the ice nucleation activity of *P. s. syringae*.

Bacterial canker is an important limiting factor in sweet cherry production in Michigan and available control procedures are inadequate. Planting of cultivars with moderate to high resistance should help reduce losses from this disease, but the selection of cultivars resistant to both pathovars has been difficult. In our study on cultivar susceptibility, Hedelfingen and Viva exhibited moderate resistance to both pathovars, whereas Valera and Vic were relatively more resistant to pathovar *syringae* than to pathovar *morsprunorum*. Although our results are in general agreement with inoculation studies carried out by Allen and Dirks (1), it is possible that variants of these pathovars exist that are more virulent on these cultivars than the isolates used in this study and in Canada.

ACKNOWLEDGMENTS

We thank C. D. Kesner for shoots of the 13 cultivars of sweet cherry, J. M. Paterson for the strains of *P. s. syringae* and *P. s. morsprunorum*, S. D. Whitcomb and G. R. Ehret for technical assistance, and J. A. Bartz for editorial assistance. We gratefully acknowledge fellowship arrangements by the Brethren Service Exchange Program through the kindness of H. L. Gibble and S. W. Zagaja.

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