The Role of Ice Formation in the Infection of Sour Cherry Leaves by *Pseudomonas syringae* pv. *syringae*

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ABSTRACT

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Infection of sour cherry (*Prunus cerasus*) leaves by ice nucleation-active (INA^+) and inactive (INA^-) strains of *Pseudomonas syringae* pv. *syringae* and *P. s.* pv. *morsprunorum* after leaf freezing was investigated. Leaves were infected only when ice previously had been formed in their intercellular spaces. This occurred between −2 and −3 C. INA^+ strains induced freezing 0.5–0.8 C above the temperature required for INA^- strains. Leaves that had been sprayed and frozen weighed 2–4% more than leaves that had not been sprayed and frozen. After freezing, 10^3–10^4 bacteria per gram of leaf tissue could be isolated from the leaf interior. This ingress of bacteria appeared to be passive. *P. s.* pv. *tabaci* cells were also absorbed by sour cherry leaves as well as *P. s.* pv. *syringae* cells. Investigations on symptom development and leaf freezing showed that the infection is not related to low temperature, but to ice formation in the presence of *P. s.* pv. *syringae*. The infection by this bacterium did not result from freezing injuries of the leaf tissue. Freezing predisposed the plants to infection if inoculation occurred within 20 min of thawing. Inoculations made 30 min after thawing did not result in any lesions.

Bacterial leaf spot of sour cherry (*Prunus cerasus* L.) incited by *Pseudomonas syringae* van Hall pv. *syringae* may cause considerable crop damage and serious economic losses under cool and wet weather conditions. In spring, the first symptoms often appear after late frosts, and this phase of the disease is the most dangerous because a great portion of the expanding leaves are damaged. In summer, the pathogen survives as an epiphyte on leaf surfaces, but in wet weather, it may also infect the foliage (7,8), causing necrotic leaf spots. The spots are small, light brown with a pale green halo or occasionally a purple halo. Young leaves are the most sensitive. As they mature, the infected areas become dry and fall out; the leaves then have a shot-hole appearance. When leaves are mature, they are no longer infected. The initiation of leaf spots is not fully understood. Recent investigations (22,25) have revealed that leaves can be successfully infected between −0.5 and −2 C, and the optimal temperature range for symptom development is 15–25 C.

Isolates of *P. s.* pv. *syringae* are active in ice nucleation and catalyze ice formation at temperatures as warm as −2 C. When ice nucleation-active (INA^+) bacteria are present on plants, frost injury may occur at −2 to −5 C (16). Frost injury has been reported as a predisposing factor in blossom blight of pear caused by *P. s.* pv. *syringae* (20). Development of cankers on inoculated peach seedlings was correlated with the INA of *P. s.* pv. *syringae* strains (24). In spite of these reports, other lines of evidence suggest that the contribution of INA^+ bacteria to frost injury of stone fruit trees is limited (9). The woody stem tissue of these plants has a source of ice nucleation material that by itself promotes ice formation at −2 to −4 C (3–5, 21).

Whether or not INA of this pathogen is related directly to its ability to induce leaf spots has not been established. The purpose of this study was to determine the relationship between ice formation and infection of sour cherry leaves by INA^+ and INA^- *P. s.* pv. *syringae* strains.

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MATERIALS AND METHODS

Bacterial strains and media. Strains of *P. s.* pv. *syringae* were isolated from epiphytic bacterial populations of healthy Heimanns Rubin sour cherry leaves at Dossenheim in August 1984. Pathogenic strains were further characterized (14,23). Strain 146 of *P. s.* pv. *morsprunorum* was obtained from R. Grimm (Switzerland). Bacteria were routinely cultured on nutrient agar (Difco) supplemented with 1 g/L yeast extract (Difco) and 12 g/L sucrose. Antibiotic plates contained streptomycin (100 mg/L) and rifampicin (50 mg/L) as specified in the text.

Plant inoculations. Bacteria were grown on slants for 48 hr at 25 C and suspended in sterile deionized water to a final cell density of about 10^8 colony-forming units (CFU) per milliliter as determined turbidimetrically and confirmed by a standard dilution plate assay. Six-year-old sour cherry cultivar Heimanns Rubin trees grown in 10-L plastic containers were used. Plants were grown in a greenhouse at 23 C and when the new shoots had 6–7 leaves, plants were transferred for 2–3 wk to a growth chamber with a 16-hr light and 8-hr dark period at 4 C and 80% relative humidity for hardening. Leaves were inoculated by spraying bacterial suspensions at low velocity to avoid forcing the pathogen into stomata. Inoculated plants were moved immediately after inoculation to the growth chamber, where the temperature was 2 C and relative humidity 95%. In cooling experiments, the temperature of the chamber was lowered at a standard rate of 1 C per hour. Freezing in leaves was identified by a sudden increase in temperature as sensed by a nickel constantan thermocouple taped to the leaf surface. The period of cooling was generally 3–4 hr. At the end of cooling, the chamber temperature was raised slowly (2–3 C per hour) and the plants were kept at 4 C overnight. On the following day, they were returned to the greenhouse, where the temperature was 23 C. Eight trees were used in each experiment, half receiving 2 C temperature treatment (control), and half exposed to the cooler treatment. On the seventh day after inoculation, leaf infections were recorded as the number of necrotic spots per square centimeter of leaf tissue on the four youngest leaves.

Ice nucleation activity of bacterial suspensions. Bacterial

suspensions were spotted on paraffin-coated aluminum foil boats and floated on a refrigerated bath at −5 and −10 C. The number of drops that froze within 2 min was recorded and the nucleation frequency was determined with regard to the bacterial concentration of the suspension (10,17,19).

Water soaking and weight increase of leaves. The lower surface of the leaves was visually observed for the appearance of water soaking continually during and after the cooling period. Weight increase of leaves was determined at different temperatures after spraying. At the desired temperature, plants were moved to a dark chamber, where the temperature was 5 C. They were left to equilibrate for 30 min, then the leaves were detached, blotted to remove any free surface suspension, and weighed immediately. To promote evaporation of the absorbed water, leaves were left in the light at room temperature until the water-soaked areas disappeared (about 15 min) and weighed again. The loss in weight was corrected with the loss in weight of control leaves (sprayed and held at 20 C).

Estimation of bacterial number in the leaves. In these experiments, streptomycin- and rifampicin-resistant derivatives of strain H9 of *P. s.* pv. *syringae* and strain NCPPB 1427 of *P. s.* pv. *tabaci* were used. Leaves were surface sterilized by dipping them for 10 sec in 80% ethanol and then for 2 min in 1% sodium hypochlorite. They were then thoroughly washed in sterile distilled water and macerated in sterile water (1 g per milliliter). Samples (0.1 ml) from a 10-fold dilution series were each spread onto nutrient agar containing streptomycin and rifampicin and colonies counted after 4 days of incubation at 28 C.

Freezing tests. Quick and slow freeze tests were conducted in parallel in climatic chambers. For the quick freeze tests, the temperature of the chamber was lowered to the desired level at a rate of 8–12 C per hour and kept at the specified level for 1 hr. When slower rates (1–4 C per hour) of cooling were desired, plants were placed in a box made of polystyrene slabs 7 cm thick. The box was placed in a climatic chamber, where the cooling rate was 8–12 C per hour. In these conditions, the temperature in the box cooled at a rate of 1–4 C per hour. Whole leaves from each treatment were tested for electrolyte leakage with a conductivity meter. Leaves were rinsed and submerged in glass distilled water (20 ml/g). To facilitate the outward movement of free ions, leaves were vacuum infiltrated in the water and slowly shaken for 2 hr. The final conductivity measurements were done on samples that were killed by holding at 80 C for 30 min. Injury was evaluated by the electrolyte leakage from the treated leaves (after 2 hr shaking) expressed as a percentage of that obtained after holding the leaves at 80 C for 30 min.

TABLE 1. Effect of postinoculation temperature on lesion appearance on sour cherry leaves inoculated with *Pseudomonas syringae* pv. *syringae* and *P. s.* pv. *morsprunorum*[x]

Temperature (C)	Strains[y]				
	H1(INA$^+$)	H9(INA$^+$)	H11(INA$^-$)	H13(INA$^-$)	146(INA$^-$)
20	0[z]	0	0	0	0
2	0	0	0	0	0
0	0	0	0	0	0
−1	0	0	0	0	0
−2	0.26 a	0.16 a	0	0	0
−3	1.30 b	1.15 b	1.86 b	1.58 b	0.13 a

[x] Plants sprayed with bacterial suspensions (10^8 cfu per milliliter) and cooled 1 C per hour. Cooling finished at different temperatures and plants incubated in a greenhouse (23 C).
[y] *P. s.* pv. *syringae*: H1, H9, H11, and H13; *P. s.* pv. *morsprunorum*: 146.
[z] Lesions per square centimeter of leaf tissue. Means followed by different letters are significantly different ($P = 0.01$) according to Duncan's multiple range test.

TABLE 2. The effect of different treatments on ice formation and symptom appearance[y]

Treatment	Freezing temperature (C)	Lesions[z] when cooling was finished (no.)		
		Before freezing	At freezing	After freezing
Water	−3.0 (−2.5– −3.5)	0	0	0
H1(INA$^+$)	−2.2 (−1.8– −2.8)	0	1.50 c	1.46 c
H9(INA$^+$)	−2.2 (−2.0– −2.9)	0	1.33 c	1.36 c
H11(INA$^-$)	−2.7 (−2.4– −3.5)	0	1.53 c	1.75 c
H13(INA$^-$)	−2.8 (−2.5– −3.5)	0	0.88 b	1.70 c
146(INA$^-$)	−2.8 (−2.4– −3.6)	0	0.26 a	0.33 a

[y] Plants sprayed and cooled 1 C per hour. When cooling was finished plants transferred to a greenhouse (23 C).
[z] Lesions per square centimeter of leaf tissue. Means followed by different letters are significantly different according to Duncan's multiple range test ($P = 0.01$).

RESULTS

Characteristics of the strains used. From the original 20 strains that we isolated, four were selected for further studies. All four strains (H1, H9, H11, H13) were *P. s.* pv. *syringae*. The INA and the syringomycin production of the strains varied. Strains H1 and H9 induced and strains H11, H13, and 146 (*P. s.* pv. *morsprunorum* from Switzerland) did not induce ice formation at −5 and −10 C. Strain H1 produced and strains H9, H11, H13, and 146 did not produce syringomycin. Symptoms induced by INA$^+$ and INA$^-$ strains were similar.

Influence of temperature on symptom appearance. To investigate the effect of temperature on symptom appearance, inoculated plants were incubated immediately after inoculation for 3–4 hr at different temperatures. The number of lesions per square centimeter of leaf tissue, resulting from different postinoculation leaf temperatures, is shown in Table 1. There were no symptoms when plants were held for 3 hr or longer after inoculation at 20, 2, 0, and −1 C. At −2 C, INA$^+$ strains H1 and H9 induced a limited number of lesions. INA$^-$ strains H11, H13, and 146 did not induce any symptoms at −2 C. At −3 C both INA$^+$ and INA$^-$ strains infected the leaves. Further decreasing the temperature to −4 to −5 C damaged the leaves and the efficiency of the infection could not be evaluated.

Effect of ice formation on symptom development. When leaves were sprayed with water they supercooled to −3 C (Table 2); however, the same water on aluminum foil supercooled to −20 C. INA$^+$ strains sprayed on the leaves increase the freezing temperature to −2.2 to −2.5 C. The freezing temperature of leaves sprayed with INA$^-$ bacteria was similar to the control (water). The difference between INA$^+$ and INA$^-$ strains was 0.2–0.6 C, but statistical analysis showed that the difference was not significant, because of variability of results. When cooling was stopped before the water in the leaf tissue froze, no symptoms appeared. In contrast, when cooling was continued to freezing or below (1 C), the typical symptoms always appeared (Table 2). That ice formation is a critical prerequisite for symptom development was further demonstrated by inducing freezing with ice crystals at −1 to −2 C. At this temperature, broken ice crystals were dropped on the leaf surfaces. The treatment generally induced freezing in the leaves within 1 min, as detected by a sudden increase of the temperature from −1 to 0 C. Freezing at −1 to −2 C had the same effect on symptom development as freezing at −2 to −3 C, i.e., symptoms were observed after freezing in the presence of bacteria. This provided further evidence that ice formation rather than low temperature is the critical factor in symptom development.

Water soaking and weight increase of leaves. When previously sprayed and frozen leaves were thawed, areas of water soaking appeared beneath the water drops (Fig. 1). During thawing, frozen leaves increased in weight by 2–4% of their fresh weight (Table 3). Weight increase depended upon ice formation. There was no water absorption at 2, 0, and −1 C, temperatures at which there was no freezing. When ice formation was induced with ice crystals at −1 C, the leaf weights also increased by 2–4% after thawing.

Bacterial numbers in the leaf tissues after thawing. Bacterial numbers were determined in the same leaves that were used for measuring water absorption. At 20, 2, 0, and −1 C, the bacterial numbers varied between 10 and 500 per gram of leaf tissue (Table

3). Freezing at −2.2 to −3 C dramatically increased the number of bacteria in leaves. Their number reached 5×10^3 per gram of leaf tissue. More bacteria were isolated from leaves that gained greater amounts of weight during thawing. When frozen leaves were sampled before thawing, the number of bacteria was less than 100 per gram of leaf tissue. The whole experiment was repeated using *P. s.* pv. *tabaci* for spraying sour cherry leaves. The same pattern was observed.

Mechanism of ingress of bacteria. One way bacteria may enter the intercellular space of the leaf tissue is via the stomata. Because most leaves have more stomata on lower than the upper leaf surfaces, the importance of stomata in the infection process was assessed by covering the lower surfaces of the selected young leaves with a plastic foil before spraying. The foil was removed after spraying and care was taken to ensure that the surface remained free of bacterial suspension during cooling to the freezing point. When the lower surface was covered and the upper surface sprayed with bacterial suspensions, only a few (1–2) lesions appeared on the leaves. In contrast, when the lower surfaces were sprayed and the upper surfaces covered, more than one lesion per square centimeter of leaf tissue appeared. Because there are many more stomata on the lower surfaces than on the upper surfaces (according to our microscopic observations), it is probable that stomata are the main avenues for bacterial entry.

Effect of freezing and mechanical injuries on symptom development. When inoculated plants were cooled to the freezing point at different rates, the results show (Table 4) a clear difference in electrolyte leaching between slow and quick freezing. In most of the cases, there was no visible damage to the leaves, but sometimes at the edges of the leaves cooled at a rate of 12 C per hour, some frost damage appeared. In spite of the obvious injury differences between the treatments, there was no real difference in the lesion numbers. The freezing injuries did not increase the number of lesions. In a separate experiment, just before inoculation, leaves were sprinkled with Carborundum (500 grit) and rubbed. No difference was found in lesion numbers between treated and nontreated plants. At 20 C, none of the leaves showed symptoms, in spite of the mechanical injuries caused by Carborundum before inoculation. This experiment shows that neither freezing nor mechanical injuries can open new avenues for infection.

Effect of freezing before inoculation. Plants were cooled to freezing and left at this temperature for 30 min. After this treatment they were brought to a 5 or a 22 C chamber and sprayed with bacterial suspensions at different times. The results (Table 5) show that inoculations were successful in the first 20 min after warming the leaves. After 30 min, no symptoms were observed. The difference between the two postfreezing treatments was due to the slower melting of ice at 5 C than at 22 C.

Effect of leaf wetness and bacterial number on symptom development. Plants were sprayed with bacterial suspensions at 20 C and left for 4 hr at this temperature (relative humidity 60%). By the end of this period, leaves were perfectly dry. Plants were then cooled to freezing, slowly thawed, and treated as earlier described. When treated by this method, none of the leaves showed any lesions. This indicates that without water on the leaf surface, bacteria are unable to gain entry to the leaf tissue, even after the leaf is frozen. Control plants were sprayed with a dilution series of bacterial suspensions and immediately cooled. Lesion counting showed that bacterial suspensions containing $1.2 \times 10^3 - 1.2 \times 10^8$ cfu per milliliter infected the leaves equally well. It was observed that the higher the bacterial number of the inoculum, the shorter the incubation period before symptom development. Less than 1.2×10^3 cfu per milliliter did not induce visible symptoms.

Fig. 1. Water-soaked areas on sour cherry leaf after freezing and thawing. Leaves were sprayed with a suspension (10^8 cfu per milliliter) of *Pseudomonas syringae* pv. *syringae*, cooled to freezing point of the leaves and left to thaw.

TABLE 3. Water and bacteria absorption of leaves after cooling and by thawing[z]

Cooling (C)	Water uptake (mg/g)	Bacteria in leaf tissue (no./g)
Before freezing		
2	0	15 d
0	0	18 d
−1	0	22 d
−2	4 a	225 e
After freezing		
−2	24 b	4,225 f
−3	42 c	5,251 f

[z] Plants sprayed with a suspension (10^8 cfu per milliliter) of *Pseudomonas syringae* pv. *syringae* (H9) and cooled 1 C per hour. At the desired temperature, plants brought to a dark chamber at 5 C, and left to thaw for 30 min. Leaves were detached, weighed, and left to evaporate in the light at room temperature until the water-soaked areas disappeared (15 min). They were weighed again, surface sterilized, and their bacterial number was determined. Results represent means of four separate experiments with two replicates each; each replicate consisted of four leaves. Means with same letter within columns were not significantly different ($P = 0.01$), according to Duncan's multiple range test.

TABLE 4. The effect of freezing injury on number of lesions[y]

Freezing rate (C/hr)	Electrolyte leakage (%)[z]	Lesions on leaf (no./c²)
1	3	1.51 c
4	3	1.55 c
8	11	0.63 b
12	24	0.11 a

[y] Plants were sprayed with a suspension (10^8 cfu per milliliter) of *P. syringae* pv. *syringae* (H9) and cooled to freezing at different rates. After thawing at 4 C, leaves were detached, rinsed, submerged in water, and vacuum infiltrated. Electrolyte losses were measured after 2 hr of shaking. The final conductivity measurement was made on the samples that were killed by treating at 80 C for 30 min.

[z] $\% = \frac{\text{electrolyte loss after 2 hr}}{\text{electrolyte loss of the killed sample}} \times 100.$

Results represent means of two experiments with two replicates each; each replicate consisted of four leaves. Means with same letter were not significantly different ($P = 0.01$), according to Duncan's multiple range test.

TABLE 5. The influence of freezing before inoculation on symptom appearance[y]

Spraying after freezing (min)	Postfreezing temperature for 2 hr[z]	
	5 C	20 C
0	1.25 c	1.55 c
10	0.76 b	0.15 a
20	0.21 a	0
30	0	0
60	0	0
120	0	0

[y] Plants cooled to freezing and left for 30 min. Then they were brought to a 5 or 20 C chamber and sprayed with suspension (10^8 cfu per milliliter) of *Pseudomonas syringae* pv. *syringae* (H1) at different times. Plants incubated in a greenhouse (23 C).

[z] Lesions per square centimeter of leaf tissue. Means followed by different letters are significantly different according to Duncan's multiple range test ($P = 0.01$).

DISCUSSION

The present investigation serves to increase our understanding of the mode of ingress of *P. s.* pv. *syringae* and *P. s.* pv. *morsprunorum* into sour cherry leaves following spring frosts. Plant tissues may freeze by two mechanisms. In spring frosts, when the temperature drop is slow, the process involves the formation of ice crystals only in the intercellular spaces (13,15). If, however, the temperature drop occurs very rapidly in the tissue, intracellular freezing eventually occurs. Intracellular freezing is invariably fatal to cells, because of a mechanical disorganization of the protoplasm caused by the ice crystals that form (6,15). On the other hand, where freezing is extracellular, the cell contents are merely concentrated by dehydration. Plant tissues generally supercool to −3 to −8 C before spontaneous nucleation occurs. Ice formation requires the presence of a suitable ice nucleus. Freezing does not occur until the crystallization of supercooled water is catalyzed by the most active ice nucleus associated with the plant (12). In deciduous fruit trees, crystallization can be induced at −2 to −3 C either by INA^+ bacteria (16) and/or by the woody part of the tree itself (3–5,11,21). The relative importance of these two inducers in the freezing of leaf tissues has not been determined. Under radiative conditions, leaves cool below air temperature, resulting in leaf temperature 1 to 2 C colder than stems at the moment of freezing (1,2,18). On the other hand, in incubators no relationship was observed between the population of INA^+ bacteria and the temperature at which ice formed in peach shoots (4).

In the leaf, as ice formation proceeds, ice crystals occupy the intercellular spaces and the air is squeezed out of the tissue. During this time, the solid matter of the tissue contracts and the protoplasm shrinks (15). By thawing the ice in these spaces and by decompressing the solid matter of the tissue, bacteria suspended in water drops on the leaf surface are sucked into the intercellular spaces. This was assumed to be the case in our study because of the increased leaf weights and bacterial numbers per leaf. This ingress of bacteria may be a passive way for host colonization, because *P. s.* pv. *tabaci*, an incompatible pathogen of sour cherry, entered into sour cherry leaves as well as *P. s.* pv. *syringae*. After freezing, relatively low populations of bacteria (1.2×10^3 cfu per milliliter) were required to incite the disease. This suggests that this type of infection is very effective and once the initial inoculum is present on the leaf, the epiphytic population of the pathogen is not a limiting factor in the infection. Other factors (moisture, ice), which limit the ingress of the pathogen into the leaf, seem to control the rate of disease progress. The optimum temperature for infection of sour cherry leaves with *P. s.* pv. *syringae* ranged between −2 and −3 C. Leaf freezing was not substantially influenced by INA^+ bacteria. Investigations of symptom development and leaf freezing clearly showed that the infection is not due to the low temperature, but rather to ice formation in the leaf tissue. When ice had been formed in the tissue, the symptoms always appeared 5–7 days later at 23 C. Without freezing no symptoms were observed. The degree of incidence of freezing injury was assessed by comparing the amount of electrolyte loss obtained after slow freezing with that obtained after rapid freezing. There was substantial difference between the two treatments, indicating that injuries had been caused by rapid freezing. In spite of this, injuries caused by either freezing or mechanical wounding with Carborundum dust apparently did not increase the number of lesions. Thus, the hypothesis that the infection resulted from micro wounds or plasmamembrane damage caused by freezing must be eliminated. Inoculations after freezing were successful only when the inoculum was applied in the first 20 min after removing plants from the cold chamber. This is in agreement with earlier results (20), when blossom blight of pear was increased by subjecting the plants to freezing. On the basis of our results, it is probable that sucking the bacterial suspension into the tissue was also the principal cause of the increased disease incidence seen in that study. Leaf wetness is a prerequisite factor in most of the leaf infections induced by bacteria. As has been reported (13) leaf infection of sour cherry may also occur when plants are maintained under high relative humidity and with free water on the leaf surfaces (at least 24 hr according to our observations). Freezing of previously sprayed leaves that had been dried resulted in no infections, demonstrating that bacteria must be suspended in water for the occurrence of infection.

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