EVALUATION OF ICE-NUCLEATING MICROORGANISMS FOR REDUCING THE SUPERCOOLING CAPACITY AND COLD-HARDINESS OF *CACOPSYLLA PYRICOLA* (HEMIPTERA: PSYLLIDAE)

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Abstract

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In laboratory studies, suspensions of killed and live ice-nucleating microorganisms were used to decrease the supercooling capacity of the winter form of pear psylla, *Cacopsylla pyricola* (Foerster) (Hemiptera: Psyllidae). Dry, untreated adults supercooled extensively before they froze at –22.7°C. Application of 1000 ppm of a preparation of the killed ice-nucleating bacterium, *Pseudomonas syringae* Van Hall 1902 (Pseudomonadaceae), significantly decreased the adults' supercooling capacity causing some individuals to freeze at temperatures as high as –3.9°C. Topical application of several live microorganisms also reduced the supercooling capacity of adults significantly; *Pseudomonas putida* (Trevisan 1989) was the most effective, causing more than 80% of *C. pyricola* adults to freeze at –15°C or higher. Furthermore, the temperature of crystallization of adults treated with *P. putida* remained significantly higher than controls for at least 11 d post-treatment. Application of ice-nucleating microorganisms also reduced the capacity of adults to survive short-term exposure to high subzero temperatures comparable to a mild frost. Realization of this approach for biological control of pear psylla will require the development of methods for the delivery of microorganisms to overwintering adults under field conditions.

Lee RE, Litzgus JD, Mugnano JA, Lee MR, Horton DR, Dunley J. 1999. Capacité des microorganismes qui favorisent la formation de cristaux de glace de réduire le pouvoir de surfusion et la résistance au froid chez *Cacopsylla pyricola* (Hemiptera : Psyllidae). *The Canadian Entomologist* **131** : 715–723.

Résumé

Au cours d'études en laboratoire, des suspensions de microorganismes morts ou vivants capables de déclencher la formation de cristaux de glace, ont été utilisées pour réduire la capacité de surfusion de la forme d'hiver de la Psylle du poirier, *Cacopsylla pyricola* (Foerster) (Hemiptera : Psyllidae). Des adultes secs, non traités, ont subi une importante période de surfusion avant de geler à –22,7°C. L'application de 1000 ppm d'une préparation de bactéries mortes, *Pseudomonas syringae* Van Hall

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1902 (Pseudomonadaceae), a diminué significativement la capacité de surfusion des adultes et certains ont gelé dès que la température a atteint –3,9°C. Une application localisée de plusieurs microorganismes vivants a également résulté en une réduction significative de la capacité de surfusion des adultes. *Pseudomonas putida* (Trevisan 1889) s'est avéré le microorganisme le plus efficace, causant le gel de plus de 80% des adultes de *C. pyricola* à –15°C ou à des températures plus élevées. De plus, la température de cristallisation des adultes traités au moyen de *P. putida* est restée significativement plus élevée que celle des témoins durant au moins 11 jours. L'application de microorganismes capables de provoquer la formation de cristaux de glace a également réduit la capacité des adultes de *C. psylla* de survivre à de courtes expositions à des températures légèrement sous zéro comparables à celles qui prévalent au cours d'un gel léger. L'utilisation de cette approche dans la lutte contre la Psylle du poirier suppose la mise au point de méthodes pour mettre en contact les microorganismes et les adultes de la forme d'hiver de la psylle sur le terrain.

[Traduit par la Rédaction]

Introduction

Pear psylla, *Cacopsylla pyricola* (Foerster) (Hemiptera: Psyllidae), is the most serious insect pest of pears in western North America. This insect causes damage either directly by feeding on plant tissues or indirectly by the production of honeydew that results in russeting of the fruit, making it unmarketable (Brunner and Burts 1981). Although current control measures rely on chemical insecticides, this pest has rapidly developed resistance against a wide range of insecticides (Croft *et al.* 1989; Pree *et al.* 1990). This development of resistance, and current trends that either prohibit or discourage the use of neurotoxic insecticides, emphasize the need to develop alternative forms of cultural and biological control for this major pest.

A recent, novel method of biological control proposes using ice-nucleating bacteria to decrease the cold hardiness of insect pests during the winter (Lee 1991; Fields 1992; Lee *et al.* 1992, 1993, 1995*a*). Most insects are freezing intolerant and survive the winter by increasing their capacity to supercool (i.e., remain unfrozen at temperatures below 0°C). However, studies on a wide range of insects, including stored product pests (Fields 1993; Fields *et al.* 1995; Lee *et al.* 1992) and the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (Lee *et al.* 1994), have shown that application of ice-nucleating bacteria dramatically decreases the supercooling capacity of overwintering stages. This decrease significantly reduces their ability to survive even relatively mild subzero temperatures.

A portion of the pear psylla population overwinters in bark crevices or beneath bark scales on pear trees (Horton *et al.* 1996). Because this species inhabits areas that can experience fairly severe winter temperatures (Horton and Lewis 1996), and because in preliminary experiments we found that this species supercools extensively but does not survive freezing, we investigated whether it was possible to reduce the capacity of its winter-form stage to supercool and survive subzero temperatures by treatment with ice-nucleating microorganisms. Also, we examined how long the supercooling capacity was diminished after treatment with the most effective bacterial suspension, live *Pseudomonas putida* (Trevisan 1889) (Pseudomonadaceae).

Materials and Methods

Overwintering adults of pear psylla were collected from pear orchards in Washington State in October 1995, January 1996, and January and February 1997, and

shipped overnight to Miami University, Ohio, where they were stored for 2 weeks or less at 4°C until used in experiments.

Determination of the Temperature of Crystallization (T_c). Adults were individually cooled in two nested 10-µL disposable pipet tips so that the insect was located in the lower pipet tip. A 36-gauge copper-constantan thermocouple was threaded through the upper pipet tip and positioned next to the adult so that it detected the insect's exotherm produced at the onset of internal freezing of body water. The temperature at which the exotherm was first detected was recorded as the temperature of crystallization (T_c). In a walk-in environmental chamber set at 4°C, adults were loaded into pipet tips using a camel hair paint brush. Each unit was placed in a glass test tube (13 × 100 mm) supported by a foam platform, and a foam plug was used to seal the upper part of each tube. The foam platform was then lowered into a Neslab RTE 140 refrigerated ethanol bath initially set at 0°C, and then cooled at 1.5°C/min until all insects froze.

Effect of Topical Application of Ice-nucleating Microorganisms on T_c. Bacterial and fungal ice-nucleating agents were applied topically to determine their effect on the supercooling capacity of adult pear psylla using methods that have been described previously (Lee *et al.* 1992, 1994, 1996; Strong-Gunderson *et al.* 1992, 1994). We used suspensions of a commercially available, freeze-dried, killed preparation of *Pseudomonas syringae* van Hall 1902 (activity = 2.02×10^4 ice-nucleating sites/g) provided by Genencor International, Inc. (San Francisco, California). Suspensions of this preparation (1, 10, 100, 1000, and 10 000 ppm) were prepared in sterile distilled water.

Suspensions (approximately 10^9 cells/mL) of four species of live ice-nucleating bacteria were prepared from colonies of *Enterobacter agglomerans* BBI Ewing and Fife 1972 (Enterobacteriaceae) (originally isolated from an insect; Lee *et al.* 1991), *P. putida* F31 and *Pseudomonas fluorescens* F12 (Trevisan 1889) (originally isolated from a frog; Lee *et al.* 1995*b*), and *P. syringae* cit7 (provided courtesy of S. Lindow, Berkeley, California). Cultures were grown aerobically on nutrient agar with glycerol (NAG; 2.5% v/v) plates at 20°C for 48 h. An ice-nucleating strain of the fungus *Fusarium avenaceum* (Corda ex Fr.) Sacc. strain 411 (Hyphomycetes) (provided by Stephan Pouleur and Claude Richard, Agriculture Canada, Sainte-Foy, Quebec) was grown on parafilmed potato flake agar (PFA) plates at 25°C in the dark. A suspension of *F. avenaceum* was made by scraping 30 mg of mycelium harvested from approximately 1 cm^2 area of the culture, which was then suspended in 0.5 mL of sterile distilled water.

The ice-nucleating activity of the microbial cultures was assayed using a freezing-droplet method (Vali 1971) as modified by Lindow (1982). Nucleating activity of each suspension was assessed for a group of 120 droplets of 10 µL each, which was applied to an aluminum pan placed in a beaker suspended in a refrigerated bath (Neslab RTE 140). As the pan was cooled (0.3°C/min) from 0° to –6°C, the temperature at which each droplet froze was recorded.

Using a camel hair paint brush, pear psylla were transferred to a 100-mm plastic Petri dish lined with filter paper (Whatman No. 5) at 4°C. Using a plastic atomizer, pear psylla were misted with approximately 0.2 mL of the appropriate microbial suspension through an inverted plastic funnel placed over the Petri dish. Pear psylla were held for a minimum of 2 h at 10°C before their T_cs were determined as described above. In the experiment examining the effects of various concentrations of killed *P. syringae*, T_cs were compared among control and treated pear psylla using a Kruskal–Wallis (KW) Nonparametric ANOVA, followed by a Dunn's multiple comparisons test. In the experiments comparing the effects of various live microbial suspensions on supercooling capacity, T_cs were compared using an ANOVA, followed by a Tukey–Kramer test.

Survival at Subzero Temperatures. We determined the effect of topical application of a killed *P. syringae* preparation (1000 ppm) relative to water on pear psylla survival at –4.4°C, a temperature selected to simulate a mild winter frost. October-collected pear psylla were loaded into a 1.5-mL microcentrifuge tube and misted (140 ± 10 µL; mean ± SE) with their respective treatment before polyester batting was placed below and on top of adults to wick away any excess moisture. Frozen treatment groups were held at –4.4°C for 24 h, whereas unfrozen (control) treatments were held at 4°C for 24 h. Survival was compared among groups using a χ^2 analysis, followed by a Bonferroni-adjusted Fisher's exact test.

A similar experiment examined survival after treating pear psylla with live ice-nucleating microorganisms. The treatments for this experiment were a wet control and suspensions of *P. syringae*, *P. putida*, *P. fluorescens*, and *E. agglomerans* (prepared as described above). A 60-mm plastic Petri dish was lined with filter paper (Whatman No. 5) and 300 µL of each treatment suspension was pipetted onto the filter paper. In an environmental chamber set at 4°C, January/February-collected pear psylla were transferred onto the treated filter paper using a camel hair paint brush; each treatment was composed of 3 Petri dishes with 10 adults per Petri dish. Petri dishes were then loaded into a glass jar suspended in an ethanol cold bath (Forma Scientific Co.) set at 0°C. Pear psylla were cooled to –5°C and held for 16 h. The adults were then warmed to 0°C, removed from the bath, and held at 4°C for 1 h before survival was assessed at room temperature (23°C). Survival was compared among treatment groups using a χ^2 analysis, followed by a Bonferroni-adjusted Fisher's exact test.

Duration of T_c Elevation Following Treatment With *P. putida*. Because of the effectiveness of *P. putida* in elevating the T_c, we also determined the duration of this effect. As described above, pear psylla were placed in a Petri dish lined with filter paper and misted with approximately 0.2 mL of either the bacterial suspension or distilled water (control). Petri dishes containing pear psylla were kept at 10°C until T_c determinations were done periodically during the following 11 d. T_cs were compared among groups using ANOVA, followed by a Bonferroni multiple comparisons test.

Results and Discussion

Effect of Topical Application of Ice-nucleating Microorganisms on T_c. Topical application of ice-nucleating microorganisms reduced the supercooling capacity of pear psylla. October-collected, dry, untreated adults (n = 19) supercooled markedly before they froze at a T_c of –22.7 ± 0.6°C (mean ± SE). There were differences among the mean T_cs of pear psylla treated with serial dilutions of killed ice-nucleating *P. syringae* (KW = 33.68, $P < 0.0001$; Fig. 1). A minimum concentration of 1000 ppm was required to reduce the supercooling capacity of pear psylla ($P < 0.01$), and a 10 000 ppm *P. syringae* preparation was no more effective than a 1000 ppm preparation ($P > 0.05$). At these highest concentrations, some adults began to freeze at temperatures as high as –3.9°C compared with untreated adults, which started to freeze at –15°C. Concentrations of 1, 10, and 100 ppm did not elevate the mean T_cs compared with untreated controls ($P > 0.05$ in all cases).

To investigate the effect of using live aqueous suspensions of ice-nucleating microorganisms on T_c, we selected five strains with relatively high ice-nucleating activity. Using the freezing droplet assay, we determined that the fungus *F. avenaceum* and the bacterium *P. syringae* preparations had the highest T_{max} (–2.0°C), and *P. syringae* had the highest T_{50} (Table 1). There were differences in mean T_cs among pear psylla treated with live microbial suspensions ($F_{6,67} = 4.50$, $P < 0.001$; Fig. 2). Topical application of

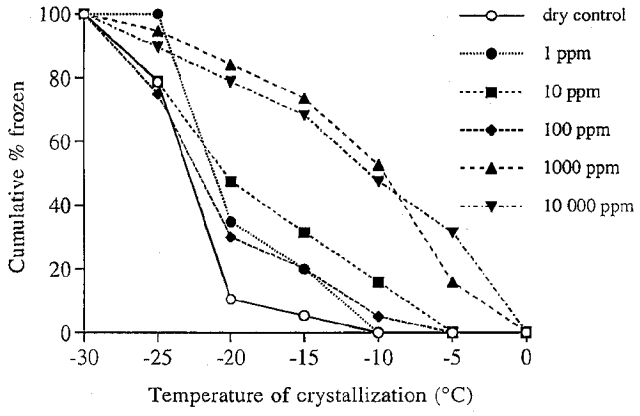

FIGURE. 1. Cumulative freezing profile of October-collected adult *Cacopsylla pyricola* treated with various concentrations of a killed preparation of the ice-nucleating bacterium *Pseudomonas syringae*. Each curve is based on n = 19–20 individuals.

TABLE 1. Ice-nucleating activity of live bacterial and fungal suspensions based on 120 droplets of 10 µL each of aqueous suspension in the freezing-droplet assay, and survival of adult pear psylla (*C. pyricola*) after treatment with suspensions (approximately 10^9 cells/mL) of these microbes, followed by exposure to –5.0°C for 16 h.

Treatment	Temperature (°C)*			% survival (alive/total)
	T_{max}	T_{50}	T_{90}	
Water (control)	—	—	—	86 (25/29)
F. avenaceum	–2.0	–3.6	–3.9	—
P. syringae	–2.0	–2.6	–2.9	70 (19/27)
P. putida	–2.5	–2.9	–3.3	47 (14/30)†
P. fluorescens	–3.3	–4.1	–4.4	50 (15/30)†
E. agglomerans	–2.5	–3.5	–4.8	70 (21/30)

*T_{max}, T_{50}, and T_{90} are the temperatures at which the first drop, and 50 and 90% of the drops froze, respectively.
†Indicates a significant reduction in survival relative to water control (χ^2 test; *P. putida*, P = 0.0022; *P. fluorescens*, P = 0.0048).

P. putida reduced the supercooling capacity of pear psylla relative to both the dry control ($P < 0.01$) and wet (treated with water only) control ($P < 0.001$) treatments. Treatment with *P. putida* caused some individuals to freeze as high as –3.3°C, which was only 0.8°C below the T_{max} for this suspension (Table 1). In addition, *P. putida* reduced the supercooling capacity of pear psylla relative to those treated with topical applications of *E. agglomerans* ($P < 0.05$) and *F. avenaceum* ($P < 0.05$). More than 80% of the pear psylla treated with *P. putida* froze at –15°C or higher compared with the other microbial suspensions in which less than 45% of the individuals froze by this temperature (Fig. 2).

Survival at Subzero Temperatures. We also conducted short-term survival tests of 24 h or less under mild subzero conditions selected to simulate a mild frost that commonly occurs during the winter in Washington State (Horton *et al.* 1996). Survival among October-collected pear psylla treated with water or killed *P. syringae* differed ($\chi^2_3 = 42.1$, $P < 0.0001$; Table 2). Adults treated with 1000 ppm of the *P. syringae* preparation had significantly reduced survival (22%) compared with water-treated controls (56%) following a 24 h exposure to –4.4°C (P = 0.0073). Treatment with water followed

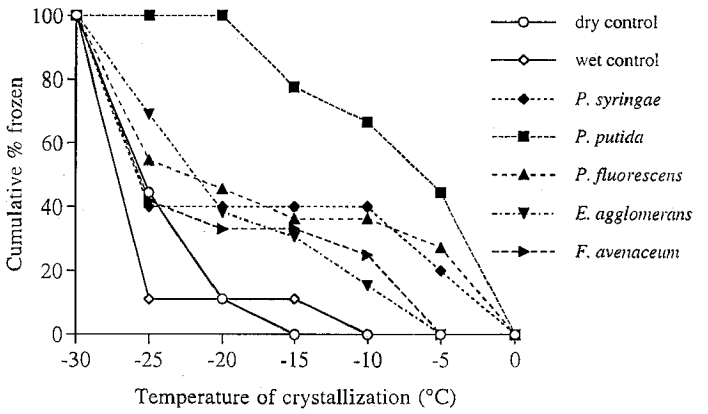

FIGURE. 2. Cumulative freezing profile of January/February-collected adult *Cacopsylla pyricola* treated with suspensions of living ice-nucleating bacteria or a fungus. Most curves are based on n = 9–12 individuals.

TABLE 2. Survival of adult *Cacopsylla pyricola* after treatment with a killed preparation of ice-nucleating *Pseudomonas syringae* (1000 ppm) compared with water. Survival was assessed after exposure to 4°C (unfrozen) or –4.4°C (frozen) for 24 h.

Treatment	% survival (alive/total)*
Water	
Unfrozen	100 (20/20)^{a}
Frozen	56 (20/36)^{b}
P. syringae	
Unfrozen	90 (18/20)^{a,b}
Frozen	22 (8/36)^{c}

*Values for survival with different superscripts were statistically distinguishable (Bonferroni-adjusted Fisher's exact test; $P < 0.0083$).

by freezing exposure also reduced pear psylla survival (P = 0.0002) relative to unfrozen controls. Survival of both groups in the unfrozen (4°C) control treatments was high (water treated, 100%; bacterial treated, 90%) and did not differ (P = 0.49).

Survival among January/February-collected adult pear psylla treated with four living microbial suspensions and held for 16 h at –5°C differed (χ^2_4 = 13.7, P = 0.0084; Table 1). Relative to the water control, *P. putida* (P = 0.0022) and *P. fluorescens* (P = 0.0048) significantly reduced the survival of adults.

Duration of T_c Elevation Following Treatment With *P. putida*. Because the *P. putida* preparation was most effective in significantly elevating the T_c of a large proportion of the adult pear psylla (Fig. 2), we investigated how long the effect would last (Fig. 3). Between 2 and 11 d post-treatment, the mean T_cs ranged from –8.2° to –16.0°C and were higher than control values, which had means of –24.2°C or lower ($F_{9,104}$ = 10.0, $P < 0.0001$). These data indicate that the T_c remains elevated for more than a week after application, and thus support the idea that the cold tolerance of this species could be compromised by field application of an ice-nucleating agent a few days before the arrival of a predicted cold front.

Application of water alone appears to be only moderately effective in promoting inoculative freezing at high subzero temperatures. Adult pear psylla supercooled

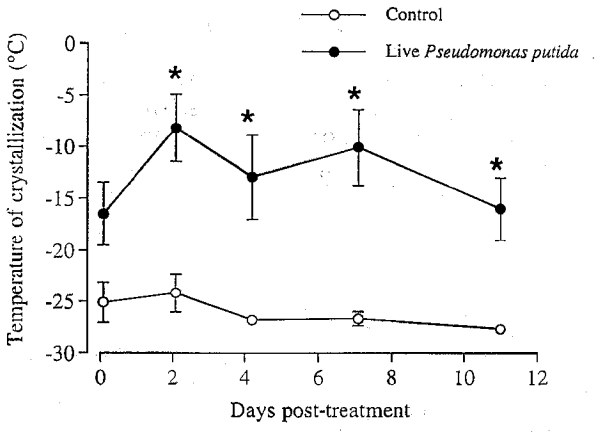

FIGURE. 3. **Duration of temperature of crystallization (T_c; mean ± SE, n = 10–14 for each time point) elevation of adult *Cacopsylla pyricola* following treatment with a live aqueous suspension of the ice-nucleating bacterium *Pseudomonas putida*. The asterisk indicates significant differences in mean T_c between the bacterial treatment and the water control at time intervals post-treatment application (ANOVA; $P < 0.01$).**

extensively (T_c = –26.7 ± 1.8°C, mean ± SE) with no individuals freezing above –10°C after misting with water (Fig. 2). These results are consistent with the moderate levels of mortality that we observed following short-term exposure to subzero temperatures (Tables 1 and 2). Our results are also consistent with those of Horton *et al.* (1996) who reported no mortality and less than 25% in water-misted adults following 24 h at –1 and –6°C, respectively.

Potential for Use in Biological Control. Temperatures that caused mortality in pear psylla misted with suspensions of ice-nucleating microorganisms in this study were within the typical range of winter temperatures occurring within the major pear-growing regions of the Pacific Northwest. Indeed, minimum winter air temperatures in central Washington pear orchards reach –25°C (National Oceanic and Atmospheric Administration). Difficulties in controlling pear psylla with conventional methods have prompted the search for new management strategies, including techniques that attack the overwintering lifestage (Krysan 1990). The application of ice-nucleating organisms in fall or winter to reduce overwintering survival may be useful for biological control of pear psylla if methods can be developed to ensure that the material contacts the overwintering insects. A possible difficulty with this approach is related to the dispersal behaviour of the pear psylla overwintering form. An unknown proportion of the overwintering population disperses from the pear orchard in late fall and early winter (Horton *et al.* 1994). Thus, these individuals would escape contact with the ice-nucleating microorganisms if the application occurred late in the season. Despite this potential difficulty, however, results reported herein suggest that a fall or winter control program involving the use of ice-nucleating microorganisms could prove to be a valuable part of an integrated pest management (IPM) program for pear psylla.

In summary, our data indicate that the supercooling capacity of the winterform of pear psylla is reduced, and low temperature tolerance is compromised by topical application of both killed and live suspensions of ice nucleating microorganisms. Because the effect of the *P. putida* application on pear psylla was retained for at least 11 d, it is possible that bacterial agents may be applied in the field in anticipation of cold weather as a means of inducing winter mortality of the resident pest population.

Acknowledgments

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