

Ice-Nucleating Active Bacteria Reduce the Cold-Hardiness of the Freeze-Intolerant Colorado Potato Beetle (Coleoptera: Chrysomelidae)

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ABSTRACT In laboratory experiments, a freeze-dried concentrated form of the ice-nucleating active bacteria, *Pseudomonas syringae*, was used to decrease the supercooling capacity of field-collected diapausing Colorado potato beetles, *Leptinotarsa decemlineata* (Say). Application of the *P. syringae* to adult beetles increased their mean supercooling point values from $-7.6 \pm 0.2^\circ\text{C}$ (untreated) to $-3.7 \pm 0.1^\circ\text{C}$ (1,000 ppm). No beetles survived cooling to temperatures below their supercooling point, indicating that this species is freeze-intolerant. During tests conducted in 1991 and 1992, the increase in the supercooling point was directly dependent on the amount of *P. syringae* added to soil containing the beetles. Cumulative freezing distributions indicated that 80% of beetles treated with 100 ppm of *P. syringae* would be expected to freeze and die when exposed to -5°C ; in contrast, none or very few of the untreated control beetles would be expected to freeze at this temperature. Other experiments demonstrated that the capacity of *P. syringae* treatments to increase the supercooling point of the beetles decreased after 2 wk of exposure at 4°C and when experiments were done at 10°C . If delivery systems were developed that would expose adult beetles to ice nucleating agents and preserve their ice nucleating activity until critical low-temperature exposure occurs in mid-winter, these nucleating agents could be used in conjunction with cultural control strategies for increasing winter mortality.

KEY WORDS *Leptinotarsa decemlineata*, cold-hardiness, ice nucleating bacteria

THE COLORADO POTATO BEETLE, *Leptinotarsa decemlineata* (Say), is the most serious defoliating pest of potatoes, *Solanum tuberosum* L., in North America. Its status as a serious pest has evolved largely as a consequence of its development of insecticide resistance and the current practice of planting extensive potato monocultures that promote the cumulative build up of Colorado potato beetle populations from year-to-year (Casagrande 1987, Ioannidis et al. 1991). Consequently, efforts to control this pest have focused increasingly on alternative approaches.

One control strategy relies on cultural methods to rapidly expose beetles to lethal subzero temperatures during the winter (Kung et al. 1992, Milner et al. 1992). Specifically, adults are induced to aggregate outside normal overwinter-

ing sites by planting trap crops late in the growing season on the edges of fields. Mulching these areas would encourage beetles to remain at the site of the trap crop to overwinter and may also reduce the depth to which the beetles burrow during the winter. Overwintering mortality of the beetles then can be increased when the mulch is removed in midwinter (Milner et al. 1992).

The supercooling point refers to the temperature at which ice nucleation occurs spontaneously within an insect (Lee 1991). For freeze-intolerant insects this value represents the absolute lower lethal temperature for survival. Many freeze-intolerant species seasonally depress their supercooling points, thereby increasing their cold-hardiness in preparation for winter. Previous studies have demonstrated that the supercooling point of cold-hardy but freeze-intolerant species may be increased by the application of ice-nucleating active bacteria (Fields 1990, 1992; Lee et al. 1991, 1992, 1993; Strong-Gunderson et al. 1990). Consequently, these bacteria may offer a novel means for the biological control of overwintering insects

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(Fields 1990, 1992; Lee 1991; Lee et al. 1993; Strong-Gunderson et al. 1990).

Our primary objective in this study was to determine whether ice-nucleating active bacteria could be used to elevate the supercooling point of diapausing adults of the Colorado potato beetle. We determined the effect of various concentrations of *Pseudomonas syringae* and the duration of its exposure on the supercooling point of beetles. To simulate overwintering conditions, we exposed beetles to ice-nucleating active bacteria mixed with soil. Our ultimate goal is to determine whether ice-nucleating active bacteria can be used in combination with the cultural control methods of Milner et al. (1992) to increase the susceptibility of overwintering populations of the Colorado potato beetle to low temperature.

Materials and Methods

Experimental Animals. Because our objective was to test Colorado potato beetles that had prepared naturally for overwintering, collections were made from fields in which the potato vines had been killed in preparation for harvesting in late August and early September from the Hancock Agricultural Station in central Wisconsin and shipped to Miami University by overnight carrier. When potato vines are killed, beetles normally leave the fields and search for suitable overwintering sites. When received at Miami University, beetles were held unfed at 15°C, 10:14 (L:D) h for 1–2 wk before they were separated into groups ($n = 25–50$), placed in plastic cups containing 200 g sand moistened with 15 ml water, and held in the dark at 4°C. Because under these conditions the beetles readily burrowed into the sand, we assumed that they were in the expected physiological state typical of overwintering individuals.

Ice-Nucleating Active Bacteria. Ice-nucleating active bacteria were formulated as a concentrated, freeze-dried, and killed preparation of *Pseudomonas syringae* provided by Genencor International, Rochester, NY. The *P. syringae* used in this study had an ice-nucleating activity of 2.02×10^4 ice-nucleating sites per gram.

The beetles were exposed to the bacterial preparation by thoroughly mixing it into moist sand to which beetles were subsequently introduced. Beetles were permitted to move about freely over this substrate for 48 h before we determined their supercooling points.

Supercooling Point Determination. Supercooling points were determined by positioning beetles in contact with a 30-gauge copper-constantan thermocouple inside a 1.5-ml polyethylene tube. The tubes were placed inside glass test tubes suspended in a refrigerated bath (5°C) and allowed to thermoequilibrate for 5 min before being cooled at $\approx 0.6^\circ\text{C min}^{-1}$. The lowest temper-

ature attained before the release of the latent heat of crystallization, caused by the formation of ice in body fluids, was recorded as the supercooling point.

Experimental Design. Three sets of experiments were conducted. The first set established whether the inert carrier portion for the dry, powdered *P. syringae* preparation had an effect on the supercooling point of *L. decemlineata*. The test involved running 2–4 replicates of $n = 11$ to 12 beetles in each of the following treatment groups: control (no additive), 100 ppm of carrier (ice-nucleating product lacking the *P. syringae* fraction), and 100 ppm of *P. syringae* in carrier.

In a second set of experiments, the dose–supercooling point relationship was determined for the *P. syringae* preparation in 1991 and 1992. These experiments involved 4–5 replicates of $n = 9$ to 12 beetles in each of the following treatment groups: control (no additive), 1, 10, 100, and 1,000 ppm *P. syringae*. The concentration of 1 ppm was tested only in 1992.

The third set of experiments examined factors related to the potential loss of ice-nucleation activity. First, the supercooling points of beetles were determined 1, 3, 7, 14, or 21 d after initial exposure to 0 ppm (control), 100 ppm, or 1,000 ppm ice-nucleating product. The beetles remained in contact with the treated substrate and were exposed to 4°C until they were tested. Testing was done using duplicate samples, except on day 21, in which case sufficient animals were available for only one test. For each treatment group, the reported mean supercooling point at each time interval was based on a total sample of $n = 8$ to 17 beetles.

The influence of incubation temperature on the effect of treatment with *P. syringae* was studied by comparing the supercooling points of beetles determined on the 7th day of their exposure to 0 ppm (control), 100 ppm, or 1,000 ppm *P. syringae* at 4°C against values from a separate set of beetles treated similarly but exposed to 10°C.

Supercooling point values were compared among groups using either one-factor or two-factor analysis of variance (ANOVA), with means distinguished ($P < 0.05$) using the least significant difference test (Snedecor & Cochran 1982).

Results and Discussion

Carrier Tests. Because the mean supercooling point of beetles treated with 100 ppm carrier was not statistically different than that of untreated beetles, we judged that the carrier used in the commercial preparation lacks nucleating activity (Table 1). In marked contrast, beetles treated with ice-nucleating product had a mean supercooling point that was 3.4 and 3.9°C higher ($F = 65.3$; $df = 2, 91$; $P < 0.001$) relative to

Table 1. Supercooling points (°C) of Colorado potato beetles incubated in sand (untreated) or sand containing 100 ppm of bacterial carrier or *P. syringae*

Treatment	n	Mean ± SEM
Untreated	46	-7.5a ± 0.34
Carrier	23	-8.0a ± 0.36
<i>P. syringae</i>	23	-4.1b ± 0.26

Within a column, means followed by the same letter are not significantly different ($P > 0.05$; Fisher's least significant differences test).

untreated and carrier-treated beetles, respectively. These results attest to the fact that ice-nucleating activity was attributable to the *P. syringae* preparation. The fact that no beetles survived cooling to temperatures below their supercooling point indicates that this species is freeze-intolerant.

Dose-Supercooling Point Relationship. Mean supercooling points of beetles treated with *P. syringae* in concentrations ranging from 0 to 1,000 ppm were determined for populations sampled in both 1991 and 1992 (Table 2). In 1991, the means of $n = 10$ to 11 beetles ranged from $-6.4 \pm 0.3^\circ\text{C}$ (untreated) to $-2.8 \pm 0.2^\circ\text{C}$ (1,000 ppm) and differed significantly ($F = 43.7$; $df = 3, 42$; $P < 0.001$) among the treatment groups. Similarly, in 1992 the supercooling point means ($n = 44$ to 58) increased when beetles were exposed to increasing concentrations of *P. syringae*, ranging from $-7.6 \pm 0.2^\circ\text{C}$ (untreated) to $-3.7 \pm 0.1^\circ\text{C}$ (1,000 ppm), and differed significantly ($F = 62.4$; $df = 4, 240$; $P < 0.001$) among the treatment groups. In these 1992 tests, a dose of 1 ppm resulted in a supercooling point that was statistically higher than that of the untreated control. However, the supercooling points of beetles treated with 1 ppm and 10 ppm could not be distinguished statistically. Additionally, doses of 100 ppm and 1,000 ppm, which were significantly more effective than doses of 1 and 10 ppm, resulted in sta-

Table 2. Effect of *P. syringae* on the supercooling point of diapausing adults of the Colorado potato beetle (shown ± 1 SEM)

Concentration	n	Mean ± SEM
1991		
0	11	-6.4 ± 0.3a
1	0	—
10	11	-4.8 ± 0.3b
100	10	-3.3 ± 0.2c
1,000	11	-2.8 ± 0.2c
1992		
0	58	-7.6 ± 0.2a
1	44	-5.9 ± 0.3b
10	46	-6.3 ± 0.2b
100	45	-4.2 ± 0.2c
1,000	58	-3.7 ± 0.1c

Means followed by the same letter are not significantly different ($P > 0.05$; Fisher's least significant differences test).

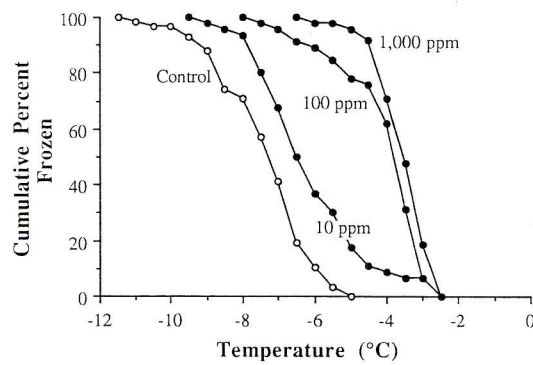


Fig. 1. Cumulative freezing profile based on individual supercooling points for Colorado potato beetles exposed to various concentrations of *P. syringae* in soil for 48 h at 4°C.

tistically indistinguishable supercooling points in both 1991 and 1992. These results suggest that the effect of *P. syringae* on the supercooling point elevation is dose-dependent, effective even at very low concentrations (e.g., 1 ppm), and most effective at concentrations of ≤ 100 ppm.

Cumulative freezing distributions based on individual supercooling point values were determined only for beetles used in the 1992 tests because sample sizes were substantially larger in this year (Fig. 1). These curves are useful because they show a profile of the theoretical, absolute lower lethal temperature for a population of beetles treated with various concentrations of *P. syringae*. For example, if beetles were exposed to -5°C , 80% of those treated with 100 ppm of *P. syringae* would be expected to freeze and die; in contrast, none or very few of the untreated control beetles would be expected to freeze at this temperature. In addition, the similarity of the 100 and 1,000 ppm curves further suggests that maximal effectiveness is achieved with 100 ppm.

Temporal and Thermal Aspects of Treatment with *Pseudomonas Syringae*. Analyses involving two-factor ANOVAs (incubation time versus *P. syringae* concentration) showed that mean supercooling point depended significantly on both incubation time ($F = 8.3$; $df = 4, 209$; $P < 0.001$) and bacterial concentration ($F = 41.2$; $df = 2, 209$; $P < 0.001$). The highly significant ($F = 6.0$; $df = 8, 209$; $P < 0.001$) interaction term indicated that the dose-response relationship varied markedly over time (Fig. 2). Mean supercooling points were significantly ($P < 0.05$) higher in 100 ppm *P. syringae* and 1,000 ppm *P. syringae* groups relative to untreated controls on days 1, 3, and 7, but not on days 14 and 21. Therefore, the nucleation activity of the bacterial preparation had been lost ≤ 2 wk after application at 4°C.

Analyses involving two-factor ANOVAs (temperature versus bacterial concentration) showed that the mean supercooling point measured 7 d

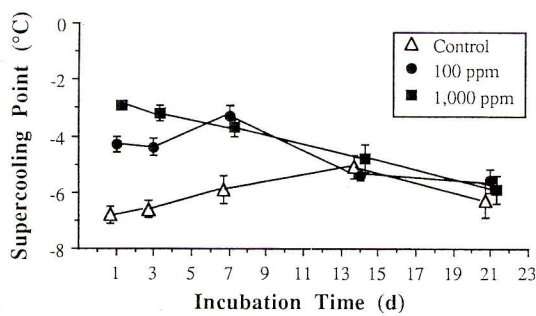


Fig. 2. Effect of the duration of exposure to *P. syringae* on the supercooling point of diapausing adults ($n = 8-17$) of the Colorado potato beetle. Beetles were exposed to 100 or 1,000 ppm *P. syringae* for up to 21 d at 4°C.

after treatment was influenced significantly by both incubation temperature ($F = 6.7$; $df = 1, 86$; $P = 0.012$) and *P. syringae* concentration ($F = 8.9$; $df = 2, 86$; $P < 0.001$). The highly significant ($F = 7.4$; $df = 2, 86$; $P = 0.001$) interaction term revealed that the dose-response relationship differed between the 4° and 10°C incubation groups (Table 3). Means for untreated beetles held at 4° and 10°C were similar. Conversely, beetles treated with 100 ppm *P. syringae* had significantly ($P < 0.05$) higher supercooling points after incubation at 4° than 10°C, although this trend was not significant with beetles treated with 1,000 ppm *P. syringae*. These results suggest that ice-nucleating activity was better retained during incubation at the lower temperature. Previous studies have reported a loss of activity with time at temperatures above 0°C (Lee et al. 1993).

The ice-nucleating activity of *P. syringae* used in these studies was sufficient at concentrations of 100 ppm to raise the supercooling point of field-collected, diapausing Colorado potato beetle adults by 2–3°C. In northern potato-growing regions, diapausing beetles overwinter at soil depths of 10–30 cm. At these depths the soil temperatures remain near 0°C when the soil surface is covered by an insulating layer of mulch or snow (Milner et al. 1992). Under such conditions, beetles rarely would be exposed to lethal winter temperatures. However, cultural manipulations designed to expose beetles to lethal low

Table 3. Supercooling points of Colorado potato beetles incubated at 4°C or 10°C untreated in sand or sand containing *P. syringae*

Treatment	4°C			10°C		
	Mean ± SEM	n		Mean ± SEM	n	
Untreated	-5.9 ± 0.5a	16		-5.3 ± 0.5a	16	
100 ppm	-3.3 ± 0.4a	14		-5.8 ± 0.3b	16	
1000 ppm	-3.7 ± 0.3a	17		-4.3 ± 0.3a	13	

Within a row, means followed by the same letter are not significantly different ($P > 0.05$).

temperatures by removing insulating layers may cause a rapid decrease in soil temperatures of 5 to 7°C at 20-cm depth (Kung et al. 1992, Milner et al. 1992). The cumulative freezing distributions (Fig. 1) clearly demonstrate the potential for ice-nucleating bacteria to increase mortality during such brief exposures to cold temperature shock. Beetles would be unlikely to experience lethal freezing at -5°C unless exposed to ice-nucleating agents that would be expected to increase mortality to 80% based on the data in Fig. 1. Consequently, ice-nucleating active bacteria would significantly increase the effectiveness of field strategies designed to expose beetles to lethal low soil temperatures. The geographical range of areas where a sufficiently low soil temperature could be attained to increase mortality would be expanded considerably if the lethal temperature were raised to -2 or -3°C using ice-nucleating microorganisms or agents. Similarly, the soil depth at which Colorado potato beetles would need to overwinter to avoid lethal exposure would be increased.

To integrate ice nucleation successfully into cultural strategies for increasing winter mortality, it will be necessary to design delivery systems that expose adult beetles to the ice-nucleating agent and preserve the agent's activity until critical low-temperature exposure occurs in midwinter.

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