Ice-Nucleating Active Bacteria Decrease the Cold-Hardiness of Stored Grain Insects

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ABSTRACT   This report provides further evidence that a freeze-dried, concentrated form of *Pseudomonas syringae*, an ice-nucleating active bacteria, reduces the cold tolerance of stored grain insect pests. Application of ice-nucleating bacteria to wheat or corn that contained insect pests decreased the insects' supercooling capacity: after treatment with 100 ppm of *P. syringae* the mean supercooling points of five insect species increased from 4.7 to 11.9°C above untreated controls. Treatment with *P. syringae* also decreased the capacity of insects to survive a 24-h exposure to subzero temperatures. Decreases in cold tolerance were observed in eight species of stored grain pests: Indianmeal moth larvae, *Plodia interpunctella* (Hübner); red flour beetle adults, *Tribolium castaneum* (Herbst); flat grain beetle adults, *Cryptolestes pusillus* (Schonherr); rusty grain beetle adults, *Cryptolestes ferrugineus* (Stephens); *Gibbium psylloides* (Czenpinski); lesser grain borer adults, *Rhyzopertha dominica* (F.); yellow mealworm larvae, *Tenebrio molitor* (L.); and granary weevil adults, *Sitophilus granarius* (L.). Results of this study provide further support for the use of ice-nucleating active bacteria as biological insecticides to kill overwintering insects by decreasing their low temperature tolerance. The approach may be particularly appropriate for the control of a variety of insect pests in restricted areas such as grain bins.

KEY WORDS   Insecta, cold-hardiness, ice nucleating bacteria, biological control

A MAJOR FACTOR in the overwintering survival of insect pests is their capacity to cold-harden (see reviews in Lee & Denlinger [1991]). Most overwintering insects are freeze-intolerant (Somme 1982). In the winter, many of these species survive by increasing their capacity to avoid freezing by supercooling, thus avoiding the lethal effects of internal ice formation (Ring 1982). Increases in supercooling capacity require the removal or inactivation of ice-nucleating catalysts (Lee 1989). Although several lines of evidence suggest that ice nucleation begins in the gut in some species, the precise nature of the heterogeneous ice-nucleating agent that regulates supercooling in freeze-intolerant insects is not clearly established (Baust & Rojas 1985, Lee 1989, Shimada 1989).

Ice-nucleating active bacteria are the most efficient heterogeneous ice-nucleating agents known. These ubiquitous bacteria are commonly found on the surface of plants and can induce freezing of plant tissues at high subzero temperatures, and it is believed that their presence causes substantial amounts of frost-related crop losses worldwide (Lindow 1983).

Ice-nucleating active bacteria can significantly decrease the supercooling capacity of insects, causing a loss of cold tolerance (Strong-Gunderson et al. 1989, 1990). Either ingestion or topical application of these bacteria to the surface of insects elevates the supercooling point (the temperature at which an insect begins to freeze internally) in a variety of freeze-intolerant insects, thereby inducing mortality. Furthermore, ice-nucleating active bacteria are normal flora of the insect gut (Lee et al. 1991). These facts suggest that ice-nucleating active bacteria may provide a novel means of biological control for freeze-intolerant insect pests during the winter (Strong-Gunderson et al. 1990, Lee 1991).

Fields (1991) recently demonstrated that the application of *Pseudomonas syringae* to the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens), not only caused a significant increase in the supercooling point of this species, but decreased its cold-hardiness when exposed to subzero temperatures. He also proposed that ice-nucleating active bacteria might function as a cold synergist for the control of insects in grain bins. The study presented here extends his observations to determine the effect of *P. syringae* on the supercooling capacity and cold tolerance of a number of freeze-intolerant stored grain insects.

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Table 1. **Effect of the ice-nucleating active bacteria *P. syringae* on the supercooling point of stored grain insects**

Species	Supercooling point (°C), $x \pm$ SEM		F	P
	Untreated control	*P. syringae* (100 ppm)		
P. interpunctella (larvae)	−10.3 ± 0.4a (n = 54)	−5.4 ± 0.5a (n = 33)	91.0	<0.001
S. granarius (adults)	−15.7 ± 1.0b (n = 16)	−8.0 ± 0.6b (n = 18)	41.8	<0.001
R. dominica (adults)	−15.2 ± 0.6b (n = 12)	−3.3 ± 0.1c,d (n = 7)	209.8	<0.001
T. castaneum (adults)	−13.9 ± 0.8b (n = 23)	−4.7 ± 0.4a,d (n = 11)	66.3	<0.001
G. psylloides (adults)	−10.7 ± 0.9a (n = 11)	−6.0 ± 0.5a (n = 11)	22.1	<0.001

Significant differences ($P < 0.05$) using one-way ANOVA tests of supercooling point means among species for untreated control and *P. syringae* treatment are indicated by different letters.

Within-species comparisons of mean supercooling points for untreated control and *P. syringae* were compared using a two-sample, one-way ANOVA test.

Materials and Methods

Insect Cultures. Laboratory strains (in culture for at least 5 yr) of the Indianmeal moth, *Plodia interpunctella* (Hubner); red flour beetle, *Tribolium castaneum* (Herbst); flat grain beetle, *Cryptolestes pusillus* (Schonherr); rusty grain beetle, *C. ferrugineus; Gibbium psylloides* (Czenpinski); lesser grain borer, *Rhyzopertha dominica* (F.); yellow mealworm, *Tenebrio molitor* (L.); and granary weevil, *Sitophilus granarius* (L.) were maintained at ≈23°C using standard methods (Bell 1982, Evans 1983).

Determination of Supercooling Points. Supercooling point values were determined by positioning insects in contact with a 30-gauge copper-constantan thermocouple within a 1.5-ml polypropylene tube. These tubes were placed into glass test tubes suspended in a 0°C refrigerated bath and allowed to equilibrate for 5 min before cooling at ≈0.6°C/min. The lowest temperature reached before the release of the latent heat of fusion was recorded as the supercooling point.

Effect of *P. syringae* on Low Temperature Survival of Stored Grain Pests. Our source of a bacterial ice nucleator was a concentrated, freeze-dried, and killed preparation of *P. syringae* (Genencor International, Rochester, N.Y.). This product provides a highly efficient source of heterogeneous ice nucleators for commercial snow-making. The *P. syringae* used in this study had an ice nucleating activity of 2.02×10^4 ice nucleating sites per gram. To evaluate the effect of ice-nucleating active bacteria on insect cold tolerance, 30–40 insects were added to 10 g of wheat inoculated with *P. syringae* and held at 23°C for 24 h. Treatment doses (100 or 1,000 ppm) were based on the weight of dry, powdered *P. syringae* to weight of grain. Control insects were added to 10 g of wheat that did not contain *P. syringae*. The samples were subsequently transferred directly to a refrigerated bath at −5 or −8°C for 24 h. After a final 24 h at 23°C, the proportion of surviving insects was determined. Survival was based on the ability of the insects to walk normally. Another similar experiment was done with corn instead of wheat.

Supercooling point values were compared using two-factor and one-factor analysis of variance (ANOVA) followed by Fisher's Least Significant Differences test to separate means (Sokal & Rohlf 1973). Chi-square analysis was used to compare survival rates of treated insects versus controls.

Results

Supercooling Points. Five species of insects were exposed to 100 ppm of *P. syringae* mixed with wheat for 24 h at 23°C (Table 1). No individual survived freezing (i.e., exposure to temperatures below its supercooling point). Using a two-factor ANOVA test, significant differences were observed among species ($F = 14.7$; df = 4, 4; $P < 0.001$) and between treatments ($F = 284.8$; df = 1, 4; $P < 0.001$). Among species for untreated controls, mean supercooling point values were higher ($P < 0.05$) for *P. interpunctella* and *G. psylloides* than for the other three species. Mean supercooling points for untreated controls ranged between −10.3 and −15.7°C. In contrast, supercooling points for insects treated with ice-nucleating active bacteria were significantly higher ($P < 0.001$) for each species tested with values ranging between −3.3 and −8.0°C. The magnitude of the treatment effect ranged from a mean increase of 4.7°C in the supercooling point value of Indianmeal moth larvae to a maximum of an 11.9°C increase in lesser grain beetle adults. *R. dominica* adults treated with *P. syringae* had significantly higher ($P < 0.001$) supercooling points than the other species.

Effect of *P. syringae* on Low Temperature Survival of Stored Grain Pests. Of the untreated control insects exposed to −5°C for 24 h in wheat, six species exhibited survival rates of 90–98% (Ta-

Table 2. Survival of larval and adult stored grain pests exposed to various concentrations of dry, powdered *P. syringae* in wheat for 24 h at 23°C before 24-h exposure to −5 or −8°C

	% Survival					
	−5°C for 24 h			−8°C for 24 h		
	Untreated control	*P. syringae*		Untreated control	*P. syringae*	
		100 ppm	1,000 ppm		100 ppm	1,000 ppm
T. molitor (larvae)	97 (29/30)	77 (23/30)*	73 (22/30)*	77 (23/30)	23 (7/30)**	53 (16/30)
S. granarius (adults)	96 (27/28)	62 (18/29)*	48 (15/31)**	84 (27/32)	23 (7/31)**	3.6 (1/28)**
C. ferrugineus (adults)	97 (28/29)	0 (0/30)**	0 (0/25)**	90 (28/31)	0 (0/29)**	0 (0/25)**
P. interpunctella (larvae)	96 (23/24)	36 (10/28)**	3.8 (1/26)**	67 (20/30)	0 (0/27)**	0 (0/30)**
C. pusillus (adults)	43 (13/30)	0 (0/29)**	0 (0.20)**	0 (0/48)	0 (0/30)	0 (0/29)
T. castaneum (adults)	19 (6/31)	0 (0/29)	0 (0/30)	0 (0/31)	0 (0/31)	0 (0/30)
R. dominica (adults)	90 (27/30)	3.6 (1/28)**	0 (0/31)**	0 (0/31)	0 (0/30)	0 (0/30)
G. psylloides (adults)	93 (28/30)	25 (8/32)**	0 (0/30)**	53 (16/30)	0 (0/30)**	0 (0/31)**

Numbers in parentheses indicate the number of survivors/total number of insects tested. *, significant difference ($P < 0.05$) using chi-square test comparing untreated control versus *P. syringae* treatment. **, significant difference ($P < 0.001$) using chi-square test comparing untreated control versus *P. syringae* treatment.

ble 2). However, two species had high levels of mortality; only 43% of the *C. pusillus* adults and 19% of *T. castaneum* adults survived. At −8°C, the control groups of three species exhibited survival rates of ≥77% (Table 3). No individuals of *C. pusillus*, *T. castaneum*, or *R. dominica* survived a 24-h exposure to −8°C.

Application of 100 or 1,000 ppm of *P. syringae* in wheat decreased survival for all eight species tested at −5°C (Table 1). For two species, *C. ferrugineus* and *R. dominica,* the application of 100 ppm reduced survival from ≥90% in the control group to <5%. In five species, no individual survived exposure to −8°C after treatment with 1,000 ppm of *P. syringae*. In the granary weevil, survival decreased from 96 to 48%. Larvae of *T. molitor* were the most resistant species to *P. syringae*, with 53% survival after treatment with 1,000 ppm.

All five species in which ≥50% of individuals in the control group survived at −8°C exhibited a statistically significant decrease in survival after treatment with 100 or 1,000 ppm (or both) concentrations of *P. syringae* (Table 2). Furthermore, in four of these five species (*S. granarius, C. ferrugineus, P. interpunctella,* and *G. psylloides*), treatment with 1,000 ppm of *P. syringae*

Table 3. Survival of adult stored grain pests exposed to various concentrations of dry, powdered *P. syringae* in corn for 24 h at 23°C before 24-h exposure to −8°C

	% Survival		
Species	Untreated Control	*P. syringae*	
		100 ppm	1,000 ppm
S. granarius (adults)	73 (22/30)	23 (7/30)*	0 (0/30)*
C. ferrugineus (adults)	83 (25/30)	0 (0/30)*	0 (0/30)*

Survival was assessed after a 24-h recovery period at 23°C. Numbers in parenthesis indicate the number of survivors/total number of insects tested. *, significant difference ($P < 0.001$) using chi-square test comparing untreated control versus *P. syringae* treatment.

reduced survival rates to ≤4% after exposure to −8°C.

In another experiment, corn was substituted for wheat and the effect of *P. syringae* on low temperature survival was tested (Table 3). Again treatment with 100 ppm *P. syringae* caused a significant decrease ($P < 0.001$) in survival following exposure to −8°C. In the rusty grain beetle, all adults died after treatment with 100 ppm, whereas no granary weevils survived exposure to 1,000 ppm.

Discussion

The use of low temperatures to control insects in stored grain is not a new idea: a significant number of studies including Knipling & Sullivan (1957), Smith (1970), Mullen & Arbogast (1979), Hunter & Taylor (1980), and Johnson & Wofford (1991) have investigated this approach for pest control in stored products. Our strategy for control differs from previous ones in that we propose to manipulate natural mechanisms of insects for freeze avoidance to make them more susceptible to cold exposure. We have shown that treatment with *P. syringae* significantly increased the supercooling point of stored-grain insects. Because these species are intolerant of freezing, an elevation of the supercooling point represents a decrease in their cold tolerance. Reduced cold tolerance was also demonstrated for insects exposed to subzero temperatures for 24 h.

The use of ice-nucleating active bacteria as biological insecticides requires that bacterial application occur concurrently with the exposure of insects to temperatures at or below the supercooling point. Although insects used in this study were not cold acclimated, other data indicate that ice-nucleating active bacteria are effective in increasing the supercooling point of cold-hardy insects including *C. ferrugineus* (Fields 1991), *Hippodamia convergens* Guérin-Méneville (Lee et al. 1991), and *Ceratoma tri-*

furcata (Forster) (unpublished data). Advantages of this biological insecticide are that it is biodegradable and its use is compatible with other control measures used in integrated pest management programs. In fact, bacterial application would occur during the winter months when other forms of control are not used.

The development of insect resistance to chemical control is well known. Insects may be slower to develop resistance to ice-nucleating active bacteria than to chemicals, because of the physical as opposed to biological nature of the mechanism by which they decrease cold-hardiness. These bacteria function as ice catalysts that limit the supercooling capacity of insect body water, causing it to freeze at temperatures only a few degrees <0°C. Even in insects with mouths that were sealed to prevent bacterial ingestion, supercooling points were elevated within minutes after topical application (unpublished data). Although, at this time, we do not know the route (e.g., spiracles, anus, pores in the cuticle) by which bacteria come in contact with insect body water, the supercooling point is elevated very rapidly, suggesting the absence of effective barriers to block ice nucleation. Consequently, it may be difficult to develop resistance because it would require the blocking of all avenues of contact between the bacteria and internal water. Thus, development of physiological mechanisms of resistance to bacterial ice nucleation may be a more complex and unlikely process compared with mechanisms of chemical resistance.

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