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Field Persistence of Ice-Nucleating Bacteria in Overwintering Colorado Potato Beetles

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Ice-nucleating bacteria are biological ice nucleators capable of elevating the temperature at which ice crystals form in the body fluids of insects, termed the supercooling point (SCP). In the freeze-intolerant Colorado potato beetle, these bacteria reduce its cold tolerance and, consequently, increase the likelihood of mortality in overwintering adults exposed to subzero temperatures. In this field study, two ice-nucleating bacteria, *Pseudomonas fluorescens* and *P. putida*, were evaluated for their persistence and efficacy against overwintering adults. Both strains significantly elevated the SCP of treated beetles immediately after ingestion. However, only beetles fed *P. fluorescens* still had significantly elevated SCPs (−4.2°C) versus control (−6.4°C) after overwintering in the field. Bacterial persistence in beetle guts was confirmed by PCR assays that positively correlated the presence of *P. fluorescens* with elevated SCPs. Despite the reduction of cold tolerance in overwintering adults with *P. fluorescens*, no significant difference was observed in the survival rates of treated versus control beetles during the winter. Because adults overwinter in the ground, the effect of this bacterium on beetle survival depends on the soil temperatures that overwintering adults experience. Nevertheless, recovery of most of the adults in the upper 15 cm of soil strata indicates that beetles with potent ice-nucleating bacteria in their guts could be subject to critically low temperatures during winter. Our results show that ice-nucleating *P. fluorescens* compromises the cold tolerance of Colorado potato beetles and suggest that by development of strategies to maximize bacterial efficacy in the field, this bacterium offers a potential biocontrol system against overwintering populations.

***Key Words: Leptinotarsa decemlineata; Pseudomonas fluorescens; Pseudomonas putida;* Colorado potato beetle; biological control; cold tolerance; ice-nucleating bacteria.**

INTRODUCTION

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is the most serious defoliator attacking potatoes in North America. Heavy defoliation by overwintered adults, and later by spring larvae and second-generation summer adults, prior to tuber formation can lead to a total loss of income for growers (Hare, 1990). Because potato crops are highly susceptible to beetle damage during the early stages of growth and the bloom stage, management of spring colonization by overwintering adults is critical in minimization of crop losses (Shields and Wyman, 1984).

Adult beetles colonize early crops when they emerge in spring from their overwintering sites and walk, and later fly, in search of host food. Within a potato field, the number of early season adults varies depending on the number of overwintering beetles that survived in the field and the number of beetles that immigrate from adjacent sites. Because Colorado potato beetles are intolerant to freezing of their body fluids (Lee *et al.*, 1994), their survival during winter is maximized by avoidance of internal ice formation. In late summer to early autumn, following crop senescence, beetles disperse in search of overwintering sites and enter into the ground to diapause (Tauber *et al.*, 1988). By burrowing into the soil, adults minimize exposure to extreme temperatures in winter and to temperature fluctuations in autumn and spring (Lashomb *et al.*, 1984). Winter survival is also enhanced by the beetle's capacity to supercool [i.e., remain unfrozen at or below the equilibrium freezing point of body fluids (Lee *et al.*, 1994)]. Diapausing beetles lower their supercooling point (SCP), the temperature at which ice nucleation spontaneously occurs in their body fluids, by evacuating their gut contents, which may contain potential ice nucleators (Lee *et al.*, 1994, 1998).

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The presence of particles, ranging from food items to ice-nucleating bacteria, in the insect gut can serve as nucleators initiating internal ice crystal formation (for reviews, see Cannon and Block, 1988; Lee *et al.*, 1998). The latter are potent biological nucleators capable of catalyzing freezing of aqueous suspensions at temperatures above −10°C. Within an insect gut, they serve as ice catalysts, reducing the insect's ability to supercool and, thus, increasing the likelihood of injury or death after exposure to subzero temperatures. Ice-nucleating activity is conferred by the presence of *ina* genes that code for ice-nucleating proteins located on the outer membrane of the bacterium (Warren, 1995). These proteins have a 16-amino-acid repeat and have structures similar to small ice crystals (Wolber and Warren, 1989). Ice-nucleating activity has been described in some members of the genera *Erwinia, Pseudomonas,* and *Xanthomonas* (Maki *et al.,* 1974; Lindow *et al.,* 1978; Kim *et al.,* 1987). Whereas most of these bacteria are epiphytes (i.e., living on plant surfaces), a few have been reported from the guts of frogs and insects (Lee *et al.,* 1991, 1995).

The susceptibility of Colorado potato beetles to low temperature has been the subject of studies attempting to enhance overwintering mortality and, thereby, reduce beetle populations (Kung *et al.,* 1992; Milner *et al.,* 1992; Lee *et al.,* 1994; Wyman *et al.,* 1994; Costanzo *et al.,* 1998). By taking advantage of the beetle's intolerance to freezing, alternative methods of control may be devised that alleviate current pest management problems brought on by the beetle's widespread resistance to chemical insecticides (Casagrande, 1987; Grafius, 1997). Milner *et al.* (1992) found that removal of insulating mulch cover from overwintering sites exposes diapausing adults to thermal shocks and increases beetle mortality. Lee *et al.* (1994) and Costanzo *et al.* (1998) showed that exposure to ice-nucleating bacteria, either by topical application or by ingestion, reduces the beetle's capacity to supercool and, thus, its cold tolerance. Because the beetle's SCP represents its lower temperature limit of survival (Lee *et al.,* 1994), application of ice-nucleating bacteria could reduce survival of overwintering adults and, consequently, delay population buildup and reduce crop damage the following crop season.

We are exploring the use of ice-nucleating bacteria as potential biological control agents for Colorado potato beetles (for review, see Lee *et al.,* 1998). By exposure of beetles to potato foliage sprayed with ice-nucleating bacteria before they burrow into the ground, overwintering beetles will ingest these potent nucleators, which will induce internal freezing at relatively high subzero temperatures (above −5°C). Previous laboratory (Costanzo *et al.,* 1998; Castrillo *et al.,* 2000a) and field (Castrillo *et al.,* 2000b) studies have already demonstrated that ice-nucleating *Pseudomonas* spp. persist in the gut and significantly elevate the SCP of overwintering beetles after a single exposure. This study was conducted to determine the potential for reducing the survival of overwintering beetles and to facilitate the design of application strategies using ice-nucleating bacteria in the field. We compared two strains of ice-nucleating *Pseudomonas* spp. for their persistence and efficacy in reducing cold tolerance of overwintering beetles and assessed their impact on overwintering survival.

MATERIALS AND METHODS

Ice-Nucleating Bacteria and Culture Conditions

Ice-nucleating *P. fluorescens* Migula strain F26-4C, isolated from the wood frog *Rana sylvatica* LeConte (Lee *et al.,* 1995), and *P. putida* (Trevisan) Migula strain Hr6-1, isolated from the bean beetle *Cerotoma trifurcata* Forster (M. R. Lee, unpublished data), were selected for this study because of their ability to initiate freezing of aqueous suspensions at relatively high temperatures (−3.2 and −2.0°C, respectively). Laboratory tests also showed that these two strains could significantly elevate the SCP of Colorado potato beetles. These bacterial strains were cultured on nutrient agar with 2.5% glycerol for 4 days at 22°C to enhance ice-nucleating activity (Lindow *et al.,* 1982). Aqueous suspensions were prepared and bacterial cell counts were determined by use of a hemocytometer and by the plating of serial dilutions on nutrient agar plates. Ice-nucleating activity of bacterial suspensions was determined as described in Castrillo *et al.* (2000a).

Experimental Insects and Field Study

Adult Colorado potato beetles were collected in early August 1998 from cultivated potato fields at the Hancock Agricultural Station in central Wisconsin. Beetles were shipped overnight to Miami University where groups of 250 beetles were transferred to plastic shoe boxes containing autoclaved sand moistened with deionized water (Castrillo *et al.,* 2000a). Beetles were held at 15°C, 12:12 (L:D) h, and fed slices of potato tubers.

Prior to exposure to ice-nucleating bacteria, beetles were starved for 24 h at 23°C. Then, groups of 20 beetles were transferred to petri dishes (100 × 15 mm) containing a thin slice of potato tuber that had been evenly coated with 0.4 ml of bacterial suspension (10^8 bacteria/ml). Beetles were allowed to feed for 24 h before being combined with other similarly treated beetles into replicate groups of 200, with three replicates for each of the two bacterial treatments and the untreated control. Control beetles were fed potato slices moistened with 0.4 ml sterile deionized water. Additional beetles were treated with ice-nucleating bacteria for SCP measurements immediately after feeding. Bee-

tles were then shipped overnight to Wisconsin for release into test arenas at the Hancock Agricultural Research Station.

Groups of 200 beetles were released into each of nine arenas composed of bottomless, square steel boxes (58 × 58 × 25 cm) that were driven 25 cm into the soil. Beetles were placed on the soil surface in arena centers and provided with slices of potato for food. Arenas were then covered to prevent beetle escape. After 1 month, arena covers were removed and soil surfaces were covered with a 4- to 5-cm layer of pine needles to simulate the natural mulch cover present on soils in adjacent field edges, the primary overwintering site of most Colorado potato beetles in the study area (Milner *et al.*, 1992). The test arenas were left undisturbed throughout winter.

Temperature loggers (Onset Computer Corp., Pocassat, MA) were placed in one arena to record ground temperature from the soil surface to 30 cm deep, at 5-cm increments, for the duration of the field experiment. In early May of the following year, all arenas were excavated to a depth of at least 30 cm and the soil removed was sieved using a 1/8-inch wire mesh to recover the beetles. Intact adults were sorted based on viability and counted before being shipped to Miami University for further studies. Survival rates (arcsine square root-transformed) were analyzed using analysis of variance (ANOVA) (Statview 5, SAS Institute, NC).

Supercooling Point Measurements

In laboratory experiments, SCPs of Colorado potato beetles were determined by positioning a 30-gauge copper-constantan thermocouple in contact with an adult beetle inside a 1.5-ml polyethylene tube (Lee *et al.*, 1994). Tubes were plugged with foam and placed inside a glass test tube suspended in a refrigerated bath initially set at 0°C. After thermal equilibration for 5 min, the temperature was decreased at a rate of 0.3°C/min. The SCP was recorded as the temperature at which the beetle froze as indicated by an exotherm (i.e., release of latent heat of crystallization) detected using a multichannel data logger (RD3752, Omega Electronics, Stanford, CT). The SCPs of live beetles recovered from the field (N = 36; 12 beetles sampled/replicate/treatment) were determined and compared to values taken from beetles sampled prior to field release (N = 30; 10 beetles sampled/replicate/treatment). Data were analyzed using a two-way ANOVA with bacterial treatment and sampling time as factors. Means were separated at the 5% level of significance by Fisher protected least significant differences (LSD) (Statview 5, SAS Institute, NC).

Detection of Ice-Nucleating Bacteria

Presence of the ice-nucleating bacteria in the beetle gut was detected using a polymerase chain reaction (PCR) technique (Castrillo *et al.*, 2000a,b). Five beetles from one replicate of each treatment were aseptically dissected and their digestive tracts triturated in 200 μl of sterile saline in a 1.5-ml polyethylene tube by use of a sterile wooden stick. To grow bacteria from beetle guts for DNA extraction, 100 μl of each gut suspension was cultured overnight in 10 ml of nutrient broth with 2.5% glycerol and shaken at 150 rpm at room temperature (~23°C). Bacterial cells from 1 ml of overnight cultures were used for DNA extraction using QIAamp tissue kit (Qiagen Inc., Valencia, CA). Presence of either ice-nucleating *P. fluorescens* or *P. putida* was detected in DNA extracts using primers and PCR conditions developed to probe for the *inaW* gene in *P. fluorescens* and the *ina* gene in *P. putida* (Castrillo *et al.*, 2000a). Three amplifications were carried out for each sample. A positive control consisting of pure DNA from either *P. fluorescens* F26-4C or *P. putida* Hr6-1 and a negative control consisting of sterile water were used for each PCR run.

RESULTS

Effect of Ice-Nucleating Bacteria on the Supercooling Capacity of Colorado Potato Beetles

The SCP of Colorado potato beetles was significantly affected by bacterial treatment (F = 148.7; df = 2, 12; $P < 0.001$) and the interaction between bacterial treatment and sampling time (F = 63.7; df = 2, 12; $P < 0.001$), but not by sampling time (F = 6.3; df = 1, 12; P = 0.27). Beetles fed ice-nucleating *P. fluorescens* F26-4C and *P. putida* Hr6-1 had significantly elevated SCPs 1.5 h after feeding compared to control beetles (F = 96.7; df = 2, 6; $P < 0.001$) (Fig. 1). The mean SCPs exhibited by treated beetles were −4.4 ± 0.2°C (range −3.0 to −7.6°C, N = 30) and −4.3 ± 0.3°C (range −2.9 to −8.1°C, N = 30) for those fed *P. fluorescens* and *P. putida*, respectively. For control beetles, the mean SCP was −9.5 ± 0.4°C (range −6.4 to −14.2°C, N = 30).

After 7 months overwintering in the field, the effect of ice-nucleating bacteria on beetle SCP varied with bacterial strain (F = 240.7; df = 2, 6; $P < 0.001$). Beetles fed *P. fluorescens* had a mean SCP (−4.2 ± 0.1°C) significantly higher than those in beetles fed *P. putida* (−6.2 ± 0.1°C) and in the untreated controls (−6.4 ± 0.1°C) (Fig. 1). The range of individual SCPs are presented in Fig. 2. As beetles were slowly cooled, more than 95% of beetles fed *P. fluorescens* were frozen by the time the temperature reached −5.0°C, and at −5.5°C all were frozen (Fig. 2). In contrast, none of those fed *P. putida* nor the untreated control beetles began to freeze until they reached −5.6 and −5.5°C, respectively (Fig. 2).

Comparison of beetle SCPs taken 1.5 h after feeding

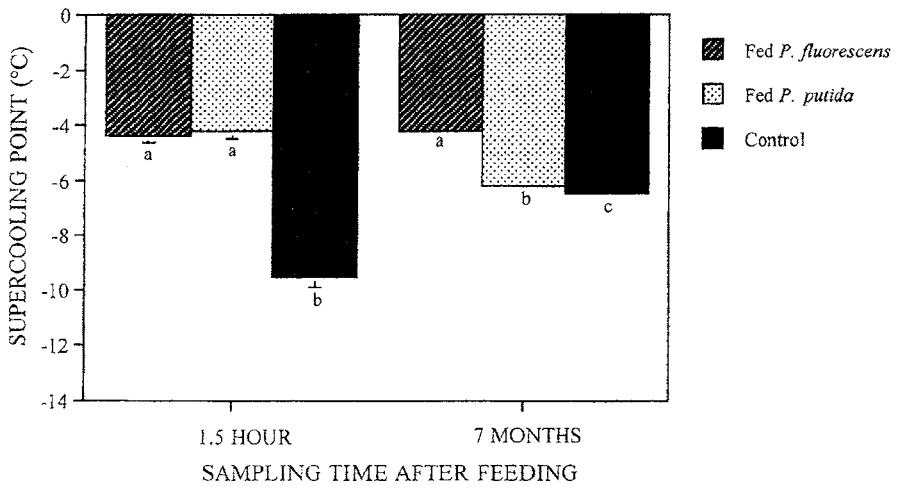

FIG. 1. Supercooling points (mean ± SE) of Colorado potato beetles fed ice-nucleating *Pseudomonas fluorescens* F26-4C or *P. putida* Hr6-1 measured 1.5 h after feeding (N = 30, with 10 beetles/replicate/treatment) and after 7 months overwintering in the field (N = 36, with 12 beetles/replicate/treatment). Bars within the same sampling time with the same letter are not statistically significant at the 5% level (Fisher protected LSD).

and after overwintering in the field for 7 months showed comparable values in those fed *P. fluorescens* (F = 5.1; df = 1, 4; P = 0.08). In contrast, the SCP of those fed *P. putida* significantly declined after 7 months (F = 74.7; df = 1, 4; P = 0.001). For untreated controls, diapausing beetles in autumn had significantly lower SCPs than beetles in spring (F = 40.1; df = 1, 4; P = 0.003), reflecting the ability of these beetles to depress their SCP in preparation for winter.

Detection of Ice-Nucleating Bacteria Using PCR

The presence of ice-nucleating bacteria in the digestive tract of treated beetles was confirmed by detection of bacterial ice nucleation genes with PCR. A band of ~4.5 Kb, corresponding to the *P. fluorescens inaW* gene, was observed in gut cultures from all beetles fed *P. fluorescens* F26-4C 1.5 h after ingestion and after 7 months in the field (Fig. 3A). In contrast, gut cultures from adults treated with *P. putida* showed the >4.5-Kb

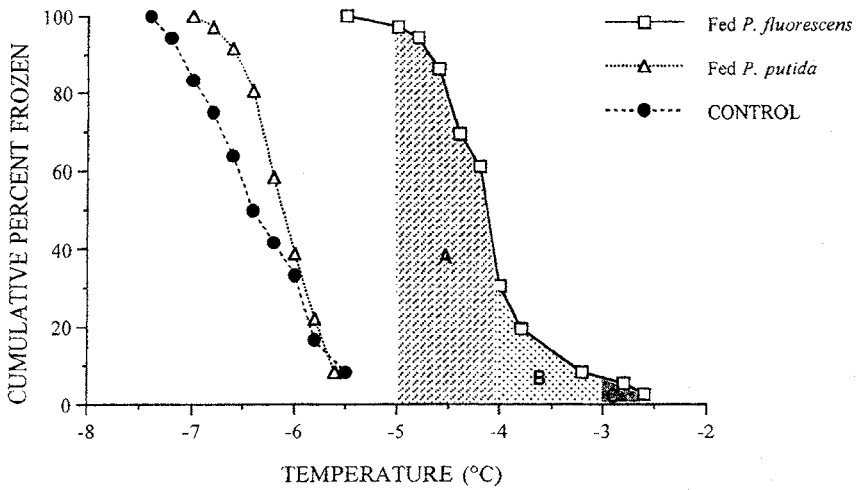

FIG. 2. Cumulative freezing profile based on supercooling points of individual Colorado potato beetles treated with ice-nucleating *Pseudomonas fluorescens* F26-4C or *P. putida* Hr6-1 and measured after overwintering in the field for 7 months (N = 36, with 12 beetles/replicate/treatment). In beetles fed *P. fluorescens* F26-4C, hatched areas represent expected mortality at subzero temperatures based on the range of supercooling values exhibited by recovered beetles in May: A, >95 to 50% mortality at −5 to −4°C; B, ~50 to 10% at −4 to 3°C; C, <10% at −3°C.

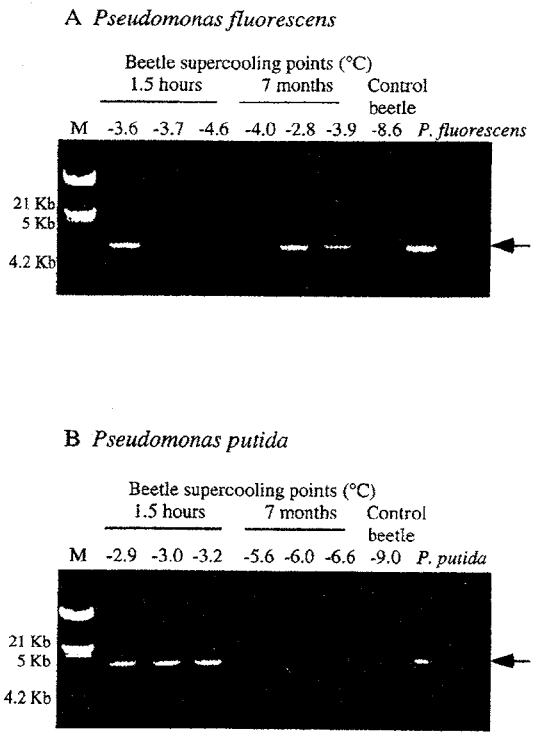

FIG. 3. PCR detection of ice-nucleating bacteria in gut cultures from Colorado potato beetles sampled 1.5 h and 7 months after feeding on potato slices treated with ice-nucleating bacteria. *Pseudomonas fluorescens* F26-4C (A) was detected using primer pair based on the *inaW* gene from *P. fluorescens,* and *P. putida* Hr6-1 (B) was detected using a primer pair based on the *inaZ* gene from *P. syringae.* Text above each lane indicates the individual beetle's supercooling point (°C) and sampling time. Supercooling point values for control beetles were measured 1.5 h after they were fed. DNA from ice-nucleating bacteria were used as positive controls. Molecular marker (M) used was lambda restricted with *Hind*III and *Eco*RI. Arrows indicate bands corresponding to *ina* genes.

band, corresponding to its *ina* gene, in beetles sampled 1.5 h after ingestion but not in beetles recovered in spring (Fig. 3B). The absence of *ina* gene, which suggests loss of *P. putida* in treated beetles, correlated with the decrease observed in SCPs for beetles collected in May. None of the control beetles exhibited a PCR product corresponding to the *inaW* gene from *P. fluorescens* or to the *ina* gene from *P. putida* when assayed before field release or after recovery in May (Fig. 3).

Impact of Ice-Nucleating Bacteria on Beetle Overwintering Survival

The effect of ice-nucleating bacteria on survival of overwintering beetles at the Hancock Agricultural Station in Wisconsin was determined by assessment of mortality rates of intact beetles recovered from arenas in May 1999. Of 200 beetles released in each test arena in September 1998, 57 to 61% were recovered from the soil the following spring (Table 1). Most of these beetles were found in the upper 15 cm of the soil strata. Among recovered beetles, those fed *P. fluorescens* and *P. putida* exhibited 31.8 and 37.4% overwintering survival, respectively. Survival of control beetles was 42.8%, and no significant differences were observed in the survival data among treatments ($F = 1.2$; $df = 2, 6$; $P = 0.36$) (Table 1).

Recordings of soil temperatures at the Hancock Agricultural Station in central Wisconsin in the winter of 1998–1999 showed values that fluctuated between 0 and −5.2°C from mid-December to mid-March (Fig. 4). Seasonal daily changes in the soil temperature at different depths followed the same pattern except for greater oscillation at the soil surface. At increasing soil depth, temperature variation decreased (Fig. 4). In the presence of deep snow cover, which persisted during most of January, the differences in soil temperature at different strata were dampened and remained relatively constant.

The minimum soil temperatures, which occurred in early January, for depths of 5, 10, and 15 cm below the soil surface were −5.2, −4.0, and −3.3°C, respectively. For the months of February and March, at depths of 5, 10, and 15 cm below the ground, the lowest temperatures recorded were −4.0, −3.1, and −2.1°C, and −3.8, −2.8, and −1.3°C, respectively. By the fourth week of March and into May, when beetles were excavated, temperatures below the ground were consistently above 0°C.

DISCUSSION

As we explore the potential of ice-nucleating bacteria for biological control of overwintering Colorado potato beetles, field experiments evaluating efficacy of bacterial strains are crucial not only in selecting candidate strains for further study but also in determining environmental factors that could affect bacterial efficacy under field conditions. Results of our field study showed that of the two ice-nucleating bacteria, *P. fluo-*

TABLE 1

Recovery and Field Survival (Mean ± SE) of Overwintering Colorado Potato Beetles Fed Ice-Nucleating Bacteria

Treatment	No. of recovered beetles[a]	Live beetles	% Survival[b]
Pseudomonas fluorescens	114 ± 20	35 ± 22	31.8 ± 12.4
P. putida	122 ± 6	46 ± 10	37.4 ± 41
Control	122 ± 28	51 ± 10	42.8 ± 3.9

[a] Based on three replicates with 200 beetles initially released in each replicate per treatment.

[b] Based on number of live beetles/number of recovered beetles in May, 1999.

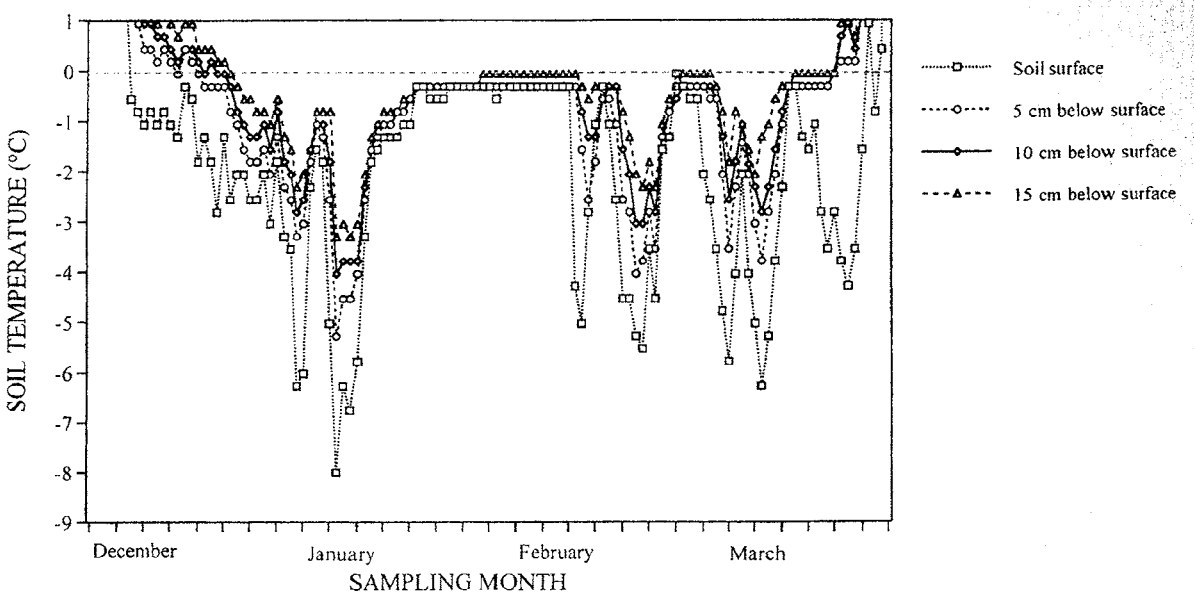

FIG. 4. Minimum soil temperatures (°C) recorded at depths of 0 to 15 cm, from December 1998 to March 1999 at the Hancock Agricultural Research Station in central Wisconsin.

rescens F26-4C and *P. putida* Hr6-1, only the former persisted and maintained its ice nucleating activity in the gut of overwintering beetles throughout the winter. This result confirmed our field study conducted during the winter of 1997–1998, which showed persistence of *P. fluorescens* as evidenced by elevated SCPs and by detection with PCR assays (Castrillo *et al.,* 2000b). In the present study, the relatively high recovery rates of treated and control beetles in spring showed that most of the beetles fed *P. fluorescens* retained the bacterium throughout winter. These results indicate that ice-nucleating *P. fluorescens* F26-4C is an excellent candidate for further development as a biological control agent.

This field study also corroborated laboratory studies conducted to test persistence of various strains of ice-nucleating bacteria in Colorado potato beetles. Laboratory screening of various ice-nucleating *Pseudomonas* spp. showed the persistence and efficacy for 2 to 12 weeks of *P. fluorescens* compared to other species against overwintering Colorado potato beetles (Castrillo *et al.,* 2000a). This confirmation provides evidence that laboratory results may accurately reflect bacterial activity in the field and that strains that persist for 2 weeks postingestion in diapausing beetles are likely to persist through the course of winter in the field.

Even though fewer beetles fed *P. fluorescens* survived winter (31.8 %), mortality did not significantly differ from that of untreated controls (42.8%). However, since beetles recovered from the soil were not separated by soil depth (i.e., by 5-cm increments), any differences in mortality at different soil depths among treatments cannot be determined. Although most of the beetles were recovered in the upper 15 cm of the soil strata, the subzero temperatures that the beetles were exposed to varied with depth of burrowing. Since beetles closer to the soil surface were subject to greater temperature fluctuations and more severe cold (Milner *et al.,* 1992; Wyman *et al.,* 1994), they would be more likely to experience temperatures low enough to initiate ice formation if *P. fluorescens* was present in their gut.

The relatively mild winter conditions that prevailed during this study period did not decrease soil temperatures within the upper 20 cm of soil strata to sufficiently impact beetle survival. Nonetheless, the relatively shallow depth at which most of the beetles were found suggests that most overwintering beetles with potent ice nucleators in their gut could be subject to lethally low temperatures. Although the depth of burrowing in sandy soils can reach up to 60 cm compared to that in heavy clay soils (0 to 20 cm) (Minder, 1966), beetles in our study burrowed to relatively shallow depths in sandy loam soil. This observation is similar to those made by Milner *et al.* (1992) in which they found 50 to 80% of the overwintering beetles in the upper 15 cm of the soil strata.

Under winter conditions in which temperatures in the upper 15 cm of soil strata drop below −5.0°C, the presence of ice-nucleating *P. fluorescens* in overwintering beetles could initiate internal freezing and cause significant mortality in a substantial portion of the population. If we assume that beetles would die at the SCP values exhibited by these beetles recovered in

May (Fig. 2), 90% mortality would be expected in beetles within the top 5 cm since at that depth the soil temperature decreased to −5.2°C in January (Fig. 4). Correspondingly, at 10 to 15 cm, overwintering mortality would be 50 to 10% in treated beetles within those depths (Fig. 2).

The significant difference in SCPs between control beetles and those with *P. fluorescens* indicated that the cold tolerance of the latter group was compromised. Comparison of the SCP in diapausing control beetles in September (−9.5°C) to the SCP in beetles ready to emerge in May (−6.4°C) confirmed that adult Colorado potato beetles depress their SCPs in preparation for winter (Boiteau and Coleman, 1996). However, in the presence of ice-nucleating *P. fluorescens* F26-4C in their gut, overwintering beetles lost the ability to depress their SCP, making them susceptible to injury or mortality at subzero temperatures above their normal winter SCP. This effect persisted through spring, demonstrating the potency and persistence of *P. fluorescens*.

Because overwintering mortality was assessed only in May, any significant difference among treatments that may have resulted shortly after minimum soil temperatures occurred in January cannot be determined. An additional sampling taken in February, after the coldest part of winter, would have provided comparison of mortality due to freezing initiated by *P. fluorescens* and mortality due to natural factors accrued in overwintering adults through winter. Since the survival rates of overwintering populations could vary significantly due to a number of environmental and physiological factors (Wyman *et al.*, 1994), the effect of ice-nucleating bacteria may be masked by high natural mortality rates.

The impact of ice-nucleating bacteria on survival of overwintering beetles is likely affected by a number of physical factors (i.e., soil type and soil moisture) that affect depth of burrowing and, thus, the soil temperatures that beetles experience. Heavy soils, which tend to have high moisture levels, not only limit depth of burrowing but also increase the beetle's sensitivity to cold by increasing their susceptibility to inoculative freezing (Minder, 1966; Kung *et al.*, 1992; Costanzo *et al.*, 1997). With these factors in mind, additional field experiments should evaluate possible interaction among soil depth, soil type, and moisture level on the efficacy of ice-nucleating bacteria in the field.

The Colorado potato beetle's ability to avoid freezing by burrowing into the soil and by depressing its SCP has allowed this freeze-intolerant insect pest to expand its habitat range from central Mexico to southern parts of Canada (Boiteau and Coleman, 1996). Since cold tolerance is an important factor determining the survival of overwintering beetle populations, manipulation of its cold hardiness using ice-nucleating bacteria could provide an effective means for controlling this pest that is complementary to existing pest management systems. Our results show that ice-nucleating *P. fluorescens* F26-4C compromises the cold tolerance of these beetles and suggest that by development of strategies that would maximize bacterial efficacy in the field, this bacterium could significantly reduce survival of overwintering populations.

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