INSECT TIMING: CIRCADIAN RHYTHMICITY TO SEASONALITY

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Using ice-nucleating bacteria to reduce winter survival of Colorado potato beetles: development of a novel strategy for biological control

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A major factor in the overwintering survival of insect pests is their ability to seasonally enhance their cold tolerance by increasing their capacity to supercool and, thus, avoid the lethal effects of internal ice formation. It has now been established that the supercooling capacity of a variety of insects can be significantly reduced by ingestion or surface application of ice-nucleating active (INA) microorganisms. Our recent studies use the Colorado potato beetle, *Leptinotarsa decemlineata,* (Coleoptera: Chrysomelidae) as a model system to address basic questions regarding the seasonal regulation of cold tolerance and insect-microbial interactions. These studies provide a foundation for novel approaches in biological control by manipulating insect cold-hardiness and overwintering survival using INA microorganisms.

1. INTRODUCTION

Most insects are not able to survive internal ice formation. Thus, a key factor in their winter survival is the regulation of the temperature at which they freeze. This temperature is termed the supercooling point (SCP) or the temperature of crystallization (Lee, 1991). As an insect is cooled below the melting point of its body fluids, freezing usually is not immediate. Instead, insects typically supercool many degrees below 0°C before ice nucleation occurs.

A seasonal pattern in the supercooling capacity is common among insects. For many species summer SCP values are often between -4 and -8°C, but gradually decrease in the autumn to -15°C or lower. Many freeze-intolerant species increase their cold tolerance by synthesizing antifreeze proteins and/or

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by accumulating large amounts of glycerol and other low molecular weight polyols and sugars (see review by Lee et al., 1996).

Various mechanisms play a role in regulating supercooling capacity (Zachariassen, 1992; Duman et al., 1995; Lee and Costanzo, 1998). For many overwintering insects, seasonal increases in supercooling capacity are correlated with increases in glycerol production (Salt, 1968; Somme, 1982). The supercooling limit is determined by the presence of ice catalysts, termed ice-nucleating agents. These compounds function as seeds for ice crystal growth in supercooled liquids. A number of authors have suggested that food material or dust within the gut may function as ice-nucleating agents (see review by Cannon and Block, 1988). Accordingly, cessation of feeding and emptying of the gut are often associated with increases in supercooling capacity. Inoculative freezing, in which contact with external ice initiates freezing of the body fluids, may result in little or essentially no supercooling of the body fluids prior to ice formation. Obviously, this markedly reduces cold tolerance for freezing-intolerant species, including most insect pests.

2. ICE-NUCLEATING ACTIVE MICROORGANISMS

An important implication of these relationships for biological pest control is that any agent that limits the supercooling capacity of a freeze-intolerant insect will increase the likelihood of injury or death following exposure to subzero temperatures. In the 1970s a unique class of biological nucleators, ice-nucleating active (INA) bacteria, was discovered (Maki et al., 1974; Lindow et al., 1978). These bacteria are remarkable for their ability to catalyze ice nucleation at temperatures as high as -1 to -2°C. Ice-nucleating activity is conferred by the presence of *ina* genes (also called *ice* genes), which code for ice-nucleating proteins localized on the bacterium's outer membrane (Warren, 1995). Ice-nucleating proteins have a 16-amino acid sequence repeat and may aggregate to function as templates for the formation of small ice crystal seeds termed "ice nuclei" (Yankofsky et al., 1981; Wolber and Warren, 1989). Ice nuclei activity has been classified by the range of temperatures in which they initiate freezing: type 1 are active between -2 to -5°C, type 2 are active between -5 to -7°C, and type 3 between -7 to -10°C (Yankofsky et al., 1981).

2.1. INA bacteria in insects and other animals

Although most of the reported ice-nucleating bacteria are epiphytic (Gurian-Sherman and Lindow, 1993), some strains have been isolated from the gut of frogs (Lee et al., 1995) and insects (Kaneko, 1991; Lee et al., 1991; Takahashi et al., 1995; Olsen and Duman, 1997). It has been proposed that these microorganisms enhance the survival of freeze-tolerant organisms in winter by triggering freezing at relatively high sub-zero temperatures, a strategy that decreases the chance of osmotic shock and intracellular freezing (Lee, 1991). In freeze-intolerant insects, however, the presence of these ice-nucleating bacteria in the gut generally reduces cold hardiness and increases the likelihood of mortality at subzero temperatures.

2.2. Potential of INA bacteria as biological control agents

The potential use of ice-nucleating bacteria as biological control agents against insect pests first became apparent with the demonstration of the ability of these microorganisms to elevate the SCP of the lady beetle *Hippodamia convergens* (Strong-Gunderson et al., 1990). For freeze-intolerant insect pests whose SCP can be elevated by exposure to INA bacteria, and which are exposed to sufficiently low environmental temperatures to initiate freezing of their body fluids, these microorganisms offer an alternative means of control (Lee et al., 1998).

Several studies have shown that ice-nucleating bacteria may be used to decrease the cold tolerance of a variety of insects. Target insects include storage pests (Fields, 1992, 1993; Fields et al., 1995; Lee et al., 1992), the Russian wheat aphid, *Diuraphis noxia* (Armstrong et al., 1998), the pear psylla, *Cacopsylla pyricola* (Lee et al., 1999), the mulberry pyralid, *Glyphodes pyloalis* (Watanabe et al., 2000), and the Colorado potato beetle, *L. decemlineata* (Lee et al., 1994; Costanzo et al., 1998; Castrillo et al., 2000a, b).

3. USING INA BACTERIA AS BIOLOGICAL CONTROL AGENTS AGAINST COLORADO POTATO BEETLES

We have used the Colorado potato beetle, *L. decemlineata*, as our primary model for these studies because it appears to be an exceptionally well-suited candidate for applications of INA bacteria. This beetle is the most serious defoliating pest of potatoes, *Solanum tuberosum* L., in North America (Hare, 1990). Adults overwinter after burrowing shallowly into the soil in late summer or early autumn (Ushatinskaya, 1978). When they emerge from dormancy they can significantly reduce yields by defoliating the early growth stages of potato plants (Shields and Wyman, 1984). This pest is notorious for rapidly developing resistance to a wide range of pesticides, including synthetic pyrethroids (Casagrande, 1987). Consequently, alternative methods that are compatible with other pest management strategies are urgently needed for controlling this pest.

In our initial study, we determined that the Colorado potato beetle is a freeze-intolerant species that dies when it freezes (Lee et al., 1994). However, overwintering beetles survive to temperatures immediately above their supercooling point, indicating that death is due to the onset of internal ice formation, and not low temperature *per se*. This result also indicates that in this species the SCP may be used as a measure of the lower lethal temperature.

Considering the relatively high SCP of overwintering adults (-7 to -9°C), it is clear that this species lacks exceptional cold tolerance (Lee et al., 1994). Furthermore, because overwintering beetles burrow shallowly (7-14 cm) in

certain soils, they may be exposed to subzero temperatures. The elevation of beetle SCPs to as little as 2 to 4 °C could be of major significance in decreasing the proportion of beetles surviving the winter. We demonstrated that exposure of Colorado potato beetles to INA *Pseudomonas syringae* significantly increased SCPs from -7.6 ± 0.2 °C to -3.7 ± 0.1°C (Lee et al., 1994).

3.1. Selection of INA bacterial strains for use in biological control

Although killed preparations of INA *P. syringae* sprayed onto CPB adults resulted in elevated SCPs, the effect persisted for only seven days (Lee et al., 1994). Consequently, we considered the feasibility of colonizing the gut of overwintering beetles with INA bacteria to achieve longer lasting effects, since temperatures low enough to kill beetles will occur long after application of INA bacteria. To explore this possibility, we first conducted a survey of the types and prevalence of bacterial flora in the digestive tract of Colorado potato beetles. Our results show a diverse flora that was present in actively feeding beetles in summer, as well as in overwintering adults (see Table 1). The relative abundance of bacterial gut flora was lower in overwintering adults than in actively feeding ones, suggesting that gut evacuation prior to winter, which eliminates food contents, also eliminates some of the gut flora.

Table 1
Bacterial flora isolated from the gut of summer and winter populations of Colorado potato beetles. (M.R. Lee et al., unpublished data)

Bacterial species	Relative abundance	
	Summer	Winter
Alcaligenes sp.		+
Acinetobacter spp.		+
Citrobacter freundii	+++	
Enterobacter agglomerans	++++	+
E. cloacae	++++	
E. taylorae	++++	+
Enterobacter spp.	++++	+
Flavobacterium odoratum	++++	+
Klebsiella oxytoca		+
K. pneumonia	++	
Pseudomonas aeruginosa	+++	
P. fluorescens		+
P. maltophilia	+	
P. paucimobilis	++	
P. stutzeri		+
Xanthomonas maltophilia		+

Ice-nucleating strains that are of the same species as the bacteria retained during the voiding process and which thrive in gut conditions of overwintering beetles are likely candidates for use against Colorado potato beetles.

The feasibility of colonizing the gut of overwintering adults with INA bacteria was confirmed when it was shown that ingestion of living ice-nucleating strains of *P. fluorescens* and *P. putida* persisted for 10 weeks after initial exposure (Costanzo et al., 1998). To simulate natural overwintering conditions, field-collected beetles in autumn were fed INA bacteria before they burrowed into the soil. Beetles were assayed for SCP and gut flora 1.5 hours after feeding, at the conclusion of a 2-week diapause induction regimen, and in mid-winter after the beetles had been in diapause for 2.5 months. Ingestion of INA bacteria caused an immediate increase in the beetle's SCP. Most noteworthy, however, is the fact that *P. fluorescens* and *P. putida* caused not only an initial SCP elevation, but that the SCP remained elevated for 2.5 months. Also of significance is the fact that the SCP remained elevated even after the beetles extensively purged their gut contents in preparation for burrowing in the soil and overwintering in reproductive diapause. Thus, this result demonstrated that natural defense mechanisms in the gut against freezing could be overcome.

Additional laboratory experiments identified two other strains of *P. fluorescens*, frog-derived F26-4C and insect-derived 88-335, able to persist and maintain activity in the beetle gut for 2 to 12 weeks after exposure (Fig. 1). Moreover, positive correlation between elevated SCP in treated beetles and the presence of INA bacteria was confirmed by use of Polymerase chain reaction (PCR) technique (Castrillo et al., 2000a). This method provided molecular evidence for the presence or absence of ice-nucleating bacteria fed to beetles. Using primers specific for the *ina* gene in the different *Pseudomonas* spp. tested, persistence of the two *P. fluorescens* strains was confirmed by the presence of their ice-nucleating gene, *inaW*, in beetles up to 12 weeks after initial exposure (Fig. 2). A band of approximately 4.5 Kb, corresponding to the *inaW* gene, was detected in treated beetles exhibiting elevated SCPs.

Consequently, *P. fluorescens* strains F26-4C and 88-335 were selected for further studies as potential biological control agents for the following reasons:

1. high levels of ice-nucleating activity with some cells active at -2°C,
2. initiate freezing in beetles at temperatures as high as -2.6°C,
3. natural isolates from the gut of frogs and insects (i.e., non-genetically engineered),
4. persist in the gut of overwintering beetles,
5. relatively easy to culture and grow at low temperatures (4°C), and
6. detectable with cultural and molecular (PCR) methods.

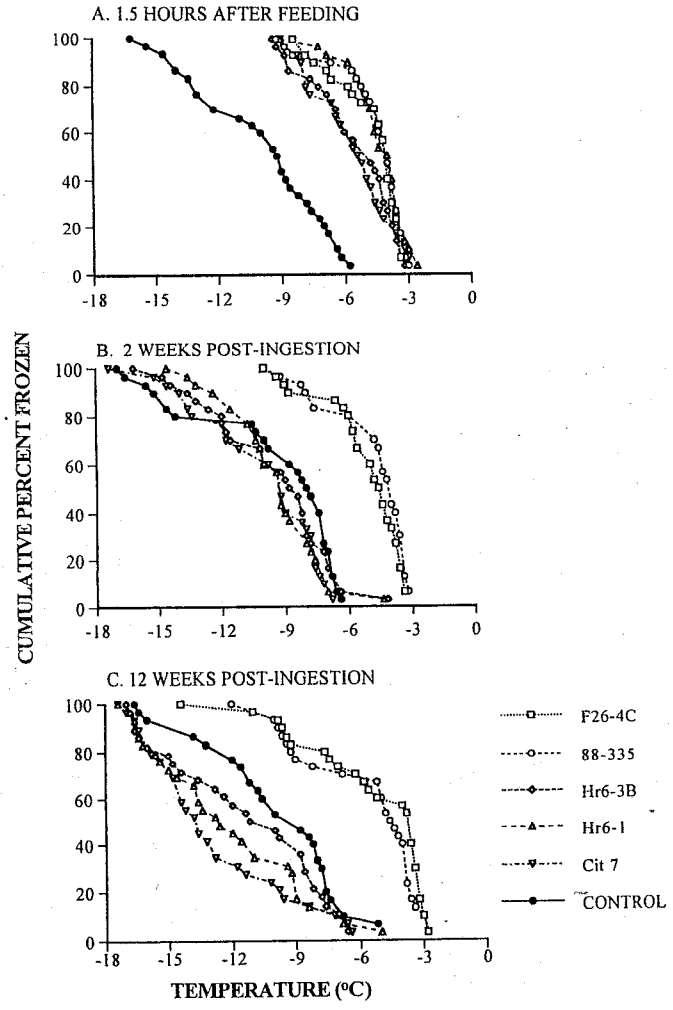

Figure 1. Cumulative freezing profiles based on individual supercooling points of Colorado potato beetles fed potato slices coated with ice-nucleating active *Pseudomonas* spp. Supercooling point values of beetles treated with each bacterial strain, along with control beetles, were measured 1.5 hours (A), 2 weeks (B), and 12 weeks (C) after ingestion. (From Castrillo et al., 2000a.)

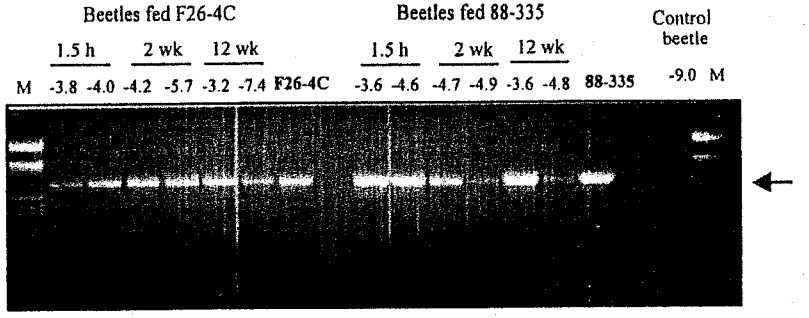

Figure 2. Detection of ice-nucleating active *Pseudomonas fluorescens* strains F26-4C and 88-335 in the gut of Colorado potato beetles at different sampling times after feeding: 1.5 hours, 2 weeks, and 12 weeks. Arrow indicates bands corresponding to the *inaW* gene. (From Castrillo et al., 2000a.)

3.2. Evaluation of bacterial efficacy and persistence in the field

Our field studies during the past two winters support the contention that it is possible to establish INA bacteria in the gut of overwintering adults (Castrillo et al., 2000b; unpublished data). In early autumn, beetles were fed slices of potato tubers coated with INA *P. fluorescens* F26-4C and released in the test arenas in the field. In spring of the following year, before beetles emerged from the ground, the soil was excavated and beetles were recovered. After overwintering for 7 months in the soil, treated beetles still had elevated SCPs, (-4.2 ± 0.1°C) compared to control beetles (-6.4 ± 0.1°C) (Fig. 3, Castrillo et al., unpublished data). Furthermore, SCP values of the recovered treated beetles were comparable to those observed 1.5 hours after exposure to INA bacteria (-4.4 ± 0.2°C) indicating that gut conditions in overwintering adults were favorable for expression of ice-nucleating activity. These data also provide evidence that laboratory results may accurately reflect bacterial activity in the field and that strains persisting for 2 weeks post-ingestion in diapausing beetles are likely to persist through the winter.

Even though the supercooling capacity of overwintering beetles was compromised by the presence of INA bacteria in their gut, overwintering survival of treated beetles with *P. fluorescens* F26-4C (31.8%) remained statistically indistinguishable from control beetles (42.8%) (Castrillo et al., unpublished data). Our field studies show that depth of burrowing determines the temperature overwintering beetles experience, which in turn determines the effect of INA bacteria on beetle survival. Given the unseasonably mild winters during which our field studies were conducted, soil temperatures did not drop low enough to affect a significant proportion of the population. Most of the recovered beetles were in the upper 15 cm of the soil strata and only beetles closer to the soil surface were subject to extreme temperature fluctuations and

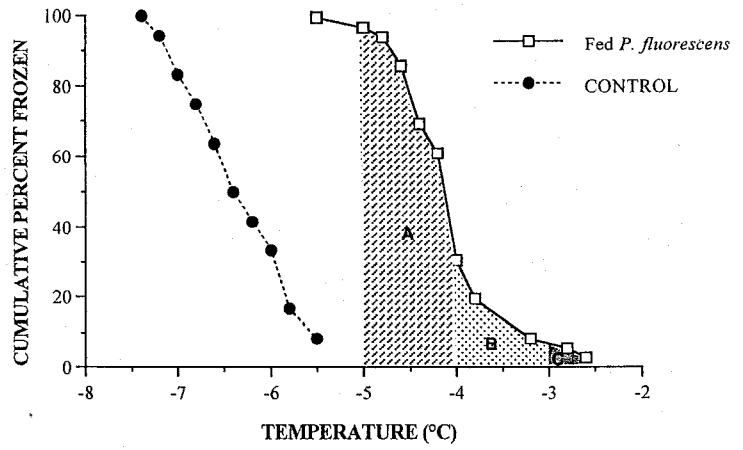

Figure 3. Cumulative freezing profile based on supercooling points of individual Colorado potato beetles fed *Pseudomonas fluorescens* F26-4C and measured after overwintering in the field for 7 months. Hatched areas in treated beetles indicate theoretical mortality at subzero temperatures: A) >95 to 50% mortality at -5 to -4°C; (B) ~50 to 10% at -4 to -3; and (C) <10% at above -3°C. (L.A. Castrillo, R.E. Lee, J.A. Wyman, M.R. Lee, and S.T. Rutherford, unpublished data.)

severe cold. Nevertheless, the relatively shallow depth of burrowing at which most beetles were found suggest that most overwintering beetles could be subject to lethally low temperatures during normal winters. For example, when temperatures in the upper 15 cm of the soil strata drop below -5.0°C, the presence of ice-nucleating *P. fluorescens* in the gut of overwintering adults could initiate internal freezing and, thus, cause notable mortality in the population. Given the range of SCPs of beetles recovered in spring (Fig. 3), 50 to 95% mortality would occur in overwintering adults exposed to soil temperatures as low as -4 to -5°C. In contrast, milder winters with warmer temperatures will reduce the likelihood of mortality due to ice-nucleating bacteria.

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. Heavy soils, which tend to have high moisture levels, limit depth of burrowing and 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). Thus, in moist soils where beetles burrow

shallowly, the effect of ice-nucleating bacteria on overwintering beetles would be enhanced during a severe winter.

3.3. Enhancement of bacterial activity

Our previous studies indicated that the degree of SCP elevation in treated beetles was affected not only by the number of bacterial cells retained in the gut, but also by the variability in the ice-nucleating activity of individual cells (Castrillo et al., 2000 a, b). Consequently, we conducted experiments to maximize ice-nucleating activity in *P. fluorescens* F26-4C by enhancing the expression of type 1 cells. Although these cells are most desirable in increasing beetle SCP, they generally make up only a small fraction of the bacterial population. In addition to our goal of maximizing bacterial activity, it was also our objective to develop a liquid medium for mass production for future field applications. Previously, bacterial culture in our laboratory was limited to a solid medium (nutrient agar plates with 2.5% glycerol), known to enhance expression of bacterial ice-nucleating activity (Lindow et al., 1982).

We adapted culture conditions used previously to enhance ice-nucleating activity in epiphytic strains (Nemecek-Marshall et al., 1993; Fall and Fall, 1998), and found that *P. fluorescens* F26-4C grown in liquid media could be induced to increase expression of type 1 ice nuclei by shifting the growth medium from 23°C to either 4 or 15°C (Castrillo et al., unpublished data).

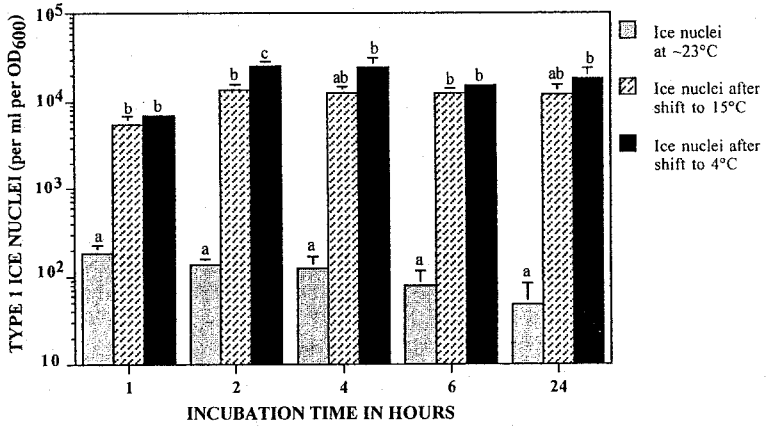

Figure 4. Effect of induction time and temperature on expression of type 1 ice nuclei in *Pseudomonas fluorescens* F26-4C grown in nutrient broth at ~23°C. Bacteria were grown to the stationary phase and then transferred to 4 or 15°C, or maintained at 23°C. Bars with the same letter are not significantly different. (L.A. Castrillo, S.T. Rutherford, R.E. Lee, and M.R. Lee, unpublished data.)

Table 2
Efficacy of ice-nucleating active *Pseudomonas fluorescens* with induced type 1 ice nuclei against overwintering Colorado potato beetles. (L.A. Castrillo, S.T. Rutherford, R.E. Lee, and M.R. Lee, unpublished data.)

Bacterial Culture Conditions			Beetle SCP (°C)*	
Medium	Phase	Induction†	1.5 Hour	2 Weeks
Nutrient agar w/ 2.5% glycerol	Solid	No	−4.2 ± 0.2a	−4.5 ± 0.3a
Nutrient agar w/ 2.5% glycerol	Solid	Yes	−3.8 ± 0.3a	−4.6 ± 0.6a
Nutrient broth w/ 2.5% glycerol	Liquid	Yes	−4.1 ± 0.3a	−4.3 ± 0.1a
L broth with limiting N and P, and with 5% dextrose	Liquid	Yes	−4.3 ± 0.1a	−4.1 ± 0.3a
CONTROL			−6.9 ± 0.9b	−7.5 ± 0.3b

*Mean values ± SEM ($N = 30$) within a column followed by the same letter are not significantly different at $\alpha = 0.05$ (Fisher protected LSD test).
†Type 1 ice nuclei expression in *P. fluorescens* was induced at 4°C for 2 hours.

Ice-nucleating activity can be enhanced during the exponential phase and into the stationary phase of growth, with greater levels of enhancement during the exponential phase. During the stationary phase optimal induction was achieved after shifting the bacteria to 4°C for 2 hours (Fig. 4). Growth in a defined medium with 5% dextrose or galactose and with limited levels of nitrogen or nitrogen and phosphorus, coupled with a temperature shift to 4°C resulted in the greatest induction of type 1 ice nuclei (Castrillo et al., unpublished data)

Bacterial cells grown in liquid media with induced type 1 ice nuclei were observed to be comparable to cells grown on solid medium in their efficacy against overwintering beetles (Castrillo et al., unpublished data). Beetle SCPs were significantly elevated 1.5 hours after feeding and even after 2 weeks post-ingestion (Table 2). Because *P. fluorescens* F26-4C will be growing under conditions prevailing in the gut of overwintering beetles in the field, induction of bacterial ice-nucleation activity prior to field application may not be necessary. Elevated SCPs of treated beetles were maintained for at least two weeks indicating that the conditions within the beetle gut permit bacterial growth and expression of ice-nucleating activity. Therefore, the low temperature shift that induces bacterial ice-nucleating activity will be provided naturally as soil temperatures decrease during winter.

4. FUTURE STUDIES

4.1. Trap-crop application strategy

One of the challenges that must be met if INA microorganisms are to be used for pest control is to effectively deliver INA microorganisms to the target insect. We envision the following scenario in using INA bacteria for the biological control of Colorado potato beetles. In late summer and early fall, shortly before entry into diapause, a high proportion of the adults disperse from the crop to seek overwintering sites in protected areas, often in close proximity to the crop (Milner et al., 1992; Weber and Ferro, 1993). During dispersal, adults typically congregate in large numbers on surviving pockets of crop foliage where feeding continues until vines are defoliated. Since the vines in virtually all fall potato fields are artificially killed 2-3 weeks prior to harvest, adult beetles migrate in large numbers to areas of fields where vine desiccants are not used (Wyman et al., 1994).

We propose to use unkilled strips of potato vines on field edges as trap crops where INA bacteria can be delivered. Trap crops will be sprayed with suspensions of INA bacteria that will be ingested by the beetles as they feed on the leaves. Shortly after feeding, beetles burrow into the soil to overwinter. Retention of the ingested INA bacteria will reduce cold tolerance, and, thus, increase mortality when soil temperatures decrease to subzero temperatures in midwinter.

4.2. Environmental persistence

Before any large-scale applications of ice-nucleating *P. fluorescens* are conducted, studies on environmental contamination and possible non-target effects need to be considered. How long do INA bacteria persist in the environment? Possible contamination of potato tubers harvested several days after bacterial application and/or possible soil accumulation leading to contamination of crops and tubers the following year are issues of concern that need to be addressed. Although *P. fluorescens* F26-4C is a gut-derived strain and may not survive outside of its overwintering host, sampling of the soil in the trap crop area and in adjacent sites, along with harvested crops should determine its persistence in an environment outside of the insect gut.

4.3. Non-target effects on beneficial insects

Because INA bacteria will be applied to potato foliage where beetles will actively feed, possible contamination of other insects, specifically parasitoids and predators, is a major concern. Do other insects become contaminated with INA bacteria applied in the field? If so, do they acquire sufficient numbers of bacteria to increase their SCPs? Are any such effects retained during the following weeks or into the winter? Although our previous experience (Lee et al., 1996) with the regulation of supercooling and ice nucleation in insects suggests that casual contact with ice-nucleating agents is unlikely to affect the

cold tolerance of non-target insect species found in potato fields, further studies are needed.

The use of ice-nucleating bacteria as biological control agents is based on their potential for reducing cold hardiness in freeze-intolerant insects. In the Colorado potato beetle, a pest that has expanded its habitat range from Mexico to southern Canada (Boiteau and Coleman, 1986) through its burrowing behavior and ability to supercool, the use of these bacteria may provide an additional means of control in regions with cold winter conditions. The feasibility of this control method is further enhanced with the identification of INA strains that are efficacious in elevating beetle SCPs and that persist in the gut of overwintering adults, and with the development of growth media for bacterial production.

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