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Biological Control of Insect Pests Using Ice-Nucleating Microorganisms

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Supercooling and Ice Nucleation in Insects

One of the most fascinating aspects of overwintering insects is their capacity for extensive supercooling of body fluids. Many species remain unfrozen to −20°C or lower. Several Alaskan gall-forming species have exceptional supercooling capacities, extending their limits to −60°C (Miller, 1982). Due to their small size, and therefore small water volume, insects have the innate capacity to supercool extensively as long as efficient ice nucleators are absent (Lee, 1991; Chapter 1). The accumulation of low molecular mass polyols and sugars such as glycerol, sorbitol, and trehalose, in sometimes multimolar concentrations, also enhances supercooling in insects (Lee, 1991).

The limit of supercooling, termed the supercooling point or the temperature of crystallization, is a significant temperature for both freeze-tolerant and freeze-intolerant insects. For those few insects able to survive extensive internal ice formation, this temperature represents a major transition to a physiological state of anaerobiosis, cellular dehydration due to freeze concentration of solute, and depressed metabolism (Storey and Storey, 1988). However, the vast majority of insects are freeze-intolerant, unable to survive the freezing of their body water. For these species, including most pest insects, the supercooling point represents the absolute, lowest temperature for survival (Lee, 1989, 1991). Nonetheless, it should be recognized that without empirical testing, this value cannot be assumed to be the lethal low temperature because some species die from chilling or cold shock injury at temperatures above the supercooling point (Lee and Denlinger, 1985; Bale, 1987).

Insects' supercooling capacity may be limited by inoculative freezing and the action of several classes of heterogeneous ice nucleators (Lee et al., 1993; Chapter 11). Inoculative freezing may occur when an insect comes in contact with external ice (Layne et al., 1990). Interestingly, in diapausing drosophilid larvae of *Chymomyza costata*, freezing is tolerated only if it occurs by inoculation at high subzero

temperatures, but not if larvae supercool extensively before freezing (Shimada and Riihimaa, 1988). Fields and McNeil (1986) reported similar results with overwintering larvae of an arctiid moth. Endogenous, heterogeneous ice nucleators include ice-nucleating proteins and lipoproteins (Zachariassen and Hammel, 1976; Duman et al., 1991). Internal inorganic nucleators may also play a role in regulating the supercooling point of insects; calcium phosphate crystallike compounds located in the Malpighian tubule have ice-nucleating activity sufficient to explain the supercooling point of the freeze-tolerant gall fly, *Eurosta solidaginis* (R.E. Lee et al., 1992a). Lastly, ice nucleation-active microorganisms have been reported recently as normal flora in the gut of insects (Lee et al., 1993). This chapter will focus on the natural relationships between ice nucleation–active microorganisms and insects and their potential use for the biological control of insect pests.

Discovery of Ice-Nucleating Microorganisms Naturally Associated with Insects

Although a number of other anatomic locations have been reported, the insect gut is the one most commonly associated with the onset of ice nucleation in insects (Lee et al., 1993). As early as 1936, Salt linked an elevation of the insect supercooling point with feeding. Various investigators have reported enhanced supercooling capacity in insects that had emptied their gut (see review by Cannon and Block, 1988). Johnston and Lee (1990) reported that the gut was the most frequent site of efficient nucleators in mealworm larvae, *Tenebrio molitor*. In prepupae of *Trichiocampus populi*, Shimada (1989) directly observed ice nucleation in the gut and the growth of the ice lattice across the gut wall using cryomicroscopy. Although Salt and others suggested that dust, accidentally ingested with food, might serve as ice nucleators in the gut (Somme, 1982; Baust and Rojas, 1985; Cannon and Block, 1988), this notion has been difficult to confirm directly. Furthermore, questions remain as to whether the ice-nucleating activity of dust particles and food materials could explain the observed supercooling points at high subzero temperatures. Nonetheless, these observations strongly suggest the presence of efficient ice nucleators in the insect gut.

Until recently, ice nucleation-active microorganisms had only been associated with plants. In the early 1970s, Ina$^+$ bacteria were first discovered in decaying leaves (see Chapter 2 and other chapters in this volume). These potent biological nucleators are restricted to gram-negative, rod-shaped asporogenous bacteria displaying ice-nucleating activity as high as −1°C. Several lichen species also have ice-nucleating activity associated with their fungal component as well as several *Fusarium* spp. (Kieft, 1988; Chapter 9).

Consequently, although no previous reports had directly linked any animal with Ina$^+$ bacteria, in the mid-1980s, we began studies to determine whether Ina$^+$ bacteria were present naturally in the insect gut (Strong-Gunderson et al., 1990a; Lee et al., 1991). During the summers of 1988 and 1989, we isolated *Enterobacter taylorae* and *E. agglomerans* from the gut of a field-collected bean leaf beetle, *Cerotoma trifurcata*, and the convergent lady beetle, *Hippodamia convergens* (Table 1). Both *Enterobacter* species exhibited maximal ice nucleation thresholds of approximately −2°C that were only slightly less than that of *Pseudomonas syringae*.

Independent investigations by Kaneko et al. (1991a,b) identified an Ina$^+$ bacterium in the gut of the diamondback moth, *Plutella xylostella*. Previously, they observed that pupae that were fed germinating radish seeds had higher supercooling points than ones fed aseptic seeds (Kaneko et al., 1989). Furthermore, supercooling points increased when pupae were held at a lower temperature. This response is consistent with the effect of low-temperature conditioning, which results in increased ice-nucleating activity in Ina$^+$ bacteria (Rogers et al., 1987). In turn, this observation led to the isolation and identification of *Erwinia herbicola* from the gut of diamondback moth pupae (Kaneko et al., 1991a,b).

Table 1. Ice nucleating active microorganisms isolated from the gut of insects

Microorganism	Insect	Reference
Bacteria		
Enterobacter agglomerans	*Hippodamia convergens*	Strong-Gunderson et al. (1990a)
	Cerotoma trifurcata	Lee et al. (1991)
E. taylorae	*H. convergens*	Strong-Gunderson et al. (1990a)
	C. trifurcata	Lee et al. (1991)
Erwinia herbicola	*Plutella xylostella*	Kaneko et al. (1991a,b)
Fungi		
Fusarium sp.	*Chilo suppressalis*	Tsumuki et al. (1992)

In 1992, a particularly interesting report described an Ina$^+$ fungus (*Fusarium* sp.), isolated from the gut of the rice stem borer larvae, *Chilo suppressalis* (Tsumuki et al., 1992). Ingestion of a mycelial suspension caused supercooling points to increase from −20 to −5°C. This report is significant for two reasons. First, at the time this work was being done, ice-nucleating activity in a free-living fungus was unknown. (However, an independent study by Pouleur and co-workers (1991) reported ice-nucleating activity in two species of free-living fungi, *Fusarium avenaceum* and *F. acuminatum*.) Secondly, the presence of an ice-nucleating microorganism in freeze-tolerant larvae suggests a mutualistic relationship between the fungus and its host (Lee et al., 1993). Here, as is believed for ice-nucleating proteins and lipoproteins (see Chapter 11), the ice-nucleating microorganisms may function to ensure that ice nucleation occurs at high subzero temperatures and to promote slow growth of the ice lattice in the extracellular space. In fact, ice-nucleating microorganisms might function even better in this regard, since they have greater ice-nucleating activity than ice-nucleating proteins and lipoproteins.

In summary, only since 1990 have Ina$^+$ bacteria and a fungus been reported as normal flora in the gut of insects (Table 1). These investigations also identified novel ice-nucleating microorganisms with previously unreported ice-nucleating activity. Further, we have identified three species of Ina$^+$ bacteria, *Pseudomonas fluorescens, E. agglomerans* and *P. putida*, from the gut of a freeze tolerant frog (M.R. Lee et al., 1992a). Considering the few research laboratories that have searched for Ina$^+$ microorganisms associated with animals, the association of these organisms may be much more common than previously recognized.

Biological Control of Insect Pests Using Ice-Nucleating Microorganisms

A number of investigators with primarily basic approaches to the study of insect diapause and cold-hardiness have wondered, at least as a secondary objective, whether it might be possible to interfere with these natural adaptations for overwintering survival and, thereby, diminish populations of insect pests. In the mid-1980s,

we began to wonder whether the natural capacity of overwintering insects to escape lethal freezing by supercooling might be artificially reduced by introducing potent nucleators into their bodies or onto their cuticles. Although at this time there was no evidence that Ina$^+$ microorganisms were associated with insects, or any animal for that matter, we wondered whether these biological ice nucleators, whose role in promoting frost damage in plants had been so clearly established, might be used to manipulate insect supercooling points (Lee, 1990).

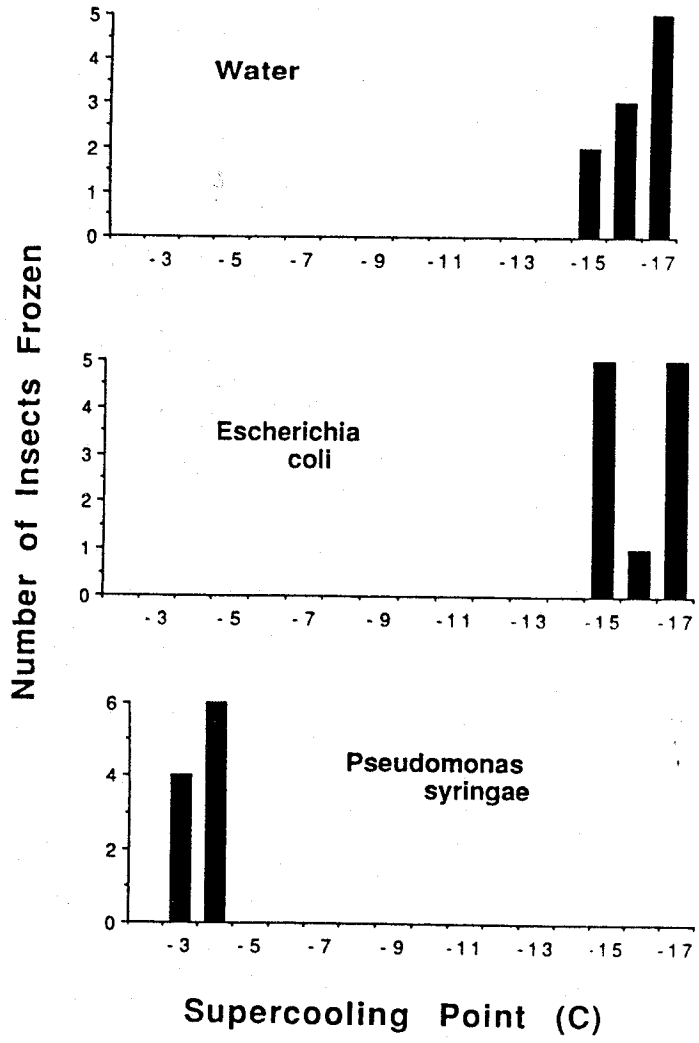

Figure 1. Supercooling points of adult lady beetles (*Hippodamia convergens*) determined immediately after ingesting water, or suspensions of a non–ice nucleation-active bacterium, *Escherichia coli*, or the epiphytic ice nucleating active bacterium, *Pseudomonas syringae* (both at 10^9 bacteria/ml of water).

In our initial study we decided to use the lady beetle, *Hippodamia convergens*, as an insect model because we were familiar with its cold tolerance (Lee, 1980). This freeze-intolerant species enhances its supercooling capacity to approximately −16°C in winter. Ingestion of water or a solution of a non-Ina$^+$ bacterium had no effect on the supercooling point; however, within seconds of ingesting a solution of *P. syringae*, supercooling points increased by as much as 14°C to −2°C (Fig. 1) (Strong-Gunderson et al., 1990b). Supercooling points remained elevated for at least 7 days after ingestion. In additional experiments, we were surprised that the supercooling point of beetles, whose mouths had been sealed, could be readily increased when sprayed with solutions of Ina$^+$ bacteria (Strong-Gunderson et al., 1989, 1992). A possible explanation for this unexpected result comes from studies in our laboratory demonstrating that topical application of *P. syringae* to the thoracic spiracles was more effective for increasing supercooling points values than application to the abdomen and other external anatomic sites (Steigerwald et al., 1993). The elevation of insect supercooling points using Ina$^+$ microorganisms has now been documented in more than 15 species from four orders of insects using five species of Ina$^+$ bacteria and the fungi *Fusarium acuminatum* and *F. avenaceum* (Lee et al., 1993; Fields et al., 1993).

Biological Control of Stored-Product Pests

Although control of stored product insects currently relies on chemical means, concerns for the toxic effects on nontarget species, the development of resistance, the removal of widely used insecticides from the market, and the problem of potential residues in food have raised interest in alternative forms of control and the use of integrated pest management strategies (Fields, 1992; Hagstrum and Flinn, 1992). Exposure to low temperature has long been used as a means for control of stored product pests because of its obvious advantages over chemical means of control (see review by Fields, 1992). Some stored product pests are very susceptible to low temperature and die even when exposed to temperatures above 0°C or only a few degrees below zero. Other stored product pests, such as the rusty grain beetle, *Cryptolestes ferrugineus*, are very cold tolerant, but not freeze tolerant, surviving weeks or months of exposure to subzero temperatures (Fields, 1990).

As a consequence, Fields (1990) proposed using Ina$^+$ bacteria as a cold synergist to increase the mortality of stored product insects exposed to cold. Obviously, this approach requires that the environmental temperature of the insect pest must be lowered to that of the supercooling point either by aerating storage bins with ambient air or by refrigeration. This strategy is supported by the fact that all of the major stored product pests are freeze-intolerant. In a laboratory study, Fields (1990) used a freeze-dried, killed preparation of *P. syringae* to reduce the supercooling capacity and cold tolerance of non-cold-acclimated rusty grain beetles. Untreated control beetles have a mean supercooling point of −17°C. However, for individuals treated with 100 or 1,000 ppm of *P. syringae*, the supercooling point increased by approximately 6 and 8°C, respectively. Similar treatments caused significantly greater mortality of beetles treated with *P. syringae* than of controls when exposed to −10°C for 24 hours. In another study, Fields (1993) found that although cold-acclimated individuals of *C. ferrugineus*, *Sitophilus granarius* and *Oryzaephilus surinamensis* were more cold tolerant than non-acclimated ones, treatment with Ina$^+$ bacteria also decreased their tolerance of subzero exposure.

Table 2. The effect of the Ina⁺ bacteria *Pseudomonas syringae* on the supercooling point (mean ± SE) of stored grain insects[a,b]

Species	Supercooling point (°C)	
	Untreated control	*P. syringae* (100 ppm)
Plodia interpunctella		
Indianmeal moth larvae	−10.3 ± 0.4	−5.4 ± 0.5
Sitophilus granarius		
Granary weevil adults	−15.7 ± 1.0	−8.0 ± 0.6
Rhyzopertha dominica		
Lesser grain beetle adults	−15.2 ± 0.6	−3.3 ± 0.1
Tribolium castaneum		
Red flour beetle adults	−13.9 ± 0.8	−4.7 ± 0.4
Gibbium psylloides		
Shiny spider beetle adults	−10.7 ± 0.9	−6.0 ± 0.5

[a] Adapted from R. E. Lee et al. (1992b).
[b] Insects were treated with 100 ppm of *P. syringae* in wheat for 24 h at 23°C. For each species, treatment with *P. syringae* caused a statistically significant increase in the supercooling point (Student's *t* test, $P < 0.01$).

Table 3. Survival of larval and adult stored grain pests exposed to various concentrations of dry, powdered *Pseudomonas syringae* in wheat for 24 h at 23°C before 24 h of exposure to −5°C[a,b]

Species	Survival (%)		
		Pseudomonas syringae	
	Control	100 ppm	1,000 ppm
Tenebrio molitor			
Yellow mealworm larvae	97	77	73
Sitophilus granarius			
Grainery weevil adults	96	62	48
Cryptolestes ferrugineus			
Rusty grain beetle adults	97	0	0
Cryptolestes pusillus			
Flat grain beetle adults	43	0	0
Plodia interpunctella			
Indianmeal moth larvae	96	36	3.8
Tribolium castaneum			
Red flour beetle adults	19	0	0
Rhyzopertha dominica			
Lesser grain borer adults	90	3.6	0
Gibbium psylloides			
Shiny spider beetle adults	93	25	0

[a] Adapted from R. E. Lee et al. (1992b).
[b] All treatments ($n = 30$) were significantly different ($P < 0.05$) from controls.

We conducted additional experiments using eight species of stored grain insects (R. E. Lee et al., 1992b). In five species, supercooling points of control insects ranged from −10.3 to −15.7°C; however, following treatment with 100 ppm of *P. syringae* preparation in wheat, mean supercooling points increased for all species, with *Rhyzopertha dominica* having the highest value of −3.3°C (Table 2). Complementary studies examined insect survival at subzero temperatures following treatment with Ina⁺ bacteria. Although some species were intolerant of −5°C exposure

Table 4. Cumulative mortality (%) of cold-acclimated rusty grain beetle (*Cryptolestes ferrugineus*) treated with 1,000 ppm of dry, powdered *Pseudomonas syringae* in aerated Manitoba granaries[a]

Time after bacterial application (days)	Granary temperature (°C)	Mortality (%)	
		Untreated	Treated
7	>0	3-5	3-7
15	−9 to −15	32-53	98-99
22	−9 to −15	62-69	98-100
30	−9 to −15	98-99	100

[a] Data from Fields (1993).

for 24 hours even when not treated with bacteria, treatment with either 100 or 1,000 ppm of *P. syringae* in wheat caused a significant, and a dose-dependent reduction in survival for all species tested (Table 3). When the temperature of exposure was decreased to −8°C, this trend continued and survival rates decreased still further (R.E. Lee et al., 1992b).

Fields (1993) also examined the efficacy of *P. syringae* in reducing the cold tolerance of *C. ferrugineus* under field conditions in Manitoba granaries. Groups of cold-acclimated rusty grain beetle adults were treated with 1,000 ppm of *P. syringae* and placed in wheat granaries in early December. On days 15 and 22 treated beetles had significantly lower survival rates than untreated groups (Table 4). However, by day 30 nearly all individuals in both treated and untreated groups had died.

From these initial studies with stored product pests, it is clear that Ina⁺ bacteria may be used to decrease the supercooling capacity and cold-hardiness even of cold-acclimated insects. In an attempt to standardize protocols for future studies of survival of stored product insects at extreme temperatures, Fields (1992) recommended the following: 1) tests should be conducted with insect strains that have been in the laboratory no more than 2 years, 2) the most temperature-resistant developmental stage of the pest should be used, 3) temperature-acclimated insects should be used, 4) a range of extreme temperatures should be tested so that data may be analyzed using probit analysis and that fiducial limits may be reported, and 5) the results of laboratory studies should be confirmed with field tests. Future investigations using Ina⁺ microorganisms for the control of stored product pests should follow these criteria.

Prospects for the Biological Control of the Colorado Potato Beetle

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is the most serious defoliating pest of potatoes, *Solanum tuberosum* L., in North America. This species overwinters by burrowing into the soil in late summer or early autumn. When overwintering adults emerge from dormancy, they can significantly reduce yields by defoliating the early growth stages of the potato plants (Shields and Wyman, 1984). This pest is known for the wide range of pesticides, including synthetic pyrethroids, to which it has rapidly developed resistance (Casagrande, 1987). The current agricultural practice of planting extensive monocultures of potatoes further promotes the cumulative buildup of Colorado potato beetle populations from year to year. Because of these factors, current research efforts have increasingly begun to

focus on alternative forms of control.

One novel approach to management of the pest uses cultural methods designed to expose Colorado potato beetles to lethal low temperatures during the winter (Milner et al., 1992; Kung et al., 1992). Trap crops of potatoes are planted in midsummer, and because these younger plants are more attractive to the adults, the beetles concentrate in these areas. In late summer, trap plants are mulched to encourage the beetles to remain at these sites over winter rather than dispersing from the fields. Insect mortality is induced by removing the mulch in midwinter immediately prior to a cold front, which causes the soil temperatures to drop rapidly. Mulching may also reduce the depth to which the beetles will burrow during the winter, increasing further their susceptibility to the lowering of soil temperature. The feasibility of this approach was recently supported by the work of Milner et al. (1992). They found that adult beetle survival was significantly lower at sites where mulch was removed in midwinter.

In collaboration with Jeffrey Wyman and Phil Kaufman, University of Wisconsin, we have begun investigating the use of Ina$^+$ microorganisms to increase the susceptibility of the overwintering beetles to low temperatures (Lee et al., 1994). Our rationale is to decrease the cold-hardiness of the beetles using these biological nucleators when applied in conjunction with the cultural control approach (Milner et al., 1992).

In our initial study, we determined that the Colorado potato beetle is a freeze-intolerant species that dies when cooled to its supercooling point (Lee et al., 1994). However, the 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 the supercooling point may be used as a measure of the lethal low temperature, at least during short-term exposure to cold.

Considering the relatively high supercooling point of approximately −7°C for overwintering adults, it is obvious that this species lacks exceptional cold tolerance. Their limited capacity for supercooling is not surprising, however, considering their thermally protected overwintering site in the soil (Lee, 1991). Nonetheless, an elevation in the supercooling point of as little as 2 to 4 degrees in the lethal low temperature would be of major significance in decreasing the proportion of beetles surviving the winter. Colorado potato beetles exposed to −4°C had a survival rate of 54.8%, whereas only 6.2% of those exposed to −6°C survived (Kung et al., 1992).

To simulate overwintering conditions in the laboratory, we tested whether supercooling points increased in beetles that were exposed to a concentrated, freeze-dried and killed preparation of *P. syringae* mixed with soil (Lee et al., 1994). Mean supercooling points of beetles treated with *P. syringae* in concentrations ranging from 0 to 1,000 ppm were determined (Fig. 2A). In both 1991 and 1992, the supercooling point means increased significantly when beetles were exposed to increasing concentrations of *P. syringae*, ranging from −7.6 ± 0.2°C (untreated) to −3.7 ± 0.1°C (1,000 ppm). In the 1992 tests, as little as 1 ppm resulted in a supercooling point that was statistically higher than that of the untreated control. These results indicate that the effect of Ina$^+$ microorganisms on the supercooling point is dose-dependent as has been reported previously in other insects (Fields, 1990; R.E. Lee et al., 1992b).

The cumulative freezing distributions, comparable to the ice nucleation spectra that are typically used to describe the ice-nucleating activity of Ina$^+$ bacteria, were also determined for these beetles (Fig. 1B). These curves are a useful form of data presentation because they show a profile of the theoretical lethal low temperature for a population of beetles treated with various concentrations of *P. syringae* (Lee et al., 1994). 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. The similarity of the 100 and 1,000 ppm curves further suggests that, under these conditions, the elevation of the supercooling point reaches a maximum near 100 ppm.

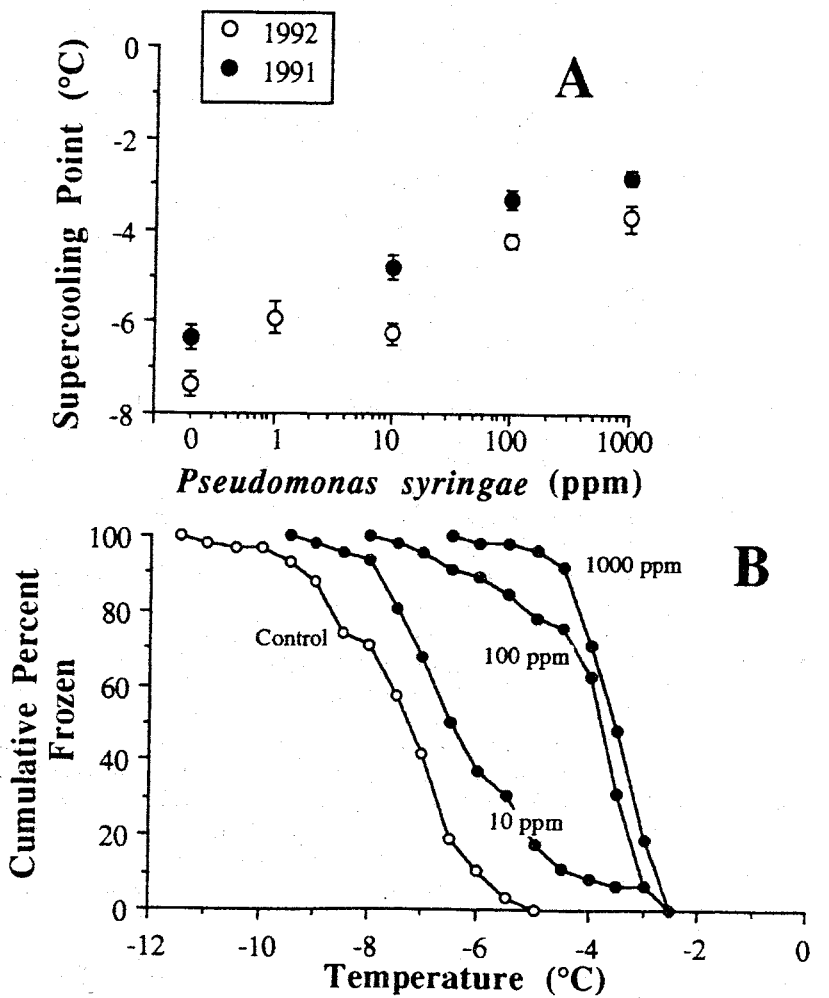

Figure 2. A, Effect of *Pseudomonas syringae* on the mean (± standard error) supercooling point of diapausing adults of the Colorado potato beetle. Beetles were exposed to various concentrations (0–1,000 ppm) of *P. syringae* in soil for 48 hours at 4°C. In 1991, sample sizes were n = 10–11, and in 1992, n = 44–58. **B,** Cumulative freezing profile for beetles exposed to various concentrations of *P. syringae* in 1992 . (Adapted from Lee et al., 1994.)

Another critical factor in the development of a biological control strategy for this species is the duration of the supercooling point elevation after application of microorganisms. The supercooling point of beetles treated with *P. syringae* was elevated significantly for 7 days after application at 4°C but was similar to that of untreated beetles by day 14 (Lee et al., 1994). Previous studies have reported a loss of bacterial ice-nucleating activity with time at temperatures above 0°C (Goodnow et al., 1990). Treatment temperature also affected supercooling point elevation of beetles following treatment with *P. syringae*. Although after 7 days the mean supercooling points for untreated beetles incubated at 4 or 10°C were similar, the values for those treated with *P. syringae* remained significantly higher at 4 than at 10°C. These data suggest that ice-nucleating activity was better retained during incubation at the lower temperature. In contrast, Fields et al. (1993) reported long-term stability of a *P. syringae* preparation held at 30°C for 8 weeks. Obviously, additional study is needed regarding the effects of temperature and duration of exposure on supercooling point elevation by *P. syringae*.

At this time it is envisioned that northern populations of the Colorado potato beetle would be treated with Ina$^+$ bacteria in late August or early September, when adults have been attracted to feed on trap crops on the edges of fields, but before they have begun to burrow into the soil to overwinter. Since ambient temperatures are still relatively high at this time of the year, the ice-nucleating activity of the bacteria may be lost before environmental temperatures drop low enough (even after applying the cultural manipulations of Milner et al. [1992]) to kill the beetles. One alternative approach to this problem would be to find ways to maintain the ice-nucleating activity of microorganisms in the gut or on the surface of the insect or to apply other Ina$^+$ microorganisms that would be retained by the beetles until environmental temperatures in their microhabitat decrease to lethal levels. Consequently, we have begun to test the efficacy of several different species of living Ina$^+$ bacteria and fungi for their effect on the beetle's supercooling point. We have also tested the effect of suspensions of living Ina$^+$ bacteria on the supercooling point. Suspensions of living *P. fluorescens, P. syringae,* and *P. putida* sprayed onto the adults all caused a significant increase in the supercooling point, indicating that living bacterial cells may also be used for supercooling point manipulation.

Another modification that may be of value in the development of biological control methods is the use of surfactants in combination with Ina$^+$ microorganisms (Lee et al., 1993). The addition of Tween 80 to *F. acuminatum* suspensions significantly increased the amount of supercooling point elevation in the beetle, *H. convergens,* compared with use of this Ina$^+$ fungus alone (M.R. Lee et al., 1992b).

In the case of the Colorado potato beetle, the application of the Ina$^+$ bacteria would be facilitated by the use of trap cropping, which concentrates the beetles in a narrow portion of the field and, thereby, would reduce the amount and cost of biological nucleators that must be applied. The successful integration of these biological ice nucleators with the cultural control strategy of Milner et al. (1992) also would allow use of this cultural control approach in areas of the country where it otherwise could not be used because winter soil temperatures would be too mild.

Concluding Remarks

Use of Ina$^+$ microorganisms for biological control has both advantages and disadvantages. First, since it appears to be effective against a diverse range of insects, consideration must be given to avoiding detrimental effects on beneficial insects. This may be accomplished if applications can be targeted to specific areas where few nontarget insects would be exposed, such as in the trap crops for Colorado potato beetles or storage product sites. If field isolates of Ina$^+$ microorganisms can be used for control, it would circumvent problems associated with the release of genetically engineered organisms into the environment. Considering the apparent ease and rapidity with which Ina$^+$ bacteria cause an increase in the supercooling point, it may be relatively difficult for insects to develop resistance to this control measure, since it would require blocking any and all avenues of contact between the Ina$^+$ organisms and internal water. The development of resistance to transcuticular nucleation is undoubtedly more complex and is expected to be an unlikely or at least slower process than common mechanisms of resistance, such as alteration in the structural target for a toxin or the production of toxin-destroying enzymes. On the other hand, if insects move to areas (e.g., burrow more deeply into the soil) sufficiently warm to remain above the supercooling point, this approach would be ineffective. Another advantage of this treatment is the biodegradability of these preparations, which are unlikely to leave behind contaminating residues. Lastly, this approach is fully compatible with other control measures that might be used concomitantly for integrated pest management of a given species.

Although the initial studies related to the potential use of ice-nucleating microorganisms are encouraging, considerably more work is needed to determine whether this approach will prove useful for biological control.

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