Temperature Sensitivity in Insects and Application in Integrated Pest Management

EDITED BY
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As concerns continue to mount regarding the environmental and human health consequences of using chemical controls for insect pests, a wide variety of alternative approaches are receiving increased attention. Crop rotation, tillage practices, genetically-engineered crop varieties, and the use of predators, parasites, and pathogens as agents of biological control are representative of these strategies. In this chapter we describe the initial results of research that may lead to a novel strategy for the control of insect pests that naturally overwinter in exposed sites or whose environment can be artificially cooled. This approach relies on the use of ice nucleating active microorganisms to increase the likelihood that pests will experience lethal internal freezing.

Supercooling and Ice Nucleation

A pure liquid or solution that remains unfrozen at temperatures below its equilibrium freezing point is said to be supercooled (Angell 1982). In the absence of ice nucleating agents small volumes of water (i.e., on the order of a few microliters) readily supercool, sometimes many degrees below their freezing point. In fact, pure water droplets can approach a limit of \(-40^\circ C\) before the random clustering of water molecules spontaneously forms an ice embryo upon which an ice lattice can form, a process termed homogeneous ice nucleation.

In biological systems, ice nucleation almost always occurs at temperatures that are above \(-20^\circ C\) (Vali 1995). In this situation it is thought that nucleation occurs via a heterogeneous process in which a non-water substrate functions as the embryonic seed crystal initiating freezing. Relatively inefficient ice nucleators are active at temperatures below \(-10^\circ C\), while a few inorganic and organic substances are active at \(-5^\circ C\) or warmer. A few bacteria and fungi (discussed later in this chapter) have the unique capacity to catalyze ice nucleation at temperatures near \(-2^\circ C\).

The specific subzero temperature at which ice nucleation occurs is determined by a stochastic process that is influenced by both volume and the duration of exposure (Vali 1995). As volume increases, the capacity of a solution to supercool decreases, whereas increasing the duration of exposure to low temperature increases the likelihood that heterogeneous ice nucleation will occur.

Supercooling and Ice Nucleation in Insects

With respect to volume, insects are, in one sense, small bags of water and consequently, in the absence of endogenous ice nucleators, have an inherent capacity to supercool, sometimes extensively (Lee 1989). Many small species and insect eggs supercool by 20 to 30°C before they spontaneously freeze (Somme 1982). In insects the temperature at which ice nucleation occurs is termed the supercooling point. Experimentally, this value is readily determined by monitoring an insect’s body temperature with thermistors or thermocouples as it is cooled to detect the abrupt appearance of an exotherm caused by the release of the latent heat of crystallization as body water freezes. The temperature at which the exotherm begins is the supercooling point.

At the organismal level, the supercooling point is significant for a number of reasons. In the many insects that are unable to survive the freezing of their body water, this value represents the lower lethal temperature. However, some insects are lethally injured when they are cooled to temperatures considerably above their supercooling point (Bale 1987, Lee & Denlinger 1985). Freezing-intolerant insects commonly depress their supercooling points during the autumn in preparation for winter, thereby decreasing the chance that they will freeze internally. Some insects are freezing tolerant and can survive the freezing of 65% or more of their body water (Lee 1991). In contrast to freezing-intolerant species, freezing-tolerant insects often undergo physiological changes that increase their supercooling point during cold-hardening. It is generally believed that promoting internal ice formation at relatively high subzero temperatures functions to slow the rate of extracellular ice formation and consequent cellular dehydration which thereby allows the insect to more easily adjust to this radical change in its internal milieu (Lee 1991).

Many insects can physiologically regulate their supercooling capacity. During cold-hardening (the acquisition of increased cold tolerance) many insects accumulate high concentrations of low molecular mass sugars and polyhydric alcohols, sometimes reaching multimolar levels in the hemolymph (Lee 1991). Glycerol, sorbitol, and trehalose are the most commonly accumulated substances, although others such as fructose, glucose, and mannitol have been reported. One effect of these compounds, sometimes termed low molecular mass antifreezes, is to colligatively depress not only the freezing point, but also the supercooling point. In insects with these antifreezes the supercooling point is depressed by approximately twice as much as the freezing point (Zachariassen 1985). Antifreeze proteins also appear to play a role in promoting supercooling in insects (Duman et al. 1995).

Several sites of ice nucleation and types of endogenous ice nucleators have been identified in insects (Cannon & Bloch 1988, Lee et al. 1993a, Zachariassen 1992). The gut is the most commonly identified site of ice nucleation. Cessation of feeding or emptying of the gut in preparation for overwintering is often associated with an increased capacity for supercooling. Freezing-tolerant insects commonly produce ice nucleating proteins and lipoproteins that function to limit supercooling and promote freezing at relatively high subzero temperatures (Zachariassen & Hammel 1976). These proteins are efficient ice nucleators inducing freezing at temperatures between \(-6\) to \(-9^\circ C\) (Duman et al. 1995). Recently, another
class of crystalloid deposits was described that function as heterogeneous nucleators in insects. In larvae of the freezing-tolerant gall fly *Eurosta solidaginis* spherules of calcium phosphate in the Malpighian tubules exhibited ice nucleating activity similar to the temperature at which the intact larvae froze (Mugnano et al. 1996).

Another way in which ice nucleation within the body fluids of an insect may begin is by inoculative freezing (Lee et al. 1996a). In this case, ice external to the insect makes contact with body water and initiates internal freezing. Because this type of freezing may occur with little or no supercooling of body fluids, it has been suggested that the term temperature of crystallization is more universal and appropriate than supercooling point (Wasylyk et al. 1988). Furthermore, inoculative freezing appears to be an important factor for low temperature survival in a number of freezing-tolerant species (Fields & McNeil 1986, Gehrken & Southon 1992, Gehrken et al. 1991) but is deleterious in freezing-intolerant species.

We should emphasize that the supercooling point as determined in the laboratory under idealized conditions necessarily represents the best-case scenario for supercooling capacity. Under field conditions, various factors undoubtedly constrain an individual's potential for supercooling. For example, supercooling capacity of larvae of the goldenrod gall fly changes seasonally in accordance with the amount of moisture within tissues of the gall it inhabits, because this soft-bodied larva is highly susceptible to inoculative freezing (Layne et al. 1990). Early in winter, when moisture is abundant, larvae within galls may freeze at only several degrees below 0°C. In contrast, supercooling point values determined for this species under idealized (i.e., dry) conditions in the laboratory may be as low as -10°C (Layne et al. 1990). This example underscores the importance of using care in estimating lower lethal temperatures from laboratory supercooling point data (Bale 1987).

**Ice Nucleating Active Microorganisms**

In the 1970's, ice nucleating active bacteria were discovered in association with plants and decaying leaves (for a historical review see Upper & Vali 1995). Taxonomically, these bacteria are restricted to only a few genera of Gram-negative rods within the Pseudomonadaceae and Enterobacteriaceae. Several recent reviews have summarized molecular and biochemical aspects of bacterial ice nuclei (Fall & Wolber 1995, Kajava 1995, Warren 1995, Wolber 1993, Wolber et al. 1995). The ice nucleating phenotype is due to a minor outer membrane-bound protein whose activity is generally lost during cell fractionation. Both free-living fungi and lichen mycobionts with ice nucleating activity are known (Ashworth & Kieft 1995), however, their highest levels of ice nucleating activity are less than those of bacterial strains. Fungal ice nuclei exhibit greater stability at high temperatures and extremes of pH than bacterial ice nucleators (Pouleur et al. 1992, Fields et al. 1995).

Even if a bacterial strain carries the gene for ice nucleating activity, its phenotypic expression generally varies considerably from cell to cell, even in the same culture (Lindow et al. 1978). Few cells from a given population will exhibit the highest levels of ice nucleating activity at temperatures near -2°C, whereas others exhibit considerably less activity. To quantitatively characterize the ice nucleating activity of a bacterial population, Vali (1971, 1995) developed a freezing droplet assay. Various cultural conditions including the composition of the medium and the incubation at low temperature sometimes cause an increase in the expression of the ice nucleating phenotype (Fall & Wolber 1995, Kajava 1995, Warren 1995, Wolber 1993, Wolber et al. 1995).

Because most strains of epiphytic ice nucleating bacteria are not only plant pathogens but are also responsible for extensive frost-related crop losses, they have received considerable study (Hirano & Upper 1991, 1995, Lindow 1983, 1995). When these epiphytic bacteria nucleate water on their own surface they also induce freezing and may facilitate their invasion of their hosts' tissues (Lindow 1983). One novel approach that has considerable promise for controlling these frost-related crop losses uses non-ice nucleating active bacteria to competitively displace or colonize the surface of plants before ice nucleating active bacteria do so (Lindow 1995).

**Natural Associations Between Ice Nucleating Active Microorganisms and Insects**

For nearly 20 years ice nucleating active microbes were known only from free-living or epiphytic strains. Early in the 1990's, reports appeared describing ice nucleating active microbes that had been isolated from the gut of ectothermic animals, primarily insects (Table 4.1). Strains of *Enterobacter taylorae* and *E. agglomerans* isolated from beetles exhibited
maximal thresholds of ice nucleating activity at approximately 2°C, only slightly less than the highly active epiphytic strains (Lee et al. 1991). When these bacteria were fed to an insect model, the lady beetle *Hippodamia convergens*, its supercooling point increased by 12-13°C above its unfed control level of -16°C (Lee et al. 1991). Similarly, Kaneko and colleagues (1991a,b) isolated *Erwinia herbicola*, which had significant ice nucleating activity, from the diamondback moth, *Plutella xylostella*.

Recent investigations with the rice stem borer, *Chilo suppressalis*, described an ice nucleating active fungus isolated from its gut flora that had sufficient ice nucleating activity to explain fully the supercooling point (-8.4°C) of the intact larvae (Tsumuki 1992, Tsumuki & Konno 1991). Unlike the cases of ice nucleating bacterial strains that were isolated from freezing-intolerant insects, the rice stem borer is freezing tolerant. Consequently, this result indicates that the fungal ice nucleator functions like ice nucleating proteins to insure that protective freezing will begin at high subzero temperatures and suggests a mutualistic association between the fungus and its insect host (Lee et al. 1993b, 1995b, Tsumuki 1992). Of particular note, an ice nucleating active *Pseudomonas putida* has been isolated from the gut of the wood frog, *Rana sylvatica*, and may, under certain conditions, serve a similar function for this freezing tolerant species (Costanzo & Lee 1996, Lee et al. 1995a).

**Manipulation of Insect Supercooling Using Ice Nucleating Active Microorganisms**

It is now evident that the supercooling capacity of a wide variety of insects is readily manipulated using ice nucleating active microorganisms (Lee et al. 1993a, 1995b). Either living or killed preparations of these ice nucleators have been used to significantly increase the supercooling point of adults and/or larvae of five insect orders: Coleoptera, Diptera, Hemiptera, Hymenoptera, and Lepidoptera (Table 4.2). Depending on the species, these treatments increase the supercooling point by a few degrees to more than 15 degrees Celsius. Ingestion of ice nucleating active bacteria causes an immediate elevation of the supercooling point (Strong-Gunderson et al. 1990); after an insect drinks for only a few seconds from a solution of ice nucleating bacteria we have observed an elevation within the few minutes required to make a supercooling point determination.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Bacteria</th>
<th>Host Animal</th>
<th>Nucleating Activity (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Enterobacter agglomerans</em></td>
<td>C. trifurcata (Colleoptera)</td>
<td>-2</td>
<td>Strong-Gunderson et al. 1990</td>
</tr>
<tr>
<td></td>
<td><em>Erwinia herbicola</em></td>
<td><em>Hippodamia convergens</em> (Colleoptera)</td>
<td>-2</td>
<td>Lee et al. 1991, Lee et al. 1995a</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas fluorescens</em></td>
<td><em>Plutella xylostella</em> (Lepidoptera)</td>
<td>&gt;-10</td>
<td>Lee et al. 1991, Kaneko et al. 1995a</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas putida</em></td>
<td>Dendroides canadensis (Colleoptera)</td>
<td>-2</td>
<td>Tsumuki et al. 1992</td>
</tr>
<tr>
<td></td>
<td><em>Fusarium sp.</em></td>
<td>Rana sylvatica (wood frog)</td>
<td>-5</td>
<td>Kaneko et al. 1991, Kaneko et al. 1995a</td>
</tr>
<tr>
<td></td>
<td><em>Chilo suppressalis</em> (Lepidoptera)</td>
<td></td>
<td></td>
<td>Tsumuki et al. 1992</td>
</tr>
</tbody>
</table>

**TABLE 4.1** Ice nucleating active bacteria and fungi from the gut of animals.
TABLE 4.2 Effect of treatment with ice nucleating active microorganisms on the supercooling point of insects. (Expanded and modified from Lee et al. 1993a)

<table>
<thead>
<tr>
<th>Insect (Stage)</th>
<th>Microorganism</th>
<th>Supercooling Point (°C) (± SEM)</th>
<th>Untreated</th>
<th>Treated</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coleoptera</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptolestes ferrugineus (adult)</td>
<td><em>Pseudomonas syringae</em>¹</td>
<td>-17.0 ± 1.0</td>
<td>-8.1 ± 0.5</td>
<td></td>
<td>Fields 1990</td>
</tr>
<tr>
<td>C. pusillus (adult)</td>
<td><em>P. syringae</em>²</td>
<td>-14.0 ± 1.0</td>
<td>-12.0 ± 1.5</td>
<td></td>
<td>Fields 1992</td>
</tr>
<tr>
<td>Diabrotica undecimpunctata</td>
<td><em>P. syringae</em>³</td>
<td>-7.5 ± 0.8</td>
<td>-3.2 ± 0.2</td>
<td></td>
<td>Strong-Gunderson et al.</td>
</tr>
<tr>
<td>howardi (adult)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>unpub. data</td>
</tr>
<tr>
<td>Gibbium psylloides (adult)</td>
<td><em>P. syringae</em>³</td>
<td>-10.7 ± 0.9</td>
<td>-6.0 ± 0.5</td>
<td></td>
<td>Lee et al. 1992b</td>
</tr>
<tr>
<td>Hippodamia convergens (adult)</td>
<td><em>P. syringae</em>¹</td>
<td>-16.0 ± 0.5</td>
<td>-2.8 ± 0.2</td>
<td></td>
<td>Strong-Gunderson et al. 1990</td>
</tr>
<tr>
<td></td>
<td><em>Erwinia herbicola</em>⁴</td>
<td>-16.0 ± 0.5</td>
<td>-4.4 ± 0.6</td>
<td></td>
<td>Strong-Gunderson et al. 1990</td>
</tr>
<tr>
<td></td>
<td><em>Enterobacter agglomerans</em>⁴</td>
<td>-16.0 ± 0.5</td>
<td>-3.1 ± 0.1</td>
<td></td>
<td>Lee et al. 1991</td>
</tr>
<tr>
<td></td>
<td><em>E. taylorae</em>⁴</td>
<td>-16.0 ± 0.5</td>
<td>-4.3 ± 0.4</td>
<td></td>
<td>Lee et al. 1991</td>
</tr>
<tr>
<td></td>
<td><em>Fusarium acuminatum</em>⁷</td>
<td>-14.9 ± 0.5</td>
<td>-11.0 ± 0.7</td>
<td></td>
<td>Lee et al. 1992b</td>
</tr>
<tr>
<td></td>
<td><em>P. syringae</em>³</td>
<td>-13.7 ± 1.9</td>
<td>-11.0 ± 1.3</td>
<td></td>
<td>Fields 1992</td>
</tr>
<tr>
<td>Oryzaephilus surinamensis (adult)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhyzopertha dominica (adult)</td>
<td><em>P. syringae</em>³</td>
<td>-15.2 ± 0.6</td>
<td>-3.3 ± 0.1</td>
<td></td>
<td>Lee et al. 1992b</td>
</tr>
<tr>
<td>Sitophilus granarius (adult)</td>
<td><em>P. syringae</em>³</td>
<td>-14.3 ± 0.8</td>
<td>-7.8 ± 0.5</td>
<td></td>
<td>Fields 1992</td>
</tr>
<tr>
<td>S. granarius (adult)</td>
<td><em>P. syringae</em>³</td>
<td>-15.7 ± 1.0</td>
<td>-8.0 ± 0.6</td>
<td></td>
<td>Lee et al. 1992b</td>
</tr>
<tr>
<td>Tenebrio molitor (larva)</td>
<td><em>P. syringae</em>³</td>
<td>-16.0 ± 0.7</td>
<td>-5.4 ± 0.7</td>
<td></td>
<td>Strong-Gunderson et al. unpub. data</td>
</tr>
<tr>
<td>T. molitor (adult)</td>
<td><em>P. syringae</em>³</td>
<td>-15.1 ± 0.6</td>
<td>-2.7 ± 0.3</td>
<td></td>
<td>Strong-Gunderson et al. unpub. data</td>
</tr>
<tr>
<td>Tribolium castaneum (adult)</td>
<td><em>P. syringae</em>³</td>
<td>-13.9 ± 0.8</td>
<td>-4.7 ± 0.4</td>
<td></td>
<td>Lee et al. 1992b</td>
</tr>
<tr>
<td></td>
<td><em>P. syringae</em>³</td>
<td>-12.3 ± 1.0</td>
<td>-5.8 ± 0.3</td>
<td></td>
<td>Fields 1992</td>
</tr>
<tr>
<td>Diptera</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sarcophaga crassipalpis (larva)</td>
<td><em>P. syringae</em>³</td>
<td>-13.8 ± 0.9</td>
<td>-3.6 ± 0.1</td>
<td></td>
<td>Strong-Gunderson et al. unpub. data</td>
</tr>
<tr>
<td>Hemiptera</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lygus sp. (adult)</td>
<td><em>P. syringae</em>³</td>
<td>-20.0 ± 0.5</td>
<td>-8.7 ± 1.0</td>
<td></td>
<td>Strong-Gunderson &amp; Lee, unpub. data</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solenopsis invicta (adult)</td>
<td><em>P. syringae</em></td>
<td>-7.9 ± 0.6</td>
<td>-4.1 ± 0.9</td>
<td></td>
<td>Landry &amp; Phillips 1996</td>
</tr>
<tr>
<td>Lepidoptera</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chilo suppressalis (larva)</td>
<td>*Fusarium sp.*⁶</td>
<td>-20.1 ± 0.9</td>
<td>-5.7 ± 0.6</td>
<td></td>
<td>Tsumuki 1992</td>
</tr>
<tr>
<td>Galleria mellonella (larva)</td>
<td><em>P. syringae</em>³</td>
<td>-10.4 ± 0.1</td>
<td>-4.0 ± 0.3</td>
<td></td>
<td>Strong-Gunderson et al. unpub. data</td>
</tr>
<tr>
<td>Plodia interpunctella (larva)</td>
<td><em>P. syringae</em>³</td>
<td>-10.3 ± 0.4</td>
<td>-5.4 ± 0.5</td>
<td></td>
<td>Lee et al. 1992b</td>
</tr>
</tbody>
</table>

¹ Misted with 10⁹ bacteria/ml water.
² Misted with 10⁸ bacteria/ml water.
³ Treated with 100 ppm dry, powdered bacteria.
⁴ Ingestion of 2 x 10⁹ bacteria/ml water.
⁵ Treated with 1,000 ppm dry, powdered bacteria.
⁶ Ingestion.
⁷ Misted with 3 mg/ml water.
Of particular interest is the fact that the supercooling point of insects, whose mouths have been sealed to prevent ingestion, is readily elevated by applying various preparations of living and dead ice nucleating active microorganisms to the cuticle (Strong-Gunderson et al. 1992). Steigerwald et al. (1995) recently explored several non-oral avenues by which surface application of nucleating agents might make contact with, and initiate the freezing of, the body water of insects. Using cold-hardy adults of the beetle *H. convergens* which consistently maintain low supercooling points of approximately −16°C, a suspension of *P. syringae* was applied to four anatomical sites (Fig. 4.1). Compared with control treatments, aqueous suspensions of either cultured or killed *P. syringae* suspensions produced significantly higher mean values when applied to the thoracic spiracle of the insect, −7.7°C and −5.6°C, respectively. Similarly, application of the ice nucleating active fungus *Fusarium avenaceum* to the thoracic spiracle significantly elevated the supercooling point from −16°C to approximately −10°C. Consequently, the spiracles may provide direct access to the body water of insects and explain, at least in part, the relative ease with which the supercooling capacity of insects is diminished using surface application of ice nucleating microbes.

Potential Use of Ice Nucleating Active Microorganisms for Biological Control

The fact that the supercooling point of a wide variety of insects can be elevated using ice nucleating active microorganisms supports the proposition that these ice nucleators may be used for the biological control of insect pests (see reviews by Fields 1992, Lee 1991, Lee et al. 1993a, 1995b). Because most of such species are intolerant of internal freezing, these microbial ice nucleators could be used to reduce these insects’ capacity to supercool and thereby compromise their ability to survive the low temperatures of winter. Obviously this strategy for control is only feasible if insects naturally experience temperatures below this elevated supercooling point in their overwintering site. Another significant problem to be overcome is how to deliver the ice nucleating active microorganism to the insect pest and have it retain its activity until low temperatures are experienced. Nevertheless, the use of ice nucleating active microbes for pest control has the advantages of avoiding toxic chemicals or the release of

FIGURE 4.1 Distribution of supercooling points of the freezing intolerant beetle, *Hippodamia convergens*. Inoculum volume was 0.5 ml of 20,000 ppm UVI *Pseudomonas syringae*. Site of inoculation listed with corresponding graph (from Steigerwald et al. 1995).
genetically altered microorganisms into the field, and it is biodegradable and compatible with other forms of pest management (Lee et al. 1993a).

In this decade several research groups have worked on problems directly related to the potential use of this approach for biological control (Fields 1992, Hong et al. 1994, Landry & Phillips 1996, Lee et al. 1991, Strong-Gunderson et al. 1990). Several studies have focused on controlling pests of stored products, particularly those infesting grain by exploiting the fact that the supercooling point of a variety of these insects is readily elevated using ice nucleating active bacteria and fungi (Fields 1990, 1993, Fields et al. 1995, Lee et al. 1992b). However, in some geographic locations the temperature within grain storage bins may not normally fall low enough to induce internal freezing of the insects even in the presence of biological ice nucleators, or the electrical costs of cooling the grain may be prohibitive (Fields 1993). Because these studies have been reviewed recently elsewhere (Fields 1992, Lee et al. 1993a, 1995b), the remainder of this chapter will focus on our recent efforts to answer basic and applied questions related to the efficacy of killed and living ice nucleating active microbes in elevating the supercooling point, modes of delivery of these agents to the pest insects, and the significance of microclimatic conditions within the hibernaculum, using the Colorado potato beetle, *Leptinotarsa decemlineata*, as an insect model system. Unlike pests infesting stored products located in relatively controlled environments, this beetle is representative of species that naturally experience subzero temperatures in natural habitats. If ice nucleating active microbes are to be used for control of these species, they must be able to function under a variety of environmental conditions.

*Regulation of Ice Nucleation in the Colorado Potato Beetle*

Well-known for rapidly developing resistance against a wide range of pesticides, including synthetic pyrethroids, the Colorado potato beetle is the most serious pest of potatoes in North America (Casagrande 1987, Ioannidis et al. 1991). The current agricultural practice of planting extensive monocultures of potatoes further exacerbates the problem of progressive population growth of the beetles from year to year (Casagrande 1987). Consequently, alternative methods are needed urgently for the control of this pest.

In late summer or early autumn adult beetles enter shallow burrows in the soil where they overwinter (Mail & Salt 1933, Uschatinskaya 1978). Burrow soil temperature and moisture influence overwintering survival (Fink 1925, Lashomb et al. 1984, Weber & Ferro 1993). Recent attempts to devise cultural control methods used trap crops on the edges of fields late in the summer as a means of concentrating adults in restricted areas (Kung et al. 1992, Milner et al. 1992). These areas were covered with an insulating layer of mulch in an attempt to limit the depth to which the beetles would burrow as they prepared to overwinter. It was hypothesized that removal of mulch, and therefore the insulation it provided, in midwinter would cause a rapid decrease in soil temperature and kill the beetles which had remained in superficial burrows. In fact, Milner et al. (1992) reported greater mortality in sites where the mulch was removed relative to control plots.

Our initial idea was to complement this cultural approach by using ice nucleating active microbes to further increase the susceptibility of the beetles to low temperature. In our first attempt to elevate the supercooling point of the Colorado potato beetle we exposed beetles to a concentrated, freeze-dried, and killed preparation of *P. syringae* (Genencor International, Rochester, NY) mixed with soil (Lee et al. 1994). Untreated beetles had mean supercooling points of $-7.6 \pm 0.2^\circ C$ (Fig. 4.2A). During both years of the study, treatment with 1 to 1,000 ppm of the *P. syringae* preparation elevated supercooling points in a dose-dependent manner as reported for other insects (Fields 1990, Lee et al. 1992a). The highest values of $-3.7 \pm 0.1^\circ C$ resulted from treatment with 1,000 ppm; however, application with as little as 1 ppm resulted in a significant increase in the supercooling point as compared to untreated control beetles. When these data were plotted as the cumulative percentage of beetles frozen versus the exposure temperature (Fig. 4.2B), it clearly showed the population range of supercooling point values following a given treatment. It also allowed us to predict the proportion of the beetles expected to survive exposure to a given subzero temperature. For beetles treated with 10 ppm, approximately 75% would be expected to freeze by the time the environmental temperature was lowered to $-7^\circ C$ (Fig. 4.2B).

The fact that field-collected adults have a relatively high supercooling point ($-7^\circ C$) and do not survive prolonged freezing at this temperature indicates that this species has rather limited cold-hardiness that is consistent with their thermally buffered hibernaculum within the soil (Lee et al. 1995b). Nonetheless, these data suggest that an elevation of as little as
2-3 degrees Celsius in the supercooling point, caused by ice nucleating active microbes, would cause a significant decrease in the chance that they would survive the winter.

**Site of Bacterial Application Affects Supercooling Point**

Using an approach similar to that of Steigerwald et al. (1995), we investigated the effect of topical application of *P. syringae* on the supercooling capacity of the Colorado potato beetle (Lee et al. 1996b). Application of *P. syringae* to the ventral abdomen did not significantly increase the supercooling point (−5.5 °C) compared with beetles treated with the non-ice nucleating active (control) bacterium *Escherichia coli* (Table 4.3). In contrast, application of *P. syringae* to the thoracic spiracle, ventral cervix, or abdominal spiracle significantly elevated supercooling point values. Taken together with the data from *H. convergens* (Fig. 4.1), these results indicate that application of ice nucleating active microbes to a number of non-oral sites can be used to elevate the supercooling point of insects. These data also suggest that it may be relatively difficult, compared to the development of resistance to traditional chemical insecticides, for an insect to develop resistance to the action of these agents for biological control.

**TABLE 4.3** Supercooling point values after application of an aqueous suspension of either a non-ice nucleating active bacterial control *Escherichia coli* or the ice nucleating active *Pseudomonas syringae* to four anatomic sites on the Colorado potato beetle. Values identified by different letters are statistically distinguishable (Lee et al. 1996b)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Anatomic Site of Application</th>
<th>n</th>
<th>Supercooling Point (°C) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>Thoracic spiracle</td>
<td>28</td>
<td>−6.5 ± 0.2a</td>
</tr>
<tr>
<td><em>P. syringae</em></td>
<td>Ventral abdomen</td>
<td>32</td>
<td>−5.5 ± 0.2ab</td>
</tr>
<tr>
<td></td>
<td>Abdominal spiricle</td>
<td>32</td>
<td>−4.7 ± 0.2bc</td>
</tr>
<tr>
<td></td>
<td>Ventral cervix</td>
<td>20</td>
<td>−5.1 ± 0.3bc</td>
</tr>
<tr>
<td></td>
<td>Thoracic spiracle</td>
<td>26</td>
<td>−4.5 ± 0.3c</td>
</tr>
</tbody>
</table>

**FIGURE 4.2** (A) Effect of *Pseudomonas syringae* on the mean (±SEM) supercooling point of overwintering adults of the Colorado potato beetle. Beetles were exposed to various concentrations (0 to 1,000 ppm) of *P. syringae* in soil for 48 h at 4°C. In 1991 sample sizes were n = 10-11; in 1992, n = 44-58. (B) cumulative freezing profile for beetles exposed to various concentrations of *P. syringae* in 1992 (from Lee et al. 1994).
Surfactant Enhances Ice Nucleating Active Fungus

Recent studies in our laboratory demonstrated that surface application of the filamentous ice nucleating active fungus *Fusarium acuminatum* elevates the supercooling point of *H. convergens* (Lee et al. 1992a). Using an aqueous suspension of *F. acuminatum* (0.03 g/ml) in the freeze-drop assay, 50% of 10 µl drops froze at -6.1°C or higher. When beetles were misted with this suspension, their supercooling points increased slightly from -14.9°C (misted with water only) to -11.0°C. In an attempt to further increase the supercooling point, we added surfactants to the fungal suspension under the assumption that greater contact between surface water and the cuticle might be achieved if the surface tension was reduced. Notably, when fungi suspended in a 1% solution of the surfactant Tween 80 were applied, the supercooling point increased from -14.9°C to -5.8°C. These results suggest that surfactants used in combination with ice nucleating active microbes may be useful in the development of protocols for the control of insect pests.

Effect of Soil Moisture and Composition on Colorado Potato Beetle Cold Hardiness

To use ice nucleating active microbes under field conditions it is necessary to have a thorough understanding of the natural mechanisms of cold-hardiness of the insect within its natural hibernaculum. For the Colorado potato beetle, soil moisture appears to play an important role in its overwintering biology. Although Tauber et al. (1994) indicated the importance of soil moisture levels in regulating dormancy and subsequent emergence of Colorado potato beetles in spring, little is known concerning the interaction between substrate moisture and cold hardiness in insects that overwinter within the soil.

Consequently, we recently investigated the role of soil moisture and hydric variables in the winter cold hardiness of the Colorado potato beetle (Costanzo et al. 1997). Diapausing adults chronically exposed to sandy soil exhibited body mass and body water content changes that were dependent on soil moisture content. These changes in body water content, in turn, influenced the supercooling point (Fig. 4.3; range, -3.3°C to -18.4°C), indicating that environmental moisture indirectly determined supercooling capacity. Tests involving acute chilling of beetles showed that specimens chilled in dry sand readily tolerated a 24-h exposure to temperatures as low as -5°C, but beetles tested in even slightly damp sand (e.g., water content: 1.7% of dry mass) incur high mortality (Fig. 4.4). Apparently, burrowing in dry soils not only promotes supercooling via its effect on water balance, but also inhibits inoculative freezing of Colorado potato beetles.

Costanzo et al. (1997) also reported that mortality of beetles was strongly influenced by substrate texture, because survival of beetles exposed to -5°C for 24 h was higher in substrates composed of sand, clay, and/or peat (48-64%) than in pure silica sand (22%). They concluded that not only moisture, but also texture, structure, water potential, and related physico-chemical attributes of soil may strongly influence the cold hardiness and overwintering survival of burrowing insects. Furthermore, this work indicated that manipulating the moisture levels of the soil surrounding Colorado potato beetles during winter may complement the action of ice nucleating active microbes applied to this species.
Seasonal Characterization of the Normal Gut Flora in the Colorado Potato Beetle

As an alternative delivery method to the addition of ice nucleating active microbes to the surface of the beetles before they burrow into the soil or to adding them to the soil directly, we are evaluating the feasibility of colonizing the beetle's gut with living bacteria or fungi. Our first step, which is nearly complete, in this line of investigation was to characterize seasonal gut flora in the Colorado potato beetle. In addition to identifying the bacterial flora, we also screened them for ice nucleating activity. Adult beetles collected from potato plants in the summer revealed a predominance of Enterobacteriaceae and E. agglomerans strains. Low levels of ice nucleating activity were detected in multiple strains of both microbes from the gut of the Colorado potato beetle. These data are consistent with previous reports that the ice nucleating active phenotype in bacteria isolated from insects has only been found in Gram-negative, aerobic rods in the Pseudomonadaceae and Enterobacteriaceae (Lee et al. 1991, 1993a). Less abundant bacterial species present as normal flora included Serratia marcescens, Klebsiella pneumoniae, Klebsiella oxytoca, and Xanthomonas maltophilia; ice nucleating activity was not detected in these strains. The common ice nucleating active epiphyte P. syringae was not found. Of particular interest was the fact that even field-collected overwintering beetles that did not feed for at least three months, and whose gut appeared shrunken and relatively or completely empty, retained a gut flora similar to that found in summer adults. Although the bacterial populations were apparently reduced compared to summer-collected adults, they did retain a similar diversity of normal flora through the winter. This result supports our idea to establish and maintain ice nucleating active microbes in the gut flora through the winter.

Isolation and Characterization of Pseudomonas putida

In related studies our research group isolated ice nucleating active bacteria from the gut of winter-collected, freezing-tolerant wood frogs (Lee et al. 1995a). Multiple strains of P. fluorescens, P. putida, and E. agglomerans with ice nucleating activity were identified. The P. putida strains exhibited substantial levels of ice nucleation activity ranging from -1.6° to -3.0°C, which places them among the most potent of known

Longevity of Ice Nucleating Active Pseudomonas syringae Preparation in the Soil

In another series of experiments we examined the length of time that a freeze-dried, killed preparation of P. syringae could retain its ice nucleating activity in soil under simulated field conditions. To test the effect of temperature and soil moisture on the ice nucleating activity, a 100 ppm P. syringae preparation was added to soil and held for 16 weeks at 4 or 15°C. Periodically, diapausing Colorado potato beetle adults were added to the soil for 1-2 hours, removed and their supercooling points determined. Overall ice nucleating activity was retained better in dry, as compared to moist, soil and at cooler versus warmer conditions, results that are consistent with a previous study that reported a loss of activity for this material at relatively high temperatures (Goodnow et al. 1990).
microbial nucleators. This activity was confirmed in vivo by feeding them to another freeze-tolerant frog *Pseudacris crucifer* resulting in a decreased capacity for this frog to supercool and remain unfrozen at -2°C (Lee et al. 1995a). Similar to the report by Tsumuki (1992) these bacteria may play a role in enhancing winter survival by promoting ice nucleation at high subzero temperatures. This research is germane to this project because we have isolated ice nucleating active *P. putida* from an insect previously, this strain has high levels of ice nucleating activity, and, as described in the next section, ingestion of one of these *P. putida* strains by the Colorado potato beetle caused a significant elevation of the supercooling point for at least 2.5 months (Table 4.4).

### Persistence and Activity of Ice Nucleating Active Bacteria in the Colorado Potato Beetle

One of the challenges that must be met if ice nucleating active microorganisms are to be used for pest control is to find ways to deliver these microorganisms to the insect pests. Consequently, we tested the efficacy of several different species of living ice nucleating active bacteria and fungi for their effect on the Colorado potato beetle's supercooling point. Suspensions of living *P. fluorescens*, *P. syringae*, and *P. putida* sprayed onto adults all caused a significant increase in the supercooling point, indicating that living bacterial cells also may be used for supercooling point manipulation.

Recently we completed a preliminary study demonstrating that ingestion of living ice nucleating active bacteria caused an elevation of the beetle's supercooling point for at least ten weeks under conditions that simulated natural overwintering. Beetles were collected in early autumn and fed on various ice nucleating active bacteria added to potato tuber before they entered diapause. Beetles readily fed on a slice of tuber that was coated with the bacterial solution. Beetles were assayed for supercooling capacity and gut flora within 1.5 h after feeding, at the conclusion of a 14-day diapause induction regimen, and in mid-winter, after the beetles had been in diapause 2.5 months.

As expected, ingestion of ice nucleating active bacteria immediately caused an increase in beetle supercooling points (Table 4.4). Most noteworthy was the fact that ingestion of *P. putida* derived from the freeze-tolerant frog and *P. fluorescens* caused not only an initial supercooling point elevation, but the supercooling point remained elevated for the entire 2.5 months of this study. It is also significant that the supercooling point remained elevated even though the beetles purged their guts extensively in preparation for burrowing beneath the soil and overwintering in reproductive diapause. These results confirm and extend findings of Chapco & Kelhn (1994) in which ingested bacteria were retained in the gut of grasshoppers for more than three weeks. Furthermore, these data lend credence to the idea of establishing ice nucleating active bacteria in the insect gut as a strategy for delivering ice nucleating active microorganisms to insect pests.
Future Directions

These studies represent only the first steps toward improving our understanding of both the natural flora of insects as well as the potential for establishing ice nucleating active microbes in insect pests. The establishment of microbes in insect pests has a wide range of possible applications for biological control. For example, the use of self-generating entomopathogenic bacteria is a likely control strategy (Chapco & Kelln 1994). Because our long-term goal is to use these bacteria for biological control, it is critical that we know the identity and ice nucleating activity of naturally occurring ice nucleating active microorganisms and their potential for manipulating their association with insect pests.

Our progress thus far provides a solid foundation for field tests with overwintering Colorado potato beetles that will attempt to elevate their supercooling point by augmenting soil moisture levels and by using a freeze-dried, killed and/or live preparations of P. syringae. In a complementary line of investigation we will continue to study the use of living ice nucleating active microorganisms to colonize the insect gut or insect surface so that they function to diminish the overwintering beetle’s supercooling capacity and promote death by freezing. It may be possible to not only colonize the gut but to develop strains of microbes with higher levels of ice nucleating activity, or ones whose expression of the ice nucleating active phenotype increases as environmental temperatures decrease during the autumn.

If practical protocols can be developed for manipulating insect cold hardness, this approach has the potential for controlling a variety of insect pests. Pests of stored products in which it is possible to manipulate the environmental temperature in conjunction with microbial treatments may be particularly good candidates for this type of control. Furthermore, the results of laboratory investigations with ice nucleating active microbes thus far suggest that methods can be developed for control of selected insect pests in agricultural environments.

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