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# Methods of Preventing Frost Injury Caused by Epiphytic Ice-Nucleation-Active Bacteria

Frost-sensitive plants, including herbaceous annuals, flowers of deciduous fruit trees, and fruit of many plant species, cannot tolerate ice formation within their tissues. Ice forming in or on frost-sensitive plants spreads rapidly both intercellularly and intracellularly, mechanically disrupting the tissues. This disruption is usually manifested as flaccidity and/or discoloration (Fig. 1) when the plant is warm again (2,17).

Classical methods of frost control for frost-sensitive plants are many and varied, but all have the same goal: to maintain the temperature of a frost-sensitive plant part above the temperature at which ice can form. These methods include mixing the cold layer of air nearest the ground with warmer air aloft by use of stationary wind machines or helicopters, by heating the air around the plants with heaters, or by watering the soil with sprinklers or by furrow irrigation. More recently, various methods have been used to reduce the radiative cooling of plants, ie, direct loss of heat in the form of infrared radiation to space during clear, calm nights. Using artificially generated fogs or foamlike insulation to cover plant materials reduces heat loss from plants and retains heat otherwise lost from the soil.

Another commonly used method of frost control is application of water directly to the plant parts during freezing temperatures. Ice forms but is limited to the exterior of the plant. Frost damage does not result so long as water is added to the ice-covered plant parts during the entire period the temperature is below 0 C (32 F). The latent heat of fusion, released when water freezes to form ice, maintains the ice-water mixture on leaves at 0 C. This mixture will remain at 0 C as long as sufficient water is continuously available to freeze. Since all plant parts contain a small amount of dissolved salts and other soluble components, the freezing point of the plant tissue is slightly lower than 0 C (−0.4 C or 30.8 F), and ice held at 0 C on the surface of the plant will not penetrate and damage the plant.

**Fig. 1. Frost injury: (A) Mature citrus trees shortly after a severe frost. (B) Immature Bartlett pear fruit with internal discoloration typical of mild injury (right) compared with uninjured fruit. (C) Newly emerged potato leaves after exposure to −4 C. (D) Avocado foliage after a mild radiative frost.**

Classical methods of frost control have many problems. Sprinkler irrigation of leaves for frost control requires large amounts of water and is ineffective when wind or poor sprinkler coverage prevents continuous wetting of the plants. The other methods mentioned require large amounts of energy and water and are rapidly becoming prohibitively expensive. Artificially generated fogs can create safety hazards, and burning large quantities of fossil fuels can deteriorate environmental quality.

Most frost-sensitive plants have no significant mechanisms of frost tolerance and must avoid ice formation to avoid frost injury. The physical methods of frost protection that warm the plant tissue above 0 C have many limitations. Frost injury may also be avoided by exploiting the supercooling property of water. Small volumes of pure water can be supercooled to approximately −40 C before the spontaneous catalysis of ice formation called homogeneous ice nucleation occurs. Even relatively large quantities of water readily supercool to −10 C. Nonaqueous catalysts for ice

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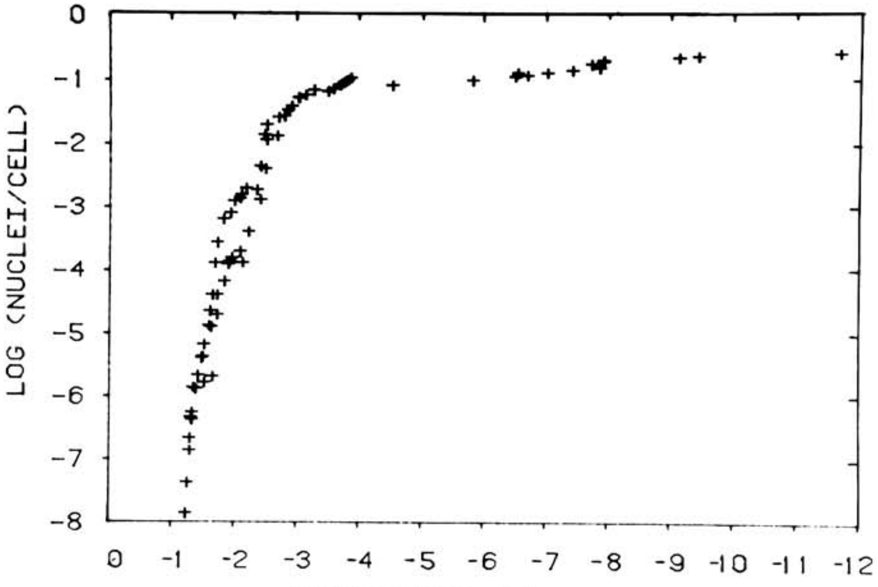


**Fig. 2. Ice nucleation activity of *Pseudomonas syringae* (logarithm of fraction of cells active in ice nucleation) as a function of temperature.**

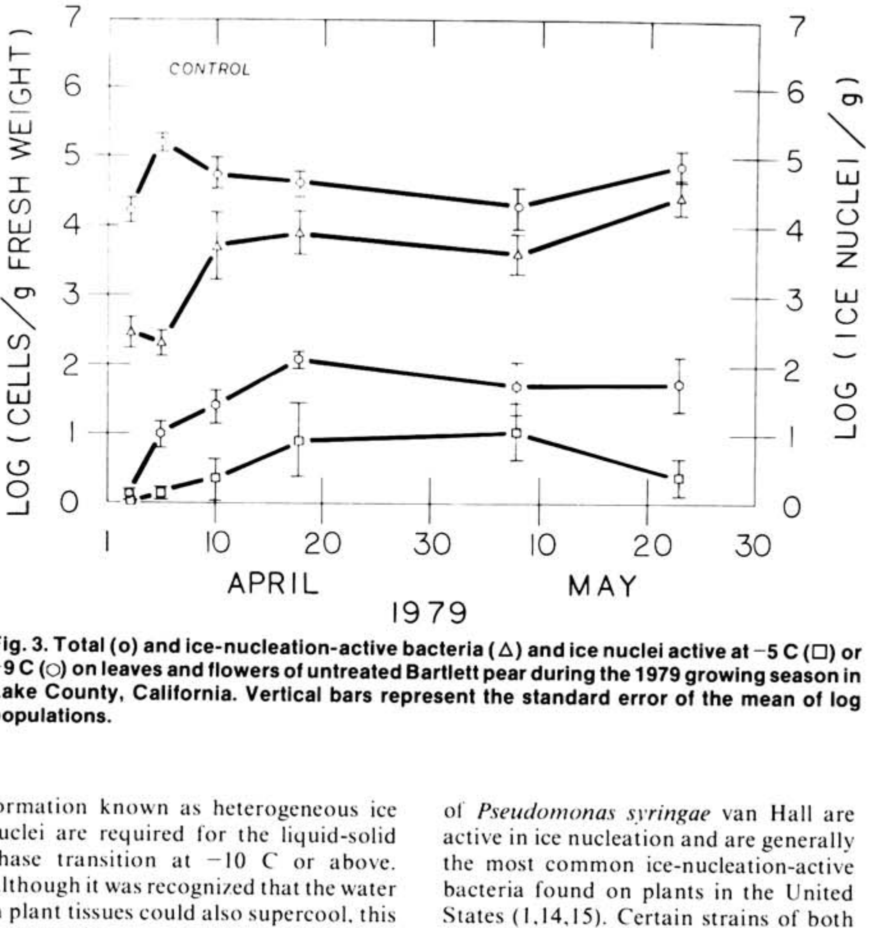


**Fig. 3. Total (o) and ice-nucleation-active bacteria (△) and ice nuclei active at −5 C (□) or −9 C (○) on leaves and flowers of untreated Bartlett pear during the 1979 growing season in Lake County, California. Vertical bars represent the standard error of the mean of log populations.**

formation known as heterogeneous ice nuclei are required for the liquid-solid phase transition at −10 C or above. Although it was recognized that the water in plant tissues could also supercool, this supercooling was generally believed to be of little practical importance, particularly under field conditions (5). Very little was known of factors influencing the supercooling ability of plant tissue (3,4,17).

Recently, three species of bacteria commonly found on leaf surfaces as epiphytes have been shown to be active catalysts for ice formation. Many strains of *Pseudomonas syringae* van Hall are active in ice nucleation and are generally the most common ice-nucleation-active bacteria found on plants in the United States (1,14,15). Certain strains of both *Erwinia herbicola* (Löhnis) Dye and *P. fluorescens* (Migula) are also active in ice nucleation and have been detected on the surface of many plants (13,14). The strains of *P. syringae* and *E. herbicola* studied to date are among the most efficient naturally occuring ice nuclei yet discovered, catalyzing ice formation at temperatures as warm as −1 C (Fig. 2). Most other organic and inorganic materials, such as dust particles, nucleate ice only at temperatures lower than −10 C and thus do not appear to be important in limiting the supercooling of plant tissue.

As can be seen in Figure 2, not every cell of *P. syringae* serves as an ice nucleus at a given time. The fraction of cells active as ice nuclei increases with decreasing temperature. Approximately one cell in 10 is an ice nucleus at −4 C or lower. At least 95% (and probably all) ice nuclei active at temperatures above −5 C on leaf surfaces are of bacterial origin (6,7).

Most plant materials are very inefficient ice nuclei themselves; significant ice-nucleation activity is observed on greenhouse-grown plants only at temperatures lower than −8 to −10 C (1,6,7,16). Ice-nucleation activity in most axenically grown plants appears to be very rare at temperatures above −5 C (6). Most field-grown plants, however, are colonized by large epiphytic populations of various ice-nucleation-active bacteria (14) that limit their supercooling ability.

Under California conditions, a large seasonal variation in the numbers of epiphytic ice-nucleation-active bacteria on both annual and perennial plants is observed. The bacterial populations found on healthy pear flowers and leaves (Fig. 3) are typical of this variation. Populations of ice-nucleation-active bacteria are generally low (less than 100 cells/g fresh weight of leaf or bud tissue) on overwintering plant tissues of deciduous plants or on emerging cotyledons or leaves of annual plants. However, large epiphytic populations of such bacteria (principally *P. syringae*) are present on emerging flowers and/or leaves of these plants. A 100-fold increase in bacterial populations occurred on pear during the 2-week period following bud break (Fig. 3). It is important to note that populations of ice-nucleation-active bacteria were largest during April through May, coinciding with the period of maximum frost hazard to pear in this location. Although not shown, populations of ice-nucleation-active bacteria decreased after late May, declining with the onset of hot, dry weather to 100 cells/g by late summer. Abundant ice nuclei were contributed by these epiphytic bacterial populations (Fig. 3). The inability of untreated pear tissue to supercool extensively in natural situations can be rationalized with the observation of over 30 ice nuclei active at temperatures warmer than −5 C per gram of leaf and flower tissue during most of April and May. Another important observation is that, as in vitro, not all bacterial cells are active as ice nuclei while on plant surfaces. In the example of pear shown in Figure 3, only about one *P. syringae* cell in 1,000 acts as an ice nucleus at −5 C while on leaf surfaces.

The frost sensitivity of most plants can be explained by the fact they harbor very large numbers of ice-nucleation-active bacteria that catalyze the ice formation that will kill tissues. Low temperatures of short duration will not damage these plants if no ice forms; the plants can be cooled to temperatures as low as −7 C (20 F) for several hours with no apparent damage. In the field, however, these bacteria on plant surfaces will cause ice to form on and in the plants, and the plants will die before this temperature is reached. In fact, the amount of frost damage at a given temperature (the chances of a given plant part freezing) increases directly with the logarithm of the numbers of ice-nucleation-active bacteria on that plant (7,8). This finding indicates that a reduction in the numbers of these bacteria will lead to a corresponding decrease in frost injury.

Various species of ice-nucleation-active bacteria have therefore been shown as both necessary and sufficient to account for the frost sensitivity of the plants examined to date. In addition to documenting their fascinating ecological role, this information has suggested several new methods of frost control

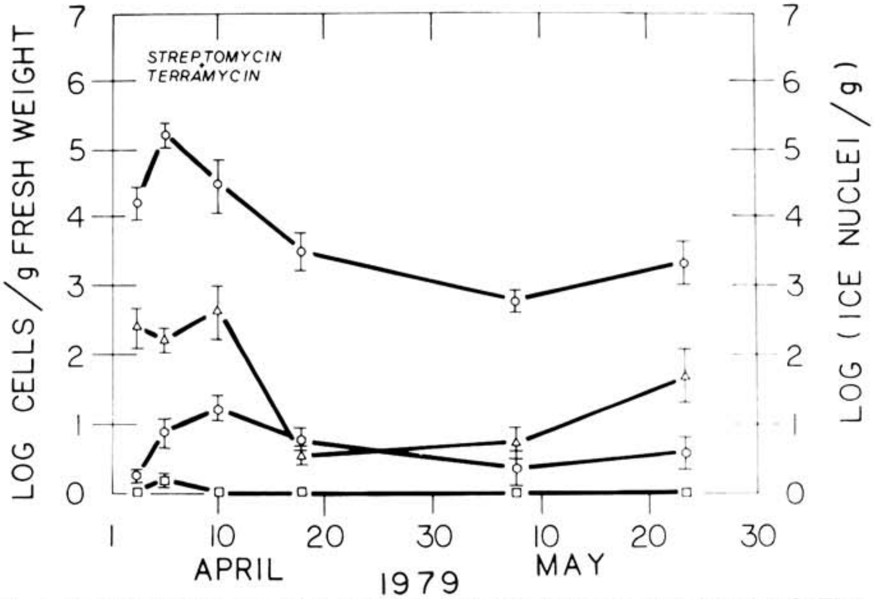


**Fig. 4.** Total (o) and ice-nucleation-active bacteria (△) and ice nuclei active at −5 C (□) or −9 C (○) on leaves and flowers of Bartlett pear treated weekly with a mixture of 100 ppm streptomycin and 75 ppm oxytetracycline (Terramycin) during the 1979 growing season in Lake County, California. Vertical bars represent the standard error of the mean of log populations.

**Table 1.** Frost injury to immature Bartlett pear fruit after a mild natural radiative frost of −3 C in Lake County, California

| Trial | Treatment frequency$^z$ | Frost injury (fraction of fruit) |
|---|---|---|
| Na$_2$CO$_3$ (0.1M) | c | 0.11 |
| Antagonist A510 | b | 0.12 |
| Urea (0.5M) + ZnSO$_4$ (0.05M) | c | 0.16 |
| Antagonist A509 | b | 0.18 |
| Streptomycin (200 ppm) + oxytetracycline (Terramycin) (100 ppm) | a | 0.25 |
| Antagonist A507 | b | 0.27 |
| Urea (0.5M) + NaCO$_3$ (0.1M) | c | 0.29 |
| Antagonist A506 | b | 0.33 |
| Kasugamycin (100 ppm) | a | 0.39 |
| Triton QS-44 (0.1%) + tartaric acid (0.05M) | c | 0.41 |
| Streptomycin (100 ppm) + oxytetracycline (Terramycin) (50 ppm) | a | 0.42 |
| Kocide 101 (2 lb/100 gal) + maneb (2 lb/100 gal) | a | 0.43 |
| Hyamine 2389 (0.1%) + Na$_3$PO$_4$ (0.05M) | c | 0.48 |
| Antagonist A508 | b | 0.51 |
| Triton XQS-20 (0.1%) | c | 0.66 |
| Control | a | 0.95 |
| LSD 5% | | 0.12 |

$^z$a = Bactericide sprayed at 5–7 day intervals to runoff; b = antagonistic bacteria (about $10^8$ cells/ml) applied once, at 10% bloom; c = bacterial ice-nucleation inhibitor applied once, 12 hours before frost.

based on enhancing the natural supercooling ability of plants by reducing the numbers of ice nuclei contributed by various ice-nucleation-active bacteria. Treatments that reduce the numbers and/or activity of ice-nucleation-active bacteria have proved promising as alternate methods of frost control.

## Bactericides

One obvious method of frost control is to employ commercially available bactericides, including copper-containing fungicides, such antibiotics as streptomycin and oxytetracycline, and various experimental organic bactericides. Significant frost control has been achieved with experimental application of bactericides on several different crops, including corn, beans, potatoes, squash, tomatoes, pear, almond, citrus, and avocado.

Fig. 5. Formation of ice in a citrus leaf during a natural radiative frost of approximately −4 C near Exeter, California, in December 1978.

Although much work remains to be done to determine the most effective rate, type, and application frequency, bactericides appear to control frost when applied before bacterial populations develop naturally on plants (eg, as a dormant spray). Bactericides may be applied often enough to act as a protectant material and prevent establishment of bacteria on plant surfaces throughout the growing season. Large reductions in epiphytic ice-nucleation-active bacterial populations are observed after protectant bactericide applications (Fig. 4). Because of the large (1,000- to 10,000-fold) reductions in epiphytic ice-nucleation-active bacterial populations on treated plants, the numbers of ice nuclei were also greatly reduced (Fig. 4), thereby lessening the chances for successful nucleation of a given plant part at temperatures above −5 C. The numbers of ice nuclei active at −5 C on treated plants were at or below the limits of detection (one ice nucleus/g fresh weight) for much of the growing season.

A single ice nucleus is currently thought sufficient to cause ice formation and thus frost injury to an entire leaf, fruit, or flower or even to groups of leaves or flowers, depending on the degree ice propagation is restricted within a plant. Figure 5 shows ice propagation in a field-grown navel orange leaf exposed to a natural radiative frost of about −4 C (26 F). Typical symptoms of frost injury are apparent. Ice formation was observed initially only in an isolated water-soaked area and had not yet propagated to the periphery of the leaf, as evidenced by ice formation in dew droplets only in the vicinity of the water-soaked area. The water-soaked lesions, presumably initiated by a single nucleus, expanded over a period of several minutes. In this particular instance, water-soaked lesions were common only on leaves of untreated trees and were nearly absent on leaves of trees treated with one of several bactericides. Fruit on treated trees also escaped ice formation (Fig. 6). The frequency of frost-damaged fruit was much lower on bactericide-treated trees than on control trees.

Since a plant part either does or does not escape ice formation, frost injury might best be considered a quantal response. Large quantitative reductions in the incidence of frost injury have been observed on bactericide-treated plants, compared with untreated plants (Table 1). Effective bactericides have reduced frost injury significantly in most trials and appear promising as frost control agents.

Although most bactericides kill ice-nucleation-active bacteria rapidly on contact in vitro, these bacteria appear to lose their ability to nucleate ice in vitro very slowly. A similar phenomenon appears to operate on leaf surfaces. Therefore, frequent applications of bactericides to act primarily as protectants may be more effective than killing bacteria that have become well established on leaf surfaces. Should this second option be adopted, sufficient time apparently must be allowed for ice-nucleation activity (associated with the dead bacterial cells, or the cells themselves) to be lost before expected freezing temperatures. Much work remains to determine which bactericides might be

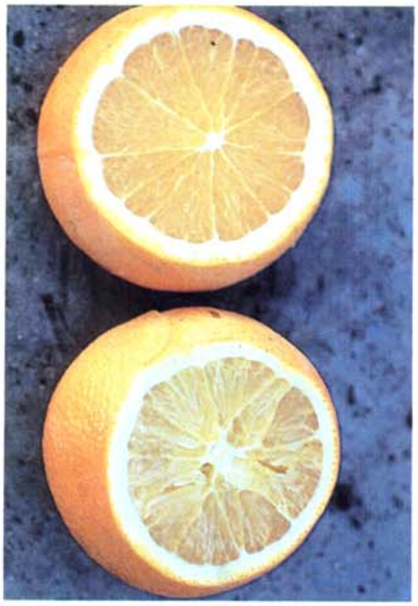


Fig. 6. Undamaged navel orange fruit (top) was treated with a cupric hydroxide formulation 3 weeks before a radiative frost of approximately −6 C. Untreated frost-damaged fruit (bottom) shows dryness and crystalline inclusions in segment membranes.

Fig. 7. Frost injury to corn at −5 C with and without leaf populations of antagonistic bacteria. The plant in the center was sprayed with a suspension of approximately $10^7$ cells/ml of the antagonistic bacterium A510 4 days before freezing. The plants at the right and left were sprayed with water and placed in a mist tent until 2 days before freezing, when they were sprayed with a suspension of approximately $10^5$ cells/ml of *P. syringae* and returned to the mist tent until just before freezing at −5 C.

most efficacious for use as frost control agents.

## Antagonistic Bacteria

Typically, only about 0.1–10% of the bacteria found on leaf surfaces are strains capable of producing active catalysts for ice formation and thus involved directly in ice nucleation. In addition, as shown in Figure 3, not all cells of strains capable of producing ice nuclei actually do so at a given time. The occurrence of some degree of competition or other form of antagonism between these strains and other epiphytic bacteria on leaf surfaces is likely, as in other ecological niches. As already noted, the degree of natural competition on most plants appears insufficient to prohibit buildup of significant populations of epiphytic ice-nucleation-active bacteria on most plants. This natural antagonism can be augmented, however, by altering the leaf surface ecology to facilitate increased populations of non-ice-nucleation-active bacteria on plants. These bacterial competitors may then occupy a position on the plant that could otherwise be colonized by ice-nucleation-active bacteria.

Bacterial competitors have been selected on the basis of their prolific production of antibiotics in vitro and/or their effective colonization of leaf surfaces. These antagonists are established by foliar application to plant parts before colonization by ice-nucleation-active bacteria (11,12). The net effect is to reduce the population of ice-nucleation-active bacteria on plants at the time of low temperatures and thereby reduce the likelihood of frost injury (Fig. 7).

Current strategies have emphasized treatment of newly exposed plant tissue or perennial leaves with bacterial competitors at the onset of favorable environmental conditions (cool, moist weather), before significant colonization and/or multiplication of ice-nucleation-active bacteria. In the example shown in Figure 8, antagonistic bacterium A506 was applied to pear trees as a foliar spray at approximately 20% bloom on 3 April 1979. A comparison of Figures 3 and 8 shows that populations of ice-nucleation-active bacteria were over 1,000-fold greater on expanding untreated leaves than on treated ones. The primary effect of treatment was prevention of the increase of ice-nucleation-active bacterial populations and ice nuclei active at −5 C or warmer that occurred after early April on untreated trees. Although the number of ice nuclei active at −9 C was also reduced, dead bacterial cells or other sources contributed these nuclei. Because of their inability to nucleate ice at −5 C or warmer, they were not involved in frost injury to plants.

Most antagonistic bacteria probably influence the frost sensitivity of plants in the same manner as a protectant bactericide, by limiting the populations of ice-nucleation-active bacteria on leaf surfaces throughout a period of freezing conditions. Reductions in frost damage to treated plants vary in magnitude but are directly related to reductions in populations of ice-nucleation-active bacteria (Fig. 8, Table 1) (11). Efficient bacterial antagonists, such as A506, effectively colonized emerging and mature tissues for relatively long periods after a single foliar application. Antagonist A506 was the predominant bacterium on plants for up to 45 days after inoculation, so frequent applications were unnecessary.

Some degree of host specificity is observed among antagonistic bacteria and may account for differences in colonization of a given host. While some bacterial strains readily colonize many different hosts, certain strains effectively colonize only the host from which it was originally isolated. This phenomenon requires much more study before any generalization can be made as to reliability of a given bacterium as a biological control agent for a given plant species.

## Chemicals that Inhibit Ice Nucleation by Bacteria

While the first two categories of frost control are similar in their attempts to rid plants of bacterial cells causing ice nucleation, a third, more subtle method of frost control may exist. A log-linear relationship has been found between frost injury to plants at a given temperature and the number of ice nuclei associated

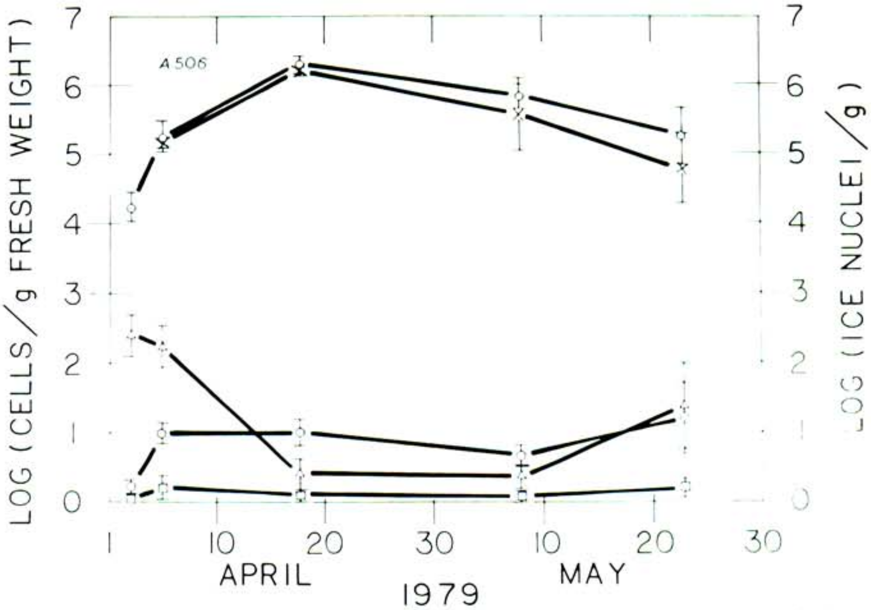


Fig. 8. Total (o) and ice-nucleation-active bacteria (Δ) and ice nuclei active at −5 C (□) or at −9 C (○) on leaves and flowers of Bartlett pear treated at 20% bloom with rifampicin-resistant antagonistic bacterium A506 (×) during the 1979 growing season in Lake County, California. Vertical bars represent the standard error of the mean of log populations.

Fig. 9. Frost injury to tomato at −5 C with and without pretreatment with bacterial ice-nucleation inhibitors. Plants in the center and on the right were sprayed with a suspension of approximately $10^5$ cells/ml of *P. syringae* 3 days before freezing and placed in a mist tent. Five hours before freezing, plants in the center were sprayed with a dilute solution of a quaternary ammonium surfactant and the plants on the right, with water alone.

with those plants (8). This relationship is mechanistically more fundamental than that between numbers of bacteria and frost damage, because ice-nucleation activity is not an intrinsic character of all ice-nucleation-active bacterial cells. Laboratory tests have shown that the ice nucleus associated with ice-nucleation-active bacteria is sensitive to various physical and chemical stresses, such as extremes of pH, specific heavy metal ions in a soluble state (including copper and zinc), and certain cationic detergents (unlike most commercial anionic agricultural surfactants or sticker-spreaders) (9). Chemicals that quickly inactivate the ice nucleus associated with ice-nucleation-active bacteria without necessarily killing bacterial cells have been termed "bacterial ice-nucleation inhibitors." Even though viable bacterial cells may remain on plants after treatment with some bacterial ice-nucleation inhibitors, the cells no longer can contribute nuclei and cannot be responsible for initiating damaging ice formation (Fig. 9).

Bacterial ice-nucleation inhibitors inactivate bacterial ice nuclei within minutes to a few hours after application to the plant (10). These chemicals are more analogous to eradicative pesticides than to bactericides or antagonistic bacteria, which are primarily protective. Significant reductions in frost injury have been achieved by applying bacterial ice-nucleation inhibitors under field conditions within a few hours of an expected frost (Table 1). Bacterial ice-nucleation inhibitors may offer a "day before" type of frost prevention and thus may be useful in areas where frost is infrequent and routine use of bactericides or antagonistic bacteria less desirable.

Although bacterial ice-nucleation inhibitors appear attractive as frost control agents, certain problems concerning their use in agriculture must be addressed. A number of different chemicals have been shown to inactivate the ice nuclei associated with ice-nucleation-active bacteria, but many are incompatible with foliar applications because of high phytotoxicity. In addition, all bacterial ice-nucleation inhibitors discovered to date are water-soluble and therefore likely to weather rapidly from foliar surfaces. Fortunately, preliminary data indicate that bacterial ice nuclei treated with nucleation inhibitors may remain inactivated even after the chemicals are removed.

## Future Directions

Ice-nucleation-active bacteria have only recently been shown to have a causal role in frost injury. The frost injury problem requires that much must yet be learned in many different fields of research, including agronomy-horticulture, plant physiology, cloud physics-meteorology, microbiology, and biochemistry as well as plant pathology, to devise and assess methods of frost control. The findings and knowledge must be exchanged between disciplines. Many questions need to be more fully addressed:

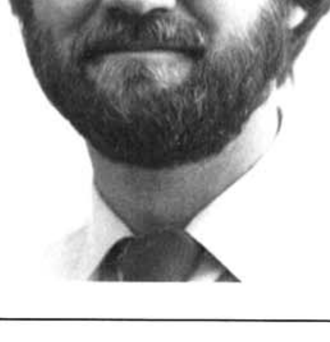
1. Where are "epiphytic" bacteria located on or in plants? Are there preferential sites of colonization and, if so, does this affect the ability of the bacteria to nucleate ice? What other factors play a role in determining the efficiency of ice nucleation by bacteria on leaf surfaces? If controlling factors can be found, could growing practices or plant varieties be developed to minimize the numbers of ice nuclei on plants?

2. Over what temperature range are bacterial ice nuclei the important limiting factor in plant supercooling, ie, below what temperature do various plant tissues or other substances limit supercooling of water in plant tissues?

3. What are the sources of epiphytic ice-nucleation-active bacteria, or, rather, why are these bacteria so ubiquitous in nature? This answer may suggest answers to many of the epidemiological questions concerning the role of the epiphytic stage of many pathotypes of *P. syringae*. Since *P. syringae* is an important plant pathogen as well as an ice-nucleation-active bacterium inciting frost injury, is the ice nucleation activity of *P. syringae* important in causing the freeze stress required for disease development in certain situations? Similarly, studies of the development and movement of epiphytic populations of *P. syringae* may ultimately be important in understanding factors triggering a disease outbreak from an epiphytic bacterium living in a commensal relationship with its host. What role do ice-nucleation-active bacteria play in atmospheric precipitation processes? Several phytopathogenic bacteria have now been reported to be transported in the atmosphere via aerosols, yet the importance of ice-nucleation-active bacteria in contributing ice nuclei active at warm temperatures in the upper atmosphere for the formation of rain and snow is as yet largely unexplored. The role of these bacteria may be potentially of critical importance in climatology studies.

4. What are the mechanisms of antagonism among epiphytic bacteria? Antagonistic bacteria are being investigated for foliar and soilborne disease control in addition to biological control of frost injury, but little is known of the major mechanisms of antagonism. Elucidation of such mechanisms might allow efficient in vitro screening procedures for effective biological control agents, improving existing antagonists and speeding the development of antagonists for different crops or different diseases. Integrated control of certain foliar diseases and frost injury might also be possible by judicious selection of antagonistic bacteria or other microorganisms.

5. How do ice-nucleation-active bacteria catalyze ice formation at only a few degrees of supercooling? This question is currently under study in our laboratory not only to find the answer but also in the hope that further knowledge of the process of ice nucleation by these bacteria may allow the development of more effective frost control agents than those discussed here. One example might be the selection of specific chemical or biological agents that would inactivate the ice-nucleation activity of these bacteria in low concentrations or for long periods without resorting to the "sledgehammer" tactics of the general biocides or chaotropic agents used to date. Increased knowledge of the ice-nucleating principle of ice-nucleation-active bacteria



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might allow speculation into the ecological and evolutionary significance of the property and may increase our understanding of the taxonomy of the diverse group of bacteria grouped under the synonym *P. syringae* or *E. herbicola*.

6. What will be the economics of frost control using the new methods discussed? Assuming one or more of these agents can be shown to be effective under a wide range of geographical, environmental, and agronomic variables, much will need to be learned about optimum types, rates, frequencies, timing, and modes of application of various frost control agents. The chances of freezing temperatures in a given location will also have to be analyzed before the complex cost-benefit relationships can be determined.

If answers to some of these and other questions can be found, significant progress will be made in alleviating one of man's oldest and largest abiotic diseases—frost injury.

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