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THE ROLE OF BACTERIAL ICE NUCLEATION IN FROST INJURY TO PLANTS

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INTRODUCTION

The science of plant pathology is largely a study of the mechanisms, quantification, and alleviation of plant stresses due to biological agents. Of obvious importance are the stresses to plants directly caused by infection by various plant pathogenic fungi, bacteria, viruses, nematodes, insects, etc. In such cases, plant stress is due either to direct damage to plant tissue or to an alteration in normal plant metabolism (43). Some biological agents, such as the fungi that form mycorrhizae with plant roots, may even reduce plant stress in certain situations and increase stress in others (4).

Plants may also be stressed directly and indirectly by various physical factors such as high or low temperatures (57) or air pollutants (36). A wealth of information exists on the direct effects of low temperatures (57) or air pollutants (36) on plant health. Physical stresses such as temperature (57) or air pollutants (36) may also influence the subsequent damage of plants incited by certain plant pathogens. The converse is also true in some situations. Root-infecting plant pathogens, for example, can make plants more susceptible to damage due to high temperatures or drought. It is clear that the interaction of the biological and physical environments of plants determines the extent of plant stress.

Many more subtle interactions between microorganisms and plants have also been reported. Bacteria living on the surfaces of healthy leaves and roots have been reported to increase plant growth, possibly by production of one or more plant growth regulators (86). Conversely, some bacteria isolated from root surfaces have been shown to be detrimental to root and plant growth (117).

Frost injury is a serious abiotic disease of plants. Losses in plant production

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in the United States due to frost injury are estimated at over one billion dollars yearly (127). Frost injury has been described as one of the main limiting factors to crop production in many locations in the temperate zone. Little attention has been paid to the mechanism of frost injury to frost-sensitive agricultural plants that are damaged at temperatures warmer than −5°C (12, 13, 92). Frost injury was considered an unavoidable result of physical stress (low temperatures) to these plants (12, 13, 92).

Some plant frost injury recently has been shown to involve an interaction of certain leaf surface bacteria as well as low temperature stress. Some bacteria cause the frost-sensitive plants on which they reside to become more susceptible to freezing damage by initiating the formation of ice that is required for frost injury (2, 67, 70, 71, 74, 76, 129). In this review, the importance of some epiphytic bacteria that initiate ice formation on plants (ice nucleation active bacteria) are discussed in reference to their significance to the frost sensitivity of many plants, and to initiation of disease. Some aspects of plant physiology and physics relevant to frost damage in frost-sensitive plants are also reviewed to elucidate further the unique role that ice nucleation active bacteria play in causing, although indirectly, the world's most destructive abiotic disease.

MECHANISMS OF PLANT FROST INJURY

Frost-sensitive plants are distinguished from frost-hardy plants by their relative inability to tolerate ice formation within their tissues (9, 13, 57, 93, 97). Examples of frost-sensitive plant tissues include herbaceous annual plants, flowers of deciduous fruit trees, fruit of many plant species, and shoots and stems of certain forest trees such as Eucalyptus. Ice formed in or on frost-sensitive plants spreads rapidly both intercelluarly and intracellularly, causing mechanical disruption of cell membranes (9, 57). This disruption is usually manifested as a flaccidity and/or discoloration upon rewarming of the plant. Thus, most frost-sensitive plants have no significant mechanisms of frost tolerance and must avoid ice formation to avoid frost injury (9, 57, 93).

CURRENT METHODS OF CONTROL OF PLANT FROST INJURY

Current practices for management of plant frost injury involve physical warming of plant tissue to at least 0°C to avoid internal ice formation (7) or by planting frost–sensitive plants in sites which do not have a history of cold temperatures. Physical methods of frost prevention include the use of stationary wind machines or helicopters to mix the cold layer of air nearest the ground with warmer air aloft during radiation frost conditions when inversions typically occur (7). Heaters have also been employed to heat the air in the vicinity of plants in need of protection (7). Water applied to soil by sprinklers or by furrow

irrigation has been used to heat the air during periods of cold temperature. More recently, various methods have been used to reduce the radiational cooling of plants, i.e. direct loss of heat in the form of infrared radiation to space during clear nights. Radiative heat losses can be reduced by the application of artifically generated fogs or foam-like insulation to cover plants (7). These methods reduce loss of heat from plants and retain heat otherwise lost from the soil. Another commonly used method of frost management is the application of water directly to plant parts during periods of freezing temperatures (7, 57). Although ice may form during such a process, it is limited to the exterior of the plant. Frost damage does not result so long as additional water is applied to ice-covered plant parts during the entire period the air temperature is below 0°C. The latent heat of fusion, released when water freezes to form ice, warms the ice-water mixture on leaves to 0°C. This mixture will remain at 0°C as long as water is continuously available to freeze on plant surfaces (7). Since all plants contain dissolved salts and other soluble components, the freezing point of the plant tissue is slightly lower than 0°C (31, 57). Ice held at 0°C on the surface of the plant will not penetrate and disrupt plant tissues.

Although current physical methods of frost prevention can sometimes protect plants from frost damage at ambient air temperatures of −3°C or above, these methods have many limitations. 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 (7). Application of water may result in an accumulation of ice on some plants that can cause mechanical breakage of limbs and other plant parts. The large amounts of energy and/or water required to implement many physical methods of frost control are rapidly becoming prohibitively expensive. Artificially generated fogs can create safety hazards to motorists and the burning of large quantities of fossil fuels can deteriorate environmental quality.

SUPERCOOLING OF WATER AND ICE NUCLEATION

Many liquids, including water, do not invariably freeze at the melting point of the solid phase. These liquids can be supercooled (undercooled or subcooled) to several degrees C below the melting point of the solid phase and will freeze only upon the spontaneous formation of, or addition of, a suitable catalyst for the liquid-solid phase transition. Catalysts for the water-ice phase transition are known as ice nuclei. Two general types of ice nuclei exist: heterogeneous and homogeneous. Homogeneous ice nuclei are of primary importance at low temperatures whereas heterogeneous nuclei are more important at temperatures approaching 0°C. Small volumes of pure water can be supercooled to approximately −40°C before the spontaneous homogeneous catalysis of ice formation occurs (5). Even relatively large quantities of water readily supercool to −10°C

to −20°C (5). Catalysis of ice formation in water involves a transient ordering of water molecules into a lattice resembling ice (5, 42). The number of water molecules that must be ordered to trigger macroscopic ice formation in supercooled water is governed by thermodynamic and geometric considerations and decreases with decreasing temperature (25, 42). At very low temperatures (approaching −40°C), random grouping of water molecules can efficiently trigger homogeneous ice formation within short time intervals (118).

At warmer temperatures, nonaqueous catalysts for ice formation known as heterogeneous ice nuclei are required for the water-ice phase transition. The mechanism of ice nucleation of all heterogeneous ice nuclei is due to ordering of water molecules into an ice-like lattice, perhaps in the case of inorganic salts, by aggregation of water molecules onto the face of fractured crystals with lattice structures similar to ice (11). The efficiency (defined by relatively warm threshold ice nucleation temperatures) of heterogeneous ice nuclei presumably increases with increasing numbers of water molecules oriented in a rigid ice-like array (5, 42).

Nonbiological Sources of Heterogeneous Ice Nuclei

The most common and the most thoroughly studied source of heterogeneous ice nuclei are mineral particles, particularly silver iodide (123). These mineral particles efficiently nucleate ice only at temperatures lower than −8°C to −15°C (123, 133). Most organic and inorganic materials such as dust particles nucleate ice only at temperatures lower than −10°C to −15°C (89, 90). Dust particles, particularly certain mineral clays, have long been considered as primary sources of ice nuclei (91, 113). Mineral particles of meteoric origin are also considered abundant atmospheric ice nuclei (113). Although abundant, these minerals are active as ice nuclei primarily at temperatures colder than −15°C, and therefore are quite unlikely to account for ice nucleation at relatively warm subfreezing temperatures. Kaolinite is among the most active mineral ice nucleus sources, but it is active in ice nucleation only at temperatures below about −9°C (89). Silver iodide, used in weather modification studies as a cloud seeding agent, is active in ice nucleation only at temperatures warmer than −8°C (133). Its abundance in nature is also very low (111). Crystals of a number of inorganic compounds, however, are ice nuclei at temperatures warmer than −10°C (11, 30, 102). Crystals of several organic compounds also have ice nucleation activity, including steroids (28, 34), amino acids (3, 103, 105), proteins (133), terpenes (107), metaldehyde (27), α-phenazine (35), and others. Although these organic compounds are active in ice nucleation at relatively warm temperatures (warmer than −5°C), they are active as ice nuclei only in a crystalline form (102). When solubilized, these compounds lose ice nucleation activity. The natural occurrence of the crystalline form of these organic compounds is likely to be small.

Ice Nucleation on Plant Surfaces

The supercooling of plant tissues is limited by the heterogeneous ice nucleus that is active at the warmest temperature. Therefore, the number and activity of heterogeneous ice nuclei in or on plants can be determined by analysis of the supercooling points of plant tissue.

The ability of many frost-sensitive plants to supercool has been recognized for some time (13, 81, 92). Modlibowska has shown that flowers of small fruit trees supercool to only −2°C before ice formation occurs (95). Extensive supercooling has been reported for lemon, grapefruit, and other *Citrus* species (37, 130–132). Wheat leaves have been reported to supercool to −4.5°C to −5.0°C (115). Several recent reports also indicate variability in the degree of supercooling, which ranged from −2°C to −14°C for a large number of different plant species (44–48, 88, 106). It is apparent that frost-sensitive plants, particularly when grown under greenhouse conditions, have the ability to supercool. Plant materials are very inefficient ice nuclei themselves; significant ice nucleation activity is observed on greenhouse-grown plants only at temperatures lower than −8°C to −10°C (44–48, 87, 88). Ice nucleation activity in plants grown axenically appears to be very rare at temperatures above −5°C (2, 70, 76).

Since ice formation and subsequent frost damage most often occur at temperatures warmer than the determined temperature limits of plant supercooling, efficient heterogeneous ice nuclei must limit supercooling under field conditions in most cases. These heterogeneous ice nuclei have been associated with the surface of plants (81). Kaku (44–48) has shown that ice nuclei are not uniformly distributed on a given leaf and that these nuclei vary in quantity both with maturity of leaves and among plant species. Similar results have been presented by other scientists (88). Atmospheric ice nuclei were once assumed to be the sources of ice nuclei on plants; however, Marcellos & Single have presented evidence that ice nucleation on leaves by air-borne particles is unlikely (87).

Bacterial Ice Nuclei

Recent research has focused on the search for biological sources of ice nuclei. The concentration of ice nuclei in the atmosphere at a given location was observed to increase with increasing organic matter content of the soil at that location (119). Decaying vegetation is a source of abundant ice nuclei (111–113, 121). The bacterium *Pseudomonas syringae* van Hall, associated with decaying leaf material, was shown to be an active ice nucleating agent (82, 121). Recently, three species of bacteria commonly found as epiphytes on leaf surfaces have been shown to be catalysts for ice formation. Many pathovars (19) of *P. syringae* are active in ice nucleation and are (generally) the most common ice nucleation active bacteria found on plants in the United States (2,

41, 64–66, 70). Certain strains of both *Erwinia herbicola* (Lohnis) Dye, and *Pseudomonas fluorescens* Migula are also active in ice nucleation (38, 39, 74, 75, 83–85, 104, 128, 129). Other scientists have reported that certain strains of other *Pseudomonas* spp. and *Erwinia stewartii* (Smith) Dye are active in ice nucleation (85, 104, 124), but these reports have not yet been verified. Approximately 50% of the many pathovars of *P. syringae* examined, including *P. syringae* pv. *coronafaciens* (Elliot) Young et al, *P. syringae* pv. *pisi* (Sadatt) Young et al, and *P. syringae* pv. *lachrymans* are active in ice nucleation (38, 39, 104). Because most strains of a given pathovar of *P. syringae* tested for ice nucleation consistently yielded either a positive or a negative reaction, the ice nucleation phenotype has been suggested as a possible taxonomic tool in differentiating the many pathotypes of *P. syringae* (38, 39, 104). Many of the ice nucleation active strains of *P. syringae* detected in nature have been isolated from nonsymptomatic plants (66–68, 74, 75, 129) and therefore represent, in most cases, unknown pathovars of *P. syringae*. Therefore in this review, except where appropriate, no attempt is made to distinguish among pathovars of *P. syringae*, and mention of ice nucleation active strains of *P. syringae* likely includes one or more pathotypes active in ice nucleation.

The strains of *P. syringae* and *E. herbicola* studied to date are the most active naturally occurring ice nuclei yet discovered. These bacteria catalyze ice formation at temperatures as warm as −1°C (63, 64, 67, 74, 82, 109, 110). Not every cell of *P. syringae*, *E. herbicola*, or *P. fluorescens* is active as an ice nucleus at a given time (70, 71, 74, 78, 82, 121, 128). The fraction of cells that are active as ice nuclei increases rapidly with decreasing temperatures below −1°C (64, 67, 70). In many *P. syringae* strains studied in vitro, approximately one cell in ten contains an ice nucleus active at −4°C or below (64, 70). Many isolates of *P. syringae* exhibit significant ice nucleation activity at −4°C or warmer and contain no additional ice nuclei activity at temperatures lower than −4°C (39). However, other isolates exhibit a reduced frequency of expression of ice nucleation at temperatures above −4°C or −10°C and/or nucleate ice only at colder temperatures (38, 39). The frequency of ice nucleation among cells of ice nucleation active strains of *E. herbicola* examined to date are approximately 10^4-fold lower than the most active strains of *P. syringae* or *P. fluorescens* at −5°C and about 100-fold lower at −9°C when grown under similar in vitro cultural conditions (64, 70, 74, 128, 129).

In vitro cultural conditions, including medium composition, solid versus liquid growth medium, aeration, and growth temperature were found to affect profoundly the ice nucleation efficiency of cells of many ice nucleation active strains of *P. syringae* and *E. herbicola*, as well as the temperature at which ice nucleation is expressed in these cells (64, 74, 79, 128, 129). However, Maki and associates (82) reported that the cell to ice nucleus ratio of their *P. syringae* isolate was constant under different growth conditions. Although many isolates of *P. syringae*, *P. fluorescens*, and *E. herbicola* are active in ice nucleation at

temperatures above −2°C, ice nucleation activity is detectable in other bacterial strains only at temperatures approaching −10°C (38, 39, 85, 104). Ice nucleation activity also does not appear to be a phenotypic characteristic expressed at a given time by every cell of a bacterial isolate capable of ice nucleation. It is not clear whether every cell of an ice nucleation active bacterial isolate expresses ice nucleating activity at some time during its life. It is also unknown whether the low frequency of ice nucleation among a population of cells represents a low frequency of association of nucleating material with the cells or whether a more dynamic yet stochastic process involving infrequent activation of more abundant existing ice nucleation active material is occurring. This activation may be controlled by physiological changes associated with cell metabolism and maturation. This point is reviewed in more depth below.

Measurement of Bacterial Ice Nuclei

The study of the ecological role of bacterial ice nuclei has been facilitated by the development of a number of rapid quantitative assays for their presence and qualitative activity. Measurements of the cumulative number of ice nuclei active above a given temperature are reported by several scientists (64, 67, 69, 70, 76, 80, 82, 84, 128). A droplet freezing assay developed and tested by Vali (120, 122) has been the basis for most measurements of bacterial ice nuclei. The droplet freezing procedure yields an estimate of the number of freezing nuclei, defined as those heterogeneous ice nuclei that are active when suspended in water (42). Since bacterial cells often occur in an aqueous environment, this method should reliably estimate ice nuclei contributed by bacteria in most situations. An accurate measurement of the temperature dependence of ice nucleation has been obtained using ice nucleus spectrometers, in which the freezing temperatures of a large collection of droplets containing bacterial cells can be measured as the droplets are slowly cooled (70, 74, 76, 80, 82, 84, 85, 120, 129). A modification of this method in which aqueous suspensions of bacteria are placed in calibrated capillary tubes has been reported to increase slightly the accuracy of determination of ice nucleation temperatures (85). The activity of bacteria as contact ice nuclei, in which dry bacterial cells contact and nucleate supercooled water droplets suspended in an isothermal cloud chamber, has also been reported (84, 113).

A rapid estimate of minimum populations of ice nucleation active bacteria present on plant samples known to contain only bacterial ice nuclei was obtained from determination of the supercooling point of individual leaves (41). The supercooling point of leaves would be expected to occur at warmer temperatures with increasing populations of ice nucleation active bacteria. Although the accuracy of this method of estimating bacterial populations may be low, the speed of this procedure may be useful in estimating the distribution of bacterial populations on plants, which has recently been shown to be log-normal (40).

More quantitative and nonselective estimates and recovery of populations of ice nucleation active bacteria have been facilitated by the use of a replica freezing technique (75). Hundreds of individual bacterial colonies isolated on nonselective media can be scored rapidly for the potential to produce ice nuclei and ice nucleation active strains can be isolated directly from frozen colonies held at constant subfreezing temperatures (75). As will be seen, this technique has proven valuable for the quantitative assessment of the ecological impact of ice nucleation active bacteria.

Ice Nucleation Active Bacteria on Plants

Most field-grown plants are colonized by large epiphytic populations of one or more species of ice nucleation active bacteria (58, 60, 63–67, 70–76, 129). Nearly all of 95 species of agricultural and native plants sampled from several locations in North America, with the exception of conifers and smooth leaved crucifers, harbored detectable populations of epiphytic ice nucleation active bacteria (75). Ice nucleation active bacteria on plants have also recently been reported from Israel and Japan (85, 128, 129).

The numbers of ice nucleation active bacteria on plant surfaces vary among species as well as temporally on a given species. The maximum populations of ice nucleation active bacteria ranged from approximately 100 cells/g fresh weight of valencia and navel orange (*Citrus* sp.) leaf tissue to over 10^7 cells/g fresh weight on leaves of English walnut (*Juglans regia* L.) or almond (*Prunus amygdalus* L.) (64, 65). Large seasonal variation in the numbers of epiphytic ice nucleation active bacteria on both annual and perennial plants has been observed (64, 75). Low populations of ice nucleation active bacteria (less than 100 cells/g fresh weight of leaf or bud tissue) generally are found on overwintering plant tissues of deciduous plants or on emerging cotyledons or leaves of annual plants (64, 75). However, large epiphytic populations of ice nucleation active bacteria (principally *P. syringae*) are present on emerging flowers and/or leaves of these plants. Bacterial populations found on healthy pear (*Pyrus communis* L.) flowers, leaves, and fruit under California growing conditions are typical of this variation (33, 61, 64, 65). A thousand-fold increase in ice nucleation active bacterial populations occurred on pear during the three-week period immediately following bud break. Populations of ice nucleation active bacteria decreased with the onset of hot dry weather after late May to less than 100 cells/gram by late summer (64). Ice nucleation active strains of both *P. syringae* and *E. herbicola* can be detected on most plants. *P. syringae* is the predominant species on the majority of plant species investigated in California (64) and Wisconsin (75), whereas only strains of *E. herbicola* have been found in Israel (129). Ice nucleation active strains of *P. fluorescens* are only rarely found on plants in California (Lindow, unpublished data). Because bacterial ice nucleation has been reported only recently, few

laboratories have investigated the populations of ice nucleation active bacteria on plants. Many more plant pathologists have studied leaf surface populations of phytopathogenic bacteria or their antagonists, including species now known to nucleate ice. Populations of *P. syringae,* and *E. herbicola* have been reported on a variety of plants throughout the world and are ubiquitous epiphytes on nearly all plants studied (6, 26, 32). The occurrence of ice nucleation activity among strains of *E. herbicola* is as yet largely unknown, but is probably low (74, 85, 129). However, the observation that at least half of the pathovars of *P. syringae* are active as ice nuclei (38, 39, 104) indicates that ice nucleation active bacteria have a worldwide distribution. Similarly, strains of *P. fluorescens* are common soil and water inhabitants. Even if a low percentage of *P. fluorescens* strains are active in ice nucleation, this species may also be an important source of ice nuclei.

Not all bacterial cells that are active as ice nuclei in vitro are also active on leaf surfaces. Since dead as well as living bacterial cells have the potential for expression of ice nucleation activity, and because it is difficult to determine quantitatively the fraction of dead and viable cells on a plant surface at a given time, only estimates of the fraction of cells active in ice nucleation while on leaf surfaces can be determined. Assuming that living cells contribute the majority of ice nuclei on leaf surfaces, an average of only one ice nucleus is expressed in a leaf surface population of 300–1000 bacteria with the potential for ice nucleation activity (64). The ice nucleation activity of a *P. syringae* strain was lower on leaves than in culture (64). However, because different strains of ice nucleation active bacteria have different nucleation frequencies in vitro, the measurement of the efficiency of ice nucleation among a collection of leaf surface ice nucleation active bacteria probably also includes genetic differences in ice nuclei expression between strains.

The fractions of both *P. syringae* and *E. herbicola* cells on leaf surfaces that are active as ice nuclei increase sharply with decreasing temperature from -1.5°C to -6°C (64). At the warmest temperature of -1.5°C, only a very low fraction (less than one cell in 10^8 is active as an ice nucleus; however, at -5°C, nearly 10% of the *P. syringae* cells on leaf surfaces are active in ice nucleation (64). Over 1000 bacterial ice nuclei active at temperatures above -5°C per gram of leaf tissue have been measured on plant surfaces (64). Thus epiphytic populations of the phytopathogenic bacterium *P. syringae* are a major source of ice nuclei active at low levels of supercooling on leaf surfaces as well as a reservoir of inoculum for disease initiation (53).

BACTERIAL ICE NUCLEATION AND FROST INJURY

A single ice nucleus is currently thought to be sufficient to initiate ice formation and subsequent frost injury to an entire leaf, fruit, flower, or even groups of

leaves or flowers, depending on the degree of restriction of ice propagation within a plant (115). Since frost-sensitive plants must avoid ice formation to avoid frost damage, frost injury to these plants might best be considered a quantal response—either a plant part escapes ice formation or it does not.

Although early investigators recognized that the water in plant tissues could supercool, this supercooling was generally believed to be of little practical importance, particularly under field conditions (57). Until recently very little was known of factors influencing the supercooling ability of plant tissue (12, 13, 92).

The frost sensitivity of most plants can now be explained by the fact that they harbor very large epiphytic populations of ice nucleation active bacteria, which limit their supercooling ability (64, 75). Low temperatures of short duration will not damage these plants if no ice formation occurs. Plants were cooled to temperatures as low as −7°C for several hours with no apparent damage or internal ice formation under greenhouse conditions where ice nucleation active bacteria were absent (1, 2, 56, 70, 76). In the field, however, the presence of these bacteria on plant surfaces will cause ice formation to occur on and in the plants, with subsequent injury at temperatures above −5°C (37, 95, 106, 130–132). The inability of plant tissues to supercool extensively in natural situations can be explained by the detection of up to 1000 ice nuclei/gram of plant tissue which are active at temperatures warmer than −5°C (64). Most other organic and inorganic materials such as dust particles nucleate ice only at temperatures lower than −10°C. It is unlikely that these nuclei are important in limiting the supercooling of plant tissue at temperatures above −5°C, the temperature range at which most frost-sensitive plants are injured (57).

Studies have shown that at least 95% (and probably all) ice nuclei on leaf surfaces active at −5°C or above are of bacterial origin (67, 70, 76). The extent of frost damage at a given temperature (the chances of a given plant part freezing) increases with increasing populations of ice nucleation active bacteria on that plant (67, 70, 76). Frost injury at a given temperature is more directly related to the numbers of actual bacterial ice nuclei on the plant at the time of freezing than to the population of ice nucleation active bacteria (64). Various species of ice nucleation active bacteria have been demonstrated to be both necessary and sufficient to account for the frost sensitivity of all frost-sensitive plants examined to date.

Management of Bacterial Ice Nucleation and Frost Injury

The discovery of bacterial ice nuclei on plants has suggested several new methods of frost protection based on enhancement of the natural supercooling ability of plants by controlling ice nuclei contributed by ice nucleation active bacteria. Treatments that reduce the numbers and/or the ice nucleation activity of ice nucleation active bacteria are promising alternate methods of frost

management. The use of these new methods of frost management is reviewed below.

BACTERICIDES One new alternative method of frost management has included the use of commercially available bactericides to reduce populations of ice nucleation active bacteria on plants. Large (100- to 1000-fold) reductions in populations of epiphytic ice nucleation active bacterial are observed following protectant bactericide applications when compared with untreated plants (59, 60, 61, 65, 66, 70). The numbers of ice nuclei on bactericide-treated plants was also significantly lower than on untreated plants (60, 64, 70), thereby reducing the chances of frost injury to a given plant part at temperatures above −5°C. Large reductions in the incidence of frost injury have been observed on bactericide-treated plants compared with untreated plants (59, 60, 61, 64–66, 70). Significant frost control has been achieved with experimental applications of bactericides on several different crops such as corn (*Zea mays* L.), beans (*Phaseolus vulgaris* L.), potatoes (*Solanum tuberosum* L.), squash (*Cucurbita* spp.), and tomatoes (*Lycopersicon esculentum* Mill.) (59, 60, 61, 64–66, 70). Much research remains to be done to determine the most effective rate, type, and application frequency of bactericides for frost management. However, bactericides appear to be effective frost management agents when applied before bacterial populations develop naturally on plants.

Some problems also exist in the use of bactericides for frost prevention. Most bactericides such as streptomycin kill growing ice nucleation active bacteria rapidly on contact in culture, but these bacteria lose their ability to nucleate ice very slowly in vitro (69), although exceptions have been reported (129). A similar phenomenon may operate on leaf surfaces. Strategies to minimize the potential for development of resistance of ice nucleation active bacteria to bactericides must also be developed if agents such as streptomycin are to be used frequently for frost management. Without proper deployment, development of resistance to effective bactericides such as streptomycin or oxytetracycline, as seen in other phytopathogenic bacteria (18, 21, 96, 114), may quickly preclude the use of such materials for either disease or frost management.

ANTAGONISTIC BACTERIA Only about 0.1% to 10.0% of the total bacteria found on plant surfaces are active as ice nuclei and are therefore involved directly in frost injury (64, 75). Competition or other form(s) of antagonism between these and other epiphytic bacteria and other microorganisms on leaf surfaces appears likely based on studies of other ecological niches. The degree of natural competition among epiphytic microorganisms is insufficient to prohibit buildup of significant populations of epiphytic ice nucleation active bacteria on most plants. However, this natural antagonism may be augmented by altering the leaf surface microbial ecology so as to favor increased populations of non-ice nucleation active bacterial competitors. These bacterial com-

petitors may then occupy a niche on the plant that might otherwise be colonized by ice nucleation active bacteria.

Bacterial competitors have been selected on the basis of their effective colonization of leaf surfaces (59–61, 64, 65, 72, 73, 77, 78). These antagonists have been established by application to seeds or by foliar application to plant parts prior to colonization of these plants by ice nucleation active bacteria (59–61, 64, 65, 72, 73, 77, 78). Populations of ice nucleation active bacteria are generally lowest on young vegetative tissues (64). The effect of treatment with non-ice nucleation active bacteria is to reduce the populations of ice nucleation active bacteria on plants during periods of low temperatures and therefore to reduce the probability of frost injury. Many bacterial antagonists have effectively colonized emerging and mature plant tissues for a relatively long period of time (one to four months) following a single foliar application (59–61, 64, 65, 72, 73, 77, 78). Populations of ice nucleation active bacteria have been decreased under field conditions from 10- to 1000-fold on plants treated with antagonistic bacteria when compared with untreated plants (59–61, 64, 65, 72). Reductions in frost damage to treated plants was related directly to reductions in the logarithm of populations of ice nucleation active bacteria on plants (59–61, 64).

Integrated management of fire blight and frost injury of pear have recently been reported (61). Antagonistic non-ice nucleation active bacteria applied at 10% bloom to pear trees colonized pear flowers and leaves for over three months and reduced significantly the epiphytic populations of *P. syringae* and *Erwinia amylovora* (61). The incidence of both frost injury, and, later, fire blight, was reduced significantly compared to untreated trees (61). The control of frost injury and fire blight from a single application of antagonistic bacteria was nearly as good as from weekly applications of a mixture of streptomycin and oxytetracycline or cupric hydroxide (61).

Mechanisms determining effective biological control of frost injury are not well known. Properties of only a few non-ice nucleation active bacterial antagonists have been studied (62). Some, but not all, non-ice nucleation active bacterial antagonists to ice nucleation active bacteria on plants are antagonistic to these bacteria in vitro (62). Similarly, some, but not all, bacteria that reduced the incidence of fire blight of pear were inhibitory to *E. amylovora* in culture (61).

Fungal and bacterial plant pathogens have been shown to be inhibited on plants as well as in vitro by certain antagonistic bacteria used as biological control agents. However, not all bacteria or fungi applied to plant foliage as biological control agents of plant disease exhibit in vitro antibiosis toward the foliar pathogen. Although antibiosis has been reported to be important in interactions of microorganisms in the rhizosphere and antibiotic production is widely used as a prerequisite for testing of potential biological control agents of

both foliar and root diseases, little is known of the importance of antibiosis in the interactions of microbes on leaves. Knowledge of the primary mechanisms of such interactions would be important in designing selection procedures for identification of potential antagonistic bacteria as biological control agents. Antibiosis was indicated in a recent report to play a minor role in antagonism of *P. syringae* on leaves by other bacteria (62). Nearly all mutants of antibiotic-producing antagonistic bacteria deficient in production of antibiotics in vitro did not differ from the parental strain in antagonism of *P. syringae* on plants (62). Similarly, antagonistic bacteria that controlled both *P. syringae* and *E. amylovora* were not inhibitory, in vitro, to both species [(61), Lindow, unpublished data]. Therefore, antagonism on leaf surfaces may be a general phenomenon such that nonspecific control of more than one target microorganism may be possible.

Control of frost injury with non-nucleation active bacteria is a good model system with which to study biological control processes for a number of reasons: 1. Frost injury is an important, worldwide problem; 2. The target microorganisms are well known and can be well quantified based on their phenotype of ice nucleation activity; 3. Subtle microbial interactions on leaves may be expressed and therefore quantified as altered ice nucleation activity of bacteria on leaves; and, 4. Even in the absence of frost injury, information gained on the ecology and control of ice nucleation active pathovars of *P. syringae* could be exploited to achieve management of the disease initiated by these and other bacteria by reduction of epiphytic inoculum sources on host plants.

ICE NUCLEATION INHIBITORS 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" (68, 69). Laboratory tests have shown that the ice nucleation site 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 surfactants used in agriculture) (50, 60, 68, 69, 82). Even though viable bacterial cells may remain on plants after treatment with bacterial ice nucleation inhibitors, the cells no longer catalyze ice formation and cannot be responsible for initiating frost damage.

Bacterial ice nucleation inhibitors inactivate bacterial ice nuclei within minutes to a few hours after application to the plant (68, 69). Significant reductions in frost injury have been achieved under field conditions after application of bacterial ice nucleation inhibitors within a few hours prior to a frost (60, 65, 68, 69). The use of bacterial ice nucleation inhibitors may offer a "day before" or eradicative type of immediate frost prevention not offered by

bactericides or antagonistic bacteria. Although many chemicals can inactivate bacterial ice nuclei, many are too phytotoxic for use on plants, and all are water soluble and may weather rapidly from plant surfaces (68, 69). The irreversibility of this effect must be investigated further. Therefore, more work needs to be done on this promising method of frost protection.

MOLECULAR BASIS OF ICE NUCLEATION

Several recent reports on the location and partial characterization of compound(s) responsible for bacterial ice nucleation activity have appeared. The ice nucleation activity of *P. syringae, E. herbicola,* and *P. fluorescens* is associated with the intact bacterium of these species (63, 82, 84, 116, 128). Ice nucleation activity is not detected in extracellular byproducts of these bacteria (82, 84, 116, 128). Several pieces of evidence indicate that the ice nucleating material in these species is membrane-bound and not a soluble cell component (82, 84, 116). Recent work indicates that the ice nucleating material in *P. syringae* and *E. herbicola* is located in or on the outer cell membrane of these gram-negative bacteria (116). Ice nuclei active at temperatures of −4°C or higher are eliminated by treatment of cells with respiratory inhibitors or with many reactive chemicals such as borate compounds, urea, by extremes of pH, or by disruption of the cells by physical processes or phage lysis (50, 68, 82, 84, 116, 128). However, these same treatments usually do not affect the numbers of ice nuclei active at colder tempeatures, e.g. below −7°C (50, 68, 82, 84, 116, 128). Small membrane fragments (0.2 μ) generally can initiate ice nucleation activity only at colder temperatures (lower than −7°C) (128). It appears that with a few possible exceptions, bacterial ice nuclei active at temperatures warmer than −4°C require a physically intact or physiologically normal cell for their expression, while those active only at colder temperatures do not. Whether ice nuclei active at different temperatures represent different substances or collections of compounds, or simply an alteration in their conformation or environment, and thus a different threshold ice nucleation activity, is as yet unclear.

The nucleating material in isolated membranes of both *P. syringae* and *E. herbicola* is sensitive to proteases (116) and many protein denaturing agents, including sulfhydyl reagents (50), suggesting that an outer membrane protein determines or is involved in ice nucleation in these species. Reagents that react with carbohydrates, including borate compounds and certain lectins, reduced the ice nucleation activity of both *P. syringae* and *E. herbicola* (50).

Genetically induced lipid changes in fatty acid auxotrophs of *P. syringae* also affected the ice nucleation activity of this bacterium (Lindow, unpublished data). Similarly, the decrease in ice nucleation activity of *E. herbicola* prior to lysis by a virulent phage was attributed to phage-induced changes in the cell

wall (50). Although any one or all of the above components of cell membranes may be involved at the active site or indirectly involved in expression of ice nucleation activity, no firm cause-and-effect relationship has yet been shown for any one substance. Some evidence from the study of antifreezing glycoproteins (24) suggests that more than one substance, such as protein and carbohydrates or protein and lipids, may be required for ice nucleation.

Recent studies of the genetic determinants of ice nucleation in *P. syringae* and *E. herbicola* should improve the understanding of this process. The gene(s) for ice nucleation in strains of both *P. syringae* and *E. herbicola* have been cloned and are expressed in *Escherichia coli* (99, 100). The expression of ice nucleation in *E. coli* was largely similar quantitatively and qualitatively to that in the original DNA source strains (99, 100), which suggests that the gene products may largely determine the expression of ice nucleation activity in a biological membrane, although other possibilities also exist.

Cloned DNA sequences conferring ice nucleation complemented ice nucleation deficient mutants of *P. syringae* that were derived by chemical mutagenesis (80, 100). Because the genes conferring ice nucleation activity were cloned on a single restriction fragment, it is obvious that: (*a*) the gene(s) for ice nucleation are not dispersed throughout the bacterial chromosome, and (*b*) a limited number of genes (one to five) are sufficient for determination of the ice nucleation phenotype. Work in progress should soon identify the gene product(s) coded by cloned ice nucleation gene(s) and whether the gene product(s) are primary determinants of the ice nucleation phenotype. The factors regulating ice nucleation activity can be more fully elucidated utilizing the cloned ice nucleation genes. The use of the cloned DNA sequence determining ice nucleation will also allow in vitro construction of site-directed deletion mutants for the ice genotype of *P. syringae* and *E. herbicola* for possible deployment as antagonists of wild type ice nucleation active strains.

BACTERIAL ICE NUCLEATION IN PLANT DISEASE

Many pathogenic strains of *P. syringae* have been reported to survive in large numbers as epiphytes on a variety of symptomless host plants, including stone fruits (10, 14–17, 20, 23, 29), olive (*Olea europaea* L.1) (22), bean (23, 41, 58, 75), and soybean (*Glycine max* (L.) Merr.) (52, 54, 55, 94). Since infection by *P. syringae* often occurs after injury to a host plant, this observation may attest to the ubiquitous presence of this bacterium as an epiphyte (53). In fact, frost injury has often been reported as a predisposing factor for infection of some plants by *P. syringae* (8, 49, 51, 98, 101, 108). Panagopoulos & Crosse (101) reported that pear blossoms supercooled to approximately −2°C. If flowers were sprayed with a bacterial suspension after freezing, infection by *P. syringae* pv. *syringae* was severe, whereas infection of inoculated unfrozen

flowers was minimal. *P. syringae* pv. *syringae* was found to occur in large numbers on flowers from branches from field sources but was not found on greenhouse-grown trees. Therefore, frozen field-grown flowers which were sprayed with water after freezing sustained severe infection by *P. syringae* whereas greenhouse-grown flowers did not. Frost injury has also been implicated in outbreaks of bacterial blight of pea (*Pisum sativum* L.) incited by *P. syringae* pv. *pisi* in South Africa (8). Freezing injury has also been found to aid in the development of bacterial canker of poplar (*Poplar* spp.) (51, 108) caused by *P. syringae* pv. *syringae,* and an unidentified pathogen of barley (98). Klement (49) also found a strong relationship between frost injury to apricot (*Prunus armenica* L.) and development of bacterial canker incited by *P. syringae* pv. *syringae*. He recognized that both cold temperatures and the bacterium were required for the development of cankers. Weaver (125) showed that both freezing injury and *P. syringae* pv. *syringae* were required for development of bacterial canker of excised peach (*Prunus persicae* (L.) Batsch.) twigs. Neither freezing nor presence of *P. syringae* pv. *syringae* alone was sufficient to cause typical bacterial canker symptoms. The ice nucleation activity of *P. syringae* pv. *syringae* strains was well correlated with development of cankers on inoculated peach seedlings frozen at −10°C (126). These authors suggested that ice nucleation activity by *P. syringae* pv. *syringae* was important in the development of bacterial canker of peach. The use of an ice nucleation-deficient mutant of *P. syringae* (80) in this study, however, would help clarify the role of ice nucleation in canker development.

Many diseases induced by *P. syringae* require or are favored by ice formation in plants prior to disease development. Because of the ubiquity of *P. syringae* it is likely to be present on plants at the time of freezing temperatures. As most bacteria including *P. syringae* do not effectively invade noninjured plant tissues, it is tempting to speculate that *P. syringae* has evolved the capacity to predispose plant tissues to ice damage and subsequent bacterial penetration and disease development.

CONCLUSIONS AND FUTURE DIRECTIONS

Ice nucleation active bacteria are present in large numbers in all temperate regions of the world and contribute a unique source of ice nuclei that are active catalysts of ice formation in non-coniferous plants at temperatures only slightly below 0°C. These bacteria are both necessary and sufficient to account for the frost sensitivity of the frost-sensitive plants on which they reside. These bacteria may also be involved in the survival of frost-tolerant plants (63) and may be important in global climatology due to their possible importance in initiating rain and snow (for review see 63). As can be judged from this review, the study of ice nucleation active bacteria requires input from many different

disciplines. Because of the diversity of roles ice nucleation active bacteria may play in nature, many workers from very diverse fields of the biological and physical sciences are now beginning to investigate this unique process and its implications for man.

As is typical of any active field of research, many more questions are raised in response to the few answers already found. Some of the more obvious questions that must be addressed include: What will be the economics of plant frost management using bactericides, nucleation inhibitors, and antagonistic bacteria? What will be the optimum types, rate, and frequency of application of these materials? What are the mechanisms of antagonism among epiphytic bacteria? Will common mechanisms of biocontrol of epiphytic microorganisms exist to allow simultaneous management of ice nucleation active bacteria and other pathogens? Where are "epiphytic" ice nucleation active bacteria located on or in plants? Are there preferential sites of colonization on plants, and does this affect the ability of the bacteria to nucleate ice? Is it possible to wash a plant free of ice nucleation active bacteria? Will the location of ice nucleation active bacteria dictate procedures to alter its ability to colonize plants? What other factors play a role in determining the efficiency of ice nucleation by bacteria on leaf surfaces? Can growing practices or plant varieties be developed to minimize the numbers of bacterial ice nuclei on frost-sensitive plants? Do other substances or plant tissues limit supercooling of water? In what temperature range are bacterial ice nuclei the important limiting factor in plant supercooling? What role do ice nucleation active bacteria play in atmospheric precipitation procedures? How important is aerosol dispersal in epiphytic colonization of ice nucleation active bacteria on plants? Will laboratory-produced ice nucleation active bacteria be of potential value as cloud seeding agents to augment rain or snowfall or to suppress hail formation? Are ice nucleation active bacteria important colonists on frost-tolerant native plants, and if so, do these bacteria play a significant role in the cold tolerance of these plants? What are the molecular determinants of ice nucleation activity? Does the ice nucleation site of bacteria have other physiological functions in these bacteria? What genetic and physiological control mechanisms are involved in expression of bacterial ice nucleation activity? Can a molecular determination of the bacterial ice nucleation site contribute to a general understanding of ice nucleation and allow development of specific inhibitors of ice nucleation? Is the gene(s) for ice nucleation similar among all ice nucleation active species and strains of these species? Can genetically engineered strains of bacteria be useful in biological control of frost injury?

Research on ice nucleation active bacteria is still in its infancy. Hopefully, as more is known of these bacteria their ecological role will be further clarified. With this knowledge, we may develop new and better methods of managing harmful associations of ice nucleation active bacteria with plants.

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