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Ice Nucleation Temperature of Individual Leaves in Relation to Population Sizes of Ice Nucleation Active Bacteria and Frost Injury

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ABSTRACT

Ice nucleation temperatures of individual leaves were determined by a tube nucleation test. With this assay, a direct quantitative relationship was obtained between the temperatures at which ice nucleation occurred on individual oat (*Avena sativa* L.) leaves and the population sizes of ice nucleation active (INA) bacteria present on those leaves. In the absence of INA bacteria, nucleation of supercooled growth-chamber grown oat leaves did not occur until temperatures were below approximately −5°C. Both nucleation temperature and population size of INA bacteria were determined on the same individual, field-grown oat leaves. Leaves with higher ice nucleation temperatures harbored larger populations of INA bacteria than did leaves with lower nucleation temperatures. Log_{10} mean populations of INA bacteria per leaf were 5.14 and 3.51 for leaves with nucleation temperatures of −2.5°C and −3.0°C, respectively. Nucleation frequencies (the ratio of ice nuclei to viable cells) of INA bacteria on leaves were lognormally distributed. Strains from two very different collections of *Pseudomonas syringae* and one of *Erwinia herbicola* were cultured on nutrient glycerol agar and tested for nucleation frequency at −5°C. Nucleation frequencies of these bacterial strains were also lognormally distributed within each of the three sets. The tube nucleation test was used to determine the frequency with which individual leaves in an oat canopy harbored large populations of INA bacteria throughout the growing season. This test also predicted relative frost hazard to tomato (*Lycopersicon esculentum* Mill) plants.

Certain bacteria are active as catalysts for ice formation in supercooled water at temperatures approaching −1.0°C (17). Most INA[2] bacteria belong to either one of two species, *Pseudomonas syringae* van Hall or *Erwinia herbicola* (Löhnis) Dye (3, 11). These two bacterial species commonly are found as epiphytes on leaf surfaces of a wide range of plant species (12) and in different geographic areas (12, 25). Their role as incitants of frost injury to sensitive plants has been well documented (1, 3, 11, 13, 14). Some strains of *Pseudomonas fluorescens* biotype G Migula (16) and *Pseudomonas viridiflava* (18) also have ice nucleation activity.

Although the ability to act as an ice nucleus is an inherent genotypic property of INA bacteria, not every cell in a given suspension of an INA bacterial strain is likely to serve as an ice nucleus at any given temperature and time (17). Bacterial ice nucleation frequency (NF = ratio of number of ice nuclei to number of bacterial cells) is dependent on the conditions under which the cells are grown, including composition of the medium and temperature during growth and on the assay temperature itself (15). Nucleation frequency for any set of growth conditions differs among different INA bacterial strains (6, 18). For example, approximately 45% of 348 *P. syringae* strains representing 40 pathovars caused nucleation of supercooled water at −5.5°C (6). The nucleation frequencies of these strains ranged from 10^{-1} (one ice nucleus per 10 bacterial cells) to 10^{-8}. The limit of sensitivity was approximately one ice nucleus per 10^8 cells.

Frost injury to sensitive plants is a function of both the number of INA bacteria present on leaf surfaces and the nucleation frequency of those bacterial cells (15). Under growth chamber conditions, the nucleation frequency of *P. syringae* isolate no. 31 was 10^{-6} to 10^{-7} at −4°C, 36 h after it was applied to corn seedlings. Under optimal conditions on nutrient glycerol agar medium, however, the nucleation frequency of isolate no. 31 was approximately 10^{-2} at −5°C; 10^3 to 10^5 times higher than that measured for cells growing on corn leaf surfaces in the growth chamber. Nucleation frequencies at −5°C for INA bacteria associated with bulked samples of potato, tomato, almond, and pear leaves ranged from 10^{-2} to 10^{-4} as determined by a droplet freezing assay of leaf washings (10). However, these average values of NF determined from bulked samples of several field-grown leaves may not be particularly representative of bacterial nucleation frequencies within a population of individual leaves, since population sizes of INA bacteria may vary by >1000-fold from one individual leaf to another (7).

In this report, we describe a method for determining the temperatures at which ice nucleation will occur on individual leaves. We illustrate the use of the method to (a) estimate nucleation frequencies of INA bacteria on individual field-grown leaves, (b) estimate the frequency with which individual leaves within a plant canopy bear relatively high populations of INA bacteria, and (c) predict frost hazard.

MATERIALS AND METHODS

Determination of the Nucleation Temperature of Individual Leaves. *Tubes and Equipment.* Nine ml of sterile K-phosphate (10 mM, pH 7.0, prepared with glass-distilled H_2O) were placed in test tubes (18 or 16 × 150 mm). The buffer-containing tubes were tested for the absence of heterogeneous ice nuclei active at −10°C by placing them in a constant temperature bath at −10°C

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[2] Abbreviations: INA, ice nucleation active; NF, nucleation frequency; CFU, colony forming unit; NT, nucleation temperature.

for 30 to 60 min. At the end of this period, the tubes were shaken vigorously. All tubes in which the buffer had frozen were discarded. The others were allowed to equilibrate to ambient temperature (about 24°C) before use in the tube nucleation test.

Refrigeration for constant temperature baths was provided by Endocal-850 Flow-Through-Coolers (Neslab Instruments, Inc., Portsmouth, NH).[3] Circulation and temperature regulation were provided by Exacal-300 Bath circulators with dual suction/force pumps (also Neslab). The working area of 25.4 × 30.4 cm of the baths supplied with the Exacal-300 systems provided space for up to 100 tubes without degradation of temperature uniformity. With insulated tubs that we have built, the Exacal-Endocal system provided adequate temperature uniformity for up to 180 tubes in a 43 × 44 cm working area. During tube-nucleation tests, temperatures were maintained within 0.05°C of the stated temperatures throughout the incubation period. Temperatures were monitored continuously with Digitec model 5810 thermistor thermometers equipped with model 703A tubular stainless steel probes (United Systems Corp., Dayton, OH).

The Tube Nucleation Test. Oat leaves (*Avena sativa* L.) and tomato (*Lycopersicon esculentum* Mill) and snap bean (*Phaseolus vulgaris* L.) leaflets were used as the test materials. Leaves from field plots were harvested at random from the top of the canopy, placed in sterile paper bags, and transported to the laboratory in a cooler. Each leaf was placed in a test tube prepared as described above.

For each set of test leaves, sterile forceps were used to completely submerge one leaf in the buffer solution in each test tube. The tubes were chilled to 0°C in an ice bath. Next, groups of these test tubes (90–100 in smaller baths, up to 180 in the larger ones) were placed in test tube racks and positioned in a refrigerated constant temperature bath so that the buffer level in each tube was below the bath surface. After the required time at the first test temperature (*e.g.* −2.5°C), the number of tubes in which the water had frozen was determined. An ice nucleation event had occurred in each of these frozen leaf-containing tubes. The tubes containing unfrozen leaves were transferred to a second bath maintained at a lower temperature (*e.g.* −3.0°C) and the process was repeated. Most sets of leaves were tested at three temperatures in the range of −2.0 to −4.0°C. A separate bath was used for each test temperature to allow for the testing of many individual leaves in a given day. With three of the larger baths, we have processed up to 1,620 leaves in 1 d. A single bath in which the temperature is lowered step-wise can also be employed for smaller sample sizes. Tubes with leaves that did not freeze at the lowest test temperature (*e.g.* −4.0°C) were placed in a −10°C bath, where they froze, so that all leaves were subjected to a freezing event prior to processing by dilution plating for determinations of INA bacterial population sizes. The temperature at which nucleation occurred was recorded for each leaf.

Determination of INA Bacterial Population Sizes on the Same Leaves as Assayed by the Tube Nucleation Test. Frozen leaves were allowed to thaw at room temperature (about 24°C) and the contents of each tube (*e.g.* one oat leaf + 9 ml buffer) were transferred to a sterile 125-ml Erlenmeyer flask. The test tube was rinsed with 1 ml of sterile buffer and the washings were added to the flask for a total of 10 ml of washing buffer volume. Each leaf was cut into about 1 to 2 cm^2 segments. The leaf segments were washed for about 2 h on a gyratory shaker set at 250 rpm. Portions (0.1 ml) from the original wash and appropriate 10-fold serial dilutions thereof, prepared in 0.01 M phosphate buffer (pH 7.0), were plated onto King's medium B (8) supplemented with cycloheximide (100 mg/l) to inhibit growth of fungi. Bacterial colonies were counted after 3 to 4 d of growth at ambient temperature (about 24°C). The number of INA colonies was estimated by the replica freezing method of Lindow *et al.* (12).

Determination of Ice Nucleation Frequencies of Bacterial Strains in Culture. Epiphytic strains of *Pseudomonas syringae* and *Erwinia herbicola* were recovered from dilution plates of washings from leaves of oats, tomato, corn (*Zea mays* L.), pepper (*Capsicum frutescens*), and snap bean. INA bacterial strains were categorized as *P. syringae*-like if they were fluorescent on King's medium B (8) and were oxidase (9) and arginine dihydrolase (23) negative. Yellow-pigmented INA strains with the appropriate colony morphology and odor were categorized as *E. herbicola*-like. The nucleation frequency of each isolate was determined at −5°C according to the method of Lindow *et al.* (11), and as described in Hirano *et al.* (6). Each isolate was grown for 2 d at 24°C on nutrient agar supplemented with glycerol (NGA: 2.7 g Difco nutrient broth, 2.0 g Difco yeast extract, 3.3 g Bacto peptone [Difco], 25 ml glycerol, and 15 g agar/l of glass-distilled H_2O). A cell suspension (~10^8 colony-forming units per ml [CFU/ml]) was prepared in sterile K-phosphate (pH 7.0, 0.1 M). Ten-fold dilution series in phosphate buffer were prepared from the suspension and 20 10-μl droplets of each dilution were placed on paraffin-coated aluminum foil 'boats' floating on a refrigerated constant temperature bath maintained at −5°C. The number of droplets that froze was recorded and the ice nucleus concentration was calculated according to Vali (24). The nucleation frequency for each strain was determined from the ice nucleus concentration and the cell density of the suspension as determined by dilution plating.

Field Plots. Field plots of oats (cv Mackinaw, Marathon, Dal, Froker, and Wright) were drill planted on April 25, 1980. The five cultivars were planted in a randomized complete block design with three blocks (*i.e.* replicates). Each replicate plot was 4.5 × 6 m. The test leaves included in this study were harvested from plots of cv Mackinaw and Marathon.

Tomato (cv Heinz 1350) seedlings were transplanted on May 9, 1979 in 6 × 6 m plots with rows 1.2 m apart. The seven treatments were planted in a randomized complete block design with three blocks. Streptomycin (11.19 kg Agristrep/ha), Kocide 101 (11.19 kg/ha), and Maneb (3.36 kg Dithane 45/ha) were applied weekly with a field sprayer starting on May 10, 1979.

RESULTS AND DISCUSSION

Duration of Incubation. A 1-h incubation at each temperature was arbitrarily used for initial experiments. We noted, however, that most of the nucleation events had occurred in the first few minutes of incubation at each test temperature. Time course experiments were conducted to determine if the incubation times could be shortened. In the experiment illustrated in Table I, individual bean leaflets were placed in a bath maintained at −2.5°C. The number of tubes in which a nucleation event had occurred was counted at various times. Ninety-five% of the nucleation events that occurred during the 4-h long incubation period were detected within the initial 30 min of incubation. In a second experiment (Table II), a set of 180 leaflets was placed directly into a −3.0°C bath and held there for 30 min. The total length of time at which this set of leaflets was at subzero temperatures was 32 min. A second set of 180 leaflets was subjected to 30-min incubations at a series of decreasing temperatures such that the leaflets were at subzero temperatures for approximately 3.5 h by the end of a 30-min incubation at −3.0°C. The same portion of each set (73.3%) of 180 leaflets froze by −3.0°C, regardless of the length of time the leaflets were subjected to subzero temperatures.

Thus, we concluded that the duration of time below freezing

[3] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

Table I. *Effect of Duration of Incubation on the Frequency with Which Individual Snap Bean Leaflets Bore Active Ice Nuclei*

A total of 180 individual leaflets was harvested between 0815 and 0825 h. Each leaflet was submerged in buffer in a test tube. All tubes were placed in a refrigerated constant temperature bath maintained at −2.5 ± 0.05°C. The number of tubes that contained frozen leaflets was scored at 15-min intervals during the 1st h and then at 30-min intervals for the subsequent 3 h of incubation.

Sampling Time (Duration of Incubation at −2.5°C)	Percentage of Leaflets Frozen	
	Incremental[a]	Cumulative[b]
min	%	
15	56.7	56.7
30	7.8	64.4
45	1.7	66.1
60	0.0	66.1
90	1.7	67.8
120	0.0	67.8
150	1.1	68.9
180	0.0	68.9
210	0.5	69.4
240	0.0	69.4

[a] Incremental percentage of a total of 180 leaflets that froze per 15- or 30-min interval.

[b] Cumulative total frozen since beginning of experiment.

Table II. *Effect of Duration of Incubation on the Frequency with Which Individual Snap Bean Leaflets Bore Active Ice Nuclei*

A total of 360 individual snap bean leaflets was harvested between 0815 and 0830 h. Each leaflet was submerged in buffer in a test tube. The tubes were then divided into two equal sets of 180 each. The first set of leaflets was subjected to decreasing temperatures from −2.0 to −3.0°C at 0.2°C steps. The leaflets were maintained at each test temperature for 30 min. The additional time per temperature interval was that required for the bath to equilibrate to the target temperature after the tubes had been placed in it. The second set of 180 leaflets was placed directly into a bath maintained at −3.0°C.

	Temperature	Duration of Incubation	Cumulative Percentage of Leaflets Frozen
	°C	*min*	%
Set 1	−2.0	36	3.3
	−2.2	71	8.3
	−2.4	106	32.2
	−2.6	145	55.6
	−2.8	178	64.4
	−3.0	210 (3.5 h)	73.3
Set 2	−3.0	32	73.3

is a less important determinant of the frequency of nucleation events than is the test temperature. Similarly, since ≥95% of the nucleation events were detected in the first 30 min, longer incubation was not justified for routine experiments. Further, since finite probability of nucleation occurs each time a tube bearing supercooled water in contact with plant material is tapped, jiggled, or otherwise handled, presumably due to cavitation, prolonged incubation may provide an overestimate of the number of ice nuclei present. Reproducible estimates of numbers of ice nuclei require careful and gentle handling of the tubes.

Nucleation Temperature of Leaves That Do Not Harbor INA Bacteria. To determine the nucleation temperature of leaves in the absence of INA bacteria, oat leaves from plants grown in a growth chamber under conditions that were not favorable for bacterial growth (*i.e.* RH <30%) were assayed by the tube nucleation test in one degree steps from −2.0°C to −10°C (Fig. 1). Nucleation events associated with these oat leaves did not occur until temperatures approached −4.0°C to −5.0°C in each of two separate experiments. In the first trial, 1.4% of a total of 500 leaves froze by −4.0°C. In the second trial, no leaves of 400 tested froze at −4.0°C (Fig. 1). A nucleation event occurred on each of two leaves by −5.0°C. No INA bacteria were detected by dilution plating on any of a subsample of these leaves. Since our limit of detection for these bacteria was ~100 CFU/leaf, none of the leaves tested had populations of INA bacteria that exceeded 100 CFU/leaf.

Leaves or leaflets from growth-chamber grown corn and tomato plants were also assayed by the tube nucleation test. Nucleation events near −5°C were detected at frequencies of about 0.7% for corn (*i.e.* two of 299 leaves froze by −4.5 to −5°C) and about 0.9% for tomato (*i.e.* one of 116 leaflets froze by −4.5°C).

Other workers have reported threshold nucleation temperatures of leaf tissue in the absence of INA bacteria. Results include: −4°C and −5°C for growth-chamber-grown tomato and soybean plants, respectively (1); −6.9 to −8.1°C for various *Solanum* species (20); −6.0°C for corn (10); and −8 to −9°C for pumpkin, barley, and tomato (13). Thus, evidence is growing that in the absence of INA bacteria, nucleation events on or in leaves occur very infrequently at temperatures greater than approximately −5°C. Pathovars of *P. syringae* are known to be seed borne in many plant species, including tomato, oats, and soybeans. Taken together with the difficulty of detecting very low populations of bacteria (*e.g.* one to ten) associated with a leaf, it is difficult to

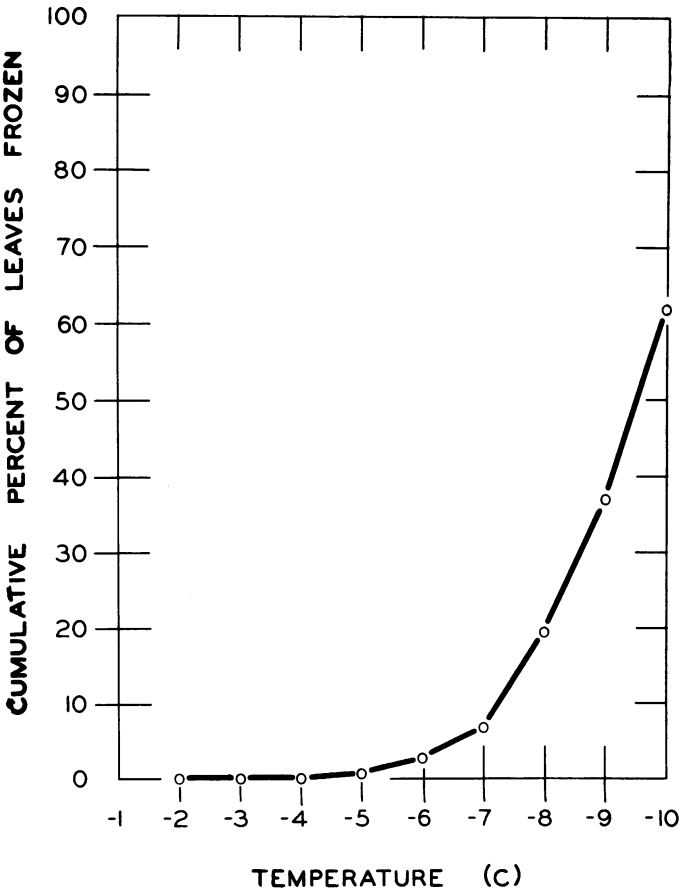

FIG. 1. Ice nucleation spectrum of oat leaves with no detectable INA bacteria. A total of 400 individual leaves from 23-d-old oat plants grown under controlled conditions were assayed by the tube nucleation test in the temperature range of −2.0°C to −10°C. The leaves were maintained at each test temperature for 1 h. The cumulative percentage of leaves that froze is plotted as a function of decreasing temperature.

be entirely sure that all of these ice nuclei are intrinsic to the leaves. However, ice nuclei intrinsic to leaves appear to be active at or below about −5°C. Woody parts of some trees in the genus *Prunus* may have ice nuclei of unknown origin active at −2°C (2, 19). These ice nuclei do not seem to be present in the leaves of the herbaceous plant species examined.

Nucleation Temperature of Leaves That Harbor INA Bacteria. Ninety individual oat leaves (cv Mackinaw) that appeared to be uniform with respect to size, age, and position in the plant canopy were harvested 35 d after planting for the tube nucleation test. The leaves were tested at −2.5, −3.0, and −4.0°C. Of the 90 leaves, 17 froze by −2.5°C, 45 froze between −2.5 and −3.0°C, and 22 froze between −3.0 and −4.0°C. Thus, only approximately 7% of the leaves from the field-grown oat plants did not exhibit a nucleation temperature higher than −4.0°C. Population sizes of INA bacteria were subsequently determined for each of the 90 oat leaves by dilution plating. The results are presented in Figure 2 where INA bacterial population sizes, expressed as $\log_{10}$ CFU/leaf, are plotted on a cumulative probability scale (see Ref. 4). (A straight line on this scale is indicative of a normal distribution. Since $\log_{10}$ transformed data were plotted, these data can be said to approximate a lognormal distribution, as expected [7].)

Among the 90 leaves, population sizes of INA bacteria ranged from less than 100 CFU/leaf (*i.e.* none detected), to greater than 10 million CFU/leaf. The overall mean and variance of the lognormal distribution were 3.60 and 1.18, respectively. When the INA bacterial population sizes on this set of 90 oat leaves were grouped according to the temperature at which nucleation occurred on the associated leaf, the bacterial population frequency distribution within each temperature group also approximated a lognormal (Fig. 3). The mean and variance of INA bacterial populations for the different leaf ice nucleation temperature groups are summarized in Table III. From the cumulative probability plots (Fig. 3), the frequency with which a given INA bacterial population size is met or exceeded can be estimated. For example, for this set of 90 oat leaves, approximately 52% of the leaves that had nucleation temperatures above −2.5°C also had population sizes of INA bacteria that exceeded 10^5 CFU/leaf. In contrast, less than approximately 5% of the leaves with lower nucleation temperatures had such high populations. Thus, although INA bacterial populations may vary over 1,000-fold on leaves with ice nucleation temperatures within the same range, the probability of occurrence of a large INA bacterial population (*e.g.* $>10^5$ CFU/leaf) is much higher on leaves with warmer nucleation temperatures. Alternatively, the frequency of high INA bacterial populations on individual leaves appears to be proportional to the fraction of leaves with relatively high nuclea-

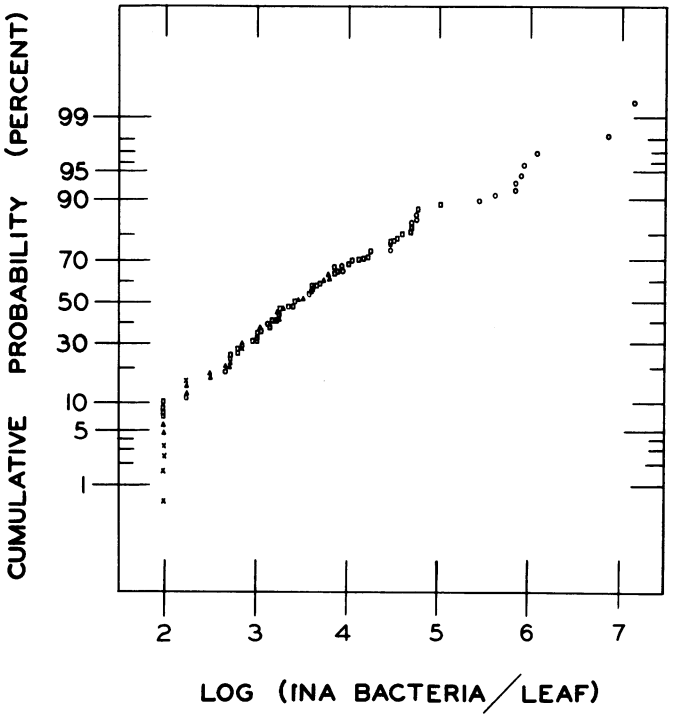

FIG. 2. Cumulative probability plot of INA bacterial populations on each of 90 individual oat leaves. Ice nucleation temperature (NT) for each leaf was determined by the tube nucleation test. Corresponding INA bacterial population size was determined for each thawed leaf by washing, dilution plating, and replica freezing and is expressed as $\log_{10}$ (INA bacteria per leaf). NT ≥ −2.5°C (○); −2.5°C > NT ≥ −3.0°C (□); −3.0°C > NT ≥ −4.0°C (△); and −4.0°C > NT (×).

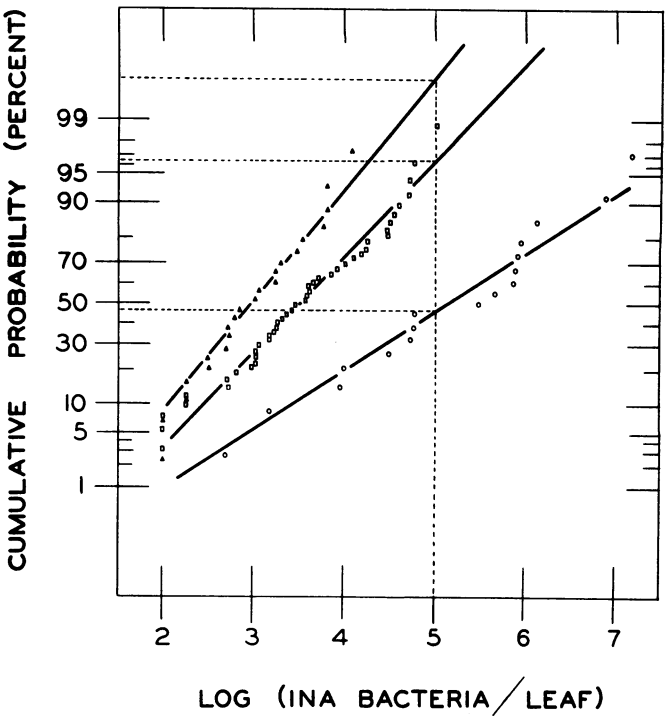

FIG. 3. Cumulative probability plots of populations of INA bacteria per oat leaf grouped according to their respective leaf ice nucleation temperature (NT). Groups are: populations of INA bacteria on leaves with NT ≥ −2.5°C (○); −2.5°C > NT ≥ −3.0°C (□); and −3.0°C > NT ≥ −4.0°C (△).

Table III. *Means and Variances of INA Bacterial Populations in Relation to the Temperature at Which Nucleation Occurred on Individual Oat Leaves*

Nucleation Temperature (NT)	No. of Leaves	Mean	Variance
		$\log_{10}$ CFU/leaf	
NT ≥ −2.5°C	17 (0)[a]	5.14	1.52
−2.5°C > NT ≥ −3.0°C	45 (4)	3.51	0.69
−3.0°C > NT ≥ −4.0°C	22 (2)	2.99	0.37
−4.0°C > NT	6 (4)	2.09	—[b]
Overall	90 (10)	3.60	1.39

[a] The numbers in parentheses denote the number of leaves of the total within each group with no detectable INA bacteria. These leaves were assigned a value of $\log_{10}$ (CFU/leaf) = 2.0 (our limit of sensitivity for the dilution plating assay) in calculations of the sample statistics.

[b] Variance not calculated due to censoring.

tion temperatures.

Regression analysis of nucleation temperature on $\log_{10}$ INA bacterial population for the 84 oat leaves that froze by −4.0°C (from Fig. 3) was highly significant ($r = 0.551$; $P < 0.001$, $df = 82$; $y = -4.1 + 0.25x$). The regression equation estimates the ice nucleation temperature (y) of a leaf given $\log_{10}$ INA bacterial population (x). If $x = 0$ (*i.e.* if the population averages one INA bacterium per leaf), $y = -4.1$°C. Thus, if a nucleation event occurs on a leaf at a temperature above approximately −4°C, viable INA bacteria can be expected to be present on that leaf. For a nucleation event to occur on a leaf at temperatures below −4 to −5°C, the presence of INA bacteria does not appear to be necessary. Taken together with the ice nuclei intrinsic to plants at temperatures below −5°C discussed above, the implication with regard to frost protection, is that under optimal conditions a reduction of INA bacterial populations on leaf surfaces to very low (*i.e.* undetectable) levels may allow approximately 2 to 3 degrees of protection (*i.e.* from −2 to −4 or −5°C) on plant species that have intrinsic nuclei active at about −5°C.

Distribution of Ice Nucleation Frequencies among INA Bacteria on Oat Leaves and in Culture. Bacterial ice nucleation frequency at a given temperature is the ratio between the number of ice nuclei and the number of bacterial cells (13). We can begin to estimate nucleation frequencies of INA bacteria on individual leaves from the data obtained by the sequential testing for leaf nucleation temperature and INA bacterial population size on the same set of leaves. In the tube nucleation test, what we actually detect is a nucleation event. If a nucleation event occurs at a given temperature on a given leaf, we know that at least one ice nucleus that was sufficiently efficient to be active at the test temperature was associated with that leaf. More ice nuclei may be present that might be active at the test temperature. We can only detect the presence of at least one. Thus, the number of ice nuclei present on a given leaf may be underestimated. The narrower the temperature range between test temperatures, the more likely it will be that a single nucleation event will represent the effect of a single ice nucleus. If we assume that each nucleation event represents only one ice nucleus, then the nucleation frequency on each individual leaf will be represented by the reciprocal of the number of INA bacteria present on that leaf.

Since INA bacterial populations followed a lognormal distribution among individual oat leaves within a test temperature range (*cf.* Fig. 3), bacterial nucleation frequencies among these individual leaves can also be said to be lognormally distributed. From Table III, a mean nucleation frequency for each test temperature can be estimated from the reciprocal of the untransformed mean INA bacterial populations. For example, the mean log nucleation frequency at −2.5°C was −5.14 (or 7.24×10^{-6} ice nuclei/cell) for INA bacteria on oat leaves. Thus, on the average at least one INA bacterium was active as an ice nucleus at −2.5°C in a population of approximately 10^5 INA bacteria. By −3.0°C only an average of 10^3 cells were necessary for the occurrence of a nucleation event. These nucleation frequency values for INA bacteria on oat leaves are mean values. In fact, it is evident from Figure 3 that for a given temperature (*i.e.* −2.5°C) nucleation frequencies can vary from 10^{-3} to 10^{-7} on individual leaves. The underlying reasons for the variability in INA bacterial population size and in nucleation frequency on individual leaves have not been determined. If nutrient availability and/or microclimate differ among leaves, then this could affect both bacterial population size and nucleation frequency. The INA bacterial populations on individual field-grown leaves probably contain highly variable genotypes that may differ in nucleation frequencies. Thus, different leaves may harbor INA bacterial populations with very different ice nucleation frequencies.

Nucleation frequency can vary greatly among different INA strains cultured under the same set of laboratory conditions (Fig. 4). The strains tested were grouped into three categories. (a) One hundred seventy-eight strains of *P. syringae* from the National Collection of Plant Pathogenic Bacteria (NCPPB), Harpenden, England. These strains were isolated as pathogens from many different hosts and geographic locations (*cf.* 6). (b) One hundred fifty-nine putative strains of *P. syringae* isolated as epiphytes from nondiseased leaves of various plants in Wisconsin. (c) Seventy-seven putative strains of *E. herbicola* also from various plant species in Wisconsin. All strains were tested for ice nuclea-

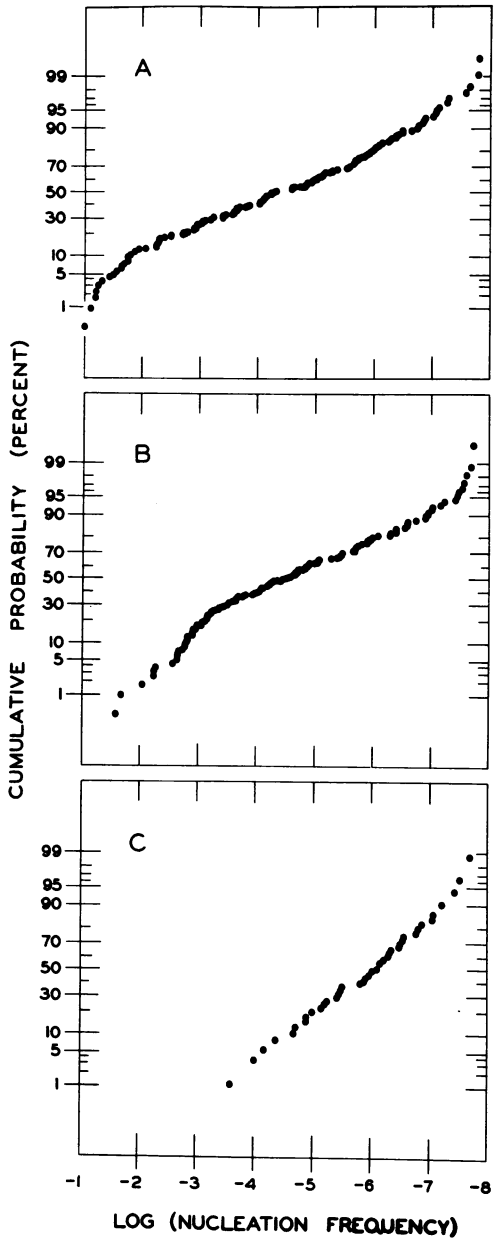

FIG. 4. Lognormal distributions of nucleation frequencies for each of three groups of INA bacteria. A, 154 strains of phytopathogenic *P. syringae* from the National Collection of Plant Pathogenic Bacteria, Harpenden, England. B, 144 strains of putative *P. syringae* isolated from nondiseased leaf surfaces of several plant species in Wisconsin. C, 42 putative strains of *E. herbicola* isolated from several plant species in Wisconsin. All strains were grown for 2 d on NGA at 24°C. Nucleation frequency for each strain was calculated from the concentration of ice nuclei active at −5°C as determined by a droplet freezing assay and the density (CFU/ml) of the test bacterial suspension as determined by dilution plating.

tion activity at −5°C and −10°C to measure ice nuclei active in regions I (−2 to −4°C) and II (−7.5 to −9°C) (13). The percentages of strains within each group that were active at −10°C but not at −5°C were 13.5% and 10.1% for the pathogenic and epiphytic collections of *P. syringae*, respectively, and 45.4% for *E. herbicola*.

It is interesting to note that analogous to the distribution of nucleation frequencies of INA bacteria on individual oat leaves, nucleation frequencies of different strains of *P. syringae* and *E. herbicola* also could be described by the lognormal distribution. The distribution of nucleation frequencies of each of the two *P. syringae* collections were judged lognormal by the Kolmogorov-Smirnov D statistic (22) [NCPPB collection: D = 0.066, P > D = 0.099; Wisconsin Collection: D = 0.087, P > D = <0.01]. The distribution of nucleation frequencies of the *E. herbicola* collection was lognormal by the Wilk-Shapiro W statistic (21) [W = 0.974, P < W = 0.570]. The mean $\log_{10}$ NF values of the two large sets of *P. syringae* were similar (−4.37 for the NCPPB collection *versus* −4.63 for the Wisconsin collection) as were the variances of the distributions (1.75 for NCPPB *versus* 1.56 for Wisconsin). A median $\log_{10}$ nucleation frequency of −4.0 at −5.0°C was reported for 82 *P. syringae* strains isolated from fruit tree orchards in Washington and Oregon (5). The similarity of the distributions of nucleation frequencies of these large sets of *P. syringae* collected in very different ways from different locations is remarkable. Perhaps a large, random sample of −5°C ice nucleating *P. syringae* from any source can be expected to exhibit a mean log NF of about −4.5.

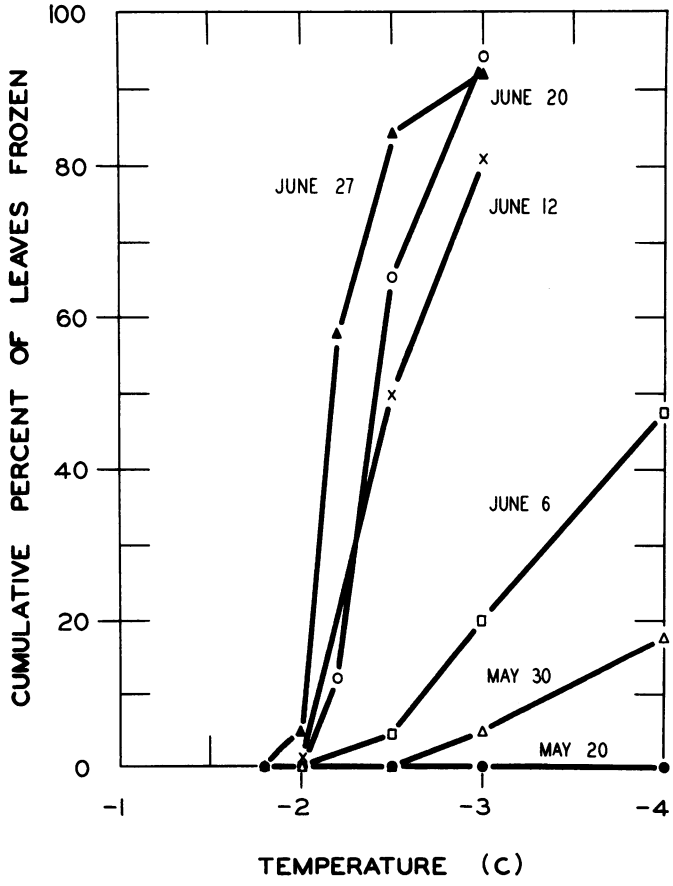

FIG. 5. Seasonal trend of INA bacterial populations on oat leaves estimated by the tube nucleation test at several temperatures. Thirty leaves from each of three replicate plots (4.5 × 6 m) were harvested at each sampling date. Data are expressed as the cumulative percentage of 90 total leaves which froze at the test temperature.

Table IV. *Relationship between the Tube Nucleation Test and Frost Injury to Tomato under Field Conditions (October 1979)*

Treatment	Frost Injury[a]	Tube Nucleation Test (Percentage of Leaflets Frozen[b])		
		−3.0°C	−3.5°C	−4.0°C
			%	
Control	65	22.2	40.0	60.0
Maneb (M)	41	15.6	28.9	31.1
Kocide (K)	37	6.7	13.3	31.1
M + K	34	4.4	8.9	24.4
M + S	32	6.7	11.1	33.3
M + K + S	31	6.7	13.3	33.3
Streptomycin (S)	23	4.4	17.8	26.7
r[c]		0.922	0.827	0.904
P		<0.01	<0.05	<0.01

[a] Expressed as percentage of leaves injured out of a total of approximately 50 to 60 leaves per treatment. Leaves with any visible water soaking were rated as frost injured.

[b] Total of 45 leaflets tested per treatment.

[c] Correlation coefficient for frost injury on results from the tube nucleation test.

Strains of *E. herbicola* were less efficient as ice nuclei than strains of *P. syringae*. This is in agreement with results of Lindow *et al.* (11, 13, 14). Almost half of the *E. herbicola* strains tested were active at −10°C but not at −5°C. Among those *E. herbicola* strains that exhibited ice nucleation activity at −5°C, the mean $\log_{10}$ NF was −5.93 with a variance of 1.00.

Application of the Tube Nucleation Test to Estimating the Frequency with Which High Populations of INA Bacteria Occur in a Given Plant Canopy. If leaf ice nucleation temperatures above −4.0°C are a function of population levels of INA bacteria, then the tube nucleation test could be used as a rapid means of estimating the frequency with which high INA bacterial populations occur on individual leaves. Thus, the tube nucleation test was used to monitor the frequency with which leaves with high populations of INA bacteria occurred on oat leaves throughout the 1980 growing season (Fig. 5). At each of six sampling dates, 90 leaves of approximate equivalent age and position in the canopy were selected at random and assayed by the tube nucleation test at each of four temperatures. At the first sampling date, May 20, 25 d after planting (DAP), none of the 90 leaves had ice nucleation temperatures higher than −4.0°C. As the season progressed, the frequency with which individual oat leaves froze at each of the test temperatures increased. By June 27, 63 DAP, 85% of the leaves had nucleation temperatures equal to or higher than −2.5°C. The increased frequency with which oat leaves froze at the test temperatures as the season progressed, can be taken as an indication of an increase in numbers and hence growth of INA bacteria. This is in agreement with studies of Lindow *et al.* (11) wherein INA population levels on annual plants such as corn, snap beans, soybeans, pumpkins, and tomatoes increased substantially as the season progressed.

The tube nucleation test is less precise than the dilution-plating procedure in quantitating the frequency of high epiphytic INA bacterial populations. However, it has the distinct advantage of being a rapid means of obtaining estimates of such frequencies on a large number of individual leaves. A sample size of over 1,000 individual leaves can be tested on a given day, and the results are available in a few hours. These features of the tube nucleation test should make it extremely useful in frost hazard assessments.

Application of the Tube Nucleation Test to Predicting the Frost Hazard to Sensitive Plants. The tube nucleation test was used to assess the frost hazard to tomato plants in field plots in

1979. On October 2 and 3, 1979, leaflets from tomato plants in field plots that had been treated with various chemicals to alter populations of INA bacteria were assayed by the tube nucleation test at −2.5, −3.0, −3.5, and −4.0°C. The frequencies with which leaflets froze at each of the test temperatures for each of seven treatments are presented in Table IV. Nucleation events at −2.5°C occurred on less than 5% of the leaflets per treatment. Apparently, relatively low levels of INA bacteria were present on those leaflets. The frequency with which leaflets froze increased with decreasing test temperature. On October 5, 1979, a light radiation frost occurred. The correlations between percentage of leaflets frozen at each of the assay temperatures and percentage of leaves injured under the natural frost, 3 d later, were significant for −3, −3.5, and −4.0°C. Thus, the tube nucleation test may be useful in assessing the frost hazard to sensitive crops.

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