Bacterial Ice Nucleation as a Predictor of Bacterial Brown Spot Disease on Snap Beans

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ABSTRACT

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Snap bean plants that harbored different population sizes of *Pseudomonas syringae* pv. *syringae* were established in field plots by several combinations of seed and foliage treatments. The tube nucleation test was used to determine ice nucleation temperatures of individual bean leaflets within each of the plots at 3- or 4-day intervals throughout each of two growing seasons. The frequency with which bean leaflets harbored large population sizes of ice nucleation active *P. s.* pv. *syringae* was estimated by the frequency with which ice nucleation events occurred between −2.0 and −2.5 C. Incidence of brown spot was assessed every 3 to 4 days on the same sets of field plots. The proportion of leaflets frozen at −2.0 and −2.5 C was significantly correlated ($p < 0.0001$) with the incidence of brown spot assessed 3–8 days later. The frequency with which ice nucleation events occur on individual bean leaflets at relatively high temperatures appears to be a sufficiently reliable predictor of large pathogen populations and, therefore, of disease. The tube nucleation test shows potential for use in disease management schemes for foliar blights caused by ice nucleation active pathovars of *P. syringae*.

Although the fact that plant leaf surfaces are colonized by epiphytic phytopathogenic bacteria has been recognized for the past 25 yr (1,7,12), a quantitative relationship between pathogen population size and subsequent disease incidence has been found only recently (14,19). Lindemann et al (14) demonstrated that, although mean pathogen population size was not a reliable predictor of bacterial brown spot on snap beans (*Phaseolus vulgaris* L.) caused by *Pseudomonas syringae* Van Hall pv. *syringae*, the frequency with which populations of *P. s.* pv. *syringae* exceeded approximately 10^4 colony-forming units (cfu) on individual symptomless bean leaflets was a good predictor of brown spot incidence 1 wk after flowering.

Rouse et al (19) have developed a stochastic model that relates pathogen population size on individual leaves within a plant canopy to subsequent disease incidence. In this model, the distribution of pathogen population sizes in a canopy was coupled with the conditional probability of disease given pathogen population size. Application of the model to the *P. s.* pv. *syringae*-brown spot disease system yielded an ED_{50} value of approximately 10^5 cfu per bean leaflet under field conditions.

In the studies of Lindemann et al (14) and Rouse et al (19), population sizes of *P. s.* pv. *syringae* were estimated by dilution plating of washings of a large number of individual bean leaflets. Although dilution plating provides relatively precise estimates of population sizes of *P. s* pv. *syringae* on individual leaflets, it is not a rapid procedure. A 3- to 4-day period is required between leaf harvest and enumeration of bacterial colonies. Moreover, dilution plating is time-consuming and places a physical constraint on the number of individual leaf samples that can be processed in a given day. Although the model developed by Rouse et al (19) is conceptually important because it assigns proper epidemiological weight to the epiphytic populations of the pathogen, dilution plating limits its practical application. For pest management purposes, it would be desirable to have an assay for bacterial pathogen population sizes that is reliable, fast, and easy to perform.

P. syringae is active as an ice nucleus at relatively high temperatures (16,18). Maki et al (18) and Lindow et al (16) demonstrated that for a given ice-nucleation active (INA) bacterial strain, not every cell is active as an ice nucleus at any given time and/or temperature. The nucleation frequency (i.e., the ratio of ice nuclei to viable cells) of many strains of *P. syringae* increases rapidly as the temperature decreases between about −2 to −3.5 C (16–18). We have examined the quantitative relationship between the temperature at which ice nucleation is likely to occur on an individual leaf or leaflet and the population size of INA bacteria present on that leaf (3). The tube nucleation test (3,9) and dilution plating were used to estimate ice nucleation temperatures of leaves and INA bacterial population sizes, respectively. For oat leaves, mean $\log_{10}$ populations of INA bacteria per leaf were 5.14 for leaves with ice nucleation temperatures between −2.0 and −2.5 C and 3.51 for nucleation temperatures between −2.5 and −3.0 C. The frequency with which leaves froze at −2.5 C, as measured with the tube nucleation test, was used to estimate the frequency with which large populations of *P. syringae* occurred on individual oat leaves throughout the growing season (3). The tube nucleation test has also been used to assess the frost hazard to sensitive plants (3).

In this paper, we discuss the use of the tube nucleation test to estimate the frequency with which large populations of *P. s.* pv. *syringae* occur on individual bean leaflets and the quantitative relationship between this frequency and subsequent incidence of bacterial brown spot disease.

MATERIALS AND METHODS

Experimental design. In 1981 and 1982, experimental plots of snap bean were established at the University of Wisconsin Experimental Farms, Arlington, WI. The cultivar Eagle (Asgrow Seed Co., Kalamazoo, MI) was planted on 27 June in 1981 and on 17 July in 1982. In both years, a set of nine treatments was established in a randomized complete block design, with three blocks (i.e., 3 replicate plots per treatment). Each plot consisted of

eight rows spaced 76 cm apart. The plot length was 7.6 m in 1981 and 6.1 m in 1982.

The treatments were the nine possible combinations of three seed treatments and three foliage treatments (Table 1). In each year, a single seed lot was used. The seed treatments included: no treatment, seeds inoculated with the pathogen at the time of planting by incorporation of dry, ground brown-spot diseased bean leaves in the seed hopper (2), and seeds soaked in a 100-mg/L streptomycin sulfate (Agri-Strep, 21.2% A.I., Merck & Co., Inc., Rahway, NJ) solution for 20 min, then air dried before planting. All seeds had had prior commercial treatments of captan 75 and streptomycin. Foliage treatments included: no treatment, weekly spray applications of copper hydroxide (either Kocide 606 at 4.67 L/ha or Kocide 101 at 2.24 kg/ha [Kocide Chemical Co., Houston, TX]), and spray application of copper hydroxide when the number of bean leaflets frozen by −2.5 C was equivalent to or greater than a specified threshold. In 1981, the threshold was five or more leaflets frozen out of 90 (about 5%). In 1982, the threshold was 13 or more leaflets frozen out of 90 total (about 15%).

Sampling procedure. Bean leaflets for estimation of *P. s.* pv. *syringae* population sizes with the tube nucleation test were harvested every 3 or 4 days (i.e., each Monday and Thursday) for 7 wk starting approximately 15 days after planting. Sampling was always conducted between 0730 and 0830 hours. Thirty individual leaflets of similar size were taken at random from the top of the canopy of each plot per sampling time. Each set of 30 leaflets was placed in a No. 5 Kraft paper bag. The samples were kept in an ice chest until they could be assayed with the tube nucleation test.

Determination of the ice nucleation temperature of individual bean leaflets. The ice nucleation temperature of individual bean leaflets was estimated with the tube nucleation test, which has been described in detail elsewhere (3,9). Each leaflet was totally immersed in 9 ml of sterile, ice nucleus free, potassium phosphate buffer (0.01 M, pH 7.0) in a 16-× 150-mm test tube. The test tubes with leaf samples were placed in a refrigerated constant temperature bath (Neslab Instruments, Inc., Portsmouth, NH) maintained at −2.0 C. After 30 min, the tubes in which the buffer had frozen were removed and counted. The remaining tubes were gently transferred to a bath maintained at −2.5 C. The process was repeated for each of three test temperatures (−2.0, −2.5, and −3.0 C). Temperature within each bath was maintained within ± 0.05 C of the target temperature.

The data are expressed as cumulative percent leaflets frozen at each of the test temperatures. The cumulative number of leaflets frozen at −2.5 C is equal to the number of leaflets frozen during the 30 min at −2.0 C plus the number frozen during 30 min at −2.5 C. The cumulative number of leaflets frozen by −2.5 C divided by the total number of leaflets yields the cumulative percent leaflets frozen at −2.5 C.

For selected sampling times, 30 tubes of the 90 allocated per treatment were randomly marked before the leaflets were placed in them. The temperature at which ice nucleation occurred for these leaflets was recorded. Marked tubes with leaflets that did not freeze by −3.0 C were placed in a bath maintained at −10 C to initiate freezing. The selected leaflets were stored frozen at −20 C until they could be processed by dilution plating for more precise estimates of *P. s.* pv. *syringae* population sizes.

Determination of *P. s.* pv. *syringae* population sizes on individual bean leaflets. Frozen leaflets were allowed to thaw at room temperature (about 24 C) and the contents of each tube (e.g., one bean leaflet and 9 ml of buffer) were transferred to a sterile 125-ml Erlenmeyer flask. The test tube was rinsed with 10 ml of sterile potassium phosphate buffer (0.1 M, pH 7.0) supplemented with Bacto peptone (1% w/v). The rinse was added to the flask for a total of 19 ml of washing buffer. Each leaflet was cut into approximately 1- to 2-cm^2 segments. The leaf segments were washed for 2 hr 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 (11) supplemented with cycloheximide (100 mg/L) to inhibit growth of fungi. Fluorescent, oxidase negative colonies (i.e., putative *P. s.* pv. *syringae*) were counted after 3 days of incubation at ambient temperature (about 24 C). The replica freezing procedure of Lindow et al (15) was used to estimate the population sizes of INA bacteria on bean leaflets. Bacterial population sizes are expressed as $\log_{10}$ (colony-forming units per leaflet).

Over a thousand fluorescent, oxidase negative putative *P. s.* pv. *syringae* strains were isolated from dilution plates that were representative of leaflets sampled throughout the growing season. The isolates were tested for pathogenicity to bean with the pod inoculation assay described by Lindemann et al (13). Pods from growth chamber grown bean plants (cultivar Eagle) were used for the test.

Disease assessment. Brown spot disease incidence on all leaflets of randomly selected plants within each plot was assessed every 3 or 4 days throughout the growing season in 1981 and 1982. The number of plants that were rated per plot depended on the number of leaves present per plant at that sampling time. On the average, a total of approximately 100–150 leaflets, representing four to eight plants, were scored per plot. Disease incidence is expressed as percent leaflets diseased.

Data analyses. The relationship between the frequency with which individual bean leaflets bore "large" populations of *P. s.* pv. *syringae* as estimated by the frequency with which they bore bacterial ice nuclei and disease incidence was examined by linear regression analyses. Brown spot disease incidence for each of the 27 plots (i.e., nine treatments × three plots per treatment) measured at a given time was regressed on the percent leaflets frozen at −2.0, −2.5, or −3.0 C, determined 4–8 days before the disease assessments. Regression analyses were also performed on the arc sine-square root transformed values of proportion of leaflets diseased and frozen. Because population sizes of *P. s.* pv. *syringae* and disease incidence were monitored at frequent intervals throughout the growing season, disease incidence values could be regressed on the frequency with which leaflets froze at each of the three test temperatures determined either 3 or 4 days or 7 or 8 days before the time when disease measurements were made.

RESULTS AND DISCUSSION

Relationship between population size of *P. s.* pv. *syringae* and ice nucleation temperature of individual bean leaflets. Replica freezing of dilution plates from washings of approximately 2,000 individual bean leaflets harvested throughout the growing season in 1981 indicated that *P. s.* pv. *syringae* was the predominant ice nucleation active bacterium present on bean leaflets in our experimental plots. Of a total of 1,076 fluorescent, oxidase negative putative *P. s.* pv. *syringae* strains isolated from dilution

TABLE 1. Treatments applied to bean seed and/or foliage to establish different leaf-associated population sizes of *Pseudomonas syringae* pv. *syringae*

Seed	Foliage
Untreated	Untreated
Untreated	Copper hydroxide (Kocide) weekly application[a]
Untreated	Copper hydroxide (Kocide) freezing trigger[b]
Pathogen inoculated[c]	Untreated
Pathogen inoculated	Copper hydroxide weekly application
Pathogen inoculated	Copper hydroxide freezing trigger
Streptomycin treated[d]	Untreated
Streptomycin treated	Copper hydroxide weekly application
Streptomycin treated	Copper hydroxide freezing trigger

[a] Either Kocide 101 at 2.24 kg/ha or Kocide 606 at 4.67 L/ha was applied to the foliage at weekly intervals from plant emergence to harvest.
[b] Either Kocide 101 at 2.24 kg/ha or Kocide 606 at 4.67 L/ha was applied to the foliage when the percent leaflets frozen at −2.5 C exceeded 5% of 90 leaflets (i.e., 30 leaflets per plot × three plots per freezing trigger treatment) in 1981 and 15% in 1982.
[c] Seeds were mixed with a powder of ground-up brown spot diseased-bean leaflets at the time of planting.
[d] Seeds were soaked in a streptomycin solution (100 mg/L) for 20 min then air dried before planting.

plates of bean leaf washings, 98.8% of the strains were active as ice nuclei at −5 C, whereas 97.6% produced brown spot symptoms in a pod inoculation assay. All *P. s.* pv. *syringae* strains were ice nucleation active. However, not all of the INA *P. syringae* strains were pathogenic to beans.

TABLE 2. Means and variances of *Pseudomonas syringae* pv. *syringae* population sizes in relation to the temperature at which ice nucleation occurred on individual bean leaflets

DAP[a]	Nucleation temperature (NT)	N[b]	C[b]	Mean[c] (log_{10}cfu/leaflet)	Variance[c] (log_{10}cfu/leaflet)
23	NT ≥ −2.0 C	142	15	5.53	2.87
	−2.0 C > NT ≥ −2.5 C	99	42	2.95	5.64
	−2.5 C > NT ≥ −3.0 C	21	15	1.31	3.56
44	NT ≥ −2.0 C	41	0	5.50	0.97
	−2.0 C > NT ≥ −2.5 C	163	55	3.17	3.17
	−2.5 C > NT ≥ −3.0 C	0			
	−3.0 C > NT	50	43	0.51	2.78

[a] DAP = Days after planting.
[b] N = Total number of observations; C = number of censored observations (i.e., leaflets on which *P. s.* pv. *syringae* was not detected at a limit of detection of 2.2 as log_{10} [colony-forming units per leaflet]).
[c] For data sets with censored observations, means and variances were estimated according to the maximum likelihood procedure of Rouse et al (19).

Population sizes of *P. s.* pv. *syringae* in relation to the temperature at which ice nucleation occurred on individual bean leaflets are presented in Table 2. For leaflets sampled at 23 and 44 days after planting, the mean *P. s.* pv. *syringae* population size was 300-fold higher on leaflets with ice nucleation temperatures ≥ −2.0 C as compared with leaflets with nucleation temperatures between −2.0 and −2.5 C. The median *P. s.* pv. *syringae* population on leaflets that did not freeze by −3.0 C was less than about 10 cfu per leaflet. Thus, within a range of 1 C, the tube nucleation test separated groups of leaflets on which the median *P. s.* pv. *syringae* population sizes differed by more than five orders of magnitude. Relatively small shifts in nucleation temperature can be related to relatively large changes in INA *P. s.* pv. *syringae* bacterial population sizes.

To compare pathogen population sizes, as estimated by the tube nucleation test, to disease, it was necessary to establish plots in which pathogen population sizes varied. Different population sizes of *P. s.* pv. *syringae* were successfully established on bean leaflets by the various seed and foliage treatments. For example, at 23 days after planting, population sizes of *P. s.* pv. *syringae* ranged from 1.72 to 6.76 log (colony-forming units per leaflet) (Table 3). In addition, there was a positive association between *P. s.* pv. *syringae* population size and the frequency with which individual bean leaflets froze by either −2.0 or −2.5 C. This relationship was observed for other sampling times as well.

TABLE 3. Cumulative percentage of leaflets frozen by −2.0 and −2.5 C and means and variances of *Pseudomonas syringae* pv. *syringae* population sizes for each of nine treatments sampled at 23 days after planting in 1981

Treatment		Cumulative % frozen[a]		*P. s.* pv. *syringae*[b]	
Seed	Foliage	−2.0 C	−2.5 C	Mean	Variance
Pathogen	Untreated	88.8	100.0	6.76	0.24
Pathogen	Copper hydroxide weekly	91.1	100.0	6.65	0.25
Pathogen	Copper hydroxide trigger	84.4	100.0	6.64	0.13
Untreated	Untreated	64.4	98.9	4.39	2.62
Untreated	Copper hydroxide weekly	53.3	97.8	4.26	2.09
Untreated	Copper hydroxide trigger	21.1	75.6	3.45	2.29
Streptomycin	Untreated	12.2	62.2	2.49	5.91
Streptomycin	Copper hydroxide weekly	14.4	72.2	1.11	5.76
Streptomycin	Copper hydroxide trigger	18.9	75.6	1.72	4.41

[a] Ninety leaflets per treatment were assayed with the tube nucleation test.
[b] For data sets with censored observations, means and variances were estimated with the maximum likelihood procedure of Rouse et al (19). The limit of detection for the dilution plating assay was 2.2 as log_{10} (colony-forming units per leaflet).

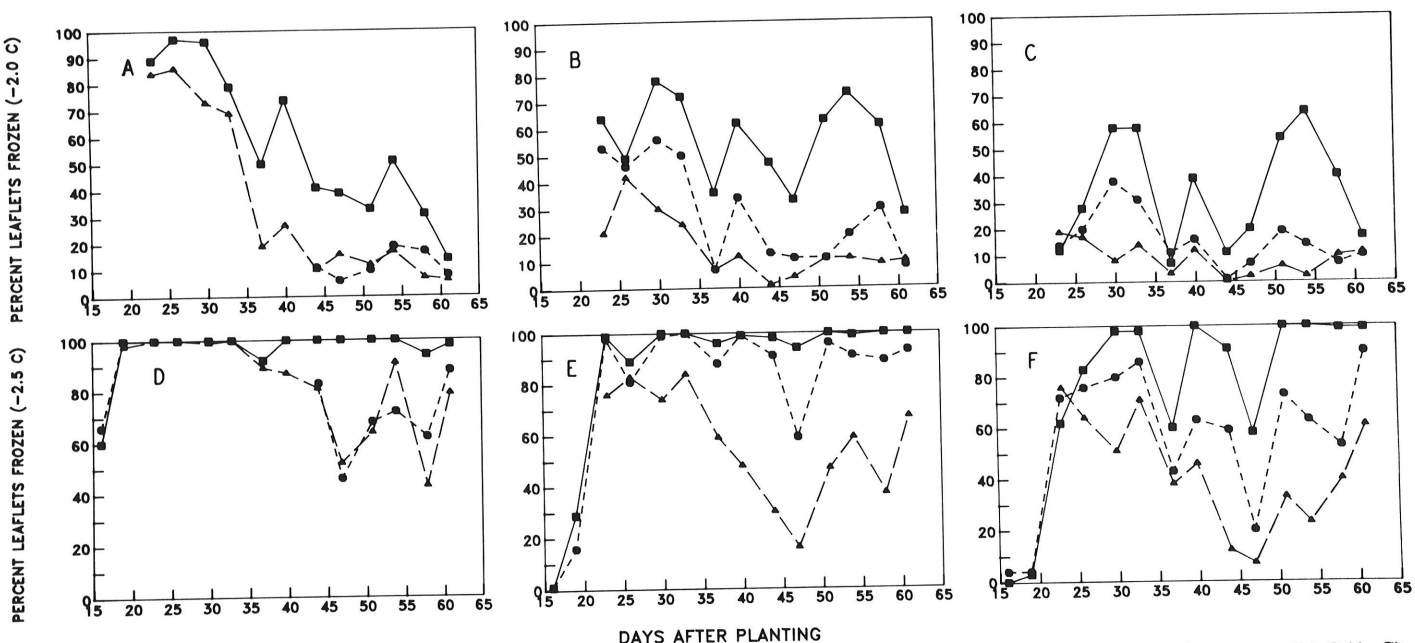

Fig. 1. 1981 seasonal trends of relative population levels of *Pseudomonas syringae* pv. *syringae* as monitored with the tube nucleation test at −2.0 C **(A–C)** and −2.5 C **(D–F).** Seed treatments were: A & D: pathogen-inoculated; B & E: untreated, and C & F: streptomycin-treated. Foliar treatments were: none (■); application of copper hydroxide at weekly intervals (•); and application of copper hydroxide when the cumulative percentage of leaflets frozen at −2.5 C was ≥ 5% of 90 total (▲).

In total, these results support the conclusion that between −2.0 and −3.0 C the temperature at which ice nucleation occurs on individual bean leaflets and the frequency with which these events occur are quantitatively related to the population size of *P. s.* pv. *syringae* present on those leaflets. The system is highly variable from leaflet to leaflet in terms of pathogen population sizes, and at this time we are not able to estimate the precise population sizes of *P. s.* pv. *syringae* associated with a given leaflet from its nucleation temperature. For populations of leaflets, however, the tube nucleation test does allow us to estimate relative population sizes of *P. s.* pv. *syringae* that are likely to be present within canopies from the proportions of leaflets that freeze at carefully chosen temperatures in this range. Estimates of actual population sizes of *P. s.* pv. *syringae* from nucleation data require consideration of other environmental factors and are beyond the scope of this paper.

Seasonal trends in population sizes of *P. s.* pv. *syringae* as monitored with the tube nucleation test. The proportions of leaflets that froze by −2.0 and −2.5 C at each sampling time for each of the nine treatments in 1981 are presented in Figure 1. Differences in pathogen population sizes were present at 23 days after planting (Table 3) and throughout most of the growing season (Fig. 1). In general, the proportion of leaflets frozen at the test temperatures was highest for plots planted with pathogen-inoculated seeds (Fig. 1A and D) and lowest for plots planted with streptomycin-treated seeds (Fig. 1C and F).

In the second growing season (1982), the proportion of leaflets frozen at −2.0 C was lower than in the first (Fig. 2). The percentages of leaflets frozen at this temperature were less than 25% for most of the treatments and at most of the sampling times. The absence of substantial rainfalls during plant emergence in 1982 as compared with 1981 may have accounted for the differences in *P. s.* pv. *syringae* population sizes in 1981 and 1982. Although overall population sizes of *P. s.* pv. *syringae* were lower in 1982 than in 1981, differences in pathogen population sizes were once again established with the various seed and foliage treatments. These differences, as monitored with the tube nucleation test, were more noticeable at −2.5 than at −2.0 C. In both years, frequencies of frozen leaflets were lower in plots where copper hydroxide was applied than in those that received no bactericide application.

Population sizes of *P. s.* pv. *syringae* can fluctuate dramatically within a 24–72-hr period in response to changes in the physical environment (6,8,10). In both 1981 and 1982 (Figs. 1 and 2), large changes in the number of leaflets that froze at the test temperatures were frequently observed from one sampling time to the next. The tube nucleation test, thus, is suitable for monitoring a bacterial pathogen that has the potential for rapid population increase in response to changes in the physical environment.

Seasonal trends in bacterial brown spot disease incidence. Disease progress curves for the nine treatments established in 1981 and 1982 are presented in Figures 3 and 4, respectively. The seed and foliage treatments resulted in different levels of brown spot disease in 1981. The effects of the seed treatments were evident early in the 1981 growing season. When grouped according to seed treatment, disease incidences (expressed as percent leaflets diseased) at 24 days after planting ranged from 61 to 73, 24 to 30, and 5 to 8% for pathogen, untreated, and streptomycin seed treatments, respectively. Differences in disease incidence due to foliage treatments became evident as the season progressed. When grouped according to foliage treatment, disease incidences at 55 days after planting ranged from 42 to 52, 13 to 35, and 9 to 27% for untreated, weekly, and twice weekly applications of copper hydroxide, respectively.

Early in the growing season, brown spot disease incidence was substantially lower in 1982 than in 1981. The large differences in disease incidence among the various seed treatments observed early in the season in 1981 were not detected in 1982. The most significant differences in disease incidence in 1982 were noted late in the season. Plots that received either weekly or twice weekly (i.e., copper hydroxide freezing trigger treatments) applications of copper hydroxide had lower levels of brown spot as compared with untreated plots.

Relationship between the frequency with which leaflets froze at −2.0 and −2.5 C and brown spot disease incidence. Examples of the quantitative relationship between results from the tube nucleation test and subsequent brown spot disease incidence are presented in Figure 5. The percentage of leaflets frozen at −2.0 C at 30 days after planting in 1981 was highly correlated with the percentage of leaflets diseased 4 (Fig. 5A) and 8 (Fig. 5B) days later. Values of r^2 were 72.6 and 68.6% ($p < 0.0001$) for disease assessed at 34 and 38 days after planting, respectively. Regression analyses of brown spot disease incidence on the results from the tube nucleation test for all other sampling times are summarized in Tables 4 and 5 for data collected in 1981 and in Table 6 for 1982. In general, highly significant coefficient of determination values (i.e., r^2) ($p < 0.0001$)

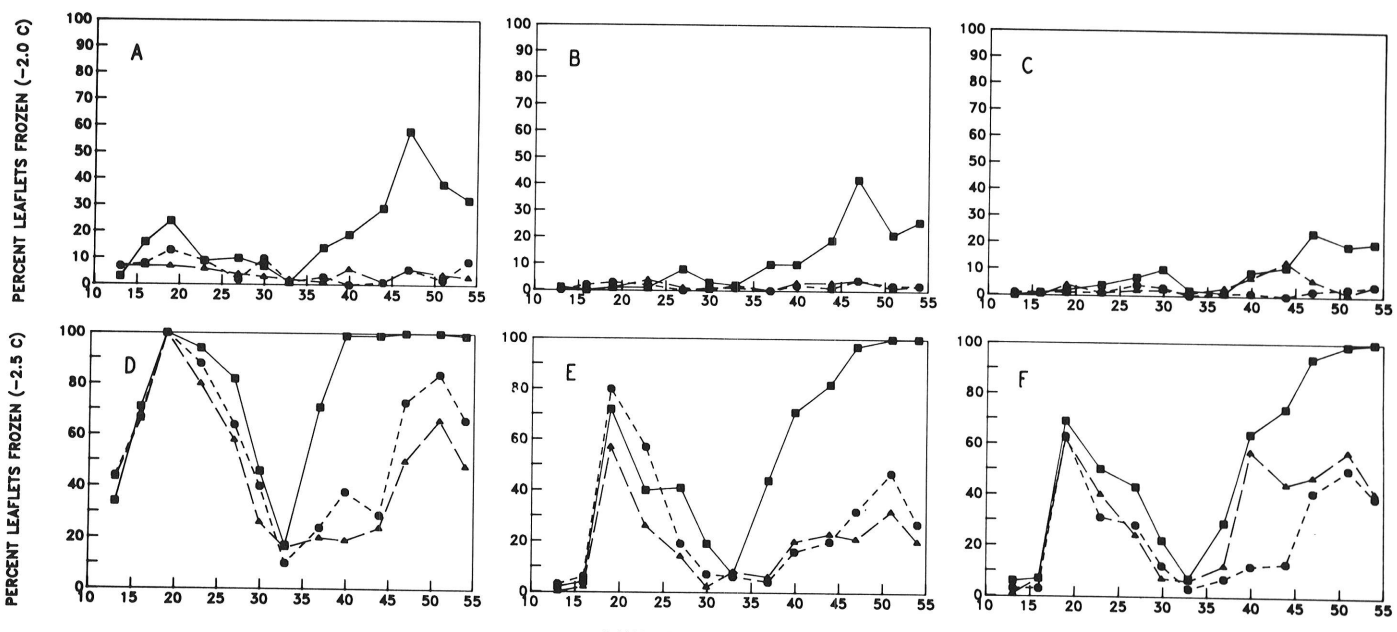

Fig. 2. 1982 seasonal trends of relative population levels of *Pseudomonas syringae* pv. *syringae* as monitored with the tube nucleation test at −2.0 C **(A–C)** and −2.5 C **(D–F).** Seed treatments were: A & D: pathogen-inoculated; B & E: untreated, and C & F: streptomycin-treated. Foliar treatments were: none (■); application of copper hydroxide at weekly intervals (●); and application of copper hydroxide when the cumulative percentage of leaflets frozen at −2.5 C was ≥ 15% of 90 total (▲).

were obtained when disease incidence was regressed on the percentage of leaflets frozen at either −2.0 or −2.5 C in both years. In 1981, regression analyses on the arc-sine transformed data did not improve the r^2 values for the regression of disease incidence on freezing frequencies measured at −2.0 C. However, at −2.5 C, r^2 values were generally higher when the data were transformed since the percentages of leaflets frozen for several treatments remained at or near 100% throughout most of the growing season (Fig. 1D–F). Although r^2 values for data collected in 1982 were lower than those obtained in 1981, the coefficients of determination were, in most cases, significant at the 0.01% level. Regression analyses on the arc-sine square root transformed data did not improve the r^2 values. The lower r^2 values for the data collected in 1982 can be attributed to the smaller differences in the number of leaflets frozen and in disease incidence among the various seed and foliage treatments (Figs. 2–5). The case in which r^2 was not significant occurred when freezing data at 34 days after planting, the end of a prolonged period of bacterial population decline, was compared to disease at 41 days after planting, immediately after a substantial flush of bacterial growth.

The data in Figure 5 and Tables 4–6 clearly demonstrate that the ice nucleation activity of *P. s.* pv. *syringae* is highly correlated with

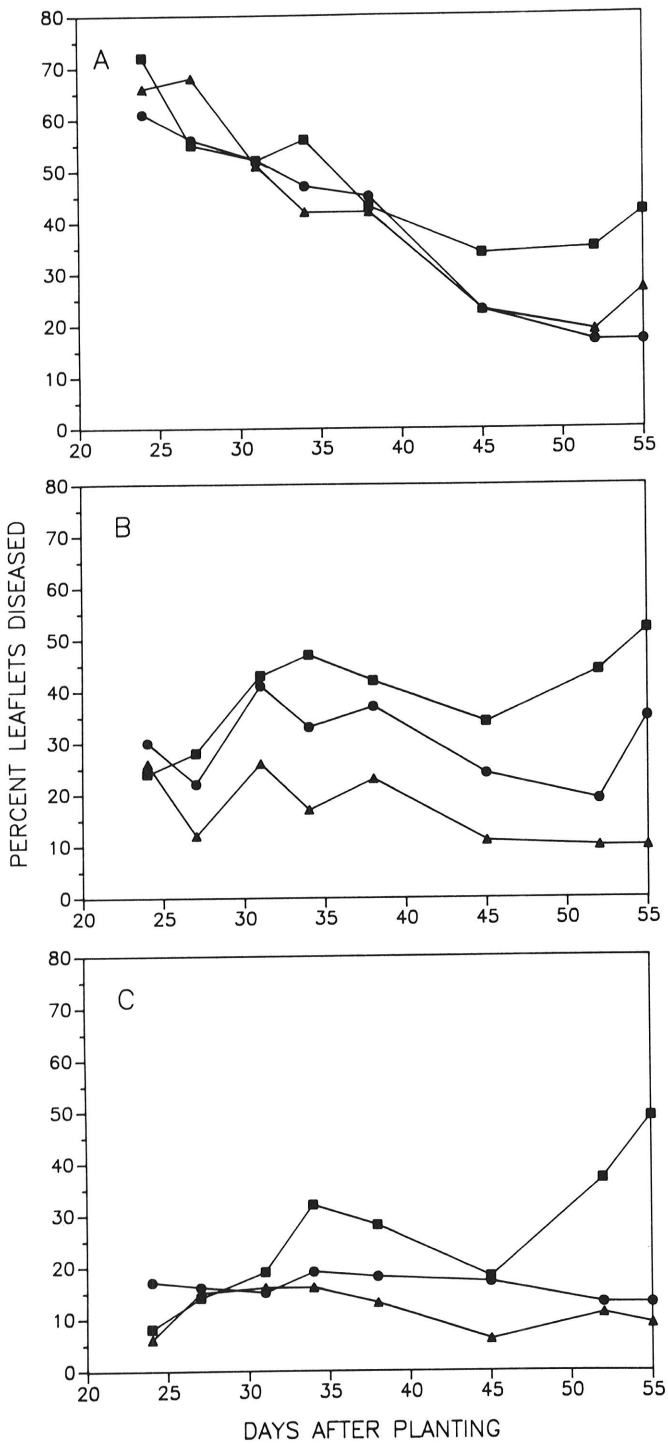

Fig. 3. 1981 seasonal trends of bacterial brown spot disease incidence. Seed treatments were: **A,** Pathogen-inoculated; **B,** Untreated, and **C,** Streptomycin-treated. Foliar treatments were: none (■); application of copper hydroxide at weekly intervals (•); and application of copper hydroxide when the cumulative percentage of leaflets frozen at −2.5 C was ≥ 5% of 90 total (▲).

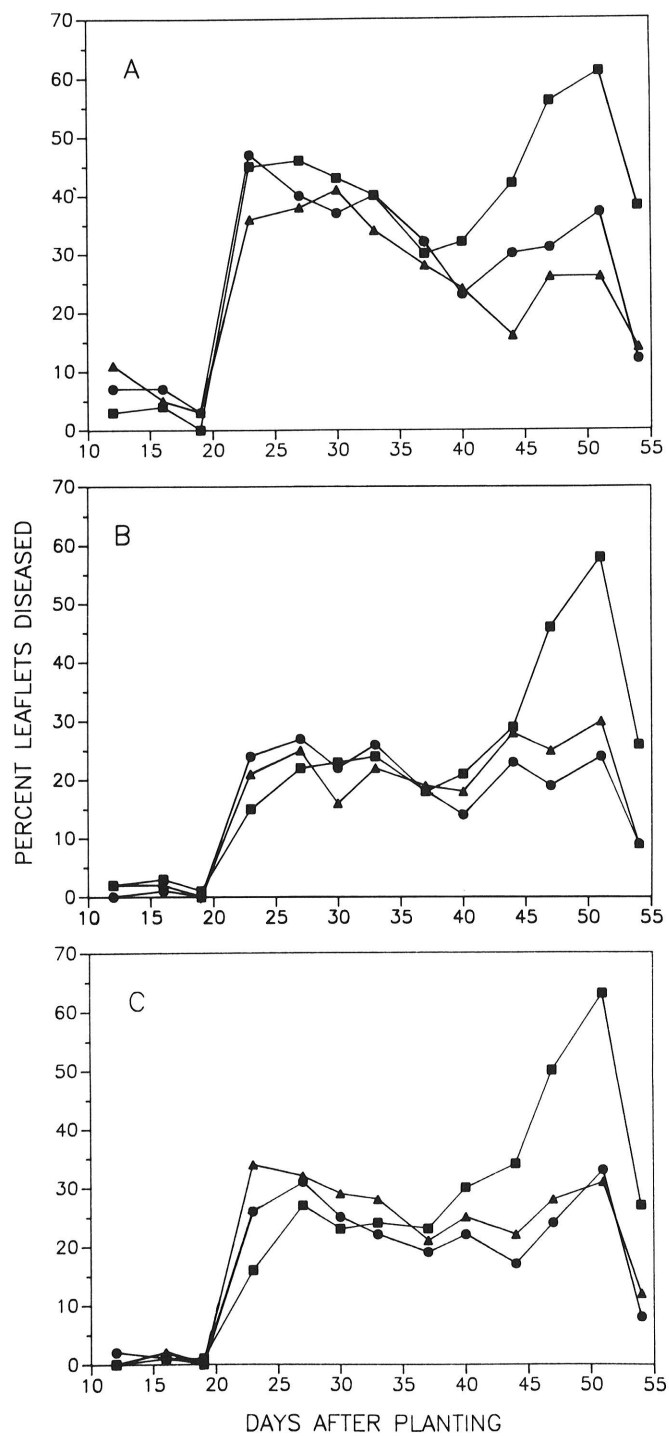

Fig. 4. 1982 seasonal trends of bacterial brown spot disease incidence. Seed treatments were: **A,** Pathogen-inoculated; **B,** Untreated, and **C,** Streptomycin-treated. Foliar treatments were: none (■); application of copper hydroxide at weekly intervals (•); and application of copper hydroxide when the cumulative percentage of leaflets frozen at −2.5 C was ≥ 15% of 90 total (▲).

brown spot disease incidence. Thus, ice nucleation is a sufficiently accurate indicator of pathogen populations to serve as a predictor of bacterial brown spot disease incidence. The relationship between results from the tube nucleation test and subsequent disease is not a deterministic one. The percentage of leaflets frozen at −2.0 or −2.5 C will not provide an estimate of the precise amount of disease that will be present a week later. It will, however, provide an estimate of the probability of disease occurring 4–8 days later. The higher the proportion of leaflets frozen, the higher the probability that disease is likely to occur.

The tube nucleation test—its utility and limitations in disease management. The tube nucleation test should be applicable, not only to bacterial brown spot of snap bean, but to other diseases caused by INA plant pathogenic bacteria (4), such as angular leaf spot on cucumbers. The test has been used successfully to predict the incidence of halo blight on oats caused by *P. s.* pv. *coronafaciens* (5; Hirano et al, *unpublished*). As a disease management tool, the tube nucleation test has several advantages: It is relatively easy to perform and should provide reliable and rapid results when used carefully. The precautions that must be taken into consideration have been discussed in detail elsewhere (3,9). Large numbers of individual leaflets (or leaves for other crops) can be tested in a given day. With the appropriate number of refrigerated baths and student helpers, we have been able to test up to 2,000 bean leaflets in a day, 3 or 4 days a week. Within several hours of leaf harvest, we can estimate the probability with which brown spot disease is likely to occur in the following week. There have been occasions when results of the tube nucleation test predicted a high probability of disease occurring, but disease incidence a week later was negligible. From other ongoing work (6,8,10), we have found that population sizes of *P. s.* pv. *syringae* can increase rapidly to levels greater than 10^5 cfu per leaflet (i.e., the ED_{50} value according to Rouse et al [19]) within a very short time period given favorable environmental conditions. However, populations can also decrease rapidly if conditions do not remain favorable to maintain the high pathogen populations. Hence, false positives can occur. However, we have not observed false negatives since these studies were begun. That is, we have not encountered the situation where low numbers of frozen leaflets were followed by subsequent high levels of brown spot disease.

TABLE 4. Regression analysis of 1981 brown spot disease incidence on the frequency with which individual bean leaflets froze at −2.0 C[a]

DAP[b]						Regression coefficients	
Pathogen population	Disease	Lag[c] (days)	r^2	df	p	Intercept (b_0)	Slope (b_1)
23	27	4	0.749	25	0.0001	0.038	0.558
23	31	8	0.781	25	0.0001	0.118	0.464
26	31	5	0.679	22	0.0001	0.100	0.476
26	34	8	0.597	22	0.0001	0.118	0.435
30	34	4	0.726	22	0.0001	0.056	0.497
30	38	8	0.686	22	0.0001	0.113	0.357
33	38	5	0.551	22	0.0001	0.122	0.372
37	45	8	0.668	22	0.0001	0.128	0.463
40	45	5	0.751	22	0.0001	0.079	0.373
44	52	8	0.545	25	0.0001	0.142	0.567
47	52	5	0.448	25	0.0001	0.127	0.637
47	55	8	0.410	25	0.0003	0.160	0.782
51	55	4	0.631	25	0.0001	0.127	0.637

[a] For each set of disease incidence data, the proportion of leaflets diseased for each plot was regressed on the corresponding proportion of leaflets frozen at −2.0 C, determined 4, 5, or 8 days before disease incidence measurements. Although there were 27 plots (nine treatments × three plots per treatment), not all of the plots were assayed with the tube nucleation test at each sampling time. Hence, the degrees of freedom (df) were not always 25.
[b] DAP = Days after planting when the tube nucleation test was performed or disease incidence was assessed.
[c] Number of days between tube nucleation test and subsequent disease assessment.

TABLE 5. Regression analysis of 1981 brown spot disease incidence on the frequency with which individual bean leaflets froze at −2.5 C[a]

DAP[b]						Regression coefficients	
Pathogen population	Disease	Lag[c] (days)	r^2	df	p	Intercept (b_0)	Slope (b_1)
16	24	8	0.701	16	0.0001	0.431	0.508
19	24	5	0.812	19	0.0001	0.343	0.388
19	27	8	0.759	19	0.0001	0.374	0.320
23	27	4	0.613	25	0.0001	−0.277	0.655
23	31	8	0.767	25	0.0001	−0.192	0.619
26	31	5	0.381	22	0.0029	0.028	0.450
26	34	8	0.366	22	0.0019	0.043	0.437
30	34	4	0.702	22	0.0001	−0.142	0.553
30	38	8	0.767	22	0.0001	0.019	0.421
33	38	5	0.794	22	0.0001	−0.177	0.538
37	45	8	0.759	22	0.0001	−0.249	0.505
40	45	5	0.680	22	0.0001	0.095	0.295
44	52	8	0.514	25	0.0001	0.187	0.269
47	52	5	0.618	25	0.0001	0.246	0.287
47	55	8	0.570	25	0.0001	0.268	0.335
51	55	4	0.692	25	0.0001	0.043	0.423

[a] For each set of disease data, the arc sine of the square root of the proportion of leaflets diseased for each plot was regressed on the arc since of the square root of the proportion of leaflets frozen at −2.5 C, determined 4, 5, or 8 days before disease incidence measurements. Although there were 27 plots, not all of the plots were assayed with the tube nucleation test at each sampling time. Hence, the degrees of freedom (df) were not always 25.
[b] DAP = Days after planting when the tube nucleation test was performed or disease incidence was assessed.
[c] Number of days between tube nucleation test and subsequent disease assessment.

TABLE 6. Regression analysis of 1982 brown spot disease incidence on the frequency with which individual bean leaflets froze at −2.5 C[a]

DAP[b]					Regression coefficients	
Pathogen population	Disease	Lag[c] (days)	r^2	p	Intercept (b_0)	Slope (b_1)
12	19	7	0.174	0.030	0.001	0.047
16	19	3	0.126	0.069	0.001	0.025
16	23	7	0.528	0.0001	0.211	0.332
19	23	4	0.175	0.030	0.135	0.206
19	27	8	0.378	0.001	0.141	0.227
23	27	4	0.580	0.0001	0.167	0.274
23	30	7	0.722	0.0001	0.101	0.338
27	30	3	0.505	0.0001	0.156	0.313
27	33	6	0.429	0.0001	0.195	0.221
30	33	3	0.427	0.0001	0.234	0.270
30	37	7	0.618	0.0001	0.180	0.247
33	37	4	0.225	0.012	0.179	0.582
33	40	7	0.035	0.353	0.206	0.301
37	40	3	0.204	0.018	0.189	0.177
37	44	7	0.400	0.0001	0.203	0.265
40	44	4	0.489	0.0001	0.180	0.198
40	47	7	0.511	0.0001	0.211	0.292
44	47	3	0.720	0.0001	0.178	0.355
44	51	7	0.669	0.0001	0.217	0.408
47	51	4	0.646	0.0001	0.151	0.409
47	54	7	0.562	0.0001	−0.007	0.290

[a] For each set of disease data, the proportion of leaflets diseased for each plot was regressed on the proportion of leaflets frozen by −2.5 C, determined 3–7 days before disease incidence measurements. Degrees of freedom = 25.

[b] DAP = Days after planting when the tube nucleation test was performed or disease incidence was assessed.

[c] Number of days between tube nucleation test and subsequent disease assessment.

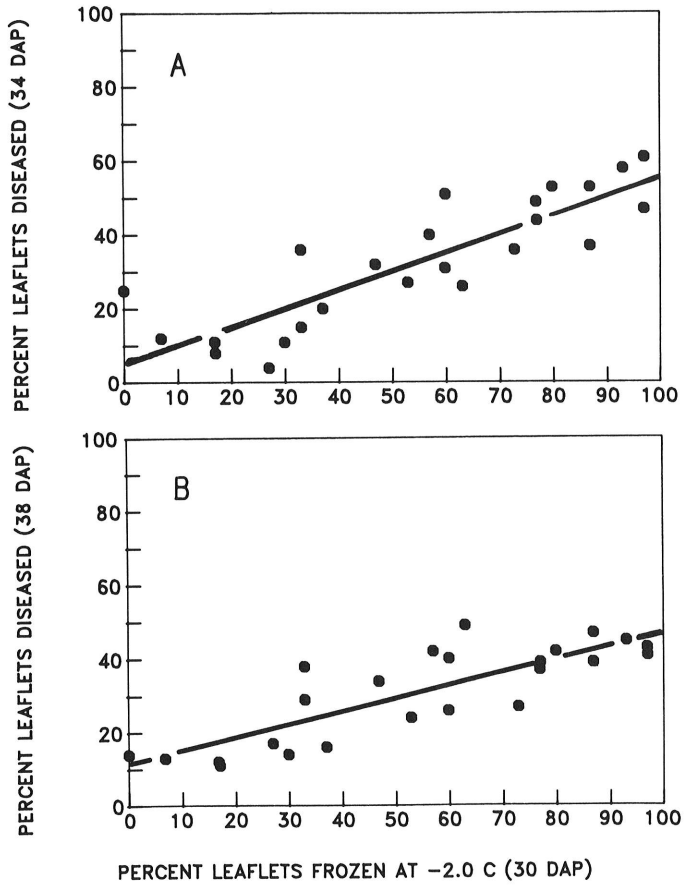

Fig. 5. Regression analysis of the percentage of leaflets diseased on the percentage of leaflets frozen at −2.0 C estimated at 30 days after planting (DAP). Disease measurements were made at, **A**, 34 DAP and, **B**, 38 DAP, 4 and 8 days, respectively, after the tube nucleation test was performed. Regression statistics were: **A**, $r^2 = 0.726$, $p < 0.0001$, $y = 5.6 + 0.497x$; **B**, $r^2 = 0.686$, $p < 0.001$, $y = 11.3 + 0.357x$.

LITERATURE CITED

1. Crosse, J. E. 1959. Bacterial canker of stone-fruits. IV. Investigation of a method for measuring the inoculum potential of cherry trees. Ann. Appl. Biol. 47:306-317.
2. Hagedorn, D. J., Rand, R. E., and Saad, S. M. 1972. *Phaseolus vulgaris* reaction to *Pseudomonas syringae*. Plant Dis. Rep. 56:325-327.
3. Hirano, S. S., Baker, L. S., and Upper, C. D. 1985. Ice nucleation temperature of individual leaves in relation to population sizes of ice nucleation active bacteria and frost injury. Plant Physiol. 77:259-265.
4. Hirano, S. S., Maher, E. A., Kelman, A., and Upper, C. D. 1978. Ice nucleation activity of fluorescent plant pathogenic pseudomonads. Proc. 4th Int. Conf. Plant Pathogenic Bacteria, Angers, France. 2:717-724.
5. Hirano, S. S., Rouse, D. I., Arny, D. C., Nordheim, E. V., and Upper, C. D. 1981. Epiphytic ice nucleation active (INA) bacterial populations in relation to halo blight incidence in oats. (Abstr.) Phytopathology 71:881.
6. Hirano, S. S., Rouse, D. I., and Upper, C. D. 1984. Epidemiology of bacterial brown spot and ecology of *Pseudomonas syringae* pv. *syringae* on *Phaseolus vulgaris*. Pages 24-27 in: Proceedings of the Second Working Group on *Pseudomonas syringae* pathovars. C. G. Panagopoulos, P. G. Psallidas, and A. S. Alivizatos, eds. Hellenic Phytopathological Society, Athens, Greece.
7. Hirano, S. S., and Upper, C. D. 1983. Ecology and epidemiology of foliar bacterial pathogens. Annu. Rev. Phytopathol. 21:243-269.
8. Hirano, S. S., and Upper, C. D. 1986. Ecology and physiology of *Pseudomonas syringae*. Bio/Technology 3:1073-1078.
9. Hirano, S. S., and Upper, C. D. 1986. Bacterial nucleation of ice in plant leaves. Pages 730-738 in: Methods in Enzymology. Protons and Water: Structure and Translocation. Vol. 127. L. Packer, ed. Academic Press, New York. 830 pp.
10. Hirano, S. S., and Upper, C. D. 1986. Temporal, spatial and genetic variability of leaf associated bacterial populations. Pages 235-251 in: Microbiology of the Phyllosphere. N.J. Fokkema, and J. Van den Heuvel, eds. Cambridge University Press, London.
11. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.
12. Leben, C. 1965. Epiphytic microorganisms in relation to plant disease. Annu. Rev. Phytopathol. 3:209-230.
13. Lindemann, J., Arny, D. C., and Upper, C. D. 1984. Epiphytic populations of *Pseudomonas syringae* pv. *syringae* on snap bean and nonhost plants and the incidence of bacterial brown spot disease in relation to cropping patterns. Phytopathology 74:1329-1333.
14. Lindemann, J., Arny, D. C., and Upper, C. D. 1984. Use of an apparent infection threshold population of *Pseudomonas syringae* to predict incidence and severity of brown spot of bean. Phytopathology 74:1334-1339.
15. Lindow, S. E., Arny, D. C., and Upper, C. D. 1978. Distribution of ice nucleation-active bacteria on plants in nature. Appl. Environ. Microbiol. 36:831-838.
16. Lindow, S. E., Arny, D. C., and Upper, C. D. 1978. The role of bacterial ice nuclei in frost injury to sensitive plants. Pages 249-263 in: Plant Cold Hardiness and Freezing Stress–Mechanisms and Crop Implications. P. H. Li and A. Sakai, eds. Academic Press, New York. 416 pp.
17. Lindow, S. E., Hirano, S. S., Barchet, W. R., Arny, D. C., and Upper, C. D. 1982. Relationship between ice nucleation frequency of bacteria and frost injury. Plant Physiol. 70:1090-1093.
18. Maki, L. R., Galyan, E. L., Chang-Chien, M., and Caldwell, D. R. 1974. Ice nucleation induced by *Pseudomonas syringae*. Appl. Microbiol. 28:456-459.
19. Rouse, D. I., Nordheim, E. V., Hirano, S. S., and Upper, C. D. 1985. A model relating the probability of foliar disease incidence to the population frequencies of bacterial plant pathogens. Phytopathology 75:505-509.