Diel Variation in Population Size and Ice Nucleation Activity of *Pseudomonas syringae* on Snap Bean Leaflets

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The extent to which diel changes in the physical environment affect changes in population size and ice nucleation activity of *Pseudomonas syringae* on snap bean leaflets was determined under field conditions. To estimate bacterial population size and ice nucleation activity, bean leaflets were harvested at 2-h intervals during each of three 26-h periods. A tube nucleation test was used to assay individual leaflets for ice nuclei. Population sizes of *P. syringae* were determined by dilution plating of leaflet homogenates. The overall diel changes in *P. syringae* population sizes differed during each of the 26-h periods. In one 26-h period, there was a continuous increase in the logarithm of *P. syringae* population size despite intense solar radiation, absence of free moisture on leaf surfaces, and low relative humidity during the day. A mean doubling time of approximately 4.9 h was estimated for the 28-fold increase in *P. syringae* population size that occurred from 0900 to 0900 h during the 26-h period. However, doubling times of 3.3 and 1.9 h occurred primarily within each 26-h period. These large diel changes were inversely correlated primarily with the diel changes in air temperature and reflected changes in nucleation frequency rather than changes in population size of *P. syringae*. Thus, the response of bacterial ice nucleation activity to the physical environment was distinct from the changes in population size of ice nucleation-active *P. syringae*.

Aerial parts of plants exist in a continuously fluctuating physical environment. Temperature, relative humidity, radiation, wind speed, and other weather parameters change diurnally. The occurrence of dew at night and drying during the day are cyclical events. Rain or irrigation may change the environment of the leaf surface suddenly and drastically. Thus, the time scale on which the physical environment of leaf surfaces can change substantially is often on the order of seconds to hours. Despite the diel (24-h day-night) fluctuations in the physical environment of the leaf surface and the known rates at which bacteria are likely to respond to such changes (23), most field studies of the population dynamics of leaf-associated bacteria have been based on sampling intervals of a few days, a week, or several weeks (6, 10, 17, 24, 27–29). Although these seasonal studies have provided some understanding of the general conditions that affect long-term trends in the sizes of leaf-associated bacterial populations, the time scale on which significant changes in bacterial population size actually occur in the phyllosphere has not been addressed. In addition, the extent to which diel fluctuations in the physical environment affect changes in bacterial populations has not been examined.

To estimate rates, it is essential to sample at an appropriate frequency and in a way that will provide the desired information. Clearly, if bacterial growth in the phyllosphere occurs with generation times of only a few hours and if these growth rates change in response to daily changes in the physical environment, then weekly sampling can provide at best a picture of the integral of the overall growth processes. This sampling will not yield useful estimates of growth rates and their changes. Since the phyllosphere contains many different bacterial species (12), it would be informative to measure rates of growth of component bacterial populations in addition to those of the total bacterial population. Microbial ecologists have measured rates of total bacterial growth in aquatic environments quite successfully (4, 13, 25). However, the methods that have been used are not well suited to estimating growth rates of specific bacterial components. The objective of this study, therefore, was to examine the short-term population dynamics of leaf-associated bacteria, focusing on a specific bacterium-plant system, *Pseudomonas syringae* van Hall on snap bean (*Phaseolus vulgaris* L.) leaflets. *P. syringae* pv. *syringae* is the causal agent of bacterial brown spot disease on snap beans (7). Infectivity titration experiments conducted under field conditions have demonstrated that relatively large populations (ca. 105 CFU per leaflet) of this bacterium are necessary before disease is likely to occur (15, 26). It is of interest, therefore, to determine the rates at which pathogen populations are likely to increase to a level that places the crop at hazard to disease. Ice nucleation-active (INA) *P. syringae* is also responsible for frost injury to sensitive plants (2, 18, 20). Although ice nucleation activity is an inherited trait in *P. syringae*, not every bacterial cell in a population is active as an ice nucleus at any given time and temperature (21, 22). The ratio of ice nuclei to viable cells or nucleation frequency has been used to express the relative ice nucleation activity of a given suspension of INA bacteria (21). The nucleation frequency of *P. syringae* varies among strains and with the conditions of growth for a given strain (8, 21). Lindow et al. demonstrated that the extent to which sensitive plants are injured by frost at a given temperature is a function not of the population size of INA bacteria present but also of their nucleation frequency (21). Thus, diel changes in ice nucleation activity of *P. syringae* on snap bean leaflets and

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the effect of the physical environment on this activity are also of substantial interest and were included in this study.

MATERIALS AND METHODS

Experimental design. Seeds of the snap bean cultivar Cascade (Sunseeds Genetics, Inc., Hollister, Calif.) were planted on 6 June 1983 in a plot (30 by 30 m) at the University of Wisconsin Arlington Experimental Farm. Rows were 76 cm apart and contained approximately 10 plants per meter. Three 26-h periods 14 days apart were selected in advance to provide an unbiased sample of weather. The diel experiments were conducted 30 to 31 days after planting (DAP) on 6 to 7 July, 44 to 45 DAP on 20 to 21 July, and 58 to 59 DAP on 3 to 4 August. For each experiment, 100 individual leaflets were harvested randomly from the top of the canopy at 2-h intervals starting at 0900 h and ending at 1100 h the next day. The leaflets were placed in no. 5 Kraft paper bags (25 leaflets per bag) and transported in a cooler to the laboratory for immediate processing with the tube nucleation test. Leaf harvesting was completed within 10 min, and initiation of the tube nucleation test occurred about 5 to 10 min later.

Determination of ice nucleation activity associated with individual bean leaflets. The tube nucleation test (8, 11) was used to estimate the frequency with which bean leaflets froze at −2.0, −2.2, and −2.5°C. Each leaflet was submerged in 9.0 ml of sterile, ice nucleus-free potassium phosphate buffer (0.01 M, pH 7.0) in a 16-mm test tube. The leaflet-containing tubes were held in ice until all leaflets had been processed; this took approximately 15 min. The tubes were then placed in a bath (Neslab Instruments, Inc., Portsmouth, N.H.) maintained at −2.0°C. After 30 min at −2.0°C, tubes with frozen buffer were counted. A nucleation event had occurred in these tubes. The remaining tubes were placed in a second bath held at −2.2°C. The process was repeated at the third test temperature, −2.5°C. Bath temperature precision was ±0.05°C of the target temperature. Ice nucleation activity was expressed as the cumulative percentage of leaflets that froze at each test temperature. For example, the cumulative percentage of leaflets frozen at −2.5°C is equal to the sum of the number of leaflets that froze at −2.0, −2.2, and −2.5°C divided by the total number of leaflets tested.

At each sampling time, a random subset of 30 of the 100 leaflets was marked before the leaflets were placed in them. After the tube nucleation assay, the selected leaflets were stored frozen at −20°C until they were processed by dilution plating for estimation of population sizes of P. syringae and other bacteria.

Determination of bacterial population sizes on individual bean leaflets. Population sizes of P. syringae and other bacteria were determined by dilution plating of homogenates of individual leaflets. Each leaflet was allowed to thaw for 20 to 30 min at ambient temperature (approximately 23 to 24°C). The leaflet and 9 ml of buffer in each tube were transferred to a sterile 50-ml beaker. The test tube was rinsed with 10 ml of sterile potassium phosphate buffer (0.1 M, pH 7.0) supplemented with Bacto-Peptone (0.1%, wt/vol). The rinse buffer was added to the beaker. Next, each leaflet was cut into 1- to 2-cm² segments (approximately) and comminuted for 10 s with a Polytron homogenizer equipped with a model PTA 20 TS probe (Brinkmann Instruments, Westbury, N.Y.) set at speed 5. Four 10-fold dilutions of each leaflet homogenate were made with sterile potassium phosphate buffer (0.01 M, pH 7.0). A 0.1-ml portion from the original homogenate and from each 10-fold dilution was spread on King’s medium B (14) containing 100 mg of cycloheximide per liter of medium to prevent fungal growth. Colonies of P. syringae had a characteristic appearance and were counted after 3 days of incubation at ambient temperature. Colonies of all other bacteria were counted 7 days after plating. Pseudomonas mesophila was easily identified on King’s medium B by its relatively small, pink colonies (3). Bacterial population sizes were log₁₀-transformed and expressed as log CFU per leaflet.

Environmental monitoring. Environmental parameters monitored in the plot area throughout the growing season included relative humidity (ventilated psychrometer; Delta-T Devices, Cambridge, England), air temperature (thermistor), solar radiation (LI-COR silicon-cell pyranometer; Campbell Scientific, Logan, Utah), wind speed (MET-ONE cup-type anemometer; Campbell Scientific), and wind direction (wind vane; Campbell Scientific). Thirty-minute averages for all parameters were recorded automatically with data loggers (models CR-5 and CR-21; Campbell Scientific). Rainfall was sensed with a Sierra-Misco tipping-bucket rain gauge (Campbell Scientific) and recorded every 30 min. Presence of dew was assessed visually.

RESULTS AND DISCUSSION

Diel variation in population sizes of P. syringae. From experiments that lasted 26 h, we were able to determine the changes that occurred within periods of 24 h (i.e., diel changes) from 0900 to 0900 h. The overall patterns in diel changes in population sizes of P. syringae differed during each of the 26-h periods sampled (Fig. 1). The net change from 0900 h on 6 July to 0900 h on 7 July was a significant 5.8-fold increase in population size (Fig. 1A and 2A). Although two- to threefold changes in population sizes appeared to occur between consecutive 2-h samples, these population sizes were not significantly different from each other. Thus, if fluctuations in bacterial population sizes of this magnitude, frequency, and regularity do occur, our methods were not sufficiently sensitive to detect them.

During sampling period 2, starting at 0900 h on 20 July, an almost continuous logarithmic increase in P. syringae population size occurred (Fig. 1B). Twenty-four hours after the start of the experiment, the population size of P. syringae had increased 25-fold (Fig. 1B). If the net increase was due solely to multiplication of the bacterium, then populations of P. syringae doubled about every 4.9 h, as estimated from the slope of the regression line of population versus time for all datum points from 0900 h on 20 July to 0900 h on 21 July (r² = 0.89). Doubling times as short as 3.3 and 1.9 h were estimated from the slope of the regression lines for the period from 1700 to 2300 h (r² = 0.98) and the period from 0100 to 0700 h (r² = 0.97), respectively.

In experiment 2, the increase in population size on the median leaflet (about 10⁷ CFU in 24 h) was 2 to 5 orders of magnitude greater than that expected for changes due to either immigration or emigration (16). Lindemann and Upper (16) estimated a mean daily upward flux and deposition of bacteria over a bean canopy of approximately 4.36 and 3.03 log CFU per leaflet per day, respectively. Thus, immigration and emigration probably did not have a quantitatively important impact on changes in P. syringae population sizes during experiment 2. On the other hand, population sizes of P. syringae were increasing at rates of the same order of magnitude as would be expected under optimal laboratory conditions (30). Thus, it is apparent that growth of P. syringae was the dominant factor influencing changes in
FIG. 1. Diel variations in population sizes of *P. syringae*. Population sizes of *P. syringae* were estimated for each of 30 individual bean leaflets per sampling time. Each datum point represents the mean log$_{10}$ *P. syringae* population for each set of 30 leaflets harvested on 6 and 7 July (30 to 31 DAP) (A), 20 and 21 July (44 to 45 DAP) (B); and 3 and 4 August (58 to 59 DAP) (C). Error bars represent standard errors of the mean.

population size during this period. The extent to which cell death was occurring, and hence attenuating net growth rate, cannot be estimated for this experiment.

Despite the high leaflet-to-leaflet variability in population sizes of *P. syringae* observed at each sampling time (Fig. 2A) during experiments 1 and 2, the estimated population variances were not significantly different from each other within each 26-h period. When substantial growth of *P. syringae* did occur during experiment 2, the population variances did not change significantly (Fig. 2B). Thus, during periods when increases in population size did occur, the overall change in *P. syringae* population size appeared to be the same as that which would have occurred if the population on every leaflet had increased by an identical proportion. This would be the expected result if *P. syringae* on every leaf had grown exponentially at exactly the same rate during these periods and if death, immigration, and emigration were insignificant relative to growth. Although identical growth rates on all
leaves does not seem plausible, an average growth rate that is not affected by initial population sizes on leaves may be.

During sampling period 3, from 3 to 4 August, population sizes of *P. syringae* decreased during the day (1300 to 1700 h) and then increased at night (1700 to 0100 h) (Fig. 1C), resulting in no significant net change in the mean log of the population during the 26-h period (Fig. 2C). The frequency distribution of *P. syringae* population sizes estimated for 0900 h on 3 August did not conform well to the expected lognormal (9) (Fig. 2C). The frequency of population sizes below about $10^4$ CFU per leaflet was greater than expected. By 0900 h on 4 August, the frequency of leaflets with *P. syringae* population sizes of less than $10^4$ CFU per leaflet appeared to have decreased. This indicates that although there was no net change in *P. syringae* population size over the 24-h period, population sizes of the bacterium apparently underwent greater change on some leaflets than on others.

Although net increases in *P. syringae* population sizes were measured for two of the three 24-h periods, net decreases must have occurred during the 13-day periods between experiments. For example, compare the initial bacterial populations in Fig. 1C with the final populations in Fig. 1B. In addition, substantial changes in the population sizes of other bacteria and large changes in the composition of the overall bacterial community on bean leaves occurred during the 4-week period encompassed by these experiments (Fig. 3). *P. syringae* was the predominant bacterium in the leaf-associated bacterial communities on leaflets harvested during the first 26-h period (Fig. 3A). This bacterial species constituted about 50 to 70% of the total bacterial population. For this reason, short-term changes in the total bacterial population appeared to be nearly parallel to those exhibited by *P. syringae* alone.

During the 2 weeks that elapsed between the end of sampling period 1 and the beginning of period 2, *P. syringae* populations declined slightly, while the population sizes of other bacteria on bean leaflets increased substantially. Thus, by 0900 h on 20 July, only 5% of the total bacterial population was *P. syringae* (Fig. 3B). By 3 August, *P. syringae* was less than 1% of the total bacterial population (Fig. 3C). In contrast, population sizes of *P. mesophilica*, a common saprophyte on bean leaflets, increased substantially during the same 4 weeks (Fig. 3). During experiment 1, population sizes of this species were too low to allow reliable enumeration. By 20 July, *P. mesophilica* was a dominant component in the bean leaflet bacterial community.

Although *P. syringae* populations increased rapidly during experiment 2, most of the other bacterial populations associated with the bean leaves did not. For example, *P. mesophilica* decreased in population size during the day and increased at night to achieve no net change, in a pattern very similar to that observed for *P. syringae* during experiment 3. Thus, the rapid bacterial multiplication observed during experiment 2 was relatively specific.

**Relationship between changes in *P. syringae* population sizes and weather conditions.** Thirty-minute averages of air temperature, relative humidity, solar radiation, and wind speed for the 26-h periods starting at 0900 h on 6 and 20 July and 3 August are presented in Fig. 4. Air temperature and relative humidity were lower during sampling period 1 than during periods 2 and 3. The average daytime (0900 to 2100 h) and nighttime temperature were 21.1 and 13.5, 30.6 and 24.4, and 25.3 and 21.2°C for periods 1, 2, and 3, respectively. Relative humidity was highest and solar radiation was lowest during experiment 3. Highest average daytime and nighttime wind speeds were measured during 26-h periods 3 and 1, respectively.

Dew was present on the top leaves of the canopy by 2100 h on each of the 3 nights sampled and persisted until about
0830 to 0900 h the next morning. Although rain did not fall during sampling periods 1 and 2, 22 mm of rain fell between 2000 h on 19 July to 0400 h on 20 July, 5 to 11 h prior to the start of experiment 2. On 3 August, 2 mm of rain fell between 1005 to 1030 h, followed by a slight drizzle between 1248 and 1310 h. Less than 1 mm of rain fell during the second rainfall, and the rain gauge did not sense the event. As a result of the rain, moisture was present on the top of the canopy from about 1005 to 1430 h on 3 August.

The diel variations in population sizes of _P. syringae_ that occurred during sampling periods 1 and 3 could not be readily explained by short-term changes in the weather. Low solar radiation, high relative humidity, and presence of moisture on the leaves are among the conditions that generally have been assumed to be favorable for growth of leaf-associated bacterial populations. These conditions prevailed during sampling period 3, when populations of _P. syringae_ tended to decrease during the day, and no net increase occurred. During the other two experiments, when measurable increases in _P. syringae_ populations did occur, leaves were dry during the day, radiation was more intense, and humidities were lower than during experiment 3.


**Diel variation in ice nucleation activity associated with individual bean leaflets.** Overall diel trends in leaf-associated bacterial ice nucleation activity were qualitatively similar for all three sampling periods (Fig. 5). Bacterial ice nucleation activity, expressed as the cumulative percentage of bean leaflets that froze at each of three test temperatures, was lower during the day than during the night. The rates and magnitudes of the changes in ice nucleation activity differed, however, for the three sampling periods. For example, the decreases in the cumulative percentage of bean leaflets that froze by $-2.5\,^\circ\mathrm{C}$ between 0900 and 1700 h were from 92 to 80% and from 100 to less than 20% on 6 and 20 July, respectively.

In previous studies (8, 10), a quantitative relationship was established between the temperature at which ice nucleation occurs on individual bean leaflets and the population sizes of _P. syringae_ on those leaflets. In this study, however, the diel variation in leaf-associated bacterial ice nucleation activity did not appear to parallel the short-term changes in population sizes of _P. syringae_ (Fig. 1 and 5). Instead, diel changes in bacterial ice nucleation activity appeared to follow the diel periodicity in air temperature, relative humidity, and solar radiation. Stepwise multiple regression analyses with $y$ representing the cumulative percentage of bean leaflets that

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**FIG. 4.** Diel variations in weather parameters on 6 and 7 July ( ), 20 and 21 July ( ), and 3 and 4 August ( ). Values represent 30-min averages.
froze by −2.2 or −2.5°C and x representing the population size of *P. syringae*, average air temperature, relative humidity, and solar radiation 30 min prior to leaf harvest were conducted to identify the relationship of these parameters to ice nucleation activity. Average ambient temperature 30 min to 2 h prior to leaf harvest appeared to have the most significant effect on nucleation activity (Table 1 and Fig. 6). Highly significant correlation coefficients were obtained for the relationship between cumulative percentage of leaflets frozen by −2.2°C and air temperature (for period 1, \( r = -0.938 \) and *P* < 0.001; for period 2, \( r = -0.633 \) and *P* < 0.05; and for period 3, \( r = -0.802 \) and *P* < 0.001). Similar trends were obtained for the −2.5°C test temperature. Thus, the nucleation frequency (ratio of ice nuclei to viable cells) of those cells appeared to be strongly affected by ambient temperature immediately before sampling. A similar phenomenon has been reported by Anderson et al. (1) for INA bacteria in suspension. Relative humidity increased the coefficients of determination for periods 2 and 3. Population sizes of *P. syringae* increased the coefficients of determination for periods 1 and 3. Thus, relative humidity and *P. syringae* population size appeared to have some effect on the diel variation in leaf-associated ice nucleation activity.

The absence of a significant relationship between INA *P. syringae* population size and ice nucleation activity in this study may be further explained by the observation that the nucleation frequency of *P. syringae* is lower when cells are in the exponential phase of growth than when they are in the stationary phase (5, 19; S. S. Hirano, unpublished data).

![Figure 5](image1.png)

**FIG. 5.** Diel variations in ice nucleation activity expressed as cumulative percentages of leaflets frozen at −2.0 (■), −2.2 (●), and −2.5°C (▲). One hundred leaflets were harvested at each sampling time: 6 and 7 July (A), 20 and 21 July (B), and 3 and 4 August (C).

![Figure 6](image2.png)

**FIG. 6.** Relationship between average ambient temperature 30 min prior to leaf harvest and the cumulative percentage of leaflets frozen by −2.2°C.

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**TABLE 1. Effects of ambient temperature, relative humidity, solar radiation, and population size of *P. syringae* on bacterial ice nucleation activity**

<table>
<thead>
<tr>
<th>Sampling period no. (dates)</th>
<th>Variable</th>
<th>( r^2 )</th>
<th>Increase in ( r^2 ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (6–7 July)</td>
<td>Temp</td>
<td>88.0</td>
<td>88.8</td>
</tr>
<tr>
<td></td>
<td><em>P. syringae</em> population size</td>
<td>93.4</td>
<td>5.4</td>
</tr>
<tr>
<td>2 (20–21 July)</td>
<td>Temp</td>
<td>40.1</td>
<td>40.1</td>
</tr>
<tr>
<td></td>
<td>Relative humidity</td>
<td>65.0</td>
<td>24.9</td>
</tr>
<tr>
<td>3 (3–4 August)</td>
<td>Temp</td>
<td>64.3</td>
<td>64.3</td>
</tr>
<tr>
<td></td>
<td>Relative humidity</td>
<td>73.9</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td><em>P. syringae</em> population size</td>
<td>80.3</td>
<td>6.4</td>
</tr>
<tr>
<td>1 + 2 + 3</td>
<td>Temp</td>
<td>63.0</td>
<td>63.9</td>
</tr>
<tr>
<td></td>
<td><em>P. syringae</em> population size</td>
<td>71.4</td>
<td>7.5</td>
</tr>
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</table>

* Stepwise multiple regression analysis was performed with Minitab (Minitab, Inc., State College, Pa.) to examine the effects of ambient temperature, relative humidity, solar radiation, and population size of *P. syringae* on the cumulative percentage of bean leaflets that froze by −2.2°C. The weather parameters were averages of ambient temperature, relative humidity, and solar radiation during the 30-min period that ended at leaf harvest.
Thus, in experiment 2, in which differences in population sizes of INA \textit{P. syringae} were large enough that detection of an effect of population size on ice nucleation activity might have been expected, the effect was probably confounded by decreasing nucleation frequencies during exponential growth. In the other two experiments, differences in \textit{P. syringae} population sizes were sufficiently small that only minor effects of population size of INA bacteria were detected (Table 1).

In a previous study (10), ice nucleation activity on bean leaflets at temperatures higher than about -3°C was found to be highly dependent on the population sizes of INA bacteria present on those leaflets when leaf samples were harvested between about 0800 and 0830 h. This sampling protocol minimized the effect of the diel fluctuation in temperature on ice nucleation activity and facilitated establishment of the quantitative relationship between population size of INA bacteria and ice nucleation activity. We therefore recommend very strongly that leaf samples tested for ice nucleation activity for the purpose of forecasting the hazard of frost or disease caused by INA \textit{P. syringae} be harvested at the same time each morning to minimize the diel fluctuation in ice nucleation frequency of \textit{P. syringae} on leaves.

In this paper we describe bacterial populations and ice nucleation activities during only 3 days of the approximately 50-day life span of a snap bean crop from plant emergence to pod harvest. Thus, the picture that emerges from our research must be considered only a limited look at the overall dynamics of \textit{P. syringae} and \textit{P. mesophilica} populations on leaf surfaces. It is apparent that the frequency with which populations are sampled has a profound influence on the conclusions that can be drawn. In both sampling periods 2 and 3, bacterial populations were found to decrease during the day and increase at night, which led to no net change over a 24-h period. Samples taken as frequently as once each day would have led to the conclusion that there was no change in bacterial populations during these two 24-h periods. Very little change in population size of \textit{P. syringae} occurred between 0900 h on 7 July and 0900 h on 20 July. Does this mean that neither death nor growth of this species occurred during this 13-day period? Or were rapid increases in \textit{P. syringae} population sizes followed by equally dramatic decreases likely to describe the unobserved dynamics of these 13 days?

Most descriptions of the dynamics of bacterial growth on plant surfaces have been based on samplings too infrequent to measure the rapid increases reported here. The growth rate of a specific bacterial component may be much higher than the average growth rate for the entire bacterial community (Fig. 3B).

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LITERATURE CITED


