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Effect of Sampling Scale on the Assessment of Epiphytic Bacterial Populations

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Abstract. Bacterial populations on above-ground plant surfaces were estimated at three different biological scales, including leaflet disks, entire leaflets, and whole plants. The influence of sample scale on the estimation of mean bacterial population size per unit and per gram and on the variability among sampling units was quantified at each scale. Populations were highly variable among sampling units at every scale examined, suggesting that there is no optimal scale at which sample variance is reduced. The distribution of population sizes among sample units was sometimes, but not consistently, described by the lognormal. Regardless of the sampling scale, expression of population sizes on a per gram basis may not reduce variance, because population size was not generally a function of sample unit weight within any single sampling scale. In addition, the data show that scaling populations on a per gram basis does not provide a useful means of comparing population estimates from samples taken at different scales. The implications of these results for designing sampling strategies to address specific issues in microbial ecology are discussed.

Introduction

Epiphytic bacteria are common inhabitants of plant leaflet surfaces and can have important effects on the plants on which they live. Accurate estimates of epiphytic bacterial population sizes are needed to assess their role in several important biological phenomena. For example, reliable disease forecasts [19, 31], prediction of frost damage [6, 20, 21, 23–25], and assessment of the risks associated with the release of genetically engineered microorganisms into the open environment [4] depend on accurate estimates of bacterial population structure.

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Epiphytic bacterial population sizes are usually estimated from one of several possible units. Three such units include small leaf segments, entire leaves, or whole plants. Most commonly, individual leaves or leaflets have been chosen as the sampling unit [30]. Estimated bacterial population sizes are usually scaled on a per unit weight (or sometimes per unit area) basis to allow population estimates to be directly compared with those from other studies or from those taken at different scales (e.g., leaf disks vs. whole leaves). Scaling of population data by weight or by unit area is often done assuming that variability in bacterial population sizes among leaves is partly a function of weight and that bigger leaves or bigger sampling units will support larger populations in relation to their greater weight. Thus, in addition to permitting comparisons among samples of different unit size, scaling on a per unit weight basis has been perceived to provide a reduction in sample variance when samples contain units covering a range of sizes. However, neither the variability in bacterial population sizes relative to the variability in weight among sampling units nor the influence of sampling scale on the estimation of bacterial population sizes has been rigorously quantified. Specifically, the manner in which the size and weight of the sampling unit may influence the distribution of bacterial population sizes among units and the mean–variance relationships at each scale have not been investigated.

Although some information is available on the distribution of microbial population sizes among sampling units for whole leaves [1], such information is missing for smaller and larger sampling scales. Previous work has shown that the distribution of total and fluorescent pseudomonad population sizes among individual leaves can be described by the lognormal [7, 9, 10]. However, although some qualitative studies on bacterial spatial pattern on leaves have been published [3, 17, 18, 28, 29, 34, 35], accurate quantitative distributions of bacterial population sizes among small segments of a single leaf or different leaves have not been well described. Motile bacteria can relocate to different sites on the leaf surface under conditions of leaf wetness [16], but the extent to which different portions of a leaf may vary as a habitat for bacterial growth or survival is not known. Thus, though studies suggest that bacterial populations are aggregated on leaves, the scale of the aggregation and its influence on the variance of bacterial population size among leaf subunits are not known. In addition, although it may be expected that the physiological state of plants is an important biological factor that could influence bacterial population sizes, little information is available on the distribution of bacterial populations among plants.

Overall, the lack of information on the influence of sampling scale on the estimation of bacterial populations seriously restricts our ability to select optimal sampling strategies and scales logically. If the variability in population size among sampling units differs greatly as a function of sampling scale, statistical analyses of differences in bacterial population sizes among treatments and the ability to quantify interactions among coexisting bacterial strains may be significantly influenced by the scale at which the bacterial populations are sampled. Additionally, population estimates obtained using samples taken at different scales may not be directly comparable. Thus, a critical evaluation of the influence of sampling scale on the estimation of population parameters, such as the mean and variance, for epiphytic bacteria is needed. The implications of this information for designing sampling strategies to address specific issues in risk assessment and microbial ecology are discussed.

Materials and Methods

Bacterial Strains

Pseudomonas syringae strain B728A was isolated from a bacterial brownspot lesion on a bean leaflet. The characteristics and source of this strain have been reported [27]. *Pseudomonas syringae* strain TLP2 was isolated from an asymptomatic potato leaflet. This strain was found to be non-pathogenic on all plant species tested [22]. Both strains were resistant to 100 μg/ml of rifampicin. Laboratory cultures were stored at −80°C in sterile 15% (v/v) glycerol in 25 mM potassium phosphate buffer (pH 7.0). Strains were cultured on King's medium B (KB) at 24°C for 24 h for inoculum production. Cells were harvested from plates with a sterile loop and suspended in sterile distilled water. Cell concentrations were determined turbidimetrically and diluted with sterile water to the appropriate dilution.

Plant Growth and Bacterial Inoculations

In the greenhouse, potato plants (*Solanum tuberosum* cv. Russet Burbank) were grown from surface-sterilized potato tubers. Tubers were grown in a sterilized sand–peat mixture for approximately 28 days, when plants were about 20 cm in height. Snapbean plants (*Phaseolus vulgaris* cv. Bush Blue Lake 274) were grown from seed in a sand–peat mixture for approximately 14 days or until primary leaves were fully expanded and the first trifoliate leaves had just emerged. Bacterial suspensions (ca. 10^6 cells per milliliter in sterile distilled water) were spray-inoculated onto leaflets until runoff. Each leaf received between 1 and 2 ml of bacterial suspension, and a substantial fraction (>50%) of this dripped from the leaflets. The plants were then enclosed individually in clear plastic bags and incubated under fluorescent lights (800 lux) at 21°C for 24 h. In some cases, as indicated in the results, plants were subsequently exposed to cyclic changes in physical conditions. During the sampling, individual leaflets were excised from the plants, retaining as little petiole as possible. Leaflets that showed no damage or defects were chosen randomly from among the inoculated plants.

Potato plants were established in the field in a randomized complete block design at the University of California Kearney Agricultural Research and Extension Center on 3 September 1987. Surface-sterilized potato tubers (cv. Russet Burbank) were cut into pieces approximately 4 cm in diameter, each of which contained at least one eye. Approximately 50 such seed pieces were planted in each of four replicate blocks. On 23 November, when plants were approximately 25 cm tall and had at least seven leaves, plants were spray inoculated with a suspension of *P. syringae* strain TLP2 (ca. 10^7 cells per milliliter). Bacteria were applied to plants to runoff, using a CO_2-pressurized hand-held sprayer operated at 40 psi, as in other studies [26]. Plants were subsequently exposed to ambient field conditions (maximum daytime temperatures ca. 18°C, minimum nighttime temperatures 6°C). After 1 week, individual leaflets were sampled from each of two plants in each block, and bacterial populations were estimated as described below.

Estimation of Bacterial Populations

Bacterial populations were estimated for individual leaflet segments, whole leaflets, and entire plants. To quantify the distribution of bacterial populations within a single leaflet, individual leaflets were cut into pieces, and population sizes were estimated independently for each piece. A total of 12 individual leaflets from different potato plants that were inoculated and maintained in a greenhouse were examined. Each leaflet was sliced into 0.3 cm x 0.3 cm squares using a flame-sterilized razor blade. Bacterial population sizes were estimated for all segments of each leaflet. The total number of segments for each leaflet ranged from 50 to 120. For each leaflet, the location of each segment was noted as coordinates that produced a leaflet map. Individual leaflet segments were placed in sterile Eppendorf microfuge tubes containing 0.2 ml of sterile washing buffer (0.1 M potassium phosphate buffer, pH 7.0, containing 0.1% Bacto peptone). Leaflet segments were macerated with sterile disposable

Table 1. Bacterial populations (strain B728a) on individual leaflets, populations per 9-mm^2 leaflet segment, numbers of segments per leaflet, and variance/mean ratios for populations per segment

Leaflet No.	Total population[a]	Mean per segment	N[b]	Variance/mean[c]
1[d]	27,900	340	82	260
2	3,337	68	49	158
3	3,883	56	69	143
4	24,816	528	47	81
5	14,628	318	46	166
6	19,380	380	51	404

[a] Total number of bacterial colony-forming units from all segments. All segments were sampled on each leaflet.
[b] Number of segments per leaflet.
[c] Variance/mean ratio for number of bacterial colony-forming units per leaflet (nontransformed data).
[d] Leaflets 1, 2, and 3 were sampled 24 h after bacterial inoculation; leaflets 4, 5, and 6 were sampled 48 h after inoculation.

plastic pestles. The entire leaflet macerate was then spread on the surface of KB containing 100 μg/ml of cycloheximide and 50 μg/ml of benomyl to inhibit fungal growth, and 100 μg/ml of rifampicin (KBR). Bacterial populations on each leaflet segment were determined from colony counts on plates after incubation at 28°C for 3 days.

To estimate bacterial populations on entire leaflets, individual leaflets were randomly selected from bean or potato plants. Individual leaflet samples were collected both from greenhouse-grown bean and potato plants and from field-grown potato plants. Sample sizes varied among experiments and are indicated in Tables 1 and 2. Leaflets were weighed, and each was immersed in 20 ml of washing buffer in a large test tube. Tubes were sonicated in an ultrasonic cleaner (Bransonic 52) for 7 min, and 10-fold serial dilutions were plated on KBR. Bacterial populations on each leaflet were determined based on bacterial colony counts on plates after 3 days incubation at 28°C.

For whole plant population estimates in the field, all the fully expanded leaves from each of eight individual field-grown potato plants were sampled. The population size of strain TLP2 was evaluated on each individual leaflet, as described above. For whole-plant population estimates in the greenhouse, all the primary leaves and all leaflets from first trifoliates were processed individually at varying time intervals following inoculation of bean plants with strain B728a. Plant incubation conditions were as described above.

Statistical Methods

All statistical calculations were performed using SAS (version 6.03) (SAS Institute Inc., Cary, N.C.). Analysis of variance of samples of different unit size was done on nontransformed and log-transformed estimates of population size (log per gram and log per sampling unit) using the General Linear Models procedure. Variance/mean ratios for nontransformed data provided an index to aggregation of populations among units [2]. The goodness-of-fit of the normal distribution to the log-transformed and nontransformed estimates of bacterial populations for samples of different unit size was tested by the Shapiro-Wilk W statistic or the Kolomogorov D-statistic [32, 33] using the Univariate procedure in SAS.

Results

Leaflet Segments as Sampling Units

The population sizes of strain B728a on individual 9-mm^2 segments of three potato leaflets (representative of the leaflets sampled) are illustrated in Fig. 1. Mean

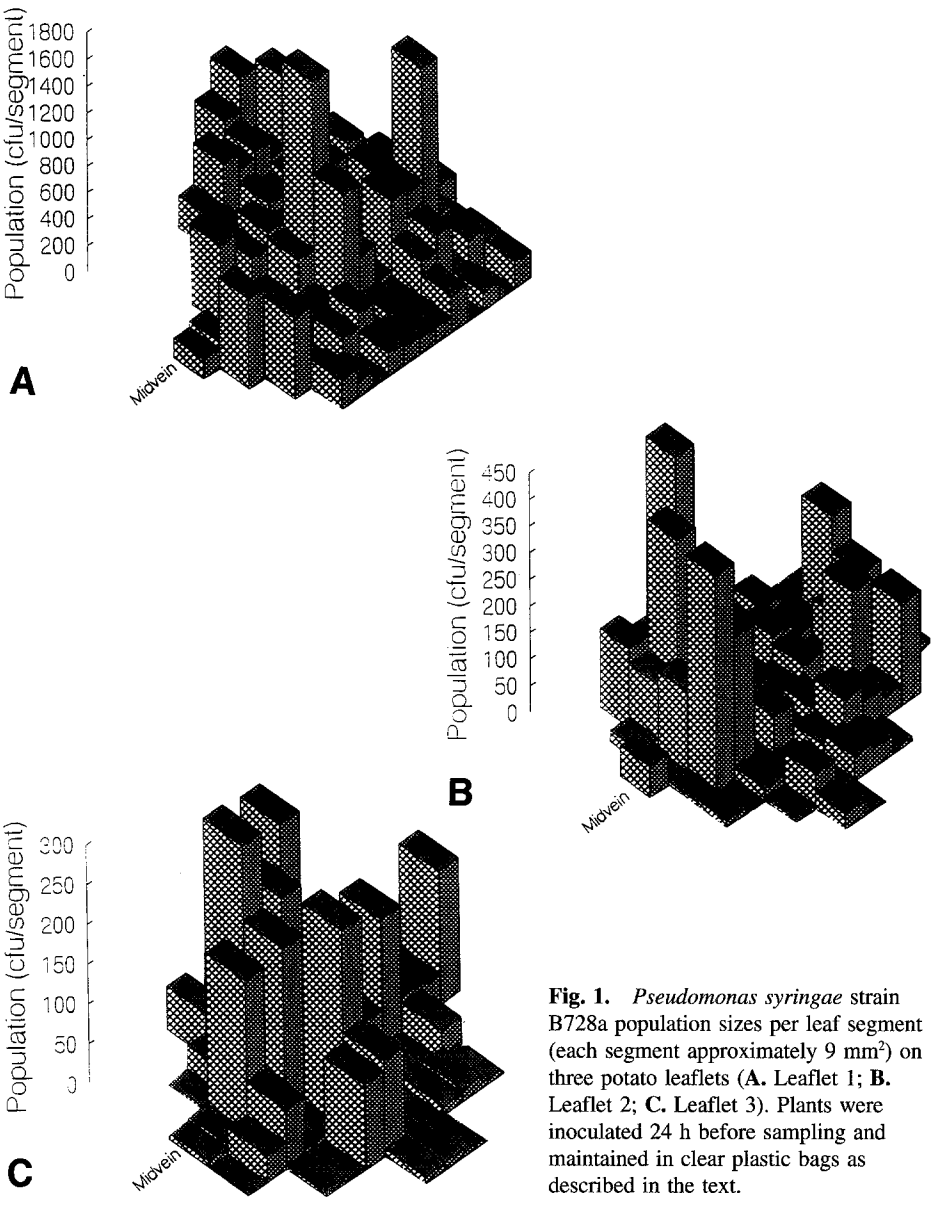

Fig. 1. *Pseudomonas syringae* strain B728a population sizes per leaf segment (each segment approximately 9 mm^2) on three potato leaflets (**A.** Leaflet 1; **B.** Leaflet 2; **C.** Leaflet 3). Plants were inoculated 24 h before sampling and maintained in clear plastic bags as described in the text.

population size per segment on different leaflets ranged from 56 to 528, though within a given leaflet, there were segments with much larger population sizes and segments with no detectable populations. Populations on the central vein of the leaflets tended to be larger than those on the leaflet edges (mean population size along the central vein was greater than that on the edge segments for five of six leaflets).

Bacteria were clustered on segments within leaflets, as evidenced by the large variance/mean ratios observed (Table 1, mean population per segment for leaflets

Table 2. Influence of scaling factors on population assessments for epiphytic bacteria

Sampling unit	Mean	Variance among units	Variance/Mean	*P*-value Normality[a]	n[b]
Leaflet segments,[c] strain B728a, greenhouse					
Leaflet 1					
Pop. per segment	340.000	88,270.000	260.000	0.0001 (W)	82
Log (pop. per segment)	2.348	0.222	—	0.0002 (W)	82
Pop. per gram	100,071.000	7.64×10^9	76,346.000	0.0001 (W)	82
Log (pop. per gram)	4.816	0.222	—	0.0002 (W)	82
Leaflet 2					
Pop. per segment	68.000	10,773.000	158.000	0.0001 (W)	48
Log (pop. per segment)	1.420	0.463	—	0.2529 (W)	48
Pop. per gram	20,030.000	9.32×10^8	46,525.000	0.0001 (W)	48
Log (pop. per gram)	3.89	0.463	—	0.2529 (W)	48
Leaflet 3					
Pop. per segment	56.000	8,002.000	143.000	0.0000 (W)	69
Log (pop. per segment)	1.407	0.434	—	0.1415 (W)	69
Pop. per gram	16,551.000	6.92×10^8	41,828.000	0.0000 (W)	69
Log (pop. per gram)	3.875	0.434	—	0.1415 (W)	69
Whole Leaflets,[c] strain TLP2 greenhouse					
Sample 1 (immediately after inoculation)					
Pop. per leaflet	3,543.000	5.04×10^6	1,422.000	<0.0100 (D)	100
Log (pop. per leaflet)	3.460	0.080	—	>0.1500 (D)	100
Pop. per gram	4,500.000	1.57×10^6	3,482.000	<0.0100 (D)	100
Log (pop. per gram)	3.550	0.080	—	>0.1500 (D)	100
Sample 2 (24 h incubation under cool, wet conditions)					
Pop. per leaflet	228,109.000	7.68×10^{10}	33,682.000	<0.0100 (D)	100
Log (pop. per leaflet)	5.150	0.186	—	>0.1500 (D)	100
Pop. per gram	269,863.000	1.18×10^{11}	435,777.000	<0.0100 (D)	100
Log (pop. per gram)	5.240	0.157	—	>0.1500 (D)	100

Sample 3 (24 h incubation under hot, dry conditions)					
Pop. per leaflet	49,843.000	3.46×10^{10}	694,179.000	<0.0100 (D)	100
Log (pop. per leaflet)	4.283	0.309	—	0.0250 (D)	100
Pop. per gram	79,538.000	9.65×10^{10}	1,213,256.000	<0.0100 (D)	100
Log (pop. per gram)	4.454	0.306	—	0.0210 (D)	100
Sample 4 (24 h incubation under cool, wet conditions)					
Pop. per leaflet	2,776,325.000	6.13×10^{12}	2,207,955.000	<0.0100 (D)	100
Log (pop. per leaflet)	6.220	0.297	—	<0.0100 (D)	100
Pop. per gram	4,339,287.000	2.10×10^{13}	4,837,200.000	<0.0100 (D)	100
Log (pop. per gram)	6.409	0.042	—	<0.0100 (D)	100
Whole leaflets,[d] strain TLP2, field					
Pop. per leaflet	22,961.300	2.41×10^{9}	104,960.585	<0.0001 (W)	40
Log (pop. per leaflet)	2.915	3.595	—	<0.0001 (W)	40
Pop. per gram	9,912.419	9.82×10^{7}	9,906.764	<0.0001 (W)	40
Log (pop. per gram)	2.484	2.671	—	<0.0001 (W)	40
Whole plants,[c] strain B728a, greenhouse					
Pop. per plant	5,715.000	8.42×10^{6}	1,473.000	0.5164 (W)	6
Log (pop. per plant)	3.700	0.068	—	0.4245 (W)	6
Pop. per gram	1,216.000	4.68×10^{5}	385.000	0.0221 (W)	6
Log (pop. per gram)	3.030	0.052	—	0.0530 (W)	6
Whole plants,[d] strain TLP2, field					
Pop. per plant	114,803.300	2.35×10^{10}	204,697.949	0.0070 (W)	8
Log (pop. per plant)	4.725	0.346	—	0.8756 (W)	8
Pop. per gram	5,879.670	3.45×10^{7}	5,867.676	0.0131 (W)	8
Log (pop. per gram)	3.450	0.248	—	0.5800 (W)	8

[a] P = the probability of rejecting the hypothesis that the distribution of population sizes among units (e.g., population per segment/leaflet/plant, log population per segment/leaflet/plant, population per gram, or log population per gram) is described by the normal distribution as tested using either the Shapiro-Wilk statistic (W) or the Kolomogorov D-statistic (D).

[b] n = number of sample units (leaflet segments, leaflets, or plants).

[c] Leaflet segment, whole-leaflet, and whole-plant samples were taken from plants inoculated and maintained in the greenhouse as described in the text. See text for details of segment, leaflet, and whole-plant sampling strategies.

[d] Whole-leaflet and whole-plant samples were taken from plants inoculated and maintained in the field as described in the text. See text for details of leaflet and whole-plant sampling strategies.

shown in Fig. 1). Variance/mean ratios were greater than 50 for strains B728a and TLP2 on all leaflets sampled. The variance in bacterial population size among segments differed among leaflets, ranging from 8,008 to 153,520. Aggregation was also evident at a larger scale on some leaflets. For example, segments with high bacterial populations are clustered on leaflet C (Fig. 1C). The distribution of bacterial population sizes on segments within individual leaflets was generally described better by the lognormal than by the normal distribution (Table 2, leaflets 2 and 3; hypothesis of normality rejected for non-transformed data, not rejected for the log-transformed data). However, the lognormal did not provide a good fit for the distribution of population sizes per segment among segments for all leaflets (Table 2, leaflet 1; hypothesis of normality rejected for both nontransformed and log-transformed data).

Whole Leaflets as Sampling Units

Epiphytic bacterial population sizes and mean–variance relationships among individual leaflets in both field and greenhouse studies are shown in Table 2. The large variance/mean ratios for the nontransformed estimates of bacteria per leaflet suggest that populations are clustered on specific leaflets in a nonrandom pattern [2], or that there is a great deal of variability among leaflets in bacterial population size when expressed per leaflet. This large variability among leaflets was present directly following inoculation of leaflets (sample 1, greenhouse) and was maintained over time in the greenhouse (samples 1–4 taken at 24-h intervals). Additionally, the large levels of leaflet-to-leaflet variability were observed over a wide range of population levels (mean bacterial population sizes per leaflet ranging from 3,543 to 2,776,325; Table 2) and following incubation of plants under both cool/wet and hot/dry conditions. Variability in population size among leaflets for the log-transformed data was much larger among inoculated leaflets in the field than in the greenhouse.

Bacterial populations among leaflets have been shown to be well described by the lognormal distribution [7], and this distribution was appropriate for the per leaflet and per gram leaflet weight data in some of the whole leaflet samples (Table 2, samples 1 and 2). However, the lognormal distribution did not provide a good description for all samples (Table 2, samples 3 and 4). In these cases, an alternative transformation [11, 33] may be needed to meet the assumptions necessary for normal-based statistics.

Plants as Sampling Units

Bacterial population sizes for whole-plant samples and the variability among plants in these estimates are indicated in Table 2. On inoculated plants maintained under controlled conditions in the greenhouse, population sizes among plants were highly variable (populations ranged from 1,992–9,081 on small plants). Variance/mean ratios in both the field and the greenhouse suggest clustering of bacterial populations among plants. Variability in population size among plants was greater for inoculated plants in the field than in the greenhouse.

For whole-plant samples, the sample size was not large enough to provide a

Table 3. Relationships between leaflet weight and bacterial population per leaflet and population density (population size per gram leaflet)

	Leaflet weight and population per leaf		Leaflet weight and population density	
Sample[a]	r^b	P^c	r	P
1	0.372	0.0001	−0.278	0.005
2	0.221	0.290	−0.370	0.718
3	−0.530	0.598	−0.098	0.335
4	0.167	0.096	−0.228	0.022

[a]Sample 1 directly following inoculation. Samples 2 and 4 were following 24 h incubation under cool, moist conditions. Sample 3 followed 24 h incubation under hot, dry conditions. See text for details. These data correspond to the whole-leaflet samples 1–4 in Table 2.
[b]Pearson product moment correlation. n = 100 for all samples.
[c]Probability of obtaining a more extreme value of r, indicates the significance of the correlation.

rigorous distributional test. Neither the normal nor the lognormal could be rejected as possible descriptors of the distribution of population sizes among plants, and the lognormal was also not inappropriate for describing the population per gram among plants (Table 2; hypothesis of normality not rejected for nontransformed or log-transformed data).

Scaling Factors and Population Assessment

Population sizes are generally scaled on a per sample unit, log per sample unit, per sample unit weight, or log per sample unit weight basis. Depending on the variability in weight relative to population sizes among sampling units, scaling on a per unit weight basis may increase, decrease, or have no effect on the sample variance (Table 2). In 100-leaflet samples of inoculated greenhouse plants, leaflet weight was not consistently correlated with bacterial populations (Table 3). Additionally, there were sometimes significant negative correlations between population density (population size per gram per leaflet) and leaflet weight (Table 3). In these cases, heavier leaflets (leaflets that weighed more, though not necessarily leaflets having larger surface areas) supported smaller population densities than the leaflets with less mass.

Quantifying Differences Among Units

Data from multiple segments of individual leaflets permit a test of the hypothesis that the mean population size per segment is not significantly different among leaflets. Using data from all leaflet segments for the three leaflets described in Table 1, this hypothesis is rejected (log colony-forming units [cfu] per segment $F = 58.10$, $P = .0001$; log CFU per gram $F = 59.06$, $P = .0001$). Thus, based on these data, we conclude that the mean population per 9-mm^2 segment (per unit area) is not the same among different leaflets. Among sampled leaflets, larger leaflets having a greater area tended to have larger total populations, apparently

as a function of both more surface area and greater mean numbers of individuals per unit area (Table 1).

Multiple samples of individual leaflets from the same plant allow a test of the hypothesis that mean bacterial population size per leaflet is not significantly different among plants. Under field conditions, mean population sizes of bacterial strain TLP2 on individual potato leaflets were significantly different on different plants (log CFU per leaflet; $F = 3.27$, $P = .0098$). Thus, plants with the same or similar numbers of leaflets may support different total bacterial populations. The influence of leaflet position (height) within the potato plant canopy on bacterial population size was also investigated using these data. We found no significant influence of leaflet position on mean bacterial population size per leaflet (log CFU per leaflet; $F = .14$, $P = .9676$). In the greenhouse, mean population sizes of strain B728a per leaflet and per gram per leaflet were also significantly different on different plants (log per leaflet $F = 3.28$, $P = .0157$; log per gram $F = 4.63$, $P = .0024$). Thus, in both the field and in controlled environmental conditions in the greenhouse, individual plants appear to differ in their ability to support inoculated bacterial populations.

Comparing Population Data Among Samples Taken at Different Scales

At similar sampling intensities (sample size n = 5), no clear reduction in variance could be obtained by the selection of one sampling scale over the others (Table 4). Though variance in population size among segments is smaller than that among leaflets or among plants—reflecting in part the much smaller population sizes on leaflet segments versus on leaflets or whole plants—once data are appropriately transformed, differences in variance associated with scale are no longer consistent (Table 4).

Because bacterial populations were aggregated among sampling units at all scales, estimates of the mean log cells per gram based on samples taken at a small scale will underestimate the log cells per gram at a larger scale (Table 5). Alternatively, Table 5 illustrates that whole-leaflet samples will behave as "bulked" samples of leaflet segments and will overestimate the population size per gram on individual leaflet segments [12, 14]. Whole-plant samples will similarly overestimate the population size per gram on individual leaflets.

Discussion

Analysis of population sizes of introduced epiphytic bacteria based on leaflet segment, whole-leaflet, and whole-plant data provide fundamentally different sorts of biological information. For example, the data on populations on adjacent leaflet segments provide information on the level at which bacterial populations are aggregated on individual leaflets and thus on the scale at which sites for bacterial growth and survival are distributed across the leaflet surface. Data from whole-leaflet samples reflect the variability among leaflets in ability to support bacterial populations, integrating both the nutritional and the physical environment on leaflets. Finally, whole-plant samples illustrate the differences in whole-plant–mediated bacterial habitat quality and quantity. What is common to all of these sampling

Table 4. Influence of sampling unit scale (leaflet segments, whole leaflets, whole plants) on variability among units[a]

Populations	Mean	Variance
Population per unit		
Leaflet segments[b]		
Leaflet 1	151	59,633
Leaflet 2	75	17,132
Leaflet 3	52	6,160
Whole leaflets[b]		
Sample 2	221,368	7.50×10^{10}
Sample 3	26,765	7.45×10^{8}
Sample 4	2.90×10^{6}	7.67×10^{12}
Whole plants[b]		
Sample 1	5,720	8.41×10^{6}
Log population per gram		
Leaflet segments		
Leaflet 1	4.78	0.21
Leaflet 2	3.93	1.58
Leaflet 3	4.03	0.36
Whole leaflets		
Sample 2	5.25	0.17
Sample 3	4.46	0.47
Sample 4	6.40	0.19
Whole plants		
Sample 1	2.95	0.07

[a] All data are presented on a per sampling unit basis and on a log per gram basis. Data are the mean values for both the mean and variance from 20 independent samples of n = 5 (5 segments, 5 leaflets, or 5 plants) to remove influences of sample size on variance.

[b] All plants were inoculated and maintained under greenhouse conditions as described in the text. See text for details of segment, leaflet, and whole-plant sampling strategies. Leaflets 1, 2, and 3 correspond with leaflets 1, 2, and 3 in Table 2; whole-leaflet samples 2, 3, and 4 correspond with whole-leaflet samples 2, 3, and 4 in Table 2; the whole-plant sample corresponds with the whole-plant sample of strain B728a in the greenhouse in Table 2.

scales is the high degree of clustering of bacterial population sizes among sampling units. At the level of leaflet segments, leaflets, and entire plants, bacterial populations were tremendously variable among units. Thus, individual segments on leaflets vary greatly in their ability to support bacterial populations, as do leaflets and individual plants. At each scale, this variability likely integrates a variety of factors, including differences in bacterial immigration or inoculation, availability of resources such as nutrients and moisture, and protection from environmental stresses. Understanding the scales at which bacterial populations vary in space and in time is critical to determining the factors most important in bacterial colonization and thus in causing population variance.

The lognormal distribution is used commonly in the description of epiphytic populations on leaves [7]. Although bacterial populations among leaflet segments, among leaflets, and among whole plants were often well described by the lognormal distribution, this was not always the case. Even for the individual leaflet samples,

Table 5. Epiphytic bacterial population estimates per gram of leaflet tissue based on mean population per gram among all leaflet segments and on the total population summed over all leaflet segments and scaled by total leaflet weight for the same leaflets[a]

	Mean log CFU per gram among leaflet segments[b]	Total log CFU per gram (all segments)[c]
Leaf 1	4.816	5.000
Leaf 2	3.892	4.302
Leaf 3	3.875	4.219

[a]CFU, colony-forming units.
[b]Calculated as mean log (population per segment/weight per segment) for all segments.
[c]Calculated as log (total population summed over all segments/leaflet total weight).

the lognormal did not always provide an appropriate description of the distribution of population sizes among sampling units. Thus, the routine use of the lognormal distribution to summarize and analyze epiphytic populations may not be appropriate, and these data suggest that the appropriateness of the lognormal should always be evaluated before its application to a particular data set. Unfortunately, the large sample sizes used in this study are not frequently employed in ecological work, and the ability to determine whether the lognormal is an inappropriate distribution for a small data set is difficult. Nonparametric tests offer an alternative means for analyzing data in cases where distributional assumptions are not met, yet these tests may lack statistical power relative to distribution-based or normal statistics [5].

Selection of a sampling scale may be based on a variety of objectives and limitations, such as methodological considerations of the number of samples that can be processed and the ease of processing, biological features of plants, and attempts to reduce sample variance. The high level of variability in bacterial populations among sampling units at every scale investigated and regardless of the bacterial strain or plant species indicates that among leaflet segments, leaflets, or entire plants there is no optimal scale at which variance among units is significantly reduced. This contrasts with what has been observed in the sampling of rhizobacterial populations, where variability was significantly greater for root segments than for whole roots [15]. Thus, in the sampling of epiphytic bacterial populations, selection of sampling units should be based on methodological and biological reasons and not on an assumption of reduced variance at greater or smaller sampling scales (e.g., leaflets vs. leaflet segments vs. plants). For instance, the subsequent incidence of brownspot disease has been accurately described by the frequency of leaves having population sizes above a threshold population required for disease initiation [8, 19]. Because disease occurs on individual leaves, whole plant samples may have obscured such a relationship. However, simple detection of genetically distinguishable microorganisms, such as released recombinant strains of bacteria, may be facilitated by large sample units such as whole plants.

Methodological considerations in the selection of sampling strategies should center on ease of sampling and sample processing rather than any perception of

reduced variance as a function of sampling scale. However, once a sampling scale has been determined, variance estimates will be fundamental to selecting an appropriate sample size (n). The variability of bacterial population size estimates in single leaflet samples was substantially greater when leaflets were harvested from field rather than greenhouse environments (Table 2). This finding suggests that strategies that develop protocols through sampling based on greenhouse and microcosm studies may underestimate the sample sizes needed for adequate description of population sizes under field conditions. A consistently greater variance of bacterial population sizes among sampling units under field conditions may obscure our ability to detect differences among treatments. Further work will be needed to determine whether the higher variance associated with field-collected leaflets and plants is a general phenomenon or whether it is associated with particular environmental or biological features. Nonetheless, extrapolation from laboratory studies to the field must be made with caution, and appropriate estimates of sample variance should be made before selecting field sampling strategies.

Despite the high levels of variability among segments within individual leaflets, and among leaflets within individual plants, we found that these differences were sometimes significantly less than the differences among leaflets of different plants or among different plants. Individual leaflets sometimes supported significantly different populations, and separate plants also supported significantly different populations per gram in some cases. This points to a flaw in the standard scaling of populations on a per unit weight basis. When leaves or leaflets are the sampling units and populations are scaled on a per gram basis, if the leaves or leaflets support significantly different populations per gram, the scaling factor does not contribute useful biological information and may only confuse the issue. When leaflet weight and bacterial population sizes are not significantly correlated, scaling does not provide consistent reductions in, and may actually increase, sample variance. The rationale for scaling on a per gram basis thus needs to be carefully considered. Specifically, scaling should be based on biological information about the population or process under study in relation to the scaling factor. Studies on the relationship between leaf weight and nutrient availability, immigration rates, or number of colonizable sites are needed to determine the value of routine scaling of population data on a sample unit weight basis.

An additional caution relating to the scaling of population data on a per gram basis concerns the potential utility of such scaling in permitting comparisons among samples collected at different sampling scales. Scaling on a per gram basis does not result in population estimates that are comparable among samples taken at different scales. The data show that these samples behave as "bulked" samples [12, 14], and because of the clustering of bacterial populations at every scale investigated, such samples provide inaccurate estimates of populations per gram at a higher or lower scale. Specifically, mean log population per gram of leaflet segment underestimates the log population per gram of leaflet. Also, the log population per gram of leaflet overestimates the mean log population per gram of leaflet segment. Thus, scaling on a per gram basis does not provide a simple way of contrasting population data collected based on different sampling units.

Samples of different unit size provide very different sorts of information to researchers and may sometimes provide conflicting information on microbial interactions [13]. Insufficient effort has been made to understand the influence of sample

scale on the study of microbial populations and microbial interactions on plants. These data highlight the importance of understanding microbial populations at multiple scales and of designing sampling strategies and summary statistics based on clearly defined biological and methodological objectives.

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