APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1991, p. 51–56
0099-2240/91/010051-06$02.00/0

Vol. 57, No. 1

Evaluation of Methods for Sampling, Recovery, and Enumeration of Bacteria Applied to the Phylloplane

KATHERINE DONEGAN,[1]* CARL MATYAC,[1]† RAMON SEIDLER,[2] AND ARLENE PORTEOUS[2]

NSI Technology Services Corporation[1] and Environmental Research Laboratory, U.S. Environmental Protection Agency,[2] Corvallis, Oregon 97333

Received 3 July 1990/Accepted 16 October 1990

Determining the fate and survival of genetically engineered microorganisms released into the environment requires the development and application of accurate and practical methods of detection and enumeration. Several experiments were performed to examine quantitative recovery methods that are commonly used or that have potential applications. In these experiments, *Erwinia herbicola* and *Enterobacter cloacae* were applied in greenhouses to Blue Lake bush beans (*Phaseolus vulgaris*) and Cayuse oats (*Avena sativa*). Sampling indicated that the variance in bacterial counts among leaves increased over time and that this increase caused an overestimation of the mean population size by bulk leaf samples relative to single leaf samples. An increase in the number of leaves in a bulk sample, above a minimum number, did not significantly reduce the variance between samples. Experiments evaluating recovery methods demonstrated that recovery of bacteria from leaves was significantly better with stomacher blending, than with blending, sonication, or washing and that the recovery efficiency was constant over a range of sample inoculum densities. Delayed processing of leaf samples, by storage in a freezer, did not significantly lower survival and recovery of microorganisms when storage was short term and leaves were not stored in buffer. The drop plate technique for enumeration of bacteria did not significantly differ from the spread plate method. Results of these sampling, recovery, and enumeration experiments indicate a need for increased development and standardization of methods used by researchers as there are significant differences among, and also important limitations to, some of the methods used.

The monitoring of genetically engineered microorganisms released into the environment requires accurate and efficient sampling, recovery, and enumeration methods. The majority of research on detection and quantification of microorganisms has considered released microorganisms in controlled or relatively simple environments or indigenous, rather than applied, microorganisms in more complex environments. The development and comparison of standard methodologies for detecting and enumerating microorganisms applied to the phylloplane have been minimal.

Factors which must be considered in developing a sampling scheme for microorganisms applied in natural environments include the size, frequency, and manner of selection of samples. Most researchers studying microbial populations on leaves use bulk samples of varying leaf numbers (12, 13). The issue of sample size, specifically, whether leaf samples should be collected and processed individually or pooled, has been addressed by several researchers (6, 7). In their experiments the distribution characteristics of indigenous epiphytic bacterial populations have been assessed; results indicate a lognormal distribution of the indigenous bacteria and an overestimation of the median value of a population when bulk samples are used. These researchers have developed an algorithm which compensates for this overestimation by using the variance of the population that is calculated from single leaf samples. The question of whether similar distribution characteristics exist for applied bacteria and the feasibility of using such algorithms in population estimates of applied bacteria have not been addressed.

Assuming the collection of representative samples, efficient and consistent methods of recovery of the microorganisms from the samples must be developed. The recovery method needs to be consistent among samples, and the efficiency should also be independent of the species of the microorganism and of the length of time since its application. In addition, an assay method should recover the same relative proportion of microorganisms over a range of leaf densities of microorganisms (2). Four processing methods for the recovery of microorganisms from leaves, washing, blending, stomacher blending, and sonication, are commonly used by researchers (3, 11, 14, 17). Only some of these methods have been compared and may vary in their effectiveness, and yet results of studies from laboratories which use different processing methods are frequently compared.

The plating of microorganisms recovered from samples requires not only a high degree of accuracy and reproducibility but also expediency, as field sampling frequently involves large numbers of samples. The use of drop, rather than spread, plating has been suggested and has the advantage of greatly reducing the number of plates which must be prepared, inoculated, and counted (1, 9). Delayed processing of collected samples, such as by storage at room or freezing temperatures, has also been used to make large experiments more manageable (8). Experimental and statistical evaluation of these methods has been limited.

To address the aforementioned concerns, several aspects and types of methods were evaluated in experiments following application of *Erwinia herbicola* and *Enterobacter cloacae* to Blue Lake bush beans (*Phaseolus vulgaris*) and Cayuse oats (*Avena sativa*). To examine sampling methodology, differences between single and bulk leaf samples and the effect of bulk sample size were evaluated. In evaluating the recovery of microorganisms from samples, the concentration of microorganisms in the sample, the time between

* Corresponding author.
† Present address: North Carolina Agricultural Extension Service, Durham, NC 27701.

collection and processing of samples, and the mechanical device used to process samples were all considered. To determine the most efficient and accurate plating method, drop and spread plate methods were compared.

MATERIALS AND METHODS

Sampling experiments: single versus bulk leaf samples and sample size. The experiment was performed once with oat plants and twice with bean plants. Twelve trays of plants were used in each experiment. Bean and oat seeds were planted in trays (64 by 25 by 13 cm) containing Premier Brands Pro-mix Bx potting soil (Steuber Distributing Co., Seattle, Wash.) and grown in a greenhouse at 28°C. After 3 weeks, plants were sprayed with a plastic misting bottle until runoff with a suspension of *Erwinia herbicola* (EPA 81A; obtained from S. Beer, Cornell University, Ithaca, N.Y.) resistant to 500 μg of nalidixic acid per ml. The suspension was prepared from an 18-h culture of *E. herbicola* grown in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, Mich.) with 500 μg of nalidixic acid (Sigma Chemical Co., St. Louis, Mo.) per ml. The culture was washed twice in 0.01 M phosphate buffer (pH 7.2) by centrifugation for 10 min at 3,836 × *g* and resuspended in phosphate buffer to a concentration of ca. 10^7 to 10^8 CFU/ml.

For the single leaf samples, 24 leaves were aseptically collected in sterile lab bags (Tekmar Co., Cincinnati, Ohio), weighed, and processed individually in 10 ml of phosphate buffer. For the bulk samples, three samples, each consisting of five leaves, were similarly collected, weighed, and processed in 20 ml of phosphate buffer. Bulk and single leaf samples were collected and processed 0, 1, 3, 7, 14, 21, 28, and 35 days after plants were sprayed. In addition, in the oat experiment and one of the bean experiments, bulk leaf samples of 2, 5, 10, 15, and 20 leaves were similarly collected and processed in 30, 75, 100, 100, and 100 ml of phosphate buffer, respectively; these samples were collected in triplicate and processed on days 7, 14, and 21 postspray. All samples were blended for 1 min in a Stomacher Lab-Blender (a device with two reciprocating paddles that apply pressure to the bagged sample [Tekmar Co.]). Samples were then serially diluted in phosphate buffer, drop plated (10 μl/drop, 4 drops per concentration, 4 concentrations per plate) in duplicate on LB agar plus 500 μg of nalidixic acid per ml and 100 μg of cycloheximide (Sigma) per ml and incubated at 30°C for 24 h.

Colony counts from duplicate plates were averaged and converted to CFU per gram of leaf weight and log CFU per gram. The distribution of single leaf samples was evaluated with histograms of nontransformed (CFU per gram) and log-transformed (log CFU per gram) data. Mean and median values, variance, and confidence intervals for log-transformed data were calculated by using the Statistical Analysis System (SAS; SAS Institute, Cary, N.C.) and compared for the single and bulk leaf samples. An algorithm (6) was used to adjust the mean of bulk leaf samples by the variance of the single leaf samples and the adjusted values were then compared with values from single and bulk leaf samples. Variance-to-mean ratios were plotted over time and used as indicators to evaluate the changes in distribution of bacteria among leaf samples (5, 10). The variance of the bulk leaf sample population was plotted against leaf number per sample and used as an estimate of the increased precision obtained by changing the number of leaves per sample (2).

Recovery experiments: comparison of processing methods. Bean plants, 2.5 weeks old and grown in trays as described previously, were sprayed with a plastic misting bottle until runoff with a suspension of *Enterobacter cloacae* (EPA 113A; isolated from the gut of the cutworm *Peridroma saucia*) resistant to 100 μg of rifampin per ml. The inoculum was prepared as previously described (100 μg of rifampin per ml was used in the culture broth instead of nalidixic acid) and resuspended in sterile water to a concentration of ca. 2×10^7 CFU/ml. Nine six-leaf samples per processing method were aseptically collected 0 and 14 days postspray and weighed. Samples were processed by one of the following methods. (i) In washing, the six leaves were aseptically cut into ca. 3-cm strips, placed in a sterile 250-ml Erlenmeyer flask with 75 ml of 0.01 M Tris buffer (pH 7.5), and shaken for 2 h on a Psychrotherm shaker (New Brunswick Scientific Co., New Brunswick, N.J.). (ii) In blending, the six leaves were aseptically cut into ca. 3-cm strips, placed in a sterile blender (Waring Products, New Hanford, Conn.) with 50 ml of Tris buffer, and blended at high speed for 1 min. (iii) For sonication, the sample was divided into two three-leaf samples, placed in sterile lab bags with 20 ml of Tris, processed in a sonicator (Branson Equipment, Shelton, Conn.) for 7 min, and recombined. (iv) In Stomacher blending, the sample was divided into two three-leaf samples, placed in sterile lab bags with 20 ml of Tris, processed for 1 min in a Stomacher Lab-Blender, and recombined.

Samples from all processing methods were serially diluted in Tris buffer, spread plated (100 μl/plate) in duplicate on LB agar plus 100 μg of rifampin per ml and 25 μg of cycloheximide per ml and incubated for 24 h at 30°C. Colony counts from duplicate plates were converted to log CFU per gram and used in the SAS analysis of variance (ANOVA) and Student-Neuman-Keuls test to determine whether the mean log CFU-per-gram values of each of the four processing methods were significantly different.

Effect of bacterial concentration in sample on recovery efficiency. Three-week-old bean plants, grown in trays as described previously, were sprayed with a plastic misting bottle until runoff with bacterial suspensions prepared as described previously. Two experiments used *Erwinia herbicola* and two experiments used *Enterobacter cloacae* (EPA 107A; isolated from the gut of the cutworm *P. saucia*, resistant to 500 μg of nalidixic acid per ml). In each experiment, a low (ca. 10^4 to 10^5 CFU/ml) and a high (ca. 10^7 to 10^{10} CFU/ml) concentration of bacteria suspended in phosphate buffer and a sterile phosphate buffer solution were applied, with five plant trays per concentration or solution. After 2 h all leaves were removed and aseptically cut into ca. 3-cm^2 pieces and placed in sterile containers. Pieces of leaves sprayed with bacteria were mixed with pieces of leaves sprayed only with phosphate buffer in proportions so that the final samples contained 3 g of leaf material and consisted of 2, 5, 10, 20, 50, or 100% leaves sprayed with bacteria. Samples for every proportion were prepared in triplicate in sterile lab bags, processed in 20 ml of phosphate buffer in the Stomacher Lab-Blender for 1 min, and serially diluted in phosphate buffer. Dilutions were drop plated in duplicate on LB agar plus 500 μg of nalidixic acid per ml and 100 μg of cycloheximide per ml and incubated at 30°C for 24 h.

Colony counts were averaged for duplicate plates and converted to CFU per gram and used in the SAS regression analysis, with CFU per gram as the dependent variable and proportion of leaves sprayed with bacteria as the independent variable.

Effect of freezing samples on recovery of bacteria. Three-week-old bean plants, grown in trays as described previ-

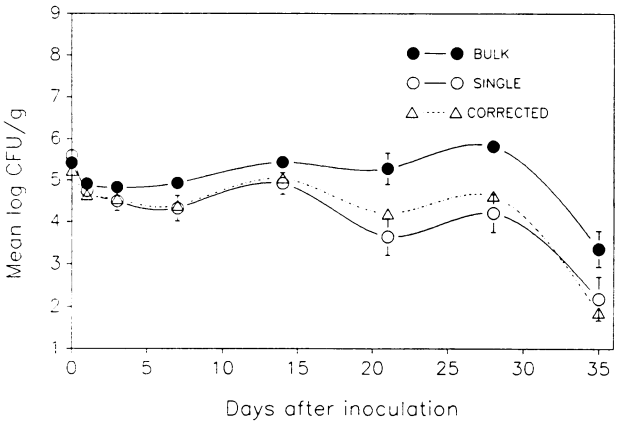

FIG. 1. Mean log CFU-per-gram values over time of *Erwinia herbicola* recovered from single and bulk leaf samples from oat plants in experiment 3. Corrected line is algorithmic adjustment of bulk sample values by variances of single samples.

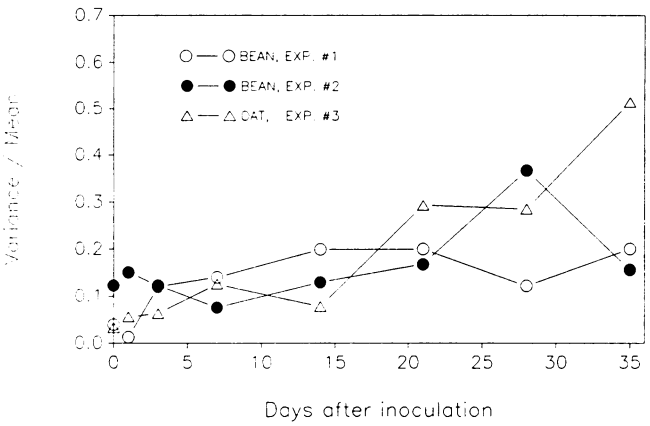

FIG. 2. Variance/mean ratio over time of single leaf samples from oat and bean plants sprayed with *Erwinia herbicola* in experiments 1, 2, and 3.

ously, were sprayed with a plastic misting bottle until runoff with a ca. 3×10^7-CFU/ml suspension of *Erwinia herbicola* in phosphate buffer (prepared as described previously). Six samples, four leaves per sample, were aseptically collected for each treatment, placed in sterile lab bags, and weighed. Treatments consisted of freezing samples at −20°C for 1, 3, or 14 days, with and without the addition of 20 ml of phosphate buffer before freezing. Two hours postspray, samples were immediately processed in a Stomacher Lab-Blender in 20 ml of phosphate buffer for 1 min and serially diluted in phosphate buffer. Dilutions were drop plated in duplicate on LB agar plus 500 μg of nalidixic acid per ml and 100 μg of cycloheximide per ml and incubated for 24 h at 30°C. On days 1, 3, and 14, frozen samples were thawed for 30 min at room temperature, 20 ml of phosphate buffer was added to those samples frozen without buffer, and samples were processed and plated as described above. Three weeks after the plants had been sprayed, samples were again collected to examine whether the effects of immediate and delayed processing on recovery were different for plants with established bacterial populations. Sample size, processing, and plating were the same as above except that samples were frozen for 0, 1, 3, or 6 days and all samples were frozen without the prior addition of phosphate buffer.

Colony counts were averaged from duplicate plates and converted to log CFU per gram and used in the SAS ANOVA and Tukey's Studentized Range Test to determine whether mean values were significantly different among treatments.

Plating experiments: comparison of drop and spread plating. Four sets of seven concentrations, ranging from ca. 10^2 to 10^8 CFU/ml, were prepared by serially diluting a ca. 10^9-CFU/ml suspension of *Enterobacter cloacae* EPA 107A (prepared as described previously) in phosphate buffer. Each drop plate contained four 10-μl drops of four of the concentrations, for a total of 16 drops per plate. Each spread plate contained 100 μl of one concentration spread onto the surface of the agar. Both spread and drop plates were prepared in duplicate on LB agar containing 500 μg of nalidixic acid per ml and incubated at 30°C for 24 h.

Colony counts were taken from spread plates with 20 to 200 colonies and from drop plates with 20 to 60 colonies. Counts were averaged for duplicate plates and adjusted for the dilution factor. Adjusted colony counts were used in the SAS ANOVA and Student-Newman-Keuls test to test for significant differences in counts between the spread and drop plate methods.

RESULTS

Sampling experiments: single versus bulk leaf samples and sample size. In all three experiments, bacterial counts from single leaf and bulk leaf samples collected 1 to 7 days after application were similar but were significantly ($P < 0.05$) higher for bulk samples collected 14 days or longer after bacterial application (Fig. 1). Over the three experiments, the bulk leaf sample values fell within the 95% confidence intervals of the single leaf samples only 33% of the time, indicating that the bulk leaf samples overestimated 66% of the time. Application of an algorithm for correcting values obtained from bulk leaf samples produced corrected bulk values that were similar to those of the single leaf samples 70% of the time (Fig. 1). In all three experiments, histograms of single leaf samples indicated a lognormal distribution and the variance/mean ratio for the single leaf samples increased over time (Fig. 2).

In the two experiments on size of bulk leaf samples, the

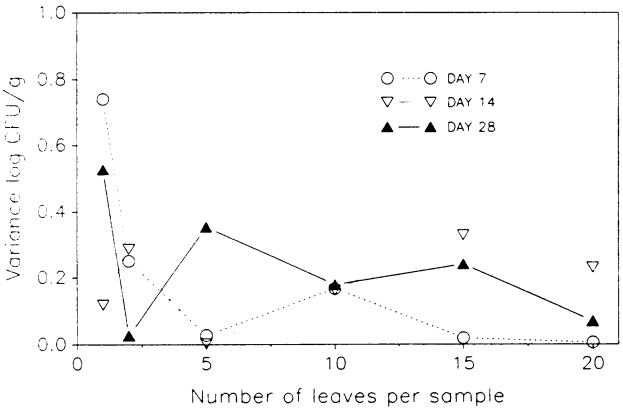

FIG. 3. Sample variance versus leaf number per sample of *Erwinia herbicola* recovered from bean plants 7, 14, and 21 days after inoculation in experiment 2.

TABLE 1. Comparison of efficiencies of processing techniques for recovering *Enterobacter cloacae* from bean leaves

Mean (log CFU/g)[a]	n	Treatment
6.59 A	18	Stomacher blending
6.39 B	18	Blending
6.00 C	18	Sonication
5.88 D	18	Washing

[a] Student-Newman-Keuls test grouping: means with the same letter are not significantly different at the 5% level. Averaged values of nine samples collected on days 0 and 15 are shown.

sample variance versus leaf number per sample, for all three sample days, declined with an increase in sample size but the decline beyond two leaves per sample was not significant ($P < 0.05$) (Fig. 3).

Recovery experiments: comparison of processing methods. The ANOVA of the log CFU-per-gram values from the four recovery methods indicated a significant ($P < 0.01$) effect of the methods on numbers of bacteria recovered on both days 0 and 15 (Table 1). The comparison of means test, for combined day 0 and 15 values, indicated that all methods were significantly ($P < 0.05$) different from one another and ranked, from best to worst, in efficiency of numbers of bacteria recovered as follows: stomacher blending, blending, sonication, washing.

Effect of bacterial concentration in sample on recovery efficiency. In all of the experiments, plotting of the CFU-per-gram values against proportion of sprayed leaves produced a straight line (Fig. 4). The linearity of the data and the high coefficient of determination values (Table 2) indicate that recovery with stomacher blending is constant over a range of leaf bacterial populations.

Effect of freezing samples on recovery of bacteria. In the experiment with samples collected 2 h after bacteria were applied to the plants, bacterial numbers declined the longer the samples were frozen and when buffer was added to the samples before freezing. Although the number of bacteria recovered from samples frozen for only 1 or 3 days, without the addition of buffer, did not significantly differ from samples that were processed immediately, the number of bacteria recovered from samples frozen for 14 days was significantly lower (Table 3). For all periods of freezing, the addition of buffer to samples before freezing, compared with that after freezing, resulted in lower recovery levels of bacteria.

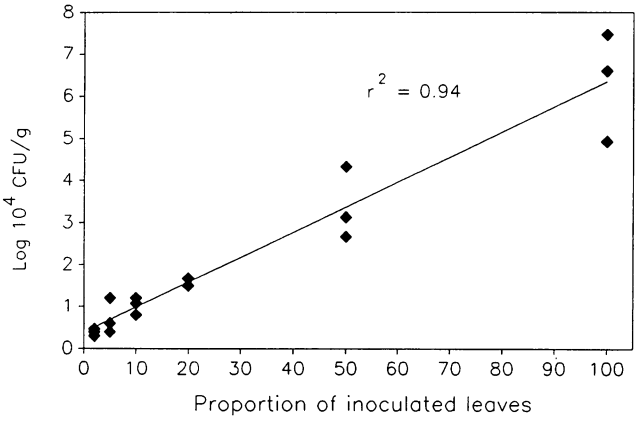

FIG. 4. Effect of bacterial concentration in sample on recovery efficiency of *Enterobacter cloacae*, applied at a concentration of 4.27×10^4 CFU/ml, from 18 3-g leaf samples containing a proportion of sprayed leaves ranging from 2 to 100%.

TABLE 2. Effect of bacterial concentration in sample on recovery efficiency

Expt	Bacterium	Spray concn (log CFU/ml)[a]	r^2[b]
1	*Erwinia herbicola*	4.81	0.94
1	*E. herbicola*	8.56	0.83
2	*Enterobacter cloacae*	4.63	0.94
2	*E. cloacae*	10.58	0.86
3	*E. cloacae*	5.32	0.94
3	*E. cloacae*	7.48	0.83
4	*E. herbicola*	4.63	0.91
4	*E. herbicola*	8.22	0.61

[a] Concentration of spray solution applied until runoff on bean plants.
[b] Coefficient of determination values from regression of log CFU-per-gram values of 18 samples (three replicates of samples with 2, 5, 10, 20, 50, and 100% proportion of sprayed leaves).

In the experiment with samples collected 21 days after application of the bacteria to the plants, the comparison of means test indicated no difference in number of bacteria recovered among the freezing treatments (Table 4).

Plating experiments: comparison of drop and spread plating. Colony counts did not significantly differ ($P < 0.05$) between drop and spread plates (Table 5).

DISCUSSION

The results for the sampling, recovery, and enumeration experiments indicate substantial variation in the efficiencies of the methods examined.

Bulk leaf sampling is commonly used in field research, rather than single leaf sampling, because it is considered to be a relatively quick yet representative process for quantifying microbial populations. The spatial pattern of microorganisms applied to plants, assuming an initial even spray, is uniform between leaves in the few days following application. The variance/mean ratios were very low in the initial phases of the experiments but increased with time. This indicates that the variation in the spatial pattern of applied bacteria increases with time and is likely due to such factors as microclimates, unequal distribution of nutrients, and competition from indigenous microbial populations, creating microniches and consequent clustering of the applied microorganisms. Such an increase in the variation of population

TABLE 3. Comparison of immediate and delayed processing of samples collected 2 h postspray on recovery of *Erwinia herbicola* from bean leaves

Mean (log CFU/g)[a]	n	Treatment[b]
6.29 A	6	0 days, − buffer
5.89 AB	6	1 day, − buffer
5.83 AB	6	3 days, − buffer
5.22 BC	6	1 day, + buffer
5.02 CD	6	3 days, + buffer
4.43 DE	6	14 days, − buffer
3.86 E	6	14 days, + buffer

[a] Tukey's Studentized Range Test grouping: means with the same letter are not significantly different at the 5% level.
[b] Treatments were 0 to 14 days of storage at −20°C, with and without the addition of phosphate buffer before freezing.

TABLE 4. Comparison of immediate and delayed processing of samples collected 3 weeks postspray on recovery of *Erwinia herbicola* from bean leaves

Mean (log CFU/g)[a]	n	Treatment[b]
4.68	6	6 days, − buffer
4.36	6	0 days, − buffer
4.13	6	1 day, − buffer
3.97	6	3 days, − buffer

[a] None of the means were significantly different at the 5% level.
[b] Treatments were 0 to 6 days of storage at −20°C, without the addition of phosphate buffer before freezing.

spatial pattern between individual leaves was observed at approximately 7 days postspray in the sampling experiments. Interestingly, approximately 7 days postspray is also when values for bulk samples began to diverge from those for single leaf samples. A possible explanation is that with a shift in a population from a normal distribution to a lognormal distribution the mean no longer represents the median (6). Hirano et al. found that the magnitude of overestimation of the mean population size by bulk sampling depends on the population variance (6). The algorithm that we tested (6) used the variance of the single leaf samples to adjust the mean of the bulk sample; when the variance is low, as in the few days after application of the bacteria, little adjustment of the bulk sample values is required. It has also been suggested that larger bulk samples result in more accurate estimates of bacterial populations (4). Our results indicate that the variance between samples containing one or two leaves is quite high but that the variance of samples with five leaves is not significantly higher than that found between samples containing up to 20 leaves. For bulk samples containing a minimum of three leaves, an increase in the number of samples collected, rather than in the number of leaves in each sample, is more important in precise estimation of bacterial populations.

The significant difference in the number of microorganisms recovered by the four processing methods is important as all four methods are commonly used by researchers (3, 11, 14, 17). Our results suggest that, under the conditions tested, processing of samples in a Stomacher Lab-Blender allows the greatest recovery of microorganisms from the leaf surface along with minimal adverse effects on viability. In addition, the method is also the most expeditious because (i) in the field samples can be collected directly into sterile processing bags; (ii) the processing time of 1 min is relatively quick; and (iii) disposal of the bagged samples is simple. Both the blending and washing methods require sterilization and decontamination of containers, a time-consuming task in experiments with numerous samples. The sonication and, particularly, the washing methods involve long processing times per sample. Processing in a Stomacher Lab-Blender, along with the blending method, does have the disadvantage of releasing plant cell contents, which may be inappropriate in some experiments.

TABLE 5. Comparison of the spread and drop plating techniques

Mean (log CFU/g)[a]	n	Treatment
9.61	8	Spread plating
9.56	8	Drop plating

[a] The means were not significantly different at the 5% level. Concentrations of *Enterobacter cloacae* that were diluted and plated are shown.

Consistent recovery over a range of leaf microbial populations is an important attribute of a method for enumerating applied microorganisms. The effectiveness of stomacher blending in providing constant recovery regardless of sample concentration promotes confidence in using the method in extended field trials as the range of population levels of released microorganisms typically increases over time.

In large-scale experiments the collection of samples may be so time-consuming that the processing of the samples on the same day is not possible; an efficient method of delayed processing for such situations would be advantageous. Our results suggest that the freezing of collected samples, with or without the addition of buffer, may adversely affect survival or recovery of microorganisms in the samples. The lack of a significant difference in number of bacteria recovered from samples frozen without buffer which were processed 0, 1, and 3 days after collection in experiment 1 and 0, 1, 3, and 6 days in experiment 2 suggests that a delay of a few days in processing is possible but that longer storage periods are not practical, as indicated by the significant decline in recovery of samples frozen for 14 days. The adverse effect of the prefreezing addition of buffer on number of bacteria recovered may be due to bacterial injury resulting from the freezing and thawing of the buffer (16).

The lack of a significant difference between the drop and spread plating procedures is noteworthy in that drop plating, because of the large reduction in the number of plates required, is a very practical means of accommodating the large numbers of samples typically necessary in field work. Drop plating four concentrations per plate, each in quadruplicate, uses 1/16 of the number of plates required for similar spread plating. It is advisable, however, always to use at least two drop plates per sample to allow for detection of unintentional differences between plates in media and antibiotic concentration. In addition, the level of detection is 10-fold less than that possible with 100-μl spread plates because drop plating uses only 10 μl of the sample.

The frequency of the release of genetically engineered microorganisms, and of the studies designed to determine the consequent environmental effects, is likely to increase; our results indicate a need for continued development and standardization of the methods required in such studies.

ACKNOWLEDGMENTS

We thank Valerie Prince for technical assistance and Hal Kibby for support of this research. We express our appreciation to Lisa Ganio for statistical review and Steven Lindow for scientific review of the manuscript.

REFERENCES

1. **Badger, E. H., and E. S. Pankhurst.** 1960. Experiments on the accuracy of surface drop bacterial counts. J. Appl. Bacteriol. **23**:28–36.
2. **Campbell, C. C., and L. A. Nelson.** 1986. Evaluation of an assay for quantifying populations of scletoria of *Macrophomina phaseolina* from soil. Plant Dis. **70**:645–647.
3. **Conlin, K. C., and S. M. McCarter.** 1983. Effectiveness of selected chemicals in inhibiting *Pseudomonas syringae* pv. tomato in vitro and in controlling bacterial speck. Plant Dis. **67**:639–644.
4. **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.
5. **Elliot, J. H.** 1983. Statistical analysis of samples of benthic invertebrates. Freshwater Biol. Assoc. Sci. Publ. no. 25.

6. **Hirano, S. S., E. V. Nordheim, D. C. Arny, and C. D. Upper.** 1982. Lognormal distribution of epiphytic bacterial populations on leaf surfaces. Appl. Environ. Microbiol. **44:**695–700.
7. **Hirano, S. S., and C. D. Upper.** 1986. Temporal, spatial and genetic variability of leaf-associated bacterial populations, p. 235–251. *In* N. J. Fokkema and J. Van Den Heuvel (ed.), Microbiology of the phyllosphere. Cambridge University Press, New York.
8. **Hirano, S. S., and C. D. Upper.** 1989. Variation in population size and ice nucleation activity of *Pseudomonas syringae* on snap bean leaflets. Appl. Environ. Microbiol. **55:**623–629.
9. **Hoben, H. J., and P. Somasegaran.** 1982. Comparisons of the pour, spread, and drop plate methods for enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. Appl. Environ. Microbiol. **44:**1246–1247.
10. **Johnson, K. B., J. D. Apple, and M. L. Powelson.** 1988. Spatial patterns of *Verticillium dahliae* propagules in potato field soils of Oregon's Columbia Basin. Plant Dis. **72:**484–488.
11. **Knudsen, G. R., M. V. Walter, A. Porteous, V. J. Prince, J. L. Armstrong, and R. J. Seidler.** 1988. Predictive model of conjugative plasmid transfer in the rhizosphere and phyllosphere. Appl. Environ. Microbiol. **54:**343–347.
12. **Lindemann, J., H. A. Constantinidou, W. R. Barchet, and C. D. Upper.** 1982. Plants as sources of airborne bacteria, including ice nucleation-active bacteria. Appl. Environ. Microbiol. **44:** 1059–1063.
13. **Lindow, S. E., D. C. Arny, and C. D. Upper.** 1978. Distribution of ice nucleation-active bacteria on plants in nature. Appl. Environ. Microbiol. **36:**831–838.
14. **Lindow, S. E., G. R. Knudsen, R. J. Seidler, M. V. Walter, V. W. Lambou, P. S. Amy, D. Schmedding, V. Prince, and S. Hern.** 1988. Aerial dispersal and epiphytic survival of *Pseudomonas syringae* during a pretest for the release of genetically engineered strains into the environment. Appl. Environ. Microbiol. **54:**1557–1563.
15. **O'Brien, R. D., and S. E. Lindow.** 1989. Effect of plant species and environmental conditions on epiphytic population sizes of *Pseudomonas syringae* and other bacteria. Phytopathology **79:** 619–627.
16. **Rodrigues, U. A., and R. G. Kroll.** 1989. Microcolony epifluorescence microscopy for selective enumeration of injured bacteria in frozen and heat-treated foods. Appl. Environ. Microbiol. **55:**778–787.
17. **Zagory, D., S. E. Lindow, and J. R. Parmeter, Jr.** 1983. Toxicity of smoke to epiphytic ice nucleation-active bacteria. Appl. Environ. Microbiol. **46:**114–119.