Direct-Count Techniques for Enumerating *Clavibacter xyli* subsp. *xyli* Which Causes Ratoon Stunting Disease of Sugarcane

M. J. Davis

University of Florida, Institute of Food and Agricultural Sciences, Ft. Lauderdale Research and Education Center, 3205 College Avenue, Ft. Lauderdale 33314.

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ABSTRACT

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A fluorescent-antibody-direct-count-on-filters (FADCF) technique was developed to enumerate *Clavibacter xyli* subsp. *xyli* from both pure culture and sugarcane. An acridine-orange-direct-count (AODC) technique, a fluorescent-antibody-direct-count-on-microscope-slides (FADCS) technique, and a counting-chamber technique were also developed for enumeration of the bacterium from pure culture but not from sugarcane. The FADCF and AODC techniques are similar, except that instead of an acridine orange stain a fluorescent-antibody is used to obtain increased specificity. In both techniques, stained bacteria are concentrated on the surface of polycarbonate membrane filters before enumeration with epifluorescence microscopy. With *C. x.* subsp. *xyli* from pure culture, similar cell counts were obtained with the FADCF and AODC techniques over a thousandfold range of concentrations. The lowest concentrations that were conveniently measured with the FADCF and AODC techniques were between 10^4 and 10^5 cells per milliliter. The FADCS and counting-chamber techniques were tenfold and one-thousandfold less sensitive, respectively. Since counts obtained with all four techniques generally followed a Poisson distribution, the accuracy of counts was directly related to the number of cells counted. When sap samples from sugarcane infected with *C. x.* subsp. *xyli* were examined, populations estimated with the FADCF technique were consistently higher than for plate-count estimates, but the correlation between the estimates was highly significant.

Ratoon stunting disease (RSD) causes substantial yield losses in sugarcane (interspecific hybrids of *Saccharum*) in almost all cane-producing areas of the world (13,23). A small, nutritionally fastidious, xylem-inhabiting, coryneform bacterium, *Clavibacter xyli* subsp. *xyli* (Davis et al 1984) (6), causes RSD (5,7,9,20,24). No immune sugarcane cultivars are known (17,25). Screening cultivars for tolerance is difficult because infected plants have no external symptoms other than reduced growth, and internal symptoms are sometimes absent or ephemeral (23). Presently, yield trials are the only reliable method for determining cultivar susceptibility (8). However, pathogen concentration in host extracts is positively correlated to cultivar susceptibility (1,10,18); therefore, enumeration of the pathogen might be used to assay cultivar susceptibility (10).

Before the feasibility of such a screening procedure can be determined, more information on the population dynamics of *C. x.* subsp. *xyli* in sugarcane is needed. Very little is known about the spatial and temporal variability in numbers of the bacterium in sugarcane; in part, this is because appropriate enumeration procedures have not been available. Numbers of *C. x.* subsp. *xyli* in sugarcane have been based on direct-counts in wet mounts of xylem sap or crude juice extracts with phase-contrast microscopy (1,10,18). However, the accuracy and sensitivity of this counting technique has not been established. Based on sensitivity alone, the usefulness of the technique for studying the population dynamics of *C. x.* subsp. *xyli* in sugarcane is limited due to the small sample volume that can be examined effectively—typically about 5×10^{-8} ml within each microscope field when using a counting chamber (16).

The development of media for isolating the pathogen in pure culture (5,19) has made estimating viable populations based on plate counts possible (4). However, the slow growth of *C. x.* subsp. *xyli* in culture and problems with bacterial and fungal contamination (4) were primary considerations in the decision to investigate other than viable-count techniques. A fluorescent antibody technique, which has been adapted to enumerate *C. x.* subsp. *xyli* on membrane filters in this study, was more efficient for detecting *C. x.* subsp. *xyli* in sugarcane extracts than phase-contrast microscopy, conventional fluorescent-antibody staining, or isolation in culture (4).

Of the four direct-count techniques I evaluated for enumerating *C. x.* subsp. *xyli*, three involve epifluorescence microscopy. The first is an adaptation of direct-count techniques used to enumerate total bacterial populations in samples from aquatic habitats after staining with a DNA-binding fluorochrome (3,12,14,15,21,26). The second is a modified fluorescent-antibody staining technique for *C. x.* subsp. *xyli* (11) in which the bacteria are counted after being fixed and stained on microscope slides. In the third, bacteria are stained with fluorescent-labeled antibodies, concentrated on filters, and then enumerated. The fourth is a modified counting-chamber technique for which phase-contrast optics are used. The relative merits of each technique for enumeration of populations of *C. x.* subsp. *xyli* are compared.

The purpose of the research reported in this paper was to develop an enumeration technique and procedure to study the population dynamics of *C. x.* subsp. *xyli* in sugarcane.

MATERIALS AND METHODS

Sources of bacteria. The F1 strain (6) of *C. x.* subsp. *xyli* from culture and *C. x.* subsp. *xyli* extracted from naturally infected sugarcane were used throughout the study. Cultures were grown in RSD broth (6) or on SC agar (5) media. In some experiments to test enumeration methods at different cell concentrations, broth cultures of the F1 strain in late exponential growth phase were diluted in 0.01 M phosphate-buffered saline (0.85%) (PBS), pH 7.2. Volumetric pipettes or flasks were used to prepare serial twofold or tenfold dilutions. In other experiments, mature stalk internodes were sampled from plants of six sugarcane clones (CP 53-1, CP

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59-22, CP 63-588, CP 65-357, CP 72-1210, and CP 72-2086) that had been inoculated 9–12 mo earlier with the F1 strain or plants of five naturally infected clones (CL 54-378, CL 59-1052, CP 65-357, CP 70-1133, and CP 72-1210) from commercial fields. Stalks were washed and surface sterilized, and the sap was extracted from the internodes by centrifugation at 8,300 *g* for 10 min as previously described (4,5). Freshly extracted sap was used in all experiments; when fluorescent-antibody techniques were studied, the sap was sometimes stored frozen (−20 C) for several days before use. Viable counts of *C. x.* subsp. *xyli* in sap were obtained using a dilution-plate technique (4). Counts were taken at the appropriate dilution level from three spread plates of SC agar previously inoculated with 0.1-ml portions of the dilution.

Enumeration with phase-contrast microscopy. A Petroff-Hausser counting chamber (Hausser Scientific, Blue Bell, PA) was used. Suspensions of *C. x.* subsp. *xyli* were mixed with an equal volume of 0.1 N HCl to change the surface charge of the cells to allow them to adhere to the glass surfaces of the counting chamber (16). Immediately after the chamber was filled, it was inverted for 2 min and allowed to stand upright for another 3 min before cells were counted. A conventional 18 × 18-mm glass coverslip was used rather than the reinforced coverslip provided with the chamber, as the latter would not allow the phase-contrast objective to seat properly over the viewing area. All counts were made at a magnification of × 1,000 with phase-contrast optics. The grid lines etched in the counting chamber delineated an area of 0.00025 mm^2 (0.05 mm × 0.05 mm) with a depth of 0.02 mm. Thus, the volume enclosed by a square was approximately 5×10^{-8} ml at room temperature (25 C). Cells within the volume delineated by 1, 2, or 4 contiguous squares in the chamber were enumerated in a single count. Sufficient numbers of squares were viewed to obtain greater than five cells in a single count, if possible. The same number of squares per count was used throughout each sample. The microscopic field of view was changed after each count, and 30 counts were made per sample. The concentration of cells (B) in each sample was calculated by using the formula: $B = N/(7.5 \times 10^{-7}\ S)$ in which B is the concentration (cells per milliliter) of cells in each sample, N is the total number of cells that were counted in the sample, 7.5×10^{-7} is the volume in milliliters of the original sample delineated by 30 small squares and adjusted for the twofold dilution with HCl, and S is the number of small squares within the area used for a single count. Approximately one cell per four contiguous squares (10^7 cells per milliliter) was arbitrarily set as the minimum concentration of cells for counting. No attempts were made to enumerate samples with a lower concentration of cells, as determined after a preliminary scan of several microscope fields. The general rule, which was followed in all counting procedures throughout this study, was that cells touching the right or bottom sides of the counting area (volume) were included in the count; cells touching the other two sides were not.

Enumeration with epifluorescence miscroscopy. An acridine-orange direct-count (AODC) technique was based on previously described procedures (3,12,14,15,26) and modified to sample small volumes of liquid containing *C. x.* subsp. *xyli*. Cells were stained during the AODC procedure by mixing an equal volume, usually 0.1 ml, of a sample with 0.02% aqueous acridine orange (Aldrich Chemical Company, Inc., Milwaukee, WI) in a test tube. This mixture was allowed to stand for 3 min and then vigorously agitated for 5–10 sec. The mixture was then poured into one of ten sample holders of a filtration manifold (model FH202, Hoeffer Scientific Instruments, San Francisco, CA). The test tube was rinsed with about 4 ml of PBS, and the PBS was added to the mixture in the sample holder. Stained bacteria in the diluted sample were collected on the surface of a polycarbonate membrane filter (0.2-μm pore-size, 13-mm diameter; Nucleopore Corp., Livermore, CA) (12) by filtration using a vacuum of 60-mm Hg. The preparation was washed once by filtration with about 4 ml of fresh PBS. Prior to use, polycarbonate filters were stained overnight with an alcoholic solution of Sudan Black B (0.007%), briefly rinsed with deionized water, and air-dried to provide a suitable background for epifluorescence microscopy (26). A nitrocellulose filter (0.45-μm pore-size; Gelman, Ann Arbor, MI) was placed between each polycarbonate filter and the filter support of the filtration manifold (3) to prevent aggregation of bacteria and debris over pores in the filter support during filtration. A drop of PBS was applied to the edge of the filters before the sample holder was put in place, thus forcing any air from between the filters which might interfere with flow during filtration. Samples were placed in the sample holder before the filters dried.

After sample filtration and rinsing were completed, the polycarbonate filter was transferred, while still moist, to a microscope slide. The filter was placed right-side-up on the slide, and a drop of low-fluorescence immersion oil (Olympus Corp., Lake Success, NY) was used to gently mount a coverslip on top of the filter. Low-fluorescence immersion oil was found to be a satisfactory mounting medium, because cells remained stationary on the filter's surface. Such stationary cells in a single focal plane were easy to locate and count. The oil-immersion objective also could be used without a coverslip; however, cells became dislodged when the objective was placed in the oil and when the filter was moved to view different fields. Glycerol mounting medium, as used in conventional fluorescent-antibody procedures where cells are fixed to glass slides, was unsatisfactory because cells had a tendency to float which made counting difficult. An Olympus microscope (model BHA) equipped with an epifluorescence illuminator (model BH-RFL) was used to examine the filter for bacteria. The illuminator had a 100-W mercury lamp, B filter package (DM-580 dichroic mirror and 0-590 filter), BG-36 exciter filter, and 0-515 barrier filter. An Olympus UVFL 100 oil-immersion objective was used to provide a total magnification of ×1,200.

The fluorescent-antibody-direct-count-on-filters (FADCF) technique was identical to the AODC technique, except that a fluorescent-antibody staining step was substituted for the acridine orange staining step and the final rinse with PBS was omitted. Fluorescein isothiocyanate (FITC) conjugated to immunoglobulin G (IgG) with specificity for *C. x.* subsp. *xyli* was prepared and used for staining as previously described (4). The appropriately diluted (1:40) FITC-IgG conjugate in PBS was filtered before use, mixed with an equal volume of sample, and the mixture was then incubated for 30 min in the dark at room temperature.

The fluorescent-antibody-direct-count-on-slides (FADCS) technique was performed using the same FITG-IgG conjugate as used in the FADCF technique. Toxoplasmosis slides (Bellco Glass Inc., Vineland, NJ) with eight, 6-mm-diameter wells were coated with a mixture of molten gelatin (1%) and Tween-80 (0.1%) and air-dried before use. Five microliter samples were used. Each sample was smeared evenly throughout a well, air-dried, and gently heat-fixed. When more than one sample was taken from the same preparation, each sample was put on a different slide. Each well on a slide was used for a sample from a different preparation. The FITC-IgG conjugate was diluted 1:40 in PBS and used to stain the sample in the dark for 30 min at room temperature. Stained samples were rinsed twice for 10 min with PBS and once briefly with deionized water. Coverslips were mounted on the stained preparations with Aqua Mount (Lerner Laboratories, New Haven, CT) or glycerol mounting medium (50% glycerol in PBS). Slides were observed by using epifluorescence microscopy as described above.

For all epifluorescence counting procedures, a reticle was placed in one eyepiece of the microscope. The reticle was used to delineate different-sized counting areas. The grid consisted of one large square divided into 49 contiguous smaller squares. The size of the counting area was adjusted to accommodate counting about 10–20 bacteria, if enough bacteria were present. Usually, the cells in one small square or in one row, three rows, or all seven rows of small squares, or in the entire microscope field were counted. At ×1,200 magnification, a small square delineated an area of 7.35×10^{-5} mm^2. The entire microscope field had an area of 0.024 mm^2 which was equivalent to the same area as in 326.7 small squares. For the AODC and FADCF techniques, the effective surface area of the filter was 95.0 mm^2. For the FADCS technique, the area of the well on the toxoplasmosis slide was 28.3 mm^2. In most experiments, the number of counts per sample was 15 or 30. The formula used to

estimate bacterial populations (B) for the FADCF, AODC, and FADCS techniques was as follows: $B = KN/VCS$ in which B is the bacterial population (cells per milliliter), K is the number of small squares per filter area, N is the total number of cells counted, V is the volume of the sample, C is the number of counts taken for each sample, and S is the number of small squares within each counting area. For the FADCF and AODC techniques, K was 1293055.6. For the FADCS technique, K was 284845.1.

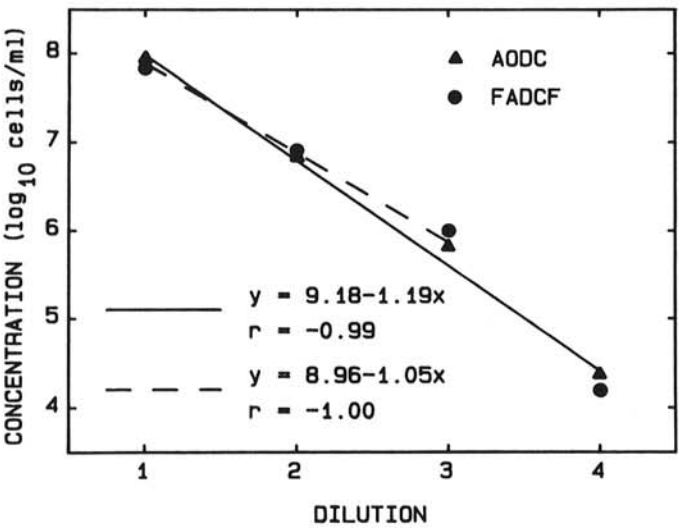

Fig. 1. Linear regression of mean cell concentrations of *Clavibacter xyli* subsp. *xyli*, obtained with both the acridine-orange direct-count (AODC) and fluorescent-antibody-direct-count-on-filters (FADCF) techniques, on a series of tenfold dilutions. Since there was no significant difference between their individual regressions, data from the two techniques were combined in the analyses. Two regressions are shown, one for the first three dilutions (- - -) and the other for all four dilutions (——). Each data point represents the mean of five independent determinations. The area in which counts were taken within the field of view of the microscope was adjusted with the aid of an eyepiece reticle to accommodate counting different cell concentrations. An approximate total of 150 cells or the cells in 15 microscope fields were counted, whichever came first.

RESULTS

While the AODC technique was being developed, background counts, due mostly to what appeared to be bacterial contaminants, were sometimes obtained even after precautions were taken to prevent this contamination. These precautions included filtering reagents immediately before use and using clean glassware rinsed with filtered deionized water. The background counts were usually equivalent to bacterial concentrations in the sample of approximately 10^5 cells per milliliter, but occasionally populations equivalent to approximately 10^7 cells per milliliter were found. No consistent source of these contaminants was identified. Thus, it became necessary to make background counts and adjust counts of *C. x.* subsp. *xyli* accordingly. When sugarcane sap samples were examined by using the AODC procedure, background fluorescence from the nonspecific staining of plant tissue fragments and other debris was an additional problem.

The effective ranges and accuracies of the AODC and FADCF techniques for counting *C. x.* subsp. *xyli* cells from broth culture were examined using a tenfold serial dilution of cells suspended in PBS. The mean AODC and FADCF concentration estimates from the first dilution and those obtained from three successive dilutions in the series were subjected to linear regression analysis with dilution as the independent variable (Fig. 1). No bacteria were observed in samples from the fifth successive dilution with either procedure, and these data were not used in the analyses. The regression equation for the AODC estimates was $y = 9.19 - 1.17x$ with a correlation coefficient (r) equal to -0.997, and the regression equation for the FADCF estimates was $y = 9.18 - 1.20x$ with $r = -0.994$. Linear regression was significant for both the AODC ($P > 0.003$) and FADCF ($P > 0.006$) data. The slopes and intercepts of the two equations were not significantly different ($P > 0.978$ and $P > 0.822$, respectively). A slope (b) equal to -1.0 for the regression of concentration on a tenfold dilution was expected. When the data from the fourth dilution was deleted from the analyses, the equations for the regression lines obtained with the remaining AODC and FADCF data were $y = 8.99 - 1.06x$ with $r = -0.999$ and $y = 8.92 - 1.05x$ with $r = -0.997$, respectively.

The counting-chamber and FADCS techniques were developed in an attempt to provide a more rapid and less expensive means than the FADCF procedure to enumerate cells of *C. x.* subsp. *xyli* in sugarcane sap. All three direct-count techniques provided a

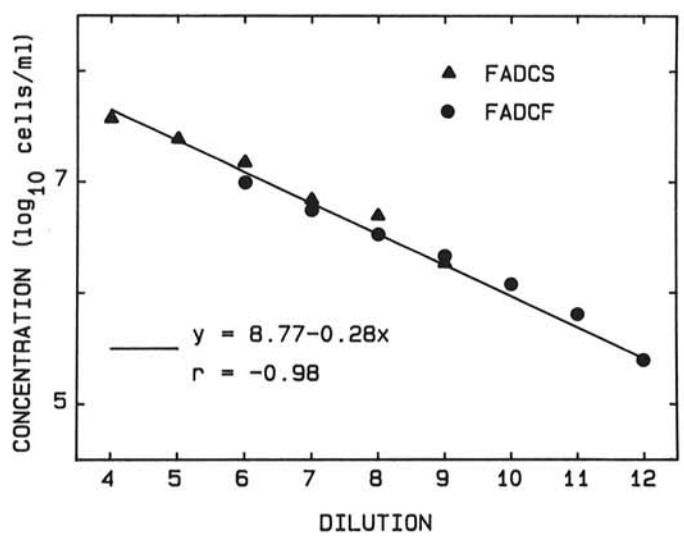

Fig. 2. Linear regression of mean cell concentrations of *Clavibacter xyli* subsp. *xyli*, obtained with both the fluorescent-antibody-direct-count-on-microscope-slides (FADCS) and fluorescent-antibody-direct-count-on-membrane-filters (FADCF) techniques, on a series of twofold dilutions. Data from the two techniques were combined in the analysis, since there was no significant difference between their individual regressions. Each data point represents the mean of five independent determinations. Cell counts were limited to the entire area delineated by an eyepiece reticle in the microscope. Fifteen counts were taken for each determination.

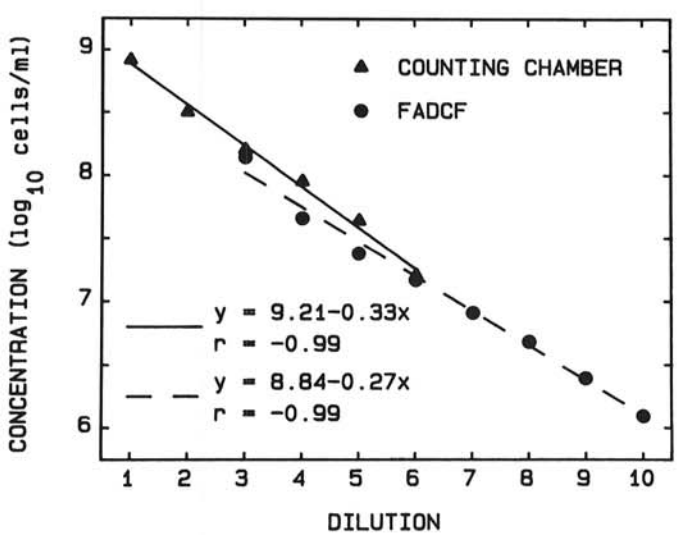

Fig. 3. Linear regressions of mean cell concentrations of *Clavibacter xyli* subsp. *xyli*, obtained with the counting-chamber (——) or fluorescent-antibody-direct-count-on-membrane-filters (FADCF) (- - - - -) techniques, on a series of twofold dilutions. Each data point represents the mean of five independent determinations. Depending on the concentration of cells, the area in which counts were taken was adjusted from one to four squares in the counting chamber and with the FADCF technique, from one to all 49 squares delineated by an eyepiece reticle in the microscope. Thirty counts were taken for each determination.

satisfactory means of estimating concentrations of the pathogen in suspensions from pure culture (Figs. 2 and 3). However, the counting-chamber and FADCS techniques were unsatisfactory to measure pathogen populations in sugarcane sap. When the counting chamber was used, debris and bacterial contaminants often interfered with *C. x.* subsp. *xyli* enumeration. In addition, many bacteria did not become attached to the glass surfaces of the chamber after treatment with HCl; consequently, it was necessary to scan different focal planes to count the bacteria. Also, because a nonreinforced coverslip was used, the depth of the chamber varied between counts, as measured with the stage micrometer. In preliminary experiments with bacteria suspended in PBS, the cells became attached to surfaces of the chamber, and subsequent changes in depth of the chamber had little effect on enumeration. However, when the bacteria were suspended in sap, changes in depth of the chamber affected the accuracy of the counts. Problems with bacterial attachment also were encountered during the FADCS procedure when sap was examined. Although attempts were made to heat-fix the bacteria to slides, counts of the bacteria were frequently lower than expected when compared to counts made with the FADCF technique suggesting that cells were lost during the staining and washing steps. Inadequate fixation was noted when stained bacteria were frequently seen suspended in the mounting medium.

The counting-chamber and FADCS techniques were used further to establish the accuracy of the FADCF technique for enumerating *C. x.* subsp. *xyli* in suspensions from pure culture. When the FADCF and FADCS techniques were compared by sampling a twofold dilution series (Fig. 2), the linear regressions of mean cell concentrations on dilutions were highly significant ($P > 0.0001$) for results of both techniques. The regression equations were $y = 8.75 - 0.27x$ with $r = -0.988$ for FADCF technique and $y = 8.72 - 0.27x > r = -0.986$ for the FADCS technique. No significant difference between the slopes ($P > 0.897$) and intercepts ($P > 0.873$) of the two regression equations was observed. Based on a chi-square test for goodness-of-fit of the variance (2) the 15 counts of each of the 35 replications with the FADCF technique and 24 of the 30 replications with the FADCS technique followed a Poisson distribution ($P > 0.05$).

When the FADCF technique was compared with the counting-chamber technique, most of the variation among mean cell concentrations was accounted for by linear regression for both techniques (Fig. 3). The dilutions examined by using the two techniques were not all the same because of the difference in effective ranges of the techniques. The intercepts ($P > 0.003$) and the slopes ($P > 0.025$) of the two regression lines were different. However, when the combined data from the two techniques were analyzed, the regression equation was $y = 9.09 - 0.306x$ with $r = -0.992$, and a significant ($P > 0.0001$) regression was obtained. The slope ($b = -0.306$) of the regression line for the combined data closely approximated the expected slope ($b = -0.301$) for a linear regression of concentration on a series of twofold dilutions. The chi-square variance test indicated that the variance of counts (30 counts per replication and five replications per dilution) was not significantly different ($P > 0.05$) than expected for a Poisson distribution in 33 of 35 replications with the FADCF technique and 25 of 30 replications with the counting-chamber technique over all dilution levels.

The FADCF technique was further tested for detection and subsequent enumeration of *C. x.* subsp. *xyli* using sap samples extracted from internodes of sugarcane stalks from inoculated or naturally infected plants. For comparison, viable populations of the pathogen were examined by using the dilution-plate technique. In one experiment, every other internode, eight per stalk, was sampled in single mature stalks from five inoculated plants of six cultivars. *C. x.* subsp. *xyli* was present in 127 samples, but not in 87 samples, as determined with both the FADCF and dilution-plate technique for 214 (90.7%) of the 236 samples. Dilution-plate data was lost for four samples due to contamination. The pathogen was detected with the FADCF technique in 21 of the 22 samples where results of that technique did not agree with those of the dilution-plate technique. The mean cell concentration in these 21 samples was 5.68 ± 0.09 $\log_{10}$ cells per milliliter, whereas the mean concentrations when the pathogen was detected by both the FADCF and dilution-plate techniques were 7.90 ± 0.06 $\log_{10}$ cells/ml and 7.30 ± 0.11 $\log_{10}$ colony-forming units (cfu) per milliliter, respectively. Linear regression of the 127 FADCF estimates on dilution-plate estimates (Fig. 4) was highly significant ($P > 0.0001$); however, the regression indicated that increasingly lower dilution-plate estimates in relation to FADCF estimates were obtained with lower cell concentrations. The possibility that the sporadic occurrence of comparatively low dilution plate estimates had biased the regression was evident upon inspection of the regression plot (Fig. 4). When 19 paired estimates differing by more than one $\log_{10}$ unit were arbitrarily omitted from the analysis, the regression equation for the remaining 108 observations was $y = 2.46 + 0.72x$. The regression was still highly significant ($P > 0.0001$) and $r = 0.91$. Increasing differences between estimates with lower cell concentrations was still indicated by the slope of the regression, but to a lesser degree. Substantial errors in FADCF estimates due to nonrandom distribution of cells on membrane filters was not evident. One-hundred thirty-five of 152 (88.8%) membrane counts (15 counts per sample) had variances which were not significantly different ($P > 0.05$) than expected for a Poisson distribution. Those counts which were not Poisson distributed generally had variances greater than the mean count, indicating a contagious distribution of cells on the membranes; however, population estimates derived from these counts were not consistently associated with large deviations from the regression line.

Regressions of FADCF estimates on dilution-plate estimates for *C. x.* subsp. *xyli* population size in samples from naturally infected sugarcane were similar to those obtained for experimentally inoculated material. The regression equation for mean estimates from 30 samples taken on 9 September 1982 was $y = 3.07 + 0.67x$ and $r = 0.77$. The regression equation for estimates from 38 similar samples taken on 26 October 1982 was $y = 4.47 + 0.48x$ and $r = 0.70$. Again, unusually low dilution-plate estimates in relation to corresponding FADCF estimates appeared to be adversely affecting the regression. When observations with estimates differing by more than one $\log_{10}$ unit were arbitrarily omitted in subsequent analyses, only 2 of 30 observations were omitted from the September data, but 21 of 38 observations were omitted from the October data. The regression equations for the adjusted

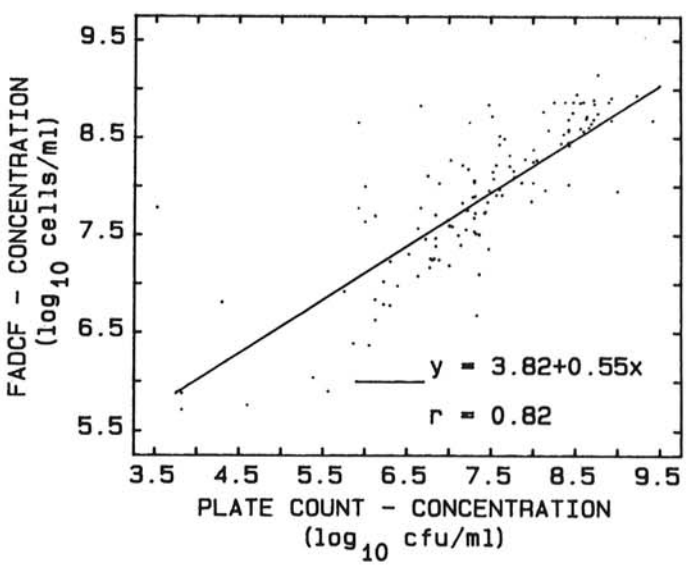

Fig. 4. Linear regressions of cell concentrations of *Clavibacter xyli* subsp. *xyli*, obtained with the fluorescent-antibody-direct-count-on-membrane-filters (FADCF) technique, on concentrations of colony-forming units obtained with a dilution-plate technique. Each of the 127 data points represents a single determination with both techniques of the concentration of the pathogen in individual sap extracts.

September and October data were $y = 1.29 + 0.91x$ and $y = 0.29 + 1.07x$, respectively, and the regressions were both highly significant ($P > 0.0001$). Correlations between the population estimates for the September ($r = 0.85$) and October ($r = 0.99$) data were greater than before, and the slope of the regression was nearer unity. Variation among FADCF estimates from the same sample was examined for the September data. Three subsamples were taken from each sap sample. Two were used to obtain independent FADCF estimates, and a third was used to obtain dilutions for plating. Plate-count data was taken from three spread plates at the appropriate dilution of each sample. The pooled standard deviation within the 30 paired FADCF estimates was 0.043 $\log_{10}$ cells per milliliter, while the pooled standard deviation within the 30 triplicate plate-count estimates was 0.103 $\log_{10}$ cfu per milliliter. The mean square error for the within sampled variance of FADCF estimates was significantly ($P > 0.01$) lower than that for the plate-count estimates.

DISCUSSION

Numerous preliminary experiments were conducted to refine individual aspects of the counting techniques developed in this study. At their present state of development, the AODC, FADCS, counting-chamber, and FADCF techniques were all useful for enumerating cells of *C. x.* subsp. *xyli* in suspensions from pure culture; however, only the FADCF technique appeared to be suited for use with sugarcane sap without further improvement. The principal disadvantages of the AODC technique with sap samples were nonspecific staining of plant debris and contaminating bacteria. The former caused unacceptable background fluorescence that interfered with counting, and the latter increased the potential for erroneous counts by adding to the population estimates of *C. x.* subsp. *xyli*. The primary disadvantage of the FADCS technique with sugarcane sap was the loss of bacteria during the procedure, which might be overcome by improved fixation methods. The counting-chamber technique was deemed inadequate because bacteria were frequently suspended in the sap and not attached to a surface where they could be easily located and counted. Also, unlike the other techniques, stains were not used, thus making resolution of the bacteria more difficult. Additionally, the counting-chamber technique was the least sensitive of the techniques studied.

To overcome problems of nonspecificity associated with the AODC procedure, an FITC-IgG conjugate specific for *C. x.* subsp. *xyli* was substituted for the acridine orange stain in the FADCF technique. The near identity of AODC and FADCF population estimates (Fig. 1) indicated that the FITC-IgG and acridine orange stains were equally effective for staining *C. x.* subsp. *xyli*. With the FADCF technique, background fluorescence after filtration of the stained sample was minimal; therefore, a rinse to remove excess stain was not necessary. The fluorescence of FITG-IgG stained *C. x.* subsp. *xyli* faded during excitation by ultraviolet light such that the amount of time for making counts within an individual microscope field was limited to less than 10–20 sec, depending upon the intensity of the light and the contrast of stained cells against the background. Adjustment of the area in which counts were made by use of any eyepiece reticle in the microscope facilitated counting of fewer cells. In most experiments, counts were limited to 10–20 cells before changing microscope fields, thereby avoiding problems associated with fading of the fluorochrome. When the individual count was limited in this manner, it was easier to remember which cells had been counted, and eye and mental fatigue were thereby reduced.

To facilitate the development of a counting protocol, an effort was made to obtain a random distribution of bacteria to count. A departure from randomness that was frequently observed in early experiments on the AODC and FADCF procedures was the tendency of bacteria to be more concentrated toward the outside edge of filters. This problem was overcome by diluting the sample immediately before filtration. The advantage of an appropriate filtration volume has been previously noted for other applications of the AODC technique (15). Similar problems with distribution were observed when samples were air-dried on slides for the FADCS procedure. The distribution of bacteria on slides was improved by using slides coated with a gelatin-Tween 80 mixture and by using a small sample volume. This allowed samples to spread thinly throughout the sample wells and to dry faster.

After procedures of the different direct-count techniques were refined to their present state, counts obtained with the techniques had an apparent Poisson distribution, indicating that the bacteria were randomly dispersed (22). In a Poisson distribution, the total count of discrete objects (i.e., cells) provides both the best estimate of the mean value and of the precision of the estimate (16), and there is a negative hyperbolic relationship between the mean and the number of individual counts required to maintain a given level of precision (14). Therefore, more microscope fields would need to be counted at lower cell concentrations to achieve the same precision. A protocol was adopted in which the total count was limited to a specified approximate number of cells, except when the concentration of cells was too low to obtain this total count from a specified number of microscope fields or less; then only the cells in the specified number of microscope fields were counted. This protocol allowed for a uniform level of precision at most concentrations, and reduced the time spent making counts at high and low cell concentrations where the laws of diminishing returns apply. This protocol was used to obtain the data shown in Fig. 1 comparing the FADCF and AODC techniques.

A major consideration when evaluating each of the direct-count techniques was their sensitivity with respect to enumerating low concentrations of *C. x.* subsp. *xyli*. The theoretical sensitivities of the techniques can be compared by calculating the population estimate derived from the same mean count with each technique given the maximum area or volume examined within a microscope field. With the AODC and FADCF techniques, a mean count of one cell per microscope field would be equivalent to 3.95×10^4 cells per milliliter when a 0.1-ml sample size is used. By comparison, a mean count of one cell per microscope field would be equivalent to 2.36×10^5 cells per milliliter by using the FADCS technique (0.005 ml sample size), and one cell per four contiguous squares in the counting chamber would be equivalent to 1.00×10^7 cells per milliliter. Thus, the FADCS technique, and more so, the counting-chamber technique are not theoretically as sensitive as are the FADCF and AODC techniques for enumeration of low populations of *C. x.* subsp. *xyli*. Actual population estimates obtained with the AODC and FADCF techniques (Fig. 1) at the greatest dilution where cells were detected were 2.36×10^4 cells per milliliter and 1.64×10^4 cells per milliliter, respectively. These estimates compared very favorably with the plate-count estimate of 2.35×10^4 cfu/ml for the same suspension which was obtained with a hundredfold greater dilution. However, these AODC and FADCF population estimates were lower than expected (5.75×10^4 cells per milliliter) based on an extrapolated estimate obtained from the other dilutions. Although dilution error might account for this discrepancy, a more likely explanation is that the population estimates were lower than expected because of errors associated with estimating populations from counts with a median and mode equal to zero as was the situation with these data. The near identity of the plate-count estimates with the FADCF and AODC estimates at low cell concentrations might be accounted for if fewer viable cells or cfus were present than total cells.

When the counting-chamber, AODC, FADCF, and FADCS techniques were examined, populations differing twofold or tenfold were readily differentiated by all four techniques (Figs. 1–3). Practically identical regression equations were obtained when the FADCF technique was compared with the FADCS or AODC technique. However, when the FADCF and counting-chamber techniques were compared, significantly different regression equations were obtained. The slopes of the regression equations for data obtained by the FADCF and FADCS techniques (Figs. 2 and 3) consistently had a value of $b = -0.27$, or $b = -0.28$ which was significantly different ($P > 0.001$) than the expected $b = -0.30$. The slope ($b = -0.33$) of the regression of counting-chamber estimates on a twofold dilution was also significantly different ($P > 0.001$) than $b = -0.30$. These differences between observed and expected

slopes were due to an unidentified source of error. This situation was not observed for FADCF and AODC data (Fig. 1) from a tenfold-dilution series which covered a larger range of concentrations, and which was obtained by using a different counting protocol. Comparison of the FADCF technique with the other direct-count techniques, notwithstanding observed discrepancies, indicated that it might be useful for estimating actual populations of *C. x.* subsp. *xyli* in sugarcane.

Estimates of the total cell population in sugarcane extracts obtained by using the FADCF technique compared favorably with corresponding plate-count estimates, especially at higher cell concentrations. A greater total cell estimate than viability estimate was expected due to dead bacteria being included in the total cell count. This will result in regression equations with intercepts greater than zero, as were consistently observed. However, a divergence between total cell and viability estimates at lower cell concentrations was also observed repeatedly. This added to the predicted intercepts of the regressions and produced slopes that were significantly less than one. Some lack of fit of the data to the regression can possibly be explained by error due to inconsistent loss of pathogen viability in sap samples. Loss of viability was difficult to assess, but examination of within stalk variation of pathogen populations (*unpublished*) suggested that extreme differences between corresponding FADCF and plate-count estimates were partially due to unusually low plate-count estimates. When paired estimates differing by more than one $\log_{10}$ unit were omitted from the regression analyses, intercepts were smaller and slopes approached unity, but some divergence of estimates at lower cell concentrations was still evident with some data. Therefore, some other factors, such as dilution error or the presence of growth inhibitors in extracts, might have also been involved. The low variation between subsample population estimates obtained by using either technique indicated that error associated with reproducibility of estimates within samples was not a major factor.

In a previous study (4), the FADCF technique was found to detect more infections by *C. x.* subsp. *xyli* in sugarcane than did phase-contrast microscopy, conventional fluorescent-antibody staining, or isolation of the pathogen in pure culture. In this study, the usefulness of FADCF technique to enumerate the pathogen in sugarcane was demonstrated. The ability to specifically detect and enumerate *C. x.* subsp. *xyli* at relatively low concentrations with the FADCF technique imparts an additional advantage over other techniques. Thus, the FADCF technique can be used to both detect and enumerate *C. x.* subsp. *xyli* in sugarcane extracts with a higher degree of confidence than is presently provided by other techniques.

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