Precision and Bias of Three Quantitative Soil Assays for *Verticillium dahliae*

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ABSTRACT

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Three widely used techniques to quantitatively assay field soil for *Verticillium dahliae* were compared for precision, bias, and time required to assay a sample. The techniques compared were dilution plating, wet sieving, and the Andersen sampler technique. Precision was measured by the standard error of the mean for repeated assays of 10 samples of naturally infested soil. Bias was measured by the deviation between the inoculum density estimates obtained with each of the techniques and the true number of microsclerotia introduced into samples of steamed silica sand. Wet sieving was the most precise method, but also the most biased and time consuming, and gave the lowest recovery of the fungus from field soil. On the average, only 64% of the microsclerotia introduced into samples of steamed silica sand were detected with the wet sieving assay, and estimates of inoculum levels in naturally infested soil samples were nearly two to three times higher with the Andersen sampler and dilution plating than with wet sieving. The Andersen sampler technique was unbiased and efficient in detecting the fungus in field soil, but it was the least precise technique. Finally, the dilution plating technique was the least biased with a recovery rate of nearly 100% in steamed silica sand amended with a known number of propagules. It was the least time consuming, had the highest recovery rate of the fungus in naturally infested soil, and was moderately variable.

A wide variety of techniques have been developed to detect and quantify *Verticillium dahliae* Kleb. in soil (5,21). Although bioassays with indicator plants have been used (11,15), most of the quantitative methods reported rely on spreading a known mass or volume of soil on a selective substrate and recording numbers of colony-forming units. The soil can be spread directly on the substrate (2), through a modified Andersen sampler (6,7,9,17), or after dilutions in water (8,10,12,20,24). Various techniques, including washing and decanting (12), dry or wet sieving (1,2,6,18), flotation on cesium chloride or sucrose (4), or combinations of the preceding (19), have been used to concentrate the fungus in the fraction of soil assayed, before spreading over the substrate.

Because the samples of soil taken by researchers in the field usually consist of large volumes of soil, it is often impractical to assay each sample in its entirety. Instead, one or several fractions with a known mass or volume are taken at random or arbitrarily from each soil sample and assayed separately. Such fractions will be referred to as subsamples in the present study. The results from the assay of every subsample taken from a sample are usually averaged to provide an estimate of the inoculum density of the fungus in the sample. As with any quantitative technique, the significance of this estimate depends on two critical qualities of the soil assay: precision and bias. Precision, as an indication of the degree of agreement of repeated measurements of the same quantity, determines the level of confidence attached to the repeatability of the soil assay (30). Bias measures the nearness of a result obtained by a particular method to the true value or the value accepted as true (30). If a soil assay were to be biased, it would be of interest to determine whether the true soil inoculum density of a pathogen was systematically over- or underestimated.

The worker starting a research program that requires some information about soil inoculum levels of *V. dahliae* faces a difficult choice. The large number of techniques developed to quantitate the fungus in soil suggests that no particular method has been identified as totally satisfactory. Comparative studies are rare, and conflicting evidence has been reported on the efficiency of some techniques to recover *V. dahliae* from soil (6,26,27). Information on the precision and bias is scant or lacking for most soil assays for *V. dahliae*.

The most complete information available about the qualities of a

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soil assay concerned a cesium chloride flotation technique (4). In that study, the bias of the flotation technique was evaluated by assaying soil samples to which known numbers of microsclerotia of *V. dahliae* had been added. The reproducibility of the technique was discussed, and repeated assays of various soils were conducted, although no quantitative measure of precision was presented (4). In other studies, the precision of assays was measured by the standard error of the mean, or by a coefficient of variation (ratio of the standard deviation to the mean), calculated from the results of repeated assays of a few soil samples (2,6). A possible variation of the precision of the assay with increasing inoculum density of *V. dahliae* in soil samples was postulated, but no attempt was made to quantify the relationship. Few workers have examined the bias of soil assays for *V. dahliae* by adding known numbers of microsclerotia or conidia to soil before the assay (4,20). An alternate method involved the assay of 10-fold dilutions (with heat sterilized soil) of artificially infested samples (9). The recovery of the fungus in each dilution was compared with its expected value, calculated on the basis of the dilution rate, and the inoculum density found in the original sample. However, the observed and expected inoculum levels in each dilution could be identical as long as the assay consistently detected a certain percentage of the propagules present in a sample, even if this percentage was quite low. To be detected, even with this method, that particular type of bias would require that one knew the true number of propagules of the fungus present in the original sample.

In an attempt to provide information relevant to the choice of a soil assay for *V. dahliae*, the present study was initiated to estimate the precision and bias and to compare the cost in time of three of the most widely used techniques for quantifying *V. dahliae* in soil: wet sieving, Andersen sampler, and soil dilution techniques.

MATERIALS AND METHODS

Soil assay techniques. We used the wet sieving technique of Huisman and Ashworth (18) and the modified Andersen sampler technique of Butterfield and DeVay (6). Only the upper sieve plate of the Andersen sampler (stage 1; 1.18-mm-diameter holes) was used in the present study, as it was reported that less than 5% of the propagules are captured on the lower plate (stage 2; 0.91-mm-diameter holes) (6). The soil dilution plating technique used 10-g subsamples of air-dried soil. The subsamples were mixed with 100 ml of water in 250-ml Erlenmeyer flasks. The soil suspension in each flask was continuously stirred as 1-ml aliquots were drawn with a pipette and evenly spread over a selective agar medium as described below.

For each of the techniques, the agar plates supporting the fraction of soil assayed were incubated for 2–3 wk in the dark at 20–22 C. The surface of the agar medium was gently washed under a stream of water to remove soil particles before counting numbers of microsclerotial colonies of *V. dahliae* with the aid of a dissecting microscope (magnification 15×). The estimate of soil inoculum of the fungus in a sample was expressed as an average number of colony-forming units (cfu) of *V. dahliae* per plate, or converted into an average number of propagules per gram of dry soil (ppg), based on the soil dilution factor of each technique.

Choice of the selective medium. Many selective media have been used to isolate *V. dahliae* from soil (29). They differ mostly in the type of carbon source and compounds used to enhance the growth and detectability of *V. dahliae* against that of other soil microorganisms. Carbohydrates such as sucrose (2,3), various sugars (13,22), polygalacturonic acid or sodium polygalacturonate (1,6,14,16), and cellophane (2) are often used as a carbon source, but they are absent in a few media (24,25). Compounds used as selective agents include soil or plant extracts (14,16,24), biotin (1,23), guanidine (1,6), a surfactant (Tergitol NPX, Sigma Co.) (1,6), ethanol (3,25), pentachloronitrobenzene (3,13,14), and antibacterial compounds such as streptomycin sulfate (1–3,6,13,14,16,22,24,25), chloramphenicol (3,6,14,16,24), and chlorotetracycline (6,14,16,24).

The enhancement of growth and detection of *V. dahliae* and the suppression of growth of other soil microorganisms have been compared for only a few of these very diverse media (1,6,14,16). Thus, three commonly used media were compared in a preliminary study: the pectate medium (PM) of Huisman and Ashworth as modified by Butterfield and DeVay (6), the ethanol agar (EA) of Ausher et al (3), and the soil extract medium (SM) of Menzies and Griebel as modified by Green and Papavizas (16).

These three media were compared for the assay of samples of soil naturally infested with *V. dahliae*, with the Andersen sampler and the dilution plating techniques. Five soil samples were assayed with the Andersen sampler technique (10 50-mg subsamples plated on each medium for each sample) and five samples were assayed with the dilution plating technique (two aliquots from each of five 10-g subsamples per medium for each sample).

Detection of inoculum in naturally infested soil. Ten soil samples were collected from commercial potato fields with a history of Verticillium wilt in the Central Sands region of Wisconsin. The soils were a Plainfield loamy sand (sand, mixed, mesic, typic Updipsamment, Entisol: 22% coarse sand, 30% medium sand, 31% fine sand). Each sample was air dried for 4–5 wk at 30–50% relative humidity and 20–24 C to eliminate drought-sensitive conidia and mycelial fragments (6) and stored in plastic bags at room temperature until processed. Before the assay of each sample, the soil aggregates were gently broken up by hand, and the soil was homogenized by hand shaking for 1 min.

Each of the 10 samples of field soil was assayed with each of the three techniques, using the following design. Fifty 50-mg subsamples were plated from each sample assayed with the Andersen sampler technique, resulting in 50 observations. With the wet sieving technique, five 10-g subsamples were taken per sample of field soil and sieved separately. For each subsample, all the residue from the 37-μm-mesh sieve was collected and spread over 10 plates. The number of colony-forming units counted on the 10 plates per subsample was added, resulting in five observations. Finally, five 10-g subsamples were taken from each sample assayed with the dilution plating technique, and each suspended in 100 ml of water in a 250-ml Erlenmeyer flask. Ten 1-ml aliquots were randomly taken from each flask and each plated separately. The numbers of colony-forming units growing from the 10 aliquots from each subsample were averaged, resulting in five observations. In total, 50 petri plates per technique were used for each sample.

For each of the 10 samples of field soil examined, the estimates of inoculum density of *V. dahliae* (one for each assay technique) and their associated standard deviation were calculated, based on the replicate observations for each technique. The data from this experiment were used in two ways: to evaluate the precision of each assay technique and to test whether the three techniques provided similar estimates of inoculum density in the samples.

Transformation of the data. The data obtained in the present study, from the assay of naturally or artificially infested samples, were square-root transformed (unless otherwise mentioned) before the statistical analyses. This transformation was performed to stabilize the variance, because it appeared that the means and variances calculated with the raw data were usually not independent. Such a phenomenon was not unexpected because a random dispersion of inoculum in the homogenized soil would be associated with a Poisson distribution (for which mean and variance are equal) of the number of microsclerotia per subsample (30). An appropriate transformation to stabilize the variance of Poisson-distributed data, or data for which mean and variance are approximately proportional, is the square-root transformation (30).

Production of inoculum. Large numbers of free microsclerotia were produced in a sterilized mixture of rye flour and silica sand (1:5, v:v) by seeding with a 7-day-old liquid shake culture of *V. dahliae* in Czapek-Dox broth. After a 2–3-wk incubation at 24 C, the sand inoculum was air dried at room temperature for 3 wk. The microsclerotia were separated from the sand by decantation, collected on a filter paper (Whatman No. 1) over a Büchner funnel, and rinsed with sterile distilled water. The microsclerotia were then dried and stored at 4 C until used. In repeated assays, 500 individual microsclerotia were plated on PM and 99–100% germination was observed.

Detection of artificial inoculum in steamed silica sand. To evaluate the absolute recovery of *V. dahliae* in absence of competition from other soil microorganisms, samples of steamed silica sand infested with known amounts of microsclerotia were processed for each technique. The test was done for 10 10-g samples with wet sieving (the residues from the 37-μm-mesh sieve were spread over 10 plates for each sample), and 30 50-mg samples with the Andersen sampler. Ten 1-ml aliquots were plated for each of three 10-g samples processed by dilution plating. To prevent overcrowding in the petri plates, appropriate numbers of microsclerotia were introduced into the samples to obtain a total of 10 expected colony-forming units in each plate. Thus, 10, 100, and 1,000 microsclerotia were transferred under a dissecting microscope into each sample to be processed with Andersen sampler, wet sieving, and dilution plating, respectively.

RESULTS

Comparison of three media selective for *V. dahliae*. For all but one of the samples assayed, the inoculum levels of *V. dahliae* detected on PM and EA were higher than on SM (Table 1). The difference between SM and either PM or EA was significant based on a two-way analysis of variance of the square-root transformed colony counts per petri plate. Although no significant difference was found between the results for EA and PM, slightly larger numbers of propagules of the pathogen were detected, on the average, on PM than on EA.

Bacterial colonies were frequently observed on EA but not on SM or PM. During incubation at room temperature, the plates of EA sustained a rapid and abundant growth of many different fungi present in the soil samples, and microsclerotial colonies of *V. dahliae* were at times difficult to identify. In contrast, growth of fungal colonies on PM was slow and restricted, and microsclerotial colonies of *V. dahliae* were easily detected. PM was used as the selective medium for *V. dahliae* for the rest of this study.

Precision. Precision was measured for each sample by the standard error of the mean associated with the estimate of inoculum density in a sample (mean of 5, 5, or 50 observations per sample for wet sieving, dilution plating, and Andersen sampler, respectively). The standard error of the mean (standard deviation divided by the square root of the number of observations), rather than the standard deviation, was used to compare the precision of the three techniques, because of the unequal number of observations per sample for each technique. For each technique, the standard error of the mean increased approximately proportionally to the square root of the average soil inoculum density (Fig. 1). The correlation coefficients were 0.96, 0.95, and 0.92 for Andersen sampler, dilution plating, and wet sieving, respectively. The slopes of the regression lines forced through the origin relating the variance of the sample mean to average soil inoculum density were 0.588, 0.219, and 0.071, for Andersen sampler, dilution plating, and wet sieving, respectively. The wet sieving technique consistently had the lowest standard error for a given inoculum density, and the Andersen sampler technique tended to have the highest.

Whereas the Andersen sampler technique had one source of variability associated with taking subsamples for the assay of each sample, wet sieving and dilution plating had two: With wet sieving, the residues obtained for each subsample were spread over 10 plates, and with dilution plating, each 10-g subsample was in turn randomly subsampled when 1-ml aliquots were taken from each flask. Depending on the contribution of each source of variability, the researcher might be able to maximize the precision of the

TABLE 1. Effect of three selective media on the recovery of *Verticillium dahliae* in naturally infested soil samples assayed with the Andersen sampler technique or the dilution plating technique

	Sample	Inoculum density (ppg)[w]		
		Selective medium		
		SM[x]	PM[y]	EM[z]
Andersen sampler assay	1	7 a	6 a	4 a
	2	30 a	41 ab	61 b
	3	26 a	54 b	42 ab
	4	42 a	62 a	58 a
	5	43 a	150 b	126 b
	Average	29.6	62.6	58.2
Dilution plating assay	6	5 a	10 a	11 a
	7	15 a	40 b	51 b
	8	44 a	123 b	93 b
	9	21 a	49 b	48 b
	10	39 a	54 b	47 ab
	Average	24.8	55.2	50.0

[w] The estimates of inoculum density (expressed as numbers of propagules per gram of dry soil) for each sample and each technique are averages based on colony counts observed from 10 petri plates. Within each line, inoculum densities followed by a different letter were significantly different ($P < 0.05$; Newman-Keuls multiple range test on the square-root transformed numbers of colony-forming units).

[x] Soil extract medium of Menzies and Griebel as modified by Green and Papavizas (16).

[y] Pectate medium of Huisman and Ashworth as modified by Butterfield and DeVay (6).

[z] Ethanol agar of Ausher et al (3).

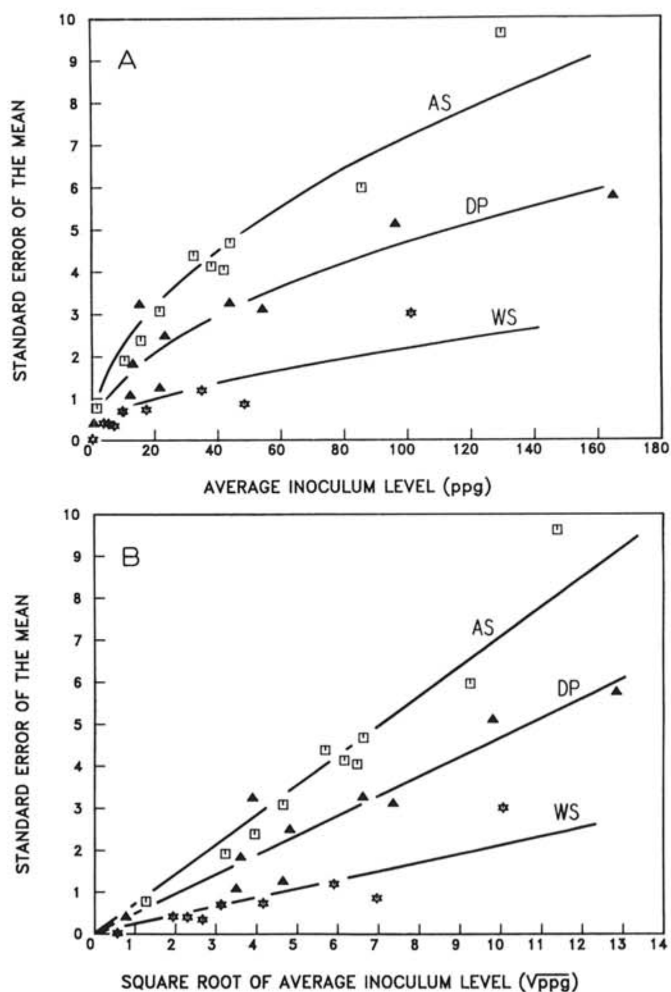

Fig. 1. Relationship between precision of three soil assays for *Verticillium dahliae* (expressed as the standard error of the mean for repeated assays of naturally infested soil samples) and soil inoculum density of the fungus (expressed as an average number of propagules per gram of dry soil [ppg]). **A,** Samples were assayed with the Andersen sampler, dilution plating, and wet sieving techniques. **B,** A linear relationship was observed if the standard error of the mean was plotted against the square root of average inoculum.

dilution plating technique, for a given total number of petri plates used per soil sample, by allocating a larger number of replicates to the more variable of the two steps. Thus, for dilution plating two sources of sampling variability existed, namely, variability between 10-g subsamples and variability between 1-ml aliquots within each 10-g subsample. The contribution of each source of variation to the variance of the sample mean was evaluated for dilution plating with the following model:

$$\sigma^2 = (n_2\sigma_1^2 + \sigma_2^2)/n_1n_2 \quad (1)$$

where σ^2 was the variance of the sample mean; σ_1^2 and σ_2^2 were the between-flask and between-aliquot (within-flask) components of variance, respectively; n_1 and n_2 were the number of flasks per sample and number of aliquots per flask, respectively (28). In the present study, n_1 and n_2 were 5 and 10, respectively. To estimate σ_1 and σ_2, an analysis of variance was performed on the counts of *Verticillium* (raw data, expressed as propagules per gram) from the 50 petri dishes used to plate 10 aliquots from each of five subsamples (Table 2). This analysis was performed separately for each of the 10 soil samples. The mean square experimental error (MSEE) (between-flask effect) and the mean square sample error (MSSE) (between-aliquots, within-flask effect) from each analysis of variance table are estimates of ($\sigma_2^2 + n_2\sigma_1^2$) and σ_2^2, respectively (28). A different estimate of σ_1 and σ_2 was obtained with each of the 10 samples. The values of the estimates for a given sample were found to be related to the average density of soil inoculum in that sample, as both the MSEE and the MSSE of the raw data increased approximately linearly as the sample mean increased (with coefficients of correlation of 0.95 and 0.94, respectively). The slopes of the regression lines forced through the origin were 10.919 and 9.039 for MSEE and MSSE, respectively, leading to $s_1^2 = 0.188\mu$ and $s_2^2 = 9.039\mu$ as estimates for σ_1^2 and σ_2^2, respectively, where μ was the average inoculum density in the sample. To test whether σ_1^2 was significantly different from zero for each sample, an F test was performed on the ratio F = MSEE/MSSE obtained from the analysis of variance of either raw or square-root transformed data (28). The ratios MSEE/MSSE were comparable for the raw data and the square-root transformed data (Table 2). They were significantly greater than 1.0 in only one case, indicating that there was little contribution of the between-flask effect to the variance of the sample mean. This also reflected the efficacy of the soil homogenizing method.

Bias. A significant bias ($P < 0.05$) was observed for the wet sieving and Andersen sampler techniques, for which an average of 64.3% and 88%, respectively, of the introduced inoculum was detected in the assay (Table 3). The "absolute" inoculum density (based on the known number of microsclerotia introduced in the samples of steamed silica sand) was slightly overestimated with dilution plating, but the bias was not statistically significant (Table 3).

Recovery of natural inoculum in field soil. The estimates of inoculum density obtained by wet sieving were consistently lower than those obtained by the Andersen sampler or dilution plating technique (Table 4). The differences between wet sieving and either dilution plating or Andersen sampler were significant ($P = 0.05$) for all but one of the individual samples (based on pairwise comparisons on the square-root transformed data), and globally, based on two-way analysis of variance on the square-root transformed data. Over the whole inoculum range, the densities detected by the Andersen sampler technique or dilution plating were up to five times and, on average, 2.8 and 2.2 times higher, respectively, than those detected by wet sieving. The ratios, dilution plating/wet sieving and Andersen sampler/wet sieving varied from 1.09 to 5.00 for the 10 samples of field soil examined in this study (Table 4). The dilution plating technique tended to detect slightly more propagules per gram of soil than the Andersen sampler technique at higher soil inoculum densities and less than the Andersen sampler technique at lower densities of soil inoculum. On the average, over the whole inoculum range, similar estimates were obtained with the Andersen sampler and dilution plating techniques (Table 4).

Time cost. A few steps of the soil assay, such as drying and homogenizing the soil samples and incubating the petri plates before counting colonies, are similar for all three techniques. Only the steps for which the techniques differed were timed. The time required for processing one sample was the shortest with dilution plating and the longest with wet sieving (Table 5). The time required for counting colonies of *Verticillium* varied slightly with the inoculum density of the fungus in the samples. This step took longer for wet sieving than for dilution plating and Andersen sampler as the expected concentration of the fungus on wet sieving plates was 10 and 20 times larger than that for plates from the dilution plating and Andersen sampler techniques, respectively (Table 5).

DISCUSSION

Among the three selective media tested in this study, SM was less efficient than the two others in enhancing the growth and detectability of *V. dahliae* in soil assays. For practical reasons, the plates of EA were not incubated at 18 C as recommended by Ausher et al (3) but at room temperature (20–22 C). Although the difference in temperature resulted in an abundant growth of fungi that sometimes rendered the identification of *V. dahliae* difficult,

TABLE 2. Analysis of variance of colony counts (expressed as numbers of propagules per gram of dry soil, ppg) from repeated assays of 10 soil samples naturally infested with *Verticillium dahliae*

Sample	Inoculum[w] density (ppg)	MSEE[x]	MSSE[y]	F ratio[z]	
				Raw	Transformed
1	0.6	8	6	1.44	1.44
2	12.2	165	120	1.38	0.86
3	13.0	57	147	0.39	0.39
4	15.2	78	221	0.35	0.38
5	21.6	517	146	3.54*	2.59*
6	23.2	527	348	1.51	1.90
7	43.8	307	148	2.07	1.81
8	54.2	487	468	1.04	1.05
9	96.2	1,307	1,274	1.03	1.09
10	164.8	1,657	1,276	1.30	1.31

[w] Each number is the mean of observation from 50 petri plates (10 aliquots plated from each of five subsamples per sample).
[x] Mean square experimental error.
[y] Mean square sample error.
[z] Ratios MSEE/MSSE for raw and for square-root transformed data. An asterisk (*) indicates that the ratio was significantly greater than 1.0 at the 5% confidence density.

TABLE 3. Bias of three soil assays for *Verticillium dahliae*

	Wet sieving[v]	Andersen sample[w]	Dilution plating[x]
Colony counts[y]			
Observed range	51–72	7–10	8–12
Expected	100	10	10
Bias[z]	64.3%*	88.0%*	100.7%

[v] 100 microsclerotia were introduced in each of 10 10-g samples of steamed silica sand. The residue from the 37-μm-mesh sieve was spread over 10 plates for each sample.
[w] 10 microsclerotia were introduced in each of 30 50-mg samples of steamed silica sand.
[x] 1,000 microsclerotia were introduced in each of three 10-g samples of steamed silica sand. Ten 1-ml aliquots were plated from each sample.
[y] Counts per sample for wet sieving and Andersen sampler and for individual aliquots for dilution plating.
[z] Ratio, expressed in percent, of the estimate provided by the assay (average of 10 observations for wet sieving and 30 for Andersen sampler or dilution plating) to the known inoculum density introduced in the samples. An asterisk (*) indicates that the estimate was significantly different from the true inoculum density ($P = 0.05$, analysis of variance of square-root transformed data).

the estimates of inoculum density of the pathogen obtained with EA were comparable to those obtained with PM. The PM medium allowed both an easy identification of microsclerotial colonies of *V. dahliae* and the detection of the most propagules per sample.

Considerable differences were observed in the time needed to process a sample, in the bias, and in the precision of three commonly used soil assays for *V. dahliae*. The wet sieving technique was the most, and dilution plating the least time-consuming technique. A large part of the time required to assay one sample with the Andersen sampler technique was due to weighing precisely 50-mg subsamples from each soil sample. This step could be expedited, possibly making the Andersen sampler the least time-consuming technique, by assaying subsamples of a given volume rather than a given weight of soil.

Whereas dilution plating detected nearly 100% of the microsclerotia introduced in steamed silica sand, approximately 10 and 40% were lost when the samples were processed with the Andersen sampler and wet sieving techniques, respectively. Based on the data presented by Butterfield et al (7), a bias of 5% of the actual inoculum density was expected for the Andersen sampler in the present study, as we used only the upper sieve plate of the Andersen sample. The bias observed for the Andersen sampler technique in the present study was slightly higher than expected. In the presence of a natural soil microflora, the systematic loss of microsclerotia of *V. dahliae* in the Andersen sampler seemed to be "compensated for", as the estimates of inoculum density for the Andersen sampler technique and dilution plating were comparable in naturally infested soil samples. One possible reason for this phenomenon could be a difference in the interactions of soil microorganisms on the selective substrate. The dispersion of the dry soil particles in 400 separate spots on the surface of the agar (Andersen sampler technique) might give *V. dahliae* a better chance to compete with the rest of the microflora present in the sample, than the spreading of soil particles in a film of water (dilution plating technique). Whereas the bias of dilution plating and that of the Andersen sampler were comparable, more than 40% of the inoculum present in steamed silica sand escaped detection with wet sieving. Similarly, estimates of inoculum densities in naturally infested soil samples were on average twice as large with Andersen sampler and dilution plating than with wet sieving. These results are in agreement with the findings of Butterfield et al (7) that 30–50% of the propagules of *Verticillium* in samples of field soil passed through the 37-μm-mesh sieve and with the report of Camporota and Rouxel (8) that approximately 68% of the microsclerotia from naturally infested soil were smaller than 40 μm.

For each of the three soil assays examined in the present study, a positive correlation was found between the average inoculum density in a soil sample and the standard error of the sample mean. This relationship was quantified with linear models. Within the range of soil inoculum densities observed in this study, the estimated models were $s^2 = 0.588\mu(50/n) = 29.40\mu/n$, and $s^2 = 0.071\mu(5/n) = 0.355\mu/n$, for Andersen sampler and wet sieving, respectively, where s was the estimate of the standard error of the mean, μ the average inoculum density, and n the number of subsamples taken from the soil sample. For dilution plating, the model was

$$s^2 = (n_2 s_1^2 + s_2^2)/n_1 n_2 = (0.188 n_2 + 9.039)\mu/n_1 n_2,$$

where n_1 and n_2 were the number of subsamples per sample and number of aliquots per subsample, respectively. For the three soil assays, the precision of the estimates of soil inoculum density decreased (as the standard error increased) with increasing inoculum densities. However, the standard errors expressed as percent of the average inoculum density (ratio $s \times 100/\mu$, based on the models) were decreasing functions of μ, indicating that the relative precision of the assays increased with increasing soil inoculum densities. These results are in agreement with earlier observations on the dependence of the precision of soil assays on average inoculum densities in the soil samples (2,6). Based on the models, expected values of the standard error were computed and compared with data available in the literature. In a study of the wet sieving technique, Ashworth et al (2) reported standard errors of 0.02 and 2.11 for inoculum densities of 0.13 and 46.97 ppg, respectively. The corresponding expected standard errors based on our model were 0.02 and 2.37, respectively. Such a close agreement was not found with data available on the Andersen sampler technique: Butterfield and DeVay (6) reported that standard deviations were less than 10% of the mean propagule number for inoculum densities higher than 20 ppg. A lower level of precision was observed for the Andersen sampler in the present study, as the estimated coefficients of variation calculated from the model for Andersen sampler decreased from 121% for 20 ppg to 44% for 150 ppg. Calculated standard errors based on data presented by Ben Yephet and Pinkas (4) on a cesium chloride flotation technique were also found to be approximately proportional to inoculum densities. The estimate of the standard error was $s^2 = 0.668\mu/n$, indicating that the level of precision of the flotation technique was similar to that of wet sieving for an inoculum range of 0.1–22 ppg.

The proportionality between sample variance and average inoculum density observed for all three techniques suggested that

TABLE 4. Comparison of detected inoculum densities of *Verticillium dahliae* (in propagules per gram of soil, ppg) in naturally infested soil samples using the soil dilution plating (DP), Andersen sampler (AS), and wet sieving (WS) technique

Sample	Inoculum density (ppg)[x]			Ratio	Ratio
	DP	AS	WS	DP/WS	AS/WS
1	0.60 a[y]	1.60 a	0.32 a	1.88	5.00
2	12.20 a	15.60 a	5.26 b	2.32	2.97
3	13.33 a	10.40 a	3.76 b	3.46	2.77
4	15.20 b	32.40 a	7.10 b	2.14	4.56
5	21.60 a	21.60 a	9.74 b	2.22	2.22
6	23.20 b	44.00 a	9.78 c	2.37	4.50
7	43.80 a	42.00 a	17.30 b	2.53	2.43
8	54.20 a	38.00 b	34.86 b	1.55	1.09
9	96.20 a	85.60 a	48.44 b	1.99	1.75
10	164.80 a	129.60 b	101.12 c	1.63	1.28
Average[z]	42.58 a	42.08 a	23.76 b	2.21	2.79

[x] Estimates of inoculum density for each sample and each technique are based on colony counts from 50 petri plates.

[y] Within each line, means followed by a different letter were significantly different ($P < 0.05$; pairwise comparisons on the square-root transformed data).

[z] Averages followed by a different letter were significantly different according to the Newman-Keuls multiple range test on square-root transformed data ($P < 0.05$).

TABLE 5. Cost in time to assay one soil sample for *Verticillium dahliae*, based on a total of 50 petri plates per sample, for each of three techniques

Step	Wet sieving (min)	Dilution plating (min)	Andersen sampler (min)
Weigh subsamples	2–3	2–3	20–25
Plate subsamples			
dilution or wet sieving[x]	8–10	1	...
label plates impact soil on plate[y] dispense aliquots[x] spread soil on plates[x] change subsample	12–15	13–15	20–25
Wash flasks and tubes	2	2	...
Total soil processing	24–30	18–21	40–50
Counting colony-forming units	75–80 (90 sec/plate)	40–45 (50 sec/plate)	35–40 (45 sec/plate)
Total	100–110	58–66	75–90

[x] Dilution plating and wet sieving only.

[y] Andersen sampler only.

the counts of *Verticillium* per plate might have a Poisson distribution (30). The assumption of a random dispersion of inoculum in the homogenized soil would also lead to the hypothesis that the number of microsclerotia present in a subsample had a Poisson probability distribution (30). For each of the 10 soil samples assayed in this study, the frequency distributions of colony counts observed for the Andersen sampler technique and for the dilution plating technique (50 observations per sample for each technique) were compared with a Poisson model (Tables 6 and 7). For all but one sample, a good fit of the Poisson distribution was obtained for the observed data from both the Andersen sampler and the dilution plating techniques.

Within the range of inoculum densities observed in the present study, the standard errors associated with the estimates of soil inoculum density in a given sample were consistently lowest for wet sieving, and tended to be highest for Andersen sampler. The estimate of soil inoculum density obtained for a given sample was, therefore, the most precise with wet sieving and tended to be the least precise with Andersen sampler. These results were obtained for the particular case in which an equal number of petri plates (50 in the present study) was used per sample for each technique. As the precision of the estimate of soil inoculum density depends on the number of subsamples taken from a sample (and also on that of aliquots per subsample for dilution plating), any given level of precision could be achieved with any of the three techniques by using appropriately large numbers of petri plates. For practical reasons, the number of petri plates needed per sample to obtain a given level of precision for the estimate of inoculum density was chosen as a criterion of comparison of the three soil assays. Using the linear models described earlier, the standard error of the mean for each soil assay was expressed as a function of the number N of petri plates used per sample, rather than of the number n of subsamples taken from a soil sample. For the Andersen sampler, n and N were identical; for wet sieving, N was equal to $10\,n$ as 10 plates were used per subsample. Finally for dilution plating, N was equal to the number of subsamples taken (n_1) times the number of plates used per subsamples (n_2); the particular case $n_2 = 1$ (one aliquot taken from each subsample) was considered in the computations, as it corresponded to the highest level of precision achievable with dilution plating. The resulting expressions of the models were $S^2 = 29.40\,\mu / N$, $S^2 = 3.55\,\mu / N$, and $S^2 = 9.23\,\mu / N$, for the Andersen sampler, wet sieving, and dilution plating, respectively. It can be seen from these expressions that the cost of obtaining a given level of precision for the estimates of soil inoculum density would be highest with Andersen sampler and lowest with wet sieving.

To select the most desirable soil assay technique for *V. dahliae*, Andersen sampler, wet sieving, and dilution plating were ranked based on their performances in each of the experiments conducted in this study. The Andersen sampler technique was considered least desirable, as it was slightly biased, more time consuming than dilution plating, and required more petri plates than dilution plating or wet sieving to obtain a given level of precision of the estimate of inoculum density in a soil sample. Wet sieving was the most precise method, but also the most biased and the most time consuming. Dilution plating was the least biased and the least time-consuming technique, but it appeared less precise than wet sieving for a given number of petri plates used to assay a soil sample. The choice between wet sieving and dilution plating was based on the following considerations. Supposing that wet sieving consistently underestimated the inoculum density of *V. dahliae* in soil samples by a constant percentage, it would be possible to calculate an unbiased estimate of inoculum density for each sample by multiplying the estimate obtained from the assay by a constant "compensation factor", β. If $s^2_{\text{wet sieving}}$ was the variance of the sample mean associated with the estimate of inoculum density in a sample of soil, then the variance of the sample mean, $s^2_{\text{wet sieving}*}$, associated with the transformed, presumably unbiased, estimate of inoculum density would be $s^2_{\text{wet sieving}*} = \beta^2 s^2_{\text{wet sieving}}$ (28,30). Based on the results presented in Table 4, the estimates of inoculum density in samples of field soil were on the average 2.21 times larger with dilution plating than with wet sieving. The associated models for the variance of the sample mean were $s^2 = 3.55\,\mu / N$, and $s^2 = 9.23\,\mu / N$, for wet sieving and dilution plating, respectively, where μ was the average inoculum density in the sample and N the number of petri plates used for the assay of one soil sample. After multiplication by the "compensation factor" $\beta = 2.21$, the transformed sample mean would have a variance $s^2_{\text{wet sieving}*} = (2.21)^2 s^2_{\text{wet sieving}} = 17.34\,\mu / N$. Because of the correction for the bias of the technique, the corrected estimate obtained with wet sieving would be less precise than that obtained with dilution plating. Conversely, a greater number of plates would be needed with wet sieving than with dilution plating to achieve a given level of precision for an unbiased estimate of soil inoculum density in a sample. It should be noted that the possibility of transforming the estimates of inoculum density obtained with wet sieving, to compensate for its bias, depends on the assumption that these estimates could be multiplied by a known, constant, compensation factor. However, the variations of the ratios of dilution plating/wet sieving shown in Table 4 suggest that such an assumption may not always be appropriate. Based on these considerations, the dilution plating technique was chosen by the authors for further work on the inoculum density of *V. dahliae* in field soil.

LITERATURE CITED

1. Ashworth, L. J. Jr., Harper, D. M., and Andris, H. L. 1974. The influence of milling of air-dry soil upon apparent inoculum density and

TABLE 6. Fit of a Poisson model to the frequency of colony-forming units of *Verticillium dahliae* per plate for 10 naturally infested soil samples assayed with the Andersen sampler technique[x]

Sample	Mean[y] cfu/plate	Variance-to-mean ratio	Chi-square goodness of fit test		
			df[z]	X^2	$P(\chi^2 > X^2)$
1	1.60	0.94	1	0.18	0.69
2	15.60	0.91	1	0.11	0.74
3	10.40	0.88	1	0.04	0.85
4	32.40	1.48	2	1.22	0.55
5	21.60	1.09	1	0.17	0.70
6	44.00	1.24	3	1.83	0.62
7	42.00	0.98	3	2.00	0.58
8	38.00	1.12	3	1.52	0.68
9	85.60	1.04	4	3.14	0.54
10	129.60	1.78	5	11.64	0.04

[x] Observed frequencies are based on counts from 50 plates for each sample.
[y] Average inoculum density in the sample (propagules per gram).
[z] Degrees of freedom.

TABLE 7. Fit of a Poisson model to the frequency of colony-forming units of *Verticillium dahliae* per plate for 10 naturally infested soil samples assayed with the dilution plating technique[x]

Sample	Mean[y] cfu/plate	Variance-to-mean ratio	Chi-square goodness of fit test		
			df[z]	X^2	$P(\chi^2 > X^2)$
1	0.60	0.92	1	0.21	0.67
2	12.20	1.15	2	0.34	0.85
3	13.00	0.95	2	2.73	0.26
4	15.20	1.16	2	2.53	0.29
5	21.60	0.97	3	1.45	0.70
6	23.20	0.69	2	1.76	0.43
7	43.80	0.83	4	4.28	0.34
8	54.20	0.87	4	6.37	0.19
9	96.20	1.33	5	3.36	0.65
10	164.80	0.79	5	12.22	0.03

[x] Observed frequencies are based on counts from 50 plates for each sample.
[y] Average inoculum density in the sample (propagules per gram).
[z] Degrees of freedom.

propagule size of *Verticillium albo-atrum*. Phytopathology 64:629-632.
2. Ashworth, L. J., Jr., Waters, J. E., George, A. G., and McCutcheon, O. D. 1972. Assessment of microsclerotia of *Verticillium albo-atrum* in field soils. Phytopathology 62:715-719.
3. Ausher, R., Katan, J., and Ovadia, S. 1975. An improved selective medium for the isolation of *Verticillium dahliae*. Phytoparasitica 3:133-137.
4. Ben-Yephet, Y., and Pinkas, Y. 1976. A cesium chloride flotation technique for the isolation of *Verticillium dahliae* microsclerotia from soil. Phytopathology 66:1252-1254.
5. Bouhot, D. 1979. Estimation of inoculum density and inoculum potential: techniques and their value for disease prediction. Pages 21-34 in: Soil-Borne Plant Pathogens. B. Shippers and W. Gams, eds. Academic Press, New York. 686 pp.
6. Butterfield, E. J., and DeVay, J. E. 1977. Reassessment of soil assays for *Verticillium dahliae*. Phytopathology 67:1073-1078.
7. Butterfield, E. J., DeVay, J. E., and Garber, R. H. 1978. The influence of several crop sequences on the incidence of Verticillium wilt of cotton and on the population of *Verticillium dahliae* in field soil. Phytopathology 68:1217-1220.
8. Camporota, P., and Rouxel, F. 1977. Intérêt du broyage et du tamisage à sec dans les techniques d'isolement du *Verticillium dahliae* à partir du sol. Ann. Phytopathol. 9:77-81.
9. DeVay, J. E., Forrester, L. H., Garber, R. H., and Butterfield, E. J. 1974. Characteristics and concentrations of propagules of *Verticillium dahliae* in air-dried field soils in relation to the prevalence of Verticillium wilt in cotton. Phytopathology 64:22-29.
10. Easton, G. D., Nagle, M. E., and Bailey, D. L. 1969. A method of estimating *Verticillium albo-atrum* propagules in field soil and irrigation waste water. Phytopathology 59:1171-1172.
11. Evans, G., McKeen, C. D., and Gleeson, A. C. 1974. A quantitative bioassay for determining low numbers of microsclerotia of *Verticillium dahliae* in field soils. Can. J. Microbiol. 20:119-124.
12. Evans, G., Snyder, W. C., and Wilhelm, S. 1966. Inoculum increase of the Verticillium wilt fungus in cotton. Phytopathology 56:590-594.
13. Evans, G., Wilhelm, S., and Snyder, W. C. 1967. Quantitative studies by plate counts of propagules of the Verticillium wilt fungus in cotton field soils. Phytopathology 57:1250-1255.
14. Farley, J. D., Wilhelm, S., and Snyder, W. C. 1971. Repeated germination and sporulation of microsclerotia of *Verticillium albo-atrum* in soil. Phytopathology 61:260-264.
15. Green, R. J., Jr., 1969. Survival and inoculum potential of conidia and microsclerotia of *Verticillium albo-atrum* in soil. Phytopathology 59:874-876.
16. Green, R. J., Jr., and Papavizas, G. C. 1968. The effect of carbon source, carbon to nitrogen ratios, and organic amendments on survival of propagules of *Verticillium albo-atrum*. Phytopathology 58:567-570.
17. Harrison, M. D., and Livingston, C. H. 1966. A method for isolating Verticillium from field soil. Plant Dis. Reptr. 50:897-899.
18. Huisman, O. C., and Ashworth, L. J., Jr. 1974. Quantitative assessment of *Verticillium albo-atrum* in field soils: Procedural and substrate improvements. Phytopathology 64:1043-1044.
19. Huisman, O. C., and Ashworth, L. J., Jr. 1974. *Verticillium albo-atrum*: Quantitative isolation of microsclerotia from field soils. Phytopathology 64:1159-1163.
20. Isaac, I., Fletcher, P., and Harrison, J. A. C. 1971. Quantitative isolation of *Verticillium* spp. from soil and moribund potato haulm. Ann. Appl. Biol. 67:177-183.
21. Johnson, L. F., and Curl, E. A. 1972. Methods for Research on the Ecology of Soil-Borne Plant Pathogens. Burgess Publishing Co., Minneapolis, MN. 247 pp.
22. Jordan, V. W. L. 1971. Estimation of the distribution of *Verticillium* populations in infected strawberry plants and soil. Plant Path. 20:21-24.
23. Kaiser, W. J. 1964. Biotin requirement of the Verticillium wilt fungus. Phytopathology 54:481-487.
24. Menzies, J. D., and Griebel, G. E. 1967. Survival and saprophytic growth of *Verticillium dahliae* in uncropped soil. Phytopathology 57:703-709.
25. Nadakavukaren, M. J., and Horner, C. E. 1959. An alcohol agar medium selective for determining *Verticillium* microsclerotia in soil. Phytopathology 49:527-528.
26. Schnathorst, W. C. 1981. Life cycle and epidemiology of *Verticillium*. Pages 81-111 in: Fungal Wilt Diseases of Plants. M. E. Mace, A. A. Bell, and C. H. Beckman, eds. Academic Press, New York. 640 pp.
27. Smith, V. L., and Rowe, R. C. 1984. Characteristics and distribution of propagules of *Verticillium dahliae* in Ohio potato field soils and assessment of two assay methods. Phytopathology 74:553-556.
28. Snedecor, G. W., and Cochran, W .G. 1967. Statistical Methods. Iowa State University Press. Ames, IA. 593 pp.
29. Tsao, P. H. 1970. Selective media for isolation of pathogenic fungi. Annu. Rev. Phytopathol. 8:157-186.
30. Zar, J. H. 1984. Biostatistical Analysis. Prentice-Hall, Inc., Englewood Cliffs, NJ. 718 pp.