Evaluation of an Assay for Quantifying Populations of Sclerotia of *Macrophomina phaseolina* from Soil

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ABSTRACT

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A semiselective assay procedure for quantitative isolation of *Macrophomina phaseolina* from soil was evaluated for reliability and precision. Prior sieving of soil through mesh with 2-mm-diameter openings did not significantly affect recovery of sclerotia of *M. phaseolina*. The assay recovered a constant proportion of inoculum from soil over a range of inoculum densities (1–34 and 2–127 propagules per 10 g of soil). The optimum number of subsamples to assay per soil sample is one when variation among samples, subsample variation, and costs are considered. If costs are completely ignored, a sample size of two appears appropriate.

Several selective quantitative assays have been developed for the ubiquitous soil fungus *Macrophomina phaseolina* (Tassi) Goid. (2–5). Each assay method has the same primary goal: to obtain the best possible estimate of propagule density in the most efficient manner. Ideally, this estimate should be unbiased and have a high degree of precision. If a bias is present in the assay, it should at least be constant over a range of inoculum densities so that comparisons among fields, treatments, etc., can be made. An assay method also should be efficient with regard to the time and cost of materials required to perform the assay.

The purpose of this research was to evaluate a semiselective quantitative assay for *M. phaseolina* that included components of the assay procedure developed by Papavizas and Klag (5) and Mihail and Alcorn (4). Specific considerations were the role of soil-sieving before the assay, the ability to recover a consistent proportion of propagules (sclerotia) over a range of inoculum densities, and the effect of number of subsamples assayed per soil sample on variance estimates.

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MATERIALS AND METHODS

Assay method. Soils were prepared following the general procedure of Papavizas and Klag (5); the semiselective medium of Mihail and Alcorn (4) was used. Ten grams of air-dry soil were blended with 250 ml of tap water (Osterizer Dual Range, Oster, Milwaukee, WI) at about 9,000 rpm for 5 sec. The water-soil mixture was washed through a 177-μm pore sieve (80-mesh) in tandem with a 44-μm pore sieve (325-mesh) with running tap water for 1 min. In preliminary tests, usually 100% of viable propagules were found on the 44-μm pore sieve and never less than 95% of viable propagules. Material on the 177-μm pore sieve was discarded and material on the 44-μm pore sieve was washed into a 600-ml beaker with 100 ml of 0.525% NaClO solution. After the soil solution was stirred on a magnetic stir plate for 8 min, beaker contents were washed 1 min with running tap water on a 44-μm pore sieve to remove the NaClO residue. Soil was washed into a sterile 250-ml flask with 40–50 ml sterile distilled water and 100 ml of molten, cooled (50–55 C) potato-dextrose agar medium (200 g of washed potato, 20 g of dextrose, and 27 g of Difco Bacto agar per liter) was added. Fresh aqueous solutions of streptomycin sulfate (250 μg a.i./ml medium) and chloroneb (100 μg a.i./ml medium) were added and the flask gently swirled. The mixture was poured into five petri dishes and incubated 4–5 days at 31–32 C in the dark. Colonies of *M. phaseolina* were identified by the characteristic appearance of black sclerotia submerged in the medium.

Reliability of the assay. One goal was to make the assay procedure as cost-efficient as possible. A step in the assay recommended by Mihail and Alcorn (4) was crushing air-dried soils through a 2-mm pore sieve. Because this procedure has a relatively large cost when assaying a large number of samples, the effects of sieving on the results of the assay were evaluated.

Samples of a sandy clay loam soil (55.9% sand, 31.9% clay, and 12.2% silt) were obtained from 12 arbitrarily selected sites in a field by extracting nine cores (2.5 × 10 cm) in an area 0.6 × 0.6 m at each site and bulking the cores. All samples were hand-mixed and air-dried. Each sample was gently sieved through a sieve with 2-mm-diameter openings. The fractions that did and did not pass the sieve were weighed and each fraction was gently mixed by hand for 2 min. Two subsamples were reconstituted for each sample such that each subsample contained equal portions of each fraction by weight. One subsample was mixed gently by hand for 2 min. The other subsample was crushed through the sieve with 2-mm-diameter openings and then mixed gently for 2 min. Six 5-g portions were arbitrarily taken from the crushed and noncrushed subsamples and assayed for *M. phaseolina*. Data were analyzed by analysis of variance.

If the assay procedure is to be useful, it should recover the same relative proportion of inoculum over a range of inoculum densities. To test the consistency of recovery, two portions of a nonautoclaved and autoclaved sandy clay loam field soil (75.2% sand, 21.3% clay, and 3.5% silt) with different initial inoculum densities were mixed in two experiments to give the following percentages of nonautoclaved soil: 100, 50, 20, 10, 5, and 2. Five arbitrarily selected 10-g portions of each soil mix were assayed for *M. phaseolina*. Initial inoculum density in experiment 1 was 33.8 propagules per 10 g of soil and in experiment 2 was 127 propagules per 10 g of soil. Least-squares regression analysis was used to determine the relationship between percent nonautoclaved soil and number of colonies of *M. phaseolina* obtained.

To test the precision of the assay procedure, 28 samples of the sandy clay loam sample used in the sieving test were arbitrarily selected from among 180 samples taken by removing three cores (2.5 × 10 cm) from the center of each of 180 quadrats (6.1 × 6.1 m) in a field planted to corn. For each of the 28 samples, five replicate 10-g subsamples were assayed for *M. phaseolina*. In an analagous test, six additional soil samples

were selected and six 10-g subsamples assayed per sample for *M. phaseolina*. Data were analyzed by analysis of variance, and after estimating the variance components, the variance about the general or overall mean was calculated for one to five or one to six hypothetical subsamples assayed per sample. Costs associated with each component of the assay were calculated and optimum number of subsamples to be assayed per soil sample were calculated (1).

RESULTS AND DISCUSSION

Inoculum density in soil samples used in the sieving test differed ($P = 0.05$) and ranged from 2.7 to 23.7 propagules (colony-forming units) per 5 g of soil. The mean inoculum densities of *M. phaseolina* found were 12.1 and 11.1 propagules per 5 g of soil for sieved and unsieved soil, respectively, and these values were not different ($P = 0.05$). Thus, over a range of inoculum densities passing soil through a sieve with 2-mm-diameter openings did not affect the recovery of *M. phaseolina* from the soil used. On the basis of this result and because of the relatively high cost (in terms of time) associated with this procedure, the sieving step was removed from all subsequent assays. If soil tends to harden into clumps or blocks during air-drying, however, such clumps should be broken into small enough pieces to allow effective mixing of a soil sample before taking a representative subsample.

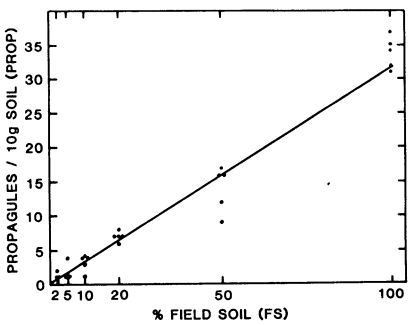

Fig. 1. Propagules (colonies) of *Macrophomina phaseolina* obtained per 10 g of soil assayed using a semiselective quantitative assay for mixtures of a sandy loam field soil and autoclaved portions of the same soil at proportions of 100:0, 50:50, 20:80, 10:90, 5:95, and 2:98 field soil/autoclaved soil (w/w). The regression equation represented by the line is: propagules per 10 g soil = 0.31 (percent field soil) $r^2 = 0.97$.

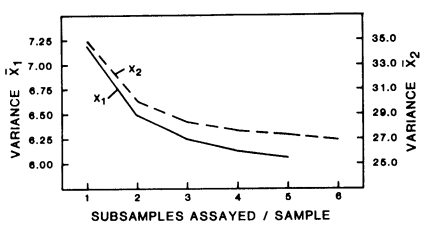

Fig. 2. Variance of the general mean number of colonies of *Macrophomina phaseolina* obtained per 10 g of soil in a semiselective, quantitative assay when assaying one to five or one to six hypothetical 10-g subsamples per sample for two runs, respectively, with different mean propagule densities of 17.2 and 40.6 per 10 g of soil for experiments 1 and 2, respectively.

The same relative proportion of *M. phaseolina* was recovered over the inoculum density range of one to 34 propagules per 10 g of soil (Fig. 1). In mixtures of autoclaved and nonautoclaved field soil with 100, 50, 20, 10, 5, and 2% nonautoclaved field soil, mean inoculum densities obtained were 33.8, 14.0, 7.0, 3.2, 1.6, and 0.8 propagules, respectively, per 10 g of soil for the first experiment. In the linear regresssion model (coefficient of determination, $R^2 = 0.97$) used to describe the data, the y-axis intercept of 0.32 was not different ($P = 0.05$) from zero and the slope of 0.31 was different ($P = 0.01$) from zero. In the second experiment, in mixtures of autoclaved soil and nonautoclaved field soil, mean inoculum densities obtained were 127.0, 59.6, 24.0, 18.2, 11.4, and 2.4 propagules per 10 g of soil with 100, 50, 20, 10, 5, and 2% nonautoclaved field soil, respectively. In this linear regression model ($R^2 = 0.91$), the y-axis intercept of 2.16 was not different ($P = 0.05$) from zero and the slope of 1.23 was different ($P = 0.01$) from zero. The significant linear effects in the data and high coefficient of determination values confirm that proportional inoculum recovery with the assay is constant and linear over a range of inoculum densities. In each experiment, the highest variance about the mean occurred in the 50% nonautoclaved soil sample and the variances may thus not be constant over propagule densities. This may be an artifact in our soil-mixing system, because the same trend toward possible nonconstant variance at varying propagule densities was not found in the tests to determine precision of the assay.

In the tests to determine the precision of the assay for *M. phaseolina*, mean inoculum densities were 17.2 and 40.6 colonies per 10 g of soil for soils assayed in the first and second run, respectively. Analysis of variance was performed on data from each run. The results of these analyses for the first run are presented in Table 1.

Table 1. Analysis of variance for number of *Macrophomina phaseolina* colonies obtained per 10 g of soil in run 1 with 28 soil samples and five subsamples assayed per soil sample with a semiselective assay procedure

Source	df[a]	Mean squares	Expectation of mean squares[b]
Sample	27	848.42	$\sigma^2 + 5\,\sigma_s^2$
Error	112	39.67	σ^2

[a] Degrees of freedom.
[b] σ_s^2 Is the among sample variance, σ^2 is the variance due to subsamples.

The expectation of the sample mean square contains a component resulting from variation among samples, σ_s^2, and one resulting from subsample variation, σ^2. By calculation for the first run, $\hat{\sigma}_s^2$ (circumflex refers to estimated value) = 161.75 and $\hat{\sigma}^2 = 39.67$, derived as follows:

$$\hat{\sigma}^2 + 5\,\hat{\sigma}_s^2 = 848.42$$
$$\hat{\sigma}^2 = 39.67.$$

Thus

$$\hat{\sigma}_s^2 = (848.42 - 39.67)/5 = 161.75.$$

For the second run, $\hat{\sigma}_s^2 = 126.92$ and $\hat{\sigma}^2 = 46.53$. With the estimated values for $\hat{\sigma}^2$ and $\hat{\sigma}_s^2$, it is possible to calculate the variance of the general or overall mean for the test with five subsamples assayed per soil sample and also to determine what this variance would be if four, three, two, or one subsample were assayed per soil sample. The change in this estimated variance would indicate the potential benefit from increasing the number of subsamples assayed per soil sample.

Thus, with five subsamples assayed per soil sample in run 1 using the values obtained in Table 1, the variance of the general mean (VGM) is:

$$\text{VGM} = [(\hat{\sigma}^2 + 5\hat{\sigma}_s^2)/140] = 848.42/140 = 6.06,$$

where 140 is n, the total number of subsamples assayed. Similarly, for four subsamples assayed per sample:

$$\text{VGM} = [(\hat{\sigma}^2 + 4\,\hat{\sigma}_s^2)/112] = 6.13.$$

The decrease in VGM with an increase in the number of subsamples assayed per sample was similar between runs 1 and 2 (Fig. 2). VGM decreased the most between one and two subsamples assayed per soil sample as one would expect from theoretical considerations. The results of the study of the precision of the assay suggest that if cost is not a consideration, then a logical number of subsamples to assay per soil sample on the basis of the curves presented in Figure 2 would be two.

Cost is often an important consideration, and this assay for *M. phaseolina* has a fairly high cost associated with it. Total time needed per sample to prepare culture medium, take samples in the field, perform assay, and count colonies after incubation for each sample is 12.6 min. Total time needed per additional subsample is 11.6 min (time to take sample is removed from this time and attributed only to the first subsample per sample). If labor cost is $6.00 per hour, then cost per initial subsample is $1.26 and per additional subsample is $1.16. Cost of materials (agar, dextrose, potatoes, antibiotics, and disposable petri dishes) is $0.73 for an assay per sample or subsample. Total cost is thus $1.99 per initial subsample and $1.89 for each additional subsample assayed per

sample. The differential cost of obtaining a sample is thus \$0.10.

Given estimates of the variances and costs associated with samples and subsamples and the optimum number of subsamples to assay per sample can be determined (1) as: optimum number of subsamples per sample (OPT) = $(\hat{\sigma}^2/\hat{\sigma}_s^2)^{1/2} \cdot (C_s/C)^{1/2}$, where $\hat{\sigma}^2$ and σ_s^2 are as previously defined and C_s is the cost to obtain a sample and C is the cost to assay each subsample. Thus, for run 1,

$$\text{OPT} = [(39.67/161.75)^{1/2} \cdot (0.10/1.89)^{1/2}] = 0.114,$$

and for run 2,

$$\text{OPT} = [(46.53/126.92)^{1/2} \cdot (0.10/1.89)^{1/2}] = 0.139.$$

Rounding up to the nearest whole subsample, one is the optimum number of subsamples to assay per sample. Thus, given the cost of the assay, the amount of variance associated with subsamples in this assay and the ability to recover a constant proportion of inoculum over a range of inoculum densities, only one subsample per soil sample should be assayed. Because of the relatively high cost to assay a sample, in order to have an optimum number of subsamples to be assayed equal to 2 (assuming $P \geqslant 1.5$ is rounded up to 2) the ratio $\hat{\sigma}^2/\hat{\sigma}_s^2$ would have to equal or exceed 18.9. These results imply that if the size of an experiment or survey is to be increased, then the direction of the increase should be to take more samples rather than to take more subsamples.

This assay procedure for *M. phaseolina* should be useful in a wide range of studies on this pathogen and is relatively simple to perform. The reliability and precision of this semiselective assay allow conclusions to be made with confidence in studies on such subjects as population dynamics, management effects on inoculum level, and survival of inoculum of *M. phaseolina*.

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