A "Most Probable Number" Method for Estimating Inoculum Density of *Aphanomyces euteiches* in Naturally Infested Soil

W. F. Pfender, D. I. Rouse, and D. J. Hagedorn

Graduate research assistant, assistant professor, and professor, respectively, Department of Plant Pathology, University of Wisconsin, Madison 53706.
Research supported by the College of Agriculture and Life Sciences, University of Wisconsin, as project 120.
We thank R. E. Rand and S. A. Vicen for technical assistance.
Accepted for publication 22 February 1981.

ABSTRACT

Pfender, W. F., Rouse, D. I., and Hagedorn, D. J. 1981. A "most probable number" method for estimating inoculum density of *Aphanomyces euteiches* in naturally infested soil. Phytopathology 71:1169-1172.

A bioassay was developed to determine the effective inoculum density of *Aphanomyces euteiches* in naturally infested field soil. Infested soil was mixed with steamed soil to produce a series of dilution levels. Each dilution level was divided into aliquots, and each aliquot was tested for the presence of the pathogen using a host bioassay under optimal conditions for infection. The proportion of aliquots found to be infested at each dilution level was used to calculate the infective inoculum density of *A. euteiches* in the original soil by means of the "most probable number" (MPN) method. A computer program was written to calculate the MPN for any series of up to five dilutions and any number of aliquots per dilution. In addition, the program calculates confidence limits for the MPNs and the statistical acceptability of the MPN. MPN estimates of infective inoculum density in field soils were consistent with the results of a standard root rot potential test and were correlated with epidemiologic and yield measurements from field plots of diseased peas.

Aphanomyces euteiches Drechs. is the principal incitant of pea root rot in Wisconsin (2). Neither genetic resistance nor chemical controls have been effective against the disease, but losses can be reduced by avoiding land heavily infested with the pathogen.

To improve our understanding of this disease and limit crop losses, a reliable technique for quantifying inoculum density of the pathogen in soil is needed. Because no selective medium has been found to permit direct counts of the population density with soil dilution plates, several other methods have been used. A greenhouse technique for assessing root rot hazard of prospective pea-field soil devised by Reiling et al (15) was improved by Sherwood and Hagedorn (17) and is a valuable diagnostic tool in a grower advisory service. While highly effective for its purpose, the method is not sufficiently quantitative for research concerning population biology of *Aphanomyces* in soil.

Several approaches toward more precise measurement of *Aphanomyces* inoculum density have been attempted. Boosalis and Scharen (1) counted oospores of *Aphanomyces* per unit volume of soil by microscopically examining organic debris separated from soil by wet-seiving. This method is tedious, however, and does not assess the viability of the propagules. Mitchell et al (10) also separated organic debris from infested soil but used a host bioassay to quantify the infectivity of the separated material. Burke et al (3) used complete soil rather than separating it into organic and mineral fractions. They estimated the *A. euteiches* inoculum level by counting plants infected when roots grew through a layer of infested soil placed over vermiculite. However, a "most probable number" (MPN) approach failed, because peas were not infected when infested soil was diluted with noninfested soil.

In our investigations of Aphanomyces root rot on the sandy soils of central Wisconsin, we devised a method for determining soil inoculum densities of the pathogen from complete soil with a host bioassay. A preliminary report has been published (12). An assay of complete soil should predict disease in the field better than an assay using only the organic fraction of the soil, and a host bioassay should measure the infective inoculum rather than the total population of the pathogen. The most probable number method of estimating populations of microorganisms seems well suited for such a measurement but has been used only rarely to determine inoculum levels of soilborne plant pathogens (6,8,14,16). The method involves testing aliquots taken from the soil to determine simply whether the organism is present or absent. The proportion of aliquots that contain the organism can be related to the number of infective propagules in a given volume of the original soil. Because the calculation of the inoculum density in the original soil depends on the proportion of aliquots that produce a zero response, several dilutions of the original soil are commonly made, and each dilution is assayed by removing and testing aliquots. In this way, at least one dilution will produce aliquots some, but not all, of which are infested.

Tables of MPNs are routinely used by bacteriologists but are usually based on a 10-fold dilution series and do not provide the flexibility needed to assess inoculum densities of soil fungi such as *Aphanomyces*. Parnow (11) developed a computer program for calculating the MPN and the confidence limits of the estimate for any range of dilutions and any number of aliquots per dilution. De Man (5) developed a computer simulation program to calculate the probability of occurrence of particular combinations of positive aliquots at several dilutions, given the MPN calculated from the observed combination. We developed a computer program similar to Parnow's, but included the option of calculating the probability of occurrence of the observed result, and a test for significance of this probability.

MATERIALS AND METHODS

Several soil dilutions were first prepared by mixing measured volumes of infested soil with steamed soil. We used Plainfield sand (91.3% sand, 4.3% silt, 4.5% clay), heated with aerated steam to 60 C for 30 min, as a diluent. Each dilution was thoroughly mixed by vigorous shaking in an inflated plastic bag. Aliquots were drawn from each dilution with a standard (4-cc) scoop.

Two plastic seed cavity trays (Jiffy Corp., West Chicago, IL 60185) nested within each other (Fig. 1) were used to test aliquots for the presence of the pathogen. Each tray consisted of 96 conical cavities measuring 2.5 × 2.5 × 7.5 cm. In each cavity of the upper

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

0031-949X/81/11116904/$03.00/0

tray, an aliquot of infested soil was placed between two layers of vermiculite. A thin layer of sand below the soil layer prevented soil from sifting down through the vermiculite. Two captan-treated seeds of Perfection peas, cultivar 8221 (Canners Seed Corp., Lewisville, ID 83431), were placed in each cell and covered with more vermiculite.

The purpose of the lower tray was to control the moisture level in the soil layers of the upper tray. Cavities of the lower tray were sealed at the bottom, and water was added to each cavity until it flowed out through the side hole, keeping the water level in the cells of the lower tray uniform. The upper tray was nested partway into the lower tray and supported by a wooden frame (Fig. 1). Thus, soil moisture level in each cell of the upper tray was held constant at approximately −1.5 mb, because the lower surface of each aliquot was 1.5 cm above water level.

Temperature was maintained at 24 ± 1 C by placing the nested trays in a water-filled pan resting in a controlled-temperature bath. Water lost from the cells of the lower tray by evapotranspiration was replaced daily with a watering tube via holes in the top surface of the upper tray.

After 16 days, plants were removed and scored as positive or negative for infection by *A. euteiches*. The water-soaked, honey yellow color characteristic of roots infected by this pathogen was distinctive and easily differentiated from the symptoms of infection caused by *Pythium* spp. and *Fusarium solani* (Mart.) Appel & Wr. f. *pisi* (F. R. Jones) Snyd. & Hans. Preliminary isolations from 300 roots confirmed that the presence of the pathogen could be determined from these root symptoms. Occasionally, plants were found whose roots were only slightly discolored; these roots were plated to determine whether the pathogen was present. The number of positive aliquots at each dilution level was used to calculate the MPN estimate of *A. euteiches* inoculum density in the undiluted soil.

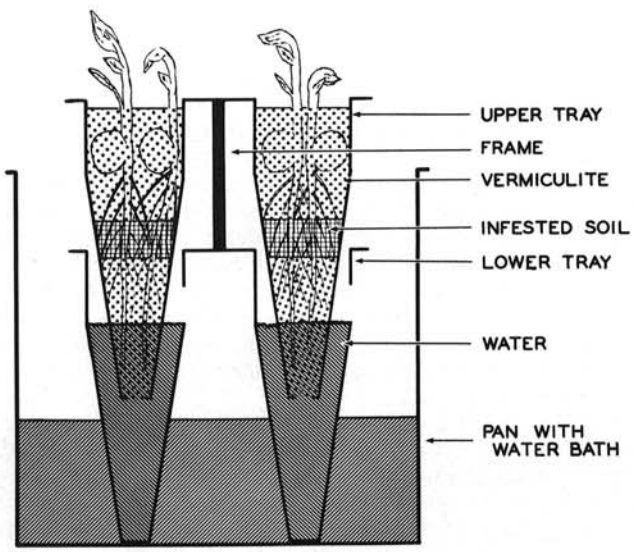

Fig. 1. Apparatus used in most probable number bioassay technique to determine whether soil aliquots contain infective propagules of *Aphanomyces euteiches*. Constant water level in cells of the lower tray maintains matric water potential of soil aliquots in upper tray at −1.5 mb. Water bath keeps temperature of system constant 24 ± 1 C.

A computer program written in Ascii Fortran (available from D. Rouse, Department of Plant Pathology, University of Wisconsin, Madison 53706) was used to calculate the MPN, the approximate confidence limits for the estimated MPN, the probability of occurrence of the observed assay results, and a test for the statistical acceptability of the estimated MPN. The program calculates the MPN with a subroutine that solves for the MPN in the maximum likelihood equation stated by Halvorson and Ziegler (7). Confidence limits for the MPN are calculated as in Parnow (11). The procedure assumes that the logarithm of the MPN is approximately normally distributed. Another subroutine calculates the probability of obtaining each of the possible combinations of positive aliquots (infected plants) at each dilution, given the estimated MPN (5). Starting with the estimated MPN, the computer routine works backwards and calculates the probability of occurrence of all combinations of positive aliquots in the test, then arranges these probabilities in descending order and adds them until the combination of positive aliquots actually observed in the experimental test is reached. If one minus the accumulated probability thus obtained was greater than 0.05 (arbitrarily chosen as the significance level), the estimated MPN was judged acceptable.

To assess the validity of the MPN estimates, three types of tests were performed. In the first, known numbers of culturally produced oospores of *A. euteiches* were added to steamed soil, and inoculum levels were estimated by the MPN method. In the second test, soil samples were assayed by Sherwood and Hagedorn's (17) root rot potential test and by the MPN method, and the results of the two assays were compared. In the third test, soil samples taken before planting from field plots with different infestation levels were analyzed by the MPN test. Peas were then planted in the plots, and the MPN estimates of inoculum density were compared with epidemiological data from the plots. The level of infestation of the field plots, in Plainfield sand, was adjusted before sampling by adding different amounts of naturally infested soil to the plots. Ten plants were removed from each plot each week and scored for disease severity. The roots were plated on water agar to determine infection by *A. euteiches*. Pod weight of 40 plants was taken at harvest as a yield measurement.

RESULTS

We were able to quantify the inoculum density of infective propagules of *Aphanomyces euteiches* in soil with the method described above. In general, the proportion of positive aliquots decreased with increasing dilution rates of a given soil, as expected. In 70 tests, we found no results that were inconsistent with the MPN model; that is, no results were unacceptable at the 5% level. The computer program for calculating the MPN performed well over a wide range of dilution rates, aliquot numbers, and

TABLE 1. Most probable number (MPN) estimates of *Aphanomyces euteiches* inoculum density in artificially infested soil

Oospores added per gram of soil	Proportion of aliquots showing infection at indicated dilution					MPN (ippg)[a]	Infectivity[b] (%)	95% Confidence interval	Acceptability[c]
	Undiluted	1/2	1/10	1/25	1/100				
530	···	···	6/9	4/9	1/9	2.2	0.42	1.2–4.2	0.922
204	···	···	5/9	1/10	1/9	1.2	0.58	0.6–2.6	0.876
51	···	7/9	2/10	0/9	···	0.45	0.88	0.2–0.9	0.487
10	2/19	3/10	···	···	···	0.04	0.41	0.02–0.1	0.398
0.8	0/29	···	···	···	···	< 0.006	···	···	···

[a] Infective propagules per gram.
[b] MPN-estimated inoculum density (ippg) divided by the number of oospores added per gram of soil.
[c] The probability (0.0–1.0) that the calculated number of infective propagules per gram would produce the observed combination of positive aliquots at the dilutions used.

population densities.

In the first test of the validity of the MPN estimates of *A. euteiches* inoculum level in soil (Table 1), the estimates increased with increasing concentration of added oospore inoculum in the soil. The 95% confidence interval was determined for each estimate, and the acceptability of each estimate was calculated. All estimates were within the acceptance range. The infectivity level (MPN-estimated inoculum density divided by number of oospores added) was relatively consistent over a wide range of inoculum densities and averaged 0.57%. That is, only one of every 175 oospores added was detectable by our method. This low infectivity level could be caused by low viability of the added oospores, low germinability of the relatively young oospores, or a low proportion of the aliquot volume actually being included in the rhizosphere of the assay plant. Estimates of inoculum in a given soil should vary with the size of the rhizosphere affecting each aliquot. We found, however, in preliminary work that the MPN estimate of *A. euteiches* in a given soil was the same whether each aliquot was assayed with one plant or two (where an aliquot was scored positive if either or both plants were infected). Therefore, the total volume of the aliquot was most likely adequately sampled by the assay plants, and the low infectivity level of our added oospores was probably caused largely by low germinability and/or viability.

Inoculum was not detected in soil infested with 0.8 oospores per gram. The threshold for detection in this particular test (29 aliquots) was 0.006 infective propagules per gram. Given an infectivity level of 0.57%, a detection threshold of 0.004 infective propagules per gram would have been required to demonstrate the presence of the pathogen in this soil sample. Such a threshold of detection would have been achieved with 45 aliquots of undiluted soil.

MPN estimates generally agreed with the assessment of root rot hazard by Sherwood and Hagedorn's (17) method (Table 2). In both sandy and loam soils, samples showing low root rot hazard (0–50) contained levels of *A. euteiches* undetectable by the MPN method. Soils rated hazardous for peas (70–100) generally had moderate to high inoculum densities as estimated by the MPN method.

The third test was the most relevant for assessing the validity of MPN estimates of *A. euteiches* inoculum density in field situations. In this test, the MPN estimate was a good predictor of disease progress, disease severity, and yield loss (Table 3).

DISCUSSION

The technique described in this paper is useful for estimating infective inoculum density of *A. euteiches* in soil. The MPN method was facilitated in part by the computer program we developed, because the program allowed the calculation of MPNs from any series of five or fewer dilutions and any number of aliquots per dilution.

In a review of the MPN method, Cochran (4) points out that two assumptions underlie the calculation of the MPN and must be met in designing an MPN procedure: the propagules of the organism must be distributed randomly throughout the original sample, and a single infective propagule in an aliquot must be sufficient to result in a positive test response (symptom expression). Therefore, in using the MPN technique to determine inoculum density of soilborne pathogens, the soil must be well mixed before use, and the conditions of the bioassay must be optimal for infection and disease development. Optimal conditions for infection by *A. euteiches* include water-saturated soil, temperature near 24 C, and growth of roots from noninfested into infested medium (3). We assume that infection by a single germinated oospore (infective propagule) is sufficient to induce visible infection under the conditions of the bioassay. We do not assume that all oospores in a population are infective; many may not be infective because of dormancy or inviability, for example. For this reason, MPN estimates of *A. euteiches* inoculum density in soil are expressed as infective propagules per gram rather than as oospores per gram.

A check for determining whether the MPN technique is appropriate (ie, whether the assumptions implicit in the use of MPNs are valid) is to observe whether the proportion of positive aliquots observed at each dilution was likely to occur given the estimated MPN. For example, suppose that five, seven, and nine of 10 aliquots were positive (infected plants) in a dilution series of 1.0, 0.5, and 0.2, respectively, of the original soil. The calculated MPN would be 0.001 with 95% confidence limits of 0.00036 and 0.0026. However, based on the assumptions of the MPN technique, the observed result is clearly improbable. The probability of occurrence of particular combinations of positive aliquots, and thus the acceptability of the estimated MPN calculated from those combinations of positive aliquots, can be calculated as described above. If unacceptable (improbable) results are frequent, the use of the MPN may be questioned.

Our results show that the MPN assay effectively measures the infective inoculum density of *A. euteiches* in infested soil. The method does not measure the total population density of *A. euteiches* in soil, as indicated by the low estimates of infective propagules compared with the total number of oospores added to soil in our tests (Table 1). However, the method does measure some fairly constant proportion of the total oospore population, as shown by the correlation between number of oospores added to soil and MPN estimates of infective inoculum density. The infective inoculum thus measured, whatever its precise nature, bears a close relationship to the inoculum that incites disease in the field. This relationship makes the MPN-estimated inoculum density of *A. euteiches* in soil a good predictor of disease levels to be expected when peas are grown in that soil (Table 3). Therefore, the MPN method should be a useful tool for evaluating control strategies designed to reduce inoculum density of *A. euteiches* in the field.

The MPN estimates of inoculum densities in several field soils

TABLE 2. Comparison of most probable number (MPN) estimates of *Aphanomyces euteiches* inoculum density with root rot potential assessments of several soils

Soil type Soil no.	MPN estimate (ippg)[a]	RRP[b]
Sand		
OC-1	9.0	93 ± 5
OC-2	5.9	71 ± 6
OC-9	< 0.007 (nd)[c]	13 ± 3
Loam		
A-1	15.2	87 ± 10
317	4.1	97 ± 5
154	0.17	80 ± 6
P-1	0.015	75 ± 17
321	0.015	56 ± 24
344	< 0.015 (nd)[c]	44 ± 8
144	< 0.015 (nd)[c]	30 ± 4

[a] Infective propagules per gram.
[b] Root rot potential, determined by Sherwood and Hagedorn's method (17). Data are means ± standard deviations.
[c] *A. euteiches* not detected by MPN test. Data are thresholds of detection for the given tests.

TABLE 3. Epidemiologic measurements and most probable number (MPN) estimates of *Aphanomyces euteiches* inoculum densities in pea root rot field plots[a]

MPN estimate (ippg)[b]	Days from planting to 50% disease incidence[c]	Disease severity index at harvest[c,d]	40-plant pod yield (g)[c]
0.006	43.4 w	5.1 x	199 x
0.092	33.8 x	7.3 y	164 x
0.54	28.2 y	8.6 y	100 y
3.6	18.0 z	9.9 z	46 z

[a] Data are means of three replicates per treatment.
[b] Infective propagules per gram.
[c] Values within a column followed by different letters differ at the 5% level according to Duncan's new multiple range test.
[d] Disease severity index: 0 = healthy, 12 = maximum severity. Computed by summing disease ratings, 0–4, for root, epicotyl, and shoot.

were correlated with the root rot potentials (RRP) (17) of these soils (Table 2). The two methods differ in important respects, however. The RRP method assigns values based on a subjective disease severity rating, whereas the MPN method uses the objective criterion of infected or uninfected. In addition, the RRP method does not distinguish between root rot incited by *A. euteiches* and that incited by other pathogens. The MPN test provides conditions that favor infection by *A. euteiches* over infection by other pathogens (3) and counts as positive only those infections typical of *A. euteiches*. For this reason, RRP values of some soils may be greater than zero even when the MPN test shows an undetectable inoculum density of *A. euteiches*. Therefore, we feel that although the RRP method is valuable in grower advisory programs, the MPN method's greater accuracy makes it more useful in research concerning the biology of *A. euteiches* in soil.

As a host bioassay, the MPN method has advantages and disadvantages. It is somewhat cumbersome, requiring time, space, and facilities for growing plants under controlled conditions. In our tests, we commonly used 10 aliquots at each of three dilutions for testing a soil sample; this required a 96-cell tray, 28 × 46 cm, to test four samples. Despite its disadvantages, however, the MPN method measures the inoculum potential of the pathogen directly, where inoculum potential is defined as "the capacity of a pathogen population to infect a population of fully susceptible host plants under conditions optimum for infection" (9). This, or some similarly standardized estimate, is the critical measurement required in most quantifications of soil pathogen populations. It avoids the problem, common to many other methods of soil pathogen enumeration, of distinguishing between pathogenic and nonpathogenic forms of the organism. For example, we found a form of *Aphanomyces*, pathogenic to beans but innocuous to peas, which would be difficult to distinguish routinely from the pea pathogen on any basis other than host response (13). The MPN bioassay permits assessment of the soil population of the pea pathogen without confusion from the bean-infecting strain or perhaps still other forms of the fungus.

LITERATURE CITED

1. Boosalis, M. G., and Scharen, A. L. 1959. Methods for microscopic detection of *Aphanomyces euteiches* and *Rhizoctonia solani* and for isolation of *Rhizoctonia solani* associated with plant debris. Phytopathology 49:192-198.
2. Burke, D. W., Hagedorn, D. J., and Mitchell, J. E. 1970. Soil conditions and distribution of pathogens in relation to pea rot in Wisconsin soils. Phytopathology 60:403-406.
3. Burke, D. W., Mitchell, J. E., and Hagedorn, D. J. 1969. Selective conditions for infection of pea seedlings by *Aphanomyces euteiches* in soil. Phytopathology 59:1670-1674.
4. Cochran, W. G. 1950. Estimation of bacterial densities by means of the "most probable number." Biometrics 6:105-116.
5. de Man, J. C. 1975. The probability of most probable numbers. Eur. J. Appl. Microbiol. 1:67-78.
6. Duncan, J. M. 1976. The use of bait plants to detect *Phytophthora fragariae* in soil. Trans. Br. Mycol. Soc. 66:85-89.
7. Halvorson, H. O., and Ziegler, N. R. 1933. Application of statistics to problems in bacteriology. I. A means of determining bacterial population by the dilution method. J. Bacteriol. 25:101-121.
8. Maloy, O. C., and Alexander, M. 1958. The "most probable number" method for estimating populations of plant pathogenic organisms in the soil. Phytopathology 48:126-128.
9. Mitchell, J. E. 1979. The dynamics of the inoculum potential of populations of soil-borne plant pathogens in the soil ecosystem. Pages 3–20 in: B. Schippers and W. Gams, eds. Soil-borne Plant Pathogens. Academic Press, New York. 686 pp.
10. Mitchell, J. E., Bhalla, H. S., and Yang, G. H. 1969. An approach to the study of the population dynamics of *Aphanomyces euteiches* in soil. Phytopathology 59:206-212.
11. Parnow, R. J. 1972. Computer program estimates bacterial densities by means of the most probable numbers. Food Technol. 26(7):56-62.
12. Pfender, W. F., and Hagedorn, D. J. 1979. Estimating *Aphanomyces euteiches* population in soil by a most probable number technique. (Abstr.) Phytopathology 69:541.
13. Pfender, W. F., and Hagedorn, D. J. 1981. *Aphanomyces* root and stem rot of snap beans. (Abstr.) Phytopathology 71:250.
14. Porter, W. M. 1979. The 'most probable number' method for enumerating infective propagules of vesicular arbuscular mycorrhizal fungi in soil. Aust. J. Soil Res. 17:515-519.
15. Reiling, T. P., King, T. H., and Fields, R. W. 1960. Soil indexing for pea root rot and the effect of root rot on yield. Phytopathology 50:287-290.
16. Ricci, P. 1972. Moyens d'étude de l'inoculum du *Phytophthora nicotianae* f. sp. *parasitica* (Dastur) Waterh., parasite de l'oeillet dans le sol. Ann. Phytopathol. 4:257-276.
17. Sherwood, R. T., and Hagedorn, D. J. 1958. Determining common root rot potential of pea fields. Wis. Agric. Exp. Stn. Bull. 531. 12 pp.