Evaluation of Methods for Estimating Inoculum Potential of *Aphanomyces euteiches* in Soil

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ABSTRACT

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Three methods were evaluated for determining inoculum potential of *Aphanomyces euteiches* inoculum in soil and sand: rolled towel (RT), most probable number (MPN), and soil indexing (SI) bioassays. The RT and MPN bioassays were evaluated using clay loam, loam, and sand that were artificially infested with a series of oospore concentrations ranging from 2 to 500/g. Oospore numbers added to soil and estimated inoculum potential measured using the RT and MPN were highly correlated ($r = 0.99$ and 0.85, respectively). These two bioassays, however, yielded highly variable results among replications, and inoculum potential estimates for sand and soil infested with equal numbers of oospores were dissimilar. When the three bioassays were compared using naturally infested clay loam soil, the SI method provided results with low variability compared with the MPN and RT bioassays. Estimates of inoculum potential obtained with the three bioassays were inconsistent between and within samples tested. The results from these bioassays must be interpreted with caution, and more accurate and sensitive methods are needed for quantitative studies of the inoculum potential and ecology of *A. euteiches* in soil.

Additional keywords: peas, *Pisum sativum*, root rot

The soilborne pathogen *Aphanomyces euteiches* Drechs. infects roots of peas (*Pisum sativum* L.), alfalfa (*Medicago sativa* L.), snap beans (*Phaseolus vulgaris* L.), and many noncrop plants (9). Root rot caused by *A. euteiches* is a serious disease of peas in many regions of the world (5,9). Annual yield losses in the Great Lakes region of the United States have been estimated at 10% (5). Currently, fungicide use is not economically feasible and pea cultivars with high levels of resistance to root rot are not commercially available (5). The most effective method for reducing this disease is to avoid planting peas into fields infested with high populations of *A. euteiches*.

An important component for control of root rot and in studies of the population biology of *A. euteiches* is the ability to accurately and efficiently measure inoculum potential and population levels in soil. Lockwood (7) described two important parts of inoculum potential as being inoculum density and energy available for fungal growth. Inoculum potential in this paper refers to an index of potential disease activity that combines propagule infectivity, propagule density, and soil factors that inhibit or promote infection of roots. Dilution plating techniques used to quantify populations of some other soilborne fungi are not effective for *A. euteiches* because oospores do not germinate consistently in vitro and because no selective media are available for isolation of this fungus from soil. Consequently, bioassays that measure inoculum potential utilizing pea seedlings are used. Rolled towel (RT), soil indexing (SI), and most probable number (MPN) bioassays are the three methods most commonly used to estimate inoculum potential of *A. euteiches* in soil (8,11,14).

The SI method has been used for over 30 yr to assess inoculum potential in pea fields (13,14). Peas are planted in pots filled with the field soil being tested, and severity of root infection is evaluated after about 4 wk to obtain a disease index rating. This method provides information to growers so they can avoid planting peas in fields with high potential for disease development. The SI method, however, lacks sufficient precision for quantitative studies of *A. euteiches* population levels in soil (11).

Two other methods were developed for more precise measurement of *A. euteiches* inoculum density. The MPN technique measures propagule density more directly than does the SI method but it also has limitations (2,11). Peas are planted for the MPN method in a series of soil dilutions, and the proportion of plants infected after 16 days at the various dilutions is used to estimate the concentration of infectious propagules in soil. The RT bioassay also provides a more direct measure of propagule density than the SI method and may be comparable to the MPN method; however, it also may have limited precision and application (6,8). Soil samples for the RT bioassay are placed onto roots of multiple pea seedlings and rolled inside wet paper towels; the percentage of plants infected after about 3 wk is used to estimate inoculum potential.

The purpose of this study was to evaluate the SI, MPN, and modified RT bioassays for estimating inoculum potential of *A. euteiches* in clay loam soils collected from pea production fields in southern Minnesota. Our goal was to determine which method was most accurate, reproducible, and practical for use.

MATERIALS AND METHODS

Infestation of soil and sand with *A. euteiches*. Oospores of *A. euteiches* isolate Ae467 (isolated from pea and provided by C. Grau, University of Wisconsin-Madison) were produced in corn broth (5 g whole dried corn per 100 ml deionized H_2O) or on cornmeal agar (CMA) incubated for 30–35 days at 23 C in darkness. Mycelium containing oospores was removed from the broth or scraped from the CMA surface, homogenized with a Polytron (Kinematica AG, Littau, Switzerland), and filtered through a 180 μm sieve. Oospores were suspended in water, counted with a hemacytometer, and mixed at concentrations from 2 to 420/g into loam greenhouse soil mix (pH 7.5, 4.0% organic matter, 30% sand, 55% silt, and 15% clay), sand (washed industrial silica), or clay loam soil (Webster clay loam) from a field (location I) near Waseca, Minnesota (pH 7.0, 3.5% organic matter, 51% sand, 24% silt, and 23% clay). Soils and sand were autoclaved 45 min at 121 C prior to infestation and air-dried for 24 hr at 24 C to 4% moisture. These artificially infested soils and sand were used for RT and MPN bioassays.

Collection of field soil naturally infested with *A. euteiches*. Soil samples for RT, MPN, and SI bioassays were collected in October from two pea production fields (designated locations I and II) with a history of pea root rot in southern Minnesota near Waseca. Four bulk samples (Webster clay loam) naturally infested with *A. euteiches* were collected from each field. A bulk sample consisted of five 2,400-g subsamples collected 3–4 m apart to a depth of 1–12 cm from one of four randomly chosen sites in each field. About 500 g of each

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of the eight bulk soil samples was dried for 24 hr at 24 C to approximately 4% soil moisture, ground to a fine consistency with a mortar and pestle, and stored at 7 C for 2–3 mo until used for the RT and MPN bioassays. Soil samples for the SI bioassays were stored at 20% soil moisture at 16 C for 2–3 mo and used without drying.

RT, MPN, and SI bioassays. RT bioassays were performed using a procedure described by Mitchell et al (8) that was modified to use all components of soil rather than only sieved organic fractions. The whole soil was used because our field samples contained very small amounts of organic material large enough to be trapped on a 200-mesh sieve. This change eliminated the need to collect and process large samples. Four 5-day-old pea seedlings (Perfection 8221, Nunhems Seed Corp., Lewisville, ID) were placed side by side on a wet paper towel (bleached, 26 × 34 cm), and 0.6 g of ground field or artificially infested soil was placed onto each exposed root about 3 cm from the tip. Seedlings and soil were then covered with another wet paper towel, gently rolled into a column, and secured with a rubber band. The process was replicated five times per soil sample for each RT bioassay (20 seedlings per RT bioassay). The assembled RT bioassays were placed inside plastic bags containing about 2 cm of water and incubated for 19 ± 1 days at 22 C. After incubation, the plants with typical symptoms of *A. euteiches* root rot infection (honey-yellow coloration and loss of turgor) were counted and results were recorded as percent infected per 20 plants. About 5% of plants with symptoms characteristic of Aphanomyces root rot were selected at random, and the fungus was isolated to confirm its presence. Roots were surface-disinfested with 0.25% sodium hypochlorite, and pieces 2 cm long were placed onto a semiselective medium (10) containing rifampicin (25 mg/L) in place of vancomycin.

The MPN bioassay was conducted as described by Pfender et al (11). Samples within each MPN bioassay tray consisted of the following: one nondiluted and five diluted samples of infested soil prepared with autoclaved, ground vermiculite (soil:vermiculite 1:1, 1:9, 1:24, 1:99, and 1:399); soil infested with 84 oospores per gram; and an autoclaved vermiculite control. After 18 days of growth in a greenhouse at 23 ± 2 C, the proportion of plants infected was determined at each dilution. The values were analyzed by a computer program (11) to estimate the number of infectious fungal propagules per gram (IPPG) of soil.

RT bioassays used to estimate inoculum potential in autoclaved loam soil artificially infested with 85, 330, and 500 oospores per gram were repeated five times, and MPN bioassays with the same soil infested with 85, 170, and 450 oospores per gram were repeated twice. The MPN and RT bioassays were also used to evaluate inoculum potential in autoclaved sand and clay loam soil artificially infested with 0, 2, 4, 16, 20, 42, 84, 170, 210, and 420 oospores per gram. Bioassays done with soil infested with 2, 4, 20, and 42 oospores per gram were repeated twice, and bioassays done with other infested soils were repeated four times.

Root rot potential in bulk soil samples from each of the four sites in field locations I and II was evaluated with two and three replicates, respectively, of the SI bioassay (14). Each SI bioassay was conducted by filling three plastic pots (10 × 15 cm) with a soil sample, and 11 pea seeds (Perfection 8221) treated with metalaxyl (Apron 25F), 1.3 ml a.i./kg seed, were sown per pot. Peas were grown for 32 days in a greenhouse maintained at 23 C and watered lightly until the first two leaves were expanded, then heavily to maintain soil near saturation for the next 14 days. The pea roots were evaluated for severity of root infection, on a 0–4 scale, and the resulting values were used to calculate a disease index as described by Sherwood and Hagedorn (14).

RESULTS

Estimation of inoculum potential in artificially infested soil and sand. Inoculum potential in autoclaved loam soil infested with known numbers of *A. euteiches* oospores was evaluated with the RT and MPN bioassays. Both methods yielded strong linear relationships between the actual number of oospores in loam soil and the inoculum potential estimates ($r = 0.99$ and 0.85 for the RT and MPN bioassays, respectively) (Fig. 1). Standard errors as a percentage of the mean tended to decrease as the number of oospores increased. For the RT data, standard errors were 106, 46, and 37% of the mean at 84, 340, and 420 oospores per gram, respectively. For the MPN data, standard errors were 28, 12, and 4% of the mean at 84, 170, and 450 oospores per gram, respectively. Thus, the results for both methods were more consistent as oospore concentrations increased in autoclaved loam soil.

The inoculum potential in autoclaved silica sand and clay loam soil from southern Minnesota (location I) artificially infested with a series of oospore concentrations from 0 to 140 per gram was estimated with the RT and MPN bioassays (Fig. 2). Mean estimates of inoculum potential from the RT bioassays tended to be higher for the sand than for the clay loam soil at all oospore concentrations except 16 and 210 per gram (Fig. 2A and B). However, the estimates of inoculum potential from the MPN bioassay tended to be greater for clay loam soil than for sand at 40 and 170 oospores per gram (Fig. 2C).

Analysis of inoculum potential in field soil samples. Inoculum potential of *A. euteiches* in soil from four sites in field location II was estimated using three replicates of the RT, MPN, and SI bioassays. The three bioassay methods did not provide similar estimates of inoculum potential for individual soil samples (Table 1). Estimates of inoculum potential obtained with one bioassay also did not relate quantitatively to estimates obtained with another bioassay. The three methods yielded variable estimates

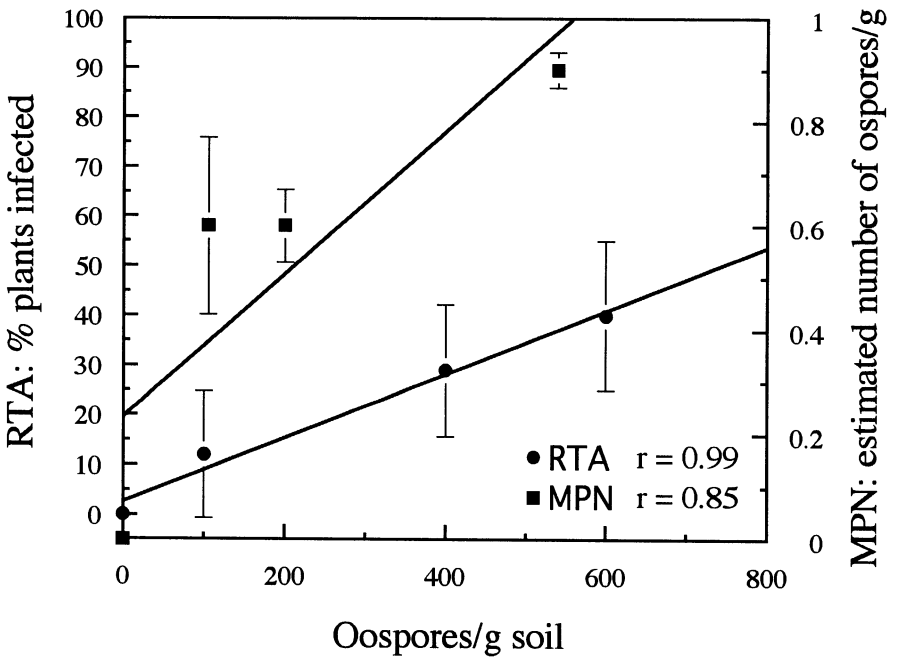

Fig. 1. Estimation of inoculum potential in autoclaved loam soil artificially infested with various concentrations of *Aphanomyces euteiches* oospores using rolled towel (RTA) and most probable number (MPN) bioassays. Data points for RTA and MPN represent the mean from five and two experimental repetitions, respectively. Vertical bars denote standard errors.

of relative inoculum potential for single soil samples; however, the trend was for SI results to be the least variable among replications (Table 1). The mean standard deviations for the SI, MPN, and RT bioassays of soil from the four sites were 15, 57, and 80% of the mean inoculum potential estimates, respectively. Similar results were obtained when four soil samples from field location I were evaluated with MPN and RT bioassays and repeated twice (Table 2). The two bioassays did not provide similar relative estimates of inoculum potential for the samples from this location. Fungi isolated from pea roots with root rot symptoms in different bioassays were characterized as *A. euteiches* on the basis of microscopic observation of hyphae and spores.

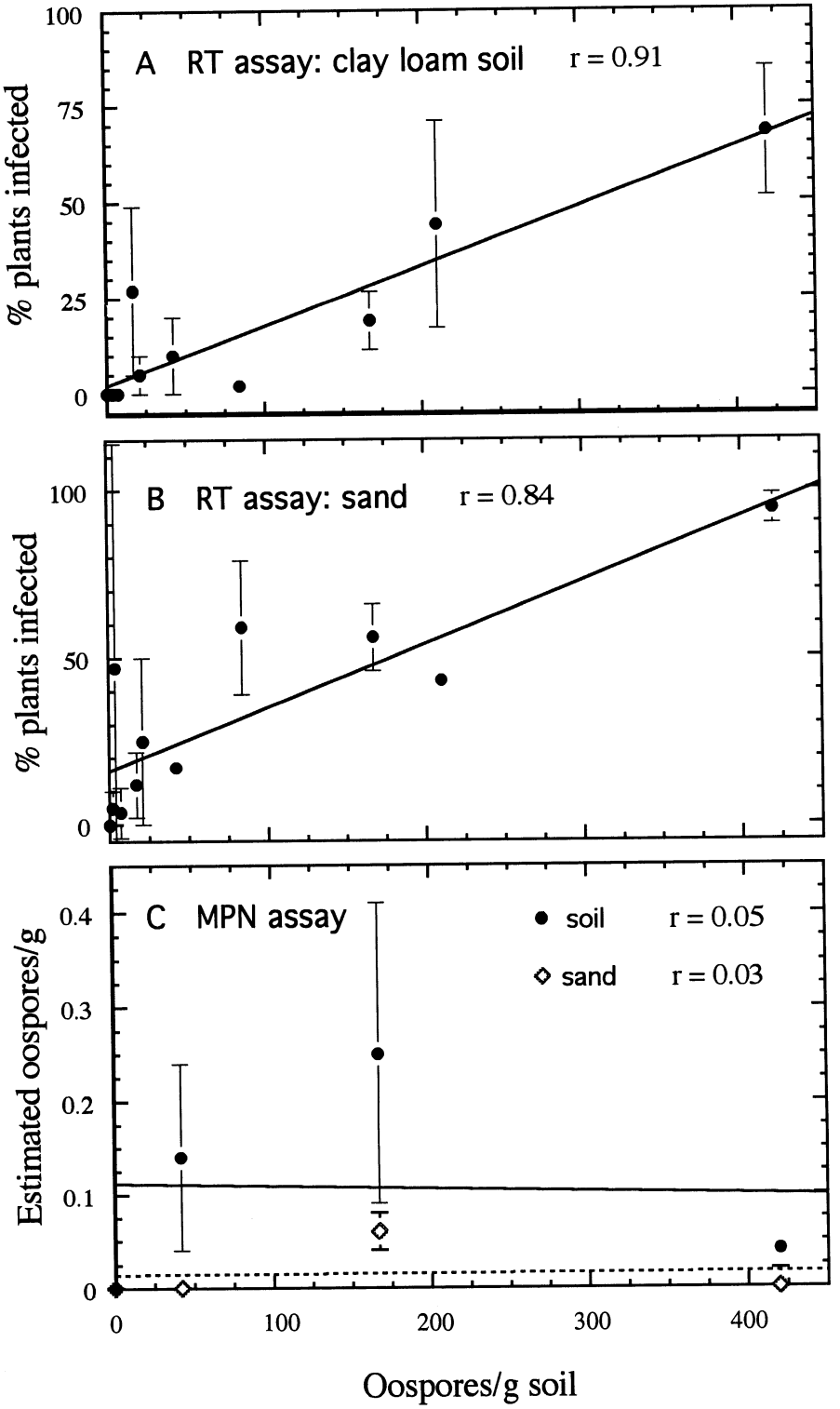

Fig. 2. Estimation of inoculum potential of *Aphanomyces euteiches* in artificially infested clay loam soil and silica sand using the **(A and B)** rolled towel (RT) and **(C)** most probable number (MPN) bioassays. Bioassays done with soil infested with 2, 4, 20, or 42 oospores per gram were repeated twice, and bioassays done with other infested soils were repeated four times. Data points represent means, and vertical bars denote standard errors.

DISCUSSION

Our results illustrate some of the problems as well as the challenges inherent in the RT, SI, and MPN bioassays. Although no single bioassay provided an accurate and reproducible measure of inoculum potential and populations of *A. euteiches*, each had its strengths and weaknesses.

The SI bioassay can be used to identify pea fields with high potential for root rot development (R. Rand, *personal communication*). It is technically the most simple bioassay, and the estimates of inoculum potential were less variable among replications than those from the MPN and RT bioassays. Potential disadvantages are the requirements for greenhouse space and large volumes of soil and the inability to quantify *A. euteiches* populations. As discussed by Sherwood and Hagedorn (14), watering practice can influence results from SI bioassays. Underwatering results in a poor estimate of disease index in soil, while moderate and heavy watering can yield similar estimates. Heavy watering, as recommended (14) and used in this study, shortens the time required to complete the test and enhances the ability of the bioassay to distinguish among soils with different levels of infestation.

An advantage of the MPN bioassay over the SI bioassay is the smaller amount of soil and greenhouse space required. Of the three bioassays, the MPN bioassay is technically the most complicated and labor-intensive. This bioassay was developed to estimate inoculum density of *A. euteiches* in soil (11). However, it actually provides estimates of inoculum potential in soil, as do the SI and RT methods, rather than the number of viable propagules, as would be obtained by a dilution plating method. Therefore, the MPN method also has limited application to population biology studies. Another disadvantage of the MPN bioassay, as reported by Pfender et al (11), was that results were more consistently related to SI bioassay results when sand was used rather than loam soil. The potential impact of soil texture on MPN results may be significant in Minnesota, where clay loam soils occur commonly in pea production areas. Results from this study suggest that soil texture influences the inoculum potential estimates obtained with MPN and RT bioassays.

Two assumptions underlying the MPN method in soil are that *A. euteiches* propagules are distributed randomly throughout samples and a single propagule infects independently (3,11). We believe the first assumption is questionable for *A. euteiches* because there is no evidence that this pathogen is distributed randomly in its natural habitat. Oospores are produced in roots, and most may

Table 1. Quantification of inoculum potential of *Aphanomyces euteiches* in clay loam field soil samples from four sites in a pea field in southern Minnesota (location II) using rolled towel (RT), most probable number (MPN), and soil indexing (SI) bioassays

Site	Percent root area infected[a]	Estimates of inoculum potential[b]		
		RT percent infected	SI disease index[c]	MPN IPPG[d]
1	29.7	15.0 (8.2)	80.4 (7.2)	0.48 (0.20)
2	19.2	17.7 (11.2)	81.5 (4.3)	0.22 (0.12)
3	47.1	1.7 (2.4)	64.8 (20.5)	0.06 (0.03)
4	...	13.0 (8.0)	82.3 (11.1)	0.12 (0.10)

[a]Twenty-five plants were evaluated at 10% bloom.
[b]Each value is the mean of three repetitions; standard deviation is shown in parentheses.
[c]Disease index >70 indicates highly infested soil.
[d]Estimated number of infectious propagules per gram (IPPG) of soil.

Table 2. Estimation by rolled towel (RT) and most probable number (MPN) bioassays of inoculum potential in clay loam soil naturally infested with *Aphanomyces euteiches* collected from four sites in a pea field in southern Minnesota (location I)

Sample	Estimates of inoculum potential[a]	
	RT percent infected[b]	MPN IPPG[c]
A	54.0 (5.7)	8.0 (7.6)
B	67.5 (46.0)	13.3 (10.2)
C	22.0 (2.8)	13.9 (7.1)
D	69.5 (43.1)	6.7 (4.0)

[a]Each value is the mean of two repetitions; standard deviation is shown in parentheses.
[b]Twenty plants per bioassay.
[c]Estimated number of infectious propagules per gram (IPPG) of soil.

remain in soil associated with root fragments (1) and perhaps in microsites where roots decompose. Tillage practices may also influence the degree of clustering of fungal propagules in field soils (16). The second assumption is also questionable. Oospores can germinate directly via a germ tube or indirectly via a zoosporangium, depending on propagule age and nutritional status of surrounding soil (9). The number of zoospores released from a single zoosporangium ranges from a few to 300 or more (9). Thus, one oospore can potentially give rise to multiple root infections. Furthermore, the number of infections that occur on a single root cannot be determined with the MPN bioassay. These assumptions as applied to *A. euteiches* raise questions concerning how MPN bioassay results should be interpreted and whether this method actually measures the number of infectious propagules per gram of soil.

The RT bioassay may provide a more direct measure of the inoculum potential of *A. euteiches* than the MPN bioassay and is not based on the same assumptions. Advantages of the RT bioassay include its technical simplicity and the requirement for smaller amounts of soil and bench space than the other two bioassays. Root infections in the RT bioassay are easier to observe, diagnose, and isolate *A. euteiches* from because of the limited contamination of roots by soil as compared with the SI and MPN bioassays. The RT bioassay is also a good method for detecting *A. euteiches* in soil samples (6,8). The RT bioassay, however, also has limited ability to quantify propagules in soil.

The accuracy of the SI, MPN, and RT bioassays for quantification of *A. euteiches* inoculum potential in soil cannot be compared directly because each of these bioassays measures inoculum potential differently. For example, the entire pea root system is exposed to infested soil for about 30 days in the SI bioassay, while only 1–2 cm of the root in the RT and MPN bioassays is exposed to infested soil for only 18–20 days. Furthermore, the types and nature of propagules of *A. euteiches* in soil are not well understood (7), and neither total nor viable oospores can be enumerated by any method.

A major concern with the bioassays evaluated in this study, especially the MPN and RT methods, was the large standard deviations among replications. Variability in results from RT bioassays that limited their quantitative value was also reported by Mitchell et al (8). Other evaluations of the RT (6) and MPN (2,11) bioassays did not clearly describe numbers of replications used or variability among replications. One explanation for the variability may be a nonrandom distribution of *A. euteiches* propagules in soil. Populations of *Pythium* and *Phytophthora* spp., which are enumerated from soil using selective media, have been shown to vary and cluster within soil of bean fields (12), in the rhizosphere of sugar beets and tobacco (4,15,16), and vertically within a soil sample (4). Boosalis and Scharen (1) observed large numbers of *A. euteiches* oospores in plant debris fragments from soil, suggesting that *Aphanomyces* propagules are clustered in soil where plant debris is concentrated. In our experience, oospores also tended to clump in artificially infested soil. Variability in the RT bioassay results from this study tended to decrease as the concentration of *A. euteiches* oospores in artificially infested autoclaved soil increased.

In summary, our results and those from other studies (2,8,11) suggest that the SI, MPN, and RT bioassays are not suitable to precisely measure inoculum potential or populations of *A. euteiches* in soil. These bioassay techniques may need to be modified to provide more accurate results. An underlying problem is an incomplete understanding of the biology of *A. euteiches* in soil, alone and in association with its host and other microbes. We have chosen the RT bioassay as a tool for our research based on the results from this study. Other methods, such as immunoassays and dilution plating, will ultimately be needed to study population dynamics of *A. euteiches* in soil. Until such improved methods are available, the bioassays evaluated in this study must be used carefully and the results interpreted with caution.

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