Journal of Nematology 28(1):50–55. 1996.

Quantification of Endospore Concentrations of *Pasteuria penetrans* in Tomato Root Material[1]

Z. X. Chen,[2] D. W. Dickson,[3] and T. E. Hewlett[4]

Abstract: Six methods for quantification of the endospore concentrations of *Pasteuria penetrans* from tomato roots are described. Mortar disruption and machine disruption methods gave the highest estimations (endospores per gram of root material) of 83.7 and 79.0 million, respectively. These methods were significantly superior to incubation bioassay (47.7 million), enzymatic disruption (32.1 million), and enzymatic disruption + flotation (25.8 million) methods. A centrifugation bioassay method gave the lowest estimation of 12.7 million.

Key words: bacterium, biological control, endospore, *Lycopersicon esculentum, Meloidogyne arenaria,* method, *Pasteuria penetrans,* root-knot nematode, tomato.

Pasteuria penetrans (Thorne) Sayre & Starr, an obligate mycelial and endospore-forming bacterial parasite of root-knot nematodes, has shown potential as a biological control agent of plant-parasitic nematodes (5). It occurs in nematode suppressive soils (6) and has suppressed nematodes in greenhouse (4,7) and field microplot experiments (13). Endospores of *P. penetrans* attach to the cuticle of second-stage juvenile (J2) of *Meloidogyne* spp. in soil. Apparently infection occurs after the J2 enters a plant root, where parasitized nematodes are able to develop into females but are incapable of reproduction (2). The females and sometimes males (9) become filled with endospores of the bacterium, which are eventually released into the soil upon host disintegration. Thus, the only life stage of *P. penetrans* for practical application in soil is the mature endospore.

Pasteuria penetrans has not been grown on artificial media (3). The parasite must be reared on *Meloidogyne* spp., which in turn must be reared on susceptible plants (16) or in a nematode-excised root system (18). Generally, endospores of *P. penetrans* are mass-produced in root-knot nematodes on tomato plants. The roots of the plants are air-dried and ground into a fine powder (16). Counting of endospores is nearly impossible in such preparations (17). The amount of endospores applied to the soil has been presented in either grams of root inoculum per kilogram of soil (7) or as the number of endospores attached per juvenile (13). These methods lack accuracy and precision because the number of endospores per unit of root inoculum varies significantly among different preparations of inocula or among inocula from different laboratories (16).

In this study, six methods for determining endospore concentrations in root material are described and compared. The objectives were to develop accurate, precise, and convenient methods for quantifying endospores in root material. Such methods are useful in field evaluations of *P. penetrans* for managing root-knot nematodes.

Materials and Methods

Nematode isolate: An isolate of *Meloidogyne arenaria* (Neal) Chitwood race 1 from Levy County, Florida, was used in this experiment. The isolate was cultured on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in a steam-pasteurized potting soil. Eggs of *M. arenaria* were extracted from roots in 0.5% sodium hypochlorite for 30 seconds (12) and caught on a sieve with 25-μm-pore openings, rinsed, and placed on Baermann funnels (1). The roots and plant debris were caught and separated

Received for publication 24 July 1995.

[1] Florida Agricultural Experiment Station Journal Series No. R-04783. Supported in part by a grant from the Southern Region IPM Grant No. 93-34103-8396. A portion of a Ph.D. dissertation by the first author.

[2] Graduate Research Associate, [3]Professor, and [4]Senior Biologist, Entomology and Nematology Department, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL 32611-0620.

E-mail: dwd@gnv.ifas.ufl.edu

The authors thank R. A. Dunn and G. C. Smart, Jr. for reviewing the manuscript.

from the eggs by a sieve with 75-μm-pore openings nested on a sieve with 25-μm-pore openings. Second-stage juveniles (J2) that hatched on the first day were discarded to avoid using nematodes that may have acquired endospores in the potting soil. The juveniles were collected by decanting the water suspension from Baermann funnels on a sieve with 25-μm-pore openings, rinsed, and transferred into a beaker. The number of J2 in the suspension was determined by counting five 1-ml aliquots with use of a compound microscope.

Pasteuria penetrans isolate: An isolate of *P. penetrans* designated P-20 (13), derived from *M. arenaria* race 1 from a peanut field in Levy County, Florida, was propagated on *M. arenaria* race 1 growing on tomato in a greenhouse. Endospore-attached J2 (up to 5 days old) were obtained by a centrifugation attachment method (10). Potted tomato seedlings (45–60 days old) were inoculated three times with the endospore-attached J2 (1,000–1,500 J2/pot/time) at intervals of 1 week. Averaged over all inoculations, 96 ± 5% of the J2 had at least one endospore attached, with 4.9 ± 2.0 endospores/J2 among those J2 with endospores. These estimates were made by counting 20 J2/aliquot. The tomato plants were grown in 15-cm-d pots and were fertilized weekly with 1 g of 20-20-20 (N-P-K)/pot. Insecticide applications and water were provided as needed. Root systems were harvested 45–60 days after the last inoculation, rinsed, and air-dried. The primary root was removed to reduce unnecessary root debris. The dried root systems were chopped into pieces of 2- to 3-cm lengths, mixed thoroughly, and weighed approximately 1 kg. Random samples of approximately 10 g each were taken for enzymatic disruption and enzymatic disruption + flotation methods.

Quantification methods: 1. Enzymatic disruption method. Six random subsamples of 1 g each were taken from the 10 g root material and incubated in 50 ml of a 12.5% cytolase solution (Genencor International, Rochester, NY). After 3 days, the material was decanted onto a sieve with 600-μm-pore openings nested in a sieve with 75-μm-pore openings and subjected to a high-pressure spray of water to dislodge the females (11). Endospore-filled females, conspicuous by an opaque and white appearance when illuminated from above, were hand-picked with a pipette (Fisher Scientific No. 13-678-20A) with the aid of a microscope at ×20. The "healthy" females appeared shiny and somewhat transparent. The females containing endospores were ground in deionized water using a glass tissue grinder. The suspension, containing endospores, was decanted into a flask and diluted to a volume of 50 ml. Concentrations of endospores in the suspensions were determined by counting five 0.1-μl aliquots using a hemocytometer (Fisher Scientific No. 02-671-10) at ×450.

2. Enzymatic disruption + flotation method. The same method as described above was used to dislodge females. Root debris with endospore-filled females in water were centrifuged at 1,000*g* for 3.5 minutes in a 50-ml centrifuge tube. The supernatant was discarded and the pellet was mixed with a 36% (w/v) sucrose solution and centrifuged again. Females floating in the sucrose solution were collected and ground in deionized water, and concentrations of endospores were determined.

3. Mortar disruption method. The chopped-root material (ca. 900 g) was ground in a Wiley mill (Model 4, Arthur H. Thomas, Philadelphia, PA, Thomas Scientific No. 3375-E10) to a powder that passed through a sieve with 5-mm-pore openings. Six random subsamples of 0.5 g of this powder plus 5 ml deionized water were placed in a 6-cm-d mortar and ground with a pestle to a slurry. The slurry was transferred to a 100-ml Erlenmeyer flask and diluted to 100 ml with deionized water. The endospore concentration in the suspension was determined with a hemocytometer at ×450.

4. Machine disruption method. The ground roots from the mortar disruption method (No. 3) were further ground in a Wiley mill (Intermediate Model, Arthur

H. Thomas, Philadelphia, PA, Fisher Scientific No. 08-338) into a powder that passed through a sieve with 250-μm-spore openings. Six random subsamples of 0.5 g each of this powder were suspended in 100 ml deionized water. The endospore concentration in the suspension was determined with a hemocytometer at ×450.

5. Centrifugation bioassay method. The remainder of the six endospore suspensions from the mortar disruption method (No. 3) were used. The suspensions were passed through a sieve with 25-μm-pore openings to separate the root debris and further diluted by pipetting 1 ml of the suspension into 9 ml water. Three subsamples from each diluted suspension were taken to determine the endospore concentrations using the centrifugation attachment method (10). A combination of 0.9 ml of the diluted suspension and 0.1 of 2-day-old J2 suspension of 290 ± 25 J2/ml was placed in a 1.0-ml microfuge tube and centrifuged at 8,700*g* for 2 minutes using a Beckman microfuge (Beckman, Palo Alto, CA). Nematodes were removed from the tubes with a pipette and placed in Corning cell wells (Corning, Corning, NY). Numbers of endospores per J2 were determined on 15 nematodes per replicate.

A standard curve was developed to interpret the numbers of endospores per J2 to endospore concentrations in the suspension. A series of endospore concentrations (500, 1,000, 5,000, 10,000, 50,000, and 100,000 endospores/ml) was prepared by serially diluting a stock endospore suspension. The stock endospore suspension was made from hand-picked females. Endospore attachment of J2 from individual concentrations was determined as described above. Each concentration was replicated three times. The endospore attachment rates per J2 were subjected to regression analysis. Endospore concentrations in the unknown samples were determined by using the regression equation.

6. Incubation bioassay method. Three subsamples from each of the six endospore suspensions that originated in the mortar disruption method (No. 3) were taken to determine the endospore concentrations. A combination of 0.9 ml of the diluted endospore suspension and 0.1 ml of the J2 suspension was placed into a Corning cell well and incubated at 22–25 C for 18 hours. Fifteen J2 per replicate were examined for the number of endospores attached.

A similar series of endospore concentrations as indicated in the centrifugation bioassay method (No. 5) was prepared. Three subsamples from each concentration were incubated with J2 at 22–25 C to determine the endospore attachment. The results were subjected to regression analysis. The concentrations of endospores in the unknown suspension were determined from the regression equation.

Results and Discussion

Differences were observed among methods tested for determining endospore concentrations of *P. penetrans* in tomato root material ($P \leq 0.05$) (Table 1). The estimations from the mortar disruption and machine disruption methods were larger than those of the other four methods ($P \leq 0.05$). The smallest estimation was from

Table 1. Comparison of six methods for quantification of endospore concentrations of *Pasteuria penetrans* from tomato roots (million endospores per gram of root material).

Method[a]	Average	SD	CV (%)
Mortar disruption	83.7 a[b]	11.6	13.9
Machine disruption	79.0 a	9.4	11.9
Enzymatic disruption	32.1 c	3.8	11.8
Enzymatic disruption + flotation	25.8 c	8.4	32.6
Incubation bioassay	47.7 b	10.4	21.8
Centrifugation bioassay	12.7 d	4.3	33.9

[a] Mortar disruption and machine disruption methods used a mortar, and a Wiley mill, respectively, to grind root material followed by hemocytometer counting of endospores. Enzymatic disruption and enzymatic disruption + flotation methods used cytolase to dislodge the females followed by hand-picking of females and centrifuge separation of females, respectively. Incubation bioassay and centrifugation bioassay methods used mortar-grinding of root material followed by an incubation bioassay at 22–25 C and centrifugation attachment method, respectively, to detect the endospore concentrations.

[b] Numbers followed by different letters are different ($P \leq 0.05$) according to Duncan's multiple-range test.

the centrifugation bioassay method ($P \leq 0.05$).

Mortar disruption and machine disruption methods provided the most precise and accurate estimations. These methods detected large numbers of endospores present in the tomato root material. The coefficients of variation were relatively small (Table 1). The similarity between the results of these methods indicated that machine grinding of root materials was effective enough to produce a fine powder containing endospores that could be used for field evaluations of *P. penetrans*. Although both methods gave satisfactory results, the mortar disruption method produced a slurry that often hindered viewing endospores in the suspension. This method was useful for handling small quantities of material. Alternatively, the root powder produced by the machine disruption method had a relatively consistent size. Endospore suspensions made from this powder appeared to facilitate viewing of the endospores, primarily due to improved light penetration of the suspension. Mortar disruption and machine disruption methods produced large amounts of plant root debris in the endospore suspension. The removal of the primary root, however, reduced approximately half of the plant root debris, thus enabling use of the hemocytometer for counting endospores. The counting was time-consuming, and experience was required to differentiate between endospores and other particles. If the endospore concentrations are low, these methods may not detect the existing endospores.

Sharma and Stirling (16) described a method similar to the mortar disruption method for quantification of endospores in root material. They detected 100–1,200 million endospores/g of root material, which is 3–35 times higher than we detected with the mortar disruption and machine disruption methods. They, however, did not provide standard deviations, so comparisons of precision cannot be made. Gowen and Channer (8) used an unknown method (probably the same method of Sharma and Stirling) to estimate endospore concentrations and reported 480–1,859 million endospores/root system, which is ca. 15–60 times higher than we obtained. They did not provide the standard deviations either. These differences in the reported endospore concentrations may reflect differences in isolates of *P. penetrans,* cultural conditions, or detection methods.

Enzymatic disruption and enzymatic disruption + flotation methods removed much of the plant root debris from the sample. This facilitated the counting of endospores in the suspensions and reduced counting time. Hand-picking of endospore-filled females, however, was tedious. Approximately 3–5 hours may be required to pick all the females from the root debris of a single plant (dry weight ca. 2 g). Enzymatic disruption + flotation, on the other hand, was rapid. It gave a slightly lower estimation than that of the enzymatic disruption method (Table 1). The lower estimation probably resulted from some females becoming trapped in root debris. Some endospore-filled females adhered to loosened cortical tissue, while others had their heads trapped inside the stele tissue. The number of endospore-filled females trapped in debris varied considerably from sample to sample. More females appeared to be trapped in root gall tissues induced from multiple nematode infections than from single nematode infections.

Compared with the enzymatic disruption method, the enzymatic disruption + flotation method required less time. Enzymatic disruption + flotation method required approximately 20 minutes to separate endospore-filled females from six samples, which was less than 3% of the total time required for the six samples processed by the enzymatic disruption method. The latter, however, had a small coefficient of variation, indicating a high precision of detection. Both methods produced low number of endospores (Table

1), implying many females had been lost or ruptured.

Although the specific weight of endospores of *P. penetrans* is reported to be 1.28 g/cm^3 (14), 36% sucrose solution (specific weight of 1.14 g/cm^3) is sufficient to float the endospore-filled females. Compared to methods described by Pableo (15) and Hussey (11), the enzymatic disruption + flotation method involved fewer steps to separate endospore-filled females from root debris. The enzymatic disruption + flotation method also used a higher concentration of sucrose solution during centrifugation because endospore-filled females are denser than the uninfected females (pers. obs.). Methods used by Pableo and Hussey, however, are more desirable for obtaining *Meloidogyne* females for biochemical studies. Enzymatic disruption + flotation method was an extension of Hussey's method for separating endospore-filled females from root debris.

Incubation bioassay method, which detected 47.7 million endospores/g of root material, was approximately half as effective as the mortar disruption and machine disruption methods. In this method nematodes acquired endospores by moving or swimming in the endospore suspension. The movement of nematodes correlates with the endospore acquisition (17). The lower estimation of endospores as compared to the mortar disruption and machine disruption methods probably was due to decreased nematode movement during the incubation process. Degradation of plant root debris at room temperature could result in lower oxygen levels, which reduces nematode activity (19). Centrifugation bioassay method gave the lowest estimation among the methods evaluated ($P \leq 0.05$) (Table 1) and was unsatisfactory. The poor results obtained with this method may be due to plant root debris covering the J2, thus separating them from endospores during centrifugation. The standard curves were developed with 'clean' suspensions prepared with hand-picked females (Fig. 1). Transformations based on such curves will underestimate the endospore concentrations in samples containing debris. Thus, standard curves made from 'dirty' suspensions may be

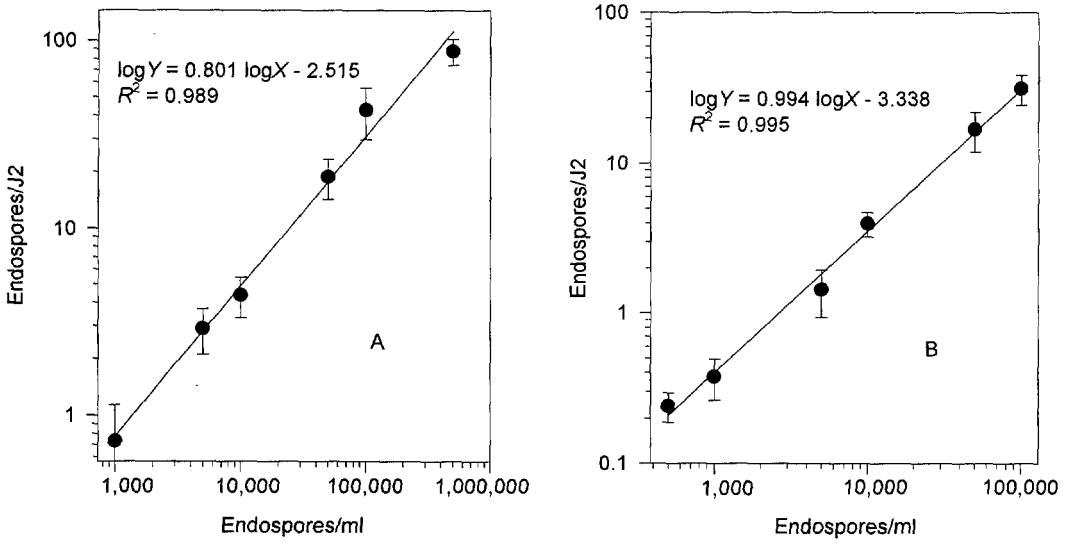

FIG. 1. Regression of the number of endospores attached to second-stage juveniles (J2) of *Meloidogyne arenaria* race 1 to endospore concentrations of *Pasteuria penetrans*. A) Centrifuge-bioassay method—J2 were centrifuged with endospore suspensions for 2 minutes at 8,700*g*. B) Incubation-bioassay method—J2 were incubated with endospore suspensions for 18 hours at room temperature (22–25 C). Bars show standard deviations. Logarithm scale.

needed to improve the estimates of endospore concentrations in practical samples.

Literature Cited

1. Baermann, G. 1917. Eine einfache methode zum auffinden con ankylostomen (Nematoden) larven in enproben. Geneeskuding Tijdschrift voor Nederlandsch-Indie 57:131–137.

2. Bird, A. F., and P. G. Brisbane. 1988. The influence of *Pasteuria penetrans* in field soils on the reproduction of root-knot nematodes. Revue de Nématologie 11:75–81.

3. Bishop, A. H., and D. J. Ellar. 1991. Attempts to culture *Pasteuria penetrans* in vitro. Biocontrol Science and Technology 1:101–114.

4. Brown, S. M., J. L. Kepner, and G. C. Smart, Jr. 1985. Increased crop yields following application of *Bacillus penetrans* to field plots infested with *Meloidogyne incognita*. Soil Biology and Biochemistry 17:483–486.

5. Dickson, D. W., M. Oostendorp, R. M. Giblin-Davis, and D. J. Mitchell. 1994. Control of plant-parasitic nematodes by biological antagonists. Pp. 575–601 *in* D. Rosen, F. D. Bennett, and J. L. Capinera, eds. Pest management in the subtropics, biological control-A Florida perspective, Hampshire, UK: Intercept.

6. Dickson, D. W., M. Oostendorp, and D. J. Mitchell. 1992. Development of *Pasteuria penetrans* on *Meloidogyne arenaria* race 1 in the field. Pp. 213–218 *in* F. J. Gommers, and P. W. Th. Maas, eds. Nematology from molecule to ecosystem. Dundee, Scotland: European Society of Nematologists.

7. Dube, B., and G. C. Smart, Jr. 1987. Biological control of *Meloidogyne incognita* by *Paecilomyces lilacinus* and *Pasteuria penetrans*. Journal of Nematology 19:222–227.

8. Gowen, S. R., and A. G. Channer. 1988. The production of *Pasteuria penetrans* for control of root-knot nematodes. Proceedings of Brighton Crop Protection Conference, Pests and Diseases. Surrey, England: The British Crop Protection Council.

9. Hatz, B., and D. W. Dickson. 1992. Effect of temperature on attachment, development, and interactions of *Pasteuria penetrans* on *Meloidogyne arenaria*. Journal of Nematology 24:512–521.

10. Hewlett, T. E., and D. W. Dickson. 1994. A centrifugation method for attaching endospores of *Pasteuria* spp. to nematodes. Journal of Nematology 25:785–788.

11. Hussey, R. S. 1971. A technique for obtaining quantities of living *Meloidogyne* females. Journal of Nematology 3:99–100.

12. McClure, M. A., T. H. Kruk, and I. Misaghi. 1973. A method for obtaining quantities of clean *Meloidogyne* eggs. Journal of Nematology 5:230.

13. Oostendorp, M., D. W. Dickson, and D. J. Mitchell. 1990. Host range and ecology of isolates of *Pasteuria* spp. from the southeastern USA. Journal of Nematology 22:525–531.

14. Oostendorp, M., T. E. Hewlett, D. W. Dickson, and D. J. Mitchell. 1991. Specific gravity of spores of *Pasteuria penetrans* and extraction of spore-filled nematodes from soil. Journal of Nematology 23:729–732.

15. Pableo, E. C. 1981. Rapid separation of female *Meloidogyne* from root debris suspension. Nematologica 27:242–243.

16. Sharma, R. D., and G. R. Stirling. 1991. In vivo mass production systems for *Pasteuria penetrans*. Nematologica 37:483–484.

17. Stirling, G. R., R. D. Sharma, and J. Perry. 1990. Attachment of *Pasteuria penetrans* spores to the root-knot nematode *Meloidogyne javanica* in soil and its effects on infectivity. Nematologica 36:246–252.

18. Verdejo, S., and B. A. Jaffee. 1988. Reproduction of *Pasteuria penetrans* in a tissue-culture system containing *Meloidogyne javanica* and *Agrobacterium rhizogenes*-transformed roots. Phytopathology 78: 1284–1286.

19. Wong, T. K., and W. F. Mai. 1973. *Meloidogyne hapla* in organic soil: Effects of environment on hatch, movement, and root invasion. Journal of Nematology 5:130–138.