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Estimating Incidence of Attachment of *Pasteuria penetrans* Endospores to *Meloidogyne* spp. with Tally Thresholds[1]

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Abstract: *Pasteuria penetrans* has been identified as an important biological control agent of root-knot nematodes. In this study the use of tally thresholds was evaluated for estimating *P. penetrans* endospore attachment to second-stage juveniles (J2) of *Meloidogyne* spp. A tally threshold (T) is defined as the maximum number of individuals in a sample unit that may be treated as absent based on binomial sampling. Three different data sets that originated from centrifugal bioassay, incubation bioassay, and field experiments were investigated. The data sets each contained 70, 33, and 111 estimates of the mean number of endospores attached per J2 (m), respectively. Empirical relationships between m and proportions of J2 with $\leq T$ endospores attached (P_T) were developed using parameters from the linear regression of $\ln(m)$ on P_T ($0 < P_T < 1$): $\ln(m) = a + b P_T$. T was set to 0, 1, 2, 3, 4, 5, 8, and 10 endospores/J2. The results indicated that the variances of linear equations tended to decrease with increasing T values for all three data sets. T values of 0, 1, 8, and 10 endospores/J2 for centrifugal bioassay and incubation bioassay, and of 0, 1, 2, and 3 endospores/J2 for field experiments were associated with an r^2 of ≥ 0.8. These T values were robust for estimating m from P_T, reducing the variability as well as the time and effort spent in estimating the mean number of endospores attached per J2.

Key words: bacterium, biological control, endospore, *Meloidogyne* spp., nematode, *Pasteuria penetrans*, root-knot nematode, sampling, tally threshold.

Pasteuria penetrans (Thorne) Sayre & Starr is an obligate, mycelial, and endospore-forming bacterial parasite of root-knot nematodes (Sayre and Starr, 1985). Endospores of *P. penetrans* attach to the cuticle of second-stage juveniles (J2) of *Meloidogyne* spp. in soil. The endospores germinate and send a germ tube through the nematode body wall and into the pseudocoel after the J2 enters a plant root and establishes a feeding site. The parasitized nematodes are able to develop into females and males but are incapable of reproduction (Bird, 1986). The females, and sometimes males (Hatz and Dickson, 1992) become filled with endospores, which are eventually released into soil upon host disintegration.

Determining the incidence of endospore attachment to J2 is important in evaluating the biological control efficacy of *P. penetrans*. The most commonly used bioassays involve counting the numbers of endospores attached to J2. Endospores of *P. penetrans* are able to attach to any part of the J2 body surface (Stirling, 1991), but the constant movement of nematodes and the contents of their intestines hinder viewing and direct counting of attached endospores under the light microscope.

Recently, binomial sampling became a useful method for estimating population densities of organisms that have a binomial distribution (Binns and Bostanian, 1990; Feng et al., 1993). In binomial sampling, the only information retained from a sample unit is whether or not the organism is present. The percentage of units without the organism is used to estimate the mean density, based on an empirical relationship between mean density and the percentage infestation level. Binomial sampling usually reduces the time and effort spent estimating the mean density (Binns and Bostanian, 1990). Shortcomings associated with binomial sampling include requiring a larger number of samples and a higher variance associated with the mean density than for methods that involve direct counting of all individuals (Binns and Bostanian, 1990; Nyrop and Binns, 1991). The application of tally thresholds in binomial sampling has

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been reported to reduce the variance greatly and thus make binomial sampling more attractive (Binns and Bostanian, 1990; Feng et al., 1993; Nyrop and Binns, 1991). A tally threshold (T) is defined as the maximum number of individuals in a sample unit that may be treated as absent (Binns and Bostanian, 1990; Nyrop and Binns, 1991). For example, when T is set to 5, samples with ≤ 5 individuals are counted as having the organism absent. Therefore, a tally threshold sampling using $T = 0$ defaults to the binomial sampling.

Before tally thresholds can be used practically, the relationship between the mean densities (m) and the proportions of no more than a predetermined tally threshold of individuals (P_T) must be determined. This can be done through a theoretical distribution, such as the Poisson (Tippett, 1932) or the negative binomial (Pielou, 1960), or through regression. Little is known about the relationship between the mean number of endospores attached per J2 (mean density) and the proportion of sample units with different tally thresholds. The objectives of this study were to establish the empirical relationships between m and P_T and determine the potential sampling errors associated with the application of the tally threshold as a quantitative tool in studies on biological control of nematodes.

Materials and Methods

Data collection: Three previously reported data sets were used for mathematical analyses: endospore attachment of *P. penetrans* to J2 of *M. arenaria* race 1 in a centrifugal bioassay, in an incubation bioassay (Chen et al., 1996a), and in a field experiment (Chen et al., 1996b). In the centrifugal bioassay, endospore-filled females were hand-picked and ground in deionized water with a glass-tissue grinder. The suspensions usually were diluted in water containing approximately 1 million endospores/ml. A combination of the diluted endospore suspensions and 2-day-old J2 suspensions were placed in a microfuge tube and centrifuged at 8,700*g* for 2 minutes (Hewlett and Dickson, 1993). Nematodes were removed from the tubes with a pipette and placed in Corning cell wells (Corning, Corning, NY). Numbers of endospores attached to individual J2 were determined on 15 to 31 nematodes per sample with the aid of a light microscope at ×450. Seventy samples with a range of endospore attachment from 0.2 to 20.9 endospores/J2 were examined.

In the incubation bioassay, tomato roots containing approximately 80 million endospores/g of root material were ground in a Wiley mill (Model 4, Arthur H. Thomas, Philadelphia, PA) to a powder that passed though a sieve with 5-mm-pore openings (Chen et al., 1996a). A 0.5-g sample of the root powder plus 5 ml of deionized water were placed in a 6-cm-diameter 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. A combination of 0.9 ml of the diluted endospore suspension and 0.1 ml of a 2-day-old J2 suspension of 290 ± 25 J2/ml was placed into a Corning cell well (Corning, Corning, NY) and incubated at 22 to 25 °C for 18 hours. The number of endospores attached per J2 was estimated on 15 J2. Thirty-three samples were examined, and the endospore attachment ranged from 0.3 to 36.0 endospores/J2.

In the field experiment, the soil was infested with *P. penetrans* endospores before the cropping season in order to evaluate its suppressiveness to root-knot nematodes (Chen et al., 1996b). Second-stage juveniles were extracted at harvest from soil with centrifugal flotation (Jenkins, 1964). Numbers of endospores attached per J2 were determined on 15 to 20 nematodes per sample. One hundred eleven samples, with a range of endospore attachment of 0.1 to 28.8 endospores/J2, were examined.

Data from the three data sets were used separately to compute the mean endospore attachment (m), variance (s^2), and the proportion of J2 (P_T) with $\leq T$ (tally threshold) endospores attached; T was set to 0, 1, 2, 3, 4, 5, 8, and 10 endospores/J2. The relationships between $\ln(m)$ (natural logarithm of

the mean) and P_T were established for each of the three data sets at different T values.

Data analysis: For each tally threshold, the empirical relationship between m and P_T was developed using parameters from the linear regression of $\ln(m)$ on P_T ($0 < P_T < 1$):

$$\ln(m) = a + b\,P_T \quad (1)$$

where m is the mean number of endospores attached per J2; P_T is the proportion of J2 with $\leq T$ endospores attached; and a and b are intercept and slope parameters from the regression, respectively. The antilogarithm form of equation 1 is:

$$m = e^{a+b\,P_T} = c\,e^{b\,P_T} \quad (2)$$

where c equals e^a. Equation 2 gives an estimate of m for a given P_T obtained in the sample.

The variance var(m), which is associated with estimation of m from P_T, consists of two components—a prediction variance, $\text{var}_p(m)$, and a sampling variance, $\text{var}_s(m)$ (Binns and Bostanian, 1990; Feng et al., 1993; Nyrop and Binns, 1991):

$$\text{var}_p(m) = m^2 s_m^2\,(1/N + (P_T - \text{avg}P_T)^2/\text{SSP}_T) \quad (3)$$

$$\text{var}_s(m) = m^2 s_m^2 \quad (4)$$

where N is the number of data points used to fit equation 1; s_m^2 is the variance about m, when values for m are generated from observed P_T values using equaton 1; $\text{avg}P_T$ is the mean value of the independent variable P_T used in the regression; SSP_T is the sum of the squares of the deviations of the independent variable P_T used in the regression.

To compare the potential sampling errors that would be involved in estimating m from P_T using the empirically determined m and P_T relationships, Karandinos' formula was used (Karandinos, 1976):

$$n = \left(\frac{Z_{\alpha/2}\,s}{dm}\right)^2 \quad (5)$$

where $Z_{\alpha/2}$ is the standard normal deviate such that $P(Z > Z_{\alpha/2}) = \alpha/2$; d is the predetermined confidence interval half-width (CI) as a proportion of the mean (d = CI/m); and s^2 is the variance estimated for prediction of m, here $s^2 = \text{var}_p(m) + \text{var}_s(m)$. When α is set to 0.05 (95% confidence), then $Z_{\alpha/2}$ equals approximately 2 and the sampling error ($E = d/Z_{\alpha/2}$) is approximately half of the estimated d value. Equation 5 was rearranged and the terms of $\text{var}_p(m)$ and $\text{var}_2(m)$ incorporated:

$$d = \frac{Z_{\alpha/2}}{m}\sqrt{\frac{\text{var}_p(m) + \text{var}_s(m)}{n}} \quad (6)$$

This equation gave a d value that could actually be attained when an estimate of P_T, obtained in the sample reading and based on a desired sample size n, was used to estimate the corresponding m. It should be noted that d would change with n and P_T in relation to m. The α level used for calculation of d was always 0.05.

The relationships of means (m) and variances (s^2) associated with the means for endospore attachment data from the three data sets were subjected to analyses by Taylor's power law (Taylor, 1961):

$$\ln(s^2) = \ln(a) + b\ln(m) \quad (7)$$

In Taylor's power law, the relationship between $\ln(s^2)$ and $\ln(m)$ is linear. The slope value, b, indicates the kind of distribution that m may possess. For example, a slope value of greater than 1 indicates a contagious distribution (Taylor, 1961). The tally threshold sampling requires that the mean (m) has a contagious distribution (Binns and Bostanian, 1990; Feng et al., 1993; Nyrop and Binns, 1991).

Results and Discussion

The results of Taylor's power law analyses on the three data sets are shown in Fig. 1. The variances (s^2) regressed better to the means of data from the incubation bioassay and field experiments, yielding higher r^2 values, than to the centrifugal bioassay. The slopes from all three data sets were >1, indicating a contagious distribution of the mean, regardless of the different methods used for endospore attachment. The slopes from data attained from the incubation bioassay and the field experiment were 1.6, which was greater than the slope of 1.3 at-

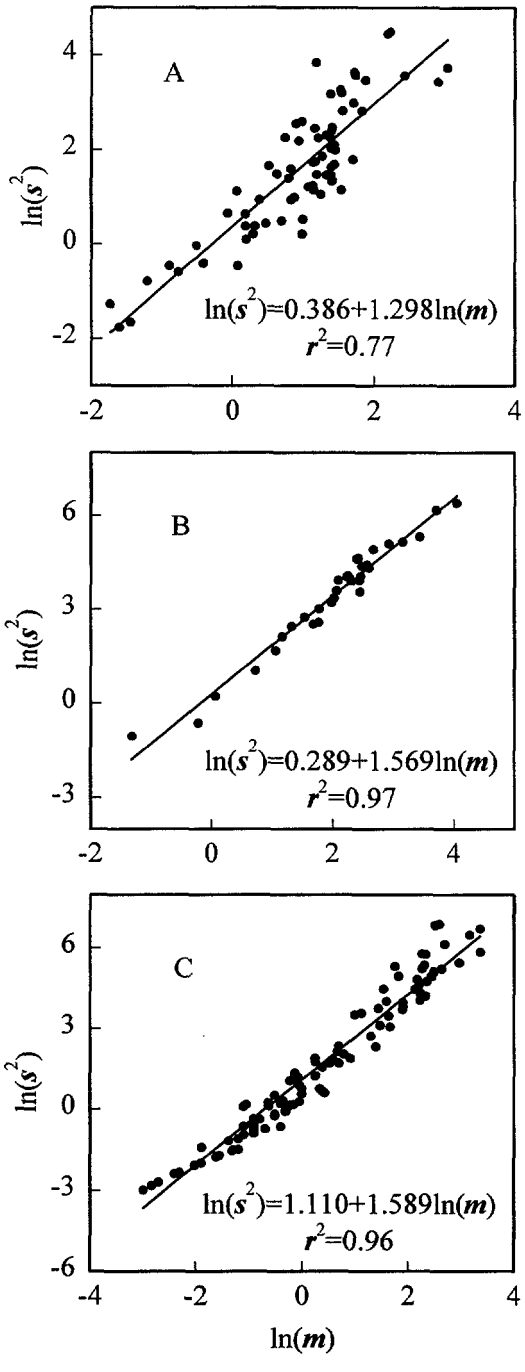

FIG. 1. Analysis of the relationships between the means (m) and the variances (s^2) associated with m using Taylor's power law. The means were the number of *Pasteuria penetrans* endospores attached to the second-stage juveniles of *Meloidogyne arenaria* race 1. A) Endospore attachment from centrifugal bioassay. B) Endospore attachment from incubation bioassay. C) Endospore attachment from field experiment.

tained with the centrifugal bioassay ($P \leq 0.01$). In all cases, Taylor's intercepts $\ln(a)$ were >0 (Fig. 1), indicating that the ratio of s^2/m, which was equal to am^{b-1} ($a>1$, $b>1$), was >1 and changed with m for each of the data sets. Therefore, a logarithmic transformation of the data is needed before the analysis of variance and means separation test are conducted (Southwood, 1978).

Results from the linear regression of $\ln(m)$ on P_T from the three data sets are listed in Table 1. Equation 1 fitted the data sets well with coefficients of determination (r^2) of approximately 0.8 for most of the tally thresholds, regardless of endospore attachment incidences obtained from the three data sets. The regression was not significant for $T = 10$ for the field experiment data set, and the results were omitted. In practice, P_T was estimated from samples based on a particular tally threshold. The mean number of endospores attached per J2 (m) was calculated from P_T according to Equation 2; a and b were the intercept and slope, respectively, of the linear regression (Table 1).

The variances of Equation 1 (s_m^2) tended to decrease with increasing T values for all three data sets. The variances also were greater for endospore attachment incidences from the field experiment than for those from the incubation bioassay and centrifugal bioassay (Table 1). Under field conditions, the age structure of J2 and the endospore concentration distribution in soil probably were highly variable. Many environmental conditions and ecological factors could affect the attachment incidence of *P. penetrans* to *Meloidogyne* spp. (Bird et al., 1989; Chen et al., 1996a; Hatz and Dickson, 1992; Stirling et al., 1990). Second-stage juveniles with different numbers of endospores attached also may have different fates in soil. The non-significant regression at $T = 10$ under field condition suggested that the mortality of J2 with >10 endospores attached probably increased abruptly, hence yielding a randomized P_T that no longer correlated with the change of $\ln(m)$.

The m range increased with increasing T values, because P_T was preset at $P_T > 0$ and

TABLE 1. Parameters from linear regression of the number of endospores attached per second-stage juvenile ($\ln(m)$) as a proportion of juveniles with $\leq T$ endospores attached (P_T) at different T values.[a]

T	N[b]	a	b	r^2	s_m^{2}[c]	SSP_T[d]	m ranges	$avgP_T$[e]
			Endospore attachment from centrifugal bioassay					
0	42	1.4152	−3.1962	0.84	0.1273	2.6802	0.2–4.8	0.2990
1	64	1.9203	−2.7149	0.83	0.1076	4.2924	0.2–9.3	0.3779
2	60	2.2311	−2.2555	0.73	0.1007	3.1751	0.4–9.3	0.5203
3	57	2.5065	−2.1201	0.68	0.0853	2.2571	0.9–9.3	0.6480
4	54	2.9478	−2.3389	0.75	0.0755	2.1780	1.1–20.9	0.7215
5	49	2.9858	−2.1742	0.71	0.0740	1.8028	1.1–20.9	0.7565
8	33	3.3366	−2.1758	0.79	0.0616	1.5118	2.1–20.9	0.8203
10	26	3.3801	−2.1473	0.81	0.0634	1.3835	2.1–20.9	0.8180
			Endospore attachment from incubation bioassay					
0	21	2.3833	−4.8348	0.81	0.1948	0.6768	0.3–11.6	0.1810
1	26	2.7040	−3.9780	0.87	0.1250	1.2891	0.3–14.4	0.2538
2	26	2.7605	−2.8491	0.74	0.1126	0.9372	1.1–14.4	0.2855
3	28	2.9342	−2.5732	0.79	0.1033	1.4879	1.1–23.4	0.3535
4	27	2.9657	−2.2376	0.75	0.0847	1.2851	2.1–23.4	0.3881
5	27	3.0922	−2.2197	0.76	0.0830	1.3146	2.1–23.4	0.4483
8	26	3.3248	−2.1519	0.83	0.0471	1.1811	2.9–23.4	0.5459
10	25	3.5408	−2.1616	0.86	0.0356	1.1053	3.7–36.0	0.5783
			Endospore attachment from field experiment					
0	102	3.0253	−5.5510	0.84	0.4405	7.5461	0.1–23.7	0.5805
1	83	3.2173	−4.3385	0.85	0.2784	6.5330	0.2–23.7	0.6014
2	70	3.4490	−3.9330	0.83	0.2397	4.9922	0.3–23.7	0.6277
3	63	4.0946	−4.3654	0.83	0.2292	3.4860	0.3–28.8	0.6643
4	52	3.9604	−3.7388	0.79	0.2052	2.7392	0.7–28.8	0.6559
5	49	4.1211	−3.6924	0.75	0.2149	2.2544	0.8–28.8	0.6823
8	41	4.0781	−3.1223	0.68	0.1880	1.6388	1.3–28.8	0.6885

[a] The linear equation is $\ln(m) = a + b\,P_T$, where m is the mean number of endospores attached per J2; P_T is the proportion of J2 with $\leq T$ endospores attached; all regression equations were significant at $P \leq 0.001$; T is the tally threshold, defined as the maximum number of individuals in a sample unit that may be treated as absent; and a and b are intercept and slope parameters from the regression analysis, respectively.

[b] N is the number of data points used to fit the equation.

[c] s_m^2 is the variance of the equation.

[d] SSP_T is the sum of the squares of the deviations of the independent variable P_T used in the regression.

[e] $avgP_T$ is the mean value of the independent variable P_T used in the regression.

$P_T < 1$. Data pairs of $\ln(m)$ and P_T with $P_T = 1$ were excluded from regression analysis to reduce the bias. When estimations were based merely on a binomial sampling ($T = 0$), the variances were greater than those for the other T values (Table 1). Thus, choosing a rational T value would give rise to a precise estimation of m, along with a desirable detection range of m. Accordingly, in Table 1, T values of 0, 1, 8, and 10 were important for estimating the endospore attachment (m) from P_T in the centrifugal bioassay and incubation bioassay, whereas 0, 1, 2, and 3 were important for the field experiment data. These T values were associated with both high coefficients of determination (r^2) and different m ranges that were ideal for detecting both low and high numbers of endospores attached per J2.

Half-widths of 95% confidence intervals as a proportion of the mean (d) (Eq. 6) varied from 0.04 to 0.37 among the T values for data sets from the centrifugal bioassay, incubation bioassay, and field experiment (Table 2). The d values also were variable with changing of P_T in relationship to $avgP_T$ (Eq. 3); hence, they are presented as a range of d. The d value for a given T value would be reduced by increasing the sample size (n). High d values were associated with the estimation of m from P_T in the data set from the field experiment, whereas low d values were associated with the estimation from the centrifugal bioassay and medium d values were attained from the incubation bioassay. In most cases, increasing the T values would reduce d. A d value of 0.2 is probably adequate for most research purposes. This re-

TABLE 2. Confidence interval half-widths as a proportion of the mean (d) associated with the estimation of number of endospores attached per second-stage juveniles (J2) (m) from the proportion of J2 with $\leq T$ endospores attached (P_T) at different T values with different sample sizes (n).

	Ranges of d[a]				
T[b]	n = 15	n = 25	n = 50	n = 100	m ranges
	Endospore attachment from centrifugal bioassay				
0	0.20–0.22	0.15–0.17	0.10–0.11	0.08–0.08	0.2–4.8
1	0.18–0.19	0.14–0.14	0.09–0.10	0.07–0.07	0.2–9.3
2	0.18–0.19	0.14–0.14	0.09–0.10	0.07–0.07	0.4–9.3
3	0.16–0.18	0.12–0.14	0.08–0.09	0.06–0.07	0.9–9.3
4	0.15–0.17	0.11–0.13	0.08–0.09	0.05–0.06	1.1–20.9
5	0.15–0.17	0.11–0.13	0.08–0.09	0.05–0.06	1.1–20.9
8	0.14–0.17	0.10–0.12	0.07–0.08	0.05–0.06	2.1–20.9
10	0.14–0.17	0.11–0.13	0.07–0.09	0.05–0.06	2.1–20.9
	Endospore attachment from incubation bioassay				
0	0.25–0.35	0.19–0.26	0.13–0.18	0.09–0.12	0.3–11.6
1	0.20–0.24	0.15–0.18	0.10–0.12	0.07–0.08	0.3–14.4
2	0.19–0.23	0.14–0.17	0.09–0.12	0.07–0.08	1.1–14.4
3	0.18–0.20	0.13–0.15	0.09–0.10	0.06–0.07	1.1–23.4
4	0.16–0.19	0.12–0.14	0.08–0.10	0.06–0.07	2.1–23.4
5	0.16–0.18	0.12–0.13	0.08–0.09	0.06–0.06	2.1–23.4
8	0.12–0.14	0.09–0.10	0.06–0.07	0.04–0.05	2.9–23.4
10	0.11–0.12	0.08–0.09	0.05–0.06	0.04–0.04	3.7–36.0
	Endospore attachment from field experiment				
0	0.37–0.37	0.27–0.28	0.18–0.19	0.13–0.13	0.1–23.7
1	0.29–0.30	0.22–0.22	0.15–0.15	0.10–0.11	0.2–23.7
2	0.27–0.28	0.20–0.21	0.14–0.14	0.10–0.10	0.3–23.7
3	0.27–0.28	0.20–0.21	0.13–0.14	0.09–0.10	0.3–28.8
4	0.25–0.27	0.19–0.20	0.13–0.14	0.09–0.10	0.7–28.8
5	0.26–0.28	0.19–0.21	0.13–0.14	0.10–0.10	0.8–28.8
8	0.24–0.27	0.18–0.20	0.12–0.14	0.09–0.10	1.3–28.8

[a] The α level used for computation of d is 0.05 (95% confidence) for all situations.
[b] T is the tally threshold, defined as the maximum number of individuals in a sample unit that may be treated as absent.

quired the sample sizes of 25 J2 for the centrifugal bioassay, 25 to 50 J2 for the incubation bioassay, and 50 J2 for the field experiment (Table 2).

Estimation of the number of endospores attached per J2 from the proportion of J2 with $\leq T$ endospores attached was feasible both theoretically and practically. Use of tally thresholds would reduce considerably the time and effort to determine the number of endospores attached per J2. Although this method required a large sample size (25 to 50 J2/sample), it was relatively easy and rapid to determine a J2 with $\leq T$ or $\geq T$ endospores attached, thus making the method less time-consuming. In practice, some J2 had several dozen to several hundred endospores attached. Obtaining an accurate count when a large number of endospores were attached per J2 was both difficult and tedious. The T values in this study were small, and using a tally threshold would obviate counting these large numbers. Thus, by choosing an appropriate T value, especially with high r^2 (Table 1) and a desirable sample size, the number of endospores attached per J2 could be determined easily and precisely.

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