Journal of Nematology 34(1):62–65. 2002.

Precision and Selection of Extraction Methods of Aphelenchid Nematodes from Maritime Pine Wood, *Pinus pinaster* L.

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Abstract: Four extraction methods for *Bursaphelenchus xylophilus* and other aphelenchid nematodes were compared on the number of nematodes per gram recovered, and on the precision of the mean number of nematodes per gram of pine wood. The number of nematodes per gram recovered by each method, in addition to its inherent shortcomings when the actual number of nematodes is unknown, failed to provide clear rankings among the extraction methods. The precision of the mean number of nematodes per gram did provide clear guidelines for selection. Selection of the method may be based on prior knowledge about the range of nematodes to be expected or the independence of precision from the mean number of nematodes.

Key words: Bursaphelenchus xylophilus, extraction methods, maritime pine, pinewood nematode, precision.

The order Aphelenchida includes several economically important species, mainly within the genera *Aphelenchoides* and *Bursaphelenchus*. A number of *Bursaphelenchus* species are frequently associated with insects and trees, and *B. xylophilus* is an important pest and pathogen of conifers of the genus *Pinus*. It may be responsible for ''pine wilt disease'' and has been reported to cause major damage in native pines in Japan, China, and Korea as well as some exotic species in the United States and Canada (Evans et al., 1996). Recently (Mota et al., 1999), *B. xylophilus* was found for the first time in Europe in maritime pine, *Pinus pinaster,* from southern Portugal.

The selection of extraction methods and quantification are important in ecological studies, and the Baermann funnel is often used. Selection of the most appropriate extraction method usually involves the comparison of different methods using a number of samples or, when feasible, subsamples followed by comparison of statistical analyses to establish which method extracts more nematodes. For a number of reasons, *a priori* knowledge of how many nematodes are present in a sample is the exception (Griesbach et al., 1999; Hoshino and Togashi, 1999; McSorley and Parrado, 1987), and different counts may result from differences in the efficiency of the methods or from differences in the number of nematodes actually present in samples.

Attempts to account for this uncertainty involve the mixing of soil prior to extraction, but mixing does not necessarily improve the spatial homogeneity of nematodes in a sample (McSorley and Parrado, 1987). Reference samples with low numbers of nematodes (Griesbach et al., 1999) might reduce but not eliminate the uncertain. Addition of nematode inoculum has been employed (McSorley and Frederick, 1991; Stetina et al., 1997) in the hope that final numbers of nematodes will accurately reflect the amount of inoculum.

However, an alternative approach based on precision rather than efficiency is available and does not require knowing the actual number of nematodes present in a sample *a priori*. Precision is defined as the closeness of repeated measurements of the same quantity (Sokal and Rohlf, 1995) and, for a given sample of size *n,* can be expressed (McSorley, 1987) by

$$D = (t^2 s^2 / n)^{1/2} \quad (1)$$

where D is the half-length of the 1-α confidence interval of the mean, t is the value of Student's *t* distribution with *n*-1 degrees of freedom and a type-I error probability α, and s^2 is the variance of the sample. Expressing D as a proportion of the mean ($\overline{Y}$), D′ is

$$D' = (t^2 s^2 \overline{Y}^{-2}/n)^{1/2} \quad (2)$$

which implies that the greater the value of D′, the lesser the relative precision of the mean.

When a series of samples are available, the relation between their variances and means can be described in a number of ways, including the negative binomial distribution or Taylor's power law. Taylor's power law covers a wider range of distributions, whereas the negative binomial distribution, which has severe ecological limitations (Taylor et al., 1979), is not independent of sample size, with its parameter k reaching ± infinity at randomness (Elliot, 1979).

Taylor's power law can be expressed by

$$s^2 = a \overline{Y}^b \quad (3)$$

where a is a sampling factor and b can be interpreted as an index of aggregation ranging (when a = 1) from a near-regular (b → 0) through random (b = 1), to a highly aggregated (b → ∞) distribution of organisms (Taylor, 1961).

Fitting Taylor's power law to a set of samples by regression techniques is better accomplished in linear form, usually taking the decimal logarithms of both terms of eq. (3),

$$\log s^2 = \log a + b \log \overline{Y} \quad (4)$$

Even if the aggregation index b frequently appears to

Received for publication 26 February 2001.

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This study was supported in part by Fundação para a Ciëncia e Tecnologia (FCT), PRAXIS XXI, no. 11189/98.

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This paper was edited by E. P. Caswell-Chen.

be independent from the mean (Elliot, 1979), as implied by eqs. (3) and (4), independence may break down at low densities (Taylor, 1961) and a straight line may not be the best description of the relationship between the logarithm of the variances and of the means. Therefore, a curvilinear model could be more appropriate, with the relationship between the mean and the variance expressed by

$$\log s^2 = \log a + b \log \bar{Y} + c (\log \bar{Y})^2 + d (\log \bar{Y})^3 + \ldots + w (\log \bar{Y})^x + \ldots \quad (5)$$

where b, c, d, . . ., w, . . . can be equal or different from zero. Once fitted, the model described in eq. (5), can be used to replace s^2 in eq. (2) by its value, providing the general equation

$$D' = \{t^2 a \bar{Y}^{[-2 + b + c \log \bar{Y} + d (\log \bar{Y})^2 + \ldots + w (\log \bar{Y})^{x-1} + \ldots]}/n\}^{1/2} \quad (6)$$

Therefore, the change of the relative precision of any extraction method can be investigated for the whole range of the expected mean number of nematodes $\bar{Y}$ for a given type-I error probability and sample size.

In addition to the selection of extraction methods, eq. (6) can be used to determine the number of samples needed to attain a desired precision with a stated type-I error probability. For this, an iterative solution of eq. (6) should be made to obtain the desired value of D′, with the appropriate value of t for each sample size *n* or type-I error, α.

The objectives of this study are to investigate both approaches (number of nematodes extracted and precision of nematode extraction), without adding nematodes or assuming reference samples, for their adequacy and usefulness to compare and select extraction methods of *B. xylophilus* and other aphelenchid nematodes from maritime pine wood.

Materials and Methods

Branches or trunks of maritime pine were collected from Quiaios (site 1, branches only) and Pegões (sites 2 and 3, branches only; site 4, only a portion of the trunk), Portugal. In total, four trees were sampled. The number of branches varied, depending on tree size and weight of wood extracted. Distribution of selected branches was casual, with no particular pattern. The material collected at each site was cut in 1 to 2-cm pieces, thoroughly mixed, randomly divided in 20 portions with the same weight (200 g in site 1, 100 g in site 2, 15 g in site 3, and 8 g in site 4), and randomly assigned to four extraction methods (five replicates per method per mixed site sample). A single operator performed all procedures, at 18–22 °C.

Method 1, trays (TR): A three-layered plastic net, nylon tissue, and paper tissue were fitted to a plastic tray and covered with wood samples. Water was added until the wood was completely soaked. After 48 hours, nematodes were collected on a 38-μm-pore-size sieve and counted.

Method 2, Baermann funnels with a plastic net and paper tissue (BP): Wood samples were soaked and immersed in water over paper tissue and a plastic net. After 24 hours, the first sample of nematodes was taken directly from the funnels (16-cm-diam.), the water was replaced, and a second sample taken after another 24-hour period.

Method 3, Baermann funnels with nylon tissue (BN): Method 3 was similar to method 2, except that nylon tissue with a 90-μm-pore-size was used instead of paper tissue and a plastic net.

Method 4, flasks (FL): Wood samples were immersed in water in 1-liter-capacity plastic flasks for 48 hours and sieved through 710 and 38-μm-pore-size sieves. Nematodes collected in the smaller-pore sieve had to be separated from the wood material using the procedure described for method 2, but only after 24 hours.

All replicates of all methods were microscopically examined for the presence of nematodes and to obtain a preliminary evaluation of abundance. Whenever preliminary counts exceeded 100 nematodes per replicate, the suspensions were diluted with water and nematodes were counted in 1-ml aliquots. Abundance was always expressed on a per-gram basis.

Data analysis of number of nematodes: Extraction methods were compared independently at each site by one-tailed Student's *t* tests after data transformation by Box-Cox transforms (Box and Cox, 1964) or, when transformation failed to homogenize variances, by one-tailed Mann-Whitney U tests. An experiment-wise type-I error of 0.05 was adopted for the six, one-tailed comparisons of each site, using the Dunn-Šidák method (Sokal and Rohlf, 1995). A least-squares regression-based approach was also followed, involving the fit of linear models to Box-Cox transformed data. Forward stepwise selection with replication was used. The candidate model included qualitative variables only, namely sites and extraction methods (coded as 1, 0) as well as all interactions among them. An experiment-wise type-I error of 0.05 was adopted for the coefficients.

Data analysis of precision: Samples were jackknifed by calculating *n* means and variances for each sample after removing one different value each time (Efron, 1982) and logarithms taken to fit a regression model by the least-squares method. The candidate model included up to the third degree of log $\bar{Y}$, plus four qualitative variables in which the extraction methods were coded as 1, 0, and all interactions among the independent variables. Model selection was done by forward stepwise selection with an experiment-wise error rate of 0.05 for the coefficients. After replacing the qualitative variables by their values, the resulting equations were used to compare the relative precision of extraction methods, replacing s^2 in eq. (2), as described for eq. (6).

Results

Selection of extraction methods by number of nematodes: Two aphelenchid species were identified: *Laimaphelenchus pensobrinus,* Massey, was present in all four samples and *Bursaphelenchus xylophilus,* Steiner & Buhrer (Nickle), in samples 2, 3, and 4. Mean values and standard errors of nematodes per gram in each site and extraction method are shown in Table 1, together with the results of mean comparisons for site by Student's *t* or Mann-Whitney U tests. Significant differences were found only at sites 2 and 3. In site 2, FL and BP did not differ in nematode extraction and neither did TR and BN, and TR and BN extracted significantly more nematodes than the former. In site 3 the lack of ''transitivity'' prevented a consistent interpretation of data (Chew, 1976) in the sense that, for example, FL is not lower than TR, TR is not lower than BP, but FL is significantly lower then BP.

Therefore, a different approach that does not allow this lack of ''transitivity'' to occur was followed, and a least-squares regression model was fit to the data. The resulting equation included site variables only and was written as

$$Y = (0.924 - 0.083\ S_1 + 0.738\ S_3 + 0.557\ S_4)^{1/\lambda} \quad (7)$$

where Y is back-transformed data of nematodes per gram, S_i are the sites, and $\lambda = 0.075$ is the estimated value of the Box-Cox transformation. The significance levels of the model and coefficients were near 0, the significance level of lack of fit was 0.06, and the adjusted coefficient of determination was $R^2_{adj} = 0.984$. Because the qualitative variables where coded as 1 when present and as 0 otherwise, eq. (7) reduces to $Y = 0.841^{1/\lambda}$ for site 1, $Y = 0.924^{1/\lambda}$ for site 2, $Y = 1.662^{1/\lambda}$ for site 3, and $Y = 1.481^{1/\lambda}$ for site 4.

Selection of extraction methods by precision: The selected model can be written as

$$s^{2\prime} = a + b\,\bar{Y}' + c\bar{Y}'^2 + d\ BN + e\ FL + f\bar{Y}'\ TR + g\,\bar{Y}'\ BP + h\,\bar{Y}'\ FL + i\,\bar{Y}'^3\ BP + j\,\bar{Y}'^3\ FL \quad (8)$$

where $s^{2\prime}$ and $\bar{Y}'$ are, respectively, logarithmically transformed variances and means, and TR, BP, BN, and FL are qualitative variables representing the methods. The significance level of the model was near 0 and of the coefficients was always less than 0.005, and the adjusted coefficient of determination was $R^2_{adj} = 0.993$. Because all qualitative variables were present in the selected model, the relationship between the variance and the mean was significantly different in all methods. When the qualitative variables were replaced by their value (1 when used, 0 otherwise), four different equations for variance were obtained. For trays (TR)

$$s^2 = 0.056\ \bar{Y}^{(0.948 + 0.379 \log \bar{Y})} \quad (9)$$

for Baermann funnels with a plastic net and paper tissue (BP)

$$s^2 = 0.056\ \bar{Y}^{[1.893 + 0.379 \log \bar{Y} - 0.095\ (\log \bar{Y})^2]} \quad (10)$$

for Baermann funnels with nylon tissue (BN)

$$s^2 = 0.030\ \bar{Y}^{(1.172 + 0.379 \log \bar{Y})} \quad (11)$$

and for flasks (FL)

$$s^2 = 0.129\ \bar{Y}^{[2.168 + 0.379 \log \bar{Y} - 0.183\ (\log \bar{Y})^2]} \quad (12)$$

The next step was to substitute the variances obtained in (9) through (12) into eq. (2) and express (for $n = 5$ and $\alpha = 0.05$) D′ in terms of the mean number of nematodes per gram as described in eq. (6), with the result shown in Figure 1.

Discussion

The selection of extraction methods by abundance of recovered nematodes requires that statistically significant differences are present. This approach, when applied here to the number of nematodes per gram recovered by the four methods, shows an apparent relationship between sites and extraction methods, with no differences among methods in sites 1 and 4, differences in sites 2 and 3, and with the results for site 3 preventing clear conclusions because of the lack of ''transitivity.''

Therefore, this analysis could not support conclusive selection of extraction method. An alternative analysis was done, still using the number of nematodes per gram recovered by the four methods, and least-squares regression was used to investigate differences among methods, sites, and interactions between methods and sites. According to the selected model, shown in eq. (7), neither significant differences among methods nor interactions between methods and sites were found.

Table 1. Mean and standard error of nematodes (number per gram) collected in four sites by trays (TR), Baermann funnels with a plastic net and paper tissue (BP), Baermann funnels with nylon tissue (BN), and flasks (FL).

Site 1		Site 2		Site 3		Site 4	
FL	0.077 ± 0.022 a	FL	0.236 ± 0.051 a	FL	695.333 ± 53.909 a	BP	153.500 ± 33.755 a
BP	0.129 ± 0.027 a	BP	0.274 ± 0.032 a	TR	778.000 ± 107.831 ab	BN	192.150 ± 20.458 a
TR	0.136 ± 0.063 a	TR	0.504 ± 0.090 b	BN	917.027 ± 214.973 ab	FL	211.375 ± 48.180 a
BN	0.178 ± 0.050 a	BN	0.554 ± 0.042 b	BP	1202.213 ± 231.204 b	TR	216.025 ± 12.852 a

For each site, means followed by the same letter do not differ significantly at an experiment-wise type-I error rate of 0.05. In all samples, $n = 5$, except TR, site 1, and FL, site 4, where $n = 4$.

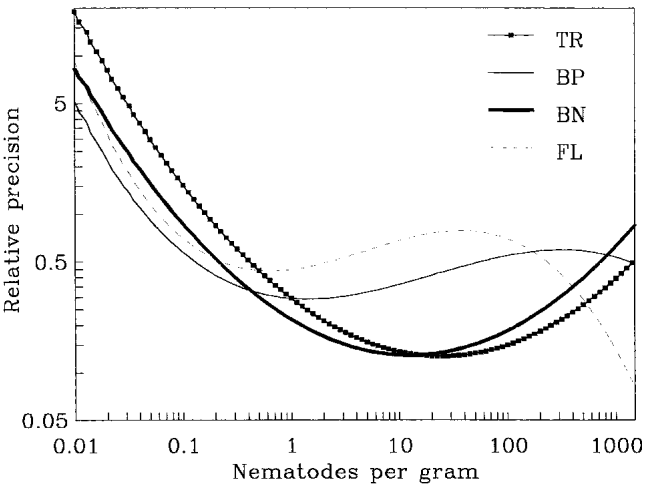

FIG. 1. Change of relative precision D′ with the mean number of nematodes per gram (for $n = 5$ and $\alpha = 0.05$) in trays (TR), Baermann funnels with a plastic net and paper tissue (BP), Baermann funnels with nylon tissue (BN), and flasks (FL).

Again, no support was available for the selection of any extraction method.

According to eq. (8), all extraction methods significantly differed in the relationship between variance and mean. Regardless of the method, variance was not independent of the mean number of nematodes per gram, and a general trend for greater precision with increasing numbers of nematodes was recognized (Fig. 1).

Nevertheless, two extraction methods groups can be identified on the basis of relative precision. In one, comprised of TR and BN, precision increases with the mean until a maximum is reached at about 22 and 12 nematodes per gram, respectively, and decreases thereafter. The other was comprised of BP and FL.

Differences of methods in terms of precision suggest that selection of the extraction method may require some prior knowledge of the approximate number of nematodes per gram to be found. If the number of nematodes per gram is less than 0.4, extraction with Baermann funnels with a plastic net and paper tissue (BP) is recommended; if between 0.4 and 14, Baermann funnels with nylon tissue (BN) should be used; trays (TR) are recommended for a range of 14 to 500 nematodes per gram; and flasks (FL) should be used for higher numbers of nematodes (500 to 1,500 per gram).

However, this *a priori* knowledge is frequently unavailable or it may be more feasible to select only one extraction method. Because the more precise method changes with the number of nematodes, a criterion other than selecting the method that maximizes precision should probably be adopted. In this case, the criterion is, undoubtedly, to select the method with a relative precision more independent from the mean number of nematodes per gram, which means that Baermann funnels with a plastic net and paper tissue (BP) should be used. After a sharp increase, precision of this method remained essentially constant when the mean number of nematodes increased. By either criteria, selecting an extraction method is possible.

We do not believe that different masses influenced the results, although this would need to be investigated in further research with more samples; the different sample weights relate to the different tree sizes and amounts of tissue required for extraction, although the results were always in reference to a weight unit.

Selection of extraction methods by precision implies that the knowledge of the exact number of nematodes present in samples is no longer a goal in itself. It also implies that the selection of a method having the least error is no longer necessarily possible. This approach provides researchers with a flexible tool to select an extraction method, though obviously not error-free, with an error distribution that has known bounds. In addition, emphasis in precision independency from the mean number of nematodes may be an important part of the experimental design, especially when predictions based on nematode numbers are desired.

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