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Short Communication

Comparing two methods for quantifying soil-borne *Entomophaga maimaiga* resting spores

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ABSTRACT

To improve usability of methods for quantifying environmentally persistent entomophthoralean resting spores in soil, we modified and tested two methods using resting spores (azygospores) of the gypsy moth pathogen *Entomophaga maimaiga*. Both methods were effective for recovering resting spores at concentrations >100 resting spores/g dry soil. While a modification of a method originally described by Weseloh and Andreadis (2002) recovered more resting spores than a modified method based on Percoll density gradients, the ability to estimate true densities from counts was similar for both methods. Regression equations are provided for predicting true resting spore densities from counts, with R^2 values for both methods $\geqslant 0.90$.

1. Introduction

Quantification of persistent pathogen stages can be important when investigating the epizootiology of insect diseases. Entomophthoralean zygospores or azygospores, commonly called resting spores, created during epizootics persist as inoculum reservoirs in soil, where only some germinate each year to cause primary infections (Hajek, 1997). For the entomophthoralean gypsy moth pathogen *Entomophaga maimaiga*, cadavers from infected later larval instars produce resting spores (Hajek, 1999). Resting spores of *E. maimaiga* are washed into the soil as cadavers decompose, mostly remaining in the organic layer at bases of trees.

Two methods for quantifying resting spores in soil have been developed but never compared. Hajek and Wheeler (1994) described extraction and quantification of *E. maimaiga* resting spores from soil by wet-sieving 5 g soil samples, followed by centrifugation in a 3-layered Percoll density gradient (PM = Percoll method). The middle density (5 mL) was transferred to a 100 mm petri dish and entirely examined at 50×. This approach to sample quantification was slow and counting one sample could take hours. Weseloh and Andreadis (2002) created a 'soil physical count' method for quantifying *E. maimaiga* resting spores in 100 mL soil samples; soil was wet-sieved, 800 mL water were added, after settling the supernatant was removed so the total volume was 200 ml, and samples were then taken for quantification (WM = Weseloh method). We modified the Percoll method (mPM) to decrease time for quantification. We modified the Weseloh method (mWM) so that the same size soil samples as the mPM were tested. In this paper, we report a comparison of these two modified methods for quantifying *E. maimaiga* resting spores in soil.

2. Materials and methods

Gypsy moth larvae were obtained as neonates from USDA APHIS CPHST, Buzzards Bay, Massachusetts, and reared following standard protocols (Bernon, 1995). Fourth instar larvae were injected with 10 μL of 1×10^5 protoplasts/mL *E. maimaiga* isolate ARSEF 6162 at the base of a proleg. Injected larvae were maintained at 20 °C for 3 weeks, allowing time for resting spores to mature within cadavers. Cadavers were soaked in water for 16 h and the resulting slurry was washed through a 63 μm sieve. The resulting resting spore suspension was quantified using a hemocytometer.

Soil was collected from three sites separated by ≈3.8 km at each of two forests in Centre Co., Pennsylvania, both with high percentage of oaks (*Quercus* spp.). The top 3 cm of soil was collected as far from tree bases as possible to minimize the potential presence of *E. maimaiga* resting spores. Soil collected from one forest contained 65.7% sand, 28.4% silt, 5.9% clay and 15.6% organic matter; soil from the second forest contained 38.5% sand, 45.5% silt, 16.0% clay and 47.3% organic matter. The resting spore suspension was used to seed soil samples at 10^1, 10^2, 10^3, 10^4 and 10^5 resting spores/g dry soil.

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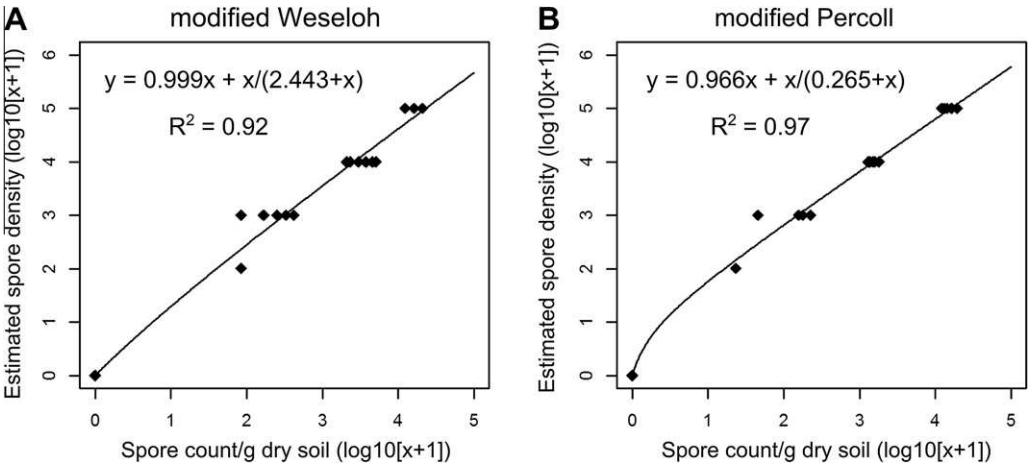

Fig. 1. Predictive regressions for calculating the true densities of *Entomophaga maimaiga* resting spores in soil samples from spore counts made from soil sub-samples using either the (A). modified Weseloh (mWM) or (B). modified Percoll method (mPM). In these regression equations both number of resting spores counted/g soil and estimated true density of resting spores/g soil were $\log_{10} + 1$ transformed.

Both modified methods began with 5 g of soil that was wet-sieved through a series of increasingly fine 7.6 cm diameter sieves (500, 250, 125, 63, and 20 μm) and material on the 20 μm sieve was processed further (see Supplementary Methods for details). For the modified Percoll method, in 30 mL Corex tubes, a 2.5 mL layer of 1.13 g/mL Percoll was overlain with a 2.5 mL layer of 1.05 g/mL Percoll. Then, a 5 mL subsample of the suspension from the 20 μm sieve was layered on top and the tube was centrifuged using a fixed angle rotor. Two milliliters of the interface between the two Percoll layers were removed and added with 2 mL 0.01% Triton-X-100 to a 6 cm diameter petri dish. Resting spores within a 10.5 mm^2 eyepiece grid were counted at 60× for 20 randomly chosen locations, for 3–6 soil samples per concentration.

For the modified Weseloh method the material on the 20 μm sieve was washed into a 500 mL beaker and water was added to 250 mL. After 30 min for settling, 200 mL were decanted. The remaining 50 mL were transferred to a 100 mL beaker, allowed to settle for 3 min and a 5 mL aliquot was removed from the middle of the liquid. The aliquot was vortexed and resting spores in six 20 μL samples were counted under 22 mm^2 cover slips.

The two modified methods for quantifying resting spores in soil were compared using a mixed model (SAS, 2002–2008), with quantification method, site and concentration of resting spores added/g dry soil as main effects, replicate sites within forests as random effects and proportion resting spores recovered as the dependent variable. Least squares means tests were used to further investigate the concentration of resting spores added/g dry soil. To predict actual densities from counts, a nonlinear regression of the form $y = mx + x/(a + x)$ was estimated using the nls() function in *R* (R Development Core Team 2012). In this regression, y is the predicted number of spores ($\log_{10} + 1$ transformed) and x is the count of resting spores/g soil ($\log_{10} + 1$ transformed). This equation forces the regression line through the origin, which is sensible since neither method will give positive spore counts when no spores are present. The parameters m and a control the asymptotic slope and how rapidly this slope is approached, respectively. Correlation coefficients for the two methods were compared using a z test (Snedecor and Cochran, 1980).

3. Results and discussion

The proportion seeded resting spores that were recovered did not differ for soils from the two forests ($F_{1,42} = 0.02$, $P = 0.9004$), so these data were merged. The overall proportion of resting spores recovered from seeded soil using the modified methods was significantly lower for the lowest resting spore concentration (10^1) than the other concentrations ($P < 0.05$). The proportion resting spores recovered differed significantly, with higher recovery for the mWM (21.6 ± 4.6%; mean ± SE) and lower recovery for the mPM (11.5 ± 1.6%) ($F_{5,48} = 4.28$, $P = 0.0027$).

Regression analysis provided conversions between count data and the true number of resting spores/g dry soil for the two modified methods (Fig. 1). Both methods suffer from decreased detection at low densities but quickly become linear, indicating fairly consistent quantification rates above approximately 100 spores/g. The mWM shows less curvature in the estimated regression line due to its slightly higher recovery rate. The correlation coefficient for the regression of estimated density to true density for the mPM was significantly greater than the correlation for the mWM ($z = 2.11$, $P = 0.035$). Using these equations, spore density predictions for counts of zero are assumed to be zero and do not relate to the R^2 values of the regressions.

The mPM produced slightly more accurate predictions, but recovered fewer resting spores from soil that the mWM, implying that the mWM could be more useful for estimating samples with lower densities of resting spores. Furthermore, the mWM required approximately 55 min per sample while the mPM required 75 min/sample (although time-efficiency would increase with more samples being run simultaneously). Overall, both methods enable good prediction of true resting spore densities from counts, with R^2 values $\geqslant 0.90$ for both methods, suggesting accurate conversions from counts to real densities, especially at densities >100 resting spores/g soil. These methods provide a viable alternative to the real-time PCR resting spore quantification method developed by Castrillo et al. (2007), especially given that high levels of organic material in surface soil samples where resting spores are located make this procedure difficult and costly, given the DNA extraction methods currently available.

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Appendix A. Supplementary material

Supplementary methods associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2012.07.021.

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