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SHORT COMMUNICATION

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New developments in the estimation of spores of *Pasteuria penetrans*

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Abstract New developments in the estimation of free spores of *Pasteuria penetrans*, a hyperparasite of plant-parasitic nematodes, including *Meloidogyne* spp., have been tested in contrasted textured soils. They were dedicated to improving the recovery of spores. Different methods of increasing energy of aggregate dispersion were compared in their efficiency in recovering spores inoculated in a sandy clay soil and a clay soil. The dispersion of the soils by the less energetic method (method A) allowed only 50% and 20% of the spore inoculum to be recovered from the sandy clay soil and clay soil, respectively. For these soils, 76% and 81% of the particle-size fraction (0–20 µm) isolated by this method were still aggregated in coarser structures. With increasing energy (methods B and C), these coarse aggregates disappeared entirely in both soils. At the same time, the recovery of spores increased sharply, representing about 87% and 75% of the inoculum of the sandy clay soil and clay soil, respectively. Therefore, at most 25% of the pool of spores remained undetectable. The formation of artificial aggregates during the enumeration procedure could not be advocated to explain this result, since the dispersion of the fraction collected for the enumeration did not improve spore recovery.

Keywords Soil dispersion · Bacteria extraction · Soil fractionation · *Pasteuria penetrans*

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Introduction

The nematode-parasitic endospore-forming bacteria *Pasteuria penetrans* (Sayre and Starr 1985) is detectable on various plant-parasitic nematodes (Sayre and Starr 1988), especially on the root-knot nematodes belonging to the genus *Meloidogyne*. Under field conditions, the extent to which it controls the development of *Meloidogyne* spp. populations largely depends on the type of host plant (Mateille et al. 1995) and on soil characteristics such as temperature (Stirling et al. 1986; Giannakou et al. 1997), humidity (Chan and Gill 1994), texture and structure (Mateille et al. 1996). The study of the epidemiology of *P. penetrans* in relation to the dynamics of the *Meloidogyne* spp. populations requires an accurate estimation of the pool of spores present in soils. Since the sustainable culture of this microorganism has been unsuccessful, its quantification relies on the numeration of infested juveniles of *Meloidogyne* spp. and of the spores attached to their cuticle (Sayre and Wergin 1977). However, this procedure does not give any information on the exact spore content of soils. Though, several methods have been proposed to fulfil this requirement, recent investigations have proved their inaccuracy in recovering spores, particularly in clay soils (Davies et al. 1988; Mateille et al. 1996).

The aim of this work was to propose a more accurate method for determining spores of *P. penetrans* in soil and to discover whether low recoveries of previous methods were mainly due the entrapment of spores either in aggregates resistant to the soil dispersion procedure or in artificial clay-size aggregates formed during the concentration of the soil suspension.

Materials and methods

Characteristics of the soils

A sandy clay soil (10.4% clay, 1.5% fine and 4.7% coarse silt, 51.6% fine and 31.4% coarse sand, and 2.9‰ organic carbon) was sampled from the experimental station of the École Nationale Sup-

érieure d'Agriculture (ENSA Thiès, Senegal). A clay soil (56.1% clay, 12.1% fine and 10.0% coarse silt, 21.7% fine and 0.2% coarse sand, and 26.8 ‰ organic carbon) was sampled from Podor (Senegal) in the west valley of the Senegal river.

Inoculation of *Pasteuria penetrans*

PVC tubes (10 cm high and 1.5 cm internal diameter), closed at the bottom by a 50-µm mesh, were filled to 9-cm height with autoclaved (24 h at 120°C), sieved (<1 mm) soil (Mateille et al. 1996). The soils were saturated by immersion in distilled water, and then drained to eliminate free water. After drainage, spores of *P. penetrans* were inoculated in 100 µl distilled water to the upper 1 cm of the soil column. Inocula varied according to the dispersion methods, i.e. 2.8 10^6, 18.6 10^6, and 16.9 10^6 spores for methods A, B and C, respectively. The tubes were watered under a drip water supply (80 µl min^{-1} for 30 min) and then the soils were gently pulled out of the tubes by air pressure and air-dried.

Soil dispersion and fractionation methods tested

Method A (Mateille et al. 1996)

A 10-g sub-sample was manually and gently shaken in 100 ml distilled water for 1 min for the sandy clay soil and for 3 min for the clay soil. After a 3–5 min period of sedimentation, the soil was sieved using a bank of sieves (200, 50, 20 µm).

Method B

A 10-g sub-sample was shaken (end-over-end at 120 rpm) for 16 h in 100 ml distilled water in the presence of three agate marbles (1 cm-diameter; Chotte et al. 1992). After a 5-min sedimentation, the soil was sieved using a bank of sieves (200, 50, 20 µm).

Method C (total soil disruption)

A 10-g sub-sample was shaken (end-over-end at 120 rpm) for 16 h in 100 ml NaOH solution (0.1 N, pH 10). After a 5-min sedimentation, the soil was sieved using a bank of sieves (200, 50, 20 µm).

Mechanical analysis

This aims at the total dispersion of the simple mineral particles in the presence of NH_4Cl (1 M final concentration) after the destruction of organic cement in boiling hydrogen peroxide. This method allows the weight distribution of the simple mineral particles of sand-, silt-, and clay-size to be known, and a comparison to that obtained by other methods to be made.

The different particle-size fractions (>200 µm, 50–200 µm and 20–50 µm) isolated by these methods were dried (105°C for 16 h) and weighed. For the fraction of particle size 0–20 µm the weight was estimated from a sub-sample and the fraction left was kept humid for spore enumeration.

Enumeration of the spores of *P. penetrans*

Enumeration of the spores was undertaken on the fraction of particle size 0–20 µm and was carried out by two different procedures:

- Procedure 1 (P1): the fraction of particle size 0–20 µm was concentrated by filtration of the suspension on a 0.6-µm filter. The spores and the mineral particles collected on that filter were then suspended in distilled water and analyzed for their spore content. This procedure was applied to the 0–20 µm fractions obtained by all disruptive methods, A, B and C.
- Procedure 2 (P2): spores and mineral particles concentrated on the 0.6-µm filter were dispersed by shaking (end-over-end at 120 rpm for 16 h) in a solution (1/4 of initial volume) of NaOH (0.1 N, pH10) prior to spore numeration. This procedure was applied to the fraction isolated by method A. Results were then compared to those obtained by P1.

The spores of *P. penetrans* were enumerated with a Malassez counting chamber (total magnification=120) and the results expressed as a percentage of the inoculum.

Statistical analysis

Five replicates were used for each soil-dispersion method combination. Percentages were transformed by Arcsin (sqrt) for analyses. A non-parametric (Mann-Whitney) test was used for comparisons between dispersion methods and enumeration procedures for the weight of soil fractions and their spore content.

Results

Weight distribution of the soil size fractions

For the sandy clay soil, weight recoveries after soil fractionation amounted to 100% (98.8% for methods A and B, 98.9% for method C), and did not differ from that of the mechanical analysis (99.2%). The size fractions >200 µm and 50–200 µm were the most abundant (Fig. 1a). They averaged 37.2% and 47.5% of the total soil weight. The weights of the size fraction >200 µm obtained by methods A and B were significantly higher than those obtained by method C and mechanical analysis. Whatever the method, the weights of size fractions 20–50 µm and 50–200 µm were not statistically different. The size fraction 0–20 µm isolated by method A was significantly less abundant than that obtained by

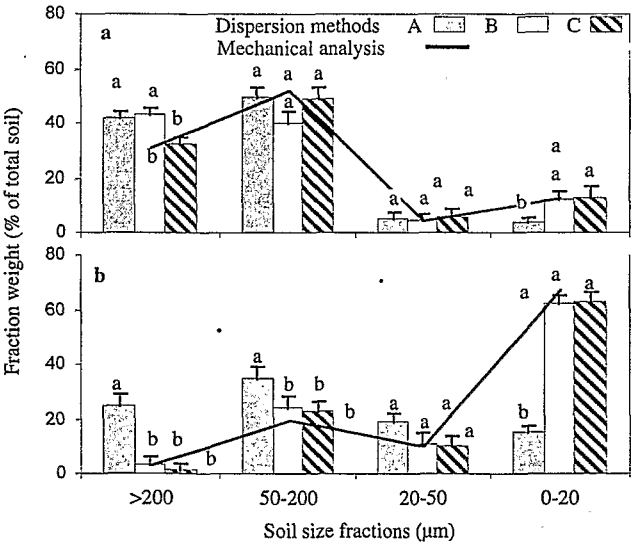

Fig. 1 Weight distribution of the particle size fractions obtained from the dispersion methods A, B and C and from classic mechanical analysis applied on the sandy clay soil (**a**) and the clay soil (**b**). For each size fraction, results with the *same letter* are not significantly different at $P > 0.05$

Table 1 Percentages of spores of *Pasteuria penetrans* recovered from soils processed by the different methods of soil fractionation and using the different procedures for spore enumeration. For each soil, results followed by the *same letter* are not significantly different at $P>0.05$ (*SE* Standard error)

Soil type	Fractionation methods						Procedures for spore enumeration			
	A		B		C		P1		P2	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Sandy clay	50 *b*	5	83 *a*	6	82 *a*	3	50 *A*	5	55 *A*	6
Clay	20 *b*	7	77 *a*	4	79 *a*	5	22 *A*	7	23 *A*	4

methods B and C. Isolated by these methods, this fraction was not statistically different from that obtained by mechanical analysis.

As for the sandy clay soil, weight recoveries after fractionation of the clay soil amounted to 100% irrespective of the dispersion method used (96.5% for method A, 97% for B, 98.3% for C), and did not differ significantly from that obtained by mechanical analysis (100%). The weight distribution of the different particle size fractions isolated by either method B or C did not differ statistically from that obtained by mechanical analysis (Fig. 1b), whereas significant differences were recorded with method A. The size fractions >200 µm and 50–200 µm isolated by the latter method were the most abundant. The size fraction 0–20 µm obtained with method A was significantly less abundant than that obtained from the other methods.

Recovery of the spores of *Pasteuria penetrans*

For both soils, the dispersion method had a significant effect on the number of spores extracted (Table 1). For soils dispersed by method A, spore recovery was lower than for soils dispersed by the two other methods. For the sandy clay soil, extracted spores amounted to 49% of the inoculum when the soil had been processed by method A, while recovery with methods B and C amounted to 87%. The gain of spores recovered after the dispersion of the soil by the latter two methods corresponded to 77% of those numbered after method A. A similar pattern was observed for the clay soil. However, the gain of spores recovered from the soil dispersed by methods B and C, compared to that obtained after method A, was much higher than that calculated for the sandy clay soil, amounting to almost to 300% of the spores recovered after method A. For this soil, the higher percentage of extracted spores was recorded when the soil had been dispersed by methods B and C, corresponding to 75% and 77% of the inoculum, respectively.

Irrespective of the soil, comparison of the spores extracted by procedures P1 and P2, after using method A, did not show any difference (Table 1). In the sandy clay soil, extracted spores, expressed as a percentage of the inoculum, amounted to 49% and 57% for P1 and P2, respectively. In the clay soil, the number of spores were much less abundant (22% of the inoculum).

Discussion

The formation of artificial aggregates was hypothesized to occur during the concentration of the 0–20 µm fraction by filtration on the sieve prior to spore enumeration. We have demonstrated that the dispersion of particles of sizes <20 µm collected on that sieve by agitation in a NaOH solution did not increase the number of spores extracted from the soils. Since the presence of NaOH is meant to disrupt the aggregates (Gavinelli et al. 1995), the formation of artificial aggregates could not be advocated. Consequently, the inefficiency in determining the pool of spores of *Pasteuria penetrans* present in heavy textured soils by the method of Mateille et al (1996) was not due to spore entrapment in artificial aggregates formed during the enumeration procedure.

It is well established that it is difficult to count bacteria present in stable aggregates formed by the interaction of organic and inorganic colloids. A few studies have already pointed out the influence of soil structure on the efficiency of biological assays (Badalucco et al. 1997; Richaume et al. 1993). In our study, the presence of stable aggregates was particularly evident for the clay soil. Indeed, particles of size fraction 0–20 µm dispersed by the less energetic method (method A) corresponded to about 20% of the simple mineral particles of the same size isolated by mechanical analysis. Thus, 80% of these particles were aggregated in coarser structures, being represented by aggregates >200 µm and 50–200 µm. When the more energetic method had been applied, these aggregates were entirely dispersed and the weights of these fractions were equivalent to those of the simple particles obtained by mechanical analysis. At the same time, spore recovery tripled when compared to that obtained by method A. At least 55% and 38% of the spores inoculated were associated with stable aggregates (>200 µm and 50–200 µm) in the clay and the sandy clay soil, respectively. The processes responsible for the presence of spores in these niches were not addressed in this study. Nevertheless, both physical entrapment or adsorption of spores on clays could be proposed, since physical (method B) and chemical (method C) disruption of these aggregates has led to the same spore recovery. As reviewed by Stotzky (1986), different processes are responsible for the interactions between mineral particles and biological entities.

In conclusion, this study clearly indicated that a reliable estimation of the pool of spores of *P. penetrans* de-

pends on the dispersion of the soil aggregates. Nevertheless, even after the entire dispersion of simple mineral particles, 10–25% of these spores remained undetected probably because of the difficulty in distinguishing spores from mineral particles in the suspension observed under the microscope (Hewlett and Serracin 1996; Marshall 1968; Ruddick and Williams 1972). Therefore, further research is needed to improve the enumeration of spores of *P. penetrans* by microscopy.

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