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Indirect effects of the bacterial soil aggregation on the distribution of *Pasteuria penetrans*, an obligate bacterial parasite of plant-parasitic nematodes

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Abstract

The effects of the inoculation of two fluorescent *Pseudomonas* strains (*P. mendocina* and *Pseudomonas* sp.) on the soil aggregation in the rhizosphere have been tested. Their indirect effects on the conservation of spores of *Pasteuria penetrans*, a bacterial endospore-forming parasite of plant-parasitic nematodes *Meloidogyne* spp., and on the infection of *Meloidogyne javanica* juveniles were investigated.

The amount of rhizosphere soil adhering to the root systems was higher in the soils inoculated with both bacterial strains *P. mendocina* and *Pseudomonas* sp. However, juveniles of *Meloidogyne* were more numerous in the soil inoculated with *Pseudomonas* sp. and the multiplication rate of *M. javanica* did not differ regardless of the treatment. Furthermore, the proportion of juveniles infested by *P. penetrans* spores was highest in soil inoculated with *P. mendocina* (B23). The spores were distributed, following a similar pattern in the soil aggregate fractions excepted in the soil inoculated with *P. mendocina* (B23). In the soil inoculated with B23, about 50% of the spores present in the rhizosphere were retained in the coarser structure characterized by aggregates > 200 μm. The role of the bacterial extracellular polysaccharides on soil and spores aggregation and on the *Meloidogyne* infection may explain these results.

Keywords: Soil; Aggregation; *Pseudomonas*; *Pasteuria penetrans*; *Meloidogyne javanica*; Biocontrol

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1. Introduction

The mycelial and endospore-forming parasite *Pasteuria penetrans* is an obligate endoparasite of nematodes and has potential as a biological control agent against the root-knot nematode *Meloidogyne* spp. (Bird et al., 1990; Stirling, 1991). Various abiotic factors such as temperature (Stirling et al., 1986; Giannakou et al., 1997) and humidity (Chan and Gill, 1994) as well as biotic factors such as rhizosphere bacteria (Duponnois et al., 1999a) are involved in the availability of this microorganism to control its nematode hosts.

Therefore, soil texture and structure were shown as important factors that are able to act on the flow of the spores of *P. penetrans* in the soil, and the capacity of the soil matrix to adsorb the spores was assumed (Mateille et al., 1996). More recently, studies on the effects of the inoculation of some soil bacteria either in suspensions containing juveniles of *M. javanica* and spores of *P. penetrans*, or in soils infested with these organisms revealed that these bacteria significantly improved the attachment of the spores of *P. penetrans* on the juvenile cuticle (Duponnois and Bâ, 1998; Duponnois et al., 1999a).

Some soil bacteria are also able to transform some root exudate into polysaccharides, which increase the aggregation and aggregate stability (Gupta and Germida, 1988; Gouzou et al., 1993). Consequently, such aggregating bacteria would help the retention of the spores of *P. penetrans* in the soil, increasing the pool of spores available for nematode attachment.

The aim of this research was to study the effects of two fluorescent *Pseudomonas* strains (*P. mendocina* and *Pseudomonas* sp.) on soil aggregation (evolution of the soil size fractions), distribution of the spores of *P. penetrans* in the aggregates, and on the infestation of *M. javanica* juveniles by *P. penetrans*.

2. Materials and methods

2.1. Nematodes

A population of *M. javanica* was cultured for 2 months on tomato plants cv. Roma in a heat sterilised soil (140°C, 40 min). The tomato roots were then harvested, cut into short pieces and placed in a mist chamber (Seinhorst, 1950) for 1 week to collect second stage juveniles.

2.2. P. penetrans endospores

Spores of *P. penetrans* were produced in females of *M. javanica* infecting the tomato plants. The females were extracted from root galls by shaking them

in a 100-ml enzyme solution (40% Celluclast, 20% Pectinex Ultra SP-L, Novo Nordisk) for 12 h (Hussey, 1971), washed in distilled water, crushed in Eppendorf tubes to release the spores of *P. penetrans*, and finally supplemented with sterile distilled water to a 1-ml final volume.

2.3. Bacteria

The bacterial strain *P. mendocina* (B23) was isolated by Duponnois et al. (1999a) from the rhizosphere of tomato plants growing in a sandy soil collected in a field heavily infested with *M. javanica* and *P. penetrans*. The strain of *Pseudomonas* sp. (strain G36) was isolated from tomato galls induced by *M. javanica* in the same soil as before (Duponnois et al., 1998). They were cultured on a 0.3% TSA medium (Tryptic Soy Agar, Difco) in the dark at 25°C for 2–3 days. Then the two bacterial strains were grown in 3 g l^{-1} liquid Difco tryptic soy broth in glass flasks (250 ml) shaken for 8 days at 25°C. The bacterial suspensions (more than 10^8 cells ml^{-1}) were centrifuged (2400 × *g*, 20 min) and the pellets were resuspended in 0.1 M $MgSO_4$.

2.4. Microbial inoculation

Two-week-old tomato cv. Roma seedlings were transplanted into 1 dm^3 polythene pots filled with an autoclaved (140°C, 40 min) sandy–clay soil. Soil physico-chemical characteristics were as follows: clay 10.3%; fine silt 1.7%; coarse silt 2.4%; fine sand 44.0%; coarse sand 40.7%; total organic carbon 0.29%; total nitrogen 0.04%, total phosphorus 1320 μg g^{-1}; pH (H_2O) 7.6. About 13.5×10^6 spores of *P. penetrans* were injected into each tomato pot. Two days later, the bacterial suspensions were inoculated into each tomato pot (2 ml per plant). Inoculation with 2 ml of $MgSO_4$ 0.1 M without bacteria was used as a control. One week after the bacterial inoculation, the tomato seedlings were inoculated with 5-ml suspensions containing 300 juveniles of *M. javanica* or 5 ml of distilled water for the control treatments without nematodes. The plants were randomly placed in a glasshouse (30°C day, 25°C night, 12 h photoperiod) and watered daily with 30 ml of sterile distilled water. Each nematode—*P. penetrans*—bacterial strain combination was replicated eight times (Table 1).

2.5. Soil aggregation analysis

2.5.1. Macroaggregation

Five weeks after the inoculation of nematodes, the plants were harvested and the roots vigourously shaken for 30 s in order to eliminate the non-rhizospheric soil. The root-adhering soil was manually recovered, air-dried and weighed.

Table 1
Presentation of the bacterial strains—nematodes—*P. penetrans* combinations tested

Treatments	*P. penetrans*	Bacterial strains	*M. javanica*
Control (C)	–	–	–
B23	–	*P. mendocina*	–
G36	–	*Pseudomonas* sp.	–
M	–	–	300 juveniles plant^{-1}
B23 M	–	*P. mendocina*	300 juveniles plant^{-1}
G36 M	–	*Pseudomonas* sp.	300 juveniles plant^{-1}
MP	13.5×10^6 spores plant^{-1}	–	300 juveniles plant^{-1}
B23 MP	13.5×10^6 spores plant^{-1}	*P. mendocina*	300 juveniles plant^{-1}
G36 MP	13.5×10^6 spores plant^{-1}	*Pseudomonas* sp.	300 juveniles plant^{-1}

2.5.2. Soil fractionation

A 25-g air-dried soil subsample was submersed in 150 ml of distilled water for 20 min and the soil suspension was sieved using a bank of sieves (200, 50, 20 μm). The different particle-size fractions (> 200, 50–200, 20–50 and 0–20 μm) were dried (105°C for 72 h) and weighed.

2.5.3. Soil dispersion

In order to evaluate the texture of the aggregates, the size fractions were shaken (end-over-end at 120 rpm for 16 h) in 150 ml of distilled water with three 1-cm agate marbles (Chotte et al., 1992). After a 5-min decantation, the suspensions were sieved using the same bank of sieves as before. The new size fractions obtained were dried (105°C for 72 h) and weighed.

2.6. Estimation of the populations of M. javanica

After 6 weeks' culture, the roots were washed with tap water and the galls induced by *M. javanica* were indexed as follows: 0, no galls; 1 = 1 to 5 galls; 2 = 6 to 20 galls; 3 = more than 20 galls; 4 = coalescing galls on the entire root system and 5 = rotten root system (Duponnois et al., 1999b). The roots were cut into 1- to 2-cm pieces and placed in a mist chamber (Seinhorst, 1950) for 2 weeks for the recovery of hatched juveniles. Moreover, the juveniles of *M. javanica* were extracted from a 150-cm^3 soil subsample, and from the soil size fractions, were separated according to the fractionation method described above. The juveniles, parasitized or not by *P. penetrans*, were counted in a 5-cm^3 counting chamber (Merny and Luc, 1969).

2.7. Detection of the Pseudomonas strains

A 2-g soil subsample taken from each pot was shaken (end-over-end 120 rpm for 24 h) in 25 ml of $MgSO_4$ 0.1 M. Then, serial dilutions of the suspensions

were plated on King B medium (King et al., 1954) and, after 48-h incubation at 25°C in the dark, the Petri dishes were observed under UV for the presence of fluorescent colonies. Then cell dimensions and morphology were determined by phase-contrast microscopy.

2.8. Distribution of the spores of Pasteuria penetrans

A 36-g air-dried soil subsample was submersed in 300 ml of distilled water for 15 min. The soil suspension was sieved using a set of sieves (200, 50, 20 μm). Three groups of spores were considered.

2.8.1. Free spores

Free spores represent the free spores in soil pores and are easily released in water. The size fraction 0–20 μm was concentrated on a 0.6-μm filter. The spores of *P. penetrans* and the mineral particles collected on that filter were suspended in distilled water for enumeration.

2.8.2. Leachable spores

Leachable spores represent the spores that are slightly adsorbed on soil particles and released by repeated water rinses of the soil. The size fractions (> 200, 50–200 and 20–50 μm) were rinsed three times with distilled water, and the filtrates were concentrated on a 0.6-μm filter for enumeration of the spores in distilled water as described above.

2.8.3. Adsorbed spores

Adsorbed spores represent the spores that are strongly adsorbed on soil particles and/or trapped in aggregates. The rinsed size fractions (> 200, 50–200 and 20–50 μm) were shaken (end-over-end at 120 rpm for 16 h) in distilled water with three 1-cm agate marbles. After a 5-min decantation, the suspensions were sieved using the same procedure as before. The 0–20 μm fraction was concentrated on a 0.6-μm filter for enumeration of the spores as described above.

The spores of *P. penetrans* were enumerated using a Malassez counting chamber (magnification × 120) (Duponnois et al., 1999a).

2.9. Statistical analysis

There were eight replicates per nematode—*P. penetrans*—bacterial strain combination. The data were subjected to a one-way analysis of variance using the Super Anova Computer program and means were compared with the Newman–Keuls multiple range test ($P \leq 0.05$). For the percentages, the data were transformed by arcsin ($\sqrt{x}$) before statistical analysis.

3. Results

3.1. Detection of the fluorescent Pseudomonas strains in the soil after 2 months' culture

No fluorescent *Pseudomonas* contaminations have been detected in the treatments without bacteria. For the bacterial treatments, some fluorescent *Pseudomonas* have been isolated from the soil suspensions. These bacterial colonies were morphologically identical to the isolates (*P. mendocina* B23 and

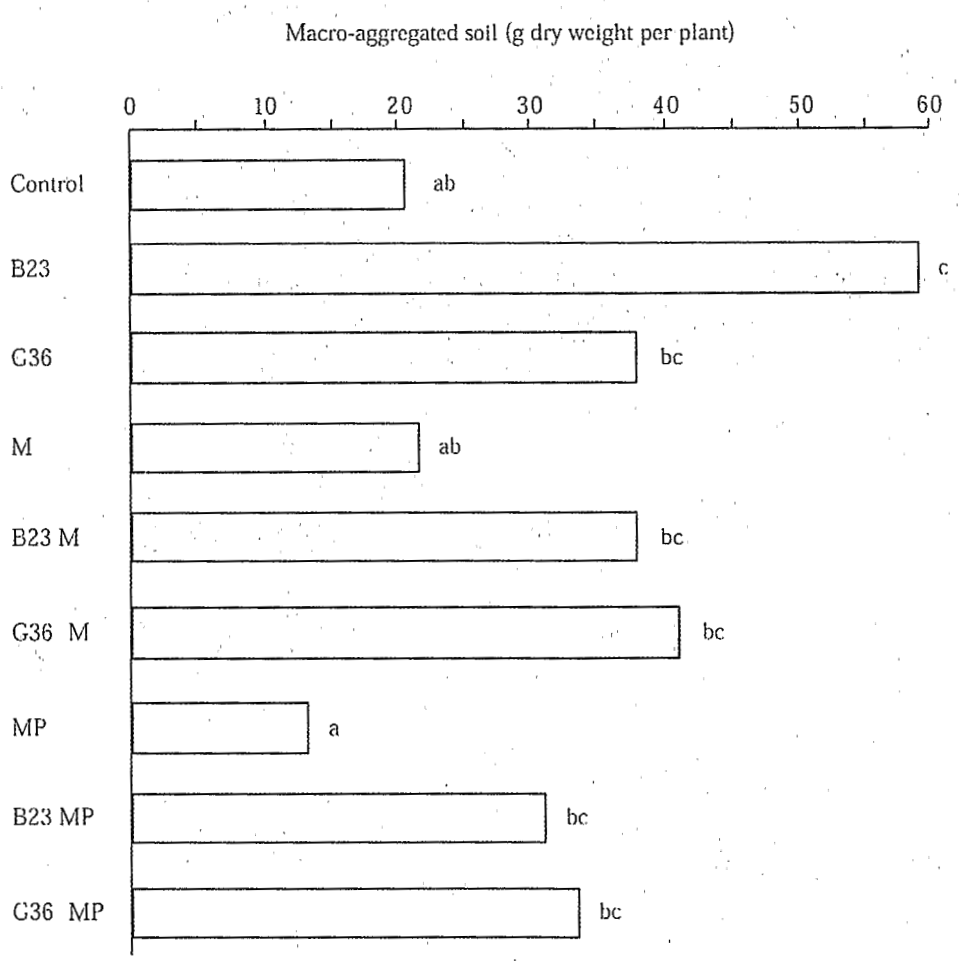

Fig. 1. Weights of aggregated soil on the roots (control: not inoculated treatment; B23: *P. mendocina*; G36: *Pseudomonas* sp.; M: *M. javanica*; B23 M: *M. javanica* + *P. mendocina*; G36 M: *M. javanica* + *Pseudomonas* sp.; MP: *M. javanica* + *P. penetrans*; B23 MP: *M. javanica* + *P. penetrans* + *P. mendocina*; G36 MP: *M. javanica* + *P. penetrans* + *Pseudomonas* sp. Data with the same letter are not significantly different according to the Newman–Keuls test ($P \leq 0.05$).

Pseudomonas sp. G36), which had previously been inoculated into the tomato pots.

3.2. Soil aggregation

3.2.1. Rhizospheric soil macro-aggregation

Compared to the control, the amount of root-adhering soil was significantly higher in the treatment B23. No significant effect of the nematode and/or *P. penetrans* inoculations have been recorded (Fig. 1). The inoculation of *P. penetrans*, *M. javanica* and *Pseudomonas* isolates, have significantly enhanced the weight of root-adhering soil compared to the MP treatment (Fig. 1).

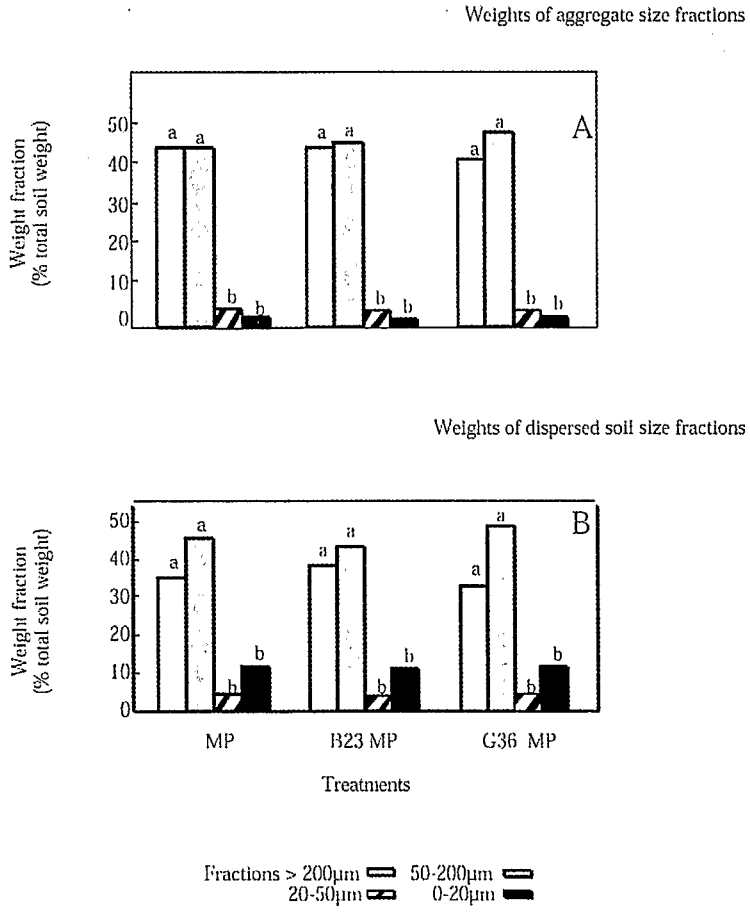

Fig. 2. Weight distribution of the soil size fractions obtained by fractionation (A) and disruption (B). For the legend, see Fig. 1. Data with the same letter are not significantly different according to the Newman–Keuls test ($P \leq 0.05$).

Table 2
Effect of *P. penetrans* and/or the *Pseudomonas* isolates on the development of *M. javanica*

Treatments	Gall index	J2/dm^3 of soil	J2/plant	Multiplication rate
M	3.8^{bc}	988.7^{a}	13823.8^{a}	49.2^{a}
B23 M	3.0^{a}	2547.5^{b}	13434.0^{a}	44.8^{a}
G36 M	3.0^{a}	1633.7^{ab}	11495.0^{a}	43.8^{a}
MP	4.0^{bc}	1640.0^{ab}	10784.7^{a}	46.3^{a}
B23 MP	3.5^{b}	2547.5^{b}	11616.3^{a}	43.0^{a}
G36 MP	4^{c}	2291.2^{b}	9453.8^{a}	39.0^{a}

Data followed by the same letter (superscript) are not significantly different according to the Newman–Keuls test ($P < 0.05$).

3.2.2. Weight distribution of the soil size fractions

3.2.2.1. Weights of aggregate size fractions. Weight recoveries after soil fractionation were: 99.9% for the soil inoculated with *P. mendocina* (B23 MP) and 99.7% either for the soil inoculated with *Pseudomonas* sp. (G36 MP) or for the soil without bacteria (MP) (Fig. 2A). Irrespective of the presence of the bacteria,

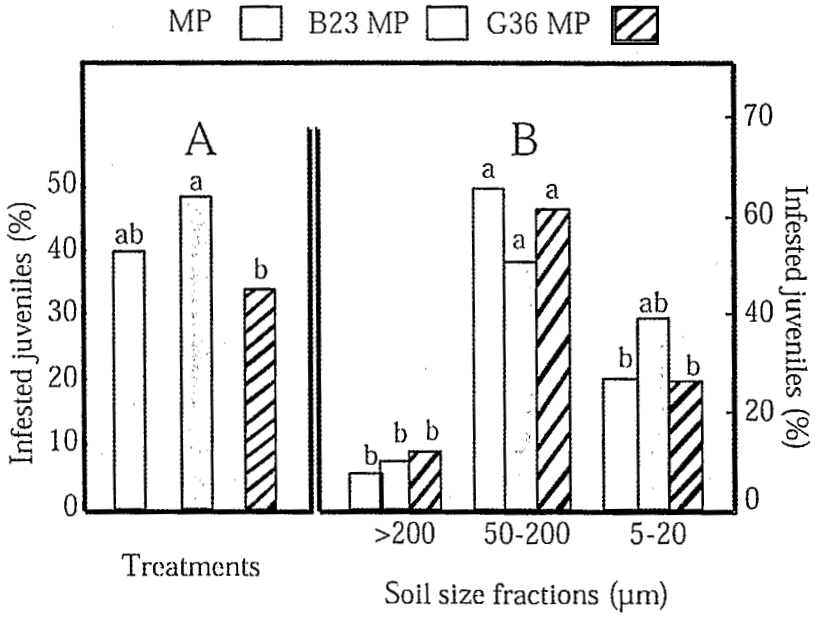

Fig. 3. Effects of the fluorescent *Pseudomonas* strains on the proportion of juveniles of *M. javanica* infested by *P. penetrans* (A) and on the distribution of the infested juveniles per soil size fractions (B). For the legend, see Fig. 1. Data with the same letter are not significantly different according to the Newman–Keuls test ($P \leq 0.05$).

the mass of the size fractions > 200 and 50–200 μm (43% to 49% of the total soil weight) was higher (×5) than that of the size fractions 20–50 and 0–20 μm (less than 10% of the total soil weight).

3.2.2.2. Weights of dispersed soil size fractions. Irrespective of the presence of the bacteria, the size fractions > 200 and 50–200 μm (34% to 50% of the total soil weight) were more abundant than the size fractions 20–50 and 0–20 μm (less than 12% of the total soil weight) (Fig. 2B).

Comparing the fractionation and the dispersion of the soils, the dispersion did not affect the size fractions 20–50 and 50–200 μm, but the size fraction > 200 μm decreased while a 10% increase of the size fraction 0–20 μm was observed.

3.3. Population of M. javanica

The root infestation and the multiplication rate of *M. javanica* did not differ, based on the treatments (Table 2). However, the juveniles were significantly more numerous in the soils inoculated with *P. penetrans* and *Pseudomonas* isolates (G36 MP, B23 MP) or B23 alone (B23 M) than in the control treatment (*M. javanica* alone). The lowest gall indexes were recorded with the bacterial treatments, B23 M and G36 M and the highest with G36 MP.

The rate of juveniles infested by *P. penetrans* did not exceed 50% (Fig. 3A). Nevertheless, the infested juveniles were significantly more abundant in the soil

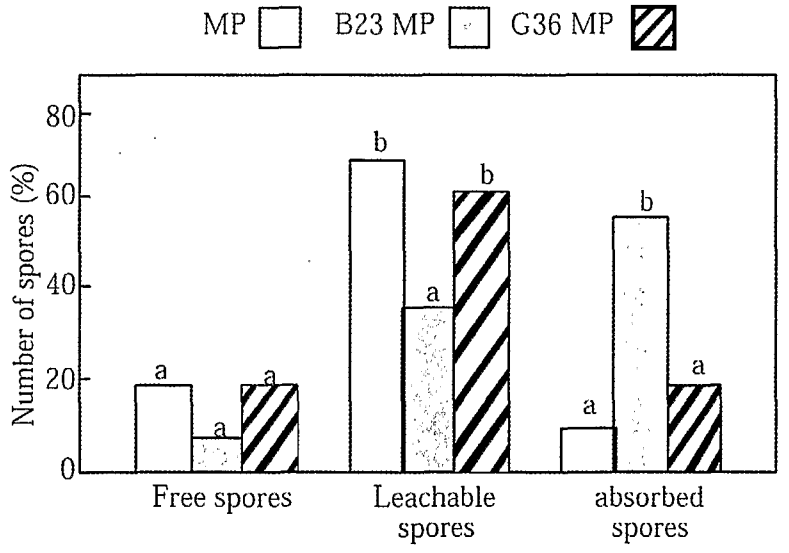

Fig. 4. Effects of the fluorescent *Pseudomonas* strains on the distribution of the different groups of *P. penetrans* spores. Data with the same letter are not significantly different according to the Newman–Keuls test ($P \leq 0.05$). For the legend, see Fig. 1.

inoculated with *P. mendocina* (B23) than in the soil inoculated with *Pseudomonas* sp. (G36). After soil fractionation (Fig. 3B), most of the infested juveniles were recovered from the size fraction 50–200 μm. Less than 12% of *Pasteuria*-infested juveniles were recovered in the coarsest size fraction > 200 μm. Among the treatments, bacterial inoculation (G36 and B23) did not modify the distribution of the infested juveniles in the soil size fractions (Fig. 3B).

3.4. Distribution of the spores of P. penetrans in the soil

The total number of spores of *P. penetrans* recovered from the soils were the same irrespective of the inoculum combination (Fig. 4). Considering the analysis of the three groups of spores (Fig. 4), the distribution of the spores was the same in the soil inoculated with *Pseudomonas* sp. (G36) and in the soil without bacteria (MP): the free spores (first group) and the adsorbed spores (third group) were less than 20% and the leachable spores (second group) were the most

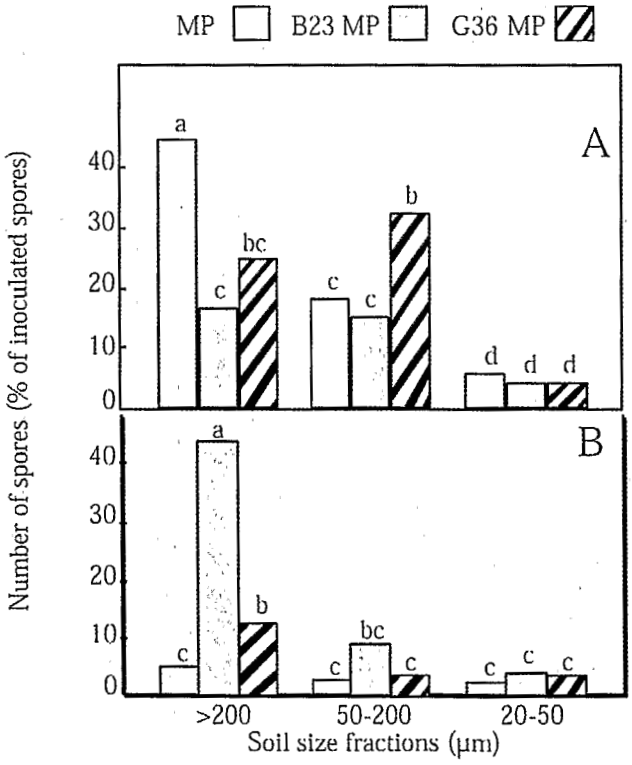

Fig. 5. Distribution of the leachable (A) and the aggregated (B) spores of *P. penetrans* in the different soil size fractions. Data with the same letter are not significantly different according to the Newman–Keuls test ($P \leq 0.05$). For the legend, see Fig. 1.

abundant (about 70%). The distribution of the spores was different in the soil inoculated with *P. mendocina* (B23): most of them (58%) were represented by aggregated spores.

Considering the analysis of the second and the third groups of spores in the soil size fractions, very few (less than 5%) leachable spores were recovered from the finest size fraction 20–50 μm (Fig. 5A). Inoculation with *Pseudomonas* had no effect on their recovery in this fraction. In the soil without *Pseudomonas* (PM), most of the spores were detected in the coarsest size fraction > 200 μm and fewer in the intermediate size fraction 50–200 μm. In the soil inoculated with *Pseudomonas* sp. (G36), the leachable spores were mostly distributed in the size fraction 50–200 μm.

In the soil without *Pseudomonas* (MP), the aggregated spores represented less than 5% of the inoculated spores (Fig. 5B), and were homogeneously distributed in the three size fractions. A few spores were detected in the size fraction > 200 μm of the soil inoculated with *Pseudomonas* sp. (G36). Most of them (45%) were extracted from the size fraction > 200 μm in the soil inoculated with *P. mendocina* (B23).

4. Discussion

No antagonistic effect of *P. penetrans* against *M. javanica* was observed in this experiment. It is possible that the biocontrol of *Meloidogyne* population by *P. penetrans* follows the latency phenomena (Chen et al., 1996). This may be ascribed to the increase of *Pasteuria* density and the scattering of the spores from infested females through the soil (Oostendorp et al., 1991; Chen et al., 1996).

The increase of the population of *M. javanica* juveniles in the soil inoculated with both *P. penetrans* and *Pseudomonas* sp. (G36 MP) may be attributed to a stimulation of the nematode egg hatching by bacterial metabolites. However, the improvement of the infection of the juveniles by *P. penetrans* spores in the soil inoculated with *P. mendocina* (B23 MP) may be due to a modification of the exosporium structure (Stirling et al., 1986) related to their hydrophobicity (Afolabi et al., 1995), increasing the attachment of *P. penetrans* spores on the nematode cuticle (Duponnois et al., 1999a).

It was usually assumed that the contribution of soil biota to soil aggregation occurs mainly by bioturbation. Microorganisms are important agents for aggregate stabilization, which is currently attributed to the production of extracellular polysaccharides (Beare et al., 1995) from root exudates and their deposition acting as soil cements (Guckert et al., 1975; Oades, 1977; Gouzou et al., 1993). The quality of the soil structure would depend on the nature of the bacterial secretion, which determines the binding action of polysaccharides on soil

particles (Tisdall, 1991). In fact, the stabilization of the aggregates depends widely on the bacterial species, which vary mostly with the nature of the available substrates (Aspiras et al., 1971). These phenomena occur more obviously in low clay content soils (as the sandy–clay soil tested) and in soils characterized by low shrink–swell capacities (Oades, 1993). According to that, the inoculation of *P. mendocina* (B23) or *Pseudomonas* sp. (G36) would provide such exopolysaccharides, which improve soil aggregation around the tomato roots.

Moreover, the extracellular polysaccharide production provided by strain B23 related to the substrate availability is relevant to improve particle binding, detaining also the aggregated spores of *P. penetrans* particularly in the fraction > 200 μm.

As the aggregates > 200 μm are not stable in water (Edwards and Bremmer, 1967), they could be disrupted by tillage and irrigation (Bertrand and Sorr, 1962; Vinten et al., 1983; Davet, 1996), releasing the spores available for nematode infestation, particularly those moving in the fraction 50–200 μm. Moreover, the *Meloidogyne* spp. juveniles move preferably through the coarse soil texture (Prot, 1986), which would be inhabited by free spores. The highest proportion of *P. penetrans*-infested juveniles observed in the soil inoculated with this *P. mendocina* (B23) could be ascribed to the specific conservation of the spores in this soil.

These results show that some rhizosphere bacteria can promote the development of *P. penetrans* and must be considered in order to promote the antagonistic effect of *P. penetrans* against the root-knot nematodes. However, the mechanisms involved in these interactions must be explained further in order to identify more efficient bacteria.

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