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Influence of soil conditions, spore densities and nematode age on *Pasteuria penetrans* attachment to *Meloidogyne incognita*

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Abstract

Several soil variables as soil texture, moisture and temperature, *Pasteuria penetrans* spore densities and nematode age were tested in the laboratory for their effects on spore attachment to *Meloidogyne incognita* juveniles. Percentage of juveniles with endospores attached and number of spores per juvenile increased with concentration of spores applied to soil. In a clay-loam soil, hundred-percent attachments were reached at densities of 5×10^5 spores per g of soil and above. Attachment was greater in loamy-sand than in sandy-loam and clay-loam soils, but lower when soil moisture was under 10% than when it was over 25%. Numbers of juveniles with spores attached were greater when soil temperatures were 25ºC and 35ºC than at 15ºC and lower in 7-30 days old juveniles than in 0-6 days old juveniles. All factors that favored nematode mobility in soil increased *Pasteuria* spore attachment to *M. incognita* juveniles.

Key words: biological control, root-knot nematode.

Resumen

Influencia de las condiciones edáficas, densidad de esporas y edad de los nematodos sobre la adherencia de *Pasteuria penetrans* a *Meloidogyne incognita*

Se realizaron diversos ensayos en el laboratorio para analizar el efecto de variables edáficas como textura, humedad y temperatura del suelo, densidad de esporas de *Pasteuria penetrans* y la edad de los juveniles de *M. incognita* sobre la adherencia de las esporas a los juveniles de *M. incognita*. El porcentaje de juveniles con endosporas adheridas y el número de esporas por juvenil de *M. incognita* se incrementó con la concentración de esporas aplicadas al suelo. En un suelo arcillo-limoso se consiguieron porcentajes de adherencia del 100% cuando las densidades de esporas alcanzaron 5×10^5 esporas por g de suelo. La adherencia de esporas a los juveniles fue mayor en suelos más arenosos, y menor cuando la humedad del suelo estaba por debajo del 10%. El número de juveniles con esporas adheridas fue mayor a 25ºC y 35ºC que a 15ºC y menor en juveniles de 7-30 días que en juveniles de 0 a 6 días de edad. Todos los factores que favorecen la movilidad de los juveniles de *M. incognita* en suelo incrementaron el porcentaje de juveniles con esporas adheridas y el numero de éstas por juvenil.

Palabras clave: control biológico, nematodo agallador.

Introduction

Pasteuria penetrans (Thorne) Sayre and Starr, the hyperparasite bacteria of *Meloidogyne* spp., has been associated with reductions in nematode damage in greenhouse and field experiments (Chen *et al*., 1996, 1997; Tzortzakakis *et al*., 1997; Siddiqui and Mahmood, 1999; Trudgill *et al*., 2000). Infection of nematodes by *Pasteuria* starts when spores attach to second stage juveniles (J2s) in soil. After juveniles enter the plant roots, germination occurs and later, sporulation takes place (Sayre and Starr, 1985). Attachment of the endospores to the nematode cuticle is therefore the first step in the life cycle of the bacterium and is essential for its reproduction. Percentages of juveniles with spores attached and number of spores per juvenile have been correlated with infection in adults (Stirling, 1984; Rao *et al*., 1997) and thus have been used as an indirect measure of biocontrol by this agent (Chen and Dickson, 1997) or as a method to estimate endospore concentrations in soil (Hewlett and Serracin, 1996; Dabire *et al*., 2001).

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Endospores of *P. penetrans* are non-motile and thus, contact with nematodes will depend on nematode movement in soil (Stirling *et al*., 1990). Therefore, any factor that increased nematode mobility should also increase spore attachment.

Although the dynamics of acquisition of *Pasteuria* spores by *Meloidogyne* juveniles in soil is not yet fully understood, several biotic and abiotic factors have been suggested to affect it in field and greenhouse experiments, as *Pasteuria* spore densities and time of exposure (Stirling *et al*., 1990; Adiko and Gowen, 1994), soil texture (Mateille *et al*., 1995), soil moisture (Dutky and Sayre, 1978) and soil temperature (Hatz and Dickson, 1992; Freitas *et al*., 1997). However, most of these experiments have been carried out in field or greenhouse trials on diverse pathosystems, involving mainly *M. javanica* or *M. arenaria*, and the influence of nematode age on spore attachment has never been tested. Therefore, experiments in laboratory under controlled conditions are necessary to confirm or refuse these hypotheses.

The objective of this work was to test and determine, under controlled conditions, the influence of several soil and environmental factors on *P. penetrans* spore attachment to *Meloidogyne incognita* (Kofoid and White) Chitwood aiming at a better understanding of the process of acquisition of *Pasteuria* spores by *Meloidogyne* juveniles in soil.

Material and Methods

Soil source

A clay-loam soil (40% sand: 30% silt: 30% clay) was collected from a fallow field at the National Agricultural Research Center (NARC), Tsukuba, Ibaraki, Japan. Sandy-loam (66% sand: 17% silt: 17% clay) and loamy-sand (76% sand: 12% silt: 12% clay) soils were prepared adding and mixing commercial sand (1-2 mm grade) to the clay-loam. Soils were sterilized twice at 120ºC for 20 min, air dried in an oven at 40ºC for two days, moistened up again to 25% soil moisture and stored for two weeks at 25ºC, before using them for experimental purposes.

P. penetrans source

Seven suspensions of *Pasteuria* endospores in water (0.67×10^7, 3.33×10^6, 0.66×10^6, 3.33×10^5, 0.66×10^5, 3.33×10^4 and 0.66×10^4 spores per ml) were prepared using a commercial powdery formulation of *P. penetrans* (10^9 spores g^{-1}) (Nematec Inc. Japan, Higashi-Ueno 6-2-1, 110-0015 Tokyo, Japan). Soils were inoculated by pouring 3 ml of spore suspension over 20 g of sterile soil placed in plastic cups (6 cm top diam.; 4.5 cm bottom diam.; 3.5 cm depth). Spore suspension droplets were uniformly distributed over the soil surface. Soil cups were sealed and incubated for three days at 25ºC to allow spore rehydration before nematode inoculation.

M. incognita source

A *M. incognita* line maintained on susceptible tomato cv. Kyoryoku-beiju (Takii Seeds, Inc. Japan) at NARC, Tsukuba, Ibaraki, Japan was used as nematode inoculum. Juveniles of *M. incognita* were collected from egg masses 24-72 h after hatching. A suspension of 200 J2s in 1 ml of water was pipetted to each cup. Soil cups were then sealed and incubated again at 25ºC for four days, before nematode recovery.

Estimation of spore attachment

Different treatments were applied to soil cups and eventually, nematodes were recovered from 20 g of soil by the Baermann method (72 h). *Pasteuria* attachment was estimated in samples of 35 J2s by percentage of J2s with spores attached and spore intensity (total number of spores in a population of 35 J2s divided by the number of J2s with spores attached). All treatments were replicated six times.

Influence of *Pasteuria* spore concentration in soil on spore attachment

Plastic cups containing 20 g of clay-loam soil with 10^6, 5×10^5, 10^5, 5×10^4, 10^4, 5×10^3 or 10^3 *Pasteuria* spores per g were prepared and inoculated with nematodes and spore attachment was estimated as previously described.

Influence of soil texture on spore attachment

Plastic cups containing 20 g of clay-loam, sandy-loam or loamy-sand soils were inoculated with *Pas-*

teuria spores (10^4 spores per g of soil) and nematodes. Nematodes were recovered four days later and spore attachment estimated.

Influence of soil moisture on spore attachment

Plastic cups containing 20 g of clay-loam soil with 10^4 spores per g were air-dried at 30ºC and then divided in three groups that received 0, 3 or 5 ml of distilled water per cup, respectively. Cups were inoculated with *M. incognita* and four days later nematodes were recovered and spore attachment estimated. Soil moisture was determined by measuring the weight loss of four soil samples of 20 g from each treatment after drying for 24 h at 105ºC, resulting in three treatments (soil moisture: 9.5%; 25.2% and 41.6%).

Influence of soil temperature on spore attachment

Plastic cups with 20 g of clay-loam soil were inoculated with *Pasteuria* (10^5 spores g^{-1}) and nematodes and then incubated at 15ºC, 25ºC and 35ºC for four days, before recovery of nematodes and *Pasteuria* attachment estimation.

Influence of juvenile age on spore attachment

Plastic cups were filled with 20 g of clay-loam soil and inoculated with *Pasteuria* (10^5 spores per g of soil). *M. incognita* J2s were recovered continuously during 30 days from egg masses and stored in a refrigerator at 10ºC. Juveniles were divided in ten age groups: [1-3], [4-6], [7-9], [10-12], [13-15], [16-18], [19-21], [22-24], [25-27] and [28-30] days old. Cups were then inoculated with 200 J2s of a specific age group, incubated for four days at 25ºC and nematodes recovered by the Baermann-funnel method. Number of J2s with spores attached and number of spores per juvenile were counted.

Statistical analysis

Data were analyzed by ANOVA using the statistical package SPSS v.8.0. When F values were significant, means were compared by the HSD Tukey test ($P < 0.05$). Kolmogorov-Smirnov and Levene's tests were performed to check for normality and homocedasticity, if significant, numerical data were $\log_{10}(x + 1)$ transformed and percentages arcsine square root transformed before being analyzed by ANOVA. Regression analyses were performed to determine the influence of endospore densities and *M. incognita* J2 age on *Pasteuria* attachment to *M. incognita* J2s. Curves with the highest R^2 were chosen as best fit.

Results

Influence of *Pasteuria* spore concentration in soil

In the clay-loam soil, hundred-percent attachments were reached at concentrations of 5×10^5 spores per g of soil and above. There were no significant differences in spore intensities from 10^3 to 10^4 spores per g of soil, but numbers increased over 10 spores per J2 at spore densities of 10^5 and at 10^6 were more than 100 per J2 (Table 1).

Percentage of attachment and spore intensity were positively correlated to *Pasteuria* spore density in soil ($P < 0.01$). Linear and exponential regression models were fit when percentage of J2S with spores attached «a» and spore intensity «b» were dependent variables, and $\log_{10}$[*Pasteuria* spore densities] «x» was the independent variable (Figs. 1 and 2).

Table 1. Influence of *P. penetrans* spore densities in soil on percentage of *M. incognita* J2s encumbered with endospores and spore intensity

Spore density in soil	Percentage of attachment (%)	Spore intensity
10^3	9.3 ± 3.6 a	1.1 ± 0.2 a
5×10^3	20.7 ± 4.3 b	1.4 ± 0.1 a
10^4	32.1 ± 4.9 c	2.2 ± 0.3 a
5×10^4	68.6 ± 4.0 d	7.7 ± 1.7 b
10^5	86.8 ± 4.7 e	11.2 ± 2.0 b
5×10^5	100.0 ± 0.0 f	71.7 ± 12.1 c
10^6	100.0 ± 0.0 f	100.0 ± 3.4 d

Values are expressed as average of six replicates ± standard deviation. Numerical data were $\log_{10}(x + 1)$ transformed and percentages arcsine square root transformed, analyzed by ANOVA and means compared by the HSD Tukey test. Values followed by the same letter within a column are not significantly different ($P < 0.05$).

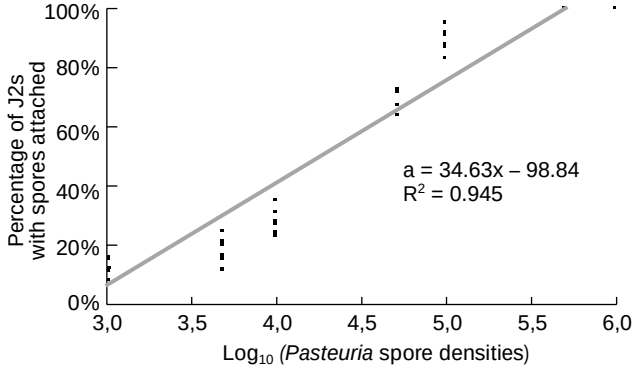

Figure 1. Regression curve for percentage of *M. incognita* J2s with *Pasteuria* spores attached to their cuticle vs Log_{10} *(Pasteuria* spore densities).

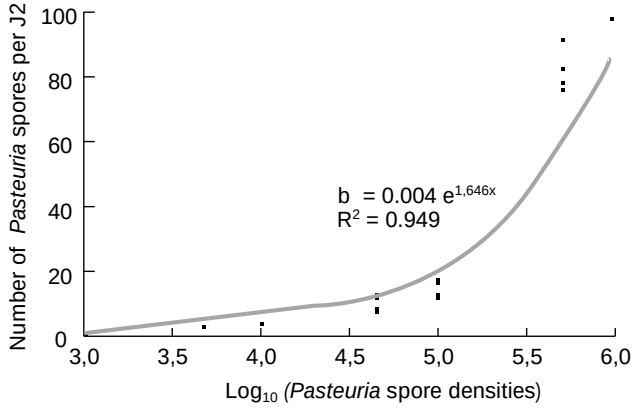

Figure 2. Regression curve for spore intensity *vs* Log_{10} *(Pasteuria* spore densities).

Influence of soil texture

In soils with 10^4 spores per g, percentage of attachment was significantly greater as proportion of sand in soil increased (Table 2). Attachment was greater in loamy-sand (62.2%) than in sandy-loam (40.0%) and in clay-loam (26.7%) soils. Spore intensity was also greater as proportion of sand increased, clay-loam (1.7) sandy-loam (2.6) and in loamy-sand (4.5).

Influence of soil moisture

Percentage of attachment increased with soil moisture but there were no significant differences in spore intensity (Table 2). At *Pasteuria* spore densities of 10^4 spores per g of soil, when soil moisture was low (9.5%) only 8.3% of J2s had spores attached, which was significantly lower than when soil moisture was 25.2% or 41.6% (23.3% and 32.2% of J2s with spores attached, respectively).

Table 2. Influence of soil conditions on percentage of *M. incognita* J2s encumbered with endospores and spore intensity

	Percentage of attachment (%)	Spore intensity
Soil texture		
Clay-loam	26.7 ± 6.4 a	1.7 ± 0.0 a
Sandy-loam	40.0 ± 8.8 b	2.6 ± 0.2 ab
Loamy-sand	62.2 ± 7.1 c	4.5 ± 0.3 b
Soil moisture		
9.5%	8.3 ± 3.5 a	1.1 ± 0.0 a
25.2%	23.3 ± 6.9 b	1.3 ± 0.1 a
41.6%	32.2 ± 5.8 c	1.2 ± 0.2 a
Soil temperature		
15°C	37.5 ± 5.9 a	2.4 ± 0.5 a
25°C	85.0 ± 6.9 b	9.2 ± 2.8 b
35°C	82.5 ± 7.6 b	5.5 ± 1.1 c

Values are expressed as average of six replicates ± standard deviation. Numerical data were $\log_{10}(x + 1)$ transformed and percentages arcsine square root transformed, analyzed by ANOVA and means compared by the HSD Tukey test. Values followed by the same letter within a factor and column are not significantly different ($P < 0.05$).

Influence of soil temperature

In soils with 10^5 spores per g of soil, percentage of attachment was greater at 25ºC (85.0%) and 35ºC (82.5%) than at 15ºC (37.5%). Numbers of spores per juvenile were also higher at 25ºC (9.2) than at 35ºC (5.5) and 15ºC (2.4) soil temperatures (Table 2).

Influence of *M. incognita* juveniles age

Percentage of attachment was significantly lower when 7-30 days old juveniles were used (72.6%) than for 0-6 days old juveniles (94.8%) ($P < 0.05$). Spore intensity was greater on 1-3 days old juveniles (9.5) than in 4-6 days old (6.2) and in 7-30 days old (3.3). Percentage of attachment and spores per juvenile were negatively correlated to the age of juveniles. Third order polynomic curves were fit for percentage of J2s with spores attached «a» and spore intensity «b», respectively when juvenile age «z» was the dependant variable (Figs. 3 and 4).

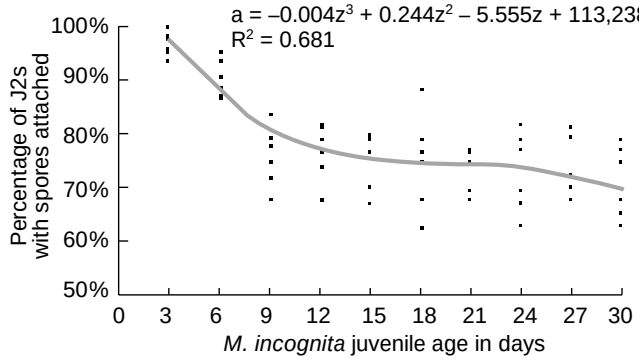

Figure 3. Regression curve for percentage of *M. incognita* J2s with *Pasteuria* spores attached *vs* juvenile age.

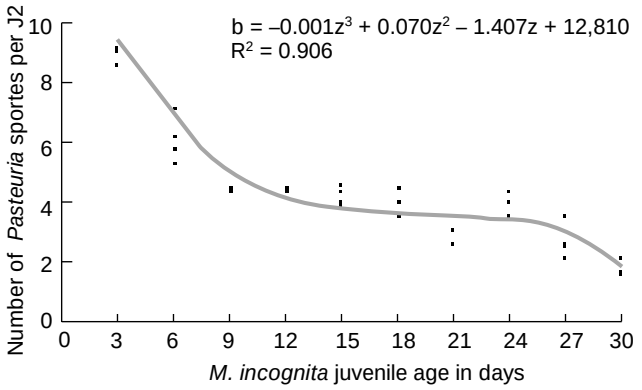

Figure 4. Regression curve for spore intensity *vs* juvenile age.

Discussion

Percentage of *M. incognita* juveniles with spores attached and number of spores per juvenile were positively correlated with *Pasteuria* spore densities in soil. Stirling *et al*. (1990) reported increases in attachment to a different species, *M. arenaria*, with greater spores concentrations in soil and suggested that endospore densities of at least 2.5×10^5 per g of soil were needed to ensure an average of 20-50 spores per nematode and reduce damage caused by *Meloidogyne* populations. *M. arenaria* J2s inoculated in soils with 10^3, 10^4, 10^5 and 10^6 *Pasteuria* endospores per g of soil gave an average of 36, 58, 92 and 100% attachment and 1.2, 3.4, 9.6 and 51.0 endospores per juvenile respectively, after three days in the soil (Hewlett and Serracin, 1996). Our results are similar to these previous reports and show that in our *M. incognita* population, 100% attachments and more than 50 spores per J2 are reached after four days in clay-loam soils, at spore concentrations of 5×10^5 per g of soil and when *Pasteuria* spores are uniformly distributed in soil.

In our experiments, both attachment parameters, percentage of attachment and number of spores per juvenile, were positively correlated with proportion of sand in soil. Mateille *et al*. (1995) found that *Pasteuria* occurrence was greater in sandy soils in a survey in Senegal and suggested that sandy soil may favor endospore attachment to *Meloidogyne* spp. A significant reduction in nematode reproduction has also been reported in sandy-loam soils with *P. penetrans* when compared to loam or clay-loam soils (Singh and Dhawan, 1992). Our results show a direct relationship between proportion of sand in soil and spore attachment to *M. incognita* juveniles. This phenomenon could be caused either by a favored mobility of nematodes in sandy soils, or related to the movement and distribution of spores in the soil pores by a more uniform distribution of spores in sandy soils or a retention of spores within clay aggregates, which would make spores unavailable for attachment to nematodes (Mateille *et al*., 1996).

Pasteuria attachment to *Meloidogyne* J2s has also been reported to increase with temperature from 15ºC to 30ºC in *M. javanica* (Stirling *et al*., 1990) and *M. arenaria* (Hatz and Dickson, 1992; Freitas *et al*., 1997). Spore attachment to *M. javanica* was double at 27ºC than at 18ºC (Stirling *et al*., 1990). In *P. penetrans* infested soils, highest attachment to *M. arenaria* J2s occurred when soil was maintained at 20-30ºC for 4 days and greater temperatures reduced endospore attachment (Freitas *et al*., 1997). In our experiment *Pasteuria* spores attached to the cuticle of *M. incognita* at 15, 25 and 35ºC but maximum attachment was obtained at 25ºC. Temperatures below 25ºC decreased percentage of attachment and number of spores per juveniles and temperatures above 25ºC reduced number of spores per juvenile. Two mechanisms have been suggested to explain this phenomenon, the increased mobility of nematodes at greater temperatures (Bird and Wallace, 1965) and activation of temperature-dependent proteins and carbohydrates involved in the attachment process (Davies and Danks, 1993).

Studies on soil moisture effects have produced variable results. Dutky and Sayre (1978) did not find any correlation between attachment and soil moisture. On the contrary, moistening dry soil for three days before adding *M. incognita* J2s increased endospore attachment (Brown and Smart, 1984). In our study soil moisture of 9.5% showed lower attachment rates than soil moistures of 25.2% or 41.6%, which can be caused by the reduced mobility of nematodes when soils are dry.

Percentage of juveniles with spores attached and number of spores per juvenile were negatively correlated to nematode age. Increasing the storage time of nematodes in water suspensions can lead to a quiescent-anoxybiotic stage (Antoniou, 1989), in which nematodes stop movement and chances of contact with *Pasteuria* spores are reduced. We observed that approximately 14.5 % of the 1-3 days old J2s were non-mobile and that 19.8% were inactive after 28-30 days storage at 10ºC. These differences were not significant at the $P < 0.05$ level. Nevertheless, it has been stated that the longer the state of quiescence the slower the return to normal conditions (De Guiran, 1977). Therefore, older juveniles would require longer time to become active and the time moving through the *Pasteuria* infested soil would be reduced. A reduction in endospore attachment caused by long-term storage of nematode suspensions in water was also reported by Freitas *et al*. (1997). These authors suggested that the surface coat of J2s, needed for endospore attachment, was lost during the water storage. According to Davies *et al*. (1991), the length of time taken for juveniles to be encumbered by spores in a water suspension was dependent on nematode age. De Silva *et al*. (1996) also showed an effect of nematode age on detachment of spores, detachment occurred only from one-day-old juveniles and not from five old days juveniles. This indicates that there may be changes in the surface coat of juveniles with age that affect attachment of spores. Changes in the cuticle with age have been observed in *Caenorhabditis briggsae* (Himmelhoch *et al*., 1977).

These results show that *Pasteuria* attachment to *M. incognita* juveniles is highly dependant on soil and nematode factors and that very favorable conditions are necessary to obtain high percentages of attachment, which would result in reductions in nematode damage. In less favorable conditions, high concentrations of spores or additional measures to increase infection rates would be necessary to be applied for a profitable application of *P. penetrans* in nematode control.

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