RESEARCH NOTE – NOTA INVESTIGATIVA

EFFECTS OF *PASTUERIA PENETRANS* ENDOSPORE RATE OF ATTACHMENT ON ROOT PENETRATION AND FECUNDITY OF *MELOIDOGYNE ARENARIA* RACE 1

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ABSTRACT

Kariuki, G. M., J. A. Brito, and D. W. Dickson. 2006. Effects of *Pastueria penetrans* endospore rate of attachment on root penetration and fecundity of *Meloidogyne arenaria* Race 1. Nematropica 36:261-267.

Pasteuria penetrans is an obligate mycelial endospore-forming bacterial parasite of root-knot nematodes that has shown great potential for their biological control. Endospores of *P. penetrans* attach to the cuticle of second-stage juveniles (J2) of *Meloidogyne* spp. when they move through soil in search of a host. The percentage of J2 with endospores attached and number of endospores per J2 are correlated with infection in adults and therefore are often used as an indirect measure of biocontrol potential. This study was carried out to test the effect of different rates of endospore attachment on the ability of root-knot nematodes to penetrate a host, produce galls and the nematode's fecundity under environmentally controlled conditions. Different attachment levels were achieved using different ratios of J2 to endospores. The number of J2 penetrating host roots was highest in the control where the J2 were not exposed to *P. penetrans* endospores and lowest at higher endospore attachment levels of 10.2 and 18.1. As few as 3.5 endospores/J2 reduced their ability to infect roots. Egg mass numbers were reduced by higher levels of endospore attachment but galling was not. This study confirms that *P. penetrans* induced soil suppressiveness is dependent on endospore concentrations and is manifested at the level of root penetration by J2 and loss of nematode fecundity.

Key words: attachment, biological control, fecundity, *Pasteuria penetrans*, penetration, suppressive soil.

RESUMEN

Kariuki, G. M., J. A. Brito, y D. W. Dickson. 2006. Efectos de la tasa de adhesión de endosporas de *Pastueria penetrans* sobre la penetración y fecundidad de *Meloidogyne arenaria* Raza 1. Nematropica 36:261-267.

Pasteuria penetrans es una bacteria micelial formadora de endosporas, parásito obligado de nematodos del nudo radical, con gran potencial para el control biológico. Las endosporas de *P. penetrans* se adhieren a la cutícula del juvenil de segundo estadio (J2) de *Meloidogyne* spp. cuando éste se mueve a través del suelo en busca de su hospedante. El porcentaje de J2 con endosporas adheridas y la cantidad de endosporas por juvenil están correlacionados con la infección en adultos y por ello son usados con frecuencia como medida indirecta del potecial biocontrolador. Este estudio se llevó a cabo para evaluar el efecto de diferentes tasas de adhesión de endosporas sobre la habilidad del nematodo del nudo radical para penetrar el hospedante y producir agallas, y el efecto sobre la fecundidad del nematodo bajo condiciones ambientales controladas. Se obtuvieron diferentes niveles de adhesión usando diferentes proporciones de J2 a endosporas. El mayor número de J2 penetró las raíces del hospedante en el tratamiento control, en donde los juveniles no fueron expuestos a las endosporas de *P. penetrans*, y la menor penetración se observó con los niveles de adhesión de endosporas más altos, 10.2 y 18.1. Se requirieron por lo menos 3.5 endosporas/J2 para reducir la habilidad de los juveniles de penetrar las raíces. Los niveles más altos de adhesión de endosporas redujeron la cantidad

de masas de huevos, pero no el agallamiento. Este estudio confirma que la supresividad del suelo inducida por *P. penetrans* depende de la concentración de endosporas y se manifiesta en el nivel de penetración de J2 y en la pérdida de fecundidad del nematodo.

Palabras clave: adhesión, control biológico, fecundidad, *Pasteuria penetrans*, penetración, suelo supresivo.

Pasteuria penetrans (Thorne) Sayre & Starr is an obligate, mycelial endospore-forming bacterial parasite of root-knot nematode that has shown potential as a biological control agent of *Meloidogyne* spp. in microplot, greenhouse, and field experiments (Chen *et al.*, 1996; Dickson *et al.*, 1994; Tzortzakakis *et al.*, 1997; Trudgill *et al.*, 2000). Attachment is the first step toward successful development of *P. penetrans* within *Meloidogyne* spp. (Afolabi *et al.*, 1995; Davies *et al.*, 1996; Talavera and Mizukubo, 2003). Percentage of J2 with endospores attached and the number of endospores attached per J2 are reported to be influenced by concentrations of endospores in soil as well as soil texture and temperature (Ahmed and Gowen, 1991; Freitas *et al.*, 1997; Hatz and Dickson, 1992; Nakasono *et al.*, 1993; Rao *et al.*, 1997; Serracin *et al.*, 1997; Stirling, 1981, 1984; Stirling *et al.*, 1979, 1990; Talavera and Mizukubo, 2003).

Endospores of *P. penetrans* are nonmotile, thus for them to come in contact with J2 of *Meloidogyne* spp. in soil is dependant on nematode movement (Stirling *et al.*, 1990; Talavera and Mizukubo, 2003). It is likely that J2 mobility in the soil profile is the single most important factor favoring attachment of endospores to the nematode cuticle, with more active J2 having a greater probability of coming in contact with endospores. However, it is known that endospores readily move downward with percolation of water thereby increasing their chance for contact with J2 (Cetintas and Dickson, 2005; Dickson and Hewlett, 1988; Oostendorp *et al.*, 1989).

The percentage of J2 with endospores attached and number of endospores per J2 have been correlated with development within the female nematode and therefore is often used as an indirect measure of biocontrol potential or as a method to estimate endospore concentrations in soil (Chen and Dickson, 1997; Dabire *et al.*, 2001; Rao *et al.*, 1997; Stirling, 1984). However, mere attachment does not mean that an endospore will germinate and enter the nematode body. It is only after penetration and development within the nematode pseudocoelom that the bacterium is considered a successful parasite.

It has been reported that endospore attachment hinders nematode mobility and infectivity and, if the bacterium ultimately infects and develops within the nematode host, it reduces nematode fecundity (Ahmed and Gowen, 1991; Davies *et al.*, 1988; Stirling, 1984; Stirling *et al.*, 1990). Three Florida isolates of *P. penetrans* (P-20, P-100, P-122) were evaluated at varying endospore concentrations for their effect on penetration and reproduction of J2 of *M. arenaria* and *M. javanica* in greenhouse experiments (Dickson *et al.*, 1994). All three isolates readily attached to their host nematode, but only medium and high densities of P-100 endospores reduced root penetration and fecundity of *M. javanica* compared with the nontreated control. All three isolates reduced fecundity at the high endospore density compared with the nontreated control. With improved methods for working with *P. penetrans* using environmentally controlled conditions, our objective was to determine the effect of endospore number on attachment, infectivity and fecundity of *P. penetrans* isolate P20 on its nematode host, *M. arenaria* race 1.

The isolate of *M. arenaria* race 1 originated from peanut grown at the former University of Florida Green Acres Agronomy Farm, Alachua County, FL, and was maintained in a greenhouse on tomato (*Lycopersicum esculentum* Mill cv. Rutgers). The speciation and race designation was confirmed by examination of morphometrics, isozyme phenotypes, and host differentials. Eggs from infected tomato roots were extracted using a 0.5% sodium hypochlorite solution (Hussey and Barker, 1973). *M. arenaria* inoculum for *in vivo* culture of *P. penetrans* was obtained by hatching J2 from eggs over a 48-hour period after discarding J2 emerging during the first 24 hours.

The isolate of *P. penetrans* used in this study, designated P-20 was isolated from *M. arenaria.* Endospores were obtained from infected female nematodes and attached to the cuticle of 1 to 3 day old *M. arenaria* J2 by centrifugation (Hewlett and Dickson, 1993). Tomato plants (45-day-old seedlings) grown in 15-cm-diameter clay pots were inoculated with 3,000 endospore encumbered J2/pot. Three days later the plants were again inoculated and maintained in a greenhouse at 27 ± 5°C. They were fertilized twice weekly by adding 50 ml of a solution containing 0.63 g/liter of 20-20-20 (N-P-K), watered daily, and insecticides and fungicides applied as needed.

Sixty days after inoculation the roots were harvested, washed with tap water, and placed in a 1,800-ml beaker containing 10% Rapidase Pomaliq 2F (Gist-Brocades Pomaliq product number 7003-A/DSM Food Specialties USA., Menominee, WI), 50 mM NaAcetate (pH 5.0), and 0.1% $CaCl_2$ at ca. 50:50 (v/v), and placed at room temperature (Charnecki, 1997). The roots were digested for 3 days and then placed on a 600-µm-pore sieve nested on a 150-µm-pore sieve and females dislodged by subjecting them to a high pressure spray of water. Endospore-filled females were hand picked with forceps under a dissecting microscope at 200× magnification and placed in 1.5-ml siliconized microtubes that contained 1-ml deionized water. They were washed with deionized water (dH2O) before rupturing using a smooth mortar and pestle and the contents passed through a woven 21-µm opening polyester filter in a 13-mm Swinnex disc holder (Fisher Scientific, Suwanee, GA). Recovered endospores were washed twice in distilled water, centrifuged at 10,000 *g* for 5 minutes, and stored at 4°C. One milliliter of the endospore suspension was placed on a hemocytometer and the number of endospores per milliliter estimated by counting at 400× magnification with a microscope.

The concentration of freshly harvested *P. penetrans* endospores was adjusted to obtain the following J2-to-endospore ratios; 1:50 (1 J2 for every 50 endospores), 1:100, 1:150, and 0 for the control, each placed in a volume of 2.5-ml deionized water and agitated thoroughly. The mixture was centrifuged at 5,500 *g* for 5 minutes (Hewlett and Dickson, 1993). Each treatment had 15,000 J2 added. A 2-ml suspension was placed on a counting slide and the first 20 J2 in each treatment were observed microscopically (400×) to determine the number of *P. penetrans* endospores attached to the cuticle.

An experiment was conducted in an environmental chamber maintained at 27°C, 60% relative humidity (RH), and 14-hours of light for 7 days (Walker *et al.*, 1993) to determine effects of spore attachment level on nematode root penetration. The treatments were the four spore attachments levels as mentioned above. Tomato cv. Rutgers seedlings growing in 8-cm-diam. pots containing autoclaved sand were inoculated with 500 J2/plant (<48 hours old) from each of the attachment

levels. The treatments were arranged in a randomized complete block design, and replicated four times. The experiment was repeated.

Seven days after inoculation, the tomato plants were harvested by gently removing the roots from the cups. The roots were washed, cleared, and stained by a root clearing technique. Individual root pieces were pressed between two glass microscope slides and the number of J2 that had penetrated the roots were counted with a microscope at 200× magnification (Byrd *et al.*, 1983).

A greenhouse experiment was conducted to determine the effects of spore attachment rate on galling and egg mass production. The same treatments as applied for the penetration experiment were used to inoculate 45-day-old tomato seedlings (cv. Rutgers) growing in 15-cm-diam. clay pots. Greenhouse temperatures averaged 27 ± 5°C. Each plant was inoculated with 3,000 J2 with endospores attached as described above. Plants were watered, insecticides and fungicides applied as needed, and fertilized weekly with a solution containing 0.63 g/liter of 20-20-20 (N-P-K). The four treatments were arranged in a randomized complete block design replicated four times. Thirty days after inoculation, the roots were harvested and the number of galls and egg masses were rated using a 0 to 5 scale where; 0 = no galls or egg masses, 1 = 1-2 galls or egg masses, 2 = 3-10 galls or egg masses, 3 = 11-30 galls or egg masses, 4 = 31-100 galls or egg masses, 5 = >100 galls or egg masses per plant (Taylor and Sasser, 1978). To aid in the visualization of egg masses the roots were washed carefully, blotted dry, and placed in a 500 ml beaker containing 20% solution of red food coloring for 15 minutes, after which the roots were rinsed in tap water and blotted dry (Thies *et al.*, 2002).

All data were subjected to analysis of variance (ANOVA) using the GLM procedure in SAS/STAT version 9.1 (SAS Institute, Cary, North Carolina). Means were separated and compared using Waller-Duncan *k* ratio *t*-test and reported as significant at the 95% confidence level. Endospores per J2 data were transformed using $\log_{10}(x+1)$ before statistical analysis. Untransformed numbers are presented in the text.

Average attachment levels of 3.5, 10.2, and 18.1 endospores/J2 were obtained from J2-endospore ratios of 1:50, 1:100, and 1:150, respectively ($P \leq 0.05$). No attachment of endospores was observed in the control.

The number of J2 penetrating roots was reduced by an increasing number of endospores attached to their cuticle ($P \leq 0.05$) (Table 1). Based on a mean attachment level of 148 J2 penetrating roots for the nontreated control at 3.5, 10.2 and 18.1 endospores/J2 there was a 53%, 79%, and 81% reduction in root penetration,

Table 1. Effect of different attachment levels of endospores of *Pasteuria penetrans* to second-stage juveniles of *Meloidogyne arenaria* race 1 on root penetration, gall formation, and egg mass production.

Attachment levels	J2 penetrating	Gall index[y]	Egg mass index
0	148.0 a[z]	5.0 a	4.8 a
3.5	68.8 b	4.5 b	4.0 b
10.2	30.4 c	4.0 c	2.8 c
18.1	28.2 c	4.0 c	1.5 d

[y]Galling and egg mass indices were rated using a 0 to 5 scale where; 0 = no galls or egg masses, 1 = 1-2 galls or egg masses, 2 = 3-10 galls or egg masses, 3 = 11-30 galls or egg masses, 4 = 31-100 galls or egg masses, 5 = >100 galls or egg masses per plant (Taylor and Sasser, 1978).
[z]Means within each column with the same letter are not different according to Waller-Duncan *k* ratio *t*-test ($P \leq 0.05$).

respectively (Table 1). The attachment levels and the number of J2 penetrating per root system were negatively correlated but the regression equation was not significant ($P \leq 0.05$). Galling and egg mass indices were greater for the control than that for all other treatments ($P \leq 0.05$). The higher attachment levels greatly reduced egg masses ($P \leq 0.05$).

These experiments demonstrated that, in addition to temperature (Freitas *et al.*, 1997; Hatz and Dickson, 1992; Serracin *et al.*, 1997; Stirling, 1981; Talavera and Mizukubo, 2003), the number of endospores attached per J2 plays a very important role in root penetration and nematode fecundity. It has been reported that the number of endospores attached to the cuticle of J2 increased in proportion to both endospore concentration and time (Stirling *et al.*, 1990). The effect of endospore concentrations in soil environments is important in understanding this organism's role in causing soil suppressiveness. Although attachment does not necessarily imply infection (Carneiro *et al.*, 2004), it is obviously an important step in the infection process (Talavera and Mizukubo, 2003). Studies have shown that attachment is important in inferring host specificity of *P. penetrans* isolates (Brito et al., 2003; Davies *et al.*, 1992; Oostendorp *et al.*, 1990).

As the mean attachment level per juvenile increases, the percentage of J2 penetrating roots decreases (Table 1). While others have shown that varying endospore concentrations had little impact on root penetration by encumbered J2 (Dickson *et al.*, 1994), our results indicate otherwise. The number of J2 entering plant host roots were reported to be reduced when they were encumbered with 15 or more endospores but, at low density, penetration was not significantly affected (Davies *et al.*, 1988). Others reported that 11 or more endospores per J2 reduced root penetration (Ahmed and Gowen, 1991). Even higher numbers of 25-30 endospores/J2 have been reported to prevent root penetration (Stirling, 1984; Stirling *et al.*, 1990). In this study, as few as 3.5 endospore/J2 reduced the ability of *M. arenaria* to enter host roots. This supports the hypothesis that *P. penetrans* induces soil suppressiveness by interfering with root penetration (Stirling, 1984; Sturhan, 1985). Generally, higher endospore attachment levels resulted in lower rates of penetration; however, linear and exponential equations to explain the relationship were not significant. The low levels of penetration (30% in control) in both experiments could be attributed to the fact that not all the J2 had penetrated the roots by day 7 when the experiment was terminated.

Galling was not affected by levels of endospore attachment although there were differences between the control and all other treatments. The infection process begins once the endospore encumbered J2 enters a plant root. The bacterium inserts a germ tube through the nematode cuticle hypodermis to reach the pseudocoelom (Sayre and Starr, 1988). The infected J2 is able to begin the formation of giant cells and galling results, but egg production is completely blocked or greatly reduced (Gowen *et al.*, 1989). This study agrees with these findings. Gall formation is the host reaction to nematode infection and is independent of *P. penetrans* infection of the nematode. It is only following subsequent cropping sequences and build up of endospore density that the nematode population in the soil is reduced and the lack of galling can be used as an indicator of *P. penetrans* suppressiveness. In contrast, reduction of egg masses serves as a good indicator of *P. penetrans* suppressiveness and is in agreement with Dickson *et al.* (1994) where all the three isolates of *P. penetrans* tested reduced egg-mass production

at all levels. This study confirms that *P. penetrans* induced suppressiveness is manifested in the form of limited penetration and egg mass production (fecundity).

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