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The cumulative build-up of *Pasteuria penetrans* spores in root-knot nematode infested soil and the effect of soil applied fungicides on its infectivity

Khalil C. MELKI*, Ioannis O. GIANNAKOU, Barbara PEMBROKE and Simon R. GOWEN

Department of Agriculture, The University of Reading, Earley Gate, Reading, Berks RG6 6AT, UK.

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Summary - The cumulative build-up of *Pasteuria penetrans* spores in field soil and pots over three crop cycles was investigated after re-incorporation of roots of cucumber and tomato plants infested with root-knot nematodes parasitised by *P. penetrans*. In a pot experiment re-incorporation of tomato roots infected with root-knot nematodes parasitised by *P. penetrans* increased the spore population to a level at which nematode invasion was prevented. In the field experiment, the second stage juveniles extracted from the soil after three crop cycles of cucumber had a mean number of eight spores attached. Neither spore attachment nor development of the parasite was affected if spores of *P. penetrans* were exposed to four commonly used fungicides. © Orstom/Elsevier, Paris

Résumé - ***Croissance cumulée des spores de* Pasteuria penetrans *dans des sols infestés par le nématode* Meloidogyne *spp. et influence sur son infestivité de l'application au sol de fongicides*** - La croissance cumulée des spores de *Pasteuria penetrans* dans le sol (en pots ou en champ) après trois cycles culturaux a été observée après ré-incorporation de racines de concombre ou de tomate infestées par *Meloidogyne* spp. eux-mêmes parasités par *P. penetrans*. Lors d'une expérience en pots, la ré-inoculation de racines de tomates infestées par *Meloidogyne* spp. parasitées par *P. penetrans* augmente le nombre de spores à un niveau tel que l'invasion par le nématode en est empêchée. Lors d'une expérience en champ, les juvéniles de deuxième stade extraits du sol après trois cycles de culture de concombre ne présentent que huit spores fixées en moyenne. Ni la fixation ni le développement du parasite ne sont affectés si les spores de *P. penetrans* sont exposées à ces quatre fongicides courants. © Orstom/Elsevier, Paris

Keywords: bacterial parasite, biological control, *Meloidogyne javanica*, *Meloidogyne incognita*, root-knot nematodes.

A biocontrol agent should give consistent, long term suppression of a nematode pest and be compatible with farming practices. The Gram-positive bacterium *Pasteuria penetrans* (Thorne) Sayre & Starr has shown some level of control of root-knot nematodes (*Meloidogyne* spp.) when it was incorporated in soil in pot experiments (Channer & Gowen, 1988) and in microplots (Tzortzakakis & Gowen, 1994*a*; Triviño & Gowen, 1996). Gowen and Channer (1988) concluded that to contrive an increase of *P. penetrans* spore concentrations to levels at which suppression might occur, it could require several cycles of root-knot nematode susceptible hosts. Oostendorp *et al.* (1991) showed that over a 2-year cropping sequence with peanut (summer) and rye, vetch or fallow (winter) there was an increase in spore attachment on *M. arenaria* from 0.11 to 8.6 spores/J2 in plots on a fine sand soil in Florida.

There is little experimental data to indicate the spore concentrations in soil that will give suppression of root-knot nematodes. The issue is made more complex if there is variability in specificity of the bacterium or susceptibility of the nematode species or populations within species (Channer & Gowen, 1992; Tzortzakakis & Gowen, 1994*b*). Gowen and Channer (1988) and Chen *et al.* (1996) suggested that a spore density of 10 000 spores per g was necessary for suppression of root-knot nematode populations, although Triviño and Gowen (1996) reported that suppression developed over six crop cycles (30 months) of root-knot nematode susceptible hosts following initial treatments with 1000 spores per g.

P. penetrans is compatible with the application of some nematicides. Reduced root galling and reproduction of *Meloidogyne* spp. were observed when carbofuran and *P. penetrans* were applied together (Brown & Nordmeyer, 1985). Mankau and Prassad (1985) tested seven nematicides at recommended field dosages to determine their compatibility with *P. penetrans* and found no effect on the bacterial parasite, only dibromochloropropane (now no longer available) was slightly toxic. There is no information on the effects of commonly used soil fungicides on spore attachment and development of *P. penetrans*.

* *Present address: Unifert, P.O. Box 11-6937, Beirut, Lebanon.*

This study followed the cumulative build-up of *P. penetrans* spores in field soil and pots after re-incorporation of roots containing *P. penetrans*-infected root-knot nematodes over a succession of three crop cycles. The effect of four widely used soil fungicides on the development of the parasite was also determined.

Materials and methods

Experiment 1. Increase of *P. penetrans* over three crop cycles in the field

A field experiment was conducted in prepared beds in a commercial plastic tunnel (50 × 8 m) at Akaibeh, Lebanon. The tunnel had been previously cultivated with tomato and cucumber. No native *P. penetrans* was present. *P. penetrans* spores contained in dried powdered tomato root (3.2×10^4 spores per mg) were incorporated in approximately 7 dm^3 (10 kg) of soil taken from planting sites along the beds. The *P. penetrans* was derived from a mixture of populations originally isolated from *M. incognita* and *M. javanica* from Malawi, USA, South Africa and Australia, and prepared on tomato plants in a glasshouse at Reading University. Different amounts of the tomato root powder were mixed in the soil in a bucket to create spore concentrations 0.5, 1.0, 2.0, and 4×10^4 spores per g of soil. After thorough mixing for 2 min, the soil was returned to the planting holes. A control treatment was amended with tomato root powder free of *P. penetrans*. Treatments consisting of eight plants were arranged randomly along the beds with four replicates.

Cucumber seedlings cv. Alruba at the three-leaf stage were transplanted in the prepared soil, 40 cm apart in the rows, 80 cm between the rows; each row was irrigated with drip irrigation. After 3 months the plants were carefully uprooted, the roots within each replicate washed free of soil, air-dried, and then ground in a coffee grinder, the resulting powder was distributed evenly in the respective treatment row along the drip line and incorporated with a hoe. Fertilizers were added as necessary. This procedure was repeated for three successive crop cycles. After uprooting the plants at the end of every crop cycle, three soil samples of 25 g each were taken from the vicinity of the root systems of each replicate. Each soil sample was air-dried, and placed on nematode extraction cylinders 6.5 cm diameter, 4 cm deep, made from PVC pipe to which a coarse plastic mesh (1 mm) had been attached at one end. The soil was moistened but was not placed in contact with water. After 48 h, when it was assumed that spores in the soil would have become hydrated (Brown & Smart, 1984), 1000 freshly hatched juveniles (J2s) from cucumber plants infested with the same mixture of *M. incognita* and *M. javanica* in the same plastic greenhouse, were added to the soil in the extraction cylinders and left for 48 h at 28-30°C. Nematodes were then extracted by adding water to the dishes on which the extraction cylinders were supported (Southey, 1986). Twenty J2s per sample were examined at 200x magnification for spore attachment using an inverted microscope.

Throughout the three crop cycles, plants were treated when necessary with foliar applications of fungicides and insecticides. Soluble fertilizers were applied in the irrigation system.

Experiment 2. Increase of *P. penetrans* over three crop cycles in pots

P. penetrans isolated from *M. javanica* on tomato from South Africa was prepared by V. Spaull and stored at room temperature as a dry root powder originally derived from infected roots. A sample from this original material was passaged on a population of *M. javanica* originating from Malawi on tomato cv. Tiny Tim and a spore-containing root powder was then prepared by the Stirling and Wachtel (1980) technique. A suspension of 1.4×10^6 spores was added to 40 000 *M. javanica* juveniles which had hatched over a 2-day period from eggs collected from a tomato root system using the hypochlorite method (Hussey & Barker, 1973). Spore attachment was monitored until 85% of J2s were encumbered with six to twelve spores. The suspension was then poured through a 20 µm sieve to separate nematodes from spores. J2s were washed from the sieves and collected in 90 ml of water. Unencumbered J2s from the same source were kept in the same conditions as the encumbered nematodes. Tomato plants cv. Tiny Tim having ten to twelve leaves (45 days old) growing in a commercially produced loam-based compost (John Innes N° 2) in 640 ml plastic pots, were each inoculated with either a 5 ml suspension containing 2000 encumbered or unencumbered J2s.

After inoculation the pots were placed in a growth room with 16 h day and 8 h night period at temperatures of 31 and 25°C, respectively. Five plants from each treatment were harvested after 37 days when 700 degree-days had accumulated (base temperature was 10°C) (Stirling, 1981). The roots were washed and placed in a phloxine B solution for 25 min (Southey, 1986) to stain egg masses and rinsed in water to remove excess stain and then cut in 1-2 cm pieces. The numbers of egg masses and females were counted by direct examination of the roots using a stereoscopic microscope. The estimation of percentage infectivity was obtained by picking 40 females per root system. Each female was placed in a drop of water and was squashed with a cover slip. These were immediately examined under a light microscope (400×). The presence of endospores or vegetative stages (Sayre & Wergin, 1977) was used to confirm

infection of females by *P. penetrans*. The remaining plants were maintained for another 10 days at the same conditions for maximum production of mature endospores for the next cycle. After that period they were uprooted and kept in a glasshouse until the soil had dried out and all stages of nematodes had been killed. After mixing the soil and the whole root system of each pot inside a paper bag, it was returned to the same pot for every replicate. To avoid loss of spores through leaching the pots were placed in water-filled saucers and kept in the same growth room for 24 h. Then the soil in each pot was inoculated with 2000 freshly hatched J2s of *M. javanica* and kept for 24 h before tomato seedlings were transplanted. This procedure was repeated once more.

EXPERIMENT 3. THE EXPOSURE OF *P. PENETRANS* SPORES TO FUNGICIDES AND ITS EFFECT ON ATTACHMENT TO ROOT-KNOT NEMATODES

Suspensions of the fungicides hymexazol (Tachigaren® 30% a.i./L), fosetyl-Al (Aliettte® 80% WP), carbendazim (Bavistin® 50 WP) and *Trichoderma* (Promot®, a formulation of equal concentrations of *T. harzianum* and *T. koningii*) were prepared at concentrations of 2000, 5000, 1500, and 1500 ppm, respectively. Twenty five ml of each suspension was mixed with 25 ml of suspension of *P. penetrans* spores containing approximately 1.02×10^6 spores. After repeated agitation to ensure thorough mixing, the combined suspensions were left for 12 h and then poured on to 50 g samples of sterile loam soil contained in extraction cylinders as described in Experiment 1. Control treatments included water without fungicide or *P. penetrans* and *P. penetrans* without fungicides. Each treatment was replicated three times and extraction cylinders were arranged in a randomised block design.

After 24 h, 1000 freshly hatched J2s were pipetted into the soil, the cylinders were then loosely covered with a polyethylene sheet to limit evaporation and left for 48 h at 28°C. After this period the cylinders were placed in contact with water and nematodes extracted. Thirty J2s collected from each dish were observed at 200× under an inverted microscope and spore attachments recorded.

EXPERIMENT 4. INFECTIVITY OF *P. PENETRANS* SPORES EXPOSED TO FUNGICIDES

The same procedure as in experiment 3 was followed but the *P. penetrans* spore suspension contained 20.4×10^6 spores in 250 ml. These were placed in deep trays and 250 ml of each fungicide suspension prepared as previously, was added. The final spores-fungicide suspensions were left for 12 h and then were slowly added to 1 kg of sterile loam soil contained in a 15 cm diameter plastic pots, so that the spore concentrations were approximately 20 400 spores per g of soil. After 24 h, 5000 J2s of a mixed *M. incognita*/*M. javanica* population were added to each pot and left for 4 days before planting with 6 week-old tomato seedlings cv. Tiny Tim. After a 50 day growing period in the greenhouse, plants were harvested and processed as described for Experiment 2. Root-knot galling was assessed according to a 0-10 scale (Bridge & Page, 1980).

STATISTICAL ANALYSIS

Data on all experiments were subjected to single factor analysis of variance (ANOVA) and where appropriate least significant differences (LSD) were calculated at either $P \leq 0.05$ or $P \leq 0.001$. The data for the first experiment were subjected to a factorial analysis of variance and again least significant differences were calculated. Results of this experiment are presented linearly but described on the ANOVA basis.

Results

EXPERIMENT 1

Spore attachment was different ($P \leq 0.001$) among spore concentrations within every cycle between the different initial spore concentrations of *P. penetrans*, as well as between the three cycles (Fig. 1). The interaction between treatments and cycles was significant ($P \leq 0.001$) with the highest attachment being recorded at the end of the third cycle in the soil that had been initially inoculated with 40 000 *P. penetrans* spores per g of soil.

EXPERIMENT 2

Fewer females produced egg masses in the *Pasteuria* treatment in the first crop cycle, while there was no difference in the numbers of nematodes invading and

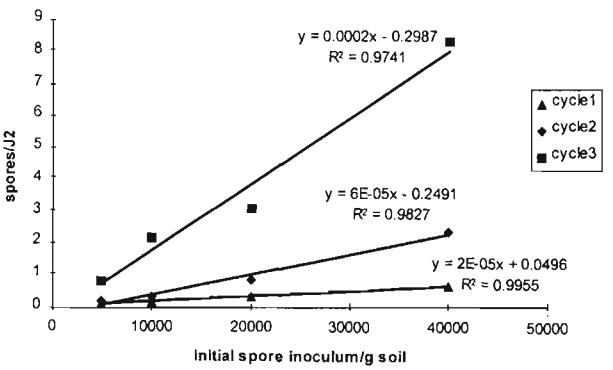

Fig. 1. *Effect of the initial rate of application of* Pasteuria penetrans *and root re-incorporation on the number of spores attached to* Meloidogyne *spp. over three cucumber crop cycles.*

developing as females (Table 1). In the second cycle 60% fewer J2s invaded root systems in the *Pasteuria* treatment while approximately 54% of them were infected by *Pasteuria*. The percentage of females which were infected by *P. penetrans* was 81.5 and 54.1% for the first and the second cycle, respectively. In the third cycle 11% of the initial inoculum of J2s invaded and reached maturity while in the *Pasteuria* treatment the tomato roots were completely free of nematode parasitism.

EXPERIMENT 3

There was no significant difference in the numbers of spores attaching to J2 between the different fungicide treatments and the untreated control ($P > 0.05$). Attachments ranged from 9.74 to 11.20 spores/J2. There was no spore attachment on J2s extracted from the soil to which neither fungicide or *P. penetrans* were added.

EXPERIMENT 4

Root galling was not affected by the different fungicide treatments although there was a difference ($P \leq 0.05$) between the treatment without *P. penetrans* and all the others (Table 2). Also, there was no difference in egg mass production among fungicide treatments, but these treatments all had fewer egg masses ($P \leq 0.001$) than the control treatment.

Discussion

The results of the cumulative effect of *P. penetrans* presented in this work agree with those reported by Oostendorp *et al.* (1991) and Triviño and Gowen (1996). The build-up of spores was pronounced in the third cycle and spore attachment increased more than ten fold in most of the treatments relative to the initial spore concentration in the soil in the first cycle. It is well documented that attachment is a density dependent phenomenon (Davies *et al.*, 1988) and is further supported by these data. Chen *et al.*, (1996) reported that root gall index was reduced to 80% when 10 000 spores per g of soil were applied as a soil drench in the top 20 cm in a peanut (*Arachis hypogaea*) field. In this study, when an initial rate of 10 000 spores per g of soil was used, even after three cycles, juveniles were only encumbered with a mean of 2.2 spores per J2, which is probably not enough for a good control (Stirling, 1985). However, in the soil that was initially infested with 40 000 spores per g, J2 were encumbered with a mean of eight spores, which should ensure good subsequent infection. In the second experiment, three cycles were enough to reduce the invasion rate of J2s to 0 when an inoculum of 2000 J2s was used for each cycle. Therefore, it might be that not only the numbers of spores per g of soil but also the volume of the soil might affect the level of control. The beneficial effects of *P. penetrans* are quite easy to demonstrate in pot experiments.

In commercial greenhouse vegetable production, large amounts of pesticides are used for the control of soil pests and diseases. It is reported that there is no effect of pesticides on *Pasteuria* spore attachment and development when they are synchronously applied with the chemicals (Mankau & Prasad, 1985). In

Table 1. *Total number of females and egg masses of* Meloidogyne *spp. per pot after three cycles of tomato cultivation in the same soil.*

Treatment	1st crop cycle		2nd crop cycle		3rd crop cycle	
	Females	Egg masses	Females	Egg masses	Females	Egg masses
Control	798*a*	781*a*	254*a*	254*a*	220*a*	220*a*
Pasteuria	649*a*	88*b*	101*b*	46*b*	0*b*	0*b*

In each column, data followed by the same letter are not significantly different at $P \leq 0.001$.

Table 2. *Effect of fungicides plus* Pasteuria *treatments on root galling and egg mass production on tomato plants.*

Treatment (with *Pasteuria*)	Root-knot index	Egg masses/root
Hymexazol	4.8 *b*	104 *b*
Fosetyl-Al	4.6 *b*	99 *b*
Carbendazim	4.8 *b*	110 *b*
Trichoderma spp	5.0 *b*	106 *b*
Water	5.0 *b*	106 *b*
Water (No *Pasteuria*)	6.8 *a*	190 *a*

In each column, data followed by the same letter are not significantly different at $P \leq 0.001$.

some cases however a positive synergism was recorded (Tzortzakakis & Gowen, 1994*a*). Our data show that the synchronous use of soil applied fungicides with *Pasteuria* does not affect the attachment and development of the parasite. Additionally, *Trichoderma* species (commercialized under the brand name Promot®) is compatible with *Pasteuria* and did not have any negative effect on infectivity of *Meloidogyne* spp.

The present study supporting the build-up of *Pasteuria* spores in soil over the crop cycles and its compatibility with other control methods employed by farmers is another encouraging step towards the integration of the use of *Pasteuria* with the common methods of pest and disease control in the field and in glasshouses.

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